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Title: Inter-individual variation of the urinary steroid profiles in Swedish and Norwegian athletes

Year: 2020

Version: Accepted version (Final draft)

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Please cite the original version:

Mullen, J., Bækken, L. V., Törmäkangas, T., Ekström, L., Ericsson, M., Hullstein, I. R., & J Schulze, J. (2020). Inter-individual variation of the urinary steroid profiles in Swedish and Norwegian athletes. Drug Testing and Analysis, 12(6), 720-730. https://doi.org/10.1002/dta.2778

Check for updates

Received: 25 October 2019 | Revised: 5 February 2020 | Accepted: 5 February 2020

DOI: 10.1002/dta.2778

RESEARCH ARTICLE

WILEY

Inter-individual variation of the urinary steroid profiles in Swedish and Norwegian athletes

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Funding information
World Anti-Doping Agency, Grant/Award Number: ISF16D12JS

Abstract

The steroidal module of the Athlete Biological Passport (ABP) aims to detect doping with endogenous steroids, e.g. testosterone (T), by longitudinally monitoring several biomarkers. These biomarkers are ratios combined into urinary concentrations of testosterone and metabolically related steroids. However, it is evident after 5 years of monitoring steroid passports that there are large variations in the steroid ratios complicating its interpretation. In this study, we used over 11000 urinary steroid profiles from Swedish and Norwegian athletes to determine both the inter- and intra-individual variations of all steroids and ratios in the steroidal passport. Furthermore, we investigated if the inter-individual variations could be associated with factors such as gender, type of sport, age, time of day, time of year, and if the urine was collected in or out of competition. We show that there are factors reported in today's doping tests that significantly affect the steroid profiles. The factors with the largest influence on the steroid profile were the type of sport classification that the athlete belonged to as well as whether the urine was collected in or out of competition. There were also significant differences based on what time of day and time of year the urine sample was collected. Whether these significant changes are relevant when longitudinally monitoring athletes in the steroidal module of the ABP should be evaluated further.

KEYWORDS

athlete biological passport, confounding factors, doping in sports, steroid profile, urinary steroids

1 | INTRODUCTION

The fight against doping in sports has changed markedly since the implementation of the Athlete Biological Passport (ABP). This method aims to detect the use of prohibited substances or methods through individual and longitudinal monitoring of selected biomarkers. Initially the ABP only included the hematological module, but from 1 January 2014 the steroidal module was added to the Anti-Doping Administration & Management System (ADAMS).[1]

The steroidal passport aims to detect doping with endogenous steroids, e.g. testosterone (T), and uses several biomarkers for this detection. The biomarkers of the steroid profile are testosterone and its metabolites androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol), and 5β-androstane-3α,17β-diol

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(5βAdiol), as well as epitestosterone (E). The markers are measured in urine as the combination of the free steroids and the glucuronidated fraction.[2] These steroids are in the passport combined into the ratios T/E, A/Etio, A/T, 5αAdiol/5βAdiol, and 5αAdiol/E.

The individual and longitudinal monitoring of the biomarkers are of interest, because the intra-individual variability is lower than the corresponding inter-individual variability.[3] Both the hematological and steroidal modules use Bayesian statistics for longitudinal profiling, and progressively switch from a population based to individually calculated reference ranges as the test numbers increase.[3] Using this approach, each athlete has his or her own reference ranges for biological markers. The goal by using Bayesian theory is to evaluate how likely the passport data are assuming a normal physiological condition.[4] However, there are factors other than doping that can affect the ratios used in the steroidal passport. The effects of these factors on all ratios in the profile need to be fully evaluated in order to improve the interpretations of these steroidal passports to better assist antidoping organizations in their testing strategy and to evaluate the likelihood of doping.

To minimize the pre-analytical and analytical variability, the World Anti-Doping Agency (WADA) has strict rules on the sample collection procedure[5] as well as the laboratory procedures.[6] In addition, much of the variability in, for example, circadian rhythm, exercise, tapering, food intake, and dehydration is reduced by the use of steroid ratios, instead of the absolute concentrations.[7-9] The largest confounders of the steroid passport are genetic factors,[10-13] bacterial contamination,[14,15] alcohol,[16-18] and certain non-prohibited drugs.[19-23] The genetic polymorphism known to have the largest impact on the steroid profile is the double deletion polymorphism (*del/del*) of uridine 5′-diphospho-glucuronosyltransferase 2B17 (UGT2B17)[12] where carriers of the del/del alleles excrete very low levels of testosterone glucuronide and hence have low T/E ratios. However, this and other genetic factors are constant, and the statistical program will adapt to this confounder after a number of tests (3–4 tests). Bacterial contamination and alcohol are detected and reported in the urine analysis and non-prohibited drugs should be reported by the athlete on the doping control form. However, after 5 years of monitoring steroid passports, large variations of the steroid ratios are still unexplained. An extensive review on the confounding factors in steroid profiling was published recently,[24] but the origin and extent of this variation in longitudinal profiles in athletes need to be evaluated. One such study has been conducted recently in a large population of male football players.[25] The study was based on 4195 urine samples analyzed prior to 2014, i.e. before the steroid module was released in ADAMS. Nevertheless, the study was a proof of principle of the usefulness of steroid profiling.

In this study, we used 11009 steroid profiles collected from more than 5400 Swedish and Norwegian athletes to determine both the inter- and intra-individual variations of all steroids and ratios in the steroidal passport. Furthermore, we investigated whether inter-individual variations could be associated with factors such as gender, age, type of sport, collection time of day, and time of year as well as whether the sample was taken in or out of competition.

2 | MATERIALS AND METHODS

2.1 | Study population

All steroidal measurements registered in ADAMS since the implementation of the steroid module in 2014 until 31 March 2017 from Swedish and Norwegian athletes were exported. 11009 steroid profiles from 5473 athletes were included in this study, of which 4180 were male athletes with a total of 7780 samples and 1293 were female athletes with 3229 steroid profiles.

Individuals that did not have Swedish or Norwegian nationality registered in ADAMS were excluded (n = 1558), as were profiles with any testing authority other than RF (Swedish Sports Confederation) or ADNO (Anti-Doping Norway) (n = 1614). Further exclusion criteria included samples where sample validity said "No", samples with analysis results "Adverse Analytical Finding" (AAF) containing substances listed in the Prohibited List, sections S1 (Anabolic agents), S2 (Peptide hormones, growth factors, related substances and mimetics), S4 (Hormone and metabolic modulators), and S5 (Diuretics and masking agents), "Atypical" (ATF) when the reason was other than T/E > 4 and those "Not analyzed". All samples with confounding factors such as ethanol consumption (detected via ethylglucuronide > 5 μg/mL) and declared use of 5α-reductase inhibitors were excluded. Lastly, profiles with comments under either section Analysis details/explanation/opinion that can possibly affect the steroid profile were excluded (n = 397). Both the Norwegian and Swedish laboratories used gas chromatography-tandem mass spectrometry (GC–MS/MS) to measure the steroids following the current version of the TD2014EAAS[26]/TD2016EAAS.[27]

2.2 | Data collection and processing

Steroid profiles were extracted from ADAMS to Microsoft Excel. All concentrations measured below the limit of quantification (LOQ) were set to LOQ for corresponding steroid using the highest LOQ of the Stockholm or Oslo laboratory. The collective LOQ used was 100 ng/mL for A and Etio, 1 ng/mL for testosterone and epitestosterone and 5 ng/mL for 5αAdiol and 5βAdiol. Ratios based on steroids lower than LOQ were not analyzed but were reported as missing values, the exception being the T/E ratio where the laboratory reported T/E ratio was used. All steroid concentrations were corrected for specific gravity according to the laboratories' measurement of specific gravity of that sample.

The sports were divided into seven sport classifications to study differences between similar sports. According to the recommendations from an exercise physiologist, the sports were divided into the categories: power/strength sports, VO_2 max endurance sports, muscular endurance sports, ball and team sports, fight sports, aiming sports, and gymnastics sports. The full list of what sports belong to what category can be found in the supplemental material (Supplemental Table S1). Sports tested less than 10 times were not included in the sports classification and are reported as missing values (n = 12 excluded sports).

2.3 | Statistical analysis

The statistical modeling and analyses were made using Mplus[28] (version 5.2, 2008) and R (version 3.3.2, 2016) and two figures were made using GraphPad Prism, version 7 for Windows (La Jolla, California, USA). Results were considered significant when $P < 0.05$ (2-sided tests).

3 | RESULTS

3.1 | Study population

After using the exclusion criteria described above, a total of 7780 samples from male and 3229 from female athletes were included in this study. 72% of the male athletes were only tested once, 15% were tested twice, and 13% were tested three or more times. The same numbers for the female athletes were 66% (1 test), 12% (2 tests), and 22% (≥ 3 tests). In total, 42% were Swedish and 58% Norwegian athletes, among those 0.8% were reported as dual citizens. The majority of the steroid profiles for men came from Norwegian athletes (62%), whereas the majority for women came from Swedish athletes (53%). 2.5% of the samples were analyzed at a WADA accredited laboratory other than the Norwegian or Swedish Doping Control Laboratory. 4652 (42%) of all samples were collected in competition (44% for men and 38% for women). The average age was 25.3 ± 5.3 years for men and 25.4 ± 5.7 for women. The top 10 most tested sports can be found in the supplemental material (Supplemental Table S2).

3.2 | Statistical modeling

All steroids except for testosterone followed a log-normal distribution. Testosterone and hence the ratios including testosterone were bimodal and required a two-group mixture model (see supplemental Table S3 for more information). For women however, the bimodal testosterone distribution was not seen and therefore the log-normal model was used for testosterone and ratios including testosterone. Additionally, due to observations below the detection threshold some observed concentrations were recoded to the threshold value and modeled using a left-censored model for the log-normal distribution. We report the quartiles of the log-normal distribution for all concentrations and concentration ratios. Because the median of the log-normal distributions coincides with the geometric mean, we also report 95% confidence intervals of the medians in figures.

3.3 | Descriptive statistics

Table 1 shows descriptive statistics for all steroids and ratios used in the steroid profile, divided only into men and women. The first part of the table gives values calculated for the whole population where the data were modeled according to the best fit model, which never was Gaussian. All values are corrected for athletes tested more than once (i.e. every athlete has equal impact on the results, regardless of how many times he/she is tested, as the individual's geometric mean for the corresponding biomarker was used). The medians and IQR (inter quartile range) in Table 1 are computed from the log-normal cumulative distribution function, these are very similar to the same values calculated for the Gaussian distribution found in the supplemental material (Supplemental Table S3). The CV, on the other hand, is calculated both from the log-normal distribution as well as the non-modeled data.

The last two columns of Table 1 show intra-individual variation expressed as CV for individuals with 10 or more samples (n = 115 for men and 70 for women). The best-fit model for intra-individual distribution was log-normal for all metabolites and ratios of the steroid profile. For the biomarker concentrations there are no missing values because values < LOQ were set to the corresponding LOQ. However, ratios based on concentrations < LOQ were excluded and are reported as missing values. For women this was a substantial number (number of samples below LOQ are shown in the text below Table 1). The variation of the ratios is lower than for corresponding concentrations and intra-individual variation was always lower than inter-individual variation.

From the testosterone distribution for men it was calculated that approximately 13.6% belonged to the low testosterone excretion group in the bimodal distribution and are therefore believed to have the double deletion polymorphism (*del/del*) of UGT2B17. The probability of most likely within this group is 0.966 and the higher testosterone excretion group is 0.989, if assigned to that group. The same estimation cannot be conducted for women since the sensitivity of the method was not sufficient to give a proper distribution for the low testosterone group.

How much of the variation that can be explained by the variables studied is illustrated in Figure 1. The variables are sport classification, test type i.e. in competition (IC) or out of competition (OOC), age, time of day, and time of year. The exact values for each ratio and concentration of the steroidal module can be found in the supplemental material (Supplemental Table S4).

3.4 | Sports classification

The sports were divided into seven different classifications based on the physiology of the sport (see supplemental Table S1 for categorization). The number of steroid profiles in each category for males and females respectively were 845 and 505 in Power/Strength sports; 2208 and 1187 in VO_2 max Endurance sports; 711 and 600 in Muscular Endurance sports; 3254 and 441 in Ball and Team sports, and 565 and 352 in Fight sports. The two last categories (Aiming sports and Gymnastic sports) had few steroid profiles (in total 199 from males and 144 from females) and were therefore excluded from the statistical calculations. Sports classification was one of the largest factors contributing to the total inter-individual variation in the steroid profile. How T, E, and T/E differ between different sport categories can be seen in Figure 2. The complete steroid profile and additional

TABLE 1 Descriptive statistics of the concentrations and ratios of the steroid profile in (A) men and (B) women. The first part of each table gives values calculated for the whole population where the data were modeled according to the best fit model, the distribution parameters can be found in supplemental Table S3. All values are corrected for athletes tested more than once. The median and IQR are computed from the model, CV is calculated based on the model as well as the non-modeled data (reported as "traditional CV"). The last two columns describe the intra-individual variations based on athletes with 10 or more tests. All concentrations below LOQ were set to LOQ, whereas ratios based on steroids lower than LOQ were reported as missing values, the exception was the T/E ratio where the laboratory reported T/E ratio was used. For testosterone and ratios with testosterone the analysis is divided into two groups based on the bimodal testosterone distribution, the same could not be done for women since so many values were below LOQ

(A)	Inter-individual (n = 7780)							Intra-individual (n = 115)	
	Model predicted values[a]								
MEN	Number missing	Best fit model[a]	Median[b] (ng/mL)	Q (25)[c] (ng/mL)	Q (75)[c] (ng/mL)	CV[d]	Traditional CV[e]	Best fit model	Traditional CV
Testosterone, low T	--	Two component mixed model	2.9	1.9	4.4	70.3%	71.1%	Log-normal	34.5%
Testosterone, high T			29.5	20.5	42.5	58.5%		Log-normal	
Epitestosterone	--	Log-normal	19.6	11.9	31.9	83.3%	72.8%	Log-normal	38.8%
5αAdiol	--	Log-normal	44.3	29.1	67.6	69.1%	65.0%	Log-normal	36.1%
5βAdiol	--	Log-normal	85.8	49.0	150.5	100%	89.6%	Log-normal	38.0%
Androsterone	--	Log-normal	2392	1659	3447	58.4%	53.5%	Log-normal	31.9%
Etiocholanolone	--	Log-normal	1610	1120	2314	58.0%	54.5%	Log-normal	34.4%
T/E, low T	16	Two component mixed model	0.13	0.09	0.17	49.4%	97.7%	Log-normal	16.4%
T/E, high T			1.43	0.95	2.16	67.1%		Log-normal	
A/T, high T	95	Two component mixed model	85	60	120	55.1%	160.7%	Log-normal	22.5%
A/T, low T			769	551	1073	52.5%		Log-normal	
A/Etio	--	Log-normal	1.49	1.08	2.05	50.9%	48.5%	Log-normal	20.4%
5αAdiol/5βAdiol	65	Log-normal	0.52	0.34	0.79	69.4%	67.4%	Log-normal	22.4%
5αAdiol/E	70	Log-normal	2.28	1.44	3.59	75.9%	87.1%	Log-normal	26.7%

(B)	Inter-individual (n = 3229)							Intra-individual (n = 70)	
	Model predicted values[a]								
WOMEN	Number missing	Best fit model[a]	Median[b] (ng/mL)	Q (25)[c] (ng/mL)	Q (75)[c] (ng/mL)	CV[d]	Traditional CV[e]	Best fit model	Traditional CV
Testosterone	--	Log-normal	3.9	2.1	7.2	116.6%	89.5%	Log-normal	44.3%
Epitestosterone	--	Log-normal	4.1	2.2	7.6	115.2%	89.4%	Log-normal	52.7%
5αAdiol	--	Log-normal	14.4	8.8	23.7	84.7%	77.1%	Log-normal	35.3%
5βAdiol	--	Log-normal	40.0	21.2	75.5	119.4%	94.5%	Log-normal	40.5%
Androsterone	--	Log-normal	1420	900	2239	76.1%	69.2%	Log-normal	34.8%
Etiocholanolone	--	Log-normal	1624	1096	2408	63.7%	61.0%	Log-normal	34.6%
T/E	608	Log-normal	1.05	0.67	1.67	76.1%	74.8%	Log-normal	39.9%
A/T	432	Log-normal	298	200	446	65.2%	242.5%	Log-normal	28.4%
A/Etio	2	Log-normal	0.87	0.62	1.24	55.1%	184.0%	Log-normal	22.7%
5αAdiol/5βAdiol	389	Log-normal	0.36	0.22	0.59	83.6%	73.1%	Log-normal	31.7%
5αAdiol/E	493	Log-normal	3.42	2.19	5.34	74.0%	70.0%	Log-normal	40.2%

[a]Distribution parameters shown in Appendix Table A-3

[b]Computed from the log-normal cumulative distribution function. The median is computed as Md(X) = Exp(μ), and it coincides with the geometric mean of log-normal distribution.

[c]Computed from the log-normal cumulative distribution function with parameters μ and σ2 in Mathematica, version 10.4.

[d]Arithmetic coefficient of variation: CV(X) = [Exp(σ2) - 1]1/2

[e]"Traditional" coefficient of variation: CV = σ/μ

Number of cases at or below detection threshold:

T: n(T ≤ 1) = 432 (13.4%)

E: n(E ≤ 1) = 315 (9.8%)

5αAdiol: n(5αAdiol ≤ 5) = 341 (10.6%)

5βAdiol: n(5βAdiol ≤ 5) = 132 (4.1%)

A: n(A ≤ 100) = 1 (0.03%)

Etio: n (Etio ≤100) = 2 (0.1%)

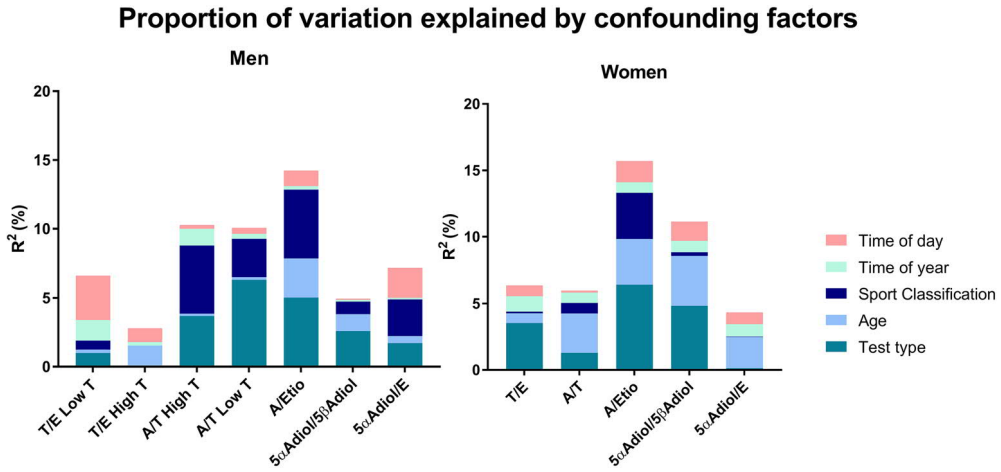

FIGURE 1 R^2-values describing how much of the inter-individual variation (percent of arithmetic CV) of the ratios of the steroidal profile can be explained by different factors. The exact numbers can be found in supplemental Table S4. The rest, up to 100%, is unknown. For men, the ratios with testosterone are divided into two groups based on where they belong in the bimodal testosterone distribution. For women this same division could not be done due to too many values below LOQ [Colour figure can be viewed at wileyonlinelibrary.com]

information about these analyses can be found in the supplemental material.

Male athletes belonging to the Ball and Team sports had the highest steroid metabolite concentration with 10–25% higher concentrations than the combined median. In particular, the 5α-steroids were higher in the Ball and Team sports compared with the VO_2 max endurance sports category, with A and 5αAdiol being 69% and 76% higher in the Ball and Team sports category (see supplemental Table S5). The T/E ratio, however, differed very little between the groups (Figure 2).

In the female sports categories, there were significantly larger differences than among the male categories. Consistent with the men, the female Ball and Team sports category had higher 5α-steroids with A and 5αAdiol being 42% and 65% higher than the VO_2 max endurance category. The Ball and Team female athletes also had significantly higher testosterone concentrations, 37% higher than the combined median and 80% higher than the Power/Strength sport. Here we could also see significant differences in the T/E ratio, with Ball and Team sports having the highest T/E ratio (1.2) and Muscular Endurance the lowest (0.96).

3.5 | In or out of competition

After correcting for all other studied factors, women show a greater difference between steroid profiles obtained in competition and out of competition compared with men. Female urine collected in competition had significantly higher concentrations of all steroids, except for 5βAdiol, than urine collected out of competition and affected all ratios significantly (Table 2). Even though the effects were not as profound for men, four out of five ratios of the steroid profiles show a significant difference in competition compared with out of competition, however, unlike the women, only one ratio was increased due to increased concentrations of the numerator (the A/Etio ratio). The other ratios differed due to the different degrees of decreased concentrations of the components of the ratios (Table 2).

3.6 | Annual variations

Men showed less annual variation for all ratios compared with women (Figure 3). There were no significant differences in T/E, 5αAdiol/5βAdiol, and 5αAdiol/E between any of the months for men. The A/T, however, was significantly lower in June and August compared with March, the medians differing by 16% at the most (between March and June, $P < 0.05$). The A/Etio was significantly lower in June compared with October, November, December, and February, the greatest difference of medians being 10% between June and December. Time of year could explain at the most 1.4% of the variation of the steroid levels in men, and this was for testosterone in the group already excreting low amounts (data can be found in supplemental Table S4).

In the female population, all ratios showed significant annual variations when comparing medians for each month. The T/E was significantly higher in September compared with July and December, the median being 30% higher in September compared with December. T/E was the ratio with the greatest difference between medians of months, A/T showing at the most 23% (between October and December), A/Etio 17% (between April and December), 5αAdiol/5βAdiol 28% (between October and November), and 5αAdiol/E 28% (between April and December). Of the total inter-individual variation of the ratios in the ABP, time of year can only explain 1.2% at the most and this was for T/E.

3.7 | Circadian variations

More than 6% of the total inter-individual variation for men in E and Etio could be explained by the time of day (data can be found

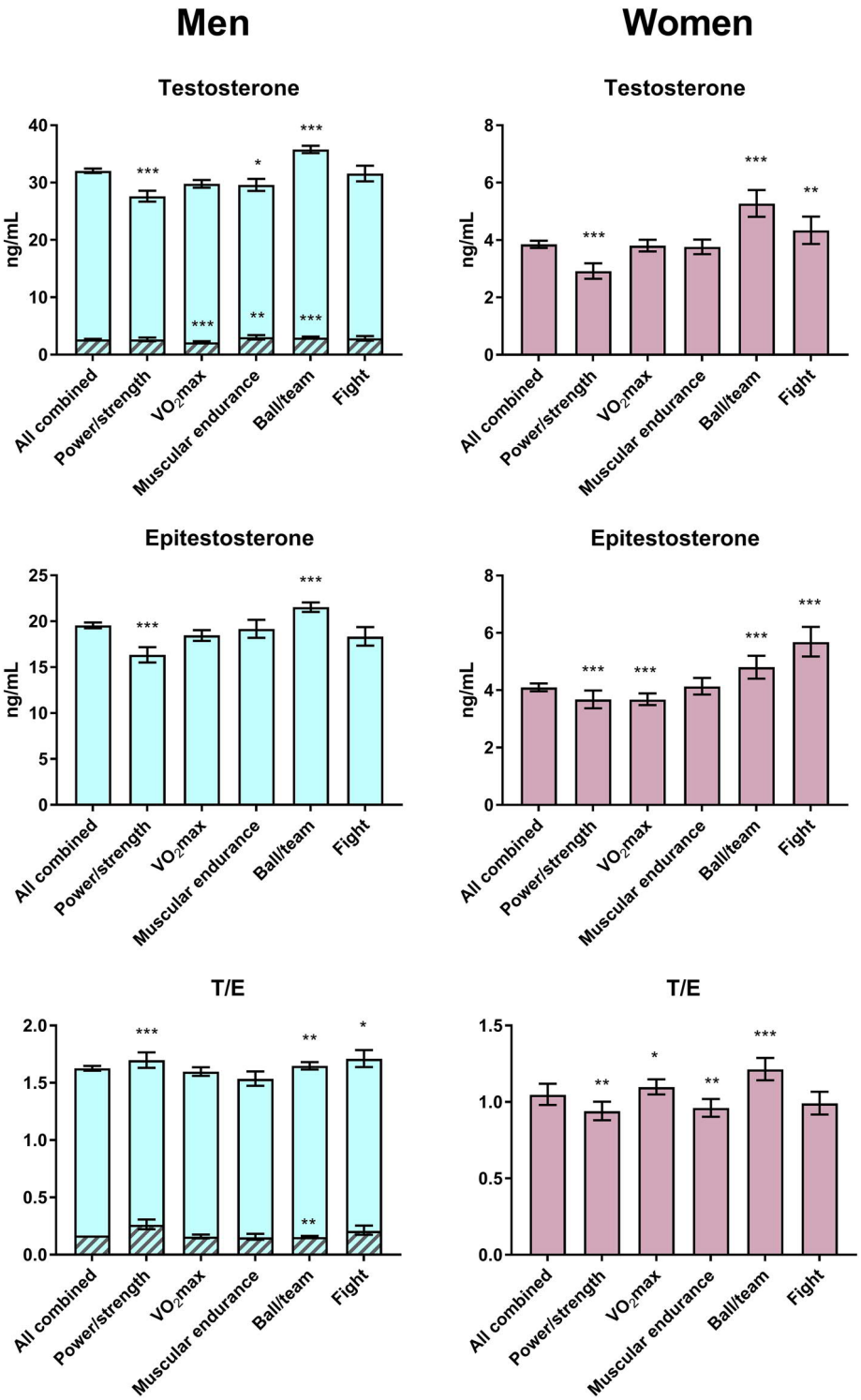

FIGURE 2 Differences in testosterone, epitestosterone, and T/E between participants in different sport categories. The height of the bar is at the geometric mean and the line represents the 95% CI. The striped bars represent the low testosterone group among the male athletes. Asterisks represent significances compared with all sports combined (first bar) where *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, after having corrected for all other confounders as well as multiple testing. All other concentrations as well as the number of tests in each category can be found in supplemental Table S5 [Colour figure can be viewed at wileyonlinelibrary.com]

in supplemental Table S4). Also part of the variations in testosterone levels were explained by at what time the urine was collected, at least for the individuals belonging to the high testosterone excretion group in the testosterone distribution ($R^2 = 0.053$). All ratios showed significant changes over the day ($P < 0.05$). The T/E was significantly lower in the morning (6:00–9:59) than at night (18:00–18:59 and 20:00–22:59) with the lowest values at 9:00 h and the highest at 18:00 h, the median differing by 33%. Urine collected in the morning (6:00–6:59) showed a significantly lower A/T than urine collected from 16:00 and forward, with 31% maximum difference in median (6:00 compared with 16:00). A/Etio was significantly lower before 10:00 compared with after 12:00, with lowest values at 9:00 and highest at 17:00 (median differing by 32.1%). 5αAdiol/5βAdiol followed a more random pattern throughout the day with a maximum difference between medians of 29% (between 10:00 and 14:00). 5αAdiol/E increased steadily over the day with maximum difference in medians of 43%.

TABLE 2 Steroid profile changes in competition (IC) compared with out of competition (OOC) with a *P*-value to describe level of significance between samples collected in and out of competition. For men, testosterone and ratios with testosterone were divided into two groups based on where they belong in the bimodal testosterone distribution. For women this same division could not be done due to too many values below LOQ

		Men		Women	
		Change IC	*P*-value	Change IC	*P*-value
T	*Low T*	−5%	0.322	+65%	<0.001
	High T	−3%	0.040		
E		−4%	0.007	+43%	<0.001
5αAdiol		−1%	0.692	+23%	<0.001
5βAdiol		−13%	<0.001	−7%	0.323
A		+16%	<0.001	+42%	<0.001
Etio		+4%	0.005	+18%	<0.001
T/E	*Low T*	+12%	<0.001	+16%	<0.001
	High T	−1%	<0.001		
A/T	*High T*	+21%	0.188	−15%	<0.001
	Low T	+25%	0.674		
A/Etio		+12%	<0.001	+21%	<0.001
5αAdiol/5βAdiol		+16%	<0.001	+26%	<0.001
5αAdiol/E		+5%	0.004	−13%	<0.001

Women show significant changes in all ABP ratios throughout the day. A/T only showed a significant change between 12:00 and 18:00, with a 28% increase. T/E varied over the day with a 36% maximum difference between medians. As for men, A/Etio was lower in the morning compared with at night with a 40% higher median at 23:00 compared with 10:00. Unlike for men, 5αAdiol/5βAdiol showed an increase over the day with highest values at 16:00 and lowest at 7:00 where the medians differed by 48%, making this the ratio with the greatest difference. 5βAdiol/E was 30% higher at 20:00 than at 7:00, where the medians differed the most.

3.8 | Age

Urinary testosterone shows peak concentrations at approximately 20 years for men and decreases slowly until about 35, the wide CI (due to fewer samples) after 35 makes it difficult to make conclusions as to what happens after this age (Figure 4). The same can be said for the T/E ratio. Women on the other hand show stable testosterone with age but a drop of T/E due to increases in E with age (data not shown). These variations with age that can be seen for the T/E explains less than 1.5% of the variation that can be seen naturally in the T/E ratio. The ratios where most of the variation can be explained by age was A/Etio for men (R^2 = 2.85) and 5αAdiol/5βAdiol for women (R^2 = 3.76).

4 | DISCUSSION

In this study, with 11009 steroid profiles from both male and female athletes, we investigated how factors reported during a doping test affect the steroid profile used in the steroid module of the ABP. This study shows that there are significant changes in the urinary steroid concentrations and ratios of the steroidal module, in urine taken in different sports, at different ages, times of day, time of year, and in or out of competition.

The steroidal levels and ratios observed in this extensive Nordic population correspond well to other studies of athletes.[25,29] We were able to confirm in this large population that the use of ratios was superior to absolute concentrations, since the ratios show lower inter- and intra-individual variation. The variability in circadian rhythm as well as annual rhythm were lower for the ratios compared with the concentrations, and the use of ratios also by-passes the need for urine dilution correction.

When modeling the data, log-normal distributions were used except for male testosterone and ratios with testosterone (i.e. T/E and A/T) which used a mixed model of two log-normal distributions. That testosterone can be modeled by two log-normal distributions was shown by Ayotte et al. in 1996.[30] The female testosterone, T/E, and A/T distribution showed a single log-normal distribution rather than a mixed model, the reason for this was likely due to the great number of missing values (Table 1). In this study, testosterone concentrations in urine from women with the double deletion of *UGT2B17* are likely excluded due to testosterone levels being below LOQ. It has been shown before that the distribution of female testosterone also is bimodal due to a deletion polymorphism in *UGT2B17*.[31]

From the testosterone distribution for men it was calculated that approximately 13.6% belonged to the low testosterone excretion group and are therefore believed to have the double deletion of *UGT2B17*.[12] To calculate the group belonging based on low or high testosterone excretion has previously been done by Sottas et al. and they, as we did, assigned 13% to the low testosterone group.[32] This

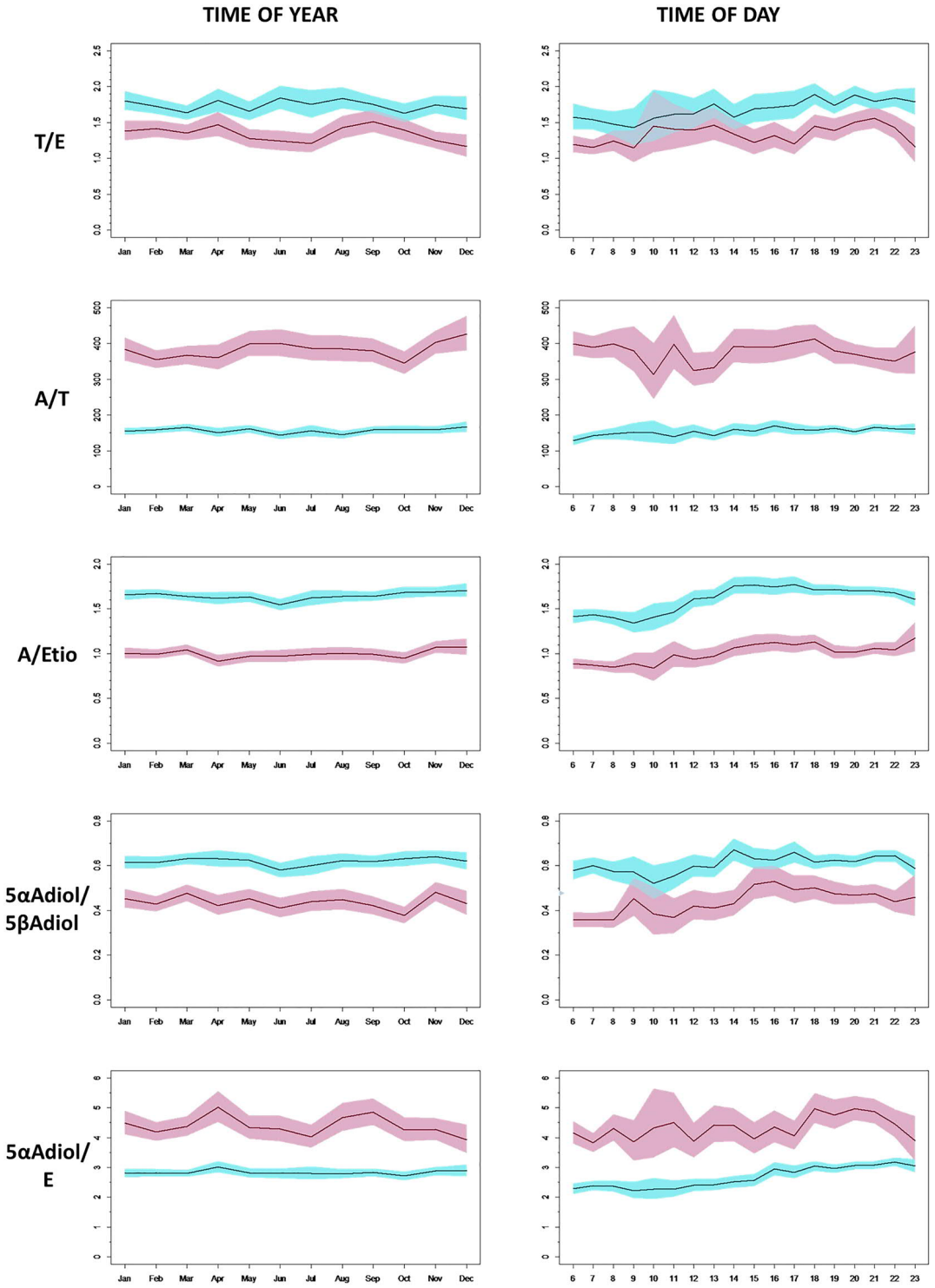

FIGURE 3 Variations in the ratios of the steroidal module during different months of the year and different times of the day. The data are shown as median with 95% CI as calculated by the model after correction for test type, sport classification, age, and time of year (for time of day) and time of day (for time of year). The pink lines are women and the blue are men [Colour figure can be viewed at wileyonlinelibrary.com]

was slightly higher than the prevalence of *UGT2B17 del/del* previously determined by genotyping in Swedish populations.[12,33]

There were large interindividual variations in the steroid profiles and only part of this variation, up to 16%, could be explained by the factors studied (Figure 1). A large portion of the unknown variation is likely due to genetic differences in the production and metabolism of steroids.

The two factors that had the largest impact on the steroid profile were sports classification and whether the sample had been collected in or out of competition (test type).

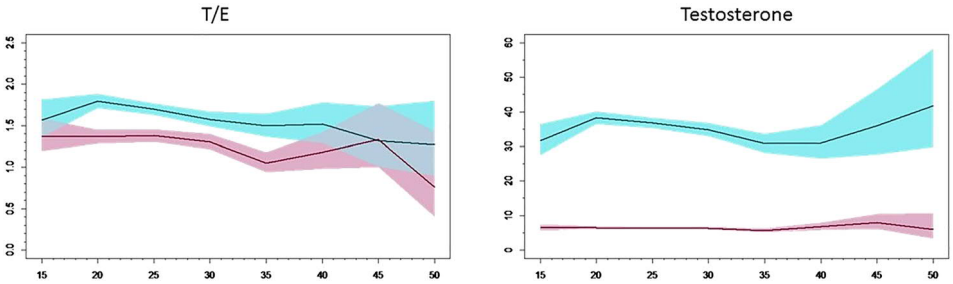

FIGURE 4 Urinary T/E and testosterone with age described as median with 95% CI. The blue is men and pink is women [Colour figure can be viewed at wileyonlinelibrary.com]

For women, all steroids showed higher levels when collected IC than OOC except for 5βAdiol. T/E, A/Etio, and 5αAdiol/5βAdiol show higher levels in competition whereas A/T and 5αAdiol/E are lower. For men all ratios but A/T were affected but to a lesser extent. The greater effect of in competition samples for women is likely due to a large portion of female steroids being produced by the adrenal cortex which is under control of the stress hormone adrenocorticotropic hormone (ACTH).[7] Interestingly, the male athletes belonging to the low testosterone group in the testosterone distribution (likely *UGT2B17 del/del*) show a greater difference in T/E in competition versus out of competition. Also interestingly, 5αAdiol/E is lower in competition for women but higher for men. When evaluating steroid profiles collected in competition from female athletes it is recommended to take into consideration that the steroid concentrations and ratios are expected to be somewhat increased compared with the samples collected out of competition.

We found small, but statistically significant, differences in urinary steroid concentrations depending on the sports category. Both male and female athletes in the Ball and Team sports had higher testosterone and 5α-steroid metabolite levels, even after correction for other confounding factors, such as whether the sample was collected in or out of competition. While this is not interesting from a longitudinal steroid passport evaluation point of view, it may be interesting from a physiological point of view.

If stress and the adrenal cortex are important players for urinary steroid levels, a much larger proportion of the athletes in individual sports are tested in a calm home environment, while Ball and Team players are most often tested in connection with training sessions together with their team, therefore, one cannot completely rule out the stress hormone factor effect described above on the higher steroid concentrations in the Ball and Team sport category.

Training induced modifications have also been implicated to affect the urinary steroids. A recent study by Amante et al.[34] found that moderate and high intensity activity alter the steroid profile compared with samples collected after rest in a male marathon runner. This could also partly explain why the athletes belonging to the Ball and Team sports have higher median steroid concentrations, as the majority of their samples are collected after training, while this is not the case with the Norwegian and Swedish athletes in participating in individual sports.

Hence, the sports category differences may, in fact, be attributed to whether the samples are collected in connection with training and in stressful situations versus after rest in calm home environments.

The annual variation of ABP ratios was less pronounced in men than in women. Men only showed significant changes in two of the ratios (A/T and A/Etio) and the median between months differed at the most by 16%. Women, on the other hand, showed significant differences in all the ratios with a maximum difference in the median of 30%. Most likely this variation is masked by the large inter and more interesting intra-individual variation.

All ABP ratios in men were lower before noon and increased over the day. The increase from the lowest to the highest was approximately 30% for all ratios except for 5αAdiol/E which varied more (43%). As the 5α-steroids were speculated to be increased due to stress and/or activity, it could also be surmised that this finding may be connected to activity, where the morning samples are predominantly collected after rest, and the 5α-steroids are always the numerator in the different ratios. Again, women show greater variation than men and also a more random pattern over the day. However, the confidence intervals were also larger making the interpretation more difficult. The larger confidence intervals were likely due to a greater variation shown in women than in men but also a lower number of test subjects. However, there seems to be a great difference in some of the ratios from the urine collected at different times of the day with up to a 48% increase in one of the ratios (5αAdiol/5βAdiol). It is possible that this variation can be seen when studying the passports longitudinally. However, because of the random variation over the day, adjustments of the current adaptive model for circadian rhythm would be very difficult to perform.

Interestingly, the ratios with 5α-metabolites divided by 5β-metabolites (i.e. A/Etio and 5αAdiol/5βAdiol) showed the largest variation with age, possibly indicating a change in preference of metabolic route with age. However, no drastic changes could be seen in any ratios indicating that age is not something to be considered when evaluating passports with continuously collected samples.

This study is the largest evaluation of steroid profiles providing additional information about the natural inter-individual variability and factors influencing the steroid profile of male and female athletes. However, the results may not be representative of a general population but rather athletes from this region. In addition, 97.5% of all samples were analyzed at either the Stockholm Doping Control Laboratory or the Norwegian Doping Control Laboratory minimizing the effect of between laboratory variability.

Unfortunately, the lists of medications were not easily accessible so there is no exclusion based on permitted drug use. Medications such as hormonal contraceptives are known to affect the steroid

profile in women and probably contribute to some of the variability, especially for epitestosterone.[20,23] Also, exclusion based on confounding factors such as alcohol and ketoconazole was only possible after these substances started being reported with the implementation of a new technical document 1 January 2016.[27] Another limitation of this study is the inclusion of some doped individuals not testing positive knowing that the prevalence of doping has been shown to be higher than the percentage of Adverse Analytical Findings reported by the WADA accredited laboratories.[35]

In this large study of over 11000 steroid profiles, we show that there are factors reported in today's doping tests that significantly affect the steroid profiles. Some of these factors, in particular test type, should most likely be taken into consideration when evaluating steroid profiles. Other factors, such as sports category and circadian variation, were also shown to be important confounders of the steroid profile, however, whether these are of importance when athletes are monitored with the steroidal module of the ABP has to be further evaluated.

ACKNOWLEDGEMENTS

This work was supported by grants from the World-Anti Doping Agency.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Mullen J, Bækken LV, Törmäkangas T, et al. Inter-individual variation of the urinary steroid profiles in Swedish and Norwegian athletes. *Drug Test Anal*. 2020;12:720–730. https://doi.org/10.1002/dta.2778