NATIONAL SPORTS ACADEMY „VASSIL LEVSKI”
SPORT FACULTY, DEPARTMENT OF PHYSIOLOGY AND BIOCHEMISTRY

Abstract of PhD Thesis

NATIONAL SPORTS ACADEMY „VASSIL LEVSKI”
SPORT FACULTY, DEPARTMENT OF PHYSIOLOGY AND BIOCHEMISTRY

Tanya Zapryanova Sheytanova

Analysis of the steroid profile of athletes and non-athletes Bulgarians

Abstract of PhD Thesis

of a dissertation for acquiring an educational and scientific degree "DOCTOR" in the scientific specialty "Theory and Methodology of Sports Science", professional field 7.6. "Sports"

Scientific Supervisor: Assoc. prof. Lubomir Petrov, PhD

Reviewers:
Assoc. Prof. Dr. Milena Georgieva Nikolova
Prof. Vihren Stoychev Bachev, D.Sc
Assoc. Prof. Milka Milcheva Mileva, D.Ph
Assoc. Prof. Dr. Albena Jordanova
Prof. Dr. Rositza Nikolova Zamfirova-Mitova

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The dissertation consists of four chapters. It contains 177 pages, 380 cited literature sources, 13 figures, 24 tables.

Subject of the dissertation for acquiring ESD Doctor:

Analysis of the steroid profile of athletes and non-athletes Bulgarians

Tanya Zapryanova Sheytanova

The defense of the dissertation will take place before

Scientific jury:

1. Assoc. Prof. Dr. Milena Georgieva Nikolova, Nalional Sport Academy “Vassil Levski”, Sofia
2. Prof. Vihren Stoychev Bachev, D.Sc, Nalional Sport Academy “Vassil Levski”, Sofia
3. Assoc. Prof. Milka Milcheva Mileva, DPh, Institute of Microbiology, Bulgarian Academy of Sciences, Sofia
4. Assoc. Prof. Dr. Albena Jordanova, Medical Faculty of Sofia University "St. Kliment Ohridski”, Sofia
5. Prof. Dr. Rositza Nikolova Zamfirova-Mitova, Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia

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2 INTRODUCTION

Anabolic-androgenic steroids (AAS) are essential for the body's adaptation to physical activity. In addition to resulting in intensive protein synthesis, accelerated muscle growth and increased physical strength, less subcutaneous adipose tissue and accelerated recovery, AAS block the glucocorticosteroid receptors (GR), thus showing they also have anti-catabolic properties (Kicman, 2008). Maintaining optimal androgen levels depends on the balance between biosynthesis and metabolic clearance and their excretion. This encourages some athletes to use steroid preparations to speed up recovery and enhance their athletic performance.

On the other hand, the accumulated evidence of proven adverse side effects from the use of AAS, along with their performance enhancing-properties, led to their inclusion in the WADA Prohibited List.

In order to detect any exogenously administered synthetic androgenic steroid analogues (testosterone, 5α-dihydrotestosterone (DHT) or 4-androstenedione (androstenedione), the changes in urinary steroid profile biomarkers shall be monitored. Initially, the differentiation between endogenous and exogenous testosterone was established by a *testosterone* over *epitestosterone* (T/E) *ratio*, and subsequently the GC/C/IRMS analysis and the steroid module of the Athlete Biological Passport (ABP) were introduced. WADA statistics from the individual longitudinal monitoring of each athlete introduced by ABP, through the steroid module, show multiple atypical results that require expert evaluation and further follow-up of the profile. In addition to doping, many other factors, such as gender, age, ethnicity, metabolic characteristics, enzyme induction or inhibition, circadian rhythms and physical activity, can affect the biosynthesis, metabolism and excretion of steroids.

The sports science is greatly interested in the effects of physical exercise on steroid metabolism. The study of this issue reveals the hormonal regulation adaptation processes at different stages of athletes' training.

Scientific literature has published many studies, in blood and urine, of the main androgens, androgenic precursors and glucocorticosteroids in the blood - testosterone, dehydroepiandrosterone (DHEA) and cortisol, cortisone and their relationships as markers for the level and balance of anabolic and catabolic processes in the body, immediately before and after different types of physical activity, by using various analytical methods. The studies of active androgens, their metabolites and urinary precursors, carried out by GC-MS, in individuals

with different intensity of physical activity, are very few and have been carried out with different methods. Application of a validated method in AAS analysis ensures reliable and reproducible results obtained in different laboratories and allows for the accumulation of a large amount of comparable data.

This research is focused on the quantification of endogenous steroids by a GC-MS method validated in accordance with requirements of ISO 17025 and WADA ISL and TD EAAS, in urine samples of non-athletes, leisure athletes and actively training women and men, in order to demonstrate the possibilities of the urinary steroid profile as a tool for analysis of changes in steroidogenesis associated with physical activity.

Interpretation of the urine steroid profile data can provide more detailed information on the athlete's adaptation to physical activity based on changes in the steroid metabolism during sports training and as a non-invasive method to help control the training process.

3 PURPOSE, TASKS AND RESEARCH METHODS

3.1 Research purpose

The goal of the study is to make a comparative analysis of the steroid profile of non-athletes, leisure athletes and actively training women and men 18 to 30 years old and to determine the appropriate biomarkers of adaptive changes in steroidogenesis associated with physical training.

3.2 Research tasks

To achieve this goal, the following tasks were formulated:

1. Analysis of the specialized sources on the topic of human steroid profile and the effect of physical training thereon.

2. Collection of urine samples from volunteers - women and men, 18 to 30 years old, non-athletes, leisure athletes and actively training individuals.

3. Quantification of steroids in urine samples by GC-MS method validated in accordance with the requirements of ISO 17025 and WADA ISL and TD EAAS.

4. Statistical processing of data obtained.

5. Characteristics and comparative analysis of the steroid profile in non-athletes, leisure athletes and actively training individuals (swimmers) men and women 18 - 30 years old.

6. Analysis of the effect of the physical activity on the biosynthesis and metabolism of steroids and drawing-up of decisive rules to determine the adaptation of steroid metabolism to different intensity training programs

3.3 Object of the study

The object of the study is the steroid profile in human urine

3.4 Subject of the study

The subject of the study is the analysis of changes in the steroid profile in the urine of non-athletes, leisure athletes and actively training Bulgarian individuals.

3.5 Subjects

The subjects were 573 women and men - non-athletes, leisure athletes and actively training 18 to 30 years old individuals.

The groups of female ana male non-athletes and leisure athletes were students from the University of Architecture, Civil Engineering and Geodesy (UACEG) and the National Sports Academy (NSA) in Sofia.

The groups of actively training women and men included swimmers training 8 to 10 times a week and regularly participating in national and international competitions.

Thus, 6 groups were formed:

F0 - 78 female non-athletes of an average age of 20,92 ± 3,17 years

F1 - 76 female leisure athletes of an average age of 21,84 ± 3,87 years

F2 - 12 active female athletes of an average age of 19,40 ± 2,27 years

M0 - 107 male non-athletes with an average age of 21,03 ± 2,95 years

M1 - 228 male leisure athletes with an average age of 21,06 ± 2,91 years

M2 - 12 active athletes with an average age of 19,50 ± 3,79 years.

Among the groups so compared, a significant age difference was found only between the female leisure and active athletes ($p<0,05$). The difference of only 2,44 years in the average age of women in their early 20s cannot be a factor significantly affecting the steroid profile.

A total of 190 urine samples from female volunteers and 383 from male volunteers were tested. Upon analysis of results and comparison with method validation parameters and steroid concentration limit values and some related ratios in accordance with criteria of WADA, the results of 149 female and 289 male samples were subjected to statistical processing (see Table 3, 4, 5, 6, 7 and 8).

3.6 Organization of research

The study was carried out in the period from 2016 to 2018. All participants filled out an informed consent form for participation in the study. Respondents with a sedentary lifestyle and non-athletes respectively identified themselves as non-athletes or leisure athletes (1 to 3 times a week).

Urine samples were collected in sterile plastic containers with a volume of 150 ml and stored at – 200C until their analysis. Samples from non-athletes and leisure athletes were collected in the morning, between 9am and 1pm.

Active athletes were providing samples once a week, on Monday morning (between 8 and 10 am), a total of 4 samples for a period of one month.

Analysis of the urine samples was performed in the Doping Control Laboratory at the Anti-Doping Centre with the Ministry of Youth and Sports. Under

Urine pH and specific gravity were measured, followed by quantification of endogenous steroids in urine.

A validated intra-laboratory method in line with the requirements of ISO 17025, WADA ISL and TD EAAS was used to determine the concentrations of endogenous Anabolic Androgenic Steroids (AAS).

3.7 Method for quantification of steroids in urine

The validated intra-laboratory method is intended for quantitative analysis by gas chromatography with mass spectrometry of urine samples after hydrolysis, liquid-liquid extraction and derivatization (TD2014EAAS).

The unit of measurement used with regard to the respective AAS concentrations is ng/ml.

Experimental protocol:

1. Sample preparation (Figure 1): 1 ml of urine was hydrolyzed with β-Glucuronidase (E. coli K12) at pH = 7.0 and 50°C for 1 hour, followed by liquid-liquid extraction (LLE) with tert-butyl methyl ether (t-BME) at pH = 9.6. The separated organic phase was evaporated to dryness, dried in vacuo and derivatized with 50 µl MSTFA/NH_4I/2-ME (500: 1: 3). d5-Etiocholanolone, d3-Epitestosterone and d3-Testosterone were used as internal standards (ISTD).

2. Loading of samples on GC/MS.

3. Analysis of samples and processing of raw data to determine the concentrations of the measured substances.

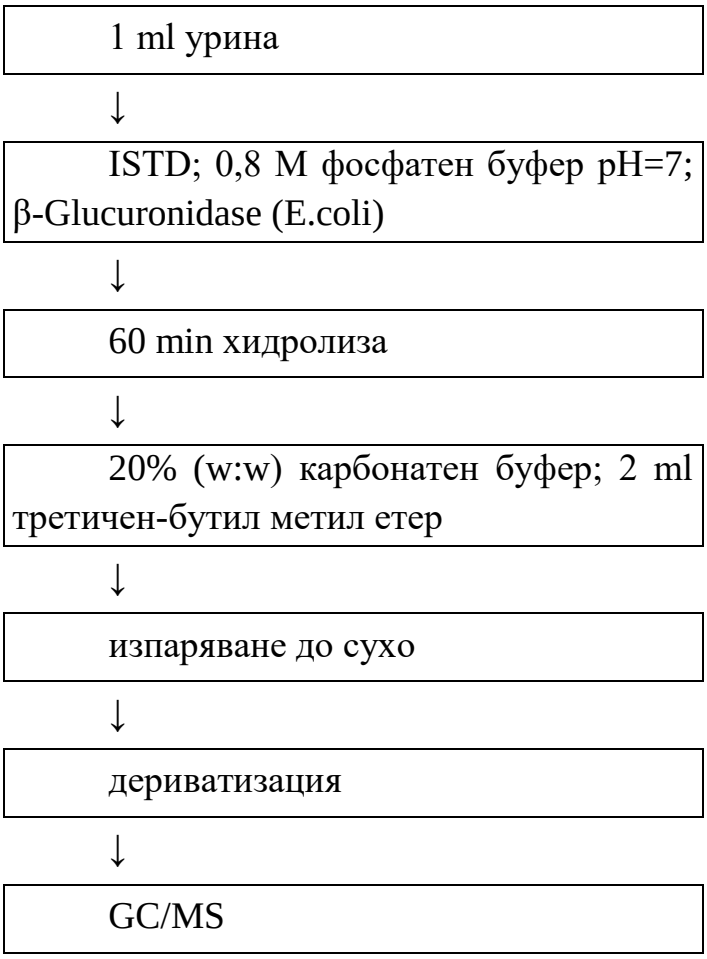

Figure 1. Sequence of sample preparation operations.

Chromatographic separation and quantification

The samples were analyzed by gas chromatography with a mass-selective detector Agilent Technologies 7890A/5975C. Agilent DB-1MS gas chromatographic column (20 m x 180 μm x 0.18 μm) - 100% Dimethylpolysiloxane was used. Each sample was tested in triplicate, injecting 2 μl. The temperature gradient of the oven starts from 190ºC with a retention of 0 min. Then heat to 218ºC with 2ºC/min and hold for 3 min. The second step of the gradient is up to 230ºC with 2ºC/min and holding 0 min. The third step of the gradient is up to 300ºC with 20ºC/min and holding for 6 min. Helium at a rate of 1 ml/min was used as the carrier gas. The mode of operation of the detector is SIM, in case of ionization with electronic impact (70 eV). The compounds were found: androsterone (m / z 434), etiocholanolone (m/z 434), 5α-Androstane3α, 17β-diol (m / z 436), 5β-Androstane-3α, 17β-diol (m/z 436), epithestosterone (m/z 432), testosterone (m/z 432), 11β-hydroxyandrosterone (m/z 522), 11β-hydroxyethiocholanolone (m/z 522), d4-androsterone (m/z 438), d5-etiocholanolone (m/z 439), d3-epithestosterone (m / z 435), d3-testosterone (m/z 435). A 5-point calibration curve was used to quantify the compounds.

3.8 Reagents and chemicals used

Methanol, HPLC
Tertiary butyl methyl ether, HPLC
Fluka potassium dihydrogen phosphate, purity> 99.5%
Dialium hydrogen phosphate Fluka, purity> 99.0%
Sigma potassium hydrogen carbonate, purity> 99.5%
Fluka potassium carbonate, purity> 99.0%
β-Glucuronidase (E. coli K12) Roche Diagnostic GmbH
N-Methyl-N- (trimethylsilyl) trifluoroacetamide (MSTFA), purity ≥ 97%.
2-mercaptoethanol (2-ME), Sigma purity ≥ 98%.
Ammonium iodide NH4I, Sigma purity ≥ 98%.

The certified reference materials (CPMs) and reference materials (PMs) used are presented in **Table 1**.

Table 1. Certified reference materials (CPM) and reference materials (PM) used.

Substance	Description	
Androsterone		PM
Etiocholanolone	CPM	
5α-Androstane-3α,17β-diol	CPM	
5β-Androstane-3α,17β-diol	CPM	
Epitestosterone	CPM	
Testosterone	CPM	
11β-Hydroxyandrosterone		PM
11β-Hydroxyetiocholanolone		PM

The operating parameters of the AAS quantification method are presented in **Table 2**.

Table 2. Detection limit (LOD), quantification limit (LOQ) and linear range of the method.

Substance	LOD, [ng/ml]	LOQ, [ng/ml]	Range, [ng/ml]	Linearity (r^2)
A	1	250	250 - 10 000	0,9978
E	1	250	250 - 10 000	0,9977
11β-OH-A	2	100	100 - 3000	0,9883
11β-OH-E	2	100	100 - 3000	0,9855
5αA3α17βdiol	2	5	5 - 300	0,9845
5βA3α17βdiol	2	5	5 - 300	0,9869
epiT	0,6	1	1 - 300	0,9984
T	0,4	1	1 - 300	0,9985

Statistical processing

The variational analysis of the obtained results and the determination of the reference limits of the steroid concentrations in the urine were performed with specialized software for statistical processing of pharmacological data Refval 4.11 and SPSS 26.

We checked the normality of the data distribution according to the Kolmogorov-Smirnov method. The conclusions about the reliability of the differences in the medians of the results obtained from the different study groups were performed with non-parametric ANOVA for independent samples - Kruskal-Wallis method using a post hoc test of Bonferroni.

4 RESULTS AND ANALYSIS

4.1 Results

4.1.1 Validation of tested samples

A total of 573 urine samples were examined, of which 190 from female volunteers and 383 from male. After comparison of the measured concentrations of the tested steroids with the validation parameters of the method for quantitative determination of steroids in urine (LOD and LOQ), samples in which a concentration of one or more steroids below LOD or LOQ was found dropped from the respective samples. For the other samples, the WADA criteria were applied for maximum permissible concentrations of A, E, 5αA3α17βdiol, epiT and T and for the limit values of the ratios T/epiT, A/T. 5α/5β and 5α/epiT. Samples in which one or more of these requirements were not met were also removed from the relevant samples. Thus, 149 samples of women and 289 samples of men remained for statistical processing.

Tables 3, 4, 5, 6, 7 and 8 present the values of the rejection criteria and the number of samples dropped as not meeting the relevant requirements. The total number of non-compliant results is higher than the number of dropped samples, because for some of the samples non-compliances were found with the criteria for more than one examined indicator.

Out of all 78 samples of female non-athletes, as shown in Table 3, 18 were removed, for which discrepancies were found for a total of 28 results. Most (8 in number) of them were removed because the concentration of 11β-OH-E was below the LOQ of the method. A total of 9 results were dropped due to measured concentrations of A or E, or epiT below LOQ. One result for 11β-OH-A was removed due to a value below LOQ. Six results were above the WADA concentration limits: 3 for 5αA3α17βdiol (above 150 ng/ml) and 3 for epiT (above 50 ng/ml). Deviations were also observed in the ratios between the concentrations of steroids included in ABP. The T/epiT ratio was increased in one sample and to 5α/epiT in 2 samples. In one sample, the A/T ratio was below 20, the minimum required by WADA. After removing the non-compliant samples of female non-athletes, the results of 60 samples (77% of the analyzed samples of female non-athletes) were used for statistical analysis.

Table 3. Removed samples (n = 18) of female non-athletes (F0), according to the validation criteria of the method (LOD and LOQ) and the WADA criteria.

Parameter	LOD [ng/ml]	LOQ [ng/ml]	WADA	F0 (samples 60/78; 77%)		
				< LOD	< LOQ	≠ WADA
A	1	250	< 10 000 ng/ml		3	
E	1	250	< 10 000 ng/ml		3	
11β-OH-A	2	100	-		1	
11β-OH-E	2	100	-		8	
5αA3α17βdiol	2	5	< 150 ng/ml			3
5βA3α17βdiol	2	5	-			
epiT	0,6	1	< 50 ng/ml		3	3
T	0,4	1	< 50 ng/ml			
T/epiT	-	-	< 4			1
A/T	-	-	> 20			1
5α/5β	-	-	< 2,4			
5α/epiT	-	-	< 10			2
SUM of rejected results:				0	18	10

Out of the analyzed 76 samples of leisure female athletes (Table 4), 14 were removed. Non-compliance showed 19 results in a total of 14 samples. As in female non-athletes, the predominant reason for the elimination of the results was the measured concentration of 11β-OH-E below the LOQ. Of the WADA scores, 4 were due to a measured T concentration above the 50 ng / ml limit for women. In two cases the concentrations of A and in one case of E exceeded the limit of 10,000 ng/ml. One result for a concentration of 5αA3α17βdiol was above 150 ng/ml. After removing non-compliant samples of leisure athletes from the sample, the results of 62 samples (82% of the analyzed samples of leisure athletes) were used for statistical analysis.

Table 4. Removed samples (n = 14) of leisure female athletes (F1), according to the validation criteria of the method (LOD and LOQ) and the WADA criteria.

Parameter	LOD [ng/ml]	LOQ [ng/ml]	WADA	F1 (samples 62/76; 82%)		
				< LOD	< LOQ	≠ WADA
A	1	250	< 10 000 ng/ml		1	2
E	1	250	< 10 000 ng/ml		1	1
11β-OH-A	2	100	-			
11β-OH-E	2	100	-		7	
5αA3α17βdiol	2	5	< 150 ng/ml			1
5βA3α17βdiol	2	5	-			
epiT	0,6	1	< 50 ng/ml			
T	0,4	1	< 50 ng/ml		2	4
T/epiT	-	-	< 4			
A/T	-	-	> 20			
5α/5β	-	-	< 2,4			
5α/epiT	-	-	< 10			
SUM of rejected results:				0	11	8

Nine, out of a total of 36 samples of active female athletes (F2) (Table 5), were removed from the sample. In these 9 samples, a total of 12 non-compliant results were found due to measured steroid concentrations below the LOQ. 3 concentrations of 11β-OH-A and 5 of 11β-OH-E lower than the LOQ of the quantification method were measured. Two results were found for T values and 2 for epiT below LOQ. As a result of the elimination, 27 samples were used for statistical processing (75% of the analyzed samples of active female athletes).

Table 5. Removed samples (n = 9) of active female athletes (F2), according to the validation criteria of the method (LOD and LOQ) and the WADA criteria.

Parameter	LOD [ng/ml]	LOQ [ng/ml]	WADA	F2 (samples 27/36; 75%)		
				< LOD	< LOQ	≠ WADA
A	1	250	< 10 000 ng/ml			
E	1	250	< 10 000 ng/ml			
11β-OH-A	2	100	-		3	
11β-OH-E	2	100	-		5	
5αA3α17βdiol	2	5	< 150 ng/ml			
5βA3α17βdiol	2	5	-			
epiT	0,6	1	< 50 ng/ml		2	
T	0,4	1	< 50 ng/ml		2	
T/epiT	-	-	< 4			
A/T	-	-	> 20			
5α/5β	-	-	< 2,4			
5α/epiT	-	-	< 10			
SUM of rejected results:				0	12	0

Table 6 shows the removed results from the analysis of samples from male non-athletes (M0). Of the 107 samples, 22 showed non-compliance with one or more of the criteria. A total of 19 results did not meet the validation criteria of the method. As in the groups already described, most (16 results) had a concentration of 11β-OH-E below LOQ. One value of A concentration and two E concentrations above 10,000 ng/ml were measured. Two results for T/epiT exceeded the limit value of the ratio 4.0 and one result for the ratios 5α/5β and 5α/epiT exceeded the limit values (2.4 and 10, respectively). Thus,

the results of 85 samples of male non-athletes (79% of the analyzed samples of non-sports men) were used for statistical analysis.

Table 6. Removed samples (n = 22) of male non-athletes (M0), according to the validation criteria of the method (LOD and LOQ) and the WADA criteria.

Parameter	LOD [ng/ml[	LOQ [ng/ml]	WADA	M0 (samples 85/107; 79%)		
				< LOD	< LOQ	≠ WADA
A	1	250	< 10000 ng/ml		1	1
E	1	250	< 10000 ng/ml		2	1
11β-OH-A	2	100	-			
11β-OH-E	2	100	-		16	
5αA3α17βdiol	2	5	< 250 ng/ml			3
5βA3α17βdiol	2	5	-			
epiT	0,6	1	< 200 ng/ml			
T	0,4	1	< 200 ng/ml			
T/epiT	-	-	< 4			2
A/T	-	-	> 20			
5α/5β	-	-	< 2,4			1
5α/epiT	-	-	< 10			1
SUM of rejected results:				0	19	9

In the most numerous group of 228 leisure male athletes (M1) (Table 7), 66 samples were dropped due to non-compliance with the criteria of a total of 98 results. 21 results with a concentration of 11β-OH-E below LOQ and one result with a concentration of E below LOQ were found. In 12 of the cases the concentration limit of A was exceeded and in 3 of the concentration of E. In 6 cases the concentration of 5αA3α17βdiol was above the limit concentration for men (250 ng/ml). There were 14 results for T with a value above the WADA upper limit for men of 200 ng/ml. There were deviations from the requirements in the ratios T/epiT, A/T, 5α/5β and 5α/epiT. For these reasons, a total of 29% of the samples in the sample were removed. The results of 162 samples of leisure athletes were used for statistical analysis (71% of the analyzed samples of leisure athletes).

Table 7. Samples removed (n = 66) of leisure male athletes (M1), according to the validation criteria of the method (LOD and LOQ) and the WADA criteria.

Parameter	LOD [ng/ml]	LOQ [ng/ml]	WADA	M1 (samples 162/228; 71%)		
				< LOD	< LOQ	≠ WADA
A	1	250	< 10000 ng/ml			12
E	1	250	< 10000 ng/ml		1	3
11β-OH-A	2	100	-			
11β-OH-E	2	100	-		21	
5αA3α17βdiol	2	5	< 250			6
5βA3α17βdiol	2	5	-			
epiT	0,6	1	< 200			
T	0,4	1	< 200			14
T/epiT	-	-	< 4			25
A/T	-	-	> 20			8
5α/5β	-	-	< 2,4			4
5α/epiT	-	-	< 10			4
SUM of rejected results:				0	22	76

6 results were removed from the samples of active male athletes (M2) (Table 8). Due to the omission of these 6 results, out of 48 analyzed samples, 42 were included in the statistical processing. Here, too, the largest share of the removed results was due to measured concentrations of 11β-OH-A (n = 3) and 11β-OH-E (n = 3) under LOQ. There were 2 results for E with values below LOQ. The eligible results of 42 samples of active male athletes (87% of the analyzed active male samples) were used for statistical analysis.

Table 8. Removed samples (n = 6) of active male athletes (M2), according to the validation criteria of the method (LOD and LOQ) and the WADA criteria.

Parameter	LOD [ng/ml]	LOQ [ng/ml]	WADA	M2 (samples 42/48; 87%)		
				< LOD	< LOQ	≠ WADA
A	1	250	< 10000 ng/ml			
E	1	250	< 10000 ng/ml		2	
11β-OH-A	2	100	-		3	
11β-OH-E	2	100	-		3	
5αA3α17βdiol	2	5	< 250			
5βA3α17βdiol	2	5	-			
epiT	0,6	1	< 200			
T	0,4	1	< 200			
T/epiT	-	-	< 4			
A/T	-	-	> 20			
5α/5β	-	-	< 2,4			
5α/epiT	-	-	< 10			
SUM of rejected results:				0	8	0

4.1.2 Reference values of steroid profile markers

Table 9 presents the reference values of the concentrations of the tested steroids in urine and their ratios calculated in Refval v.4.11, in female non-athletes (F0) and leisure female athletes (F1). Due to the insufficient number of samples from active female athletes (n = 27), it was not possible to calculate reference values for them. In addition to the 2.5th percentile and the 97.5th percentile, the table also shows the values of the 95% confidential interval. For steroids included in the ABP steroid module, the reference limits in leisure

female athletes were: from 471.10 ng/ml to 8022.87 ng/ml for A concentration, from 490.39 ng/ml to 8234.70 ng/ml for E, from 3.71 ng/ml to 40.29 ng/ml for T, from 4.74 ng/ml to 33.88 ng/ml for epiT, from 6.00 ng/ml to 84.78 ng/ml for 5αA3α17βdiol and from 8.50 ng/ml to 301.46 ng/ml for 5βA3α17βdiol. In female non-athletes, the reference range of steroid concentrations monitored in ABP was: 0.28 to 2.11 for T/epiT, 0.33 to 2.46 for A/E, 49.37 to 797.36 for A/T, from 0.13 to 1.61 for 5α/5β and from 0.53 to 5.73 for 5α/epiT.

In leisure female athletes, the reference concentrations of A were: from 596.02 ng/ml to 8873.78 ng/ml, of E - from 723.36 ng/ml to 8209.50 ng/ml, of T - from 2.27 ng/ml to 37.19 ng/ml, on epiT - from 6.72 ng/ml to 40.96 ng/ml, on 5αA3α17βdiol from 5.31 ng/ml to 85.25 ng/ml, on 5βA3α17βdiol - from 8.62 ng/ml to 311.46 ng/ml. For the ratios in ABP, the obtained reference values were: from 0.23 to 2.89 for T/epiT, from 0.36 to 2.66 for A/E, from 53.71 to 870.05 A/T, from 0.10 to 1.88 5α/5β and from 0.52 to 4.75 for 5α/epiT.

Table 9. Reference values of steroid concentrations in urine and their ratios in female non-athletes (F0) and leisure female athletes (F1).

Parameter	Fraction	F0			F1		
		Referent limit	0,95-confidence interval		Referent limit	0,95-confidence interval	
A	0,025	471,10	303,56	1067,43	596,02	466,05	1200,99
	0,975	8022,87	7348,54	8279,23	8873,78	6182,00	9581,55
E	0,025	490,39	452,97	844,44	723,36	607,16	926,62
	0,975	8234,70	6333,48	9926,07	8209,50	5960,13	8616,31
T	0,025	3,71	3,12	6,12	2,27	2,20	4,63
	0,975	40,29	29,86	49,02	37,19	28,91	45,02
epiT	0,025	4,74	2,93	7,94	6,72	6,12	8,45
	0,975	33,88	28,63	35,01	40,96	29,03	45,50
5αA3α17βdiol	0,025	6,00	5,90	9,69	5,31	4,44	9,49
	0,975	84,78	54,95	95,74	85,25	55,84	112,79
5βA3α17βdiol	0,025	8,50	8,24	8,24	8,62	6,80	14,59
	0,975	301,46	177,69	402,56	311,46	162,29	406,21
11β-OH-A	0,025	204,07	178,96	300,50	247,00	217,28	337,02
	0,975	1442,36	1280,88	1453,93	1792,62	1330,21	1963,04
11β-OH-E	0,025	104,27	103,59	118,24	106,66	105,31	120,85
	0,975	747,16	533,12	814,70	895,46	517,53	1203,88
T/epiT	0,025	0,28	0,28	0,36	0,23	0,17	0,34
	0,975	2,11	1,84	2,13	2,89	1,70	3,96
A/E	0,025	0,33	0,20	0,55	0,36	0,29	0,52
	0,975	2,46	2,03	2,50	2,66	2,05	2,90
A/T	0,025	49,37	40,22	72,67	53,71	52,60	84,27
	0,975	797,36	598,08	813,56	870,05	536,11	1210,34
5α/5β	0,025	0,13	0,12	0,16	0,10	0,07	0,16
	0,975	1,61	1,36	1,75	1,88	1,38	2,04
5α/epiT	0,025	0,53	0,51	0,66	0,52	0,48	0,60
	0,975	5,73	3,60	7,00	4,75	4,15	5,46
11β-OH-A/ 11β-OH-E	0,025	1,15	0,94	1,54	0,95	0,75	1,21
	0,975	8,71	1,54	8,98	7,38	6,06	8,22
A/ 11β-OH-A	0,025	1,21	1,13	1,55	1,40	1,22	1,87
	0,975	11,53	9,53	12,07	14,31	10,00	17,60
E/ 11β-OH-E	0,025	3,18	2,98	4,52	3,05	2,45	3,68
	0,975	40,14	27,90	46,14	46,18	24,05	50,92

The reference values of steroid concentrations in urine calculated with Refval v.4.11 and their ratios in male non-athletes (M0), and leisure male athletes (M1) and active male athletes (M2) are presented in Table 10. For concentration of A reference limits were: from 600.13 ng/ml to 7079.14 ng/ml, from 766.30 ng/ml to 9558.12 ng/ml and from 931.64 ng/ml to 5189.03 ng/ml for male non-athletes, leisure male athletes and active male athletes, respectively. The reference values for E were from 639.68 ng/ml to 8118.20 ng/ml, from 450.66 ng/ml to 7096.06 ng/ml and from 762.22 ng/ml to 5432.05 ng/ml, for T - from 8.43 ng/ml to 162.81 ng/ml, from 6.75 ng/ml to 161.28 ng/ml and from 3.79 ng/ml to 139.22 ng/ml, for epiT - from 8,94 ng/ml to 96,97 ng/ml, from 9,00 ng/ml to 140,30 ng/ml and from 3,53 ng/ ml to 80,31 ng/ml, for 5αA3α17βdiol - from 9.81 ng/ml to 173.78 ng/ml, from 12.79 ng/ml to 148.65 ng/ml and from 6.51 ng/ml to 102.62 ng/ml and for 5βA3α17βdiol - from 12, 70 ng/ml to 449.08 ng/ml, 19.58 ng/ml to 452.01 ng/ml and 8.33 ng/ml to 241.65 ng/ml for male non-athletes, leisure male athletes and active male athletes, respectively.

The T/epiT ratio had reference values from 0.22 to 3.69, from 0.19 to 3.59 and from 0.13 to 3.70 for male non-athletes, leisure male athletes and active male athletes, respectively. For the A/E ratio the reference values were from 0.46 to 3.22, from 0.47 to 3.14 and from 0.30 to 2.13, A/T - from 22.63 to 378.49, from 21 , 42 to 496.54 and from 22.23 to 327.21, 5α/5β - from 0.15 to 1.77, from 0.14 to 1.81 and from 0.07 to 1.06, 5α/epiT - from 0,49 to 5,79, from 0,54 to 5,72 and from 0,24 to 5,53 for male non-athletes, leisure male athletes and active male athletes, respectively.

Table 10. Reference values of steroid concentrations in urine and their ratios in male non-athletes (M0), leisure male athletes (M1) and active male athletes (M2).

Parameter	Fraction	M0			M1			M2		
		Referent limit	0,95-confidence interval		Referent limit	0,95-confidence interval		Referent limit	0,95-confidence interval	
A	0,025	600,13	580,70	1131,93	766,30	766,30	887,68	931,64	920,97	1137,77
	0,975	7079,14	6048,91	8912,88	9558,12	8005,73	9820,99	5189,03	3654,19	5248,64
E	0,025	639,68	427,33	837,75	450,66	450,66	708,91	762,22	760,97	921,86
	0,975	8118,20	6384,11	8819,53	7096,06	6000,31	8324,97	5432,05	3002,76	5499,31
T	0,025	8,43	3,32	10,40	6,75	4,56	10,18	3,79	3,60	7,51
	0,975	162,81	116,42	171,27	161,28	129,69	166,89	139,22	86,96	140,42
epiT	0,025	8,94	8,39	11,02	9,00	7,72	12,51	3,53	3,45	7,44
	0,975	96,97	67,72	146,43	140,30	100,48	150,29	80,31	74,01	80,67
5αA3α17βdiol	0,025	9,81	8,53	14,53	12,79	5,17	17,04	6,51	6,43	8,34
	0,975	173,78	136,64	245,03	175,97	148,65	221,07	102,62	78,60	102,70
5βA3α17βdiol	0,025	12,70	11,74	16,90	19,58	13,00	29,32	8,33	8,10	22,09
	0,975	449,08	311,73	567,95	452,01	349,07	710,81	241,65	184,81	241,68
11β-OH-A	0,025	182,09	164,18	257,05	226,39	182,93	275,60	156,67	154,37	220,60
	0,975	1516,82	1273,10	1596,21	1772,01	1402,45	2057,80	896,68	702,99	908,91
11β-OH-E	0,025	103,40	100,84	100,84	104,33	100,84	109,68	142,18	141,96	150,87
	0,975	826,67	747,46	1022,49	616,74	519,27	876,41	901,35	448,70	937,71
T/epiT	0,025	0,22	0,18	0,52	0,19	0,12	0,30	0,13	0,13	0,23

Parameter	Fraction	M0			M1			M2		
		Referent limit	0,95-confidence interval		Referent limit	0,95-confidence interval		Referent limit	0,95-confidence interval	
	0,975	3,69	3,32	3,83	3,59	3,15	3,61	3,70	3,19	3,71
A/E	0,025	0,46	0,35	0,59	0,47	0,38	0,63	0,30	0,30	0,68
	0,975	3,22	2,96	4,16	3,14	2,40	3,51	2,13	1,73	2,13
A/T	0,025	22,63	20,86	27,28	21,42	20,93	30,66	22,23	21,66	32,88
	0,975	378,49	216,52	508,82	496,54	327,46	607,73	327,21	252,84	331,45
5α/5β	0,025	0,15	0,15	0,20	0,14	0,13	0,18	0,07	0,07	0,15
	0,975	1,77	1,11	1,82	1,81	1,59	2,09	1,06	0,76	1,07
5α/epiT	0,025	0,49	0,34	0,68	0,54	0,51	0,64	0,24	0,24	0,32
	0,975	5,79	4,84	6,01	5,72	4,49	6,09	5,53	2,81	5,66
11β-OH-A/ 11β-OH-E	0,025	0,49	0,34	0,68	0,54	0,51	0,64	0,71	0,71	0,79
	0,975	5,79	4,84	6,01	5,72	4,49	6,09	5,48	2,99	5,67
A/ 11β-OH-A	0,025	1,61	0,91	1,99	1,45	1,00	1,82	2,10	2,06	2,84
	0,975	10,06	8,00	10,41	10,25	8,95	11,46	11,43	9,49	11,44
E/ 11β-OH-E	0,025	2,65	1,39	1,39	2,43	2,32	3,89	1,81	1,77	3,02
	0,975	26,25	26,25	34,24	35,61	25,66	55,16	37,60	14,35	38,58

4.1.3 Variation analysis of the studied steroid markers

Table 11 presents the variational analysis of the concentrations of the tested steroids in the urine of female non-athletes (F0) and the proven significant differences between them with the non-parametric Kruskal-Wallis test. The highest mean concentrations of androsterone and etiocholanolone were 3431.164 ng/ml and 3126.795 ng/ml, respectively. The lowest mean concentrations of all measured steroids were testosterone (15,324 ng/ml) and epithestosterone (16,896 ng/ml). 5α-Androstane-3α, 17β-diol had an average concentration of 31.30 ng/ml, and 5β-Androstane-3α, 17β-diol at 74.21 ng/ml. For non-ABP steroids, 11β-hydroxy androsterone and 11β-hydroxyethiocholanolone, the mean values were 728.49 ng/ml and 257.86 ng/ml, respectively.

Statistically significant differences, for steroids included in ABP, were found between active female athletes and female non-athletes, for the concentrations of 5α-Androstane-3α, 17β-diol (F2 <F0, p <0.010) and epithestosterone (F2 <F0, p <0.001). The values for female non-athletes were higher than those for active athletes. For those not included in ABP, 11β-hydroxyandrosterone and 11β-hydroxyethiocholanolone, differences were also found between active athletes and female non-athletes. For 11β-hydroxyandrosterone the concentrations in the samples of female non-athletes were significantly higher (F2 <F0, p <0.010), and for 11β-hydroxyethiocholanolone - lower than those measured in the samples of active athletes (F0 <F2, p <0.010).

Table 11. Variation analysis of [ng/ml] concentrations of test steroids in urine in female non-athletes (F0).

F0		A	E	11β-OH-A	11β-OH-E	5αA3α17βdiol	5βA3α17βdiol	EpiT	T
Mean		3431,16	3126,79	728,49	257,86	31,30	74,21	16,90	15,32
Std, Error of Mean		265,87	245,01	44,62	19,72	2,36	9,20	0,92	1,13
Median		3039,98	2758,90	655,85	211,39	27,50	47,25	16,31	12,58
Std, Deviation		2059,42	1897,81	345,60	152,77	18,27	71,28	7,13	8,72
Skewness		0,68	1,18	0,41	1,61	1,02	2,24	0,56	1,37
Kurtosis		-0,40	1,75	-0,76	2,71	1,45	6,97	-0,03	2,56
Range		7975,67	9473,10	1274,97	711,11	89,84	394,32	32,08	45,90
Minimum		303,56	452,97	178,96	103,59	5,90	8,24	2,93	3,12
Maximum		8279,23	9926,07	1453,93	814,70	95,74	402,56	35,01	49,02
Percentiles	2,5	622,69	524,25	225,38	104,27	6,09	8,74	6,37	4,25
	25,0	1776,79	1773,75	423,98	142,17	15,72	25,06	11,20	10,00
	75,0	4967,21	3892,35	982,91	323,92	42,62	98,43	21,63	20,46
	97,5	8022,87	7401,05	1431,89	686,05	74,86	258,41	33,88	32,58
Differences				F2<F0 p<0,010	F0<F2 p<0,010	F2<F0 p<0,010		F2<F0 p<0,001	

Independent-Samples Kruskal-Wallis Test. The significance level is 0.05. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table 12 presents the variational analysis of the ratios between urinary steroid concentrations in female non-athletes (F0) and the proven significant differences between them with the non-parametric Kruskal-Wallis test. The mean values of the ratios included in ABP were: 0.98 for T/epiT, 1.19 for A/E, 266.19 for A/T, 0.64 for 5α/5β, 2.03 for 5α/EpiT.

Of the ratios included in ABP, significant differences were found for T/epiT, A/E and 5α/5β. The T/epiT ratio in female non-athletes showed significantly lower values (F0 <F2, p <0.025), and the A/E ratios (F2 <F0, p <0.010) and 5α/5β (F2 <F0, p <0.050) - higher values compared to active athletes.

Of the ratios not included in ABP, there were significant differences between female non-athletes and active athletes in 11β-OH-A/11β-OH-E, as in non-athletes it showed higher values than in active athletes (F2 <F0, p <0.001).

Table 12. Variation analysis of the ratios between the concentrations of the studied steroids in urine in female non-athletes (F0).

F0		T/epiT	A/E	A/T	5α/5β	5α/EpiT	11β-OH-A/11β-OH-E	A/11β-OH-A	E/11β-OH-E
Mean		0,98	1,19	266,19	0,64	2,03	3,25	5,15	14,02
Std, Error of Mean		0,06	0,07	23,52	0,05	0,16	0,23	0,36	1,17
Median		0,96	1,17	213,77	0,58	1,87	2,76	4,88	11,91
Std, Deviation		0,49	0,50	182,20	0,40	1,21	1,76	2,78	9,04
Skewness		0,56	0,45	1,21	0,76	1,36	1,46	0,42	1,38
Kurtosis		-0,41	-0,16	1,17	-0,09	3,31	1,92	-0,63	2,21
Range		1,85	2,30	773,34	1,63	6,48	8,04	10,94	43,17
Minimum		0,28	0,20	40,22	0,12	0,51	0,94	1,13	2,98
Maximum		2,13	2,50	813,56	1,75	7,00	8,98	12,07	46,14
Percentiles	2,5	0,28	0,44	57,65	0,14	0,54	1,34	1,21	3,36
	25,0	0,56	0,80	127,94	0,30	1,16	2,04	2,74	7,73
	75,0	1,32	1,51	367,22	0,88	2,77	4,06	6,96	18,64
	97,5	2,11	2,42	782,70	1,50	4,59	8,46	11,05	40,14
Differences		F0<F2 p<0,025	F2<F0 p<0,010		F2<F0 p<0,050		F2<F0 p<0,001		

Independent-Samples Kruskal-Wallis Test. The significance level is 0.05. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table 13 presents the variational analysis of the concentrations of the tested steroids in the urine of leisure female athletes (F1) and the proven significant differences between them with the non-parametric Kruskal-Wallis test. Androsterone (A) had a higher mean concentration than etiocholanolone (E), 3150.01 ng/ml and 2841.33 ng/ml, respectively. The mean concentration of 5αA3α17βdiol was 29.27 ng/ml and was lower than that of 5βA3α17βdiol - 68.09 ng/ml. EpiT had a higher mean concentration than that of T (17.51 ng/ml and 14.83 ng/ml).

Significant differences were found only in active female athletes for 5αA3α17βdiol ($F2<F1$, $p <0.025$) and EpiT ($F2<F1$, $p <0.001$), which were monitored in ABP. Both steroids had higher concentrations in leisure female athletes. Of the steroids not included in ABP, proven differences were found in 11β-OH-A. The concentrations of 11β-OH-A in the samples of leisure female athletes were higher than those in active athletes ($F2 <F1$, $p <0.010$).

Table 13. Variation analysis of the concentrations [ng/ml] of the tested steroids in the urine of leisure female athletes (F1).

F1		A	E	11β-OH-A	11β-OH-E	5αA3α17βdiol	5βA3α17βdiol	EpiT	T
Mean		3150,01	2841,33	786,78	287,84	29,27	68,09	17,51	14,83
Std, Error of Mean		249,16	221,97	63,64	22,88	2,45	8,19	1,00	1,11
Median		2600,39	2404,18	699,62	251,99	25,53	45,98[a]	15,99[a]	14,44[a]
Std, Deviation		1961,86	1747,82	501,07	180,19	19,30	64,48	7,86	8,77
Skewness		1,31	1,29	3,08	2,61	1,78	2,90	1,12	0,91
Kurtosis		1,62	1,61	14,86	10,32	5,19	12,02	1,77	1,01
Range		9115,50	8009,15	3456,19	1098,56	108,35	399,42	39,38	42,82
Minimum		466,05	607,16	103,12	105,31	4,44	6,80	6,12	2,20
Maximum		9581,55	8616,31	3559,31	1203,88	112,79	406,21	45,50	45,02
Percentiles	2,5	708,92	799,50[c]	218,76	106,72	5,45	9,88	7,17	2,35
	25,0	1695,83	1512,96	511,68	164,74	14,71	30,22	11,92	7,11
	75,0	3969,37	3667,13	929,49	338,33	38,72	85,10	21,18	18,44
	97,5	8803,96	7837,82	1946,81	719,67	84,12	238,00	37,41	31,39
Differences				F2<F1 p<0,010		F2<F1 p<0,025		F2<F1 p<0,001	

Independent-Samples Kruskal-Wallis Test. The significance level is 0.05. Significance values have been adjusted by the Bonferroni correction for multiple tests

Table 14 presents the variational analysis of the ratios between the concentrations of steroids in the urine of leisure female athletes (F1) and the proven significant differences between them with the non-parametric Kruskal-Wallis test. The mean T/epiT ratio was 0.91, for A/E, A/T, 5α/5β, 5α/EpiT 1.22; 264.12; 0.62 and 1.81 respectively.

In the ABP ratios, significant differences were found between leisure female athletes and active female athletes for T/epiT, A/E and 5α/epiT. In the ratios T/epiT (F1<F2, p<0.001) and 5α / epiT (F1<F2, p<0.025) leisure female athletes had lower values than active athletes, and for A/E (F2<F1 , p<0,010) - higher.

Of the non-ABP ratios, significant differences between leisure female athletes and active female athletes were found for 11β-OH-A/11β-OH-E and A/11β-OH-A. The ratio 11β-OH-A/11β-OH-E (F2 <F1, p <0.001) had higher values in samples of leisure female athletes, and A/11β-OH-A (F1<F2, p<0.050) lower than active athletes.

Table 14. Variation analysis of the ratios between the concentrations of the studied steroids in the urine of leisure female athletes (F1).

F1		T/epiT	A/E	A/T	5α/5β	5α/EpiT	11β-OH-A/11β-OH-E	A/11β-OH-A	E/11β-OH-E
Mean		0,91	1,22	264,12	0,62	1,81	3,19	4,62	11,65
Std, Error of Mean		0,08	0,07	24,43	0,05	0,15	0,25	0,39	1,10
Median		0,79	1,12	206,26	0,49	1,48	2,77	3,71	9,76
Std, Deviation		0,59	0,54	192,36	0,42	1,17	1,97	3,06	8,68
Skewness		2,48	0,70	2,39	1,13	1,24	1,96	1,90	2,52
Kurtosis		10,37	0,37	8,67	1,28	0,91	5,64	4,69	8,15
Range		3,79	2,61	1157,74	1,96	4,98	11,35	16,38	48,47
Minimum		0,17	0,29	52,60	0,07	0,48	0,52	1,22	2,45
Maximum		3,96	2,90	1210,34	2,04	5,45	11,87	17,60	50,92
Percentiles	2,5	0,27	0,37	55,28	0,10	0,53	0,76	1,41	3,23
	25,0	0,50	0,83	144,93	0,29	0,91	2,06	2,52	6,81
	75,0	1,19	1,57	328,89	0,90	2,16	3,79	6,15	12,92
	97,5	2,10	2,46	693,65	1,74	4,54	8,18	11,87	42,05
Differences		F1<F2 p<0,001	F2<F1 p<0,010			F1<F2 p<0,025	F2<F1 p<0,001	F1<F2 p<0,050	

Independent-Samples Kruskal-Wallis Test. The significance level is 0.05. Significance values have been adjusted by the Bonferroni correction for multiple tests

Table 15 presents the variational analysis of the concentrations of the studied steroids in the urine of active female athletes (F2) and the proven significant differences between them with the non-parametric Kruskal-Wallis test. The mean concentration of etiocholanolone 3323.50 ng/ml was higher than that of androsterone 2838.01 ng/ml. 5αA3α17βdiol had an average concentration of 18.99 ng/ml, 5βA3α17βdiol 72.86 ng/ml. Testosterone had a higher mean concentration than epithestosterone, 12.47 ng/ml and 9.38 ng/ml, respectively.

For steroids not included in ABP, 11β-OH-A and 11β-OH-E, the respective mean concentrations were 507.64 ng/ml and 357.39 ng/ml.

Of the steroids included in ABP, significant differences between active athletes and the other two groups were found for 5αA3α17βdiol - ($F2 < F1$, $p < 0.025$) and EpiT ($F2 < F1$, $p < 0.001$). For both steroids, active female athletes had lower values compared to both non-athletes and leisure athletes.

Significant differences were also found for steroids not included in ABP, 11β-OH-A and 11β-OH-E. In 11β-OH-A ($F2 < F0$, $p < 0.010$; $F2 < F1$, $p < 0.010$) these differences were with both non-athletes and leisure athletes and the concentrations in active athletes were lower. For 11β-OH-E, active athletes showed a difference only with non-athletes. The values of 11β-OH-E ($F0 < F2$, $p < 0.010$) in active athletes were higher.

Table 15. Variation analysis of the concentrations [ng/ml] of the tested steroids in urine in active female athletes (F2).

F2		A	E	11β-OH-A	11β-OH-E	5αA3α17βdiol	5βA3α17βdiol	EpiT	T
Mean		2838,01	3323,50	507,64	357,39	18,99	72,86	9,38	12,47
Std, Error of Mean		367,48	353,68	49,64	29,99	2,95	14,91	1,82	2,18
Median		2084,41[a]	2764,84[a]	406,44[a]	301,31[a]	12,73[a]	43,42[a]	4,59[a]	7,72[a]
Std, Deviation		1909,46	1837,77	257,94	155,82	15,33	77,49	9,47	11,31
Skewness		1,70	1,34	0,88	0,90	1,46	2,07	1,42	1,10
Kurtosis		3,00	1,36	-0,24	-0,05	1,43	4,50	0,73	-0,11
Range		8089,35	7142,51	874,06	560,52	54,69	327,39	31,07	35,16
Minimum		934,63	1476,89	209,13	163,44	2,95	8,86	1,54	1,50
Maximum		9023,98	8619,40	1083,18	723,96	57,65	336,25	32,61	36,67
Percentiles	2,5	952,20[c]	1489,38[c]	212,27[c]	166,99[c]	3,17[c]	8,97[c]	1,56[c]	1,51[c]
	25,0	1557,69	1901,42	293,00	229,78	8,82	24,65	3,15	4,03
	75,0	3464,18	4148,13	690,18	474,81	25,13	96,82	12,04	18,96
	97,5	8495,61	8281,45	1079,73	717,67	57,05	319,30	31,98	36,42
Differences				F2<F0 p=0,010 F2<F1 p<0,010	F0<F2 p<0,010	F2<F0 p<0,010 F2<F1 p<0,025		F2<F0 p<0,001 F2<F1 p<0,001	

Independent-Samples Kruskal-Wallis Test. The significance level is 0.05. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table 16 presents the variational analysis of the ratios between the concentrations of steroids in the urine of active female athletes (F2) and the proven significant differences between them with the non-parametric Kruskal-Wallis test.

The ABP ratios, T/epiT, A/E, A/T, 5α/5β and 5α/EpiT, averaged 1.66; 0.86; 372.75; 0.39 and 2.96, respectively. For these ratios, significant differences were found in T/epiT, A/E, 5α/5β and 5α/EpiT. In the T/epiT and A/E ratios, the differences were between active female athletes and the other two groups. Active athletes had higher values of the T/epiT ratio compared to both non-athletes and leisure athletes ($F1<F2$, $p<0.001$; $F0<F2$, $p<0.001$). For the A/E ratio, active athletes showed significantly lower values compared to both non-athletes and leisure athletes ($F2<F0$, $p<0.010$; $F2<F1$, $p<0.010$). For the 5α/5β ratio, there were significant differences between active athletes and non-athletes. In active athletes the values were lower ($F2<F0$, $p<0.050$). For the 5α/EpiT ratio, differences were found compared to leisure athletes. The reported values in active athletes were higher ($F1<F2$, $p<0.025$).

Table 16. Variation analysis of the ratios between the concentrations of the studied steroids in the urine of active female athletes (F2).

F2		T/epiT	A/E	A/T	5α/5β	5α/EpiT	11β-OH-A/11β-OH-E	A/11β-OH-A	E/11β-OH-E
Mean		1,66	0,86	372,75	0,39	2,96	1,53	5,89	9,80
Std, Error of Mean		0,19	0,05	45,63	0,04	0,40	0,15	0,50	0,90
Median		1,49	0,87	256,01	0,36	2,28	1,28	6,04	8,26
Std, Deviation		0,99	0,26	237,07	0,22	2,09	0,80	2,60	4,66
Skewness		0,41	0,02	0,46	-0,02	1,11	1,73	0,47	1,28
Kurtosis		-1,20	-0,67	-1,08	-0,88	0,27	2,88	0,26	0,67
Range		2,97	0,97	781,81	0,76	7,57	3,16	10,35	16,15
Minimum		0,40	0,40	52,40	0,02	0,72	0,76	1,47	3,91
Maximum		3,36	1,37	834,21	0,78	8,30	3,92	11,82	20,06
Percentiles	2,5	0,40[c]	0,40[c]	54,22[c]	0,03[c]	0,75[c]	0,76[c]	1,59[c]	4,18[c]
	25,0	0,84	0,66	195,87	0,22	1,36	1,00	4,01	6,53
	75,0	2,45	1,04	586,41	0,56	4,22	1,74	7,01	11,35
	97,5	3,35	1,36	824,94	0,77	8,03	3,86	11,77	20,01
Differences		F1<F2 p<0,001 F0<F2) p<0,025	F2<F0 p<0,010 F2<F1 p<0,010		F2<F0 p<0,050	F1<F2 p<0,025	F2<F0 p<0,001 F2<F1 p<0,001	F0<F2 p<0,050	

Independent-Samples Kruskal-Wallis Test. The significance level is 0.05. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table 17 presents the variational analysis of the concentrations of the studied steroids in the urine of male non-athletes (M0) and the proven significant differences between them with the non-parametric Kruskal-Wallis test. The highest mean concentrations were A and E, 3277.20 ng/ml and 2641.50 ng/ml, respectively. 5αA3α17βdiol had an average concentration of 67.65 ng/ml and 5βA3α17βdiol 155.77 ng/ml. T had a higher mean concentration (55.89 ng/ml) than EpiT (34.47 ng/ml). Of the steroids not included in ABP, 11β-OH-A had an average concentration of 728.60 ng/ml and 11β-OH-E 288.46 ng/ml.

For the steroids included in ABP, significant differences were reported only for active athletes in A (M2 <M0, p <0.010), 5αA3α17βdiol (M2 <M0, p <0.001) and 5βA3α17βdiol (M2 <M0, p <0.010). In samples from male non-athletes, concentrations of these steroids were higher.

Significant differences, not included in APB 11β-OH-A, were also found between active male athletes and male non-athletes. The values of 11β-OH-A (M2<M0, p<0.001) were higher in non-athletes.

Table 17. Variation analysis of the concentrations [ng/ml] of the tested steroids in urine in male non-athletes (M0).

M0		A	E	11β-OH-A	11β-OH-E	5αA3α17βdiol	5βA3α17βdiol	EpiT	T
Mean		3277,20	2641,50	728,60	288,46	67,65	155,77	34,47	55,89
Std. Error of Mean		188,41	190,89	35,63	21,05	4,76	11,86	2,53	4,26
Median		2948,64	2179,32	681,74	236,87	64,70	128,52	28,36	46,59
Std. Deviation		1737,09	1759,96	328,45	194,11	43,86	109,32	23,34	39,28
Skewness		0,71	1,58	0,50	1,75	1,13	1,27	1,86	0,92
Kurtosis		0,21	2,45	-0,09	2,92	2,07	2,04	5,60	0,24
Range		8332,18	8392,21	1432,04	921,66	236,49	556,20	138,04	167,95
Minimum		580,70	427,33	164,18	100,83	8,53	11,74	8,39	3,32
Maximum		8912,88	8819,53	1596,21	1022,49	245,03	567,95	146,43	171,27
Percentiles	2,5	649,72	662,64	187,87	104,13	10,76	13,15	9,46	8,66
	25,0	1871,93	1535,87	517,55	154,00	31,63	85,24	16,67	26,11
	75,0	4353,43	2993,20	906,62	354,69	95,00	214,86	47,87	81,15
	97,5	6899,83	7745,30	1454,33	814,01	162,34	443,85	92,39	151,07
Differences		(M2<M0) p<0,010		(M2<M0) p<0,001		(M2<M0) p<0,010	(M2<M0) p<0,010		

Independent-Samples Kruskal-Wallis Test. The significance level is 0.05. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table 18 presents the variational analysis of the ratios between urinary steroid concentrations in male non-athletes (M0) and the proven significant differences between them with the non-parametric Kruskal-Wallis test. In male non-athletes, the mean T/epiT ratio was 1.78. The A/E, A/T, 5α/5β and 5α/EpiT ratios averaged 1.48, 87.84, 0.54, 2.30, respectively. For the ratios not included in ABP, 11β-OH-A/11β-OH-E, A/11β-OH-A and E/11β-OH-E, the corresponding mean values were: 3.29; 4.77 and 10.75.

From the ratios observed in ABP, there were significant differences only for 5α/EpiT (M2<M0, p<0.001) in non-athletes compared to active athletes. The values for non-athletes were higher.

For non-ABP steroid ratios, significant differences were found in 11β-OH-A/11β-OH-E and E/11β-OH-E compared to active male athletes. For the ratios 11β-OH-A/11β-OH-E (M2 <M0, p <0.001) and E/11β-OH-E (M2<M0, p<0.010), non-athletes had higher values compared to active athletes.

Table 18. Variation analysis of the ratios between the concentrations of the tested steroids in the urine of male non-athletes (M0).

M0		T/epiT	A/E	A/T	5α/5β	5α/EpiT	11β-OH-A/11β-OH-E	A/11β-OH-A	E/11β-OH-E
Mean		1,78	1,48	87,84	0,54	2,30	3,29	4,77	10,75
Std, Error of Mean		0,10	0,09	8,94	0,04	0,15	0,22	0,22	0,70
Median		1,67	1,28	62,63	0,48	1,97	2,68	4,46	9,09
Std, Deviation		0,93	0,79	82,46	0,35	1,41	2,04	2,06	6,44
Skewness		0,33	1,00	2,80	1,84	0,90	1,13	0,63	1,14
Kurtosis		-0,74	0,59	9,60	3,65	0,04	1,13	0,15	1,38
Range		3,65	3,81	487,96	1,67	5,67	9,80	9,50	32,85
Minimum		0,17	0,35	20,86	0,14	0,34	0,23	0,91	1,39
Maximum		3,83	4,16	508,82	1,81	6,01	10,03	10,40	34,24
Percentiles	2,5	0,22[c]	0,48[c]	23,60[c]	0,16[c]	0,52[c]	0,54[c]	1,64[c]	2,78[c]
	25,0	1,01	0,84	38,82	0,31	1,29	1,84	3,41	6,14
	75,0	2,43	1,91	95,97	0,64	3,25	4,35	5,77	14,72
	97,5	3,67	3,20	349,59	1,64	5,64	8,68	10,06	26,16
Differences						M2<M0 p<0,001	M2<M0 p<0,001		M2<M0 p<0,001

Independent-Samples Kruskal-Wallis Test. The significance level is 0.05. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table 19 presents the variational analysis of the concentrations of the studied steroids in the urine of leisure male athletes (M1) and the proven significant differences between them with the non-parametric Kruskal-Wallis test. The mean values of A and E were 3651.75 ng/ml and 2758.20 ng/ml. For 5αA3α17βdiol and 5βA3α17βdiol, the means were 67.73 ng/ml and 140.32 ng/ml. T had a higher mean concentration than EpiT, 55.51 ng/ml and 42.31 ng/ml, respectively. ABP steroids not included, 11β-OH-A and 11β-OH-E, had mean concentrations of 806.72 ng/ml and 251.79 ng/ml.

Of the steroid concentrations observed in ABP, significant differences were found only for A (M2 <M1, p <0.001), E (M2 <M1, p <0.050) and 5αA3α17βdiol (M2 <M1, p <0.001) compared to active athletes. Values for all three steroids were higher in leisure athletes.

For 11β-OH-A and 11β-OH-E, which are not included in ABP, differences were also found only between leisure athletes and active male athletes. 11β-OH-A had higher concentrations in leisure athletes (M2<M1, p<0.001) and 11β-OH-E lower than in active athletes (M2<M1, p<0.010).

Table 19. Variation analysis of the concentrations [ng/ml] of the tested steroids in urine in leisure male athletes (M1).

M1		A	E	11β-OH-A	11β-OH-E	5αA3α17βdiol	5βA3α17βdiol	EpiT	T
Mean		3651,75	2758,20	806,72	251,79	67,73	140,32	42,31	55,51
Std, Error of Mean		170,29	134,26	35,76	11,65	3,36	9,17	2,29	2,97
Median		3187,28	2498,31	748,86	211,22	57,91	110,45	33,17	46,68
Std, Deviation		2167,40	1708,83	455,09	148,33	42,79	116,68	29,13	37,76
Skewness		0,88	1,15	2,83	1,90	1,06	2,44	1,70	1,14
Kurtosis		0,38	1,40	17,13	5,72	0,92	8,77	3,33	1,27
Range		9552,66	8684,85	3991,49	984,35	219,47	783,96	143,77	190,64
Minimum		281,10	278,37	143,46	52,17	4,87	10,60	7,08	3,38
Maximum		9833,75	8963,21	4134,95	1036,51	224,34	794,56	150,85	194,02
Percentiles	2,5	772,32	484,93	233,34	105,54	12,91	19,49	9,16	6,71
	25,0	2055,77	1521,87	516,04	138,64	33,11	62,03	21,59	28,47
	75,0	5126,36	3576,61	1014,23	324,69	91,12	178,11	53,62	73,16
	97,5	9574,53	6925,53	1815,60	611,49	173,73	446,70	133,42	158,94
Differences		M2<M1 p<0,001	M2<M1 p<0,050	M2<M1 p<0,001	M2<M1 p<0,010	M2<M1 p<0,001			

Independent-Samples Kruskal-Wallis Test. The significance level is 0.05. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table 20 presents the variational analysis of the ratios between the concentrations of steroids in the urine of men in leisure male athletes (M1) and the proven significant differences between them with the non-parametric Kruskal-Wallis test. The mean T/epiT ratio was 1.55. The A/E and A/T ratios were 1.45 and 97.64, respectively, 5α/5β and 5α/EpiT - 0.65 and 1.88, respectively.

Not included in the ABP ratios 11β-OH-A/11β-OH-E, A/11β-OH-A, E/11β-OH-E averaged 3.76; 4.74; 12.39.

For the ABP ratios, significant differences were found only between active athletes and leisure male athletes at 5α/5β (M2 <M1, $p<0.010$) and 5α/EpiT (M2<M1, $p<0.001$). For both ratios, leisure athletes had higher values.

For the A/11β-OH-A ratio (M1<M2, $p<0.025$), significant differences were found between leisure athletes and active athletes. The values in leisure athletes were lower. The values of E/11β-OH-E were significantly higher in leisure athletes compared to active athletes (M2<M1, $p<0.001$).

Table 20. Variation analysis of the ratios between the concentrations of the studied steroids in the urine of leisure male athletes (M1).

M1		T/epiT	A/E	A/T	5α/5β	5α/EpiT	11β-OH-A/11β-OH-E	A/11β-OH-A	E/11β-OH-E
Mean		1,55	1,45	97,64	0,65	1,88	3,76	4,74	12,39
Std, Error of Mean		0,07	0,05	8,61	0,04	0,10	0,18	0,17	0,66
Median		1,50	1,34	62,81	0,52	1,47	3,23	4,30	10,27
Std, Deviation		0,89	0,61	109,60	0,45	1,23	2,23	2,20	8,44
Skewness		0,50	0,96	2,94	1,37	1,93	1,88	0,89	2,54
Kurtosis		-0,45	1,32	8,84	1,68	4,53	5,06	0,64	10,39
Range		3,69	3,20	590,76	2,29	7,19	13,93	11,04	59,08
Minimum		0,09	0,36	20,33	0,08	0,46	0,35	0,96	2,11
Maximum		3,78	3,57	611,09	2,37	7,65	14,29	12,00	61,18
Percentiles	2,5	0,19	0,50	21,67	0,14	0,54	1,06	1,53	2,50
	25,0	0,83	1,04	41,89	0,32	1,08	2,40	3,19	7,06
	75,0	2,10	1,77	95,64	0,81	2,39	4,52	5,84	15,63
	97,5	3,56	3,12	486,56	1,80	5,64	9,57	10,16	32,94
Differences					M2<M1 p<0,010	M2<M1 p<0,010	M2<M1 p<0,001	M1<M2 p<0,025	M2<M1 p<0,001

Independent-Samples Kruskal-Wallis Test. The significance level is 0.05. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table 21 presents the variational analysis of the concentrations of the studied steroids in the urine of active male athletes (M2) and the proven significant differences between them with the non-parametric Kruskal-Wallis test. Androsterone (A) had an average concentration of 2234.73 ng/ml, E - 2116.86 ng/ml, 5αA3α17βdiol - 35.70 ng/ml, 5βA3α17βdiol - 100.55 ng/ml. The mean T concentration (44.30 ng/ml) was higher than that of epiT (33.66 ng/ml). Non-ABP steroids, 11β-OH-A and 11β-OH-E, had mean concentrations of 417.66 ng/ml and 300.07 ng/ml.

In A, E, 5αA3α17βdiol and 5βA3α17βdiol, which are followed in ABP, active male athletes showed significant differences compared to the other two groups. For A (M2 <M0, p <0.010; M2 <M1, p <0.001) and 5αA3α17βdiol (M2 <M0, p <0.001; M2 <M1, p <0.001), differences were found for both non-athletes and leisure athletes. Active athletes showed significantly lower concentrations compared to both groups. In E, the difference was between active and recreation athletes. Active male athletes had lower E values (M2 <M1, p <0.050). For 5βA3α17βdiol, a difference was found only compared to non-athletes. Concentrations of this steroid in active male athletes were lower than in non-athletes (M2 <M0, p <0.010).

Significant differences were demonstrated for both steroids not included in ABP, 11β-OH-A and 11β-OH-E. In 11β-OH-A, active athletes showed significantly lower values compared to both non-athletes and leisure athletes (M2 <M0, p <0.001; M2 <M1, p <0.001). For 11β-OH-E, differences were found only compared to leisure male athletes. The concentrations of 11β-OH-E in active athletes were significantly higher (M1 <M2, p <0.010).

Table 21. Variation analysis of the concentrations [ng/ml] of the tested steroids in urine in active male athletes (M2).

M2		A	E	11β-OH-A	11β-OH-E	5αA3α17βdiol	5βA3α17βdiol	EpiT	T
Mean		2234,73	2116,86	417,66	300,07	35,70	100,55	33,66	44,30
Std, Error of Mean		151,84	222,61	29,13	21,05	3,98	10,22	3,52	4,95
Median		2080,28	1752,55	345,14	269,55	26,77	92,53	28,41	35,28
Std, Deviation		984,05	1442,67	188,77	136,40	25,76	66,26	22,83	32,09
Skewness		0,99	3,10	0,98	2,56	1,22	1,12	0,63	1,14
Kurtosis		0,97	12,61	0,22	10,75	0,91	1,02	-0,77	1,19
Range		4327,67	8245,68	754,54	795,75	96,28	282,60	77,22	136,82
Minimum		920,97	760,97	154,37	141,96	6,43	8,10	3,45	3,60
Maximum		5248,64	9006,65	908,91	937,71	102,71	290,70	80,66	140,42
Percentiles	2,5	999,22	774,72	171,24	143,62	6,10	9,84	4,05	3,87
	25,0	1363,20	1306,76	288,24	225,04	18,27	59,01	15,15	21,34
	75,0	2811,57	2246,46	527,02	349,65	49,49	110,71	49,30	62,08
	97,5	4811,50	7077,61	895,06	671,25	102,04	263,74	78,06	131,62
Differences		M2<M0 p<0,010 M2<M1 p<0,001	M2<M1 p<0,050	M2<M0 p<0,001 M2<M1 p<0,001	M1<M2 p<0,010	M2<M0 p<0,001 M2<M1 p<0,001	M2<M0 p<0,010		

Independent-Samples Kruskal-Wallis Test. The significance level is 0.05. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Table 22 presents the variational analysis of the ratios between the concentrations of steroids in the urine of active male athletes (M2) and the proven significant differences between them with the non-parametric Kruskal-Wallis test. The mean ratios included in ABP, T/epiT, A/E, A/T, 5α/5β, 5α/EpiT were: 1.65, 1.20, 82.65, 0.42, 1.37, respectively. The ratios 11β-OH-A/11β-OH-E, A/11β-OH-A, E/11β-OH-E had average values of 1.55, 5.88 and 8.12, respectively.

Of the ABP ratios, only 5α/5β and 5α/EpiT showed significant differences. Differences in 5α/EpiT were found between active athletes and the other two groups. The values of 5α/EpiT were significantly lower in active male athletes than in non-athletes and in leisure athletes (M2 <M0, p <0.001; M2 <M1, p <0.010). At 5α/5β, significant differences were demonstrated only in leisure athletes. Active athletes had higher values of this ratio (M2-M1, p <0.010).

Of the non-ABP ratios, significant differences were found for 11β-OH-A/11β-OH-E, A/11β-OH-A and E/11β-OH-E. The values of the ratios 11β-OH-A/11β-OH-E (M2 <M0, p <0.001; M2-M1, p <0.001) and E/11β-OH-E (M2 <M0, p <0.010; M2- M1, p <0.001) were lower in active athletes. In both cases, the values for active athletes were higher. Differences between active and leisure athletes were also demonstrated for the A/11β-OH-A ratio. The values of A/11β-OH-A were significantly higher in active athletes (M1 <M2, p <0.025). For the E/11β-OH-E ratio, significant differences were demonstrated between active athletes and non-athletes (M2 <M0, p <0.010) and leisure athletes (M2 <M1, p<0.001). In both cases, the values of active athletes were lower.

Table 22. Variation analysis of the ratios between the concentrations of the studied steroids in urine in active male athletes (M2).

M2		T/epiT	A/E	A/T	5α/5β	5α/EpiT	11β-OH-A/11β-OH-E	A/11β-OH-A	E/11β-OH-E
Mean		1,65	1,20	82,65	0,42	1,37	1,55	5,88	8,12
Std, Error of Mean		0,15	0,06	12,08	0,04	0,16	0,14	0,38	1,06
Median		1,48	1,28	51,47	0,42	1,13	1,34	5,68	6,46
Std, Deviation		0,97	0,42	78,29	0,23	1,05	0,94	2,47	6,89
Skewness		0,38	-0,06	2,20	0,90	2,22	2,52	0,46	3,02
Kurtosis		-0,52	0,21	3,93	1,24	6,37	8,41	-0,58	10,15
Range		3,58	1,83	309,80	1,00	5,42	4,97	9,38	36,81
Minimum		0,13	0,30	21,66	0,07	0,24	0,70	2,06	1,77
Maximum		3,71	2,13	331,46	1,08	5,66	5,67	11,44	38,58
Percentiles	2,5	0,14	0,32	25,63	0,08	0,25	0,73	2,34	1,79
	25,0	1,01	0,91	38,98	0,25	0,76	0,94	3,75	4,62
	75,0	2,43	1,42	83,13	0,53	1,65	1,66	7,71	7,69
	97,5	3,61	2,11	322,96	1,05	4,70	4,51	11,39	31,83
Differences					M2<M1 p<0,010	M2<M0 p<0,001 M2<M1 p<0,010	M2<M0 p<0,001 M2<M1 p<0,001	M1<M2 p<0,025	M2<M0 p<0,001 M2<M1 p<0,01

Independent-Samples Kruskal-Wallis Test. The significance level is 0.05. Significance values have been adjusted by the Bonferroni correction for multiple tests.

As a basis for the analysis of the obtained results, the observed significant differences in AAS concentrations and their ratios were summarized in Table 23.

Table 23. Compared data on the proven significant differences between the concentrations of steroids in urine and the ratios between them with the non-parametric Kruskal-Wallis test.

Parameter	F0	F1	F2	M0	M1	M2
A				M2<M0 p<0,010	M2<M1 p<0,001	M2<M0 p<0,010 M2<M1 p<0,001
E					M2<M1 p<0,050	M2<M1 p<0,050
11β-OH-A	F2<F0 p=0,010	F2<F1 p<0,010	F2<F0 p=0,010 F2<F1 p<0,010	M2<M0 p<0,001	M2<M1 p<0,001	M2<M0 p<0,001 M2<M1 p<0,001
11β-OH-E	F0<F2 p<0,010		F0<F2 p<0,010		M1<M2 p<0,010	M1<M2 p<0,010
5αA3α17βdiol	F2<F0 p<0,010	F2<F1 p<0,025	F2<F0 p<0,010 F2<F1 p<0,025	M2<M0 p<0,001	M2<M1 p<0,001	M2<M0 p<0,001 M2<M1 p<0,001
5βA3α17βdiol				M2<M0 p<0,010		M2<M0 p<0,010
EpiT	F2<F0 p<0,001	F2<F1 p<0,001	F2<F0 p<0,001 F2<F1 p<0,001			
T						
T/epiT	F0<F2) p<0,025	F1<F2 p<0,001	F0<F2) p<0,025 F1<F2 p<0,001			
A/E	F2<F0 p<0,010	F2<F1 p<0,010	F2<F0 p<0,010 F2<F1 p<0,010			
A/T						
5α/5β	F2<F0 p<0,050		F2<F0 p<0,050		M2<M1 p<0,010	M2<M1 p<0,010
5α/EpiT		F1<F2 p<0,025	F1<F2 p<0,025	M2<M0 p<0,001	M2<M1 p<0,010	M2<M0 p<0,001 M2<M1 p<0,010
11β-OH-A/11β-OH-E	F2<F0 p<0,001	F2<F1 p<0,001	F2<F0 p<0,001 F2<F1 p<0,001	M2<M0 p<0,001	M2<M1 p<0,001	M2<M0 p<0,001 M2<M1 p<0,001
A/11β-OH-A		F1<F2 p<0,020	F1<F2 p<0,050		M1<M2 p<0,025	M1<M2 p<0,025
E/11β-OH-E				M2<M0 p<0,001	M2<M1 p<0,001	M2<M0 p<0,001 M2<M1 p<0,001

4.2 Discussion

Since the study participants were divided by gender and level of their usual physical activity, the reference values obtained refer to homogeneous populations. Only with regard to the group of active female athletes the samples meeting the criteria (Table 5) were not sufficient to calculate reference values. As reported by other authors (Van Renterghem, et al., 2010; Martínez-Brito, et al., 2013) with regard to the substances included in the steroid profile, no normal distribution of concentrations measured was observed, which determined the use of nonparametric methods.

The reference values presented in Table 9 and Table 10 and the variation analysis between respective gender groups show that there are no statistically significant differences with regard to any of the indicators between the groups of non-athletes and leisure athletes in both sexes. This indicates that the regular, moderate physical activity (up to 3 workouts per week) does not affect the steroid profile.

The androgenic metabolites with the highest concentration in the urine are androsterone (5α-Androstane-3α-ol-17-one) and etiocholanolone (5β-Androstane-3α-ol-17-one). Precursors of androsterone excreted in the urine may be androstenedione and testosterone, and of etiocholanolone - androstenedione only. Along with them, 5α-Androstane-3α, 17β-diol and 5β-Androstane-3α, 17β-diol are found in the urine. 5α-Androstane-3α, 17β-diol is obtained from androsterone as a result of reversible conversion and from DHT as a result of irreversible conversion. 5β-Androstane-3α, 17β-diol is a metabolite of etiocholanolone and can also be derived from the minor and inactive metabolite of testosterone, 5β-DHT. Therefore, etiocholanolone and 5β-Androstane-3α, 17β-diol originate mainly from androstenedione and as 5β-steroids, they are mainly a product of hepatic metabolism (Chen & Penning, 2014). This is also confirmed by the fact that mutations in the liver isoenzyme AKR1D1 show decreased concentrations or lack of 5β-reduced steroids in the urine and liver failure (Gonzales, et al., 2004; Lemonde, et al., 2003; Palermo, et al., 2008).

As (1) no statistically significant differences in the A and E concentrations were reported between all female groups, but the A/E ratio in active female athletes showed lower values compared to the other two groups (Table 23) and (2) no statistically significant differences were found in the concentrations of 5β-Androstane-3α, 17β-diol measured in all three female groups, but 5α-Androstane-3α, 17β-diol in active female athletes has

significantly lower concentrations compared to the other two groups, these findings, together with the results for 11β-OH-A (see below), can be explained not by decreased hepatic metabolism (no differences in 5β-metabolites) but rather by reduced peripheral inactivation.

Evidence of an altered androgen metabolism rate in active athletes are also found in the male results. In active male athletes (1) the A concentrations are lower than those in the other groups, and (2) the E concentrations are lower only compared to the leisure athletes, which, however, does not affect the A/E ratio (there are no statistically significant differences between all male groups). The reported decreased excretion of androsterone glucuronide may be due to the conversion of androgenically inactive A in peripheral tissues through the so-called "Backdoor pathway" (Kamrath, et al., 2012; Fukami, et al., 2013; Bauman, et al., 2006; Gupta, et al., 2003) in the active androgen DHT, which is an intracrine activation to maintain the necessary androgen levels.

It is noteworthy that where the differences are more definite for A, namely in active athletes compared to leisure male athletes (M2 <M1; p <0,001), a difference is also found for E (p <0,050). Significantly lower concentrations of 5α-Androstane-3α, 17β-diol were found in active athletes compared to non-athletes and leisure male athletes and of 5β-Androstane-3α, 17β-diol in active male athletes compared to non-athletes. These findings, together with the significantly lower concentrations of 11β-OH-E (5β-metabolite of glucocorticosteroid origin) compared to leisure athletes, and of 11β-OH-A in active athletes compared to the other two male groups, may be explained by a probable decrease not only in peripheral but also in hepatic androgen metabolism (decreased 5β-metabolites).

Amid these metabolic changes, no statistically significant differences were found in both female and male groups in the measured T concentrations and A/T ratio values. There is evidence in the literature that there is no increased concentration of steroidogenic enzymes and T in the muscles as a result of physical training (Vingren, et al., 2008). In active male athletes, the lack of increased T biosynthesis is confirmed by the lack of difference in the amount of glucuronidation inactivated T and the lower concentrations of its glucuronidated metabolites compared to the other two male groups. These results show that all metabolic changes manage to maintain the normal level of active androgen in the body (T). A comparison of metabolic changes between active male and female athletes shows that women maintain the active androgen (T) levels through a reduced peripheral metabolism only, whereas in men this happens in

combination with reduced peripheral metabolism and reduced hepatic metabolism.

Since T/epiT and 5α/epiT ratios, together with other steroid profile parameters, are a criterion for an abuse of T and/or its precursors (Mareck, et al., 2010; Kicman, et al., 1995), the analysis of differences observed in the steroid profile of non-athletes, leisure athletes and active athletes, both men and women, are important to clarify any possible changes in the steroid profile as a result of active training.

No statistically significant differences were found for the T/epiT ratio between the three male groups, nor were there any differences in the T and epiT concentrations. The concentrations of 5α-Androstane-3α, 17β-diol in active male athletes are lower than in the other male groups, which, along with no differences in epiT concentrations among the three male groups, results in lower values of the 5α/epiT ratio in active athletes.

Lower concentrations of epiT were found in active female athletes, but higher values of the T/epiT ratio compared to the other two female groups, and no differences with other female groups in terms of T were observed. Things are different after we examine the dependencies for the 5α/epiT ratio in women. Epitestosterone (epiT) and 5α-Androstane-3α, 17β-diol have lower concentrations in active female athletes than in the other two groups, but with regard to the 5α/epiT ratio, active female athletes show a significant increase in values compared to leisure athletes only. This indicates differences in the rate of metabolism in peripheral tissues (5α-metabolites) between active and leisure female athletes.

Taking into account the discussed differences in active female athletes with regard to the T/epiT and 5α/epiT ratios and the fact that the production of epiT is only 3% of the production of T, and its rate of excretion is 30-50% of that of T (Wilson & Lipsett, 1966), and that the interconversion of T and epiT is negligible (Dray & Ledru, 1966), it can be assumed that the rate of decrease in epiT excretion in active female athletes is lower than the rate of decrease of 5α-Androstane-3α, 17β-diol. 1

11β-OH steroids

In urine, 11β-OH-E is a metabolite of glucocorticosteroid origin, and the 11β-OH-A present in higher concentrations is mainly derived from 11-oxo C19 steroids (Jones, et al., 2017; Shackleton, et al., 2008). On the other hand, 5β-

reduction is indicative of hepatic metabolism (Chen & Penning, 2014) and 5α-reduction is indicative of peripheral tissue metabolism (Russell & Wilson, 1994). 11β-OH-E is a 5β-steroid and 11β-OH-A is a 5α-steroid. In this respect, the concentrations of 11β-OH-A and 11β-OH-E and the 11β-OH-A/11β-OH-E ratio can be considered informative with regard to steroids originating mainly from the adrenal glands. In active athletes of both sexes, statistically significant lower concentrations of 11β-OH-A were found compared to the other respective gender groups, which indicates decreased peripheral metabolism of 11-oxogenized C19-steroids. Despite the higher concentrations of 11β-OH-E measured in active male athletes compared to leisure male athletes and in active female athletes compared to female non-athletes, always in active athletes the values of 11β-OH-A/11β-OH-E ratio remain lower compared to all other groups of the respective sex (i.e. non-athletes and leisure athletes are likely to generate more 11-oxygenated C19 steroids originating outside the adrenal glands as an inactivation mechanism; it is also an evidence of metabolic retention in active athletes).

Since androsterone is a source of active androgen produced in peripheral tissues through the “Backdoor pathway” (Kamrath, et al., 2012; Fukami, et al., 2013; Bauman, et al., 2006; Gupta, et al., 2003), and its reduced excretion in active male athletes is a testimony thereof, the A/11β-OH-A ratio can be considered as comparison of two possible ways for the production of active androgens in peripheral tissues - "Backdoor pathway" and 11-oxygenated androgen pathways (Kamrath, et al., 2012; Pretorius, et al., 2017; Swart & Storbeck, 2015). In this regard (1) the lower concentrations of A and 11β-OH-A observed in active athletes compared to the other two male groups and (2) the reported higher value of A/11β-OH-A ratio in active athletes compared to leisure athletes only, suggests the use of 11-oxygenated androgen pathways simultaneously with "Backdoor pathway". Given that (1) in women there were no differences in A concentrations across all groups, and (2) lower concentrations of 11β-OH-A were found in active athletes compared to the other two female groups and (3) higher values of A/11β-OH-A ratio in active athletes compared to leisure female athletes, it can be concluded that 11-oxygenated androgen pathways were also activated in active female athletes, probably due to the greater importance of the adrenal 11-oxogenized C19 steroids for the balance of active androgens available in women, and that the use of 11-oxygenated androgen pathways precedes the “Backdoor pathway”.

(1) statistically higher concentrations of 11β-OH-E in female active athletes compared to non-athletes, (2) no statistically significant differences in E concentrations and (3) in E/11β-OH-E ratio in all three groups, were found. In this context, if we examine the E/11β-OH-E ratio as representative of 5β-metabolism with glucocorticosteroid (11β-OH-E) and C19-steroid origin (E), it is found that the increased excretion of 11β-OH-E in active female athletes does not affect the clearance of 5β-steroids. While in active male athletes lower concentrations of E and 11β-OH-E are reported compared to leisure athletes, and although they show no differences compared to male non-athletes, the E/11β-OH-E ratio values in active male athletes are lower compared to the other two male groups. This is yet a further evidence of a decreased hepatic metabolism in active male athletes.

Analysis of steroid profile differences in the urine of participating groups of individuals showed that active athletes (10 or more workouts per week) maintained active androgen testosterone levels, despite the reduced AAS production, at the expense of reduced metabolism and excretion. From data found in the literature it can be assumed that at longer intense loads this compensatory mechanism may be insufficient and a decrease in testosterone concentration may occur.

Thus, based on the steroid profile, a state of a compensated reduced AAS synthesis can be registered, which precedes a possible adverse decrease in their levels, and allow for preventive measures to be taken with regard to training load and recovery.

Table 24 provides a systematized list of theoretically possible steroid metabolism states in the course of the training process in active athletes, detectable by changes in the urinary steroid profile. These conditions can be most reliably diagnosed by longitudinal observations.

Table 24. Systematized theoretically possible states of steroid metabolism in the course of the training process in active athletes, detectable by changes in the urinary steroid profile.

Normal testosterone excretion for the individual	Normal testosterone excretion for the individual	Decreased testosterone excretion
Normal state of androgen synthesis and metabolism	Compensated reduced androgen synthesis at the expense of reduced metabolism	Decreased androgen synthesis, e.g. after discontinuation of exogenous AAS.
Normal excretion of testosterone metabolites in the individual	Decreased excretion of testosterone metabolites	Decreased excretion of testosterone metabolites

5 CONCLUSIONS, RECOMMENDATIONS AND CONTRIBUTIONS OF THE STUDY

5.1 Conclusions

1. No statistically significant differences were found in the steroid profile of the groups of non-athletes and leisure athletes, in both sexes.

2. Testosterone concentrations and androsterone/testosterone ratio values did not show statistically significant differences between the three groups, in both sexes.

3. Decreased peripheral androgen inactivation was observed in active female athletes.

4. In active male athletes there was a decrease in peripheral and hepatic androgen metabolism.

5. In active athletes of both sexes decreased peripheral metabolism of 11-oxogenized C19-steroids was found.

6. In the active athletes' stage of training being studied, there was an adaptive decrease in metabolism and maintenance of AAS levels in response to their reduced synthesis.

7. The steroid profile studied by validated GC-MS methods can serve as a tool for the analysis of steroidogenesis-related metabolic changes in active athletes.

5.2 Recommendations

1 To expand the range of active athletes being studied with athletes practicing different sports in order to establish sport-specific reference values.

2 To conduct systematic individual longitudinal monitoring of the steroid profile changes during different stages of athlete's preparation in order to detect steroid metabolism adaptation and mis-adaptation processes.

3. In the future, the steroid profile study to be included as part of the regular athletes' checks and the interpretation of results so obtained to be applied in the training process controls in the relevant sport to achieve excellence.

5.3 Contributions

1. For the first time in Bulgaria, endogenous anabolic androgenic steroids reference values in active male athletes have been derived with a method validated in accordance with requirements of ISO 17025 and WADA ISL and TD EAAS.

2. For the first time in Bulgaria, through the analysis of steroid profiles obtained by a method validated in accordance with the requirements of ISO 17025 and WADA ISL and TD EAAS, the change in biosynthesis, metabolic clearance and excretion of endogenous anabolic androgenic steroids in active athletes compared to non-athletes and leisure athletes was established.

Publications in connection with the dissertation

1. Sheytanova T. The paradigm – athlete biological passport, Medicine and sport, 2020, 1, 48-54

2. Sheytanova, Tanya Z., Zarkova, Violeta L., Orbetzova, Maria M., Genchev, Gencho D. Assessment of Referent Values of Androgenic Steroids in Urine of Bulgarian Population at 16-30 Years of Age, Endocrinologia, 2020, 2, 100-119

Participation in congresses and symposia with materials related to the dissertation

1. Tanya Sheytanova. "The paradigm – athlete biological passport", XXth International Conference on Sports Medicine, 11.11.2019, Tsarsko selo, Sofia