

Genetic aspects of epitestosterone formation and androgen disposition: influence of polymorphisms in CYP17 and UGT2B enzymes

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Objective Testosterone is a commonly abused androgen in sports and in the gym culture of the society. Its abuse is conventionally disclosed by urinary assay of the testosterone/epitestosterone (T/E) glucuronide ratio, which should not exceed 4. A noteworthy number of athletes, however, have higher natural ratios than 4, most likely because of decreased excretion of epitestosterone glucuronide. Falsely positive doping test results are of great concern for the legal rights of the sportsman. Our objective was to study the genetic aspects of epitestosterone formation, and to elucidate the impact of genetic variation in androgen-metabolizing enzymes.

Methods Urine from different study populations was analysed for androgen glucuronides by gas chromatography-mass spectrometry. All men were genotyped for the uridine diphospho-glucuronosyltransferase (UGT) 2B17 deletion polymorphism and single nucleotide polymorphisms in the cytochrome P-450c17 α (CYP17), UGT2B15 and UGT2B7 genes. Expression of UGT2B15 mRNA in human liver samples was analysed using real-time PCR.

Results A T>C (A1>A2) promoter polymorphism in the CYP17 gene was associated with the urinary glucuronide levels of epitestosterone and its putative precursor androstene-3 β , 17 α -diol, resulting in 64% higher T/E ratios in A1/A1 homozygotes. Individuals devoid of UGT2B17 had

significantly higher UGT2B15 mRNA levels in liver than individuals carrying two functional UGT2B17 alleles.

Conclusion The CYP17 promoter polymorphism may partly explain high natural (>4) T/E ratios. Our data indicate that 5-androstene-3 β , 17 α -diol is an important precursor of epitestosterone and that CYP17 is involved in its production. In addition, we found that lack of the UGT2B17 enzyme may be compensated for by increase in UGT2B15 transcription. *Pharmacogenetics and Genomics* 18:477–485 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Anabolic androgenic steroids were added to the International Olympic Committee's list of banned substances in 1975. Since then it has always been a challenge to detect abuse of naturally occurring steroids, such as testosterone. In 1982, the urinary ratio between testosterone and epitestosterone glucuronides (EGs) was introduced as a test to detect testosterone abuse with an authorized upper limit of 6.0, which was later lowered to 4.0. The mean ratio in Caucasian populations is 1.0–2.0 [1,2], whereas in Asians the mean ratio is considerably lower [3,4]. We have shown that the reason for this ethnic disparity in testosterone/epitestosterone (T/E) ratio is strongly associated with a deletion mutation in the gene of the major catalyst of testosterone glucuronidation,

UGT2B17 [4], and that individuals homozygous for this mutation (*del/del*) may not reach a T/E ratio of 4 after a single intramuscular dose of testosterone (unpublished data). The deletion polymorphism is six times more common in a Korean compared with a Swedish population [4]. There are also individuals that have naturally higher T/E ratios than 4 [5–8], most likely because of decreased excretion of EG [5,7]. The reason behind low levels of urinary EG is unknown.

Epitestosterone is a naturally occurring 17 α -hydroxy epimer of testosterone. Although it has a central role in doping tests, very little is known about its physiological role and how it is formed in humans. No clear results have been published about the potential precursor(s)

of epitestosterone *in vivo*, although 5-androstene-3 β , 17 α -diol (Ae-17 α -diol) has been suggested to be the main precursor [9]. Weusten *et al.* [10] showed that Ae-17 α -diol is synthesized from pregnenolone in a single step, most probably by direct action of CYP17. The details in testosterone formation and the proposed epitestosterone formation are shown in Fig. 1.

CYP17 plays a central role in steroid metabolism, catalysing the 17 α -hydroxylation of pregnenolone and progesterone [11] (Fig. 1). A T > C exchange in the promoter region of the *CYP17* gene, adding a putative binding site for the Sp1 transcription factor has been described [12]. The T > C polymorphism gives rise to the A1 and A2 alleles. It has been postulated that the variant genotype (-34C, A2 allele) may increase transcription.

Testosterone is excreted mainly as glucuronide conjugates after metabolism by uridine diphospho-glucuronosyl transferases (UGT). We demonstrated that a deletion polymorphism in the gene coding for *UGT2B17* [13] is strongly associated with testosterone glucuronide levels in urine [4]. Even though 66% of the variation in the T/E ratio is explained by the *UGT2B17* polymorphism [4], other enzymes may be important in the androgen excretion and influence the T/E ratio, especially in individuals homozygous for the *UGT2B17* deletion (*del/del*).

Testosterone and the more potent androgen dihydrotestosterone (DHT) are in addition to *UGT2B17* also conjugated by *UGT2B15* [14,15]. A G to T polymorphism

in the *UGT2B15* gene, resulting in an aspartate (encoded by *UGT2B15*1*) to tyrosine (encoded by *UGT2B15*2*) at position 85 [16] exist. The polymorphism has been associated with altered glucuronidation activity *in vitro* [16] and with decreased serum levels of DHT *in vivo* [17].

Another member of the *UGT2B* enzyme family, *UGT2B7*, has been shown to have the capacity to conjugate epitestosterone [18]. Testosterone and DHT are not major substrates of this enzyme, although two of their metabolites, androsterone and androstane-3 α 17 β -diol (Aa-3 α -diol) (Fig. 1), are mainly deactivated by *UGT2B7* [19]. A histidine (*UGT2B7*1*) to tyrosine (*UGT2B7*2*) polymorphism at amino acid 268 has been described in the *UGT2B7* gene, with an allele frequency that differs significantly between Caucasians and Japanese [20]. *UGT2B7*2* has been associated with increased testosterone levels in serum, whereas no association with baseline levels of urinary epitestosterone or testosterone was found [21].

In this study, the impact of polymorphisms in the genes *CYP17*, *UGT2B7* and *UGT2B15* on androgen excretion was determined as well as the gene expression of *UGT2B15* in individuals devoid of the *UGT2B17* enzyme.

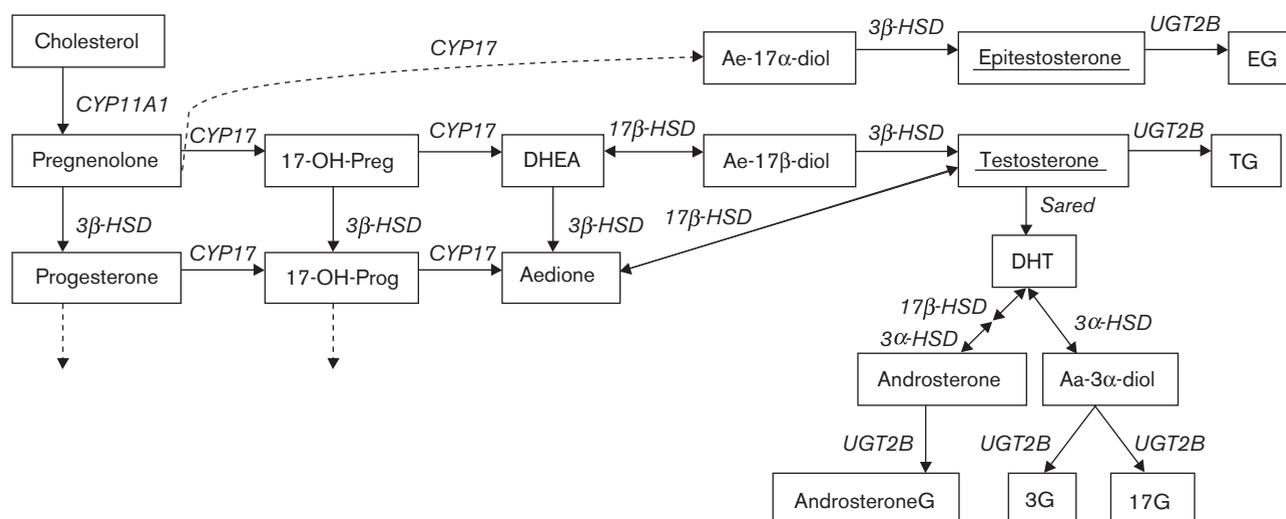
Materials and methods

Study populations

Testosterone challenge study

Study participants included 52 healthy male volunteers aged 18–50 years (mean 30.6 \pm 7.0 years) with either two,

Fig. 1



Metabolic pathways of formation and degradation of testosterone and epitestosterone. The dotted line is the suggested pathway for epitestosterone formation. 3G, Aa-3 α -diol-3 glucuronide; 17G, Aa-3 α -diol-17 glucuronide; Aa-3 α -diol, 5 α -androstane-3 α , 17 β -diol; Ae-17 α -diol, 5-Androstene-3 β , 17 α -diol; Ae-17 β -diol, 5-androstene-3 β , 17 β -diol; aedione, 4-androstenedione; DHT, dihydrotestosterone; EG, epitestosterone glucuronide; HSD, hydroxysteroid dehydrogenase; TG, testosterone glucuronide; UGT, UDP glucuronosyltransferase.

one or no allele (*ins/ins*, *ins/del* or *del/del*) of the *UGT2B17* gene. Most of them ($n = 40$) were Swedish Caucasians. The remaining 12 participants had different ethnic descent, mostly from different parts of Asia. All participants underwent a medical examination including laboratory tests before enrolment to exclude the possibility of any disease. Drugs that did not interfere with the synthesis, metabolism or excretion of steroids, were allowed. Three participants reported to have taken antidepressants throughout the study (paroxetine, escitalopram and sertraline). One participant took antibiotics (metronidazole and phenoxymethylpenicillin) on days 11–15. Three participants took one or two doses of NSAIDs (diclofenac and ibuprofen) and one patient took one dose of acetylsalicylic acid during the course of the study. 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 participant was not a member of any organization belonging to the Swedish Sports Confederation, or had had a malignancy within the past 5 years or an allergy to the study substance. All participants gave informed consent consistent with the approval of the Local Ethics Review Board in Stockholm.

In total 15 *del/del*, 23 *ins/del* and 14 *ins/ins* participants completed the study. The participants were given 500 mg of testosterone enanthate as a single intramuscular dose of Testoviron-Depot (kindly provided by Schering Nordiska AB, Solna) equivalent to 360 mg testosterone. Before administration (day 0) urine samples were collected for analyses. Urine was further collected on days 1–9, 11, 13 and 15. All samples were collected between 07:00 and 11:00 h. Adverse drug reactions were monitored from the time of screening until day 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 ICH Harmonised Tripartite Guideline for Good Clinical Practice.

Korean population

The Korean population included 72 unrelated male participants aged 21–39 years (mean 26.3 ± 3.5 years) who were recruited among medical students and personnel at Inha University Hospital. Their health status was assessed by medical questionnaire. All participants in this study participated voluntarily after giving informed consent. Venous blood was obtained from the cubital vein and collected in ethylenediaminetetraacetic acid tubes for DNA extraction. Spot urine samples were collected and immediately frozen at -20°C . The study was approved by the Institutional Review Board at Inha University Hospital and the Ethics Committee at Karolinska Institutet.

The GOOD study

The sample included 113 Caucasian men aged 18.0–20.1 years (mean 18.9 ± 0.6 years), who were selected randomly from the Gothenburg Osteoporosis and Obesity Determinants study ($n = 1068$), initiated with the aim to determine both environmental and genetic factors for bone and fat mass, in which study participants were randomly identified using national population registers, contacted by telephone, and asked to participate in the study. Venous blood was obtained from the cubital vein and collected in ethylenediaminetetraacetic acid tubes for DNA extraction. Spot urine samples were collected and immediately frozen at -20°C . The study was approved by the Ethics Committee at Gothenburg University, and written informed consent was obtained from all participants.

Genotyping

Copy number analysis of *UGT2B17*

In the participants of the Testosterone challenge study the copy number of the *UGT2B17* gene was assessed by real-time PCR analysis. Ten nanogram of genomic DNA was used in each reaction together with 2xTaqMan Universal Master Mix (Applied Biosystems, Foster City, California, USA) and *UGT2B17* exon 6-specific primers [13] and an exon 6-specific probe (VIC-CAGTCT TCTGGATTGAGTTT-MGB). Expression of albumin was quantified as endogenous control as described by Schaeffeler *et al.* [22]. Both reactions were run in 25 μl volume on the same plate. The probe concentrations were 100 nmol/l in each assay and the primer concentrations were 900 and 600 nmol/l 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 s 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 per reaction. A known *ins/del* sample was chosen as calibrator. It was set to 1 and the relative quantification (RQ) was calculated using the ddCT method [23]. When the cycle threshold (C_t) values of *UGT2B17* and albumin were plotted versus log DNA concentration, the PCR efficiency for the *UGT2B17* and albumin reaction was similar, 97 and 95%, respectively, and the difference between the slopes ($C_{t_{\text{target}}} - C_{t_{\text{ref}}}$) was less than 0.1 showing that the ΔC_t calculation could be applied [23]. 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). No overlap was observed between the groups demonstrating an unequivocal interpretation of genotyping results. The Korean participants were genotyped as described previously [4].

CYP17

The T > C substitution in the promoter region of *CYP17* in the participants of the Testosterone challenge study was investigated as described previously [24]. Briefly, the *CYP17* gene fragment was amplified by polymerase chain reaction using 300 ng of genomic DNA in a 25- μ l reaction. The PCR products were cleaved with the restriction enzyme *MspA1*, which recognizes the base pair substitution, and subsequently analysed on a 1.5% agarose gel.

The GOOD study participants were genotyped using the Sequenom MassARRAY MALDI-TOF system (San Diego, California, USA) at the SWEGENE Core Facility. The system analyses allele-specific primer extension products using mass spectrometry. Assay design was made using the SpectroDESIGNER software (Sequenom, San Diego, California, USA). Primers were obtained from Metabion (Martinsried, Germany). PCR primers 5'-ACGTTGG ATGCTAGAGTTGCCACAGCTCTT-3', 5'-ACGTTGG ATGTAAGCAGCAAGAGAGCCACG-3', and mass extend primer 5'-CAGGCAAGATAGACAGC-3' were used.

Owing to limited access of DNA, *CYP17* genotyping of the Korean population samples was conducted by a 5'-nuclease activity method using TaqMan SNP Genotyping Assay (___2852784-1) from Applied Biosystems. The PCR reaction was carried out in 12.5 μ l volume including 20 ng of genomic DNA, 2xTaqMan Universal Master Mix (Applied Biosystems). The PCR profile consisted of an initial denaturation step of 95°C for 10 min followed by 40 cycles of denaturation at 92°C for 15 s and annealing/elongation at 60°C for 1 min. The fluorescence signal was measured with an ABI 7700 Sequence Detector (Applied Biosystems).

UGT2B15

The *UGT2B15**1 and *2 polymorphism was genotyped using the primers described by Park *et al.* [25]. A 215-bp PCR product was amplified in a 30- μ l reaction containing 100 ng of genomic DNA, 2.5 mmol/l of MgCl₂, 200 μ mol/l of dNTP, 10 pmol of each primer and 0.05 U Taq Polymerase (Applied Biosystems). The cycling conditions were as follows: initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation of 93.5°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 60 s. The products were digested with *Sau3AI*, which recognize and cleave the Asp allele into a 187-bp fragment.

UGT2B15 genotyping of the Korean population samples was conducted by 5'-nuclease activity method using TaqMan SNP Genotyping Assay (___27028164-10) from Applied Biosystems. The PCR reaction was performed as described above.

UGT2B7

The *UGT2B7**1 and *2 polymorphism was genotyped using the primers and probes described by Swanson *et al.*

[21] in a 25- μ l reaction. The PCR reaction was performed as described above.

Urine analysis

Urinary unconjugated steroids (typically < 3% of glucuronide fraction) + steroid glucuronides were determined by gas chromatography-mass spectrometry after hydrolysis of the conjugates with β -glucuronidase as described [26] with minor modifications [6]. In addition to testosterone and epitestosterone, the putative epitestosterone precursor, Ae-17 α -diol, the androgen metabolites 5 α -Aa-3 α -diol, and androsterone were measured. The within and between assay coefficients of variation for all steroids analysed were less than 7 and 10%, respectively.

RNA and complementary DNA preparation

Seventeen human liver tissue samples and DNA were obtained from 14 Caucasian and three Asian human donor livers. The donor samples were genotyped for the *UGT2B17* polymorphism as described previously [4]. Total RNA was extracted from the liver specimens using Qiagen RNeasy MiniKit (Hilden, Germany) according to manufacturer's protocol, and RNA was reverse transcribed into complementary DNA (cDNA) with an oligo(T)₁₈ primer using First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Burlington, Canada) according to the manufacturer's protocol. The cDNA was diluted 10 times before real-time PCR analysis. The Ethics Committee of Karolinska Institutet at the Karolinska University hospital approved this part of the study.

Quantitative real-time PCR

Primer sequences for *UGT2B15* real-time assay have previously been described [27]. Beta-actin (Applied Biosystems) was chosen as an endogenous housekeeping control gene. Quantitative real-time PCR was performed using the ABI 7500 Fast PCR Detection System (Applied Biosystems). Reaction mixtures contained 1xPower SYBR Green PCR master mix (Applied Biosystems), 0.25 μ mol/l primers, 5 μ l cDNA template in a total volume of 25 μ l. Thermal cycling conditions included activation at 95°C (10 min) followed by 40 cycles each of denaturation at 95°C (15 s) and annealing/elongation at 60°C (1 min). Each reaction was performed in triplicate and no template controls were included in each experiment. The relative expression was calculated using the delta-deltaC(T) method [28] using an *UGT2B17 ins/ins* sample as a calibrator.

Data analyses

The between-participant variability in urine dilution was corrected by dividing the concentration values by the urinary creatinine (cr) concentration. All urinary values are expressed as the unconjugated (typically < 3% of total fraction) + the glucuronic acid conjugated fraction after correction for cr.

Caucasian participants refer to the Caucasians from the Testosterone challenge study ($n = 40$) together with the Caucasians from the GOOD study ($n = 113$) if nothing else is stated.

Existence of Hardy–Weinberg equilibrium was tested using χ^2 analysis. Urinary steroid levels were compared using the Student's t -test or analysis of variance followed by the Tukey's post-hoc test for the evaluation of differences between the individual genotypes, with P value of less than 0.05 regarded as significant. Liver cDNA levels were not normally distributed and were compared using Kruskal–Wallis analysis followed by Dunn's multiple comparison post-hoc test with P value of less than 0.05 regarded as significant.

Results

Influence of the *CYP17* polymorphism

The genotype and allele frequencies of the *CYP17* T > C (A1 > A2) promoter polymorphism in the different population samples are listed in Table 1. The genotype frequencies were in Hardy–Weinberg equilibrium.

Caucasian participants with either one or two A2 alleles had significantly higher urinary excretion of EG and the putative epitestosterone precursor Ae-17 α -diol glucuronide (Ae-17 α -diolG) [4.1 (range: 0.8–14) ng/ μ mol cr and 7.4 (range: 2.1–34) ng/ μ mol cr] than Caucasians homozygous for the A1 allele [2.6 (range: 0.5–7.2) ng/ μ mol cr, and 6.3 (range: 0–15) ng/ μ mol cr; $P < 0.001$ and $P = 0.018$] (Fig. 2). In Korean participants this difference was only observed for Ae-17 α -diolG [5.6 (range: 1.9–18.4) ng/ μ mol cr vs. 3.7 (range: 0–8.2) ng/ μ mol cr; $P = 0.0028$].

The *CYP17* single nucleotide polymorphism was also found to be associated with the T/E ratio, but only in *UGT2B17 ins/ins* and *ins/del* participants of Caucasian origin ($n = 138$). Individuals homozygous for the A1 allele exhibited 64% higher T/E ratios than the A2-carriers (2.9 and 1.8, respectively; $P = 0.0008$), whereas no association was observed in the Korean participants ($n = 16$).

Table 1 Genotype and allele frequencies of the *CYP17* A1 > A2 promoter polymorphism in a group of male Swedish Caucasian ($n = 153$) and Koreans ($n = 72$)

	Swedes % (n)	Koreans % (n)
A1/A1 genotype	35 (53)	25 (18)
A1/A2 genotype	48 (74)	44 (32)
A2/A2 genotype	17 (26)	30 (22)
A1-allele	59 (180)	47 (68)
A2-allele	41 (126)	53 (76)

The Swedish Caucasian group ($n = 153$) consisted of the GOOD study ($n = 113$) and the Caucasians from the Testosterone challenge study ($n = 40$).

Influence of the *UGT2B15* polymorphism

The genotype and allelic frequencies of the *UGT2B15* polymorphism in the different population samples are listed in Table 2. The genotype frequencies were in Hardy–Weinberg equilibrium. The allele frequencies of the GOOD study have been published elsewhere [17].

The polymorphism did not affect the baseline urinary androgen excretion in the Caucasian population sample of the GOOD study (data published previously by Swanson *et al.* [17]). Nor was there any association in the Testosterone challenge study between the *UGT2B15* genotype and any of the glucuronidated metabolites excreted in urine after testosterone administration. Among the Korean *UGT2B17 del/del* individuals, *UGT2B15**1/*1 participants had significantly higher excretion of androsteroneG and Aa-3 α -diol glucuronide (Aa-3 α -diolG) excretion than individuals with one or two *2 alleles (Table 3). No association was observed between the *UGT2B15*-specific mRNA levels in liver and the *UGT2B15* polymorphism (not shown). Our mRNA expression analysis from six *UGT2B17 del/del*, four *ins/del* and seven *ins/ins* livers, however, indicated that individuals devoid of *UGT2B17* had higher relative mRNA levels of *UGT2B15* in liver than individuals carrying the *UGT2B17 ins/ins* genotype ($P = 0.042$) (Fig. 3). Individuals homozygous for the *UGT2B17* deletion exhibited 4.5 times more *UGT2B15* mRNA than *UGT2B17 ins/ins* participants.

Influence of the *UGT2B7* polymorphism

The genotype and allele frequencies of the *UGT2B7**2 polymorphism in the different population samples are listed in Table 2. The genotype frequencies were in Hardy–Weinberg equilibrium. The allele frequencies of the GOOD study have been published elsewhere [21]. The polymorphism did not have any significant impact on urinary androgen excretion, including Aa-3 α -diolG and androsteroneG, in the Caucasians of the GOOD study (data published previously by Swanson *et al.* [21]) or Korean population sample (Table 3).

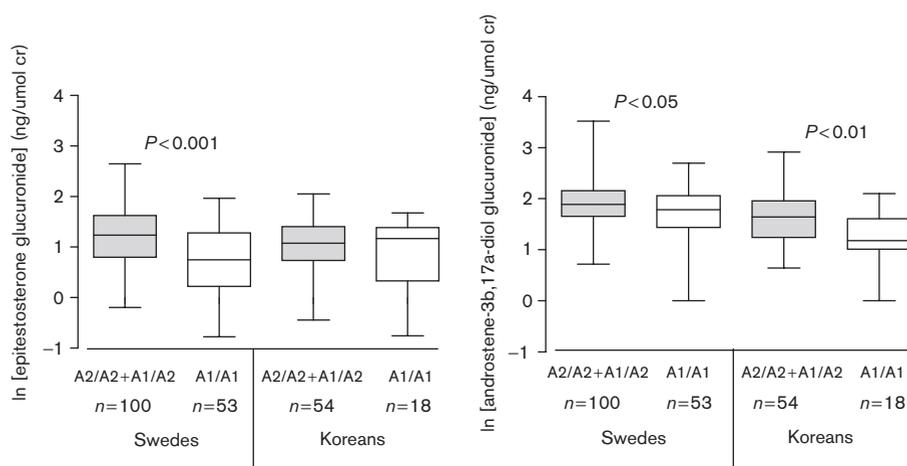
Urinary epitestosterone and Ae-17 α -diol levels after testosterone administration

After the intramuscular testosterone administration, urinary Ae-17 α -diolG and EG excretion decreased in a parallel matter (Fig. 4). The levels of urinary dehydroepiandrosteroneG did not change after the testosterone dose (Fig. 4).

Discussion

Here, we show that a T > C (A1 > A2) promoter polymorphism in the *CYP17* gene is associated with EG levels in urine in Swedish Caucasian individuals. Participants carrying the A2/A2 or the A1/A2 variant had 1.6 times higher urinary levels of EG compared with individuals homozygous for the A1 allele. The *CYP17*

Fig. 2



CYP17 A1 > A2 polymorphism and urinary epitestosterone glucuronide (EG) (left panel) and androstene-3 β , 17 α -diol glucuronide (AediolG) (right panel) levels in Swedish and Korean men. Swedes carrying the A2/A2 or A1/A2 have significantly higher EG and AediolG levels than A1 homozygous ($P < 0.001$ and < 0.5). Korean A2/A2 + A1/A2 carriers had significantly higher AediolG levels than A1/A1 carriers ($P < 0.01$), but there were no statistically significant differences in EG levels. cr, creatinine.

Table 2 Genotype and allele frequencies of the *UGT2B151, *2 and *UGT2B7**1, *2 polymorphisms in the Testosterone challenge study participants ($n = 52$) and the Korean group ($n = 72$)**

	Testosterone challenge study participants, % (n)	Koreans, % (n)
<i>UGT2B15</i>		
*1/*1	39 (20)	28 (20)
*1/*2	40 (21)	51 (37)
*2/*2	21 (11)	21 (15)
*1 allele	55 (61)	54 (77)
*2 allele	45 (43)	46 (67)
<i>UGT2B7</i>		
*1/*1	33 (17)	39 (28)
*1/*2	44 (23)	50 (36)
*2/*2	23 (12)	11 (8)
*1 allele	55 (57)	64 (92)
*2 allele	45 (47)	36 (52)

polymorphism and the low EG excretion in the A1/A1 participants may partly explain why some individuals have naturally elevated (> 4) T/E ratios [5–8]. This is of interest because falsely positive doping test results are of great concern for the legal rights of the sportsman. The urinary EG concentrations in 138 Swedes ranged from 0.5 to 14 ng/ μ mol cr (mean 3.4 ng/ μ mol cr). Out of the 14 Swedish participants with urinary EG levels below 1 ng/ μ mol cr, 10 were homozygous for the A1 allele and one was homozygous for the A2 allele.

Interestingly, the *CYP17* polymorphism is also associated with the putative epitestosterone precursor, Ae-17 α -diol in both the Korean and the Swedish participants.

There is no consensus whether the Δ^5 pathway via Ae-17 α -diol or the Δ^4 pathway via androstenedione (Fig. 1) is predominant in epitestosterone formation in humans. It

is known that urinary Ae-17 α -diolG [29] and EG [4,29–31] levels decrease after exogenous testosterone administration. Here, we show that Ae-17 α -diolG levels decrease in parallel with EG after an intramuscular dose of 360 mg of testosterone (Fig. 4), whereas dehydroepiandrosteroneG levels did not follow this pattern. This effect may be exerted by a feed-back mechanism via the follicle-stimulating hormone and luteinizing hormone, which may control the *CYP17* catalysed synthesis of Ae-17 α -diol and EG but not the adrenal formation of DHEA. These findings, and the significant association between *CYP17* and the urinary levels of EG and Ae-17 α -diolG are consistent with that the Δ^5 pathway is predominant in humans and that a large part of the formation of epitestosterone is catalysed via a direct pathway from pregnenolone to Ae-17 α -diol [10].

We were unable to show an association between the EG levels and the *CYP17* polymorphism in the Korean population. The reason for this may be lack of statistical power as we had only 72 Korean individuals in the study. Another explanation may be that we only measured the glucuronide conjugate fraction in urine, which is the favoured metabolic pathway of epitestosterone [5,32]. Epitestosterone is also excreted as sulphate conjugates and it is possible that we would have seen the effect of the *CYP17* polymorphism in the Korean population if the total urinary epitestosterone fraction was measured, as the effect was seen for the putative epitestosterone precursor Ae-17 α -diol.

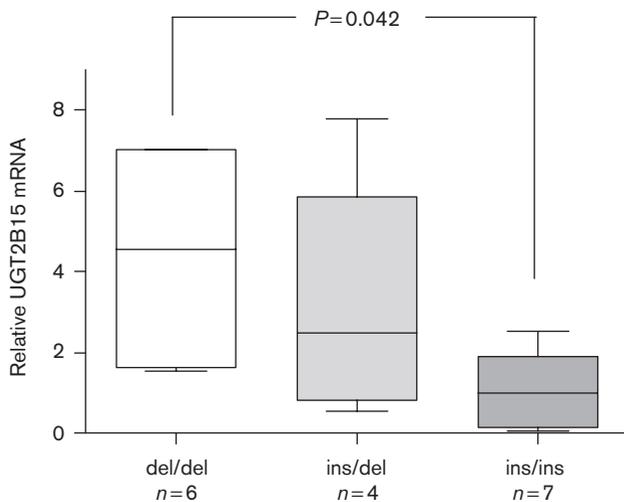
Heracek *et al.* [33] found significantly increased epitestosterone levels in prostate cancer tissues compared with

Table 3 Urinary androgen glucuronide excretion in Korean men devoid of the *UGT2B17* enzyme (*UGT2B17 del/del*) with different *UGT2B7* and *UGT2B15* genotypes

Urine parameters (ng/ μ mol cr)	UGT2B7			P value	UGT2B15			P value
	*1/*1 (n=21)	*1/*2 (n=26)	*2/*2 (n=8)		*1/*1 (n=17)	*1/*2 (n=28)	*2/*2 (n=10)	
TestosteroneG	0.32 \pm 0.12	0.29 \pm 0.10	0.31 \pm 0.15	NS	0.33 \pm 0.14	0.30 \pm 0.10	0.29 \pm 0.13	NS
EpitG	3.4 \pm 2.1	2.7 \pm 1.2	2.8 \pm 1.0	NS	3.2 \pm 1.9	3.0 \pm 1.5	2.7 \pm 1.3	NS
AndrosteroneG	235 \pm 58	204 \pm 62	198 \pm 50	NS	245 \pm 60	206 \pm 55	188 \pm 57	0.03
Aa-3 α -diolG	4.8 \pm 1.3	4.8 \pm 1.0	4.4 \pm 0.9	NS	5.6 \pm 1.6	4.4 \pm 1.5	4.4 \pm 1.2	0.03

Values are given as mean \pm SD and compared by analysis of variance.

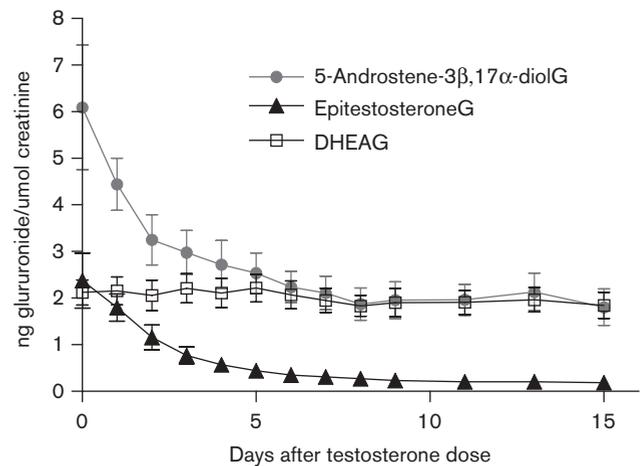
Aa-3 α -diol, 5 α -androstane-3 α , 17 β -diol; Epit, epitestosterone; G, glucuronide; NS, nonsignificant.

Fig. 3

Relative mRNA expression of *UGT2B15* in human liver samples with 0 (*del/del*), 1 (*ins/del*) or two (*ins/ins*) copies of the *UGT2B17* gene.

nontumoral prostate tissue. Animal studies have shown that epitestosterone exhibits antiandrogen properties [34,35]. Elucidation of the impact of genetic variability on the formation and excretion of epitestosterone is important to increase our understanding of the role of androgens in the pathology of endocrine organs. In addition, increased knowledge will improve the interpretation of doping test results.

Genetic variation in *UGT2B15* seems to have the largest impact on androgen disposition in participants devoid of the *UGT2B17* gene. Interestingly, in these individuals *UGT2B15*-specific mRNA hepatic levels were significantly higher than in individuals with two functional alleles of *UGT2B17*. We postulate that a lack of the *UGT2B17* enzyme is compensated for by an increase in *UGT2B15* transcription. Both enzymes are major catalysts in androgen inactivation and highly abundant in the prostate although located in different cell types [19]. Two studies have found an association with *UGT2B17* deletion and prostate cancer [36,37], whereas a large population-based study was unable to confirm these findings [38]. It

Fig. 4

Urinary levels of the 5-androstene-3 β , 17 α -diol glucuronide (G), epitestosteroneG and dehydroepiandrosteroneG (DHEAG) in the study participants of the Testosterone challenge study ($n=52$) after an intramuscular dose of 360 mg testosterone on day 0.

is possible that the intraglandular androgen homeostasis is compromised by the deletion and that backup of *UGT2B15* strives to maintain physiological levels of androgens in the prostate. We have recently shown that *UGT2B15* is mainly involved in the 17-glucuronidation of 5 α -Aa-3 α -diol and that the *1 variant of the *UGT2B15* polymorphism is associated with higher serum Aa-3 α -diol 17G [17]. In contrast, there was no significant association between the *UGT2B15* polymorphism and urinary androgen levels in the Swedish participants. Unfortunately, our urine analysis method did not allow us to separate Aa-3 α -diol 3G from Aa-3 α -diol 17G and because *UGT2B15* seems to be rather selective towards the 17 β -hydroxy position [14,17], it is possible that a more sensitive method is required to detect an association between urinary Aa-3 α -diolG levels and the *UGT2B15* polymorphism.

When considering only *UGT2B17 del/del* Korean participants ($n=55$), however, the individuals homozygous for the *1 variant had significantly higher urinary levels of Aa-3 α -diol (3G + 17G) and androsteroneG, supporting

the notion that UGT2B15 may compensate for the absence of UGT2B17, and that *UGT2B17 del/del* participants are more affected by the *UGT2B15* polymorphism than participants with a functional UGT2B17 enzyme. Earlier results on the *UGT2B15* polymorphism are conflicting. An early in-vitro report indicated that the *2 variant has higher activity than the *1 variant [16], whereas a later report claimed that the *1 variant had higher glucuronidation activity than the *2 variant [39].

Unfortunately, only two individuals in our *del/del* group in the Testosterone challenge study were homozygous for the *2 genotype, which precluded any attempts to draw any conclusions about the role of the *UGT2B15* polymorphism for testosterone excretion after a testosterone challenge.

We have recently shown that the *UGT2B7* polymorphism is independently associated with cortical bone size and serum sex steroid levels in young adult men and that participants homozygous for the *2 allele had higher serum testosterone levels than participants homozygous for the *1 allele [21]. UGT2B7 has been suggested to be a catalyst of epitestosterone glucuronidation [18], and genetic variation in this gene may, in addition to the *CYP17* polymorphism, partly explain the interindividual variation in urinary EG levels. The *UGT2B7* polymorphism, however, did not have any impact on EG levels or any other urinary androgen levels investigated here, regardless of presence or absence of the *UGT2B17* gene.

In conclusion, we have shown that the A1 > A2 promoter polymorphism in the *CYP17* gene is significantly associated with urinary Ae-17 α -diol glucuronide levels in Swedish and Korean participants, as well as EG levels in Swedes, giving rise to 64% higher T/E ratios in individuals homozygous for the A1 allele. Our data are consistent with previous results indicating that Ae-17 α -diol may be the main precursor of epitestosterone in humans and that this precursor is directly formed from pregnenolone by CYP17.

We also demonstrate that individuals homozygous for the *UGT2B17* deletion seem to compensate this deficiency with a higher expression of *UGT2B15*. As a consequence, polymorphisms in *UGT2B15* may have a larger impact on androgen disposition in individuals devoid of *UGT2B17* than in individuals with a functional enzyme. Our results may have implications for the interpretation of tests for testosterone doping.

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References

- Donike M, Rauth S, Wolansky A. Reference ranges of urinary endogenous steroids determined by gas chromatography/mass spectrometry. In: Donike M, Geyer H, Gotzmann A, Mareck-Engelke U, Rauth S, editors. *Proceedings of the 10th Cologne Workshop on Dope Analysis*. Cologne: Sport und Buch Strauß; 1993. pp. 69–86.
- Ayotte C, Goudreault D, Charlebois A. Testing for natural and synthetic anabolic agents in human urine. *J Chromatogr B Biomed Appl* 1996; **687**:3–25.
- Park J, Park S, Lho D, Choo HP, Chung B, Yoon C, et al. Drug testing at the 10th Asian Games and 24th Seoul Olympic Games. *J Anal Toxicol* 1990; **14**:66–72.
- Jakobsson J, Ekstrom L, Inotsume N, Garle M, Lorentzon M, Ohlsson C, et al. Large differences in testosterone excretion in Korean and Swedish men are strongly associated with a UDP-glucuronosyl transferase 2B17 polymorphism. *J Clin Endocrinol Metab* 2006; **91**:687–693.
- Dehennin L. On the origin of physiologically high ratios of urinary testosterone to epitestosterone: consequences for reliable detection of testosterone administration by male athletes. *J Endocrinol* 1994; **142**:353–360.
- Garle M, Ocka R, Palonek E, Bjorkhem I. Increased urinary testosterone/epitestosterone ratios found in Swedish athletes in connection with a national control program. Evaluation of 28 cases. *J Chromatogr B Biomed Appl* 1996; **687**:55–59.
- Kicman AT, Oftebro H, Walker C, Norman N, Cowan DA. Potential use of ketoconazole in a dynamic endocrine test to differentiate between biological outliers and testosterone use by athletes. *Clin Chem* 1993; **39**:1798–1803.
- Raynaud E, Audran M, Brun JF, Fedou C, Chanal JL, Orsetti A. False-positive cases in detection of testosterone doping. *Lancet* 1992; **340**:1468–1469.
- Dehennin L. Secretion by the human testis of epitestosterone, with its sulfoconjugate and precursor androgen 5-androstene-3 beta,17 alpha-diol. *J Steroid Biochem Mol Biol* 1993; **44**:171–177.
- Weusten JJ, Legemaat G, van der Wouw MP, Smals AG, Kloppenborg PW, Benraad T. The mechanism of the synthesis of 16-androstenes in human testicular homogenates. *J Steroid Biochem* 1989; **32**:689–694.
- Picado-Leonard J, Miller WL. Cloning and sequence of the human gene for P450c17 (steroid 17 alpha-hydroxylase/17,20 lyase): similarity with the gene for P450c21. *DNA* 1987; **6**:439–448.
- Carey AH, Waterworth D, Patel K, White D, Little J, Novelli P, et al. Polycystic ovaries and premature male pattern baldness are associated with one allele of the steroid metabolism gene CYP17. *Hum Mol Genet* 1994; **3**:1873–1876.
- Wilson W III, Pardo-Manuel de Villena F, Lyn-Cook BD, Chatterjee PK, Bell TA, Detwiler DA, et al. Characterization of a common deletion polymorphism of the UGT2B17 gene linked to UGT2B15. *Genomics* 2004; **84**:707–714.
- Turgeon D, Carrier JS, Levesque E, Hum DW, Belanger A. Relative enzymatic activity, protein stability, and tissue distribution of human steroid-metabolizing UGT2B subfamily members. *Endocrinology* 2001; **142**:778–787.
- Bowalgaha K, Elliot DJ, Mackenzie PI, Knights KM, Miners JO. The glucuronidation of Delta4-3-Keto C19- and C21-hydroxysteroids by human liver microsomal and recombinant UDP-glucuronosyltransferases (UGTs): 6alpha- and 21-hydroxyprogesterone are selective substrates for UGT2B7. *Drug Metab Dispos* 2007; **35**:363–370.
- Levesque E, Beaulieu M, Green MD, Tephly TR, Belanger A, Hum DW. Isolation and characterization of UGT2B15(Y85): a UDP-glucuronosyltransferase encoded by a polymorphic gene. *Pharmacogenetics* 1997; **7**:317–325.
- Swanson C, Mellstrom D, Lorentzon M, Vandenput L, Jakobsson J, Rane A, et al. The UDP Glucuronosyltransferase 2B15 D85Y and 2B17 Deletion Polymorphisms Predict the Glucuronidation Pattern of Androgens and Fat Mass in Men. *J Clin Endocrinol Metab* 2007; **92**:4878–4882.
- Coffman BL, King CD, Rios GR, Tephly TR. The glucuronidation of opioids, other xenobiotics, and androgens by human UGT2B7Y(268) and UGT2B7H(268). *Drug Metab Dispos* 1998; **26**:73–77.
- Belanger A, Pelletier G, Labrie F, Barbier O, Chouinard S. Inactivation of androgens by UDP-glucuronosyltransferase enzymes in humans. *Trends Endocrinol Metab* 2003; **14**:473–479.

- 20 Bhasker CR, McKinnon W, Stone A, Lo AC, Kubota T, Ishizaki T, *et al.* Genetic polymorphism of UDP-glucuronosyltransferase 2B7 (UGT2B7) at amino acid 268: ethnic diversity of alleles and potential clinical significance. *Pharmacogenetics* 2000; **10**:679–685.
- 21 Swanson C, Lorentzon M, Vandenput L, Labrie F, Rane A, Jakobsson J, *et al.* Sex steroid levels and cortical bone size in young men is associated with a glucuronidation enzyme UGT2B7 polymorphism (H268Y). *J Clin Endocrinol Metab* 2007; **92**:3697–3704
- 22 Schaeffeler E, Schwab M, Eichelbaum M, Zanger UM. CYP2D6 genotyping strategy based on gene copy number determination by TaqMan real-time PCR. *Hum Mutat* 2003; **22**:476–485.
- 23 Livak KJ. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet Anal* 1999; **14**:143–149.
- 24 Wadelius M, Andersson AO, Johansson JE, Wadelius C, Rane E. Prostate cancer associated with CYP17 genotype. *Pharmacogenetics* 1999; **9**:635–639.
- 25 Park J, Chen L, Shade K, Lazarus P, Seigne J, Patterson S, *et al.* Asp85tyr polymorphism in the udp-glucuronosyltransferase (UGT) 2B15 gene and the risk of prostate cancer. *J Urol* 2004; **171** (6 Pt 1): 2484–2488.
- 26 Chung BC, Choo HY, Kim TW, Eom KD, Kwon OS, Suh J, *et al.* Analysis of anabolic steroids using GC/MS with selected ion monitoring. *J Anal Toxicol* 1990; **14**:91–95.
- 27 Chouinard S, Pelletier G, Belanger A, Barbier O. Isoform-specific regulation of uridine diphosphate-glucuronosyltransferase 2B enzymes in the human prostate: differential consequences for androgen and bioactive lipid inactivation. *Endocrinology* 2006; **147**:5431–5442.
- 28 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**:402–408.
- 29 Dehennin L, Matsumoto AM. Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. *J Steroid Biochem Mol Biol* 1993; **44**:179–189.
- 30 Kicman AT, Brooks RV, Collyer SC, Cowan DA, Nanjee MN, Southan GJ, *et al.* Criteria to indicate testosterone administration. *Br J Sports Med* 1990; **24**:253–264.
- 31 Palonek E, Gottlieb C, Garle M, Bjorkhem I, Carlstrom K. Serum and urinary markers of exogenous testosterone administration. *J Steroid Biochem Mol Biol* 1995; **55**:121–127.
- 32 Borts DJ, Bowers LD. Direct measurement of urinary testosterone and epitestosterone conjugates using high-performance liquid chromatography/tandem mass spectrometry. *J Mass Spectrom* 2000; **35**:50–61.
- 33 Heracek J, Richard H, Martin H, Luboslav S, Jana S, Jitka K, *et al.* Tissue and serum levels of principal androgens in benign prostatic hyperplasia and prostate cancer. *Steroids* 2007; **72**:375–380.
- 34 Starka L, Bicikova M, Hampl R. Epitestosterone—an endogenous antiandrogen? *J Steroid Biochem* 1989; **33**:1019–1021.
- 35 Choi MH, Yoo YS, Chung BC. Biochemical roles of testosterone and epitestosterone to 5 alpha-reductase as indicators of male-pattern baldness. *J Invest Dermatol* 2001; **116**:57–61.
- 36 Karypidis AH, Olsson M, Andersson SO, Rane A, Ekstrom L. Deletion polymorphism of the UGT2B17 gene is associated with increased risk for prostate cancer and correlated to gene expression in the prostate. *Pharmacogenomics J* 2007 advance online publication, 27 March 2007; doi:10.1038/sj.tpj.6500449.
- 37 Park J, Chen L, Ratnashinge L, Sellers TA, Tanner JP, Lee JH, *et al.* Deletion polymorphism of UDP-glucuronosyltransferase 2B17 and risk of prostate cancer in African American and Caucasian men. *Cancer Epidemiol Biomarkers Prev* 2006; **15**:1473–1478.
- 38 Olsson M, Lindström S, Häggkvist B, Adami H-O, Bälter K, Stattin P, *et al.* The UGT2B17 gene deletion is not associated with prostate cancer risk. *Prostate* 2008; **68**:571–575
- 39 Court MH, Duan SX, Guillemette C, Journault K, Krishnaswamy S, Von Moltke LL, *et al.* Stereoselective conjugation of oxazepam by human UDP-glucuronosyltransferases (UGTs): S-oxazepam is glucuronidated by UGT2B15, while R-oxazepam is glucuronidated by UGT2B7 and UGT1A9. *Drug Metab Dispos* 2002; **30**:1257–1265.