



## Substantial advantage of a combined Bayesian and genotyping approach in testosterone doping tests

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### ABSTRACT

Testosterone abuse is conventionally assessed by the urinary testosterone/epitestosterone (T/E) ratio, levels above 4.0 being considered suspicious. A deletion polymorphism in the gene coding for UGT2B17 is strongly associated with reduced testosterone glucuronide (TG) levels in urine. Many of the individuals devoid of the gene would not reach a T/E ratio of 4.0 after testosterone intake.

Future test programs will most likely shift from population based- to individual-based T/E cut-off ratios using Bayesian inference. A longitudinal analysis is dependent on an individual's true negative baseline T/E ratio.

The aim was to investigate whether it is possible to increase the sensitivity and specificity of the T/E test by addition of UGT2B17 genotype information in a Bayesian framework.

A single intramuscular dose of 500 mg testosterone enanthate was given to 55 healthy male volunteers with either two, one or no allele (*ins/ins*, *ins/del* or *del/del*) of the UGT2B17 gene.

Urinary excretion of TG and the T/E ratio was measured during 15 days.

The Bayesian analysis was conducted to calculate the individual T/E cut-off ratio.

When adding the genotype information, the program returned lower individual cut-off ratios in all *del/del* subjects increasing the sensitivity of the test considerably.

It will be difficult, if not impossible, to discriminate between a true negative baseline T/E value and a false negative one without knowledge of the UGT2B17 genotype.

UGT2B17 genotype information is crucial, both to decide which initial cut-off ratio to use for an individual, and for increasing the sensitivity of the Bayesian analysis.

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### 1. Introduction

Doping with natural or exogenous androgen derivatives is a severe challenge to the vision, moral and ethics of sports all over the world. In 2006, anabolic compounds were the most frequently detected agents, accounting for about 45% of positive results in 2006. Among these testosterone, nandrolone and stanozolol were predominant (<http://www.wada-ama.org/>).

In order to discriminate exogenous testosterone from testosterone of endogenous origin the urinary ratio of testosterone glucuronide to epitestosterone glucuronide is used [1]. In 2004 a technical document from the World Anti Doping Agency (WADA) was adopted with the need of submitting the sample to IRMS anal-

ysis for determination of the <sup>13</sup>C/<sup>12</sup>C ratio of selected steroids if the T/E ratio value is equal to or greater than 4.0, as well as for altered steroid profiles (<http://www.wada-ama.org/>). The IRMS technique provides the possibility to distinguish between pharmaceutical and natural testosterone because exogenous compounds contain less <sup>13</sup>C than their endogenous homologues [2].

The T/E ratio has a much higher inter- than intra-individual variability [3] and future test programs will most likely shift from population based- to individual-based T/E cut-off ratios [4,5]. To this aim, Bayesian inference techniques look particularly promising, with limits that adapt itself in function of previous test results performed on the individual. This method has already shown its merits for the analysis of “blood passports” for the detection of blood doping [6].

There are reasons to believe that genetic variation is one of the most important causes of variation in disposition of many androgenic compounds [7,8]. We previously demonstrated that a deletion polymorphism in the gene coding for UGT2B17 [9] is strongly associated with testosterone glucuronide levels in urine [8]. All subjects

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devoid of the gene had a T/E ratio below 0.4 [8,10]. The existence of the gene does, however, not guarantee a T/E ratio higher than 0.4, because other functional mutations in the *UGT2B17* gene cannot be ruled out [8]. 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.

The aim of this study was to investigate whether it is possible to increase the sensitivity and specificity of the T/E ratio by the addition of *UGT2B17* genotype information in a Bayesian inferential framework.

## 2. Experimental

### 2.1. Subjects and design

Study subjects included 55 healthy male volunteers aged 18–50 years (mean  $30.6 \pm 7.0$  years) with either two, one or no allele (*ins/ins*, *ins/del* or *del/del*) of the *UGT2B17* gene. The study population has been described in more detail elsewhere [11]. All participants gave informed consent consistent with the approval of the Ethics Review Board. The participants were given 500 mg testosterone enanthate as single intramuscular dose of Testoviron®–Depot (kindly provided by Schering Nordiska AB, Solna) equivalent to 360 mg testosterone. Before administration, two baseline urine samples were collected for analyses. One was collected 1–3 months before and one prior to the testosterone administration (day 0). Urine was further collected on days 1–9, 11, 13 and 15. All samples were collected between 07 and 11 am. Adverse drug reactions (ADRs) were monitored from the time of screening until day 15 after administration of testosterone. No major ADRs were registered. No follow-up was needed. The study was conducted according to the Helsinki declaration and the ICH Harmonised Tripartite Guideline for Good Clinical Practice.

### 2.2. 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 maximum 48 h and then frozen at  $-20^\circ\text{C}$ .

### 2.3. Copy number analysis of *UGT2B17*

The copy number of the *UGT2B17* gene was assessed as previously described [11].

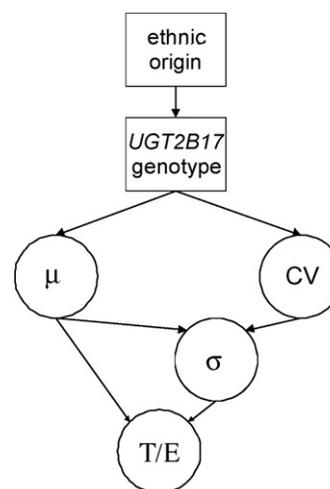
### 2.4. Urinary steroids

Urinary unconjugated steroids (typically <3% of glucuronide fraction) plus steroid glucuronides were determined by gas chromatography–mass spectrometry after hydrolysis of the conjugates with  $\beta$ -glucuronidase as described [11].

### 2.5. 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 sum of unconjugated plus the glucuronide conjugated fraction after correction for creatinine, if not specified otherwise.

The Bayesian longitudinal screening algorithm is explained in detail elsewhere [4,5]. Fig. 1 shows a graphical representation of the Bayesian model with the chosen set of variables and their probabilistic dependencies. Variables *ethnicity* and *UGT2B17* genotype have been added as discrete variables with states [Caucasian, Asian, African, others] and [*ins/ins*, *ins/del*, *del/del*], respectively. Parameters of populations were chosen from published data. For subjects



**Fig. 1.** Bayesian network for the analysis of T/E data. A circle represents a continuous variable, a rectangle a discrete variable, each arrow a causal relation between variables. Any information about the ethnicity, the *UGT2B17* genotype and/or a new T/E result can be added as new evidence in the network, with a new (posterior) distribution of the mean  $\mu$  and coefficient of variation CV obtained by Bayesian inference. The more information the better the characterization of the athlete, leading to a better specificity and sensitivity in detecting testosterone abuse.

with *ins/ins* and *ins/del* alleles, the geometric mean (GM) and geometric standard deviation (GSD) are equal to 1.40 and 1.81 for the distribution representing the population mean of the T/E and 0.176 and 1.48 for the coefficient variation (CV) of T/E. Subjects with *del/del* alleles differ by GM and GSD equal to 0.141 and 1.4321 for the mean of T/E. Prevalence of the *del/del* genotype was set to 0.10 for all ethnicities except for Asians (0.67). As in any Bayesian approach, the model can handle missing information about ethnicity and *UGT2B17* genotype. For females, naturally larger variations of the T/E ratio can also be modeled with the addition of dichotomous variable *gender* with states [male, female], and for example a GM of the CV twice as large (0.351). A software running the model is available under request to the authors.

## 3. Results

### 3.1. Bayesian test

The Bayesian analysis was conducted on all study subjects using one or two baseline T/E ratios to calculate the individual T/E cut-off ratio. The analysis was also repeated after entering the information of the *UGT2B17* genotype (obtained from DNA extracted from blood samples from the study subjects) as new evidence. In the *ins/ins* and *ins/del* individuals the genotype information did not significantly change the individual cut-off ratios after one and two baseline T/E ratios (not shown). This is not surprising, since Caucasians predominantly present an *ins/ins* or *ins/del* genotype. However, when adding the genotype information for the *del/del* group, the program returned much lower individual cut-off ratios in all subjects using one baseline T/E ratio, and in 35% (6 out of 17) of the individuals using two baseline T/E ratios (bold case) (Table 1a).

The T/E test results of one of the *del/del* individuals (subject no. 7, Table 1a) are shown in Fig. 2. If the Bayesian test is used without genotype information, he does not reach the cut-off ratio until 13 days (test no. 13) after the testosterone dose. However, if the genotype information is added to the Bayesian test, he would return a positive test 3 days (test no. 5) after the testosterone dose.

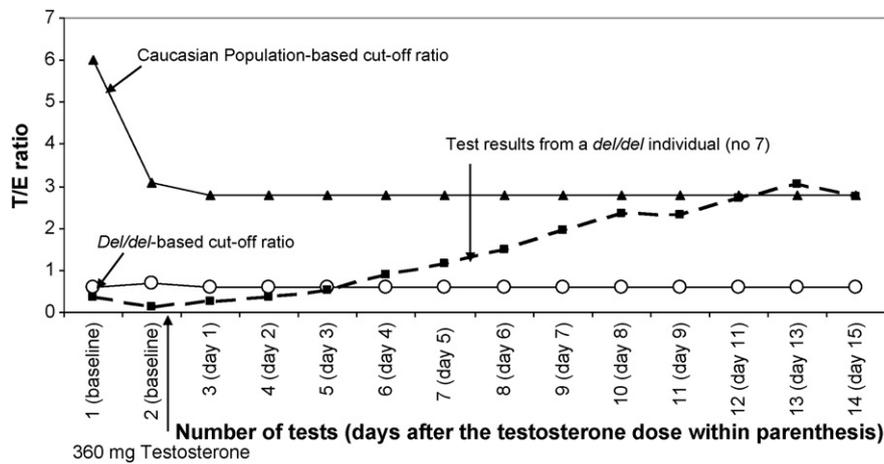
### 3.2. Sensitivity of the test after a single testosterone dose

The sensitivity of the current testosterone doping T/E test with a cut-off ratio of 4.0 is shown in Table 1b. For a theoretical rate

**Table 1a**

Individual cut-off T/E ratios in 17 *del/del* subjects using the Bayesian test *without* and *with* information on the genotype of *UGT2B17*. The Bayesian test was markedly improved using one baseline value *with* genotype information than when using two baseline values *without* genotype information in 35% of the *del/del* individuals (bold case).

Study subject	Individual T/E cut-off ratios			
	No genotype information		Genotype information	
	1 baseline value	2 baseline values	1 baseline value	2 baseline values
1	2.8	0.3	0.3	0.3
2	2.1	0.4	0.4	0.4
<b>3</b>	<b>1.7</b>	<b>1.1</b>	<b>0.4</b>	<b>0.5</b>
<b>4</b>	<b>2.9</b>	<b>1.0</b>	<b>0.5</b>	<b>0.5</b>
5	1.5	0.3	0.3	0.2
<b>6</b>	<b>2.2</b>	<b>1.5</b>	<b>0.4</b>	<b>0.6</b>
<b>7</b>	<b>3.1</b>	<b>2.8</b>	<b>0.7</b>	<b>0.6</b>
8	1.5	0.3	0.3	0.3
9	1.5	0.2	0.3	0.2
<b>10</b>	<b>3.1</b>	<b>3.0</b>	<b>0.7</b>	<b>0.7</b>
11	1.9	0.4	0.4	0.3
12	1.7	0.2	0.3	0.2
13	2.9	0.3	0.3	0.3
14	2.9	0.8	0.5	0.5
15	1.6	0.3	0.4	0.3
16	1.7	0.3	0.4	0.3
<b>17</b>	<b>6.1</b>	<b>1.3</b>	<b>0.5</b>	<b>0.4</b>



**Fig. 2.** Individual cut-off ratios based on the Bayesian analysis without and with genotype information. The upper line (triangles) shows Bayesian analysis of a *del/del* individual (subject no. 7 in Table 1a) when the model is based on a Caucasian population and the calculated cut-off ratio based on two baseline T/E values. The line with white circles shows the Bayesian analysis when the genotype information is included in the model and the individual cut-off ratio based on 2 baseline T/E values. The dashed line shows the T/E test results from the *del/del* individual after an intramuscular injection of 500 mg testosterone enanthate, equivalent to 360 mg testosterone, was given (arrow). The two first tests are the baseline T/E values. The following urine tests (numbers 3–14) are made on days 1–9, 13 and 15 after the testosterone dose and are thus positive for testosterone doping.

**Table 1b**

Sensitivity (%) of the T/E testosterone doping test with information on the deletion polymorphism of the *UGT2B17* gene. Data are shown for the test with a cut-off T/E ratio of 4.0 for all subjects or a cut-off T/E ratio of 0.6 for *del/del* and 6.0 for *ins/del* and *ins/ins* subjects, and the Bayesian test using two baseline T/E values and genotype information. An intramuscular injection of 500 mg testosterone enanthate, equivalent to 360 mg testosterone, was given on day 0.

Cut-off T/E ratio	<i>del/del</i> subjects (%)			<i>ins/del</i> subjects (%)			<i>ins/ins</i> subjects (%)		
	Day 2	Day 6	Day 11	Day 2	Day 6	Day 11	Day 2	Day 6	Day 11
4.0	5.9	58.8	29.4	62.5	100	100	100	100	100
0.6/6.0	70.6	100	100	54.2	100	100	92.9	100	100
Bayesian	88.2	100	100	70.8	100	100	100	100	100

of 1/100 false positives, the Bayesian test returned an initial limit of ~0.6 for *del/del* and ~6.0 for *ins/ins + ins/del*. Using these initial cut-off ratios the sensitivity increased substantially for the *del/del* group (Table 1b). When the Bayesian test using two known basal T/E values for each individual together with *UGT2B17* genotype information was used, the sensitivity increases even more (Table 1b). In addition there were two false positives in the *ins/ins* group (data not shown), with higher natural T/E ratios than 4.0 (4.8 and 4.3, respectively) that were eliminated using the initial cut-off level of 6.0 or the Bayesian analysis.

**4. Discussion**

This study shows the importance of *UGT2B17* genotype information in testosterone doping tests. Individuals lacking the *UGT2B17* gene excrete considerably less testosterone even after testosterone administration without the epitestosterone excretion being affected [11]. We have previously shown that 40% of the individuals lacking the *UGT2B17* gene would never reach the cut-off ratio of 4.0 used today after a single dose of 360 mg testosterone [11]. The distribution of this deletion polymorphism varies between ethnic

groups. It is common in East Asian countries but relatively rare in Caucasians. Nine percent of Swedes and 67% Koreans are devoid of the gene [8].

Future test programs will most likely shift from population based- to individual-based T/E cut-off ratios [4,5]. The latter returns a significantly higher sensitivity than the former, while returning markedly fewer false positives [5]. Nevertheless, with the extremely small changes in T/E ratios in the *del/del* group after testosterone administration the sensitivity of a longitudinal analysis remains unsatisfactory. We here show that it is considerably increased when the genotype information was entered as additional evidence in the Bayesian model.

We did not separate the *ins/ins* and the *ins/del* group in the Bayesian program, since there are substantial overlap between the T/E ratios of these two groups [8] and the difference in baseline T/E ratio between these two groups was not significant.

A longitudinal analysis is dependent on an individual's true negative baseline T/E ratio and it is important to have well designed initial cut-off ratios as guidelines before the proper amounts of urine samples have been analyzed from an individual. Here we show that the addition of the genotype information to the Bayesian model increases the sensitivity more than additional true negative baseline T/E ratios.

In some sports, especially endurance athletics, smaller doses than the 360 mg we used in this study, are common. This would result in even lesser changes of the T/E ratio in the *del/del* population. We conclude that it will be difficult, if not impossible, to discriminate between a true negative baseline T/E value and a false negative one without knowledge of the *UGT2B17* genotype. For a theoretical rate of 1/100 false positives, the Bayesian analysis returned an initial cut-off value of ~6 for a Caucasian population when genotype information was not taken into account. In contrast, when only *del/del* individuals were considered, the Bayesian analysis returned an initial cut-off value of ~0.6. We therefore conclude that *UGT2B17* genotype information may be crucial, both to decide which initial cut-off ratio to use for an individual and for making the Bayesian model more sensitive.

Further studies using smaller doses of testosterone are in progress. It is possible that additional biomarkers, such as certain testosterone metabolites, will be necessary to detect testosterone doping in individuals carrying the *UGT2B17 del/del* genotype.

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