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Doping Test Results Dependent on Genotype of UGT2B17, the Major Enzyme for Testosterone Glucuronidation

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Abstract

Context: Testosterone abuse is conventionally assessed by the urinary testosterone/ epitestosterone (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 *UGT2B17* gene challenge the accuracy of the T/E ratio test.

Objective: To investigate whether genotype based cut-off values will improve the sensitivity and specificity of the test.

Design: Open 3-armed comparative study.

Participants: 55 healthy male volunteers with either two, one or no allele (*ins/ins*, *ins/del* or *del/del*) of the *UGT2B17* gene.

Intervention: A single intramuscular dose of 500 mg testosterone enanthate.

Main outcome measures: Urinary excretion of TG after dose and the T/E ratio during 15 days.

Results: The degree and rate of increase in TG excretion rate was highly dependent on the *UGT2B17* genotype with a 20-fold higher average maximum increase in the *ins/ins* group compared to the *del/del* group. Forty percent of the *del/del* subjects never reached the T/E ratio of 4.0 on any of the 15 days after the dose. When differentiated cut-off 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.

Conclusion: 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 combatting androgen doping in sports, but also for detecting and preventing androgen abuse in the society.

Introduction

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 fiber and numbers of nuclei per fiber compared to power lifters without any exposure to anabolic steroid (3). It is possible that previous use of anabolic steroids may improve physical performance for many years after withdrawal (3).
The World Anti-Doping Agency (WADA) standardizes the rules and regulations governing anti-doping 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 stanozolol were predominant (http://www.wada-ama.org/).

Testosterone is excreted mainly as glucuronide conjugates after metabolism by uridine diphospho (UDP)-glucuronosyl transferases (UGT). 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 (5). 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) while 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) in order 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 (IOC) 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 hence have lower T/E ratios, thus increasing the risk of false-negative doping test results (12, 13). As a corollary the cut-off 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 below 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 by use of genotype based cut-off values. For this purpose 14-24 healthy volunteers each with two (*ins/ins*), one (*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 epitestosterone glucuronides and other androgens, was monitored and compared. Our findings suggest that urine analyses with combined genetic tests of the *UGT2B17* gene to considerably improve the sensitivity and specificity of the T/E test.

Materials and Methods

Subjects and design

Study subjects included healthy male volunteers aged 18-50 years. A total number of 145 were genotyped for the UGT2B17 deletion polymorphism to fill the pre-determined 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*). As 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, excretion of steroids, were allowed.Further inclusion-criteria included a negative screening for illegal drugs, anabolic androgenic steroids (AAS), 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 five years 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 prior to 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 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 07-11 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.

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 hours 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 ng genomic DNA was used in each reaction together with 2xTaqMan Universal Master Mix (Applied Biosystems) and *UGT2B17* exon 6 specific primers (14) and an exon 6 specific probe (VIC-CAGTCTTCTGGATTGAGTTT-MGB). Expression of albumin was quantified as 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 minutes followed by 40 cycles of 92° C for 15 sec and 60° C for 1 minute. 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 was calculated using the ddCT method (18).

Urinary steroids

Urinary unconjugated steroids and steroid glucuronides were analysed at the Doping Laboratory of the department. Aliquots of 2 to 8 ml (depending upon the specific gravity of the urine sample) were complemented with 1 µg methyltestosterone as internal standard. The unconjugated steroids were extracted directly

with 5 ml tert-buthyl methyl ether. The glucuronidated steroids were hydrolysed with β-glucuronidase from E. 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-trimethylsilyltrifluoroacetamide (Macherey-Nagel, Düren, Germany) and ammonium iodide as described previously (19).
Urinary steroids were determined using combined gas chromatography-mass spectrometry GC/MS. The analysis was performed with an Agilent GC-MS 5973 instrument with the Single Ion Monitoring mode (19). Analytes were identified, peaks were integrated and calculated using one point calibration with a mixture of authentic standard materials analysed 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 analysed with every batch of samples. The within and between assay coefficient of variation for all steroids analysed 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 creatinine, if not specified otherwise.
The areas under the curves (AUC) 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 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 (Ct_{target}-Ct_{ref}) was < 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 relative quantification (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 analysed 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 difference 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/del* and *ins/ins* 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.
There were no significant differences between

the genotypes in the urinary concentrations of glucuronides of androsterone, 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 testosterone glucuronide on days 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 (95 % CI, 1.4-2.6) ng/μmol cr in the *del/del* group, 18.6 (95 % CI, 12.9-24.4) ng/μmol cr in the *ins/del* group, and 41.8 (95 % CI, 27.9-55.6) ng/μmol cr in the *ins/ins* group (fig 1b).
The testosterone glucuronide area under the curve (AUC) was 16.4 (95 % CI, 13.4-19.4) ng/μmol cr × day in the *del/del* group, 162 (95 % CI, 129-195) ng/μmol cr × day in the *ins/del* group and 294 (95 % CI, 236-351) ng/μmol cr × 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 (95 % CI, 145-199) ng/μmol cr × day) than the *ins/ins* group (249 (95 % CI, 204-293) ng/μmol cr × day) ($p < 0.05$).

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 1 for the *del/del* group and 6 for the *ins/del* and *ins/ins* groups the sensitivity increased substantially for the *del/del* group and number of false positive doping tests were 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 testosterone glucuronide excretion after a single intramuscular 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 cut-off levels.
There were large differences in testosterone glucuronide (TG) excretion after testosterone administration between all three *UGT2B17* genotypes. Here, we show that 40 % of the subjects without the *UGT2B17* gene never reached the T/E cut off ratio of 4.0 on any of the 15 days after a single intramuscular dose of 360 mg testosterone. The highest average ratio for the *del/del* group (5.3) was reached on day 9. The other two groups reached their highest average ratio earlier, on day 7 (50.4) and on day 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 testosterone glucuronide than other populations (13, 20). Recently we showed that a large part of the differences in testosterone glucuronide 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 %) (15) than in Caucasians (9.3 %) (15).

The average baseline urinary TG level in the

del/del group was 0.32 (95 % CI, 0.2-0.4) ng/µmol cr. The average baseline urinary TG levels in the *ins/del* and *ins/ins* group were 8 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 analysed urine samples from 100 *del/del* individuals in this and two other studies (15, 16). All of them had TG levels below 1 ng/µmol cr. The low basal TG levels indicate that there are other UGT enzymes that catalyse 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 cut-off level for all individuals independent of UGT2B17 genotype.

Since UGT2B15 may contribute to the low levels of urinary testosterone glucuronides 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 polymorphism in the *UGT2B15* gene, resulting in an aspartate to tyrosine amino acid change at position 85 (21). However, this SNP does not seem to change the basal T/E ratios (22) or affect the T/E ratio in individuals devoid of the UGT2B17 enzyme after an exogenous testosterone dose (unpublished results). Another polymorphisms that may affect the T/E ratio is the $H^{268}Y$ polymorphism (23) in the epitestosterone conjugating UGT2B7 enzyme (7). However, we have recently shown that this polymorphism does not affect the basal T/E ratio (24). Whether polymorphisms in other genes affect the T/E ratio in addition to the UGT2B17 deletion remains to be studied.

Exogenous testosterone is known to decrease the urinary excretion rate of epitestosterone glucuronide due to suppression of the secretion of luteinizing hormone (25-27). In the present study the epitestosterone glucuronide levels decreased in all three groups after the testosterone injection. There were large inter-individual differences, but six days after the testosterone administration 92 % of all subjects had EG levels below 30 % of baseline leading to even higher increases of T/E ratios.

Today a T/E ratio cut-off limit of 4 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 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 cut-off 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 sulphate before elimination. The urinary fraction of sulphate conjugated testosterone was found to be 4 % of the glucuronidated testosterone in a reference population of 45 males aged 17-50 years. (28) Our study was not designed to investigate other excretion pathways, but it cannot be ruled out that *del/del* subjects eliminate a larger fraction of sulphate-conjugated testosterone to compensate for their compromised capacity to glucuronidate testosterone.

The determination of the $^{13}C/^{12}C$ ratio of selected steroids (IRMS analysis) provides the possibility to distinguish between pharmaceutical and natural testosterone because exogenous compounds contain less ^{13}C than their endogenous homologues (29). However, the analytical facilities and costs required preclude any routine use of this methodology for

screening in the anti-doping testing. Therefore its major use is to confirm suspected doping in samples with T/E ratios equal or greater than 4.0.

From figure 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 hours, compared to 2-4 days for the other individuals. The peak was significantly lower in the “slow rise” group as compared to 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, since 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 hydrolyse 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 since 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 is different in individuals with different genotypes. We have recently shown that the baseline serum testosterone levels are not associated with the *UGT2B17* polymorphism (22). However, the serum levels of testosterone in the different *UGT2B17* genotypes after exogenous testosterone administration remains 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 for doping tests.

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Figure legends

Fig 1a)

Urinary testosterone glucuronide excretion (ng/µmol cr) for 15 days in all subjects of the *UGT2B17 ins/ins*, *ins/del* and *del/del* group after an intramuscular injection of 500 mg testosterone enanthate, equivalent to 360 mg testosterone, on day 0. Note that the y-axes have different scales.

Fig 1b)

Average urinary testosterone glucuronide excretion (ng/µmol cr) for 15 days in the different genotype groups after an intramuscular injection of 500 mg testosterone enanthate, equivalent to 360 mg testosterone, on day 0. Vertical bars denote 95 % confidence intervals.

Fig 1c)

Average urinary epitestosterone glucuronide excretion (ng/µmol cr) for 15 days in the different genotype groups after an intramuscular injection of 500 mg testosterone enanthate, equivalent to 360 mg testosterone, on day 0. Vertical bars denote 95 % confidence intervals.

Fig 2)

Average urinary testosterone/epitestosterone ratios for 15 days in the different genotype groups after an intramuscular injection of 500 mg testosterone enanthate, equivalent to 360 mg testosterone, on day 0. Vertical bars denote 95 % confidence intervals.

Fig 3)

Sensitivity of the testosterone doping test using a cut-off ratio of 4 (left panel) or cut-off ratios of 6 for the *ins/ins* and the *ins/del* group and 1 for the *del/del* group (right panel). A single intramuscular dose of 500 mg testosterone enanthate was administered in 14 *ins/ins*, 24 *ins/del* and 17 *del/del* subjects on day 0 and the urinary testosterone/epitestosterone ratios were measured for 15 days.

Table 1)

Study population characteristics at screening

UGT2B17 Genotype	Age (yrs)	Height (cm)	Weight (kg)	BMI (kg/m^2)
del/del (n=17)	27.2 ± 4.0	179 ± 7.5	75.9 ± 8.4	23.8 ± 2.6
ins/del (n=24)	32.0 ± 7.7	180 ± 6.6	79.6 ± 9.1	24.6 ± 2.9
ins/ins (n=14)	28.7 ± 7.0	181 ± 5.4	78.6 ± 6.0	24.0 ± 2.1

Table 2)

Baseline urinary androgen glucuronide levels.

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

The values are given as the mean with the 95 % confidence interval within parentheses, or as the ratio between testosterone glucuronide and epitestosterone glucuronide levels. The asterisks denote statistical significances between the genotypes, * $p < 0.001$

Table 3)

Sensitivity (%) of the test with a cut-off T/E ratio of 4.0 for all subjects, or a cut-off T/E ratio of 1.0 for *del/del* and 6.0 for *ins/del* and *ins/ins* subjects.

	del/del Subjects (%)			*ins/del* Subjects (%)			*ins/ins* Subjects (%)		
Cut-off T/E ratio	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
1.0 / 6.0	52.9	100	100	54.2	100	100	92.9	100	100

Fig 1a)

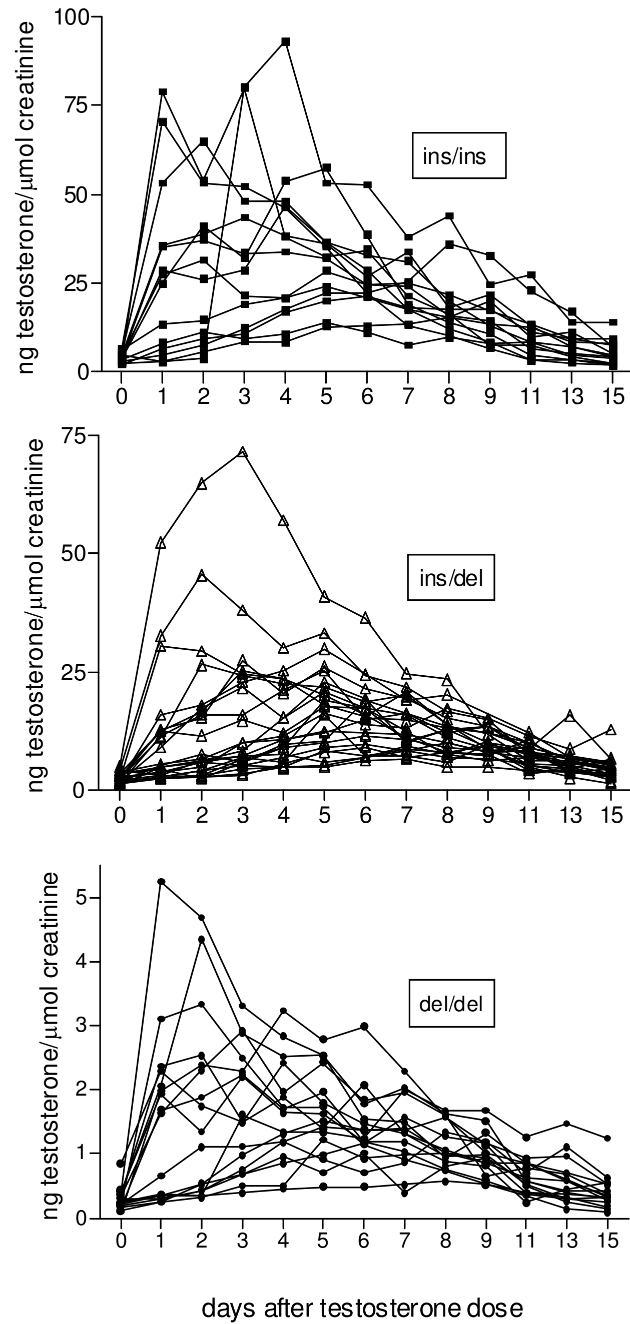

Fig 1b)

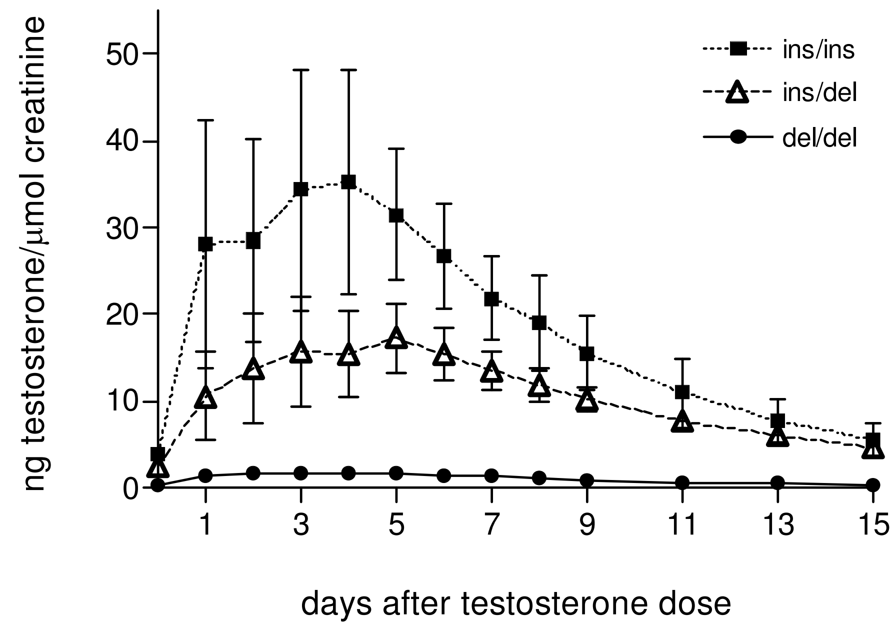

Fig 1c)

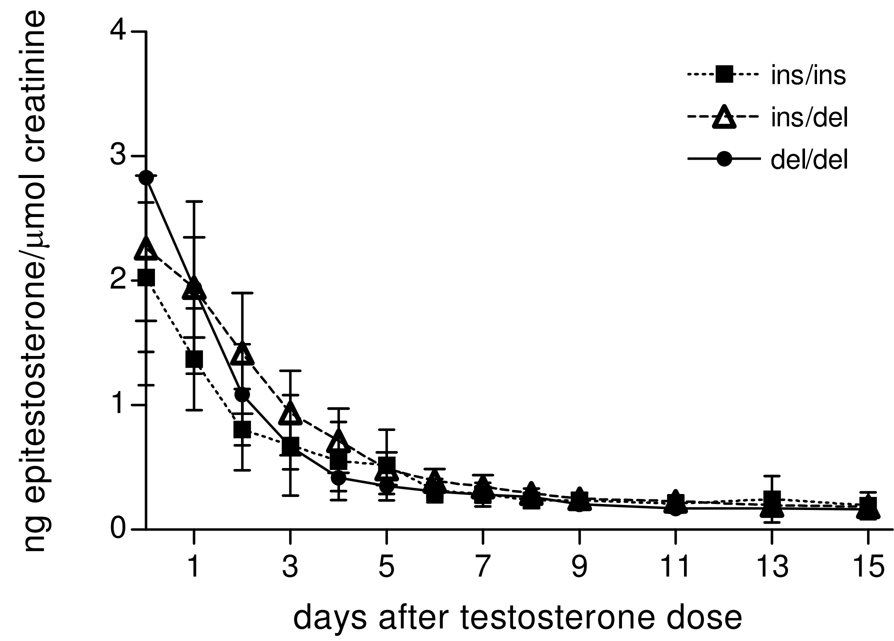

Fig 2)

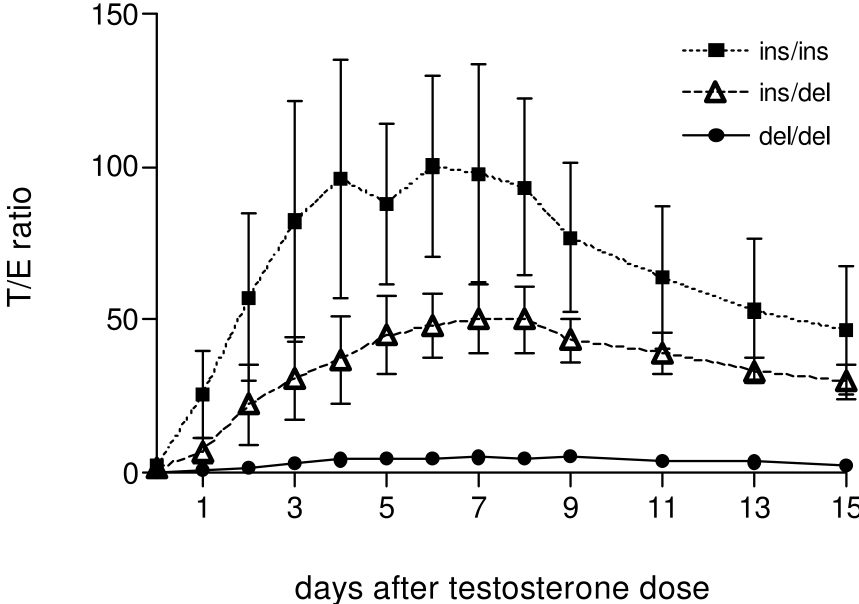

Fig 3)