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Multivariate interpretation of the urinary steroid profile and training-induced modifications. The case study of a Marathon runner

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Multivariate interpretation of the urinary steroid profile and training-induced modifications. The case study of a Marathon runner

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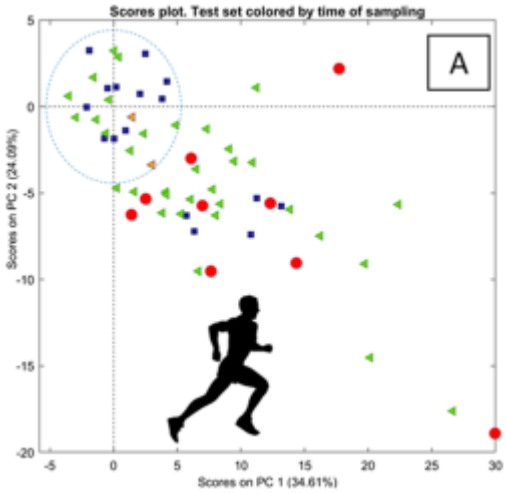
Abstract

The steroidal module of the athlete biological passport (ABP) introduced by the World Anti-Doping Agency (WADA) in 2014 includes six endogenous androgenic steroids and five of their concentration ratios, monitored in urine samples collected repeatedly from the same athlete, whose values are interpreted by a Bayesian model on the basis of intra-individual variability. The same steroid profile, plus dihydrotestosterone (DHT) and DHEA, was determined in 198 urine samples collected from an amateur marathon runner monitored over three months preceding an international competition. Two to three samples were collected each day and subsequently analyzed by a fully validated gas chromatography–mass spectrometry protocol. The objective of the study was to identify the potential effects of physical activity at different intensity levels on the physiological steroid profile of the athlete. The results were interpreted using principal component analysis and Hot~~w~~elling's T^2 *vs* Q residuals plots, and were compared with a profile model based on the samples collected after rest. The urine samples collected after activity of moderate or high intensity, in terms of cardiac frequency and/or distance run, proved to modify the basal steroid profile, with particular enhancement of testosterone, epitestosterone, and 5α-androstane-3α,17β-diol. In contrast, all steroid concentration ratios were apparently not modified by intense exercise. The alteration of steroid profiles seemingly lasted for few hours, as most of the samples collected 6 or more hours after training showed profiles compatible with the "after rest" model. These observations issue a warning about the ABP results obtained immediately post-competition.

Graphical Abstract

The urinary steroid profile of an amateur marathon runner was determined in 198 samples collected during three months of training under varying conditions. The data were interpreted by multivariate statistics revealing that intense training produces a substantial increase of the urinary steroids' level for up to 6–8 hours, but does not modify significantly their concentration ratios. Testosterone, epitestosterone, and 5α-adiol are the steroids most susceptible of varying upon intense training.



Keywords: athlete biological passport; gas chromatography–mass spectrometry; Hot~~v~~elling's T^2; principal component analysis; ~~U~~urinary ~~S~~steroidal ~~P~~profile

Figure S1. Scores plot of the PCA model built using the Reference Samples. Different symbols are used to distinguish the samples collected in the early morning after evening training corresponding to a delay of less than 12 h (green squares), from the samples collected after a day of rest, either in the morning or in the evening.

Figure S2. Box-and-whisker plot for the 8 monitored EAAS. These diagrams complement the data reported in Table 1.

Figure S3. Scores plot of the PCA model built using the Reference Samples. The eleven samples collected in 2019 during a period of rest (red diamonds) are projected into the scores plot. The sample collected at 4:05 a.m. exhibited particularly low concentration of most steroids, even if its data-point falls inside the boundary limits of the PCA model.

Table S1. Resume of the training activities, with details about the percentage time spent in the different heart rate (HR) zones (Z1 = 50 – 60 HR%; Z2 = 60 – 70 HR%; Z3 = 70 – 80 HR%; Z4 = 80 – 90 HR% and Z5 = 90 – 100 HR%), the distance run and the T^2 and Q scores.

1 INTRODUCTION

The misuse of endogenous anabolic androgenic steroids (EAAS) represents the predominant antidoping illicit practice.[1, 2] This makes the reliability of the gas chromatography–mass spectrometry (GC–MS) screening tests for EAAS misuse extremely important, particularly because the corresponding confirmatory analysis by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) is expensive and laborious.[3] On the other hand, the high inter-individual variability that characterize the endogenous production of EAAS prevents the definition of effective cut-offs for their urinary concentration.[4–7] To overcome this difficulty, in 2014, the World Anti-Doping Agency (WADA) introduced the steroidal module of the athlete biological passport (ABP).[8] This innovative anti-doping approach defines the normality limits of urinary EAAS for each individual by means of Bayesian inference after repeated controls on the same athlete (at least three), progressively excluding the influence of inter-individual variability[5, 9] and, consequently, providing higher sensitivity~~bility~~ and specificity scores to the screening tests. A pattern of six urinary EAAS is built into the ABP – testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α-androstandiol (5α-Adiol), and 5β-androstandiol (5β-Adiol)[10, 11] – together with five of their concentration

ratios: T/E, A/Etio, A/T, 5α-Adiol/5β-Adiol, and 5α-Adiol/E.[12] Lastly, dihydrotestosterone (DHT) and dehydroepiandrosterone (DHEA) are mentioned as additional constituents of the urinary steroidal panel.[12] The effectiveness of this longitudinal Bayesian approach has been verified by several studies.[5, 13, 14]

~~F~~A few research groups<<Query: AUTHOR: Few (not many) or a few (some) Ans: Some. However, also "not many" fits well.>> highlight the superior sensitivity and specificity provided to the ABP interpretation by a multivariate approach.[15–18] Recently, we compared the univariate Bayesian approach proposed by WADA for the ABP with a multivariate approach based on principal component analysis (PCA) and the diagnostic Hotelling's T^2 and Q residual scores,[19] and we demonstrated an improved performance of the ABP multivariate interpretation in relation to the identification of both deceitful sample replacement and the administration of pharmaceutical testosterone.

Since the practical efficiency of the ABP is founded on its relative constancy with time for each specific athlete, it might be questioned if significant ABP changes may arise from stressing conditions of particular intensity. As a matter of fact, the response of the hypothalamic–pituitary–adrenal (HPA) axis to physical training stress has been found to produce significant variation of the circulating pituitary and adrenal hormones level.[20–23] In particular, an acute increase of the total testosterone has been detected immediately after intense training,[24] with weak effects still detectable 24 hours and 48 hours after training.[25] Also, a rise of the DHT and DHEA basal concentration levels has been registered in some studies.[24, 26] In particular, this trend is observed once the training intensity exceeds the approximate threshold of 50% maximal oxygen consumption (VO_{2max}),[27] a parameter related to the cardiorespiratory fitness of the tested individual. However, all the cited studies[20–26] investigated the correlation occurring between intense training activity and the alteration of hormone levels by dosing the targeted analytes in blood or serum samples. Quite obviously, considerable time-lag and biochemical differences occur between biomarkers measured in blood/serum and urine with respect to the active metabolism processes and factors affecting the metabolites' excretion. Among the confounding factors that proved to modify the urinary steroid profile reported in the literature,[14, 28, 29] little attention has been paid to the training activity.

The psychological stress also proved to influence the steroids secretion from HPA and gonads.[30–32] The relationship between the stress due to competition and the increase of testosterone was demonstrated by Guezennec *et al.*, who monitored the testosterone levels in plasma before and after pistol shooting, a competition that actually requires low body energy consumption, but a consistent level of concentration and stress.[30] Either increased or decreased steroidal concentrations in the case of winning or losing, respectively, was observed in athletes. [32]

In the present case report, we explored the effect of training on the ABP of a volunteer amateur marathon runner, using the same multivariate PCA approach previously tested,[19] in order to verify the role and extent of physical training on the ABP variability. While the projection of the ABP steroid profile into a multivariate space enhances the discrimination between normal and anomalous urine samples,[19] the variability linked to natural biological factors is also likely to be concurrently emphasized. Within the limits of a case report, we intended to compare – in univariate and multivariate contexts – the extent of urinary endogenous steroid alterations due to the training activity, representing a rather unexplored subject of investigation. The steroid profile was determined on 198 samples collected over three months preceding a marathon competition and 11 further samples collected two years later, during a period of resting.

2 EXPERIMENTAL PROCEDURES (MATERIAL AND METHOD)

2.1 Chemicals and reagents

T, E, A, Etio, 5α-Adiol, 5β-Adiol, DHEA, DHT, and testosterone-d_3 were purchased from LGC Promochem SRL (Milan, Italy). Methanol, TBME, ethyl acetate, 17α-testosterone, dithioerithrol, and N-methyl-N-(trimethylsilyl) trifluoroacetate (MSTFA) were provided by Sigma-Aldrich (Milan, Italy). β-glucuronidase from

Escherichia coli was purchased from Roche Life Science (Indianapolis, IN, USA) and ammonium iodide was from TCI Co. Ltd (Tokyo, Japan).

2.2 Athlete enrolled

A voluntary, healthy, amateur male marathon runner was enrolled for this study. He was 46 years old during the period of the study, with a body mass index (BMI) equal to 20.3 ± 0.3. His aerobic capacity expressed in terms of VO_{2max} was equal to 62. Sampling of his urine took place during the whole period covered by his training activity for the 44th Berlin Marathon, ie,. from June 12, 2017 until September 24, 2017, for a total of 198 specimens. Eleven further samples were collected two years later over two days (June 1–2, 2019) within a period of resting. The athlete was an active partner of this research project, rigorously performing the sampling, the activity tracking, and the diary compilation.

2.3 Training and dietary diaries

The training activity of the athlete was routinely scheduled for the whole monitored period, as follows:

- Light activity (consisting of muscle strengthening and/or recovery run at low intensity, below the 50% of VO_{2max}): once a week.
- Short-distance run (less than 10 km): twice a week.
- Middle-distance run (between 10 and 15 km): from zero to one time a week.
- Long-distance run (more than 15 km): once a week, always in the morning.
- Interval run (fast sections of 2 to 5 minutes alternated with recovery): from one to two times a week; they are further classified on the basis of the distance: short distance (overall distance covered of less than 8 km) and middle distance (distance covered between 8 and 15 km)

All the running activities were monitored using a GPS watch Garmin® Forerunner 630 (Software 7.70.0) connected with a cardio band. The mean cardiac frequency and time spent above the aerobic threshold were recorded.

The athlete drafted a nutritional diary (mean energy intake: 2000 kcal/day), where he calculated the consumed kcal/day and took note of food supplement intake. He mainly consumed carbohydrates (eg, pasta and bread), fish, vegetables, and milk surrogates. Every morning, he took one tab of magnesium (180 mg) and potassium (300 mg) supplement and once a week, in the evening, he consumed supplements containing iron (21 mg), vitamin C (90 mg), and folic acid (0.3 mg). Alcohol consumption was extremely moderate and was monitored as a potential confounder factor; the athlete declared that he abstained from any alcohol consumption during the three weeks before the marathon competition, while in the preceding period he occasionally drank a light beer (never more than 660 mL/day). He also declared to abstain from spirit consumption at any time.

2.4 Urine sampling and processing

Depending on the training schedule, 2–3 samples per day were collected during the investigated period. In particular, the urine sampling included the collection (a) at wake-up, (b) post training, and (c) evening (approximately, at 10 pm). The post-training and evening samples coincided in the days of late (evening) training. Notably, the samples collected at wake-up after late training were not differentiated from the other "wake-up samples" even if less than 12 hours elapsed after training, since their multivariate steroid pattern turned out homogeneous (Figure S1). Due to the high temperature recorded during the training period (summer 2017) and the consequent body dehydration, the athlete was occasionally unable to provide the post-training sample shortly after the end of the run. The time elapsing between physical activity and sampling was recorded and used to interpret the experimental results.

All collected samples were stored at +4°C for a maximum of a week and then moved in a cold room at −20°C. Once collected ($N = 198$), the samples were randomly processed in six consecutive analytical sections (34 samples each). Later samples (11 samples) were treated identically. The integrity of the samples was verified using the marker 5β-androstan-3,17-dione, following WADA's guidelines[12] and no effect of degradation was detected.

2.5 GC–MS analysis

The steroids T, E, DHT, DHEA, 5α-Adiol, 5β-Adiol, Etio, and A were selected as the target analytes. 5β-androstan-3,17-dione was used as a marker of microbial degradation.[9, 12, 19]. GC–MS run under selected ion monitoring conditions was used to determine the targeted steroids, as indicated in WADA's guidelines[12] for anti-doping steroid screening. The analytical method was optimized and fully validated, following the ISO/IEC 17025 prescriptions and WADA guidelines. Briefly, the sample pretreatment involved deconjugation of glucuronide metabolites by β-glucuronidase from *Escherichia coli*, followed by liquid–liquid extraction (LLE) at basic conditions. Lastly, the dried extract was subjected to TMS derivatization. Two internal standards were used: (a) 17α-methyl-testosterone, for A and Etio, and (b) testosterone-d_3 for the other six target analytes. The instrumentation and instrumental settings for the GC–MS analysis are described in a previous publication.[19] The measured concentration levels were normalized in accordance with a standard specific gravity of 1.020 (measured by the gravimetric method).

2.6 Statistical analysis

All the urine samples collected at wake-up and in the evenings of rest days were included in the "reference population" samples (119 samples), *ie*, urine samples allegedly presenting a rather constant ABP steroid profile without any alteration related to the physical activity. The samples collected after the training and in the evening of training days constituted the "test population" (79 samples), as depicted Figure 1. Both univariate and multivariate statistical analysis were applied. The univariate approach consisted in one-way ANOVA over the two groups of samples, namely "reference population" and "test population," for each monitored biomarker. Then, a multivariate explorative investigation was run by PCA to study how the samples of the two sets distributed along the multidimensional space of the variables. Outliers were identified using the diagnostic diagram plotting the reduced Hotelling's T^2 coefficient *vs*. the reduced Q residuals. All the statistics were performed with the software Matlab® (The MathWorks, Natick, MA, USA<<Query: AUTHOR: Please ad name of town or cityin which MW is located Ans: Natick>>) version R2018b and PLS_Toolbox 8.5.[33]

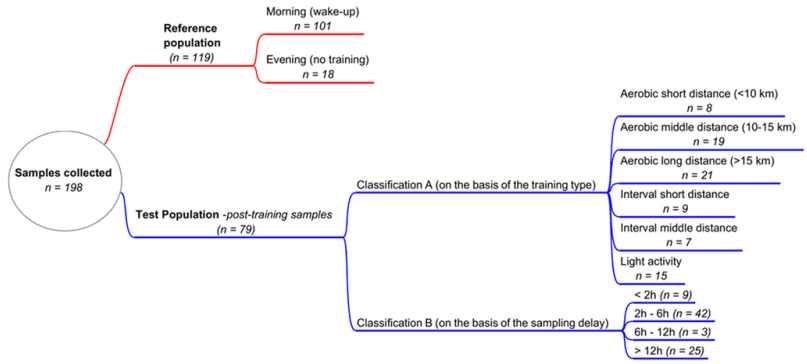


Figure 1 Graphical explanation of the samples' classification. The two main classes are indicated as "reference population" and "test population" samples. The latter are classified further on the basis of the training type (classification A) and the sampling interval (classification B) [Colour figure can be viewed at wileyonlinelibrary.com]

3 RESULTS AND DISCUSSION

The main dataset composed of 198 samples and 13 variables (six EAAS included in the ABP steroid module plus DHT, DHEA and five concentration ratios) was split into the two sample subsets of "reference population" and "test population."

3.1 Univariate analysis of the data

The one-way ANOVA revealed a significant increase of T, E, DHT, DHEA, 5α-adiol, and 5β-adiol levels (p-value <0.05, Table 1) in the post training samples with respect to the "reference" samples, while A and Etio were not modified. Also, the ratios A/T, 5α-Adiol/E, and 5α-Adiol/5β-Adiol turned out statistically different for the two groups, even if the large range of values associated with each variable made any specific conclusion questionable. Even if the possible dehydration following intense training has been compensated by data normalization using urine specific gravity, the steroid ratios are expected to be totally unaffected by such a potential bias. The boxplots reporting the urinary concentration distributions (Figure S2) showed that the samples collected after the training activity were distributed over a wider range of concentrations, with higher means and medians values. Only Etio and A presented similar distributions for the two populations with slightly lower mean values for the test samples. Mean, standard deviation, and p-values are reported in Table 1.

Table 1 Univariate analysis results. The mean values and corresponding standard deviations are reported for each analyte and ratios for the two groups of samples. The p-values are reported in the third column

Marker	Reference Samples (Mean Values ± Standard Deviation) (ng/mL)	Test Samples (Mean Values ± Standard Deviation) (ng/mL)	*p*-value
T	27 ± 11	41 ± 23	5.2×10^{-7}
E	17.0 ± 6.2	25 ± 14	4.6×10^{-7}
DHT	9.0 ± 5.9	34 ± 38	1.9×10^{-9}
DHEA	23 ± 12	44 ± 37	4.0×10^{-7}

Marker	Reference Samples (Mean Values ± Standard Deviation) (ng/mL)	Test Samples (Mean Values ± Standard Deviation) (ng/mL)	*p*-value
5α-Adiol	123 ± 85	320 ± 377	1.6×10^{-6}
5β-Adiol	51 ± 31	152 ± 160	8.1×10^{-9}
Etio	1053 ± 772	1136 ± 1200	0.59
A	625 ± 496	579 ± 700	0.61
T/E	1.60 ± 0.20	1.63 ± 0.27	0.42
A/Etio	0.58 ± 0.21	0.8 ± 2.2	0.40
A/T	25 ± 18	19 ± 22	4.6×10^{-2}
5α-Adiol/E	3.1 ± 2.0	6.8 ± 6.0	7.8×10^{-8}
5α-Adiol/ 5β-Adiol	0.44 ± 0.15	0.55 ± 0.21	5.2×10^{-5}

3.2 Multivariate approach

A PCA model was built using the "reference population" samples. The first four PCs, which explain almost 85% of the overall variance, were considered statistically significant. The third and fourth PCs provided a substantial contribution to the Reduced Hotelling's T^2 values and, to a minor extent, to the PCA model. Scores and loading plots for the first and second PCs are reported in Figure 2. Apparently, no evidence of a trend related to circadian rhythm is present, as the "wake-up" and "evening" samples appear to be almost randomly distributed in the score plot (Figure 2A). Only weak evidence of diurnal variation can be detected by calculating the separate mean PC1 and PC2 scores for the morning samples following a day of rest (coordinates: 0.50 and – 0.92) and the evening samples (0.01 and 0.11) (Figure S1). This finding is in agreement with previous studies which demonstrated the stability of urinary steroidal ratios with respect to circadian rhythm, since any diurnal pattern of steroid concentrations possibly present in blood is leveled during the urinary excretion.[34] Analogous conclusions are drawn from considering the third and fourth PC variables.

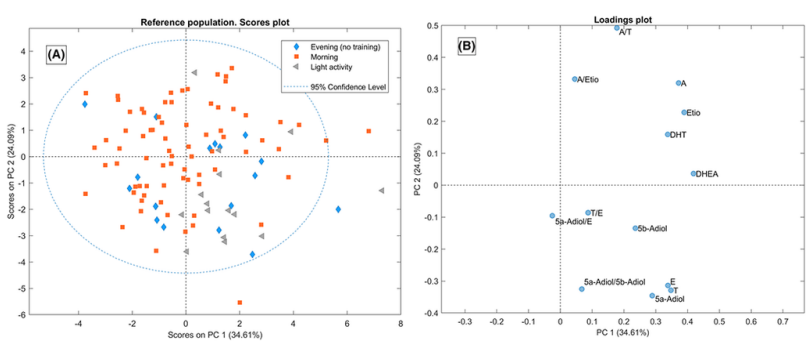


Figure 2 A, scores and B, loadings plot of the PCA model built using the "reference population" samples. The samples of the test set belonging to the subclass "*Light Activity*" (grey triangles) are projected into the scores plot of the PCA model [Colour figure can be viewed at wileyonlinelibrary.com]

Further confirmation of the steroid profile stability in the absence of perturbing factors was obtained from the 11 samples collected in June 2019 (*ie*, two years after the chief sample collection) on two consecutive days.

The projection of the corresponding data points into the former PCA model placed all of them safely inside its boundary limits (Figure S3), proving that the steroid profile of the athlete was substantially unmodified during a two-year period, provided that the "under resting conditions" were respected. A barely detectable circadian variation in the latter data was not statistically relevant, with the sole exception of a single sample collected at 4:05 a.m., in which the steroid concentrations were particularly low. Accordingly, the corresponding data point was located in the upper left corner of the PCA model (fourth quarter) (Figure S3).

The various groups of samples belonging to the "test population" (Figure 1) were projected onto the PCA model built on the "reference population." Of the 15 samples corresponding to the "light activity," as defined in Section 2.3 (Figure 2A), 14 fall within the 95% confidence boundary, demonstrating little – if any – influence of this soft activity on the urinary steroidal profile. This result was expected, due to the low physical effort required to carry out this training. Nevertheless, it is worth noting that all but one of these samples exhibited a positive score for the first PC, unlike the "reference population." This modest distribution anomaly that parallels the positive loadings observed for all EAAS, might be interpreted as a mild enhancing effect of training on steroid levels, in general. In order to ease the data visualization, all the samples belonging to the "light activity" class were excluded from the subsequent graphs.

The remaining samples of the "test population" are represented in Figure 3, where the boundaries of the model built with the "reference population" appear in the upper-left corner of Figure 3A and 3B. While some samples fall within these boundaries, some others are located quite far from the model and correspondingly produced high values of T^2 and/or Q (Figure 3C and 3D). The latter graph labels these samples as outliers, indicating that the corresponding profiles are significantly different from the "reference population" ones.

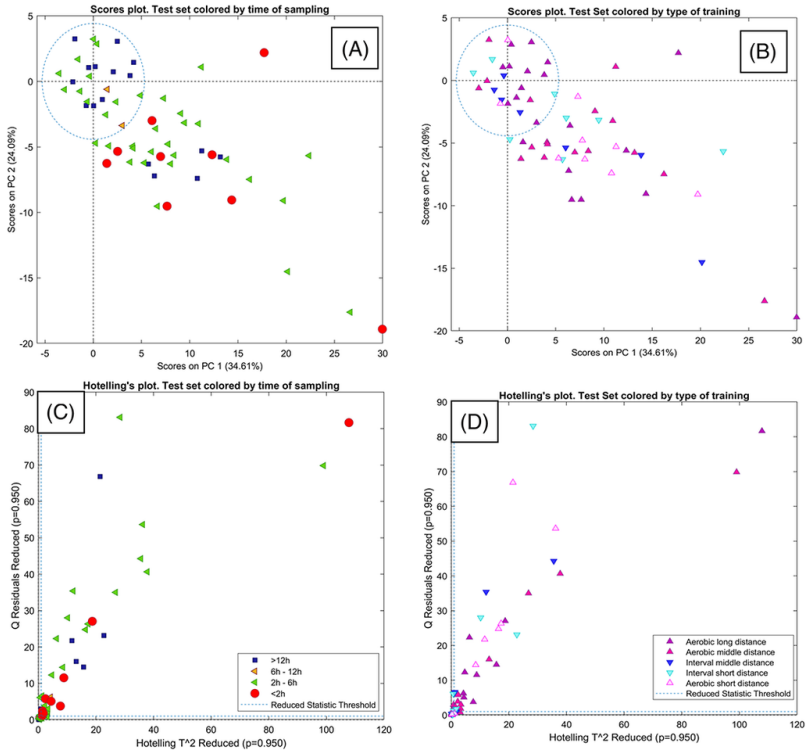


Figure 3 Projection of the "test population" samples into the space of the PCA model built using the "reference population" samples (A and B), and corresponding values of Hotelling's T^2 and Q residuals (C and D). The "test population" samples are colored on the basis of the sampling delay (A and C) or the type of training (B and D) [Colour figure can be viewed at wileyonlinelibrary.com]

To better understand the observed trends, the "test set" samples were divided into subclasses (Figure 1) according to the type of training activity or the sampling delay (*ie*, the time lapse between the training and sample collection). The percentages of samples outside the confidence boundaries are reported in Table 2. No straightforward evidence of correlation between the type of activity and the distance from the model was observed, while all the samples collected with a delay of less than two hours are located outside the boundaries of the model space. On the other hand, several samples collected after larger time intervals are still located outside the model borders. While no steady trend is observed, several important observations can be made from Figure 3 and Table 2. First, most of the samples collected more than 6 hours after the physical activity are located inside the model space or close to the boundary, suggesting the restoration of a normal EAAS profile within few hours. A striking example is given by the samples collected on August 6, 2017: the after-training steroidal profile is extremely far from the model (Reduced Hotelling's T^2 = 108 and Q = 81), while the data-point corresponding to the evening collection falls inside the model boundaries for both parameters (Figure 4). The profile measured for the sample collected after the aerobic long-distance training on July 30 shows highly negative scores along the second PC and quite high values for Reduced Hotelling's T^2 and Q (respectively, 4 and 5), while both values fall inside the normal ranges for the sample collected the same evening. Again, on June 24 (the aerobic middle-distance training day) the post-training sample produced low PC2 scores together with Reduced Hotelling's T^2 = 1.5 and Q = 2.4, but the evening sample exhibited a steroid profile compatible with the model. In the Supporting Information (Table S1), the Reduced Hotelling's T^2 vs Q residual scores for all the test samples are reported. The last interesting feature of Figure 4 is the position of the sample collected after the marathon competition (September 24) when the physical effort necessary to run 42 Km was added with the reported psychological stress given by the attendance to an international competition potentially producing significant alteration of the steroid profile. In contrast, modest displacement of the corresponding data-point from the model was observed – despite relatively high Hotelling's T^2 and Q residual values (both close to 5) – which can possibly be explained by the long delay (7 hours and 40 minutes) with which urine was sampled after the competition end. Also, in this case, it is conceivable that a smooth restoring of the physiological rest condition was underway. While it is clearly impossible to draw general conclusions from the comparison of specific sample pairs, the proposed examples are reported to underline that the perspective information provided by the PCA model holds when the data evaluation shifts from general to particular.

Table 2 Percentage of "test population" samples out from confidence boundaries (95%) in the PCA model built using the "reference population"

	Outside PCA Confidence Boundaries (%)
Time of sampling	
< 2 hours	100%
2–6 hours	68%
6–12 hours	0%
> 12 hours	24%
Type of activity	
Aerobic long distance	48%
Aerobic middle distance	68%
Aerobic short distance	78%
Interval middle distance	43%
Interval short distance	63%
Light activity	7%

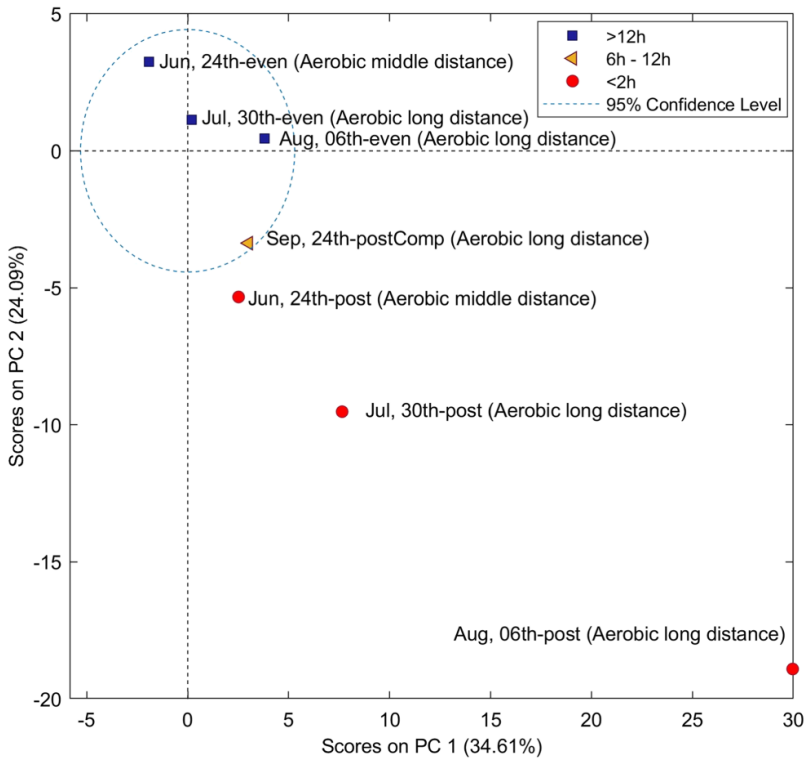


Figure 4 Projection of seven selected samples from the "test population" into the space of the PCA model, identified with a dotted blue line indicating its boundary limit [Colour figure can be viewed at wileyonlinelibrary.com]

The occurrence of possible correlations between the data-point distribution and dietary, cardiac frequency and time spent above the aerobic threshold during the workout was investigated. It was concluded that only the latter factor may have produced a detectable effect, corresponding to high scores for both T^2 and Q in most of the data-points (≅80%) relative to training sessions in which the subject had spent substantial time above the aerobic threshold. This was especially evident if the urine sampling occurred less than 3 hours after the end of the run (Table S1).

Further biological interpretation can be inferred from the comparison of scores and loadings plots reported in Figures 2 and 1B. It can be observed that the departure from the model occurs along a line crossing the second quarter of the scores plot. In particular, the extreme outliers are characterized by high values of the first PC together with high negative values of the second PC, namely a space domain corresponding – in the loadings plot of Figure 2B – to high values of T, E, 5α-Adiol and, to a minor extent, 5β-Adiol. These are the steroids more likely to show an increased concentration in the urine collected shortly after an intense physical activity. The same neat trend was reported for T in a different biological matrix (blood).[24] In contrast, the concentration ratios T/E and 5α-Adiol/E, crucial for the detection of illicit EAAS administration, appear not to be affected by physical stress induced by training, as their loadings place them close to the PC-axes intersection, *viz*. at the model centroid. While no strictly comparable studies are present in the literature, a longitudinal study investigating the blood profile of the main hormones after physical activity reported a peak concentration for DHEA and free T about 30 minutes after the end of a run. Then, the steroid levels started decreasing until lower-than-basal concentrations were observed in the timeframe of 4.5 hours.[35] Since the metabolic effects in urine are delayed and detectable over a wider time range, it is not surprising that we still observed an analogous steroid enhancement for few hours after the training.

From all these features of multivariate analysis, it can be deduced that intense physical activity may alter significantly the concentration of some steroids that are included in the ABP, at least during the first few

hours after the work. This change is not uniform but refers mainly to a concomitant increase of the urinary concentration T, E, and 5α-Adiol, as is evident in Figure 5A, 5B, and 5C, reporting the sequence of the T and 5α-Adiol concentrations, and T/E ratios, measured on the 198 urine samples vs. the corresponding Z-Scores. The ABP threshold calculated for T/E is reported in Figure 5D. Its line – initially estimated from a general reference population[19] – rapidly converges on the stable value progressively calculated from mean and standard deviation of the accumulated data. While many upper spikes of the 5α-Adiol and T data sequence are projected outside of the Z-Scores line and the 2σ-limit (two standard deviations), the same does not occur for the T/E ratio, whose data points are consistently located below the Z-Scores line. The T/E ratio depicted in Figure 5C and 5D shows limited variability with modest passing of the ABP threshold recorded only at the beginning of the series. Since uniquely the steroid ratios (not the absolute values) are considered ~~uniquely~~ during the ABP anti-doping screening, the number of false positive results to be corrected by onerous confirmatory analysis performed by isotopic ratio mass spectrometry (IRMS) is restricted. However, misleading results are expected particularly from in-competition tests, when ~~directly after the race~~ chaperones guide the athlete to the doping control station directly after the race, whereas the alteration is possibly less pronounced if more time elapses from the end of competition to the time of urine sampling.

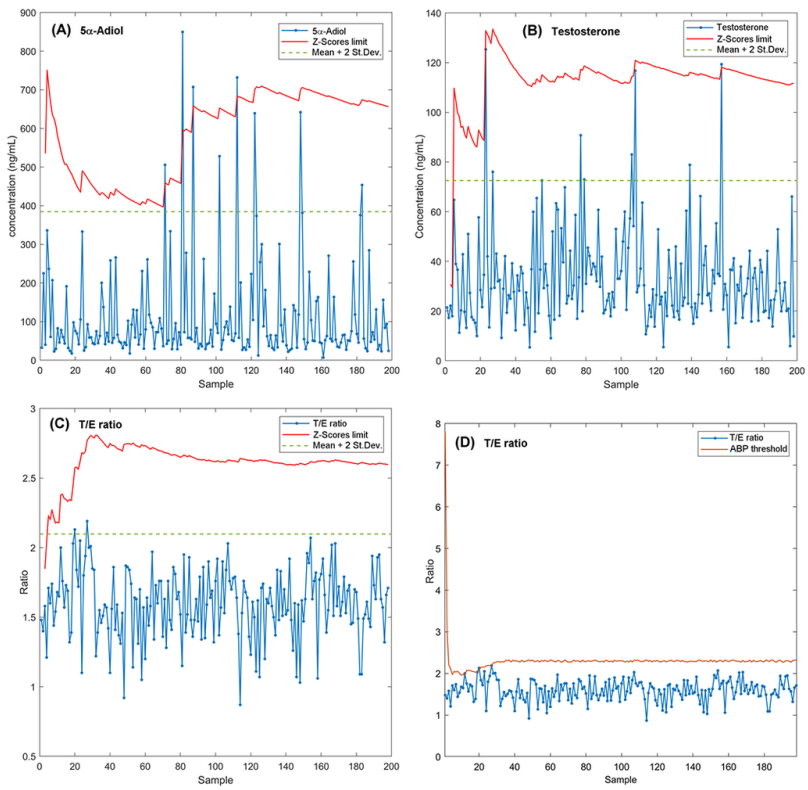


Figure 5 Series of biomarker values for the 198 urine samples (light blue dots), Z-scores (light-brown line), and 2σ-limit, calculated by adding two standard deviations to the mean value (green dotted line). A, 5α-Adiol concentration; B, testosterone concentration; C, ratio T/E; D, ratio T/E compared with the calculated ABP threshold (light-brown line) [Colour figure can be viewed at wileyonlinelibrary.com]

4 CONCLUSIONS

The obvious limitation of the present investigation is that it is based on the monitoring of a single athlete, a condition that prevents any deduction of general significance. However, this study has the worth of comparing the steroid profile recorded from a huge number of urine samples (198) collected during a short period (3 months). This opens an outlook of high statistical meaning, which is impossible to reach by the normal

practice of sequential recording under the unexpected timing typical of the ABP steroid module. <<Query: AUTHOR: please check that meaning has been retained Ans: Ok, I don't see any difference.>>Another important innovation proposed in the study is methodological, as the whole set of relevant information is deduced from a multivariate approach, which provides a different and significant perspective with respect to the classical univariate interpretation of steroid levels.

The main conclusion drawn from both the careful evaluation of large sample population data and the comparison of paired specimens is that upon physical activity of moderate intensity (*ie*, typically non-professional) the urinary steroid profile of the investigated runner is significantly modified with respect to those collected either after night-resting or several hours after work (typically, 8–12 hours). In particular, the observed concentrations of T, E, and α-Adiol turned out to be significantly higher in the urine samples collected shortly after the physical exercise. These observations issue a warning about the EAAS results obtained from urine samples collected immediately after competition, to be fed to the ABP steroid module of the tested athletes. On the other hand, this warning does not apply to the various steroid concentration ratios included in the ABP, which proved to be quite stable regardless of the sampling time. After all, the higher stability of concentration ratios with respect to the absolute steroid level is a well-known feature of the ABP[9, 10, 36] and justifies their consistent use in the ABP interpretation.[14]

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