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Evaluation of endogenous steroid sulfates and glucuronides in urine after oral and transdermal administration of testosterone. Part II: Female participants

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ABSTRACT

Detecting testosterone (T) doping remains a significant challenge, driving the search for novel biomarkers and advancements in the steroidal Athlete's Biological Passport (ABP). Phase II metabolites of endogenous anabolic androgenic steroids (EAAS) have emerged as promising biomarkers, demonstrating prolonged detection times (DTs) and greater sensitivity compared to conventional biomarkers. Studies involving male participants investigated the effect of intramuscular, oral, and transdermal administration of T on these biomarkers and proposed their integration in future urinary steroid profiling. However, before the inclusion of phase II EAAS metabolites, it is crucial to address a range of possible doping scenarios and the influence of known confounding factors, like ethnicity or sex, on the steroid profile. This study addresses this gap by investigating the impact of oral and transdermal T administration on phase II EAAS metabolites in both men and women. This second part of the study presents the results for female participants, which have not been included in prior research on this topic. Results partially confirm the trends observed in men, with sulfate ratios exhibiting prolonged detection times and higher sensitivity compared to conventional steroid profile biomarkers following multiple oral and transdermal T administration. However, the evaluation in women showed greater variability due to lower steroid concentrations and greater fluctuations influenced by the menstrual cycle. This study provides additional evidence supporting the inclusion of phase II EAAS metabolites for enhanced detection of T doping. Further, it underscores the need for further research to address the unique challenges of female steroid profiling.

1. Introduction

As reported by World Anti-Doping Agency (WADA) accredited anti-doping laboratories, anabolic androgenic steroids (AAS) are the class of prohibited substances with the highest number of positive findings each year [1]. Remaining largely unchanged in popularity over the past decade, AAS are misused by athletes to enhance their sports performance due to their anabolic effect. The substance group of anabolic agents includes endogenous anabolic androgenic steroids (EAAS), like testosterone (T), and synthetic exogenous steroids. While the intake of synthetic AAS can be confirmed by a qualitative analytical approach, identifying the exogenous administration of EAAS poses a greater challenge, as it requires distinguishing it from its endogenous origin. As the presence of EAAS alone cannot be related to an illicit drug intake, a quantitative approach including complex evaluation is required to decide on further analysis and testing [2,3]. The steroidal module of the Athletes' Biological Passport (ABP), implemented in 2014, enables the longitudinal monitoring of EAAS biomarkers in urine. Its evaluation identifies samples suspicious for the use of T or other EAAS, which are then analyzed by gas chromatography – combustion - isotope ratio mass spectrometry (GC-C-IRMS) to confirm the exogenous origin of T and/or metabolites in the sample. In addition to the automatic flagging of suspicious samples by the steroidal ABP, Athlete Passport Management Units (APMU) additionally assess steroid profiles. Based on their expert

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opinion, they provide details and recommend additional testing, such as collecting serum, blood, or further urine samples [4]. The steroidal ABP consists of the urinary biomarkers T, epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5a-Adiol), and 5β-androstane-3α,17β-diol (5b-Adiol). Metabolites excreted unconjugated and glucuronidated metabolites after enzymatic hydrolysis are quantified together as free forms using gas chromatography-mass spectrometry (GC-MS) methods. The concentrations of the biomarkers are combined into specific ratios to enhance stability and sensitivity, where T/E, A/Etio, 5a-Adiol/E, 5a-Adiol/5b-Adiol, and A/T are included in the steroid profile [3,5–7].

Although the implementation of the steroidal ABP improved the detection capabilities for T doping, it has some limitations. Exogenous confounding factors, like alcohol consumption or medication interfering with steroid metabolism, can alter the steroid profile and must be considered during the evaluation of the passports. Additionally, endogenous factors such as age, sex, and ethnicity can contribute to variations and reduced sensitivity in the steroid profile [3,8–11]. Additionally, different administration forms of T lead to different variations in the steroid profile, such as a sharp but short increase of certain biomarkers after oral administration, but minor changes and therefore low sensitivity to transdermal administration.

To further improve the steroidal ABP and to address current limitations, novel biomarkers are being explored. One promising approach is the analysis of phase II metabolites of EAAS by liquid chromatography-mass spectrometry (LC-MS), which has been thoroughly investigated in recent years [12–20]. Intact phase II EAAS metabolites, including glucuronidated and sulfated steroids, have demonstrated significant potential for extending the detection time (DT) following various forms of T administration. Recent studies have focused on sulfated metabolites, which are not considered in the current steroidal ABP, including ratios with testosterone sulfate (TS), epitestosterone sulfate (ES), androsterone sulfate (AS), and epiandrosterone sulfate (EpiAS), or combinations of sulfated and glucuronidated metabolites as suitable biomarkers. For example, after intramuscular injection, the combined ratio (CR) of testosterone glucuronide/testosterone sulfate to epitestosterone glucuronide/epitestosterone sulfate ((TG/TS)/(EG/ES)) was proposed as a promising biomarker [16,17]. In part I of this study, we evaluated the applicability of the CR in male participants [21], highlighting the differences between parenteral (transdermal, intramuscular) and enteral (oral) administration forms of T. Phase II EAAS metabolites have also been studied in both Caucasian and Asian populations to identify biomarkers unaffected by UGT2B17 gene polymorphism [18–20]. Individuals with the del/del genotype for the UGT2B17 enzyme exhibit low urinary TG levels. This deletion polymorphism, which affects the glucuronide conjugation of T, is commonly associated with Asian steroid profiles [9] and leads to a poor sensitivity of the T/E ratio to exogenous T administration.

Sex is an endogenous factor influencing the steroid profile that has not been investigated in detail. Limited data is available as women are not often included in clinical studies testing drug administration. A few studies have evaluated low-dose T gel administration across different matrices, such as urine, blood, and serum [22–26]. These studies highlight the need for additional biomarkers, a deeper understanding of female T metabolism, and improved methods for interpreting the steroid profile in women [2,27–29]. The primary challenges include the low sensitivity of the steroidal ABP in women, as steroid concentrations in female urine samples are often near the detection limits of analytical procedures. Additionally, hormonal fluctuations associated with the menstrual cycle cause significant variability in certain biomarkers, particularly those related to E. The production of EAAS also differs between men and women. In men, T is primarily produced by the testes and regulated by the hypothalamic–pituitary–gonadal (HPG) axis. In contrast, women produce much smaller amounts of T, primarily through the ovaries, adrenal glands, and peripheral tissue conversion [30]. In men, exogenous T administration triggers a strong negative feedback control from the brain on T and its epimer, E. The circulating and excreted T levels increase as a result of the sum of endogenous and exogenous T. In women, however, the lower baseline level of circulating T and the lack of strong negative feedback control mean that only the spillover of exogenous T influences biomarkers. Coupled with the naturally higher variability in female biomarkers, this makes current testing methods less effective for detecting T administration in women [29].

Research on phase II EAAS metabolites has demonstrated their significant potential for inclusion in future steroid profiling. Previous studies have primarily focused on different T administration forms or comparisons between Caucasian and Asian male participants. In this second part of our study, we investigated phase II metabolites following oral and transdermal T administration in female participants—a population that remains underrepresented in doping research. Given the limited understanding of how exogenous T affects phase II EAAS metabolites in women, our findings aim to identify additional biomarkers and enhance the detection of T doping in females. This study is the first to evaluate phase II EAAS metabolites in women after oral and transdermal T administration. We assess their impact on the conventional steroid profile, as well as glucuronidated and sulfated EAAS metabolites, and compare these results with those of male participants from the first part of the study [21]. The study included 12 male and 6 female participants.

2. Materials and methods

2.1. Chemicals and reagents

Methanol (HPLC grade), used for preparing standard solutions and sample preparation, was sourced from Chem-Lab (Zedelgem, Belgium). Milli-Q water, obtained from a water purification system (Millipore, Reference A+, Burlington, Massachusetts, USA), was used for sample preparation. Water and acetonitrile (ULC/MS-CC/SFC grade), along with formic acid (99 %, ULC/MS-CC/SFC grade) for HPLC analysis, were supplied by Bisolve (Valkenswaard, The Netherlands). Oasis HLB cartridges (60 mg/3 ml, 30 μm particle size) for solid-phase extraction were procured from Waters (Milford, MA, USA). Chemicals for preparing artificial urine and buffers were acquired as follows: potassium dihydrogen phosphate, sodium chloride, di-sodium hydrogen phosphate, and ammonium chloride from Merck (Darmstadt, Germany); urea from GE Healthcare Life Sciences (Uppsala, Sweden); and creatinine from Sigma Aldrich (St. Louis, MI, USA). Additionally, methyl-t-butyl-ether, ammonium iodide (NH4I), and ethanethiol (97 %) were also obtained from Sigma Aldrich (St. Louis, MI, USA). β-Glucuronidase (E. coli) for enzymatic hydrolysis was provided by Roche (Mannheim, Germany). N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Macherey-Nagel (Düren, Germany). For silylation, a derivatization stock solution was prepared by dissolving 200 mg of NH4I in a mixture of 10 ml MSTFA and 600 μl ethanethiol, and a derivatization working solution was created by mixing the stock solution with MSTFA (1:4, v/v), before sample preparation.

Testosterone glucuronide (TG; Batchnr.: 16S02), Epitestosterone glucuronide (EG; Batchnr.: 15S10), Androsterone glucuronide (AG; Batchnr.: 09S11), Etiocholanolone glucuronide (EtioG; Batchnr.: 15S14), Testosterone sulfate (TS; Batchnr.: 97000340), Epitestosterone sulfate (ES; Batchnr.: 97000341), Androsterone sulfate (AS; Batchnr.: 06S05), Etiocholanolone sulfate (EtioS; Batchnr.: 97001741) and the deuterated internal standards (ISTD) d3-TG (Batchnr.: 15S06), d4-EG (Batchnr.: 13S07), d3–5α-Dihydrotestosterone glucuronide (d3-DHTG; Batchnr.: 10S06), d3-TS (Batchnr.: 20S02), d3-ES (Batchnr.: 97000059), d4-AS (Batchnr.: 98000501), d5-EtioS (Batchnr.: 97001744) and d3–5α-Dihydrotestosterone sulfate (d3-DHTS; Batchnr.: 98001909) were obtained from the National Measurement Institute (New South Wales, Australia). d5-AG was synthesized by the Technical University of Vienna (Vienna, Austria). Epiandrosterone glucuronide (EpiAG; Batchnr.: C259)

and Epiandrosterone sulfate (EpiAS; Batchnr.: B2387) were purchased from Steraloids (Newport, RI, USA). The purity of all reference standards was 98 % or greater. Stock standard solutions of all analytes were prepared in methanol (100–200 μg/ml) and stored at −20 °C.

2.2. Clinical studies

The corresponding local Research Ethical Committees (Sports Medicine Association of Serbia, Belgrade, Serbia) granted the ethical approval for the study in Decision_0221 (2021). The study was conducted in accordance with the Declaration of Helsinki.

All participants enrolled in the study gave their written informed consent before inclusion and underwent a general physical examination, blood test, urinalysis, and a 12-lead electrocardiogram, which showed results within normal values. Following the completion of the study, a further medical evaluation was conducted. All participants declared not to consume any over-the-counter drugs or prescription drugs within 2 weeks before the start of the study. The moderate consumption of alcoholic beverages was allowed. Six healthy female volunteers, aged between 18 and 40, with body weight not more than 10 % above or below their respective ideal weight, were included in the administration studies. All female participants stated that they did not use hormonal contraceptives. A high compliance with the sampling protocol and overall study procedures could be ensured, as all of the participants were medical students or practicing physicians. Participants reported to engage in moderate-intensity physical activity on average three days/week. Each participant received an oral T formulation in the first study period and an additional gel formulation in the second study period. Compared to the male participants described in the first part of this study [21], female participants received half the dose of T preparations, which still represents a relatively high dose for women.

In the first study period, an oral formulation of testosterone undecanoate (TU; Andriol® Testocaps, 5 times 40 mg TU, multiple dosing) was administered as follows: 2 capsules on day 1, 1 in the morning and the evening, respectively. One capsule was administered in the morning on day 2, 3, and 4, resulting in a total T intake of 127 mg (200 mg TU) for female participants. To create baseline values, six urine samples were collected during the week before administration. After the first administration, urine samples were collected as follows, starting after the first T administration: all urine samples during week 1, one urine sample per day during week 2, and 2 urine samples per week for weeks 3, 4, and 5.

Two months after the first study period, participants received a gel formulation in the second study period. The T gel (Testavan® gel, 5 times 23 mg of T, multiple dosing) was administered in the morning on day 1–5, resulting in a total T administration of 115 mg for female participants. For estimation of baseline values, 3 urine samples were collected during the week before the gel administration. After administration, urine samples were collected as follows, starting after the first T administration: all urine samples during week 1, one urine sample per day during week 2, and 2 urine samples per week for weeks 3, 4, and 5.

2.3. Determination of the conventional steroid profile

The same sample preparation and analysis as in the first part of the study were applied [21].

2.3.1. Sample preparation

The determination of the conventional urinary steroid profile was conducted in compliance with World Anti-Doping Agency (WADA) guidelines according to the validated routine laboratory procedure [31,32]. Briefly, the procedure includes the hydrolysis of the glucuroconjugated steroids and derivatization before injection and measurement with gas chromatography–tandem mass spectrometry (GC-MS/MS) and is described elsewhere [33]. If the concentration was outside the calibration range using 2.5 ml of urine, a 100 μl urine aliquot was used.

2.3.2. GC-MS/MS analysis

The trimethylsilyl steroids were analyzed by GC-MS/MS on a Trace 1300 gas chromatograph coupled to a TSQ 8000 triple quadrupole mass spectrometer (Thermo Fisher, Waltham, MA, USA) according to the accredited procedure for quantifying the steroid profile in urine [33].

2.4. Determination of phase II metabolites

The same sample preparation and analysis as in the first part of the study were applied. For more details, refer to [21].

2.4.1. Sample preparation

As sulfates and glucuronides of EAAS metabolites are found from sub-ng/ml to several μg/ml in urine, two aliquots of urine were prepared to cover all concentration ranges. In brief, one aliquot was prepared using solid phase extraction (SPE), which was performed on Oasis HLB cartridges (60 mg, 30 μm). 2 ml of urine were spiked with 20 μl of ISTD solution (final concentration in urine samples: 40 ng/ml d3-TG, 40 ng/ml d4-EG, 400 ng/ml d5-AG, 100 ng/ml d3-DHTG, 10 ng/ml d3-TS, 10 ng/ml d3-ES, 100 ng/ml d4-AS, 100 ng/ml d5-EtioS, and 100 ng/ml d3-DHTS) After washing with water, the analytes were eluted with methanol. After evaporation, samples were reconstituted in loading solvent H2O: ACN (80:20) + 0.1 % FA and 2 μl were injected into the LC-High Resolution Mass Spectrometry (LC-HRMS) system. A second aliquot using 40 μl of urine was diluted with 360 μl aqueous ISTD solution (final concentration in urine samples: 600 ng/ml d3-TG, 6000 ng/ml d5-AG, 1500 ng/ml d4-AS, 1500 ng/ml d5-EtioS, and 1500 ng/ml d3-DHTS in 96-deep well plates. 2 μl of the mixture was directly injected (dilute and shoot, DS method) into the LC-HRMS system. 10 μl of urine was used if concentrations were outside the calibration range.

2.4.2. LC-HRMS analysis

Analysis was carried out on a Q Exactive Focus Orbitrap MS analyzer coupled to a Vanquish Horizon UHPLC System (Thermo Fisher, Waltham, MA, USA). The LC system, including a high-pressure binary pump, was equipped with a cooled sample tray (20 °C) and a column oven set at 25 °C. Shielded fused silica nanoViper™ tubing sheathed in polyetheretherketone (PEEK) from Thermo Fisher was employed in the entire system. For chromatographic separation, an InfinityLab Poroshell 120 EC-C18 column (150 ×2.1 mm i.d., 1.9 μm particle size, Agilent Technologies) was used. The mobile phase consisted of water containing 0.1 % formic acid (solvent A) and acetonitrile containing 0.1 % formic acid (solvent B). In brief, the 16-minute-long chromatographic method for SPE samples included a flow rate of 0.3 ml/min and an increase of solvent B over 10.5 min from 25 % to 39 % B. The chromatographic method for DS samples lasted 8 min, including an increase of solvent B from 40 % to 51 % over 3.8 min.

The mass spectrometer was equipped with a heated electrospray ionization (HESI) source and was operated in negative ionization mode. Source parameters were as follows: spray voltage 3.7 kV, capillary temperature 320 °C, sheath gas, and auxiliary gas (nitrogen): 30 and 10 arbitrary units, respectively. The instrument was operated in single ion monitoring (SIM) mode at 70,000 resolving power. A high mass accuracy was beneficial to confidently distinguish the target analytes from isobaric compounds.

2.5. Method validation

Methods used for quantification of the target substances TG, EG, EtioG, AG, EpiAG, TS, ES, EtioS, AS, and EpiAS were validated according to WADA guidelines [32]. The validation protocol included the determination of SPE extraction recovery, selectivity, linear range, limit of quantification (LOQ), repeatability, intermediate precision (IP), bias, measurement uncertainty (MU), robustness, extract stability, and carryover. A thorough description of the validation parameters and the results can be found in the first part of this study [21]. Corresponding

deuterated standards were used as ISTD. For EpiAG and EtioG, where no deuterated standard was available, d3-DHTG and d5-AG were used as internal standards, respectively. EpiAS was evaluated with the deuterated standard d3-DHTS. For the preparation of calibration curves, artificial urine prepared in the laboratory (2.5 g/L potassium dihydrogen phosphate, 9.0 g/L sodium chloride, 2.5 g/L disodium hydrogen phosphate, 3.0 g/L ammonium chloride, 25 g/L urea, and 2 g/L creatinine, prepared in water) was used. The calibration curves were accepted if the coefficient of determination (R2) > 0.99.

2.6. Data evaluation

Raw data processing and quantification were performed using the software Xcalibur Quan Browser (Thermo Fisher). An external calibration curve consisting of at least 5 points was used for quantification. To improve comparison, urinary concentrations of endogenous steroids were corrected by specific gravity (SG) [34]. Excluded from evaluation were samples that showed signs of extensive bacterial degradation (5α-Androstandione/A or 5β-Androstandione/Etio > 0.1, determined by GC-MS/MS) or ethyl glucuronide (ETG) (determined by LC-HRMS) higher than 5 μg/ml [31]. Values below the LOQ of the analytical method were not considered. Among all possible combinations between target sulfates and glucuronides, the ratios showing the highest sensitivity to T administration were longitudinally evaluated. The five ratios included in the conventional steroid profile determined by GC-MS/MS, namely T/E, A/Etio, 5a-Adiol/E, 5a-Adiol/5b-Adiol, and A/T, were selected as reference biomarkers. The average values of ratios obtained from the baseline samples before administration of the respective study phase, plus three times the standard deviation, were used to define the individual threshold (IT) limits [2], corresponding to a 99 % confidence interval. This approach assumed a normal distribution of data. The ratios obtained from both the administration and the post-administration samples were compared to the calculated IT. Ratios outside the IT limits indicated T administration effects. DT was calculated in post-administration samples from the last administration ($t = 0$). The last value outside the IT in the post-administration samples was defined as DT. If five consecutive values were below the IT, followed by a single value above it, this elevated value was considered an outlier and was excluded from further evaluation. Additionally, the DT was considered unreliable if less than 10 % of all samples were affected by T administration. This restriction prevented skewed ratios caused by values near the LOQ in the denominator, which could produce misleading ratio increases unrelated to T administration.

Samples from all 6 female participants were included for oral (O13-O18) and transdermal (D13-D18) T administration in the data analysis.

3. Results and discussion

3.1. General aspects

3.1.1. Comparison of EAAS metabolism in men and women

In men, the biosynthesis of EAAS takes place in the testes, with a minor contribution from the adrenals. Their production is regulated by the hypothalamic–pituitary–gonadal (HPG) axis [29]. Exogenous administration of T leads to negative feedback via the HPG, causing a reduction in endogenous T and E production. This process increases the contribution of exogenous administered T on urinary steroid profile biomarkers. Female EAAS are equally derived from the ovaries and the adrenal glands. Each produces 25 % of the circulating levels of T, while the remaining 50 % originates from peripheral conversion of androgen precursors. Production is subject to negative feedback control only to a minor extent, making the current urinary steroid profile biomarkers less effective for the detection of T administration. The concentration of circulating T in men is approximately 10-fold higher than in women [30,35]. Comparing male participants and female participants, their corresponding basal T/E ratios were 1.4 ± 0.57 and 0.67 ± 0.22, respectively. The basal T concentrations were approximately 5 times higher in male participants compared to female participants. However, due to high inter-individual differences between the participants, comprehensive sex specific conclusions cannot be drawn.

3.1.2. Influence of the menstrual cycle on biomarkers

Variability in conventional steroid profile biomarkers, but also in sulfates, has been reported among individuals [5,12,19,20]. This variability is more pronounced in women due to hormonal fluctuations associated with the menstrual cycle. Although T output varies considerably during the menstrual cycle, significant changes in urinary T concentrations have previously not been observed [28]. Among all biomarkers, E is the only parameter notably influenced by the menstrual cycle, with concentrations increasing during the ovulation and luteal phase [27]. Consequently, conventional biomarkers involving E, such as T/E and 5a-Adiol/E, exhibit substantial fluctuations, reducing their reliability. The impact of the menstrual cycle on glucuronidated and sulfated steroids has also been investigated [27,28]. No changes were observed in the EG/ES ratio, suggesting that glucuronides and sulfates are similarly affected by the menstrual cycle.

In our study, the EG/ES ratio remained stable among all participants, except for participant O13, who exhibited significant fluctuations following oral TU administration, resulting in multiple values exceeding the IT. It is important to note that menstrual cycle phases were not tracked during sample collection. Additionally, baseline samples were collected within a one-week period, which did not encompass a complete menstrual cycle. As a result, a definitive interpretation of the menstrual cycle's influence on the subsequent findings could not be established. Baseline values, however, demonstrated greater variability in individual biomarkers and ratios compared to male participants [21].

3.1.3. Evaluation

The conventional steroid profile biomarkers and sulfates AS, EtioS, EpiAS, TS, and ES determined in baseline values, were all within population reference ranges determined in females [36,37]. The observed intra-individual variation confirms previous studies and highlights the difficulties of steroidal ABP analyses in females [28]. All participants showed a basal T/E ratio ≤ 1, with participant 16 showing a very low concentration of T, corresponding to an Asian Profile. However, as no genotyping of the UGT2B17 deletion was conducted, differences compared to other participants cannot be related to this condition with certainty. T determined with GC-MS/MS was mostly $<$ LOQ, and no reliable evaluation including this biomarker was possible. Also, TG determined with LC-HRMS showed either very high RSD in baseline samples or many values below the LOQ. Therefore, ratios including T or TG were not evaluated for O16 and D16.

Among all possible ratio combinations, ratios between the target analytes that had been evaluated as sensitive biomarkers recently for male participants were assessed [19–21]. Additionally, ratios between the same glucuronides and sulfates (for example, AG/AS) and sulfates in the nominator and EpiAG in the denominator were calculated and evaluated. As EpiAG showed more values outside its IT for certain participants compared to male participants, they were included in the evaluation. However, the ratios with AS, EpiAS, and EtioS in the nominator and TS, ES, and EG in the denominator showed the highest sensitivity to T administration. Ratios between the same glucuronides and sulfates, as well as ratios including EpiAG, did not show sensitivity or prolonged DT compared to the already mentioned biomarker ratios. Therefore, the same biomarkers as in part I of the study were selected for further evaluation: the CR ((TG/TS)/(EG/ES)), and all combinations with EpiAS, AS, or EtioS in the numerator and EG, ES, or TS in the denominator. As T administration in female participants was not described in detail in the past, all conventional steroid profile biomarkers and the separate parts of the CR, namely TG/TS and EG/ES, were further included in the evaluation. Ratios including TS were evaluated for all participants, although each participant showed values $<$ LOQ in some

samples (4–39 %). Participants 13 and 15 showed less than 10 % of TS < LOQ (O13, O15, D13, D15).

3.2. Oral administration

In general, after oral administration of TU, a sharp increase in the conventional biomarker T/E is expected. The ratio A/T is considered less informative, but it improves the detection of T administration in individuals with the del/del genotype for the UGT2B17 enzyme. Other conventional steroid profile biomarkers are mainly known to be sensitive to transdermal T administration [3,11].

3.2.1. Comparison of conventional and novel steroid profile biomarkers

The mean of the basal values and the corresponding relative standard deviation (RSD) used to determine the IT are presented in Table A.1 (Supporting Information). In general, phase II metabolite ratios, including the CR, showed a higher intra-individual variability compared to conventional steroid profile biomarkers. Due to the high RSD, certain substances contained a high IT. Different ratios were affected for different participants.

In our study, TU was administered orally multiple times over 4 consecutive days. Samples were collected for five weeks. In Table 1, the number of samples affected by oral TU administration (values above the IT) is presented as a percentage of the total number of samples after the first administration. For each participant, the two ratios showing the most samples above the IT are highlighted. The ratios showing the greatest response to oral T administration among most participants are also highlighted.

The results suggest differences between each participant. The conventional steroid profile biomarkers T/E and A/Etio were most sensitive for participants O13, O15, and O16, whereas sulfate ratios with ES in the denominator showed the highest sensitivity for participants O14, O17, and O18. Most values above the IT were achieved by the sulfate ratios AS/ES, and EpiAS/ES. Although the CR did show values above the IT consistently in all samples, the sensitivity of T/E was greater. No single ratio could be clearly identified as a superior biomarker.

Table 1
Number of samples affected by oral TU administration. All samples after the first administration were considered and are presented in % of the total number of samples.

Ratio	O13	O14	O15	O16	O17	O18	Overall detection sensitivity
T/E	***47 %***	39 %	***40 %***	-	40 %	59 %	45 %
A/T	0 %	8 %	13 %	-	0 %	0 %	4 %
A/Etio	***50 %***	45 %	6 %	***55 %***	9 %	31 %	33 %
5a-Adiol/E	37 %	12 %	21 %	33 %	38 %	71 %	35 %
5a-Adiol/5b-Adiol	2 %	0 %	0 %	11 %	15 %	45 %	12 %
TG/TS	40 %	22 %	33 %	-	32 %	50 %	36 %
EG/ES	42 %	8 %	2 %	9 %	11 %	14 %	14 %
CR	32 %	22 %	35 %	-	32 %	41 %	33 %
EpiAS/EG	3 %	55 %	0 %	33 %	51 %	67 %	35 %
EpiAS/ES	23 %	***90 %***	17 %	31 %	***72 %***	69 %	***50 %***
EpiAS/TS	16 %	16 %	***50 %***	2 %	17 %	43 %	24 %
AS/EG	3 %	39 %	0 %	33 %	26 %	67 %	28 %
AS/ES	29 %	***90 %***	0 %	27 %	***58 %***	***72 %***	***46 %***
AS/TS	15 %	6 %	23 %	3 %	2 %	45 %	16 %
EtioS/EG	29 %	12 %	0 %	***42 %***	15 %	62 %	27 %
EtioS/ES	37 %	59 %	27 %	23 %	23 %	***78 %***	41 %
EtioS/TS	6 %	0 %	31 %	5 %	0 %	50 %	15 %

A key factor in assessing the suitability of a biomarker for detecting T misuse is the detection time (DT). The DT represents the period during which a ratio would be flagged as suspicious in the steroidal ABP, potentially triggering further analysis and confirmation of a doping case. DTs were calculated after the last administration of TU on day 4. Fig. 1 illustrates the DTs for T/E, the CR, and the two sulfate ratios, which exhibited the longest DTs for the majority of participants.

The ratios EpiAS/ES and EpiAS/TS (AS/TS equally) showed the longest DT for all participants. Four participants (O13, O14, O15, and O18) showed values above the IT even in the last samples. Compared to T/E, the DT was multiplied by 14–60 times. The CR showed the same or shorter DTs compared to T/E for most participants. For participant O16, ratios including T and TG could not be determined, and ratios including TS were not sensitive enough for DT evaluation. The ratio EpiAS/ES showed the most reliable results, combining sensitivity and DT.

The enteral administration of TU leads to high drug concentration in the liver [38]. The uridine 5′-diphospho-glucuronosyltransferase superfamily (UGT), which is responsible for the glucuronidation of steroids, is expressed in both the liver and extrahepatic tissues [30]. Therefore, glucuronidation is the major reaction occurring after oral drug intake. But, due to the high concentration of the orally administered drug, metabolism is most likely switching from glucuronidation to sulfation in the liver due to enzyme saturation [15,39,40]. Enzymes responsible for the sulfation of steroids in the liver are cytosolic sulfotransferases (SULTs), with dehydroepiandrosterone SULTs conjugating 3-hydroxysteroids and T. As described in the first part of the study, this enzyme shows higher activity towards A, Etio, and EpiA compared to T [41,42]. This could be a reason why we observe a higher increase in AS, EtioS, and EpiAS compared to other sulfates. Additionally, the clearance of steroid glucuronides generally proceeds faster than the clearance of steroid sulfates. It is presumed that the cause is the irreversible nature of glucuronidation, whereas sulfation is reversible [30]. These characteristics contribute to the valuable information and prolongation of DT for the detection of T administration.

T/E, CR and the phase II metabolite ratio showing the best results regarding sensitivity and DT (EpiAS/ES) are shown as examples for two participants in Fig. 2.

The expected notable increase in the conventional biomarker T/E was observed, with the CR behaving very similar, as no decreasing trend in TS was anticipated. Also, EG/ES only showed minor fluctuations without any trend. The increase in other phase II metabolite ratios was generally more moderate and values exceeded the IT for a longer period. The significant increase for participant O14 in EpiAS/ES after 253 h was due to a drop of TG, TS, EG, and ES in this sample, presumably connected to the menstrual cycle.

3.2.2. Comparison to studies including male participants

Part I of this study, including male participants, clearly indicated that sulfate ratios showed higher sensitivity to oral T administration compared to conventional steroid profile biomarkers. For women, results are less significant. The sulfate ratio EpiAS/ES showed the most reliable results. The conventional biomarker T/E showed comparable sensitivity and DT after oral TU administration between male and female participants. In men, various ratios, including EpiAS, AS, and EtioS in the numerator and TS and ES in the denominator, showed a great improvement in DT. In women, these results can be confirmed, although with less certainty. Ratios with TS in the denominator are more prone to fluctuations, as values were close to the LOQ for all participants. Results for ratios including EG and ES in the denominator have to be interpreted with caution, as values above the IT could also derive from a decrease of EG and ES caused by the menstrual cycle. The study highlights the more challenging evaluation of female steroid profiles. Nevertheless, trends observed in male participants could be confirmed, strengthening the importance of including phase II metabolites in future steroid profiling.

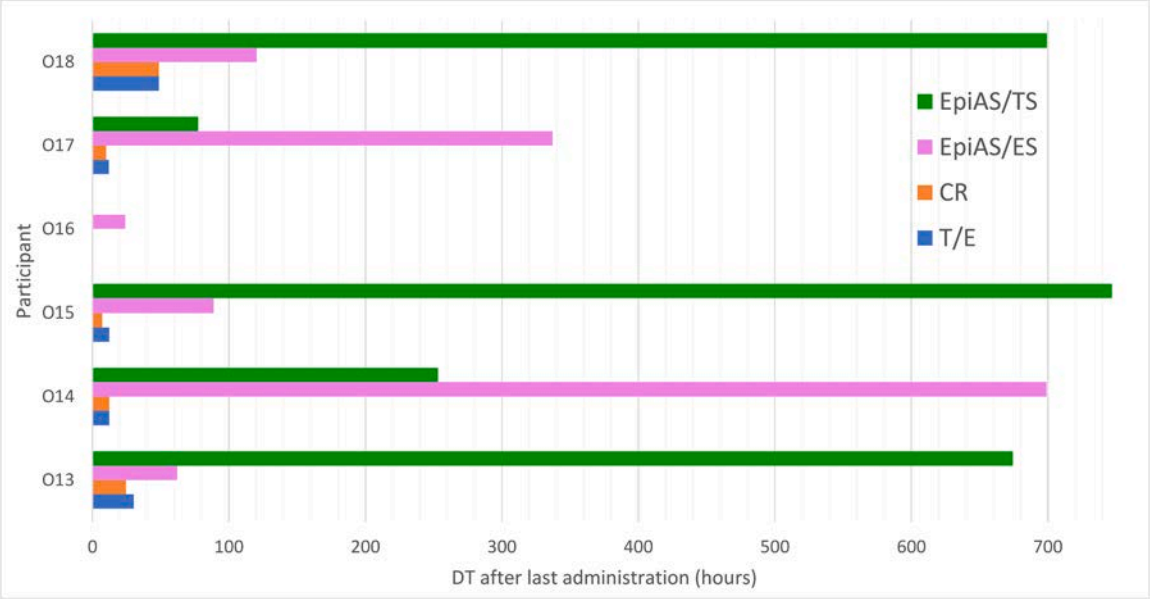

Fig. 1. DT after last administration (t = 0) of TU tablets. Comparison of T/E, CR, and the sulfate ratios EpiAS/TS and EpiAS/ES for all participants.

3.3. Transdermal administration

In general, transdermal administration of T is described to lead to a low increase in conventional biomarkers and short DT [13,43,44]. Nevertheless, T/E, 5a-Adiol/E, 5a-Adiol/5b-Adiol, and A/Etio are suitable biomarkers to detect T gel administration [7,45]. 5β-reduction is catalyzed by enzymes primarily found in the liver, 5α-reduction takes place mainly in peripheral tissues. Therefore, the higher level of 5α-reductase in the skin leads to a higher production of 5α-metabolites and an increase in 5a-Adiol and A [3,30,46].

3.3.1. Comparison of conventional and novel steroid profile biomarkers

In Table A.2 (Supporting information), the mean of the basal values and the corresponding relative standard deviation (RSD) used to determine the IT for the investigated ratios are presented. Phase II metabolite ratios, including the CR, showed a higher intra-individual variability compared to conventional steroid profile biomarkers. Due to the high RSD, certain substances contained a high IT. Different ratios were affected for different participants.

In our study, multiple administration of T gel was carried out over 5 days. Samples were collected for five weeks. In Table 2, the number of samples affected by the transdermal T administration (values above the IT) is presented as a percentage of the total number of samples since the first administration. For each participant, the two ratios showing the most samples above the IT are highlighted. The ratios showing the greatest response to transdermal T administration among most participants are also highlighted.

The conventional biomarker T/E showed the highest sensitivity in four participants (D13, D14, D15, D17). For D16, the calculation of ratios including T and TG was not possible, as the participant showed an Asian profile with concentrations < LOQ. D18 showed generally low sensitivity to T gel administration. Other conventional steroid profile biomarkers showed different sensitivity for each participant. Generally, an increasing trend in the ratios 5a-Adiol/E and 5a–Adiol/5bAdiol could be observed; A/Etio showed no increase. Ratios including EpiAS, AS, and EtioS in the numerator and TS and ES in the denominator showed generally very low sensitivity to transdermal T administration. EtioS/EG showed moderate sensitivity for D14, D15, D16, and D17. The CR did show less sensitivity to transdermal T administration compared to T/E. No ratio could clearly be selected as a superior biomarker, although conventional T/E showed the highest sensitivity in most participants.

For further evaluation of the applicability of the biomarkers, DTs were calculated after the last administration of T gel on day 5. In Fig. 3, the DT for T/E, the CR, and the phase II metabolite ratio EtioS/EG are displayed.

Although T/E showed the highest sensitivity for most participants, especially during the administration phase, it did not stay elevated as long after administration as ratios including sulfates. Although it could not be determined in all participants, the CR did show longer DT compared to the conventional T/E ratio. The ratio EtioS/EG prolonged the DT compared to T/E and other phase II metabolite ratios. The ratios T/E, CR, and EtioS/EG showed the most reliable results, combining sensitivity and DT. It must be considered that these proposed ratios all include E, which is known to be an unreliable biomarker due to its dependency on the menstrual cycle.

The slower and moderate increase in T and metabolite concentrations after transdermal T administration compared to oral administration is explained by the metabolism occurring mainly in peripheral tissues and not in the liver. Also, sulfation likely occurs at various sites [41,42,47]. Unlike after oral TU administration, the CR prolonged DT in most participants, supporting the idea that the CR is a better biomarker for parenteral T administration compared to the conventional T/E ratio.

T/E, CR, and the phase II metabolite ratio showing the best results regarding sensitivity and DT (EtioS/EG) are shown as examples for two participants in Fig. 4.

After transdermal T administration, the conventional T/E showed a steady and much less sharp increase, and values were elevated for a longer period compared to oral TU administration. The CR showed elevated values for a longer period in most participants. The ratio EtioS/EG showed a delayed increase in values compared to T/E and CR and was elevated for a longer period.

3.3.2. Comparison to studies including male participants

In the first part of the study involving male participants, ratios derived from various combinations of EpiAS, AS, and EtioS in the numerator and TS, ES, and EG in the denominator showed a significant improvement in detecting transdermal T administration compared to traditional steroid profile biomarkers. These findings could not be confirmed for women, as ratios including EpiAS and AS did not demonstrate high sensitivity or long DTs. Ratios with EG in the denominator showed longer DT compared to other sulfate ratios with ES or TS in the denominator, with EtioS/EG showing the longest DT for most

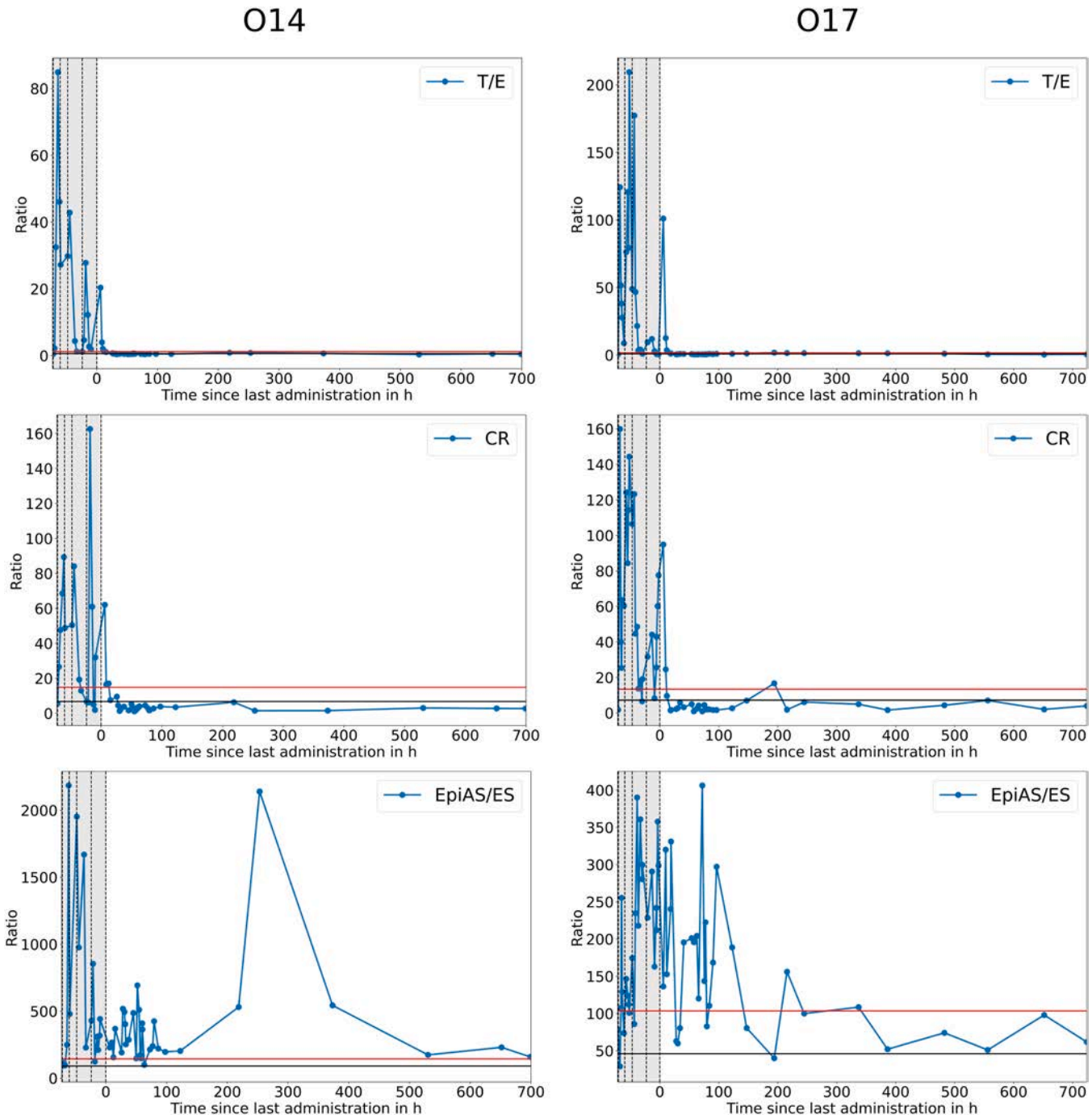

Fig. 2. Comparison of conventional T/E, CR, and EpiAS/ES for participants O14 (basal T/E 0.62) and O17 (basal T/E 0.89). The administration phase (grey) and the total period after the last administration (t = 0) of TU tablets are displayed. The mean of baseline values (black) and the IT (red) are displayed horizontally; administration of TU is displayed vertically (black, dotted).

participants. Additionally, the CR extended the DT compared to T/E. It is important to note that all ratios with high sensitivity or long DT include the parameter E, which can affect the results due to the influence of the menstrual cycle. Recent studies [19,21] conducted in males proposed ratios including AS, EpiAS, and EtioS in the numerator. In females, focusing on the conventional T/E, the CR, and EtioS provides the best results.

3.4. Overall applicability

Phase II metabolites of EAAS have been shown to prolong the DT following oral and transdermal T administration in women. Comparing the results between the sexes [21], the same trends were observed, although in women, they were less pronounced. The CR demonstrated greater sensitivity to parenteral T administration compared to enteral T administration, consistent with results from part I of the study conducted on male participants. No universal biomarker was identified, underscoring the significant intra-individual variability among participants. Nevertheless, the same biomarkers recommended for steroid profiling in men are proposed for women: ratios including EpiAS, AS, and EtioS in the numerator and TS, ES, and EG in the denominator.

However, certain limitations of the study, as well as the reliability of

Table 2
Number of samples affected by transdermal T gel administration. All samples since the first administration were considered and are presented in % of the total number of samples.

Ratio	D13	D14	D15	D16	D17	D18	Overall detection sensitivity
T/E	***70 %***	***91 %***	***79 %***	-	***82 %***	13 %	***67 %***
A/T	0 %	0 %	0 %	-	0 %	0 %	0 %
A/Etio	0 %	42 %	3 %	0 %	18 %	***30 %***	15 %
5a-Adiol/E	62 %	***72 %***	5 %	39 %	***68 %***	0 %	41 %
5a-Adiol/5b-Adiol	11 %	35 %	0 %	3 %	9 %	6 %	11 %
TG/TS	***66 %***	56 %	0 %	-	25 %	***19 %***	33 %
EG/ES	0 %	0 %	3 %	0 %	0 %	0 %	0 %
CR	49 %	42 %	33 %	-	32 %	2 %	32 %
EpiAS/EG	15 %	0 %	8 %	***74 %***	32 %	11 %	23 %
EpiAS/ES	11 %	2 %	3 %	13 %	5 %	0 %	5 %
EpiAS/TS	9 %	0 %	0 %	3 %	9 %	6 %	5 %
AS/EG	9 %	12 %	8 %	65 %	20 %	11 %	21 %
AS/ES	2 %	9 %	8 %	0 %	2 %	0 %	4 %
AS/TS	2 %	30 %	0 %	3 %	14 %	4 %	9 %
EtioS/EG	4 %	42 %	***51 %***	***87 %***	55 %	11 %	***42 %***
EtioS/ES	0 %	23 %	5 %	19 %	2 %	0 %	8 %
EtioS/TS	4 %	40 %	3 %	19 %	0 %	4 %	12 %

specific biomarkers, were more pronounced in women. One notable limitation was the lack of menstrual cycle tracking during the T administration phase, as well as insufficient baseline sampling. Future studies should plan the sampling more strategically according to menstrual cycle phases. This approach would yield more reliable results for ratios involving EG and ES, which are not reliable due to their strong dependence on the menstrual cycle. By appropriate sampling and monitoring of menstrual cycle phases, a better distinction between fluctuations caused by exogenous T administration or by the menstrual cycle would be possible. This would add further valuable data for the evaluation of female steroid profiles. Furthermore, ratios including TS showed comparable results to other biomarkers for oral TU administration, although many values showed concentrations close to the LOQ of the analytical method. As these low concentrations are not very reliable for a robust monitoring system, the results must be evaluated with caution. Nevertheless, the longitudinal evaluation of TS provides interesting information that has not been previously described. In future studies, reliable biomarkers that are independent of the menstrual cycle should also be included. Recently investigated parameters, such as dehydroandrosterone sulfate, have shown their potential after oral TU administration and should be investigated further [20].

4. Conclusions

In the presented work, phase II EAAS metabolites were examined in women after T administration for the first time. Conventional steroid profile biomarkers were compared with the CR and various ratios of glucuronidated and sulfated EAAS for the detection of oral and transdermal T administration. More pronounced than in men, high variability in sulfate concentrations was observed among most participants. The DT was extended for most participants compared to the conventional steroid profile biomarkers. Although E is known as a fluctuating and unreliable biomarker for females, the results still indicate EG and ES as the most useful parameters used in the denominator for ratio biomarkers. To improve the monitoring system, the inclusion of as many different biomarkers as possible is suggested, as inter-individual variability and different administration routes result in varying biomarker sensitivities. However, the interpretation of these novel biomarkers in women requires greater caution. Ratios involving TS may show higher fluctuations due to low concentration levels near the LOQ. Ratios including EG and ES are strongly influenced by the menstrual cycle, potentially leading to an increased number of falsely suspicious samples in women compared to men.

These findings highlight the need for a more detailed review of biological passports in female athletes, emphasizing the importance of individualized APMU evaluations. For instance, the concentration of individual biomarkers could provide valuable insights, even when conventional ratio biomarkers do not flag a passport as suspicious. Based

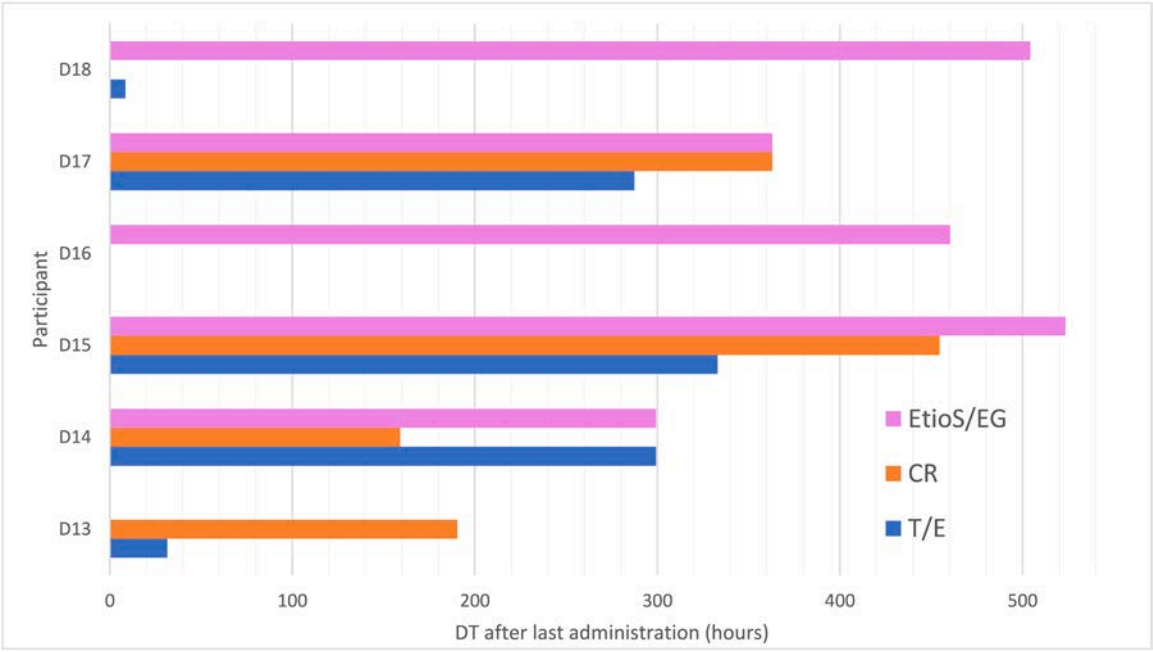

Fig. 3. DT after last administration (t = 0) of T gel. Comparison of T/E, CR, and the sulfate ratio EtioS/EG for all participants.

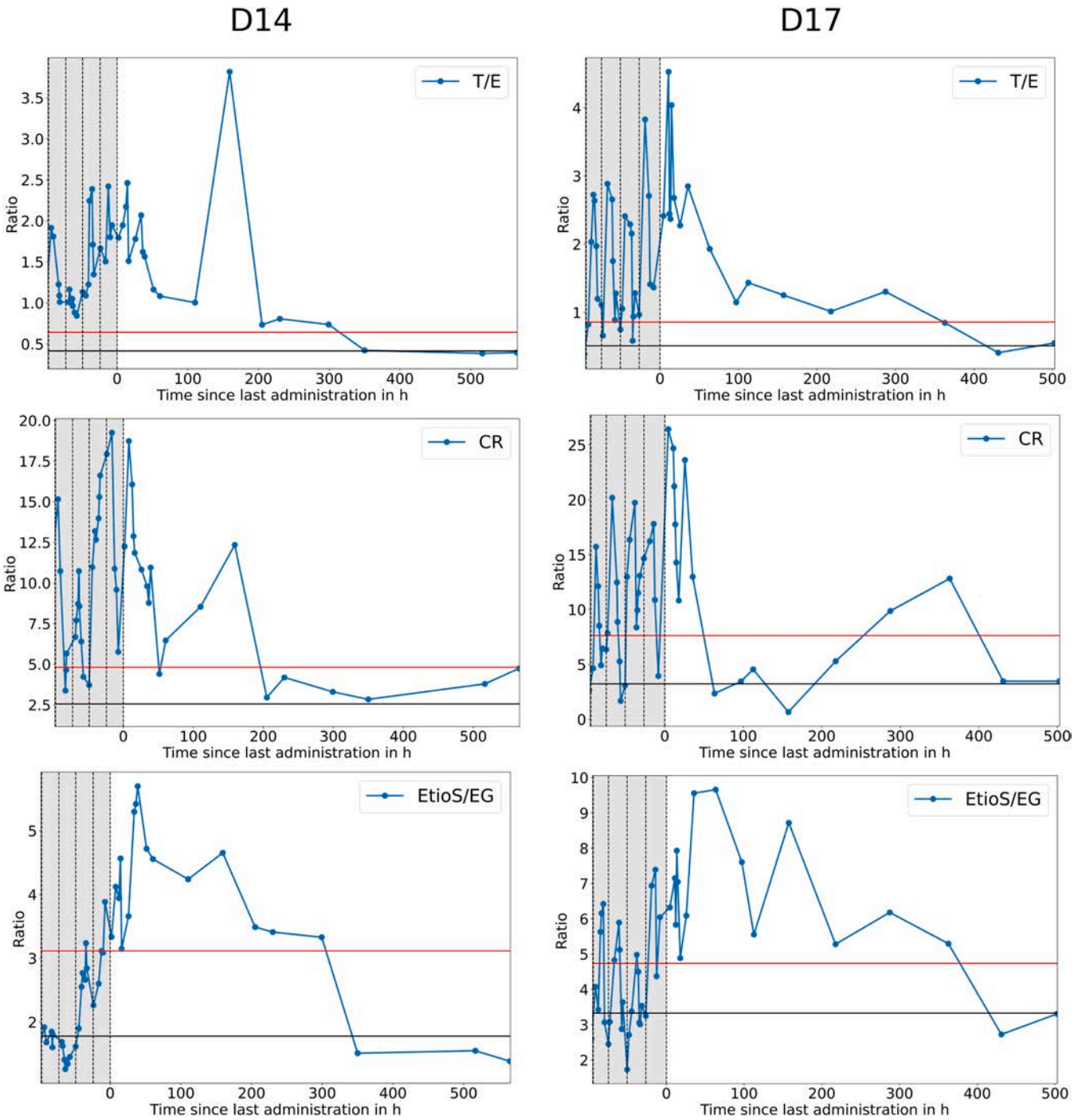

Fig. 4. Comparison of conventional T/E, CR, and EtioS/EG for participants D14 (basal T/E 0.41) and D17 (basal T/E 0.51). The administration phase (grey) and the total period after the last administration (t = 0) of T gel are displayed. The mean of baseline values (black) and the IT (red) are displayed horizontally; administration of T is displayed vertically (black, dotted).

on the combined findings of part I and part II of this study, we propose the inclusion of phase II EAAS metabolites in future steroid profiling.

The prolonged DTs of the proposed novel urinary biomarkers, combined with emerging techniques as blood steroid profiling and dried blood spot sampling, have the potential to enhance the performance of the ABP and strengthen doping case investigations by providing additional evidence [23,24,48–51]. Additional tests recommended by APMU experts, together with recommendations for long-time storage of samples, should be followed accordingly and promptly. The ability to conduct retrospective analysis, combined with the integration of multiple layers of evidence through the inclusion of different biomarkers and matrices in the ABP, plays a crucial role in detecting EAAS misuse and offers a promising approach to addressing the complexities of T doping detection.

CRediT authorship contribution statement

Marija Andjelkovic: Resources, Project administration, Methodology, Investigation, Conceptualization. **Nenad Dikic:** Resources, Project administration, Methodology, Investigation, Conceptualization. **Guro Forsdahl:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition,

Conceptualization. **Günter Gmeiner:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Conceptualization. **Sandra Pfeffer:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Gmeiner, Guenter reports financial support was provided by World Anti-Doping Agency. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2025.106905.

Data availability

Data will be made available on request.

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