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Impact of the UGT2B17 polymorphism on the steroid profile. Results of a crossover clinical trial in athletes submitted to testosterone administration

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ABSTRACT

This article studies the genetic influence of polymorphism of the UGT2B17 gen on the urinary steroid profile and its implications for the anti-doping field.

The study presents the results of a triple-blind randomized placebo-controlled crossover trial with healthy athletes submitted to a single dose of 250 mg of testosterone cypionate. Forty urine samples were collected from each participant. Mass spectrometry-based techniques commonly used in Anti-Doping laboratories, were employed to measure the urinary concentration and the $\Delta\delta^{13}C$ values of a selection of target compounds for testosterone (T) administration together with LH. Twelve volunteers were included in the study; the polymorphism was evenly distributed among them.

After T administration, the most meaningful change affected the Testosterone/Epitestosterone ratio (T/E) and the urinary concentration of LH. In relation with T/E, the wild type homozygous (*ins/ins*) group there was a mean relative increase of 30 (CI 95%: 25.2 to 36.7); in the heterozygous mutant (*del/ins*) group it was 19.8 (CI 95%:15.9 to 24.7); and in the homozygous mutant (*del/del*) group it was 19.7 (CI 95% 14.9 to 26.2). In the case of LH, ifs observed how LH values decrease significantly after the administration of Testex homogeneously among the three groups. The main outcome was related to the (*del/del*) group (homozygous mutant), where due to the depressed basal level of the steroid profile, if the longitudinal steroid profile of the athlete was not available, the analysis by GC/MS would not produce an "atypical" result according to the WADA TD2016EAAS despite the T administration. However, the genotyping of the UGT2B17 polymorphism, the follow up of LH and the use of GC-C-IRMS makes it possible to identify most of these samples as Adverse.

1. Introduction

Doping, one of the main threats facing sport, disrupts fair play and constitutes a serious risk for athletes' health [1] The most common banned substance detected in anti- doping laboratories are anabolic steroids [2]. Testosterone is biologically the most important anabolic androgenic hormone, and probably represents the molecule with the highest potential for abuse (Parr MK, 2009) [3].

In recent years, it has emerged that there is a population of individuals who have a genetic deletion of the UGT2B17 gene that alters the glucuronidation of T and some other related steroids. Consequently, T urinary excretion is significantly reduced compared to the rest of the population [4,5] and makes T misuse detection more difficult. Jakobsson et al. [6] in a previous study showed that the polymorphic gene deletion of UGT2B17 has a prevalence of 9% in Caucasians and 67% in Asiatic populations. Although other factors directly affect the steroid profile, i.e. age, gender, circadian rhythm or diet, the genetic modification of the UGT2B17 gene is by far the most influential one [7–10]. Fig. 1 shows a summary of the main metabolic pathways for a selection of endogenous steroids related to T and the genetic influence of it.

The longitudinal follow up of the individual athlete's steroid profile increases the sensitivity for detecting alterations that among other factors can be produced by T use [11,12]. So, when a case is not consistent with its baseline profile a further confirmation procedure must

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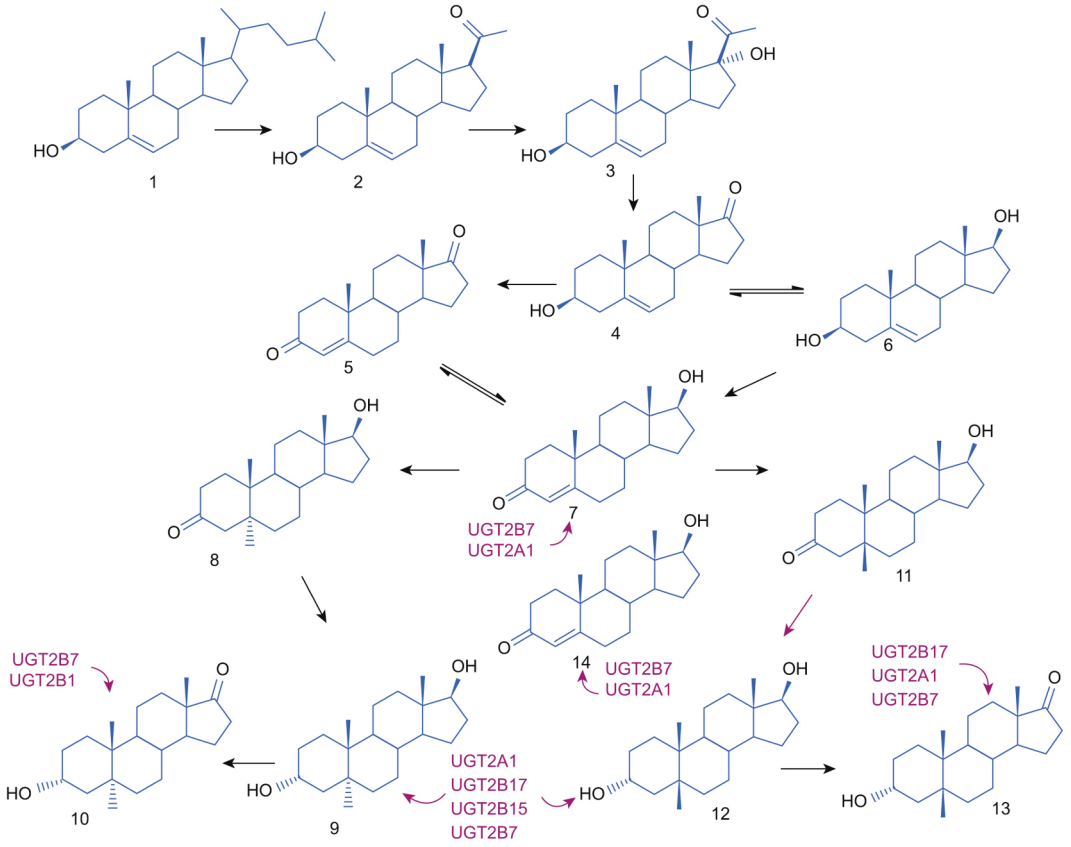

Fig. 1. Biosynthesis of endogenous anabolic steroids and genetic incidence: cholesterol [1], pregnenolone [2], 17α-hydroxypregnenolone [3],DHEA [4], 4-androstendione [5], 5-androsten-3β,17β-diol [6], testosterone [7], DHT [8], 5α-androst-3α,17β-diol [9], androsterone [10], 5β-DHT [11], 5β-androst-3α,17β-diol [12], etiocholanolone [13], epitestosterone [14].

be triggered that usually includes a GC-C-IRMS analysis [13,14] based on measuring the percentage of carbon-13 against carbon-12, Δδ13C values, in the testosterone molecule and/or its metabolites. Such percentages are significantly different between steroids generated by the human body and those produced industrially [15–19]. When the result of the GC-C-IRMS is not conclusive and there is no longitudinal steroid profile, the athlete needs to be targeted to collect more data.

The aim of this study is to explore other options on the impact in the steroid profile and the UGT2B17 gene polymorphic deletion after T single dose administration. The use of GC-C-IRMS-based methodologies with the relation between Lutenizating hormone (LH) levels and the T / LH ratio, perhaps can help to unmask results that are not clearly adverse in athletes with a mutation in the UGT2B17 gene.

2. Materials and methods

2.1. Experimental design

The study lasted 13 months, and consisted of a triple-blind randomized placebo-controlled crossover study with 12 volunteers. The athletes who agreed to take part were informed in detail about the protocol to be followed, submitted to a clinical evaluation and if they were suitable for the study, requested to sign the informed consent form.

The study was approved by the Ethics Committee of Hospital Clinico San Carlos (Madrid), Spanish Agency for Medicine and Health Products (AEMPS) and conducted according to the Helsinki Declaration. This trial was registered in the European Clinical Trials Database (EudraCT) with the number 2013-005135-24. During the study, an external monitoring company was engaged to identify any possible side effects.

The patients were selected from those included in a previous study [8] that met the inclusion criteria and were invited to participate in the present study. 146 athletes were contacted by telephone; 78 declined to participate, 40 did not answer and 16 were discarded. In Fig. 2, we present the flow diagram for the participants the average age of the all-male volunteers was 39.9 (SD 11.2) years old, and the polymorphism was homogeneously distributed with four in each of the genotypes. Table 1 shows the characterization of the group of Volunteers. All selected athletes underwent a sports-medical study. This study included a questionnaire designed to determine sociodemographic, sports characteristics, blood test and anthropometric measurements.

Each subject was administered a single dose of Testex Elmu Prolongatum, intramuscularly from a 2 ml ampoule containing 250 mg of testosterone cypionate (experimental group) and after or before a Baxter (Clear-Flex) saline solution (9 mg/mg) in a single uniform dose of 2 ml (control group). Participants were randomly assigned (1:1) to either Testex Elmu Prolongatum followed by Baxter (Clear-Flex) saline solution or the reverse.

Table 2 shows the schedule of drug/placebo administration, the type of matrix collected and its timing. At the end of the study 467 urine samples had been collected, 13 were lost.

An initial blood sample was collected from each volunteer for a clinical evaluation prior to being accepted. The urine samples were always collected the minute the volunteer woke up. Forty urine samples were collected from each participant over 7 months.

During the study period, apart from the initial one, two additional blood tests were carried out for safety purposes, at 25 days after each administration, to observe alterations and changes suggestive of intolerance to the injected medication or risk for the health, through the values of the sex hormone-binding globulin (SHBG). Finally, the last blood test was performed three months after the last administration.

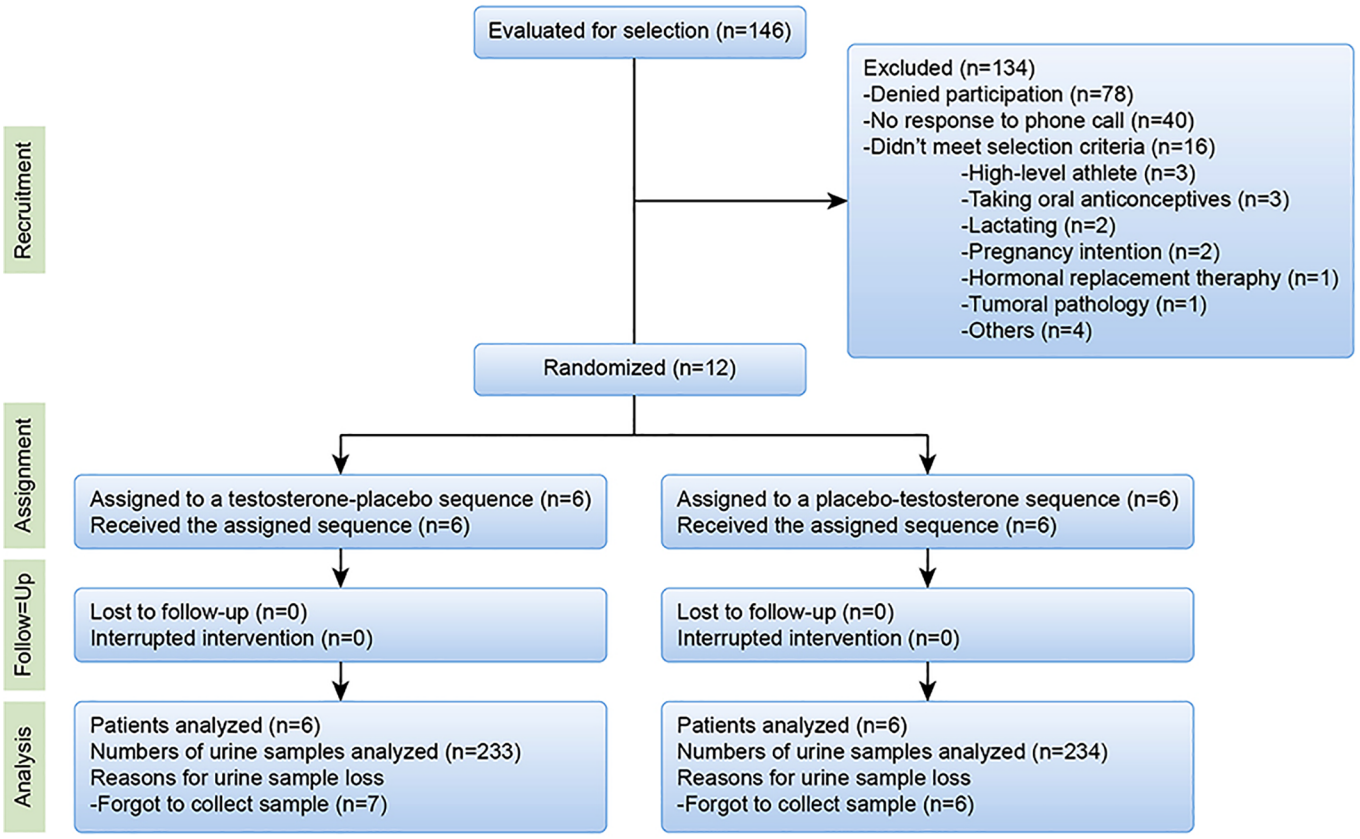

Fig. 2. Participant Flow Diagram.

Table 1
Demographic, morphological and genetic data (means and SD) of athletes who participated in the study.

	Total (n = 12) n(%)
Age*	39,9 (11,2)
Race	
Caucasian	11 (91,7)
Eastern	1 (8,3)
Genotypes	
Homozygous wt (ins/ins)	4 (33,3)
Heterozygous (del/ins)	4 (33,3)
Homozygous mutant (del/del)	4 (33,3)
Type of sport	
Aerobic	7 (58,3)
Anaerobic	2 (16,7)
Mixed	3 (25,0)
Body mass index (BMI)	25.2 (2.7)
Body composition	
Fat (%)*	15.6 (4.2)
Muscle (%)*	44.1 (3.7)
Bone (%)*	16.1 (1.3)

* Means and standard deviation.

2.2. Steroid profile characterization

The steroid profile was characterized through measurement of the urinary concentration and the $\delta^{13}C$ values for a selection of target compound metabolites of T, expanding the analytical study with the determination of LH in urine.

For the quantitative analysis, 2 ml urine were processed following the laboratory validated procedure, [20]. The target compound concentration was calculated interpolating the relative signal of each steroid with regard to its deuterated internal standard in the multi-level calibration curve; calculated concentrations were adjusted to a specific gravity (S.G.) of 1.020 g/ml.

For the measurement of $\delta^{13}C$ values, urine aliquots of 16 to 32 ml, depending on the target compound concentration, were submitted to a standard well established procedure at the laboratory [21]. The calculated 13C to 12C isotope ratio (13C/12C) was expressed as δ13C values against an international standard (Vienna Pee Dee Belemnite) [22].

The urinary LH from all urine samples was measured by IMMULITE®, an automated immunoassay system (Siemens Healthcare Diagnostics Products Ltd., Los Angeles, USA). The IMMULITE® system is a solid-phase, two-site chemiluminescent immunometric assay. The solid phase consists of a polystyrene bead that is coated with a monoclonal antibody directed against LH, which is sealed into a test unit. The urine sample and the polyclonal antibody conjugated to alkaline phosphatase are added, and after an incubation of 30 min, LH binds to the monoclonal antibody that coats the bead and to the polyclonal antibody conjugated to the enzyme in the form of a sandwich complex. The bead is washed, removing the unbound antibody. LH was measured by the addition of a chemiluminescent substrate (ester of adamantyl dioxetane phosphate) through the luminescence produced.

The quality of the analysis was controlled by means of a blank sample and a multivalent control, Lyphochek Inmmunoassay Plus Control by BioRad. This product is prepared from human serum with LH serum concentration levels (low, medium and high) [23].

2.3. Genotyping

Genomic DNA was extracted from each subject using the QIAamp® DNA Blood Mini kit (Qiagen), quantified by spectrophotometry and diluted to the concentration necessary for genotyping the UGT2B17 deletion polymorphism. Genotyping was performed by polymerase chain reaction analysis as described by Schulze et al. [24].

Table 2
Schedule of sampling and drug administration to athletes. OR codes urine samples, S codes blood samples and ADM indicates when administration took place (T or placebo).

Day	Urine sample		Blood sample	Administration	Day	Urine sample		Blood sample	Administration
1	1	OR-B-01	S1		76	20	OR-C2-05		ADM 2
11	2	OR-B-02			77	21	OR-C3-01		
21	3	OR-B-03			78	22	OR-C3-02		
31	4	OR-B-04			79	23	OR-C3-03		
41	5	OR-B-05		ADM 1	80	24	OR-C3-04		
42	6	OR-C1-01			81	25	OR-C3-05		
43	7	OR-C1-02			82	26	OR-C3-06		
44	8	OR-C1-03			83	27	OR-C3-07		
45	9	OR-C1-04			84	28	OR-C3-08		
46	10	OR-C1-05			85	29	OR-C3-09		
47	11	OR-C1-06			86	30	OR-C3-10		
48	12	OR-C1-07			91	31	OR-C4-01		
49	13	OR-C1-08			96	32	OR-C4-02		
50	14	OR-C1-09			101	33	OR-C4-03	S3	
51	15	OR-C1-10			106	34	OR-C4-04		
56	16	OR-C2-01			111	35	OR-C4-05		
61	17	OR-C2-02			121	36	OR-D-01		
66	18	OR-C2-03	S2		131	37	OR-D-02		
71	19	OR-C2-04			141	38	OR-D-03		
					161	39	OR-D-04		
					171	40	OR-D-05	S4	

2.4. Study variables

Dependent variables considered in the study:

- GC-MS analysis, urinary concentration of: androsterone (A), etiocholanolone (Etio), 5α-androstane-3α, 17β-diol (5αAdiol), 5β-androstane-3α,17β-diol (5βAdiol), testosterone (T), epitestosterone (E) and some ratios among them, especially; T/E, A/T, 5αAdiol/E, A/E and LH.
- GC-C-IRMS analysis, The determination of the $\delta^{13}C$ value of the Target Compounds (TC); androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol), 5β-androstane-3α,17β-diol (5βAdiol), testosterone (T), epitestosterone (E); together with the determination of the δ13C value of the Endogenous Reference Compound (ERC), in this case pregnanediol (PD). The calculation of the difference in δ13C values, Δδ13C value, between the ERC and the TC were also used for evaluation.

The independent variable of the study was group study and polymorphism as well to the body composition data (% fat, muscle and bone) of the subjects under study.

2.5. Statistical analysis

The sample size calculation was performed in order to compare the two-paired means for the polymorphism group, in which an unfavorable increase was expected after administration (deletion group for both alleles of the UGT2B17 gene). The sample size was calculated for an expected increase in the deletion group for both alleles of the UGT2B17 gene of 15% of the area under the curve of the testosterone to epitestosterone ratio, linearly and inversely increasing from the time of administration. It was also calculated for an unfavorable correlation coefficient (ρ = 0.40), a power of 85% and an estimated loss of 10%. The expected standard deviation from the time the study began was calculated using the median of the intra-individual standard deviation of the ratio of testosterone to epitestosterone from the previous study [8] (SD = 0.060) and an estimated post administration standard deviation (SD = 0.2). The resulting sample size was 15 for the deletion group for deletion in both alleles of the UGT2B17 gene and 15 without deletion in both alleles.

Qualitative variables are provided with their frequency distributions. Quantitative variables are expressed as their mean and standard deviation (SD) and variables not showing a normal distribution as medians and interquartile ranges (IQR = P25–P75).

For the analysis of urine sample collection, c1 and c2 periods were exchanged with c3 and c4 in volunteers whose randomization sequence began with Testex Elmu prolongatum to compare steroid profile modification after T or placebo administration. Samples corresponding to period d were not evaluated, as there was no effect of T administration on these samples. See Table 2.

The effect of administration related with genotype, randomization sequence and time delay after administration was analyzed with multiple linear regression models through generalized estimating equations (GEE). The results obtained from the same individuals were grouped into clusters, the responses of which represent an intra-group correlation, but are independent of the different groups. As the dependent variables (ratio T/E, A/T, 5αAdiol/E and A/E ratios) were not normally distributed, the data were log-transformed. To present the results the model coefficients are displayed in original scale by inverse transformation and interpreted as the ratio of means estimator between the values post-pre administration. The same statistical method was used to compare the change in LH levels and the T/LH ratio between the samples obtained in period b and the determinations after Testex.

All statistical tests were performed using STATA 12.0 software. Significance was set at $P < 0.05$.

3. Results and discussion

3.1. Sport-medical study

All of the athletes before this study carry out a sport medical questionnaire designed to determine sociodemographics, sports characteristics, previous illness, nutrition on taking medication. In this medical intervention performed a blood test and anthropometric measurements before informed consent. The results is present in Table 1.

3.2. Quantification of the steroid profile

Table 3 organized by genotype group, shows the PRE and POST administration values of the median, the ranges of variation (range), the inter quartile range (IQR) and where there is an “atypical” identification criterion, the value and the number of cases where the criterion is surpassed, for a selection of Target Compound ratios. During the pre-administration period, the median of the ratios remained stable within each polymorphism group. The results are in line with several studies that demonstrated only small intra individual variations of steroid profile parameters, especially within the ratios utilized for doping control purposes such as T/E, A/Etio, A/T, and 5αAdiol/5βAdiol, Mareck et al. [7].

It is notable that during these periods the median values of T/E were slightly higher in the (*ins/ins*) group (period b: 1.21 IQR: 1.16–1.85) compared to the (*del/ins)* group (b period: 0.85 IQR: 0.62–0.92) and much higher than the (*del/del*) group (b period: 0.09 IQR: 0.07–0.12). These results are similar to the data reported by Okano et al [25]. Consequently, the T/E ratio in the del/del (median: 0.16) group was significantly lower than that in the combined del/ins (median: 1.1) and ins/ins (median: 3.3) group. Jakobsson et al. [6] reached equivalent results to Okanós group [25], concluding that the urinary testosterone/epitestosterone (T/E) doping test is profoundly influenced by the UGT2B17 deletion polymorphism.

When the T/E ratio was evaluated against the threshold of T/E > 4, it was observed that in period c3 (urine collected within 10 days following T administration) for the (*ins/ins*) group 100% (40) of the urine samples had levels of T/E higher than 4; in the (*del/ins*) group, the proportion was 93% (37) of the samples; while in the (*del/del)* group, only 21% (8) of the samples exceeded that level. In period c4 (urine collected 10 days from administration at 5 day intervals) it was found that 24% (4) of the (*ins/ins)* group, 12% (2) of the (*del/ins)* and 0% (0) of the (*del/del*) group, showed levels of T/E above 4. Similar results were obtained by Schulze et al. [24] and Okano et al. [25] where all individuals (ins/ins) showed a ratio T/E > 4 after T administration whereas individuals (del/del) showed a ratio T/E > 4 only for some or no individuals, respectively.

As a consequence of such effects it is clear that in cases where there is no available longitudinal T/E data, there is a strong possibility that in athletes with a (*del/del)* genotype no further analysis would be activated when T is administered [26–28].

The logarithmic behavior of the T metabolites ratio was studied using a GEE model adjusted for the administration group (Testex/placebo), randomization sequence (Testex-Placebo/Placebo-Testex), measurement every day for 10 days after administration, c1 and c3 periods, and measurements every 5 days, 5 times after placebo or Testex administration, c2 and c4 periods, genotype (*ins/ins*), (*del/ins*) and (ins/ins)) and the following interactions: Group*days and group*genotype. These interactions were associated with statistically significant $p < 0.001$ and $p = 0.034$ (respectively) with the dependent variable (ln T/E), showing that the effect of the Testex administration is not constant as a function of days and genotype. The relationship of the randomization sequence was not statistically significant ($p = 0.682$) showing no periodic effect.

Given the interactions of the administration group with the genotype and the days after administration, GEE models were adjusted for variables, administration group, randomization sequence, and stratified by genotype. Samples taken within 10 days after administration of Testex (c3 samples) and placebo (c1 samples) were considered to adjust these models. Tables 3 and 4, shows the ratio of the means for variable T administration pre and post groups estimated by the models shown. In the (ins/ins) group, the means quotient between the samples taken within 10 days after T administration and within 10 days after placebo administration was 30.4 (95% CI: 25.2–36.7) while in (*del/ins)* it was 19.8 (95% CI: 15.9–24.7) and for the (*del/del)* it was 19.7 (95% CI 14.9–26.2). Since the estimated average ratio of (*ins/ins)* was not within 95% of the estimated average ratio for (*del/ins)* and (*del/del)*, the effect of T administration in the (*ins/ins)* group was greater than that produced in the other two groups.

In Fig. 3 shows the individual T/E ratio levels throughout the study according to genotype. It is a diagram of lines where each line represents the absolute values of each volunteer. The number of the line identifies each subject.

In all graphics, a baseline threshold level for labelling the case as atypical finding has been added (T/E > 4). Those results permit to ratify some of the conclusions of Okanós group [25], the overall result has demonstrated the limited effectiveness of population-based T/E ratios in screening tests for testosterone use. Subject-based steroid profiling with UGT2B17 genotyping will be an effective strategy for detecting testosterone misuse.

Another interesting observation obtained in this study was that kinetic elimination for the (*del/del)* population was more gradual and took a longer time (see Fig. 3c). In order to confirm this effect in the elimination it would be necessary to have more samples on consecutive days after the period c3.

Our study confirms the results submitted by other authors [24,25] although it presents methodological differences. The studies developed by Okano et al. [25] and Shultze et al. [24] are experimental designs not controlled before-after where only a single urine determination is available before the administration of testosterone and a number, between 9 and 12 determinations respectively, after the administration of testosterone. Our study is a “placebo-controlled triple-blind crossover study“ where 40 urine samples are collected per patient, mainly studying the change that occurs in the levels of steroid profile among the 10 determinations subsequent to the administration of testosterone against the 10 samples after placebo. In addition, our study assesses the impact of Testex on LH levels as well as the identification criteria established by WADA for the case of the IRMS analysis.

In relation to the data obtained from the determination of LH, as shown in Table 5, LH determinations and the T/LH ratio before

Table 3
Levels of the several T metabolite ratios throughout the study, depending on the genotype and period of collection of the urine (pre* or post** T administration).

			PRE T administration			POST T administration	
			b	c1	c2	c3	c4
Number of samples		(ins/ins)	20	40	19	40	17
		(del/ins)	20	40	20	40	17
		(del/del)	20	40	20	39	19
T/E	(ins/ins)	median	1,21	1,28	1,28	56,38	2,35
		IQR	1,16-1,85	1,19-1,81	1,13-2,31	45,82-66,01	1,76-3,79
		>4	3 (15%)	4 (10%)	0 (0%)	40 (100%)	4 (23%)
	(del/ins)	median	0,85	0,86	0,96	17,93	1,07
		IQR	0,62-0,92	0,62-0,99	0,71-1,06	9,54-22,56	0,61-1,29
		>4	0 (0%)	0 (0%)	0 (0%)	37 (93%)	2 (12%)
	(del/del)	median	0,09	0,11	0,1	2,93	0,16
		IQR	0,07-0,12	0,09-0,12	0,08-0,12	1,08-3,63	0,12-0,27
		>4	0 (0%)	0 (0%)	0 (0%)	8 (21%)	0 (0%)
A/Etio	(ins/ins)	median	0,91	1,03	1,29	1,21	1,09
		IQR	0,65-1,51	0,67-1,43	0,55-1,49	0,92-2,08	0,61-1,79
	(del/ins)	median	1,22	1,17	1,26	1,6	1,23
		IQR	1,01-1,61	0,96-1,60	0,94-1,34	1,30-1,75	0,99-1,63
	(del/del)	median	0,92	0,97	0,97	1,17	0,97
		IQR	0,71-1,45	0,77-1,64	0,84-2,13	1,03-2,82	0,88-1,38
A/T	(ins/ins)	median	41,8	41,6	47,1	22,2	40,7
		IQR	31,8-61,1	30,6-59,6	30,5-61,6	17,8-25,3	22,5-70,7
		<20	0 (0%)	2 (5%)	1 (5%)	17 (43%)	1 (6%)
	(del/ins)	median	75,3	76,4	67,4	41,9	79,4
		IQR	60,4-116,1	52,0-106,5	49,5-106,8	33,9-60,1	47,6-118,9
		<20	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	(del/del)	median	335	322,7	300,7	307,29	376,7
		IQR	251,9-392,0	228,6-427,2	266,5-399,9	239,7-345,7	307,3-441,7
		<20	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
A/E	(ins/ins)	median	87,7	83,7	88,6	1018,5	136
		IQR	48,8-129,2	41,9-128,1	44.8-150,2	71,9-1373,1	85,5-197,4
	(del/ins)	median	66,5	68,5	61,7	680,9	89,8
		IQR	52,0-74,6	52,8-85,4	51,5-76,5	451,2-1206,6	65,8-125,9
		range	42,1-117,5	37,2-98,3	43,0-90,5	105,6-1622,1	49,0-898,7
	(del/del)	median	34,6	39,1	39,3	880,3	81,2
		IQR	22,7-58,2	24,9-58,3	25,2-60,4	237,6-1200,3	42,8-185,6
5αAdiol/E	(ins/ins)	median	2,02	2,23	2,7	27,3	3,1
		IQR	1,20-3,47	0,97-3,07	1,16-3,30	19,55-47,26	2,26-5,44
	(del/ins)	median	1,42	1,48	1,26	20,16	1,84
		IQR	1,19-1,62	1,27-1,74	1,15-1,65	12,61-33,05	1,69-2,20
	(del/del)	median	0,93	1,04	1,02	14,91	1,8
		IQR	0,57-1,13	0,57-1,33	0,44-1,26	7,27-25,00	1,00-4,04

pre* T adm, this group of samples included all the samples collected from each volunteer when there is not T adminstration, because it corresponds to basal samples (b) or there is placebo administration.

post T adm**, this group of samples included all the samples collected from each volunteer when there is T adminstration. The samples from the period (d) were no evaluated, to much time was elasted to be detected any effect of T adm.

Table 4
Ratio of means between the T administration group and the placebo adjusted by randomisation sequence for each type of polymorphism for T/E, A/T, 5α Adiol/E and A/E ratios. For the adjustment of the models only 10 days after the administrations were selected.

		Ratio of means	IC95%		p
			low	high	
(T/E)	(ins/ins)	30,4	25,2	36,7	<0,001
	(del/ins)	19,8	15,9	24,7	<0,001
	(del/del)	19,7	14,9	26,2	<0,001
(A/Etio)	(ins/ins)	1,31	1,25	1,38	<0,001
	(del/ins)	1,21	1,15	1,29	<0,001
	(del/del)	1,42	1,34	1,51	<0,001
(A/T)	(ins/ins)	0,51	0,46	0,55	<0,001
	(del/ins)	0,58	0,54	0,62	<0,001
	(del/del)	0,95	0,88	1,02	0,14
(A/E)	(ins/ins)	12,3	10,49	14,42	<0,001
	(del/ins)	9,82	8,11	11,89	<0,001
	(del/del)	14,51	11,28	18,67	<0,001
(5αAdiol/E)	(ins/ins)	16,59	13,69	20,1	<0,001
	(del/ins)	12,12	9,91	14,82	<0,001
	(del/del)	14,3	10,79	18,95	27,11

administration (period b) were compared within each individual determinations during the 10 days after administration. The T/LH ratio presents a significant increase in the three types of populations after the administration of Testex. The increase is significantly greater in the homozygous wt group compared to homozygous mutated ($p = 0.009$), while heterozygotes do not show differences ($p = 0.116$). However, no differences were observed ($p = 0.387$) in the heterozygous group versus the mutated homozygote.

Regarding the LH values, a significant decrease was observed after the administration of Testex in each of the polymorphisms without detecting significant differences in the decrease between the three-polymorphism groups. Palonek et al. and Goebel et al. [29,30] have suggested that the measurement of levels below the lower reference in urinary LH, with the measurement of T/E values, can significantly improve the efficacy of doping detection by testosterone especially those with low T/E ratios.

3.3. Analysis by GC-C-IRMS

Due to the cost and the volume of urine available from each sample, only 5 samples from each volunteer were submitted to GC-C-IRMS analysis: one sample from the pre- administration period (period b) and samples collected on days 2, 10, 20 and 25 from the post-administration period (period c3 and c4).

In all cases the samples pre T administration (period b) provided a clear “non-consistent with steroid administration” result.

Fig. 4 summarizes the result of the evaluation according to WADA TD2016IRMS) [14] for the samples submitted to GC-C-IRMS. In such document, there are several criteria for a positive finding; in those cases where the criterion is partially fulfilled the qualification of the sample is “non-conclusive”.

During the c3 period, all samples selected from *(ins/ins)* and *(del/ins)* individuals are atypical for T/E but not those from *(del/del)* individuals. Nevertheless, the most important result is the fact that independently of the type of polymorphism, the measurement of $\Delta\delta^{13}C$ values allows detection of all the adverse cases when there is T administration during the C3 period and in some C4 samples too, avoiding the issue of the T/E ratio parameter as the criterion for T misuse in the (*del/del*) population. All samples for the c3 period produced an adverse analytical finding when WADA technical document TD2016IRMS was applied. In period c4 for (*ins/ins*) individuals only one of eight samples (13%) and for (*del/ins)* individuals two of eight samples (25%) were classified as adverse analytical findings; while for (*del/del*) individuals, six of eleven samples showed as adverse analytical findings (55%)

The detection of T abuse regardless of the polymorphism was also observed by Okano et al. [25], who obtained adverse analytical findings in 100% of the cases after 10 days of the administration of testosterone enanthate and values of $\underline{\Delta\delta}^{13}C > 3‰$ in 87% of the analyzed samples of the (*del/del*) group and 67% for the (*del/ins*) group after 15–16 days of the administration. In our case, $\underline{\Delta\delta}^{13}C$ values > 3‰ were obtained for a longer period of time probably because the administered dose was higher, since the pharmacokinetics of testosterone cypionate and testosterone enanthate are comparable when the same dose is administered intramuscularly [31].

Undoubtedly we have to recognize, the most efficient criteria after testosterone cypionate administration, independently of the UGT2B17 polymorphic group, was the $\Delta\delta^{13}C$ value of the ERC-T pair, the ERC-5αAdiol and ERC-5βAdiol pairs were both greater than 3‰.

The use of GC-C-IRMS-based methodologies has proved to be a key element for evaluating the profiles of the (*del/del*) population, because despite the T/E ratio being under 4, the threshold level for an atypical finding declaration [32,33]. In all the cases, the carbon 13 contribution allows the result to be considered as an adverse analytical finding.

4. Conclusions

The results obtained make it possible to conclude that a T single dose administration induces a significant increase in the urinary concentration of the target compounds, independently of the genotype. From the target compound ratios studied, the T/E ratio is the most efficient parameter for long-term detection of T administration.

The increase in the T/E ratio is not homogeneous among the three populations; volunteers belonging to the (*ins/ins*) group showed a higher relative mean increase than that observed in the (*del/ins*) and (*del/del*) groups. In the 10 samples of urine collected during the 10 days after the T administration, it was observed that in the (*ins/ins*) group all

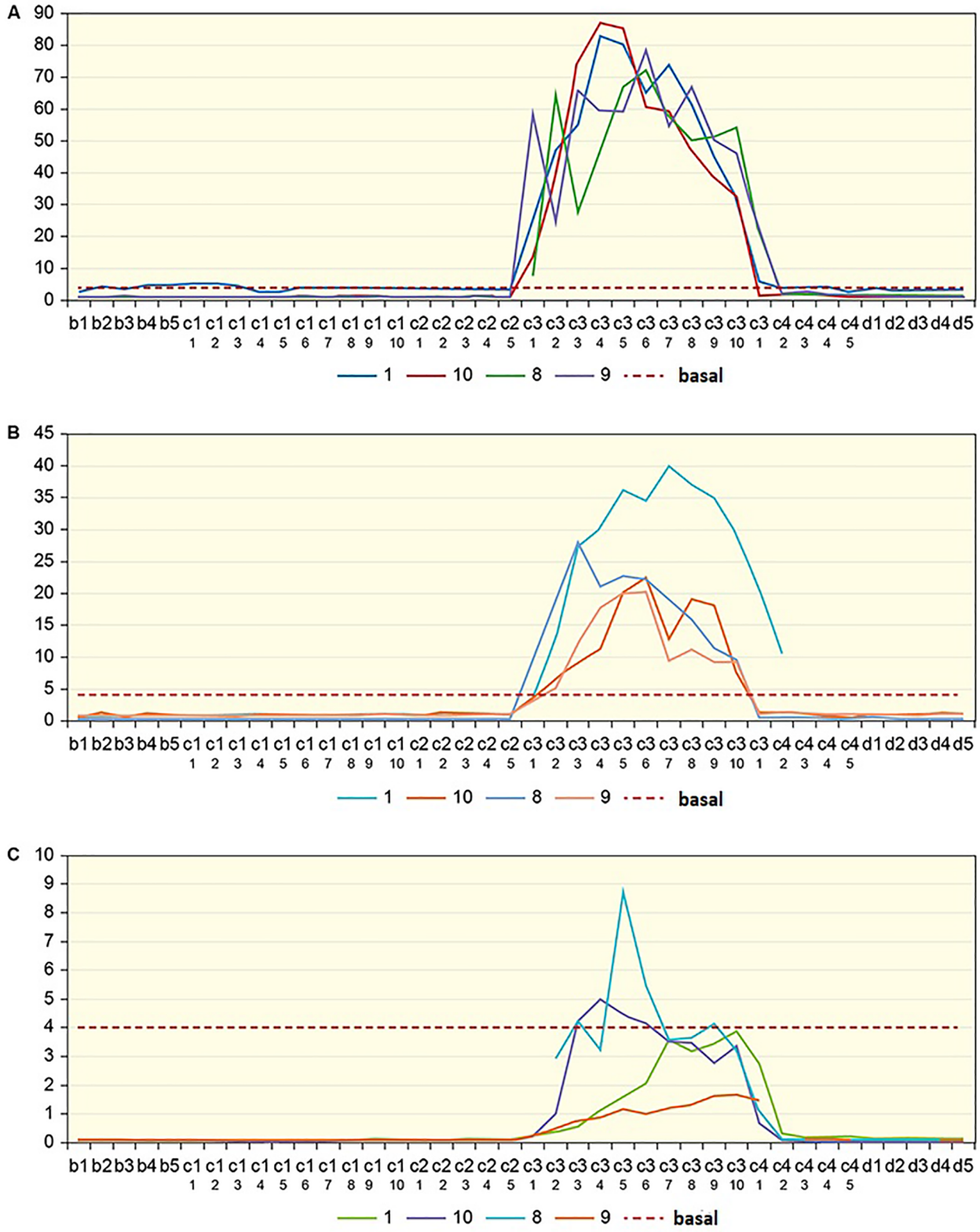

Fig. 3. Values of the T/E ratio throughout the study. Period C1 and C2 have been exchanged for C3 and C4 in periods in which the athlete randomization sequence started with Testex Elmu prolongatum in order to compare T/E levels with those starting with the placebo. (A) wt homozygous (ins/ins) population, (b) heterozygous (del/ins) population, and (C) mutant homozygous (del/del) population.

samples had levels of T/E greater than 4. In the (del/ins) group, the percentage was nearly 100% (93%) while in the (*del/del*) group only 21% of the samples exceeded that level. That fact is very important because it is the main parameter for launching the confirmatory or follow-up protocols in the anti-doping field.

The longitudinal follow-up of the LH values, independently of the genotype of the group, and despite having applied a single dose, shows a similar significant change and it́s an indicator of the administration of

Table 5
LH determinations and the T/LH ratio before administration (period b) were compared within each individual determinations during the 10 days after administration in three groups UGT2B17 genotype.

	LH						T/LH		
	Period b		Period c1		Period c1 /Period b	LH <0.8 (c1)	Period b	Period c1	Period c1 /Period b
	n	median (IQR)	n	median (IQR)	Means ratio (IC95%)	n(%)	median (IQR)	median (IQR)	Means ratio (IC95%)
Homozygous wt (ins/ins)	20	2,94 (1.33-7,57)	40	0.58 (0,18-3,60)	0.28* (0.18-0.44)	23 (57.5)	14.82 (8.22-29.25)	317.88 (63.92-1239.46)	22.44* (14.85-33,88)
Heterozygous(del/ins)	15	5,95 (1,23-25,57)	30	1,52 (0.61-11,04)	0.31* (0.20-0.48)	10 (33.3)	5.55 (2.19-10.29)	109.39 (15,74 -171,9)	13.55* (8.43-21,76)
Homozygous mutant (del/del)	20	3,63 (2,98-6,59)	38	1,29 (0.59-2,56)	0.32* (0.20-0.49)	13 (34.2)	1.06 (0.70-2.43)	14.26 (7.06-20.62)	10,26* (6.78-15.52)
* p <0.001 preriod c1 vs period b									

T.

When a GC-C-IRMS-based methodology is used, this issue is avoided, because independently of the genotype, all the samples collected 10 days after the administration were classified as Adverse Analytical Findings consistent with steroid administration according to WADA TD2016IRMS.

This group considers from the study carried out, that a practical consequence for anti-doping organizations would be to perform a genetic screening of the UGT2B17 gene, for athletes with low T/E values. This would allow to decide when to apply the methods based on GC-C-IRMS when there are few longitudinal data of the athlete's ABP or these are influenced by other pharmacological and physiological modifications [30bis].

From a medical point of view, when observing the results obtained in athletes with very low T/E and seeing that they have a great risk to their health, included increased risk of developing renal disorders [34], by accumulating more time the steroids, this can make us reconsider in the realization of a possible genetic study, as indicated by Bang et al. [35]. We are aware of the ethical conflicts involved in genetic studies applied to athletes, but we believe that the health of athletes should prevail in the face of the harmful consequences of the use of steroids by this group of athletes.

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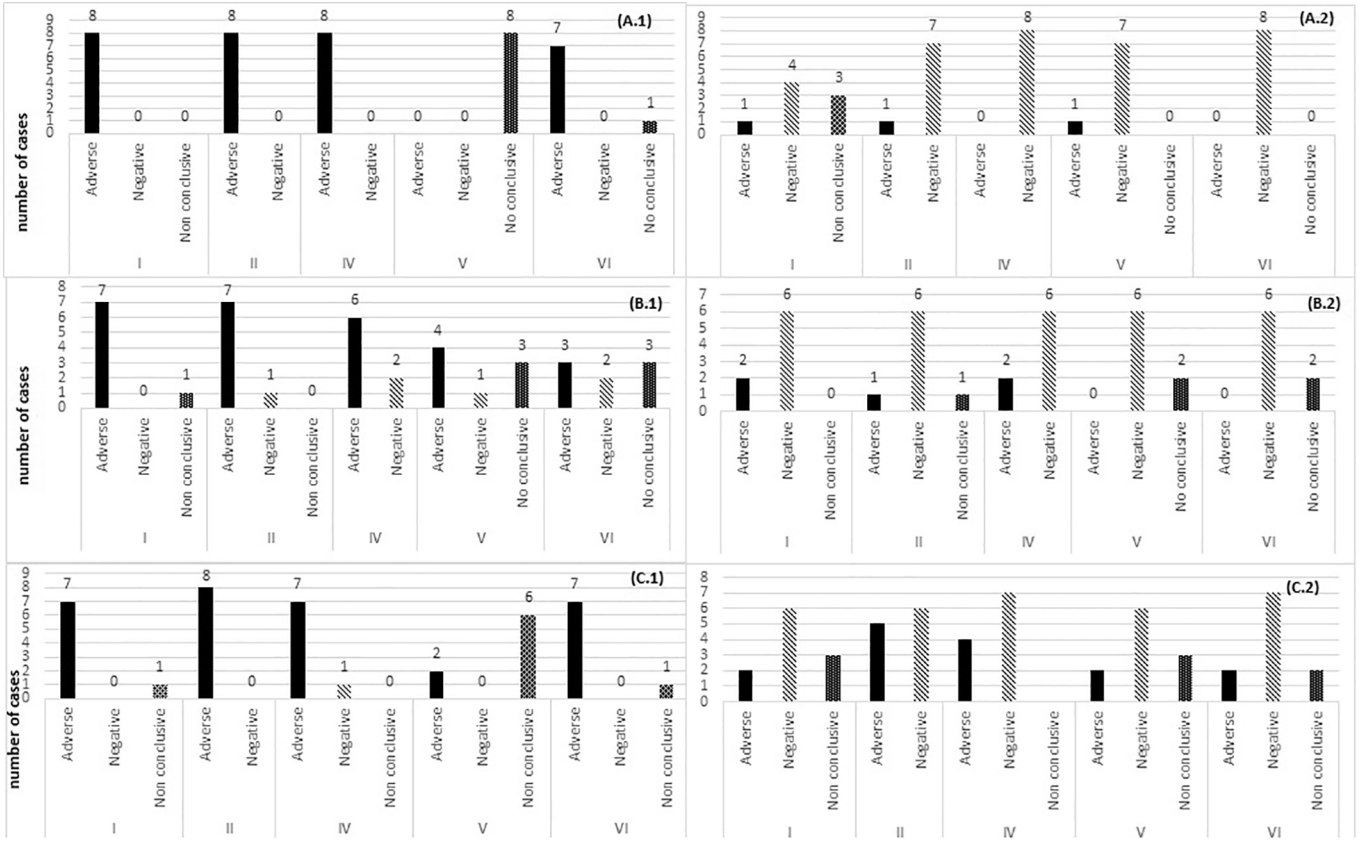

Fig. 4. Assessment of the confirmation criteria for the analysis by GC-C-IRMS according to the TD2016IRMSWADA. (A.1) ins/ins C3 period, (A.2) ins/ins C4 period, (B.1) ins/del C3 period, (B.2) ins/del C4 period, (C.1) del/del C3 period y (C.2) del/del C4 period.

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Conflict of interest

The authors declare that they do not have any financial interest or conflict of interest regarding the study.

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