Doping Test Results Dependent on Genotype of Uridine Diphospho-Glucuronosyl Transferase 2B17, the Major Enzyme for Testosterone Glucuronidation

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Context: Testosterone abuse is conventionally assessed by the urinary testosterone/epitesterone (T/E) ratio, levels above 4.0 being considered suspicious. The large variation in testosterone glucuronide (TG) excretion and its strong association with a deletion polymorphism in the uridine diphospho-glucuronosyl transferase (UGT) 2B17 gene challenge the accuracy of the T/E ratio test.

Objective: Our objective was to investigate whether genotype-based cutoff values will improve the sensitivity and specificity of the test.

Design: This was an open three-armed comparative study.

Participants: A total of 55 healthy male volunteers with either two, one, or no allele [insertion/insertion, insertion/deletion, or deletion/deletion (del/del)] of the UGT2B17 gene was included in the study.

Intervention: A single im dose of 500 mg testosterone enanthate was administered.

Main Outcome Measures: Urinary excretion of TG after dose and the T/E ratio during 15 d were calculated.

Results: The degree and rate of increase in the TG excretion rate were highly dependent on the UGT2B17 genotype with a 20-fold higher average maximum increase in the insertion/insertion group compared with the del/del group. Of the del/del subjects, 40% never reached the T/E ratio of 4.0 on any of the 15 d after the dose. When differentiated cutoff levels for the del/del (1.0) and the other genotypes (6.0) were applied, the sensitivity increased substantially for the del/del group, and false positives in the other genotypes were eliminated.

Conclusions: Consideration of the genetic variation in disposition of androgens will improve the sensitivity and specificity of the testosterone doping test. This is of interest not only for combating androgen doping in sports, but also for detecting and preventing androgen abuse in society. (J Clin Endocrinol Metab 93: 2500–2506, 2008)

Testosterone (T) was identified as the male sex hormone in the mid-1930s. It has been clinically used for nearly seven decades (1), primarily for androgen replacement therapy in men with androgen deficiency. Over the recent decades, testosterone and other androgens have been increasingly abused for muscle building and enhancement of physical performance (2). A recent study showed that power lifters with current or previous abuse of anabolic steroids have increased cross-sectional area of muscle

Abbreviations: AUC, Area under the curve; CI, confidence interval; cr, creatinine; Ct, cycle threshold; del/del, deletion/deletion; DHT, dihydrotestosterone; EG, epitestosterone glucuronide; ins/ins, insertion/insertion; ins/del, insertion/deletion; RQ, relative quantification; T/E, testosterone/epitesterone; TG, testosterone glucuronide; UGT, uridine diphospho-glucuronosyl transferase.
fiber and number of nuclei per fiber compared with power lifters without any exposure to anabolic steroid (3). It is possible that the previous use of anabolic steroids may improve physical performance for many years after withdrawal.

The World Anti-Doping Agency standardizes the rules and regulations governing antidoping in elite sports internationally. Anabolic compounds are the most frequently detected agents, accounting for about 43% of positive results in 2005. Among these testosterone, nandrolone and stanazolol were predominant (http://www.wada-ama.org/).

Testosterone is excreted mainly as glucuronide conjugates after metabolism by uridine diphospho-glucuronosyl transferases (UGTs). It is well established that UGT2B7, UGT2B15, and UGT2B17 are the principal catalysts of the glucuronidation of androgens and their metabolites in the human (4). Testosterone is mainly conjugated by UGT2B17 and, to a minor extent, by UGT2B15 (5). The main androgen substrate of UGT2B15 is androstane-3α,17β-diol. UGT2B17 shares 96% homology with UGT2B15 (6), but its substrate specificity is broader (5). UGT2B7 has been shown to have the capacity to conjugate epitestosterone (7), whereas testosterone is a poor substrate for this enzyme (5).

The tests for testosterone abuse are conducted in spot urine samples. Measuring only urinary testosterone glucuronide (TG) to detect testosterone abuse is not adequate because of large interindividual and intraindividual differences in urinary steroid concentration. The nearly constant ratio of urinary TG to epitestosterone glucuronide (EG) became the basis of the test (8). Epitestosterone is the 17α epimer of testosterone and has no known physiological function. It is not a metabolite of testosterone (9). An upper normal limit of six was calculated for the testosterone/epitestosterone (T/E) ratio based upon population studies (10, 11). In 1983 the Medical Commission of the International Olympic Committee introduced this value as a criterion for testosterone abuse. Ratios above six should be considered suspicious, and the person concerned should be subjected to further testing. Continued experience indicated that Asian individuals excrete lower amounts of TG and, therefore, have lower T/E ratios, thus increasing the risk of false-negative doping test results (12, 13). As a corollary the cutoff limit was lowered to four in 2004.

We demonstrated that a deletion polymorphism in the gene coding for UGT2B17 (14) is strongly associated with TG levels in urine (15). All subjects devoid of the gene had a T/E ratio less than 0.4 (15, 16). This polymorphism was considerably more common in a Korean Asian than in a Swedish Caucasian population, with 66.7 and 9.3% deletion/deletion (del/del) homozygotes, respectively.

Given this background we decided to monitor the testosterone excretion and the T/E ratio in healthy volunteers of different genotypes after testosterone administration. The aim was to investigate whether it is possible to increase the sensitivity and specificity of the doping test using genotype-based cutoff values. For this purpose 14–24 healthy volunteers each with two insertion/deletion (ins/ins), one insertion/deletion (ins/del), or zero (del/del) copies of the UGT2B17 gene were given a single dose of testosterone, and the urinary excretion of testosterone and EGs and other androgens was monitored and compared. Our findings suggest that urine analyses with combined genetic tests of the UGT2B17 gene considerably improve the sensitivity and specificity of the T/E test.

### Subjects and Methods

#### Subjects and design

Study subjects included healthy male volunteers aged 18–50 yr. A total number of 145 was genotyped for the UGT2B17 deletion polymorphism to fill the predetermined number of approximately 20 subjects in each of the three different genotype panels. Among the 145 genotyped subjects, 15% were homozygous for the gene deletion (del/del), 52% were heterozygous (ins/del), and 33% had two copies of the gene (ins/ins). Because the subjects originated from different ethnicities, the genotype frequencies are not representative of any particular population. In total, 17 del/del, 24 ins/del, and 14 ins/ins participants completed the study.

Study population characteristics are presented in Table 1. All participants underwent a medical examination, including laboratory tests, before enrollment to exclude the possibility of any disease. Drugs that did not interfere with the synthesis, metabolism, and excretion of steroids were allowed. Further inclusion criteria included a negative screening for illegal drugs, anabolic androgenic steroids, HIV, and hepatitis B or C virus. For inclusion it was also required that the subject was not a member of any organization belonging to the Swedish Sports Confederation, or had had a malignancy within the past 5 yr or an allergy to the study substance. All participants gave informed consent consistent with the approval of the Ethics Review Board.

Two individuals were excluded before the start of the study due to pathological laboratory tests, two due to positive virological tests, and one due to positive screening for illegal drugs. One subject was enrolled and given testosterone but was later excluded due to treatment with a substance that could interfere with analysis. The rest of the genotyped subjects that did not participate were either dropouts (n = 4) or did not match the right genotype panel. The participants were given 500 mg testosterone enanthate in castor oil as a single im dose of Testoviron Depot (kindly provided by Schering Nordiska AB, Solna, Sweden) equivalent to 360 mg testosterone. Before administration (d 0), urine samples were collected for analyses. Urine was further collected on d 1–9, 11, 13, and 15. All samples were collected between 0700 and 1100 h. Adverse drug reactions were monitored from the time of screening until d 15 after administration of testosterone. No major adverse drug reactions were registered. No follow-up was needed. The study was conducted according to the Helsinki declaration and the International Conference on Harmonisation Tripartite Guideline for Good Clinical Practice.

### Table 1. Study population characteristics at screening

<table>
<thead>
<tr>
<th>UGT2B17 Genotype</th>
<th>Age (yr)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>del/del (n = 17)</td>
<td>27.2 ± 4.0</td>
<td>179 ± 7.5</td>
<td>75.9 ± 8.4</td>
<td>23.8 ± 2.6</td>
</tr>
<tr>
<td>ins/del (n = 24)</td>
<td>32.0 ± 7.7</td>
<td>180 ± 6.6</td>
<td>79.6 ± 9.1</td>
<td>24.6 ± 2.9</td>
</tr>
<tr>
<td>ins/ins (n = 14)</td>
<td>28.7 ± 7.0</td>
<td>181 ± 5.4</td>
<td>78.6 ± 6.0</td>
<td>24.0 ± 2.1</td>
</tr>
</tbody>
</table>

Values are mean ± 10. BMI, Body mass index.
Blood and urine samples

Venous blood was obtained from the cubital vein and collected in EDTA tubes for DNA extraction. The urine samples were collected and kept refrigerated for a maximum 48 h and then frozen at −20°C.

Copy number analysis of UGT2B17

The copy number of the UGT2B17 gene was assessed by real-time PCR analysis. Ten nanograms of genomic DNA were used in each reaction together with 2× TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA) and UGT2B17 exon 6 specific primers (14) and an exon 6 specific probe (VIC-CAGTCTCTGGATTGAGTTTMGB). Expression of albumin was quantified as an endogenous control as described by Schaeffeler et al. (17). Both reactions were run in 25 μl volume on the same plate. The probe concentrations were 100 nM in each assay, and the primer concentrations were 900 and 600 nM for the UGT2B17 and albumin-specific reactions, respectively. The PCR profile consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 92°C for 15 sec and 60°C for 1 min. The effect of DNA concentration on PCR efficiency was determined using a control DNA in a dilution series of 20, 15, 10, 7.5, 5, and 2.5 ng/reaction. A known ins/del sample was chosen as a calibrator. It was set to one, and the relative quantification (RQ) was calculated using the ΔΔCT method (18).

Urinary steroids

Urinary unconjugated steroids and steroid glucuronides were analyzed at the Doping Laboratory of the department. Aliquots of 2–8 ml (depending upon the specific gravity of the urine sample) were complemented with 1 μg methyltestosterone as an internal standard. The unconjugated steroids were extracted directly with 5 ml tert-butyl methyl ether. The glucuronidated steroids were hydrolyzed with β-glucuronidase from Escherichia coli (Roche Diagnostics, Mannheim, Germany) [pH 7.0] 50°C for 1 h] and extracted in 5 ml n-pentane [pH 8.5] room temperature for 10 min. The organic phase was evaporated to dryness under a stream of nitrogen. Samples were converted into enol-trimethylsilyl ether derivatives with N-methyl-N-trimethylsilyl trifluoroacetamide (Macherey-Nagel, Düren, Germany) and ammonium iodide as described previously (19).

Urinary steroids were determined using combined gas chromatography-mass spectrometry. The analysis was performed with an Agilent gas chromatography-mass spectrometry 5973 instrument (Agilent Technologies, Inc., Palo Alto, CA) with the Single Ion Monitoring mode (19). Analytes were identified, and peaks were integrated and calculated using the mixture of authentic standard materials analyzed with every batch of samples. In addition to testosterone and epitestosterone, the androgen metabolites 5α-androstane-3α,17β-diol, androsterone, and etiocholanolone were measured. Interference with testosterone in the assay from, e.g., certain drugs, was not found in any of the samples. The day-to-day variation of the instrument was minimized using the mixture of authentic standards analyzed with every batch of samples. The within and between assay coefficients of variation for all steroids analyzed were less than 7 and 8%, respectively.

Data analyses

The between-subject variability in urine dilution was corrected for by dividing the concentration values by the urinary creatinine (cr) concentration. All urinary values are expressed as the unconjugated (typically less than 3% of the glucuronide fraction) plus the glucuronide conjugated fraction after correction for cr, if not specified otherwise.

The areas under the curves (AUCs) of the different urinary steroids were calculated using the trapezoidal rule.

Statistical analyses were performed by Kruskal-Wallis analysis, followed by Dunn’s multiple comparison post hoc test with P < 0.05 regarded as significant.

Results

UGT2B17 copy number assay

When the cycle threshold (Ct) values of UGT2B17 and albumin were plotted vs. log DNA concentration, the PCR efficiency for the UGT2B17 and albumin reaction was similar, 97 and 95%, respectively, and the difference between the slopes (Cthreshold – Creference) was less than 0.1, showing that the ΔCt calculation could be applied (18). Samples in which only albumin signal was observed were considered as homozygous for the deletion allele (del/del). Individuals with one allele (ins/del) had a mean RQ value of 1.04 (range 0.89–1.28), and individuals with two gene copies (ins/ins) showed a RQ value of 2.26 (range 1.95–2.62). There was no overlap between the groups demonstrating an unequivocal interpretation of genotyping results.

Unconjugated steroids

The unconjugated steroid fraction was analyzed in seven subjects (two ins/ins, one ins/del, and four del/del) before and after the testosterone challenge. This fraction constituted less than 3% of the glucuronidated fraction without differences between the genotypes. It was concluded that excretion of unconjugated testosterone after testosterone administration is only a minor elimination pathway even for the del/del subjects. Therefore, the unconjugated fraction was not isolated and analyzed separately in the remaining subjects.

Baseline urinary steroids

The average baseline urinary unconjugated and glucuronidated testosterone (TG) and epitestosterone (EG) concentrations and T/E ratios are presented in Table 2. The TG levels differed significantly between the del/del group and the other two groups (P < 0.001). There was no statistically significant difference in the EG levels.

The T/E ratio in the del/del group also differed significantly from both the ins/ins and ins/del group with no overlap of values (P < 0.001). The ratios in the ins/ins and ins/del groups did not differ significantly from each other.

<table>
<thead>
<tr>
<th>UGT2B17 Genotype</th>
<th>Testosterone (ng/μmol cr)</th>
<th>Epitestosterone (ng/μmol cr)</th>
<th>T/E ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>del/del (n = 17)</td>
<td>0.3 (0.21–0.39)*</td>
<td>2.8 (1.2–4.5)</td>
<td>0.14 (0.11–0.18)*</td>
</tr>
<tr>
<td>ins/del (n = 24)</td>
<td>2.6 (2.1–3.1)</td>
<td>2.3 (1.7–2.8)</td>
<td>1.4 (1.1–1.6)</td>
</tr>
<tr>
<td>ins/ins (n = 14)</td>
<td>4.0 (3.2–4.7)</td>
<td>2.0 (1.4–2.6)</td>
<td>2.3 (1.7–2.9)</td>
</tr>
</tbody>
</table>

* Statistical significance between the genotypes (P < 0.001).
There were no significant differences between the genotypes in the urinary concentrations of glucuronides of androstenedione, etiocholanolone, or 5α-androstane-3α,17β-diol, which are the major final metabolites of testosterone and dihydrotestosterone (DHT) (data not shown). These three metabolites are, in addition to UGT2B17, also conjugated by UGT2B7 and UGT2B15.

Urinary steroid profile after testosterone administration

The urinary excretion of TG on d 1–9, 11, 13, and 15 after the testosterone dose is shown in Fig. 1A. The maximum average increase in TG excretion after dose was 2.0 ng/μmol cr [95% confidence interval (CI) 1.4–2.6] in the del/del group, 18.6 ng/μmol cr (95% CI 12.9–24.4) in the ins/del group, and 41.8 ng/μmol cr (95% CI 27.9–55.6) in the ins/ins group (Fig. 1B).

![Graphs showing urinary steroid profile after testosterone administration]
The TG AUC was 16.4 ng/μmol cr (95% CI 13.4–19.4) × day in the del/del group, 162 ng/μmol cr (95% CI 129–195) × day in the ins/del group, and 294 ng/μmol cr (95% CI 236–351) × day in the ins/ins group. The differences were significant between the del/del group and the other two groups (P < 0.001), and between the ins/del and the ins/ins group (P < 0.05).

The excretion of epitestosterone glucuronide decreased to levels close to zero for all subjects without any statistically significant differences between the genotypes (Fig. 1C).

The average urinary T/E ratio increased from 0.14 (95% CI 0.11–0.18) to 5.3 (95% CI 4.1–6.5) in the del/del group, from 1.4 (95% CI 1.1–1.6) to 50.4 (95% CI 39.1–61.6) in the ins/del group, and from 2.3 (95% CI 1.7–2.9) to 100 (95% CI 70.8–130) in the ins/ins group (Fig. 2).

There were no significant differences between the individual genotypes in urinary excretion of the major testosterone and DHT metabolites etiocholanolone-G and androsterone-G (data not shown). The del/del group had a lower AUC of the major DHT metabolite 5α-androstane-3α,17β-diol-G (172 ng/μmol cr × day; 95% CI, 145–199) than the ins/ins group (249 ng/μmol cr × day; 95% CI, 204–293) (P < 0.05).

Sensitivity of the test after a single testosterone dose

The sensitivity of the current testosterone doping T/E test is shown in Fig. 3, left panel, and Table 3. When the ratio is set to one for the del/del group and six for the ins/del and ins/ins groups, the sensitivity increased substantially for the del/del group, and the number of false-positive doping tests was eliminated for the ins/ins group (Fig. 3, right panel, and Table 3).

Discussion

Our study demonstrates that the increase and the rate of increase in TG excretion after a single im testosterone dose are highly dependent on the genotype of the major testosterone glucuronidating UGT2B17 enzyme gene. Our findings have implications for interpretation of results of urinary testosterone and T/E analysis in doping tests. Genotyping as a complement to the conventional urine analysis would improve the sensitivity of the test by introduction of genotype-based differentiated cutoff levels.

There were large differences in TG excretion after testosterone administration among all three UGT2B17 genotypes. Here, we show that 40% of the subjects without the UGT2B17 gene never reached the T/E cutoff ratio of 4.0 on any of the 15 d after a single im dose of 360 mg testosterone. The highest average ratio for the del/del group (5.3) was reached on d 9. The other two groups reached their highest average ratio earlier, on d 7 (50.4) and d 6 (100) for the ins/del and ins/ins group, respectively (Fig. 2).

Previous observations have shown that individuals of Asian origin excrete lower amounts of TG than other populations (13, 20). Recently, we showed that a large part of the differences in TG excretion could be explained by genetic variation of the UGT2B17 gene (15). Because of this our study panels were based on genotypes, not ethnicity. The del/del genotype is much more common in Asian populations (66.7%) than in Caucasians (9.3%) (15).

The average baseline urinary TG level in the del/del group was 0.32 ng/μmol cr (95% CI 0.2–0.4). The average baseline urinary TG levels in the ins/del and ins/ins group were eight and 13 times higher, respectively, than the del/del group. There was no overlap of the urinary TG levels between the del/del group and the other two genotypes. In total, we have now analyzed urine samples from 100 del/del individuals in this and two other studies (15, 16). All of them had TG levels less than 1 ng/μmol cr. The low basal TG levels indicate that there are other UGT enzymes that catalyze the glucuronidation, most likely UGT2B15 (5). The TG levels did increase after injection of testosterone enanthate, but the average maximum increase was only 5% of the average maximum increase in the ins/ins group. This clearly shows the inadequacy of using the same cutoff level for all individuals independent of UGT2B17 genotype.

Because UGT2B15 may contribute to the low levels of urinary TGs in UGT2B17 del/del subjects, one could speculate that polymorphisms in this gene may also affect the T/E ratio. There is a G to T polymor-
Exogenous testosterone is known to decrease the urinary excretion rate of EG due to suppression of the secretion of LH (24–26). In the present study, the EG levels decreased in all three groups after the testosterone injection. There were large interindividual differences, but 6 d after the testosterone administration, 92% of all subjects had EG levels less than 30% of baseline, leading to even higher increases of T/E ratios.

Today, a T/E ratio cutoff limit of four gives cause for suspicion of testosterone doping. This test would misjudge over 40% of the del/del subjects even on the day when the average ratio was the highest after a single dose of testosterone (Fig. 3, left panel). The genetic variability within and between ethnic groups is a confounder, particularly when testing individuals of various ethnic descents.

On the contrary, in the ins/ins group, 14% had baseline T/E ratios above four. In our previous study (15) of a population sample of 122 young men, this limit would give a false-positive rate of 9%. False-positive results are not only of concern for the legal rights of the sportsman; they also yield an extra workload for the doping laboratories.

Baseline values of T/E ratios in the del/del subjects never exceeded 0.4. We simulated a differentiated cutoff level for the del/del (1.0) and the other genotypes (6.0), and found that at these levels the sensitivity in the del/del group increased substantially, and the false positives in the ins/ins group were eliminated (Fig. 3, right panel) in our experimental setting.

Testosterone can also undergo conjugation with sulfate before elimination. The urinary fraction of sulfate-conjugated testosterone was found to be 4% of the glucuronidated testosterone in a reference population of 45 males aged 17–50 yr (27). Our study was not designed to investigate other excretion pathways, but it cannot be excluded that del/del subjects eliminate a larger fraction of sulfate-conjugated testosterone to compensate for their compromised capacity to glucuronidate testosterone.

The determination of the $^{13}$C/$^{12}$C ratio of selected steroids (isotope ratio mass spectrometry analysis) provides the possibility to distinguish between pharmaceutical and natural testosterone because exogenous compounds contain less $^{13}$C than their endogenous homologs (28). However, the analytical facilities and costs required preclude any routine use of this methodology for screening in the antidoping testing. Therefore, its major use is to confirm suspected doping in samples with T/E ratios equal or greater than 4.0.

From Fig. 1A it seems that the TG excretion rate could be divided into at least two groups. In one group the subjects reached the peak urinary TG levels within 24 h, compared with 2–4 d for the other individuals. The peak was significantly lower in the “slow rise” group compared with the “fast rise” group. The reason for the distinct division into two parts in the testosterone excretion is not known. Genetic variation in glucuronidation enzymes is not likely because the same distribution was also observed in subjects without the UGT2B17 enzyme, which is the most important enzyme for testosterone elimination. Other candidate genes include esterases that hydrolyze the ester in the testosterone enanthate, but the particular enzyme involved in this cleavage has not been identified. The reason for this TG excretion pattern is of interest to study further because it may influence both the biological effect of testosterone treatment as well as the outcome of the doping test.

A logical follow-up of our test program is to investigate whether the effects of testosterone are different in individuals with different genotypes. We have recently shown that the baseline serum testosterone levels are not associated with the UGT2B17 polymorphism (16). However, the serum levels of testosterone in the different UGT2B17 genotypes after exogenous testosterone administration remain to be studied.

In summary, consideration of the genetic variation in androgen disposition is important in featuring the androgen urinary excretion profile, whether this is made for research purposes or doping tests.

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