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Removal of the influence of plasma volume fluctuations for the athlete biological passport and stability of haematological variables in active women taking oral contraception

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Abstract

The haematological module of the athlete biological passport (ABP) monitors longitudinal haematological variations that could be indicative of blood manipulation. This study applied a multi-parametric model previously validated in elite cyclists to compare inferred and actual PV variations, whereas the potential influence of the oral contraceptive pill (OCP) cycle on the ABP blood biomarkers and plasma volume (PV) in 14 physically active women taking OCPs was also investigated. Blood and serum samples were collected each week for 8 weeks, and the ABP haematological variables were determined according to the World Anti-Doping Agency guidelines. Transferrin (sTFN), ferritin (FERR), albumin (ALB), calcium (Ca), creatinine (CRE), total protein (TP) and low-density lipoprotein (LDL) were additionally computed as 'volume-sensitive' variables in a multivariate analysis to determine individual estimations of PV variations. Actual PV variations were indirectly measured using a validated carbon monoxide rebreathing method. We hypothesised ABP markers to be stable during a standard OCP cycle and estimated PV variations similar to measured PV variations. Measured PV variations were in good agreement with the predictions and allowed to explain an atypical passport finding (ATPF). The ABP biomarkers, Hbmass and PV were stable over 8 weeks. Significant differences occurred only between Week 7 and Week 1, with lower levels of haemoglobin concentration ([Hb]), haematocrit (HCT) and red blood cell count (RBC)(-4.4%, $p < 0.01$; -5.1%, $p < 0.01$; -5.2%, $p < 0.01$) and higher levels of PV at week 7 ($+9\%$, $p = 0.05$). We thus concluded that estimating PV variations may help interpret individual ABP haematological profiles in women.

KEYWORDS
anti-doping, haemoglobin, plasma volume, reticulocytes, women

1 | INTRODUCTION

With its ability to monitor robust indirect blood biomarkers altered by the use of performance-enhancing drugs, the athlete biological passport (ABP) is now paramount in the fight against doping.[1] Its haematological module enables the tracking of individual changes to detect deviations from a normal physiological condition. In other words, the ABP aims to monitor discrepancies in blood biomarkers expected to vary naturally within a certain range in order to identify variations that have an external cause (medical condition or doping).[2] The model relies on a Bayesian-based algorithm to compute the probability that the biomarker variations are in accordance with a normal physiological condition.[3–6] The computation is based on data generated using standardised procedures in order to harmonise pre-analytical and analytical sources of variations.[5,7–10]

However, because the ABP relies on numerous concentration-based biomarkers, such as [Hb] and OFFs, shifts in plasma volume (PV) may impair the interpretation of single ABP profiles with many sources of PV variation in athletes (e.g. exercise training, hydration, exposure to extreme environments [heat/cold or altitude] and competitions).[11–19] In this context, a multi-parametric model to remove the influence of PV shifts has been proposed[20,21] and was recently validated for the variance caused by altitude exposure in endurance athletes,[22] such as that experienced by elite cyclists during a stage race.[23] The model appeared capable of reducing the influence of PV on concentration-dependent markers of the ABP, with approximately 68% of PV variance explained, and it could also be applied in female athletes to test for actual PV shifts during the menstrual cycle. Investigating the PV variation algorithm in a female cohort is motivated by the fact that the ABP paradigm relies on the monitoring of variations in single individuals, and, to the authors' knowledge, the specific analysis of potential effects of OCPs on the ABP has not been conducted yet.

Further control of established confounding factors is hence necessary to improve the sensitivity and specificity of the ABP,[24] especially in female athletes. Notably, menses have been suggested to induce sufficient blood loss and variations in haematological parameters[25–28] to justify an atypical haematological ABP profile. Therefore, it is important to accurately describe the variations of ABP biomarkers over the course of a menstrual cycle as there is no consensus on its effect on haematological parameters.[29–36] Recently, Mullen et al[37] noted a significant increase in absolute reticulocyte count (RET#) and Ret% in the ovulatory phase (OP) and luteal phase (LP) in 17 active women with regular menstrual cycles. However, these findings were not reproduced in a recent study monitoring multiple biomarkers in 14 women with normal menstrual cycles.[36] The lack of data for possible PV shifts may restrain the interpretation of the latter atypical passport findings (ATPFs) because it was recently shown that shifts in PV can induce ATPFs.[38]

To the best of our knowledge, the haematological variations during the menstrual cycle have only been reported in women without oral contraceptives.[37] However, a large number of elite female athletes (approximately 50%) use hormonal contraception as a tool to control their cycle, with a large proportion using oral contraceptive pills (OCPs).[39–41] With the evidence that data on haematological variables in women are lacking, our study aimed to investigate virtually unknown putative variation of blood biomarkers in women during an OCP cycle.

The menstrual cycle is typically characterised by three phases: the follicular phase (FP), which starts with the menses; the OP; and the LP, which presents a high concentration in progesterone and initiates the cycle again.[42] Several hormonal chain reactions are associated with the menstrual cycle and can have an influence on female physical capacity.[41,43,44] Women taking OCPs have less amplitude in their hormonal status and cannot be considered to have 'normal' menstrual cycles even though they may have regular bleeding patterns because of the hormonal regimen of their OCPs. Thus, in this study, the terms withdrawal bleeding and standard OCP cycle are used instead of menses and menstrual cycle.

In this study, we aimed to compare actual PV shifts (calculated using CO rebreathing) with predicted variations from a multiparametric model based on a panel of 'volume descriptive' biomarkers in active women over 8 weeks. We also aimed to illustrate the efficiency of the model in evaluating potential variations in the biomarkers of the ABP in physically active women taking OCPs over two OCP cycle. To address our research question, we first hypothesised that the potential shifts in PV and the predicted variations calculated by the model would be in good agreement and that the model would allow to explain potential ATPFs. Finally, we also hypothesised that ABP biomarkers would remain stable over the course of a standard OCP cycle in active women taking OCPS.

2 | MATERIAL AND METHODS

2.1 | Study subjects

Fifteen healthy female subjects with regular bleeding patterns, aged 23.2 ± 2.4 years, volunteered to take part in this study and were monitored for 8 weeks (corresponding to two consecutive OCP cycles). Recruitment was conducted by contacting students at the Institute of Sport Sciences of the University of Lausanne (ISSUL) and continued through a snowball sampling method.[45] A minimum of 4 h of weekly physical activity was required to participate, and subjects exercised for an average of 297 ± 100 min per week during the study. All subjects had regular a cycle length of 28.5 ± 1.5 days and were taking an oral contraceptive (see Table 1). Intake of medication was recorded, and no subjects reported iron supplementation throughout the study. Procedures and risks were fully explained to the subjects, and all of them gave their written consent to participate in the study. This study was approved by the local ethics committee (CER-VD, Lausanne, Switzerland, Agreement 2018-01019) and conducted in accordance with the Declaration of Helsinki.

TABLE 1 Study cohort with cycle patterns; type of oral contraceptive pill (OCP); training data, namely, volume (min) and load (min × RPE [rate of perceived exertion]); number of samples; and cycle and bleeding length

ID	Age	Days between measure	Cycle length (days)	Bleeding length (days)	Nb of samples	Weight (kg)	Size (cm)	Training volume (min)	Training load (min × RPE)	OCP	Type of OCP	Bleeding pattern
1	22	7.3	27.5	3.5	8	57.78	157	455	3229	Elyfem 30	Combined OCP (COCP)	Low
2	22	7.0	28	4	8	63.01	180	407	3036	Belara	COCP	Low
3	22	10.3	28	3	9	60.12	160	176	662	Ologyn	COCP	Medium
4	25	7.0	28	6	8	57.66	160	349	2363	Elyfem 30	COCP	Medium
5	27	7.1	28	5	8	60.40	168	371	2118	Ologyn micro	COCP	Low
6	22	6.7	28	4	8	63.06	173	201	1496	Lysenia	COCP	Medium
7	25	6.9	28	5	8	59.80	162	310	2059	Sibilla	COCP	Medium
8	23	7.0	28	4.5	8	59.34	175	195	1279	Ologyn	COCP	Medium
9	21	7.1	28	3.5	8	63.89	165	131	936	Sue 30	COCP	Abundant
10	23	7.3	28	4	8	58.53	163	440	2911	Valette	COCP	Abundant
11	20	7.0	28	5	8	55.59	170	304	1967	Elyfem 30	COCP	Medium
12	21	7.1	28.5	5.5	8	60.36	168	303	1730	Yira 30	COCP	Medium
13	29	8.9	33.5	2.5	8	62.18	164	216	1100	Qlaira	Four phasic COCP	Low
14	22	6.7	30	7	8	55.20	162	245	1557	Elyfem 30	COCP	Low
Means	23.20	7.4	28.50	4.43	8.07	59.65	166.20	297	1885			
SD	2.43	0.9	1.49	1.18	0.26	2.63	6.26	100	764			

Note: Values are presented as means ± SD.

2.2 | Study design

The study design is illustrated in Figure 1. Over 8 weeks (corresponding to two consecutive standard OCP cycles), blood and serum samples were collected, and Hbmass and PV were measured once a week. Subjects were asked to report to the laboratory each week during the two cycles at the same time of the day (to avoid putative circadian variations). Because of the COVID-19 pandemic, one subject had to self-isolate, and thus, eight samples were collected over two non-consecutive cycles in a 3-month period. Participants were asked to record their OCP cycles through a dedicated smartphone application by reporting the days of their withdrawal bleeding (and bleeding patterns) (*Clue: Period Tracker, Ovulation & Cycle Calendar*[46,47]). Haematological variables were analysed in chronologic order over the 8 consecutive weeks independently of the cycle phase (W1–W8) with W1 representing the first visit. In addition, to assess recurring patterns, haematological variables were pooled in phases based on the first day of their bleeding and analysed. The successive phases following the week of the bleeding were defined for the analysis as follows: P0 represented the week of the withdrawal bleeding, P1 represented P0 plus 1 week, P2 represented P0 plus 2 weeks, and P3 represented P0 plus 3 weeks. Differentiating between the successive phases in weeks following the withdrawal bleeding was preferred to separating menstrual phases (FP, OP and LP) because OCP intake blunts the natural hormonal regulation of the latter phases. To monitor physical activity throughout the study, participants were asked to complete a personal training diary in which they documented each training session (date, sport, duration, rate of perceived exertion [RPE] [1–10] and physical feeling after training [1–10]) for the entire study period, starting on the first visit to the laboratory. Training volume (min), intensity (as RPE) and load (min × RPE) were then calculated from these data.

2.3 | Haematological analyses

Blood sampling was conducted by two experienced phlebotomists following the current WADA guidelines on analytical procedures[10]; however, instead of using the recommended BD Vacutainer® tubes (EDTA-K2 [K2] CE cat no. 368856/ref US 367856), we collected blood in Sarstedt S-Monovette tubes (EDTA-K2 2.7 ml, Sarstedt AG, Nümbrecht, Germany), which we considered equivalent since the same anticoagulant is being used by both brands. The participants reported to the lab having avoided any physical exercise in the 2 h preceding the sampling. The subjects then remained seated for 10 min to avoid acute PV variation.[48] Venipuncture was performed after local disinfection with a 21-G short manifold butterfly needle (Sarstedt Safety-Multifly®, Sarstedt AG, Nümbrecht, Germany) inserted into an antecubital vein, and whole blood was collected in one 2.7-ml tube (EDTA-K2 tube, Sarstedt, Nümbrecht, Germany) and one 9-ml serum tube (Sarstedt S-Monovette® Serum-Gel 9 mL, Sarstedt AG, Nümbrecht, Germany). Whole blood samples were homogenised at room temperature on a roller for 15–30 min and then analysed with an automated blood analyser (Sysmex XN-1000, Sysmex, Norderstedt, Germany). Internal quality controls provided by the manufacturer (Sysmex E-Checks, Levels 1, 2 and 3) were run twice before each batch of samples. Serum samples were kept in a vertical position for 20 min and then centrifuged (Z326K Centrifuge, Hermle Labortechnik, Wehingen, Germany) for 10 min at 2500 rpm. Finally, they were aliquoted in three samples of 800 μl, which were stored in a freezer at $-20°C$ for later analyses.

A fully automated system was used to determine HBmass using a carbon monoxide (CO) rebreathing procedure (OpCo: Detalo Instruments, Birkerod, Denmark), which is outlined in detail elsewhere.[49] Briefly, subjects were asked to breathe a 100% oxygen (O_2) bolus for 1 min in order to flush the airways of nitrogen before a bolus of 1 ml/kg of 99.997% chemically pure CO (Carbagas, Liebefeld, Switzerland) was administered in the circuit and rebreathed for 9 min in a supine position. Venous blood samples of 1.2 ml (S-Monovette Li-Heparin, Sarstedt, Nümbrecht, Germany) were taken before and after the rebreathing phase for the determination of carboxyhaemoglobin (HbCO%) in triplicate with a separate gasometer (ABL80 Co-Ox, Radiometer, Copenhagen, Denmark) for HBmass determination using formulas described elsewhere.[49] The CO remaining in the system was measured with a CO meter (Monoxor Plus, Bacharach, New Kensington, Pennsylvania, USA) and subtracted from the initial amount introduced to define the exact CO bolus received with a 0.1 ml typical error (%TE). The total red blood cell volume (RBCV), PV and blood volume (BV) were then additionally calculated from HBmass, [Hb] and HCT. In this study, the %TE for the
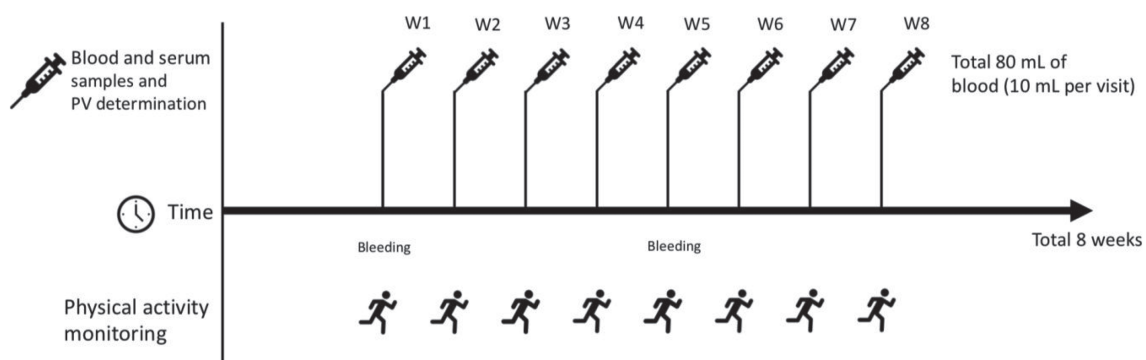

FIGURE 1 Study design with weekly blood sampling over two standard OCP cycles (Week 1 [W1] to Week 8 [W8])

determination of HBmass was 1.8%, which is in line with existing studies.[49]

2.4 | PV variation biomarkers, multivariate analysis and ABP profiling

Serological analyses were performed at the Swiss Laboratory for Doping Analyses (LAD) in Lausanne. Albumin (ALB), low-density lipoprotein (LDL), calcium (Ca), creatine (CRE), total protein (TP) and transferrin (sTFN) were analysed with a colourimetric method using a Dimension® clinical chemistry system (Siemens AG, Munich, Germany). Ferritin (FERR) concentrations were analysed with a chemiluminescence immunoassay technique (ADVIA Centaur® XP/XPT, Siemens AG, Munich, Germany).[20–23]

An individual longitudinal ABP profile was established with the values obtained from the collected blood samples using the Anti-Doping Administration and Management System (ADAMS) training environment hosted by WADA. The thresholds were set at the 99% specificity level.

From the haematological and serological analyses, analytical results from eight 'volume-descriptive' (or 'volume-sensitive') biomarkers ([Hb], platelets (PLT), sTFN, CRE, Ca, LDL, ALB and TP) were used in a multivariate analysis to determine individual estimations of PV variations (Z-scores). Next, the estimated PV was used to adjust the individual reference limits for [Hb] and OFFs, allowing for respectively higher or lower limits in case of a predicted haemoconcentration or haemodilution. The multivariate analytical model to adjust for PV based on 'volume-sensitive' biomarkers was previously described in detail elsewhere.[20] A key feature of this multivariate model is the use of a weighting function to reduce the importance of potentially abnormal values of one or more biomarkers in the PV estimation. If one of the biomarkers moves in the opposite direction to the other biomarkers, or with a different magnitude, its weight will be lower, thus reducing its importance in the computation of the PV estimation. The weighting function is crucial, as individual factors, such as pathologies, may impact the level of one or more biomarkers without being related to changes in PV. The uniformity between the eight 'volume-sensitive' biomarkers is reflected in a confidence level index (see Figure 2). Technically, the confidence level (%) is equal to the exponential sum of the weighting function, normalised between 0% and 100%. Values close to 100% have high confidence, reflecting an excellent agreement in the magnitude and direction of the eight biomarkers for this sample, whereas values close to 0% have low confidence. A higher confidence index indicates a higher probability that the model captures variations due to PV shifts and not due to other causes, such as pathology.

2.5 | Statistical analyses

Data are reported as means ± standard deviations (SD). The variability in [Hb], Hbmass, PV, RET% and OFFs over time was calculated as a

coefficient of variation (CV, %) from the mean of each individual's CV over the successive time points. One of the subjects was excluded from the analyses because of extremely low levels of FERR (<1 µg L^{-1}) as well as low [Hb] (<100 g L^{-1}) at the first measurement, which suggested a pathological condition. The normality of the distributions was tested with the Shapiro–Wilk test. Sphericity was not assumed, and the Geisser–Greenhouse correction was used. For the normally distributed variables (HBmass, PV, [Hb], RET%, RET#, OFFs, HCT, MCH, MCHC and RDW-SD), differences at the successive time points were assessed with a one-way repeated measures analysis of variance (ANOVA) with fixed and random effects to explain target variables, in which the subjects represented random effects and time was the fixed effect. The repeated measures were analysed by comparing all the time points with each other with correction for multiple comparisons using statistical hypothesis testing (Holm–Sidak test). For the variables that were not normally distributed (immature reticulocyte fraction [IRF], red blood cell count [RBC], PLT and white blood cell count [WBC]), parameters were compared using a Friedman test with Dunn's multiple comparisons. Correlations between haematological and hormonal variables were assessed with a Spearman rank-order coefficient. Linear regression analysis was used to assess the relationship between the inferred (estimated) PV variation Z-scores with the actual (measured) PV shift Z-scores after removal of the first two data points for each subject to allow the predictive model to adapt to individual specificity. A Bland–Altman plot was then used to assess the agreement between methods by calculating the bias (average of the differences) and the 95% limits of agreement, computed as the mean difference (bias) plus or minus 1.96 times its SD. The null hypothesis was rejected for $p < 0.05$. All data were analysed using dedicated software (Prism, Version 8.4.2, GraphPad Software, La Jolla, California, USA).

3 | RESULTS

3.1 | Adaptive model, PV Z-scores and intra-individual variations

Individual comparisons of the variation in PV are illustrated using the examples of two subjects in Figures 2 and 3. Individual comparisons for all subjects are available as supplementary files (Figures S6–S17). The mean confidence calculation in the PV estimation from the serum biomarkers was 59%, with a significant increase between W1 and the last week (W8) from 25% to 71% ($p < 0.001$); this is because the estimated PV calculation becomes more reliable once it is based on changes from individual samples and not from prior populations. Interestingly, one subject (S5, Figure 3) was flagged for a [Hb] reaching the upper limit, whereas the PV correction (green lines in Figure 3) accounted for a PV shift, which explains the higher concentration value.

A significant correlation was identified between the predicted PV variation (Z-scores) and the measured PV shift (Z-scores) ($r = 0.51$; $p < 0.001$), with 26% of the proportion of the estimated

FIGURE 2 Illustration of eight successive (x-axis) longitudinal biomarker values (blue lines) for one subject (S3) from blood and serum analyses with individual limits from the athlete biological passport adaptive model. The specificity was set at either 99% (red dashed lines) or 99.9% (red solid lines). Green reference limits for haemoglobin concentration and OFF-score illustrate the limits calculated after a correction for plasma volume. Bottom panels represent the confidence calculation in the PV estimation and comparison of the PV Z-score for the measured PV (blue) and inferred PV from a multi-parametric estimation (red line), as detailed in other studies[20,21] [Colour figure can be viewed at wileyonlinelibrary.com]
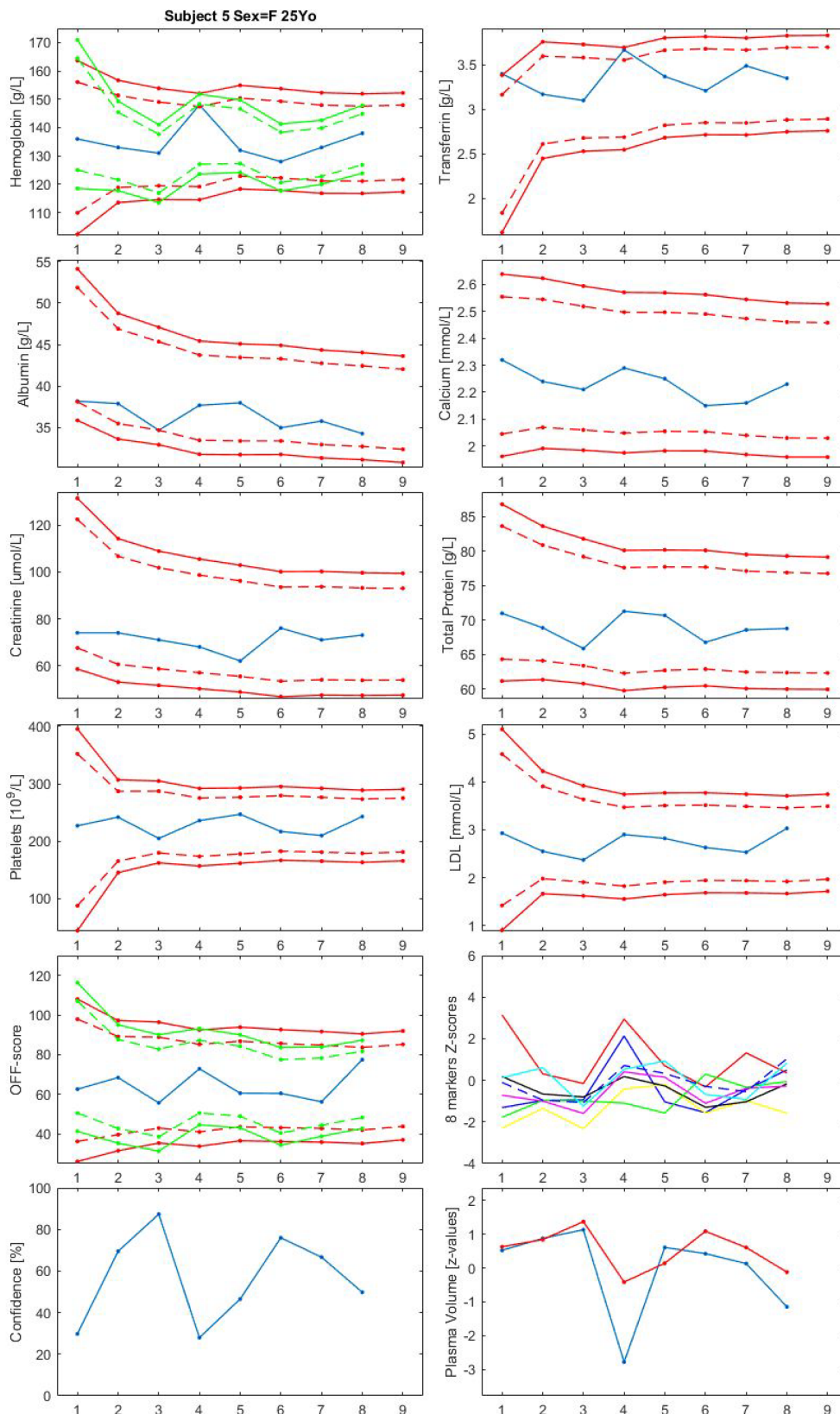
PV variation explained in the linear fit with the actual (measured) PV Z-scores. The Bland–Altman analysis illustrated a low bias (0.26) with the 95% limits of agreement between −1.3 and 1.8 (Figure 4).

To assess intra-individual variations throughout the two consecutive standard OCP cycles, CVs were computed for HBmass, PV, [Hb], OFFs, RET%, IRF, RBC and HCT. The mean CVs were 4.2%, 6.6%, 3.1%, 11.1%, 16.0%, 36.3%, 3.0% and 3.1%, respectively.
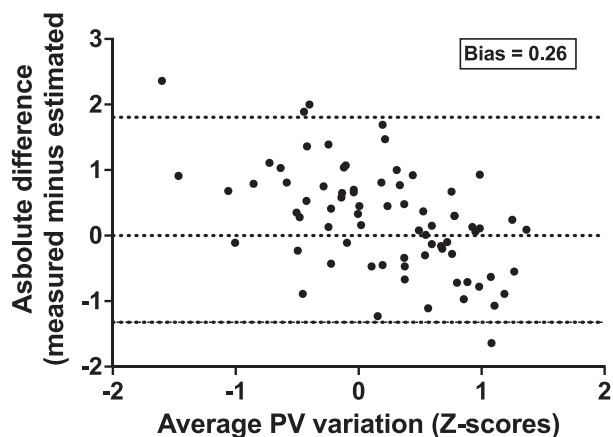
FIGURE 3 Illustration of eight successive (x-axis) longitudinal biomarker values (blue lines) for one subject (S5) from blood and serum analyses with individual limits from the ABP adaptive model. The specificity was set at either 99% (red dashed lines) or 99.9% (red solid lines). Green reference limits for haemoglobin concentration and OFF-score illustrate the limits calculated after a correction for plasma volume. Bottom panels represent the confidence calculation in the PV estimation and comparison of the PV Z-score for the measured PV (blue) and inferred PV from a multiparametric estimation (red line), as detailed in other studies.[20,21] Subject 5 flagged for [Hb] reaching the upper limit, whereas the PV correction accounted for a PV shift, which explains the higher concentration value

3.2 | Patterns of standard OCP cycle

All subjects had regular OCP cycles with a duration of 28.5 ± 1.5 days. Two subjects had longer cycles; one took a different form of OCP and had a mean cycle of 33.5 days (four phasic combined OCP), and the other had a cycle of 30 days. However, more variation occurred in bleeding patterns; the length varied from 2 to 7 days with a mean of 4.4 ± 1.2 days. Five subjects reported low blood loss over the two observed cycles (36%), whereas seven had medium bleeding (50%), and two described their bleeding as abundant (14%) (see Table 1).

3.3 | Haematological variables

Haematological and training variables for each week of measurement are reported in Figure 5 and Table 2. Statistically significant differences were observed for [Hb], with lower values during W7 compared with W1, W2 and W4 ($p < 0.01$, $p = 0.043$ and $p = 0.039$, respectively).

A concomitant difference was found for PV at W7; the PV was 9.1% higher than at W1, with a p-value just above the significance limit ($p = 0.053$). Although the difference was not significant, the [Hb] decrease at W7 corresponded with an increase in PV. Concomitantly, significant differences were also noted for HCT and RBC. HCT was significantly lower at W7 compared with W1, W2, W3, W4 and W5 (−5.1%, −3.9%, −2.2%, −4.3 and −4.3%; $p < 0.01$, $p < 0.01$, $p = 0.018$, $p = 0.013$ and $p = 0.049$, respectively) and at W6 compared with W1 (−3.3%; $p = 0.022$). In addition, the RBC at W7

was lower compared with W1, W2, W3 and W4 (−5.2%, −3.9%, −1.9%, and −3.9%; $p < 0.01$, $p < 0.01$, $p = 0.023$ and $p = 0.011$, respectively). The mean RBC at W1 was also higher than that at W3 and W6 (+3.4% and +3.5%; $p = 0.024$ and $p = 0.045$, respectively). Another significant difference was found for IRF, with the value at W1 being lower than that at W4 and W7 (−27.7% and −34.3%; $p = 0.033$ and $p = 0.015$, respectively). No other significant differences were found in any of the other measured variables.

For the data pooled in weekly phases following the week of the withdrawal bleeding (i.e. P0–P3), the RET% was significantly higher during P2 (1.31, $p < 0.001$) and P3 (1.29, $p = 0.002$) compared with the week after the bleeding (P1, 1.10). The IRF was significantly higher at P2 (5.64) compared with P0 (3.92, $p = 0.003$) and P1 (3.98, $p = 0.002$) and remained higher at P3 (5.23) versus P1 ($p = 0.007$). HCT was higher at P0 (39.5) compared with P2 (38.0, $p = 0.003$) and P3 (38.6, $p = 0.03$). The median and quartile values in the phases following the week of the bleeding for HBmass, PV, [Hb], Ret%, HCT and IRF are illustrated in Figure 5. The other biomarkers remained stable throughout a standard OCP cycle.

4 | DISCUSSION

In this study, we monitored ABP biomarkers as well as PV and Hbmass over two consecutive standard OCP cycles in 14 active women taking oral contraceptives. The main finding of this study was that a multi-parametric analysis of additional 'volume-sensitive' blood biomarkers accounted for putative PV shifts in the interpretation of the individual ABP profiles for physically active women taking OCPs. Secondly, ABP biomarkers remained remarkably stable over the two investigated OCP cycles.

4.1 | Stability of haematological markers during the standard OCP cycle

Although the haematological variables, analysed weekly, remained remarkably stable, the repeated measures ANOVA revealed a significant difference for [Hb], HCT and RBC during Week 7. Interestingly, PV tended to be higher during the same week ($p = 0.05$), whereas no significant time effect was observed over the 8 study weeks, which is in line with the small PV variations across the menstrual cycles recently reported by Aguree et al.[50] However, those results need to be put in perspective because the measured biomarkers are prone to rather high physiological variability.[51-55] Potential fluctuations induced by the OCP cycle would, thus, be inferior to the one expected from the main confounding factors such as exercise, hypoxic or heat exposure.

Furthermore, reticulocytes may also vary over an OCP cycle. To assess recurring patterns after the withdrawal bleeding, haematological variables were pooled in phases based on the first day of the bleeding and analysed, with elevated RET% and IRF 2 and 3 weeks after the start of the bleeding. Our results confirm the findings of Mullen et al,[37] who also reported elevated reticulocytosis in

FIGURE 4 Bland–Altman plot with the comparison between the estimated plasma volume (PV) variation versus the measured PV variation (Z-scores), respectively. The absolute differences are represented as a function of the average PV variation with 95% limits of agreement (dotted lines), computed as the mean difference (bias) plus or minus 1.96 times its SD
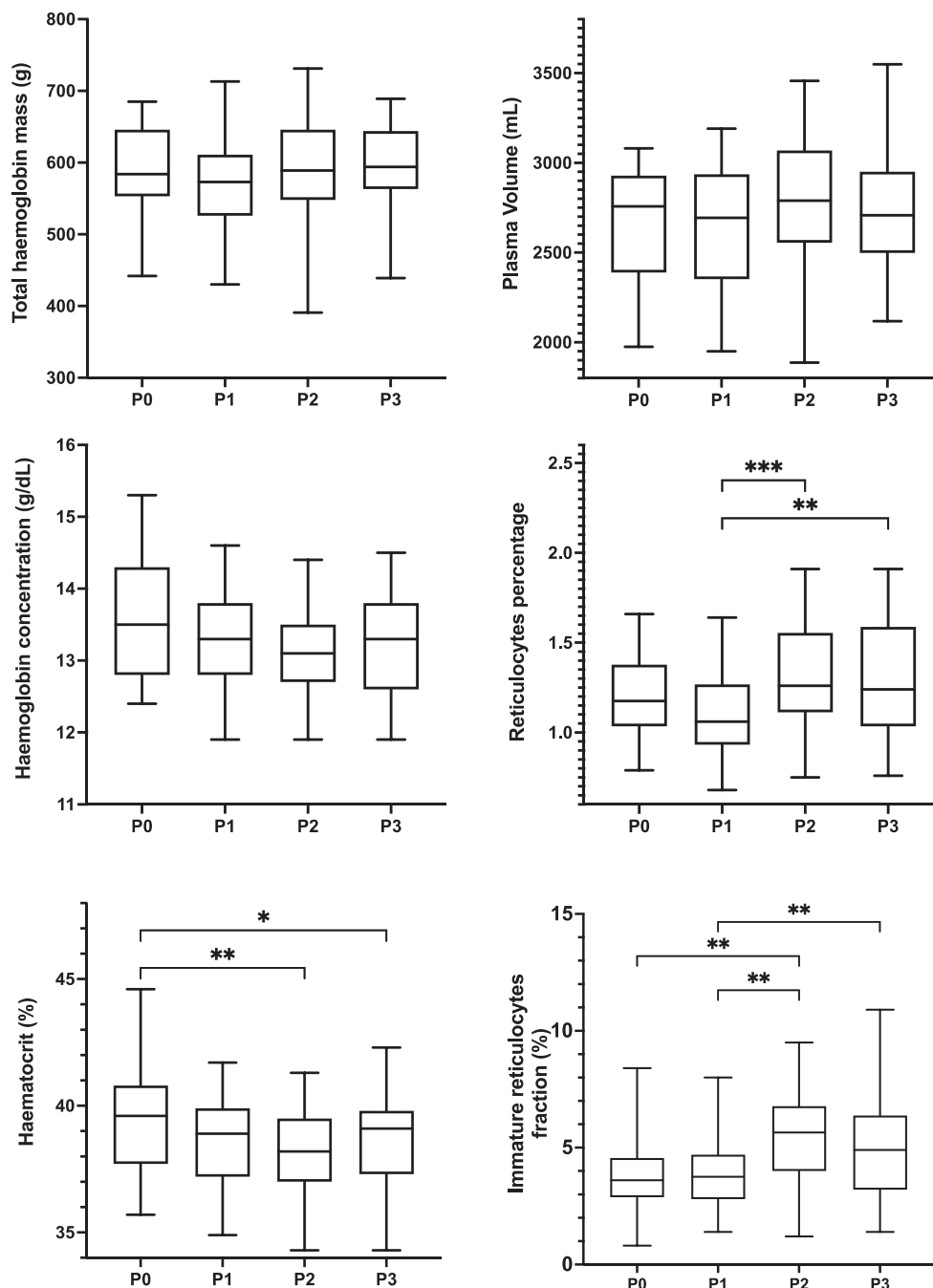
the ovulatory and LP over two menstrual cycles, but in women not taking oral contraception. This contrasts with the stable haematological variables over the successive phases of three menstrual cycles observed in women not taking oral contraception.[36] However, in the latter study, Salamin et al reported elevated RET% during a 1-month testosterone administration. Such changes, along with increased reticulocytosis, are likely induced by a depressed erythrocyte filterability[56] due to elevated progesterone (or synthetic progestative) levels.[57] Moreover, Oski et al[58] found that contraceptive intake further decreases erythrocytes filterability, which may induce greater reticulocytosis in female athletes taking OCPs, as observed in our study. However, our results should be interpreted with caution

because of the high variability that can be expected from the measurement of the RET% itself.[59] For instance, the differences reported are often close to the absolute 0.25% tolerance accepted between two consecutive measurements (for RET% above 1%) from the WADA blood analysis requirements in force.[10] Further, analytical variability also needs to be accounted for when assessing other haematological parameters such as IRF. It also is not excluded that the type or brand of OCP had an influence as subjects used 10 different brands.

On the other hand, [Hb], HBmass, RBC and PV remained steady throughout the phases. Previous studies found a significant difference in [Hb] between the FP and LP[30–33,60] Our results are however limited by the fact that our study cohort did not include a control group of
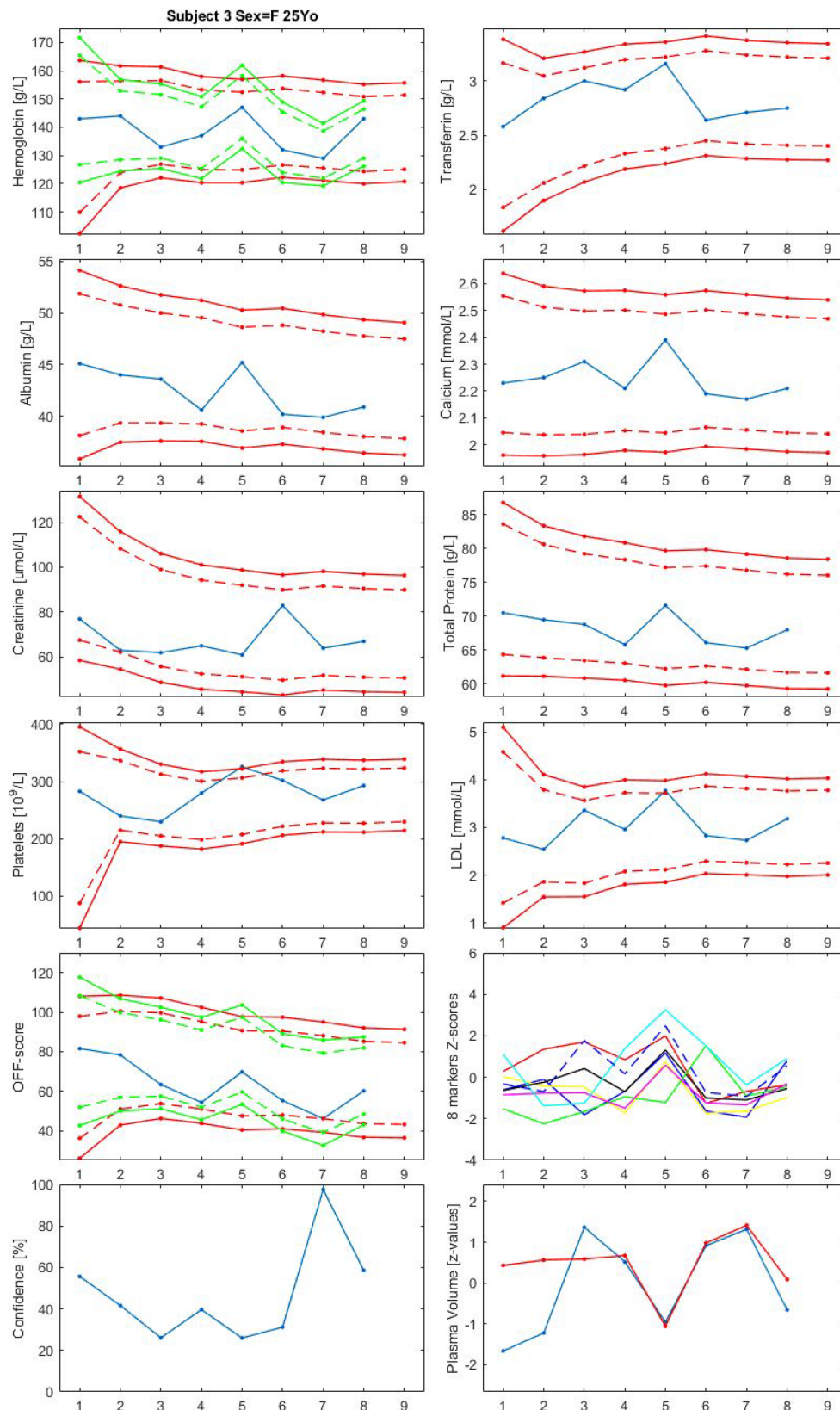
FIGURE 5 Box and whisker illustrations of the variations of total haemoglobin mass, plasma volume, haemoglobin concentration, reticulocytes percentage, haematocrit and immature reticulocyte fraction in the phases following the week of the withdrawal bleeding. P0 represented the week of the withdrawal bleeding, P1 represented P0 plus 1 week, P2 represented P0 plus 2 weeks, and P3 represented P0 plus 3 weeks. Error bars represent minimum to maximum. $^{**}p < 0.01$ and $^{*}p < 0.05$ for the difference between phases [Colour figure can be viewed at wileyonlinelibrary.com]

women not taking OCPs. Nevertheless, recently, Mullen et al[37] investigated the influence of the menstrual cycle on such biomarkers in women not taking any contraceptive and found no significant variations throughout the menstrual cycle, except from reticulocytes,

which is supported by other recent work.[29,35,37] Furthermore, Stachenfeld et al[61] investigated variations in hormones involved in fluid regulations and found no significant difference between the FP and LP. However, they found that these hormones were prone to

TABLE 2 Haematological variables and training volume over 8 weeks (W1–W8)

	W1	W2	W3	W4	W5	W6	W7	W8
Hbmass (g)	585 ± 74	573 ± 84	585 ± 71	596 ± 69	582 ± 64	576 ± 77	583 ± 65	589 ± 59
PV (ml)	2591 ± 405	2624 ± 414	2735 ± 330	2745 ± 410	2662 ± 306	2718 ± 433	2827 ± 279	2743 ± 306
[Hb] (g/dl)	13.52 ± 0.93*	13.38 ± 0.85*	13.12 ± 0.61	13.36 ± 0.79	13.36 ± 0.68	13.16 ± 0.72	12.92 ± 0.68	13.26 ± 0.65
RET%	1.20 ± 0.25	1.14 ± 0.23	1.26 ± 0.23	1.29 ± 0.30	1.23 ± 0.33	1.15 ± 0.22	1.24 ± 0.30	1.29 ± 0.40
RET# (10^6/µl)	0.055 ± 0.010	0.051 ± 0.010	0.056 ± 0.009	0.058 ± 0.013	0.056 ± 0.014	0.051 ± 0.009	0.054 ± 0.013	0.058 ± 0.017
OFFs	69.8 ± 11.6	70.1 ± 12.2	64.2 ± 10.4	65.8 ± 10.9	67.6 ± 10.4	67.4 ± 9.9	62.8 ± 10.6	65.2 ± 11.7
HCT (%)	39.4 ± 2.2*/#	38.9 ± 1.9*	38.2 ± 1.7*	39.1 ± 2.4*	39.1 ± 1.7*	38.1 ± 1.8	37.4 ± 1.6	38.4 ± 1.9
MCV (fl)	85.94 ± 3.18	86.04 ± 3.19	86.26 ± 3.25	86.39 ± 3.29	86.34 ± 3.13	85.97 ± 3.29	86.09 ± 3.43	85.93 ± 3.27
IRF (%)	3.63 ± 1.37*·◦	4.19 ± 2.46	4.87 ± 2.23	5.02 ± 1.38	5.04 ± 2.43	4.22 ± 1.84	5.52 ± 2.29	6.03 ± 2.48
MCH (pg)	29.48 ± 1.45	29.57 ± 1.43	29.63 ± 1.47	29.58 ± 1.45	29.54 ± 1.49	29.70 ± 1.54	29.76 ± 1.61	29.69 ± 1.40
MCHC (g/dL)	34.29 ± 0.92	34.35 ± 0.83	34.34 ± 1.01	34.21 ± 0.79	34.23 ± 0.96	34.54 ± 0.75	34.56 ± 0.93	34.52 ± 0.77
PLT (10^3/µl)	257 ± 37	253 ± 35	243 ± 40	253 ± 71	261 ± 52	251 ± 70	242 ± 48	254 ± 65
RBC (10^6/µl)	4.59 ± 0.32*·#·†	4.53 ± 0.29*	4.44 ± 0.29*	4.53 ± 0.36*	4.53 ± 0.26	4.44 ± 0.29	4.35 ± 0.30	4.48 ± 0.32
RDW-SD (fl)	39.48 ± 2.78	39.61 ± 2.76	39.54 ± 3.18	39.88 ± 2.54	39.42 ± 2.39	39.12 ± 2.58	38.99 ± 2.37	38.77 ± 2.27
WBC (10^3/µl)	6.62 ± 2.20	6.15 ± 1.12	6.92 ± 2.08	5.98 ± 1.53	6.28 ± 1.74	6.40 ± 1.26	6.46 ± 1.90	6.76 ± 1.89
Training volume (min)		341 ± 157	329 ± 187	326 ± 211	257 ± 98	264 ± 137	270 ± 124	292 ± 140

Note: Values are presented as means ± SD.

Abbreviations: Hbmass, total haemoglobin mass; HCT, haematocrit; HGB, haemoglobin concentration; IRF, immature reticulocyte fraction; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; OFFs, OFF-hr score; PT, platelets; PV, plasma volume; RBC, red blood cell count; RDW-SD, red cell distribution width (standard deviation); RET#, reticulocyte absolute count; RET%, reticulocyte percentage; WBC, white blood cell count.

* $p < 0.05$ for the difference compared with W7.
# $p < 0.05$ for the difference compared with W6.
◦ $p < 0.05$ for the difference compared with W4.
† $p < 0.05$ for the difference compared with W3.

natural variations, which occur independently of the menstrual phase and induce shifts in PV. Considering the results presented above, we can conclude that the ABP biomarkers as well as PV and Hbmass are likely to remain stable over a standard OCP cycle in physically active women. Potential variations due to the OCP cycle would arguably be inferior compared with the physiological fluctuations expected from the main confounding factors influencing PV.

To better investigate biomarker variability, we also computed CVs for HBmass, PV, [Hb], OFFs, RET%, RBC and HCT to control for intra-individual variations, and these biomarkers remained stable throughout the two investigated OCP cycles and did not differ between the cycles. Unsurprisingly, variations were the highest for IRF (36.3%), RET% (16.0%) and OFFs (11.1%) and are in accordance with those of Mullen et al's study.[37] A cyclical increase of reticulocytes (RET%, RET# and IRF) might occur 2 and 3 weeks after the withdrawal bleeding in a female active population, but variations remain in the same range as those induced by other physiological conditions in different studies on athletes.[52,53,62,63] However, the CVs remained low for HBmass, PV, [Hb], RBC and HCT, which is in line with results from other studies on female athletes for [Hb] and HCT.[55] More interestingly, the CVs for HBmass were similar to those reported in 130 athletes over 1 year,[64] illustrating low biological variation when considering a typical error of measurement of approximately 2%. Our results thus highlight the potential of changes in HBmass (or PV) of sufficient amplitude over a short period as a useful asset to detect blood manipulation when combined with additional blood biomarkers (e.g. OFFs or ABPS).

The bleeding cycle pattern remained stable for all subjects except one, who took a different type of OCP and whose length of withdrawal bleeding did not differ from a population with no OCP. According to Martin et al,[40] one of the positive effects of OCPs is the reduced frequency and heaviness of bleeding. In this study, two subjects described their bleeding as abundant (15.33%) (see Table 1), which is in agreement with Vannuccini et al.[65] They had CVs for HBmass, PV, [Hb], OFFs and RET% corresponding to the mean of the study population. Thus, we may conclude that the bleeding pattern did not seem to affect ABP biomarkers to a significant extent, although those patterns were self-estimated and might lack accuracy.

4.2 | Plasma volume modelling

When considering haematological variables for the ABP, the importance of BV-dependent primary markers (e.g. [Hb]) highlights the need to carefully consider PV shifts in the interpretation of longitudinal profiles. Here, we tested the validity of this multi-parametric analytical approach presented earlier in our cohort to identify that the inferred (predicted by the model) variation of PV was significantly correlated with the actual (measured by a CO rebreathing method) PV shifts. However, the coefficient of correlation was lower ($R^2 = 0.26$) than that observed by Garvican-Lewis et al.[23] The latter study underlines the importance of strict analytical procedures to reduce typical errors of measurement (e.g. with duplicate measurements) and to avoid

discrepancies in the correlation due to analytical rather than biological factors. Notably, the correlation occurred after removing the first two data points for each subject because the predictive model heavily relies on population-based priors for the first sample. This is also illustrated by the observation of an increased confidence index with the successive measurements.

In this study, we additionally compared the agreement between the estimated and measured PV variation with a Bland–Altman analysis to highlight a very low bias between methods with acceptably narrow 95% limits of agreement. Taken together with the high confidence level of the PV variation estimation model (which increased after successive iterations), we may conclude that the predicted and actual PV variations present a good agreement, especially when observing subjects with larger PV variations.

To better account for PV shifts in the interpretation of individual ABP profiles, the PV correction allowed us to explain the single ATPF identified by the ABP adaptive model when a [Hb] value reaches the upper individual limit (at a 99% specificity level); this was explained by a concomitant decrease of PV (Figure 3), indicating a pertinent approach to reduce any false positive identification of varying markers.

5 | CONCLUSION

In this study, we applied a multi-parametric model to predict putative PV shifts from additional blood biomarkers, which was in good agreement with measured PV shifts, despite relatively stable BV in our cohort of active women. The model was able to explain an ATPF, thus highlighting its validity, which accounts for PV variations due to multiple physiological or environmental confounders by using additional 'volume-sensitive' blood biomarkers.

Haematological biomarkers of the ABP, HBmass and PV were also assessed over two consecutive standard OCP cycles in an active population. Most variables remained stable, but significant variations occurred for a few concentration-based blood markers (i.e. [Hb] and HCT) during a week when PV tended to be higher. Hence, the standard OCP cycle per se did not affect the biomarkers of the ABP to a significant extent in active females taking oral hormonal contraception. Intra-individual variations remained stable from one cycle to another, whereas bleeding patterns affected neither the variation in the biomarkers of the ABP nor Hbmass and PV.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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