Garvican-Lewis Laura (Orcid ID: 0000-0002-3611-1824)

RESEARCH ARTICLE

A multi-parametric approach to remove the influence of plasma volume on the Athlete Biological Passport during a UCI cycling stage race

Laura A Garvican-Lewis[1, 2], Louisa M Lobigs[2], Tristan Equey[3], Catrin Goebel[4], Vanessa Agon[4], Andrew McCowan[5], Naomi Speers[5], Yorck O Schumacher[6]

[1]*Mary Mackillop Institute for Health Research, Australian Catholic University, Melbourne, Australia*
[2]*Australian Institute of Sport, Canberra, Australia*
[3]*World Anti-doping Agency, Montreal, Canada*
[4]*National Measurement Institute, Australian Sports Drug Testing Laboratory, Sydney, Australia*
[5]*Australian Sports Anti-doping Authority, Canberra, Australia*
[6]*Aspetar orthopaedic and sports medicine hospital, Qatar, Doha*

Running head: *Plasma volume correction during racing*

Abstract Word Count: 250

Word Count: 3257

Number of Figures: 4

Number of Tables: 4

Address for Correspondence

Laura A. Garvican-Lewis

Mary Mackillop Institute for Health Research

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/dta.2878

Australian Catholic University
Level 5, 215 Spring Street
Melbourne, Victoria 3000
Email: laura.lewis@ausport.gov.au;
Tel: +61 2 6214 1145, Fax: +61 6214 1904

ABSTRACT

Fluctuations in plasma volume (PV) present potential confounders within the concentration-based markers of the hematological Athlete Biological Passport (ABP). Here, a multi-parametric approach involving a simple blood test is applied to the current ABP adaptive model in an attempt to remove the influence of PV expansion, induced by a cycling stage race. Blood samples were obtained from 29 professional cyclists (15 female, 14 male) before, during, and after 4-5 consecutive days of racing. Whole blood was analysed in accordance with the World Anti-Doping Agency ABP guidelines for haemoglobin concentration ([Hb]) and platelets. Serum and plasma were analysed for transferrin, albumin, calcium, creatinine, total protein, and low-density lipoprotein. PV variation (Z-scores) was estimated using a multi-parametric model (consisting of the bio-markers above) and compared against calculated variations in PV (measured via CO-rebreathing). Significant reductions in [Hb] and the OFF-score were observed in females after 3 and 4 days of racing, with accompanying increases in PV, which returned to baseline values 4 days post competition. Similarly, a significant increase in PV was observed in males after 3 and 5 days of racing. When individual estimations of PV variance were applied to the adaptive model, the upper and lower reference predictions for [Hb] and the OFF-score were refined such that all outliers consistent with racing induced PV changes were removed. The PV model appears capable of reducing the influence of PV on concentration-dependent markers during competition. This is an important step towards the inclusion of the PV correction in the ABP hematological module.

Key words: Blood volume, blood doping, endurance exercise, adaptive model

INTRODUCTION

The hematological module of the Athlete's Biological Passport (ABP) is considered a fundamental tool in anti-doping [1, 2]. With the continual development of novel performance enhancing substances and blood manipulation techniques, the ABP provides a means to circumvent the need for the direct detection of a specific substance, instead providing a method of indirect direction of illegal doping practices such as erythropoietin administration and autologous blood transfusion. However, the crux of the hematological module relies on a number of concentration-dependent markers, including hemoglobin concentration ([Hb]) and the OFF-score (OFF-score = [Hb] (g/L – 60 x $\sqrt{\%\text{retic}^3}$), hence these markers can be influenced by shifts in plasma volume (PV). Plasma volume in athlete cohorts can be particularly variable since exercise, altitude, competition, heat, posture and hydration status all have the capacity to induce changes in PV by more than 10%, sometimes up to 20% [4 5 6 7 8 9]. Physical exercise influences plasma volume, with the magnitude of fluctuation dependent on the type, duration and intensity (amongst other environmental factors) [5]. In general, higher intensity exercise and longer exercise durations result in a more pronounced PV shift, such as that observed after consecutive days of endurance activity [10]. However, a recent study from Bejder and colleagues has shown that PV shifts, resulting from an increased training load, can lead to false-positive flagging by the adaptive model of the ABP [11]. Currently the adaptive model of the ABP does not account for fluctuations in PV, rather it is the role of the ABP expert to make an informed judgment on a profile based on expected changes in PV. Thus, with no objective measure, fluctuations in PV present a major confounding factor to the analysis of an ABP profile.

Recently, a model to quantify vascular volumes from a simple blood test has been proposed [12]. An optimal panel of "volume descriptive" biomarkers was identified, capable of describing ~68% of PV variance when combined. When this novel PV correction was applied to the ABP adaptive model the calculated reference limits for Hb and the OFF-score were reduced, and the model allowed for a volume fluctuation resulting from a maximal exercise effort [13]. This approach has the possibility to improve the sensitivity and specificity of the ABP. The first steps for the practical validation of the model have been initiated [14], however, further research in official sporting scenarios is required. Therefore, validation of the model in an anti-doping sporting competition context is presented here. We hypothesized that the PV

model would detect naturally occurring fluctuations in PV induced by multi-stage cycle racing; specifically a progressive PV expansion resulting in a decrease in [Hb]. Further, when the PV correction was applied to the hematological module of the ABP, we hypothesized that such fluctuations will be accounted for, thereby improving the specificity of the calculated ABP thresholds.

METHODS

Study design

Twenty-nine elite road cyclists (14 m, 15 f) competing in the 2020 edition of the Tour Down Under (Adelaide, South Australia) agreed to participate in the study, which was approved by the Australian Institute of Sport (AIS) Human Ethics committee. All athletes provided written informed consent before participating.

The women's race consisted of four stages, culminating in a criterium on the fourth day, and served as the opening professional women's race on the international cycling calendar. The men's race was also the first World Tour event of the year and consisted of six stages, preceded by a criterium two days prior to the first stage. Details of each stage as well as a timeline of all measurements are summarized in Table 1.

The athlete cohort consisted of fifteen female riders from five teams, and fourteen male riders from five teams. Rider characteristics can be found in Table 2. Riders who did not complete a stage within the allotted time or who could not continue due to illness or injury were withdrawn from the race. Baseline hematology and hemoglobin mass (Hbmass) measures were collected before the first stage and then after consecutive days of racing as per Table 1. An additional post race sample was collected from the female participants (who were available) 4 days after completion of the race. Training during the time between the completion of the race and collection of the final sample was at the discretion of each individual. We have previously shown that Hbmass remains stable throughout a 6 day cycling race [15], hence, Hbmass tests were not performed during the race.

Blood Collection and Analysis

Australian Sports Anti-Doping Agency (ASADA) Doping Control Officers supervised all venous blood collections, which followed strict WADA blood collection, transport and analysis

protocols in force [16], including a minimum of 10 mins seated rest, and with no exercise in the 2 hours prior to sample collection. All collections were performed between 0630 and 0930 at the race accommodation. At the time of sampling, athletes were asked to complete the standard ABP supplementary questionnaire, which includes details relating to previous intense competition and altitude exposure. Whole blood samples were collected into 3 mL K2EDTA vacutainers and sealed in individual BEREG-kit small blood collection containers (Berlinger, Ganterschwil, Switzerland). Whole blood samples were transported by air inside a refrigerated box at 2-12 degrees Celsius (Pelican Nanocool, Alberque, USA) to a WADA accredited laboratory (Australian Sports Drug Testing Laboratory, Sydney, Australia) for analysis. The temperature inside the box was recorded using a temperature logger (Escort iMini, Global Temperature Monitoring, Victoria, Australia). The mean temperature recorded by the temperature logger (T) and the time between collection and analysis (CAT) were used to determine a Blood Stability Score (BSS) for each sample. The BSS is = 3 * T + CAT and must be ≤85 for a sample to be considered valid. [16]

A complete blood analysis including [Hb], platelets (PLT), mean corpuscular hemoglobin concentration (MCHC) and reticulocyte percentage (RET%), was performed as per ABP operating guidelines (including all pre analytical and post analytical quality controls) using the Sysmex XN-1000 (Kobe, Japan). In addition, in order to maintain WADA accreditation, the laboratory participates in a monthly external quality assurance program. In accordance with WADA guidelines each sample was analyzed at least twice and repeated if [Hb] or RET% values were not within acceptable limits [16]. Following analysis, the samples were centrifuged, with the plasma fraction aliquoted and stored at -80 degrees Celsius for subsequent analysis. Plasma was analyzed for Albumin (ALB), Creatinine (CRE), Total Protein (TP) and Low Density Lipoprotein (LDL) with a Cobas c701 Chemistry Analyser (Roche Diagnostics, Mannheim, Germany), whilst Transferrin (Tfn), was analyzed using a Siemens Atellica CH (Tarrytown, USA).

An additional 5 mL serum vacutainer was also collected at each time point. These samples were allowed to clot at room temperature for 15 mins before transportation by car to a laboratory (Royal Adelaide Hospital, Adelaide, Australia) for analysis in a refrigerated box (Pelican Nanocool, Alberque, USA). Upon reception, serum samples were centrifuged at 4 degrees and 1500 rpm for 10 minutes. Serum was then aliquoted and analyzed on a Cobas c701 Chemistry analyser (Roche, Diagnostics Mannheim, Germany) for Calcium (Ca). The

instruments used for chemistry analysis underwent regular internal and external quality control procedures as required by the Royal College of Pathologists of Australasia (RCPA) Quality Assurance Program and met or exceeded the required standards for each specific marker.

All cyclists participating in the Tour Down Under were required to present for official in-competition anti-doping controls if requested. It is assumed, but cannot be fully excluded, that none of the riders who participated in the present study took any performance enhancing substances, including erythropoietic stimulating agents or undertook any illegal doping practices before or during the study period.

Hemoglobin Mass

Total hemoglobin mass (Hbmass) was measured using the 2 min CO rebreathing method of Schmidt and Prommer, with slight modifications [17]. Briefly, capillary fingertip samples (200 µL) were collected pre- and 7 min post-CO administration; and immediately analysed in quintuplet for percent carboxyhemoglobin (HbCO%) using a CO-oximeter (ABL80; Radiometer, Copenhagen, Denmark). Lung CO levels were determined pre- and 4 min post-CO administration using a portable gas monitor (Dräger Pac 7000, Germany). At minute 0, a body mass adapted dose of CO (1.2 mL/kg) was applied as a tracer through a closed rebreathing apparatus (glass spirometer) and rebreathed, together with pure oxygen, for 2 min. Hbmass was calculated through the difference of CO levels pre- and post-CO rebreathing, in consideration to the applied CO bolus, level of CO remaining in the lungs and environmental factors (temperature and barometric pressure). The typical error of measurement in our lab is <1.8%.

Calculation of intravascular volumes

Red cell, plasma and blood volumes were calculated as follows:

$$(1)\ \text{Red Cell Volume (mL)} = \text{Hbmass (g)} \div \text{MCHC (g/dL)} \times 100$$

$$(2)\ \text{Blood Volume (mL)} = \text{Hbmass (g)} \times 100 \div [\text{Hb}]\ \text{(g/dL)} \div 0.91$$

$$(3)\ \text{Plasma Volume (mL)} = \text{Blood volume (mL)} - \text{Red cell volume (mL)}$$

The scaling coefficient (0.91) in equation (2) refers to the F-cell ratio [18]. The percent change in PV (*ΔPV %)* was calculated relative to the first sample (S1) as follows:

$$\Delta PV\ (\%) = [(PV_{post} - PV_{pre})/PV_{pre}] \times 100$$

Where PV_{post} is the PV calculated during the race and PV_{pre} is the PV calculated before the start of the race (using S1 values).

Data Analysis

A validation of the previously described PV Bayesian model is performed here using Matlab (Mathworks, Natick). Detailed descriptions of the statistical development of the PV model [12] and its application to the adaptive model of the ABP [13] can be found elsewhere. In brief, multivariate analysis was performed on the set of 8 "volume descriptive" biomarkers: [Hb], PLT, Tfn, CRE, Ca, LDL, ALB and TP. Individual [Hb] and OFF-score values (S1-S5) were used to create individual ABP profiles using an adaptive Bayesian model. Individualized reference limits at 99% and 99.9% specificity were generated using the same code and priors as the official ABP software (ADAMS). The individual estimations of PV variation (Z-scores) derived from the panel of biomarkers (at the time of sample collection), were then applied to the adaptive model of the ABP to produce "PV corrected" [Hb] and OFF-score reference limits. Linear regression analysis was performed to compare the estimated (inferred) PV shift Z-scores with the measured PV shift Z-scores. The first 2 samples for each athlete were not included in the analysis to allow the PV model to adapt to each individual.

Separate repeated-measures ANOVAs (for men and women) with main effects of time (days of racing) were used to assess the hematological responses during the race (sample 1-4), using STATA/IC (Version 13.1, StataCorp LP, Texas, USA) with the level of significance set to $P \leq 0.05$. Pairwise comparisons were performed with Bonferroni corrections. Additional analysis was performed using data from samples 1-5 for n=10 only (due to athlete availability) and is reported in relation to changes at S5 only. Values in the text and figures are reported as means ± SD unless otherwise stated.

RESULTS

All 15 women successfully completed the race, which consisted of 382.8 km and approximately 10.2 hours of racing over the four days. Due to race schedules, only 10 riders were available for the follow up blood collection four days post race (sample 5). Thirteen of the 14 male riders completed the race (870.2 km over 6 days, ~20.5 hours), with one rider

withdrawing during stage 4 due to an injury. This rider was not available for further testing, thus, a total of 125 samples were collected. Details of the overall race performance of the male and female subjects in the study can be found in Table 2. Daily maximum temperatures in central Adelaide according to the Australian Bureau of Metreology ranged from 22.2 to 26.1°C during the women's race and 20.9 to 27.4°C during the men's race.

ABP Supplementary questionnaires

Questionnaires were completed at each blood draw. No blood withdrawals or transfusions were declared. All riders delcared three days of consecutive intense exercise prior to samples 3 and 4. One rider declared previous altitude exposure above 1500m within 2 weeks of all samples.

Blood stability scores

The mean temperature of all temperature loggers during transit was 6.9 ± 0.5 °C, and no alarms recorded for temperatures "out of range." The Collection to Analysis Time (CAT) ranged from 28 to 33 hours (mean 29.9 ± 2.2 h), with BSS ranging from 47.9 to 52.8 (mean 50.8 ±2.3).

Hematology

Table 3 quantifies the mean change from pre race levels (using sample 1) in hematological parameters. During racing, [Hb] and hematocrit (Hct) significantly decreased, with mean values for males ($p=0.018$) and females ($p<0.000$) lower than pre race in sample 4 (Table 3), but were not significantly different by 4 days post race (sample 5; [Hb] $p=0.27$, Hct $p=0.63$). RET% were significantly higher than pre race in sample 5 only ($p=0.018$). OFF-Score was significantly lower in in sample 4 in women ($p<0.000$) but not men ($p=0.085$), as well as in the post race follow up (women only, $p=0.004$)). Plasma volume increased with racing (men and women: S3 $p=0.012$; S4 $p<0.000$) but was not significantly different from pre race by four days post race (S5) in the female cohort ($p=0.56$). Individual changes in [Hb], OFF-score, RET% and PV are shown in Figure 1.

Plasma Volume Z-scores

Individual longitudinal comparisons of the variation in PV are shown for three subjects in panel L of Figures 2, 3 and 4. The blue line represents the plasma-volume variation (Z-scores) using the CO-rebreathing method (measured) and the red line represents the Z-scores using

the PV model prediction. Figure 5 shows the relationship between measured changes in PV Z-scores (x-axis) and the PV model predicted changes (y-axis). 65% of the proportion of the variance of the inferred PV Z-scores was explained by a linear fit with measured PV Z-scores ($p<0.001$).

Adaptive model

From a total of 125 observations from 29 athletes, the number of outliers (data points exceeding the upper or lower reference value) for [Hb] was reduced when the PV model was applied. Without the correction, 4 outliers in 3 female subjects (99% specificity) were flagged for [Hb] (Table 4), whereas all outliers for [Hb] were removed when the correction was applied. Only one sample in one male subject flagged for the OFF-score (Table 4) which remained with the PV correction.

Figure 2 (Subject 4) gives an example of the PV models function and describes the expected PV expansion during the race and subsequent hemodilution, followed by a slight recovery 4 days post race. With the inclusion of the PV correction the specificity of the predicted reference ranges for [Hb] and the OFF-score was improved (Figure 2 A, I, respectively), with sample 4 falling within the individual reference ranges once the PV correction had been applied. The recovery of PV (decrease) is also predicted by the model in sample 5.

Subject 7 (Figure 3) was the only subject to return an atypical passport finding (ATPF) after application of the PV model. The OFF-score for sample 1 exceeded the upper reference limit at 99% specificity both with and without the PV correction.

DISCUSSION

Plasma volume expansion

In line with previous findings, 4 and 5 days of cycle racing induced PV expansions in female and male professional cyclists, respectively. Despite the race experiencing cooler than expected temperatures for South Australia in summer [19], the observed increases in PV were not dissimilar from those measured at the same race previously [10, 15], or in other competitions [4, 10, 20]. None the less, individual variation of PV expansion is evident amongst the cyclists in

the present study. Since the magnitude of PV expansion post exercise is dependent on the duration and intensity of the exercise[9], amongst other factors, it is possible that the individual variation is partially related to the level of intensity each cyclist engaged in during the race, e.g. team tactics that required cyclists to be more active or agressive in the race may result in larger expansions vs smaller expansions in protected riders within the peleton. However, this theory falls outside of the scope of the current project and warrants further investigation. In addition, an individual difference in the magnitude of plasma volume shifts is also possible, given that the observed PV shifts at peak hemodilution ranged between 5-more than 20% in our subjects.

To our knowledge, we are the first to also describe this phenomenon in female cyclists. A rapid response was observed, with PV expansion evident after 3 stages of racing (340.3 km, ~9 hours) and a subsequent recovery to baseline observed 4 days after completion of the race. These observations confirm the importance of collecting information related to consecutive days of strenuous exercise, such as multi-stage cycle racing, at the time of sample collection for anti-doping purposes in both male and female athletes.

Plasma volume correction

Currently, in the ABP, lower reference limits for [Hb] and the OFF-score are not applied when at least three consecutive days of racing are reported by the athlete, where hemodilution is expected. Here, we demonstrate that the PV model is capable of predicting and accounting for such hemodilution, thereby allowing lower reference limits to be incorporated into the adaptive model in these instances. When the PV correction was applied to the ABP adaptive model, all outliers relating to [Hb] were removed. Further, no new outliers were recorded with the PV correction, highlighting the improved ability of the PV model to detect changes in PV, and [Hb]. In practice we anticipate that the PV model could provide an improved ability to detect artifical increases in [Hb] in the context of expanded PV, such as transfusions during stage races.

Only one ATPF for OFF-score was recorded – sample 1 of subject 7 (figure 3). The high OFF-score value was a combination of a [Hb] of 163 g/L and a low RET% of 0.61%. It has to be highlighted that the reference limits for the first sample of each profile are population derived limits, thus not the individual reference ranges of the athlete, which are only calculated once two or more samples are available. The information collected in the supplementary

questionnaire revealed that this subject is an altitude native, and prior to arriving in Australia lived permanently at 2500 m. Indeed, his Hbmass measurement was the highest of the cohort (relative to body mass), consistent with altitude adaptation [4, 21]. The slight suppression of RET% is also consistent with recent descent to sea level [22, 23]. Since RET% is not concentration dependent it is therefore not surprising that the ATPF for OFF-score remained even when the PV correction was applied. Further, Figure 3 shows that sample 2 falls within the upper reference limit, despite having near identical Hb (163 g/L) and RET% (0.68%) values because the reference limits for this sample are no longer derived from population values. In practice, ABP profiles may contain many more samples over a broader time frame, allowing for an adaptation to individual biologies, although this is not always the case. Further, the information relating to recent prior altitude exposure is available to the ABP expert reviewing the profile and in many instances can explain samples flagged as atypical by the adaptive model.

Weighting function

A strength of a multi-parametric approach over a single biomarker is the application of a weighting function for abnormal PV biomarkers.[12] In brief, if a biomarker moves in the opposite direction, and / or the variation is stronger / weaker than other PV biomarkers, then less weight is assigned to the marker in the PV correction model. Previously, an example of the potential of the weighting function has been described in the context of the model in clinical settings, allowing absolute vascular volumes to be described in patients with septic shock despite a decrease in Albumin.[12] In this respect, in the context of stage racing, changes in total protein or Albumin arising from multiple days of strenuous exercise [24], lie within the PV model's capabilities. Indeed, when the predicted plasma volume shifts were compared to the measured plasma volume shifts during the race (after allowing sufficent samples for the model to adapt to each indivdual), we found the PV model was able to predict 65% of the PV changes – in line with the ~70% reported previously [12].

Confidence of the PV model

The confidence calculation is an important component of the PV correction and is associated with the PV Z-score returned by the multi-parametric model. The confidence level (%) is equal to the exponential sum of the weighting function and is normalised between 0 and 1. Values close to 1 have high confidence where as values close to 0 have low confidence. The variance

used to calculate the z-score associated with PV is then weighted by the confidence level. In summary, the PV model prediction is related to the uniformity of the Z-scores for the various chemistry markers. In the present study, the confidence level was often the lowest for sample 1, owing to the fact that the confidence levels for for the different PV biomarkers rely exclusively on the population-based priors for the first sample. Importantly, in the majority of subjects, the confidence level did not fall below 40% in subsequent samples. One exception is subject 25 (Figure 4) who showed larger variation in the Z-scores of the 8 PV markers (Panel J), particularly in samples 3 and 4, and as a result returned a lower confidence value for sample 3 onwards. This subject presented with a viral infection but was cleared to compete by the Race Medical Doctor; therefore, it cannot be excluded that the variation observed in the PV markers was influenced by illness. In this example, despite the low confidence, the reference limits calculated by the PV model are still able to account for volumetric changes in [Hb] (panel A). In addition, this highlights the continued importance of expert review combined with the need to collate all the supplementary information pertaining to each sample, (i.e. ABP supplementary questionnaires, confidence calculation, view of the 8 biomarker z-scores) if the PV model is to be adopted by the ABP.

CONCLUSION

When applied to data collected in competition, the PV correction is able to refine the ABP reference limit calculations by removing the influence of PV on volumetric markers. This is an important step going forward for the inclusion of the PV correction in the hematological module of the ABP.

ACKNOWLEDGEMENTS

The authors wish to thank Helen Thorne, John Priestly, Margaret Anderson and Lisa Drage (ASADA) for organising and overseeing the blood collections as well as the staff of ClinPath SA for their efficient and professional phlebotomy. We thank Eileen Roberton and Sarah Woolford (South Australian Sports Insitute) for assistance with testing appartus as well as Paolo Menaspa, Jamie Stanley and Eric Haakonsen (Cycling Australia) for help with recruitment. We wish to thank Annette Eastwood and Avish Sharma for assistance during data collection and of course thank the athletes and team staff for participating in the study.

Funding for this study was received by the World Anti-Doping Agency (WADA).

FIGURE LEGENDS

Figure 1. Means ± SD and individual results for hemoglobin concentration [Hb], the OFF-score, percent reticulocytes (%RET), and percent change in plasma volume from sample 1 during the Tour Down Under in males and females. * significant change from pre race values (sample 1 / day 0). Note x axis refers to study day, with Race days 1-4 for females and 1-6 for males.

Figure 2. Subject 4 (Female). X axis refers to sample number. Red lines indicate the ABP adaptive model reference limits set at 99% (dashed lines) and 99.9% (solid lines) specificity (panels A-I). The green reference limits for [Hb] (A) and the OFF-score (I) represent the calculated limits with the inclusion of the PV correction. Panel J shows the z-scores for markers [Hb] (blue), Tfn (red), ALB (yellow), Ca (Black), CRE (green), TP (pink), PLT (cyan), LDL (dashed blue). Panel K represents the confidence calculation in the PV estimation. Panel L represents the plasma-volume z score calculations using the CO-rebreathing method (blue line) and the PV model (red line).

Figure 3. Subject 7 (Male). X axis refers to sample number. Red lines indicate the ABP adaptive model reference limits set at 99% (dashed lines) and 99.9% (solid lines) specificity (panels A-I). The green reference limits for [Hb] (A) and the OFF-score (I) represent the calculated limits with the inclusion of the PV correction. Panel J shows the z-scores for markers [Hb] (blue), Tfn (red), ALB (yellow), Ca (Black), CRE (green), TP (pink), PLT (cyan), LDL (dashed blue). Panel K represents the confidence calculation in the PV estimation. Panel L represents the plasma-volume z score calculations using the CO-rebreathing method (blue line) and the PV model (red line).

Figure 4. Subject 25 (Female). X axis refers to sample number. Red lines indicate the ABP adaptive model reference limits set at 99% (dashed lines) and 99.9% (solid lines) specificity (panels A-I). The green reference limits for [Hb] (A) and the OFF-score (I) represent the calculated limits with the inclusion of the PV correction. Panel J shows the z-scores for markers [Hb] (blue), Tfn (red), ALB (yellow), Ca (Black), CRE (green), TP (pink), PLT (cyan), LDL (dashed blue). Panel K represents the confidence calculation in the PV estimation. Panel L represents the plasma-volume z score calculations using the CO-rebreathing method (blue line) and the PV model (red line).

Figure 5. Measured changes in Plasma Volume (CO-rebreathing) versus predicted changes using the 8 biomarker PV model. Changes are given in number of deviations from an individual mean as computed by an adaptive Bayesian model.

REFERENCES

1. Schumacher YO, d'Onofrio G. Scientific expertise and the Athlete Biological Passport: 3 years of experience. Clinical chemistry. 2012;58(6):979-85. Epub 2012/04/12. doi: 10.1373/clinchem.2012.183061. PubMed PMID: 22490304.

2. Sottas PE, Robinson N, Rabin O, Saugy M. The athlete biological passport. Clinical chemistry. 2011;57(7):969-76. Epub 2011/05/21. doi: 10.1373/clinchem.2011.162271. PubMed PMID: 21596947.

3. Gore CJ, Parisotto R, Ashenden MJ, et al. Second-generation blood tests to detect erythropoietin abuse by athletes. Haematologica. 2003;88(3):333-44. Epub 2003/03/26. PubMed PMID: 12651273.
4. Garvican-Lewis LA, Schumacher YO, Clark SA, et al. Stage racing at altitude induces hemodilution despite an increase in hemoglobin mass. J Appl Physiol (1985). 2014;117(5):463-72. Epub 2014/07/06. doi: 10.1152/japplphysiol.00242.2014. PubMed PMID: 24994887.
5. Sawka MN, Convertino VA, Eichner ER, Schnieder SM, Young AJ. Blood volume: importance and adaptations to exercise training, environmental stresses, and trauma/sickness. Medicine and science in sports and exercise. 2000;32(2):332-48. Epub 2000/02/29. PubMed PMID: 10694114.
6. Ahlgrim C, Pottgiesser T, Robinson N, Sottas PE, Ruecker G, Schumacher YO. Are 10 min of seating enough to guarantee stable haemoglobin and haematocrit readings for the athlete's biological passport? International journal of laboratory hematology. 2010. Epub 2010/02/13. doi: CLH1213 [pii]10.1111/j.1751-553X.2009.01213.x. PubMed PMID: 20148988.

7. Fellmann N, Bedu M, Giry J, et al. Hormonal, fluid, and electrolyte changes during a 72-h recovery from a 24-h endurance run. International journal of sports medicine. 1989;10(6):406-12. Epub 1989/12/01. doi: 10.1055/s-2007-1024934. PubMed PMID: 2534121.
8. Bejder J, Hoffmann MF, Ashenden M, Nordsborg NB, Karstoft K, Morkeberg J. Acute hyperhydration reduces athlete biological passport OFF-hr score. Scandinavian journal of medicine & science in sports. 2016;26(3):338-47. doi: 10.1111/sms.12438. PubMed PMID: 25773052.
9. Fellmann N. Hormonal and plasma volume alterations following endurance exercise. A brief review. Sports Med. 1992;13(1):37-49. Epub 1992/01/01. PubMed PMID: 1553454.
10. Gore CJ, Morkeberg J, Schmidt W, Garvican LA, Fellman N. Plasma volume shifts during multiday racing. Clinical chemistry and laboratory medicine : CCLM / FESCC. 2013;51(6):e107-9. Epub 2012/12/18. doi: 10.1515/cclm-2012-0728. PubMed PMID: 23241592.
11. Bejder J, Andersen AB, Goetze JP, Aachmann-Andersen NJ, Nordsborg NB. Plasma volume reduction and hematological fluctuations in high-level athletes after an increased training load. Scandinavian journal of medicine & science in sports. 2017;27(12):1605-15. doi: 10.1111/sms.12825. PubMed PMID: 28138984.
12. Lobigs LM, Sottas PE, Bourdon PC, et al. The use of biomarkers to describe plasma-, red cell-, and blood volume from a simple blood test. American journal of hematology. 2017;92(1):62-7. doi: 10.1002/ajh.24577. PubMed PMID: 27737505.
13. Lobigs LM, Sottas PE, Bourdon PC, et al. A step towards removing plasma volume variance from the Athlete's Biological Passport: The use of biomarkers to describe vascular volumes from a simple blood test. Drug testing and analysis. 2017. doi: 10.1002/dta.2219. PubMed PMID: 28548390.
14. Lobigs L, Garvican-Lewis LA, Vuong VL, et al. Validation of a blood marker for plasma volume in endurance athletes during a live high: train low altitude training camp. Drug testing and analysis. 2018. Epub Feb 19. doi: 10.1002/dta.2370 PubMed PMID: 29457371.
15. Garvican LA, Eastwood A, Martin DT, Ross ML, Gripper A, Gore CJ. Stability of hemoglobin mass during a 6-day UCI ProTour cycling race. Clinical journal of sport medicine : official journal of the Canadian Academy of Sport Medicine. 2010;20(3):200-4. Epub 2010/05/07. doi: 10.1097/JSM.0b013e3181ddcd5b00042752-201005000-00007 [pii]. PubMed PMID: 20445361.

16. WADA. The World Anti-Doping Code: Athlete Biological Passport operating guidelines and compilation of required elements. Available in: https://wwwwada-amaorg/en/resources/athlete-biological-passport/athlete-biological-passport-abp-operating-guidelines. 2019;Version 7.1.
17. Schmidt W, Prommer N. The optimised CO-rebreathing method: a new tool to determine total haemoglobin mass routinely. European journal of applied physiology. 2005;95(5-6):486-95. Epub 2005/10/14. doi: 10.1007/s00421-005-0050-3. PubMed PMID: 16222540.
18. Chaplin H, Jr., Mollison PL, Vetter H. The body/venous hematocrit ratio: its constancy over a wide hematocrit range. The Journal of clinical investigation. 1953;32(12):1309-16. doi: 10.1172/JCI102859. PubMed PMID: 13108998; PubMed Central PMCID: PMC438476.

19. Meterology ABo. Monthly Mean Maximum Temperature 2020 [cited 2020 31.03.2020].
20. Schumacher YO, Pottgiesser T, Ahlgrim C, Ruthardt S, Dickhuth HH, Roecker K. Haemoglobin mass in cyclists during stage racing. International journal of sports medicine. 2008;29(5):372-8. Epub 2007/07/07. doi: 10.1055/s-2007-965335. PubMed PMID: 17614021.
21. Wachsmuth N, Kley M, Spielvogel H, et al. Changes in blood gas transport of altitude native soccer players near sea-level and sea-level native soccer players at altitude (ISA3600). British journal of sports medicine. 2013;47 Suppl 1:i93-9. Epub 2014/01/15. doi: 10.1136/bjsports-2013-092761. PubMed PMID: 24282216; PubMed Central PMCID: PMC3903154.
22. Ashenden MJ, Gore CJ, Parisotto R, Sharpe K, Hopkins WG, Hahn AG. Effect of altitude on second-generation blood tests to detect erythropoietin abuse by athletes. Haematologica. 2003;88(9):1053-62. PubMed PMID: 12969814.
23. Lobigs LM, Sharpe K, Garvican-Lewis LA, et al. The athlete's hematological response to hypoxia: A meta-analysis on the influence of altitude exposure on key biomarkers of erythropoiesis. American journal of hematology. 2018;93(1):74-83. doi: 10.1002/ajh.24941. PubMed PMID: 29027252.
24. Convertino VA, Brock PJ, Keil LC, Bernauer EM, J.E G. Exercise Training-Induced Hypervolemia: Role of Plasma Albumin, Renin, and Vasopressin. Journal of Applied Physiology. 1980;48(4):665-9. doi: https://doi.org/10.1152/jappl.1980.48.4.665. PubMed PMID: 6991463.
25. Saunders PU, Garvican-Lewis LA, Schmidt WF, Gore CJ. Relationship between changes in haemoglobin mass and maximal oxygen uptake after hypoxic exposure. British journal of sports medicine. 2013;47 Suppl 1:i26-30. doi: 10.1136/bjsports-2013-092841. PubMed PMID: 24282203; PubMed Central PMCID: PMC3903146.

TABLES

Table 1: *Tour Characteristics and timeline of testing*

Women	***Day***									
	Pre Race	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Stage		Stage 1	Stage 2	Stage 3	Stage 4					
Type		FLAT	HILLY	HILLY	CRIT					
Distance (km)		116.3	114.9	109.1	42.5					
Testing Hbmass Venous Blood	Hbmass S1	S2			S3	S4				S5
Men	***Day***									
	Pre Race	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	
Stage		Criterium	Rest	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	
Type		CRIT		FLAT	HILLY	HILLY	FLAT	FLAT	HILLY	
Distance (km)		51.0		150.0	135.8	131	152.8	149.1	151.5	
Testing Hbmass Venous Blood	Hbmass	S 1	Hbmass* S 2				S 3		S 4	

Hemoglobin mass (Hbmass) measures performed via CO rebreathing. * Note, the male cohort were tested across two days. Venous blood draws (S 1 - 5) performed in the morning following at least 10 mins of seated rest. FLAT: flat stage, HILLY: hilly and undulating stage, CRIT: criterium circuit race.

Table 2: *Subject characteristics*

	Men	Women
N	14	15
Height (cm)	182.7 ± 5.7	168.5 ± 6.1
Body mass (kg)	74.9 ± 6.8	60.1 ± 4.0
Age (years)	26.8 ± 5.1	29.3 ± 5.0
Hbmass (g)	1093 ± 122	725 ± 74
Hbmass (g.kg-1)	14.6 ± 1.0	12.0 ± 0.9
Predicted* VO2max ($mL.kg^{-1}.min^{-1}$)	72.6 ± 4.8	60.3 ± 4.1
No of finishers	13	15
Average finishing rank	76 ± 42	37 ± 21
Time behind winner	16’49” ± 13’04”	8’57” ± 9’03”

*Predicted VO2max [25], Hemoglobin mass (Hbmass) measured via carbon monoxide rebreathing pre race.

Table 3. *Hematological parameters during racing*

Women	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**

	Pre Race	*Pre Race*	*Post 3 stages*	*Post 4 stages*	*Post race (4 d)*
N	15	15	15	15	10
[Hb] g/L	136 ± 5	137 ± 4	132 ± 6	126 ± 6 *	133 ± 8
Hct (%)	41.0 ± 0.1	41.6 ± 0.1	39.9 ± 0.2 *	38.4 ± 0.2 *	40.7 ± 0.2
RET% (%)	1.39 ± 0.22	1.44 ± 0.23	1.48 ± 0.29	1.48 ± 0.28	1.65 ± 0.28 ł
MCV (fL)	96.2 ± 3.3	96.2 ± 3.4	96.6 ± 3.2	96.5 ± 3.6	97.2 ± 3.7
MCH (pg)	31.8 ± 1.0	31.7 ± 0.9	31.9 ± 0.9	31.7 ± 0.9	31.7 ± 1.1
MCHC (g/L)	331 ± 6	329 ± 8	330 ± 6	329 ± 9	327 ± 5
OFF-Score	65.1 ± 6.6	65.1 ± 6.0	59.0 ± 6.7 *	53.6 ± 7.2 *	56.1 ± 10.7 ł
PV (mL)	3686 ± 372	3623 ± 393	3855 ± 376 *	4108 ± 353 *	3553 ± 358
Men	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	
	Pre race	*Pre Race*	*Post 3 stages*	*Post 5 stages*	
N	14	14	14	13	
[Hb] g/L	149 ± 10	148 ± 11	144 ± 10	140 ± 8 *	
Hct (%)	44.9 ± 0.3	44.8 ± 0.3	43.3 ± 0.2	42.7 ± 0.2 *	
RET% (%)	1.17 ± 0.33	1.20 ± 0.33	1.20 ± 0.32	1.14 ± 0.27	
MCV (fL)	91.7 ± 3.4	91.6 ± 3.6	91.4 ± 3.6	91.8 ± 3.8	
MCH (pg)	30.4 ± 1.4	30.3 ± 1.4	30.4 ± 1.4	30.1 ± 1.4	
MCHC (g/L)	332 ± 7	331 ± 7	333 ± 6	328 ± 4	
OFF-Score	84.6 ± 13.1	83.2 ± 12.8	78.9 ± 11.5	76.3 ± 12.0	
PV (mL)	4784 ± 559	4809 ± 522	5058 ± 550 *	5207 ± 638 *	

Values are means ± SD. N, number of athletes; [Hb], hemoglobin concentration; Hct, hematocrit; RET%, percent reticulocytes; MCV, mean corpuscular volume; MCH, mean hemoglobin content, MCHC, mean corpuscular hemoglobin content; OFF-score = [Hb] g/L – 60 x (square root Reticulocytes %) (REF); PV, plama volume. * significant difference from pre race value (sample 1). ł significant difference from sample 1 (10 subjects)

Table 4. *Outliers at 99% specificity for [Hb] and OFF-score with and without the plasma volume correction*

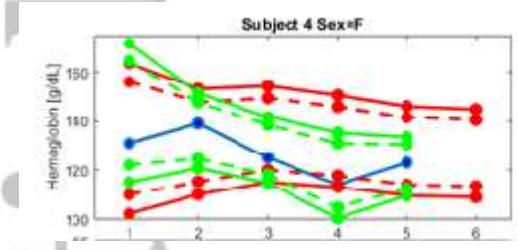
[Hb]			**OFF-score**		
Subject	Test	Outlier (upper or lower)	Subject	Test	Outlier (upper or lower)
Without Correction			Without Correction		
Female 4	4	Lower	Male 7	1	upper
Female 24	4	Lower			
Female 25	3	Lower			
Female 25	5	Upper			
With Correction			With Correction		
-	-	-	Male 7	1	upper

(*) Reference limits set at 99.9% specificity were also flagged.

Graphical abstract

Fluctuations in plasma volume (PV) present potential confounders within the Athlete Biological Passport. Here, a multi-parametric approach involving a simple blood test is applied

to the ABP adaptive model in an attempt to remove the influence of PV expansion, induced by a cycling stage race. When individual calculations of PV were applied to the ABP, the upper and lower reference predictions for [Hb] and the OFF-score were refined and all outliers consistent with racing induced PV changes were removed.



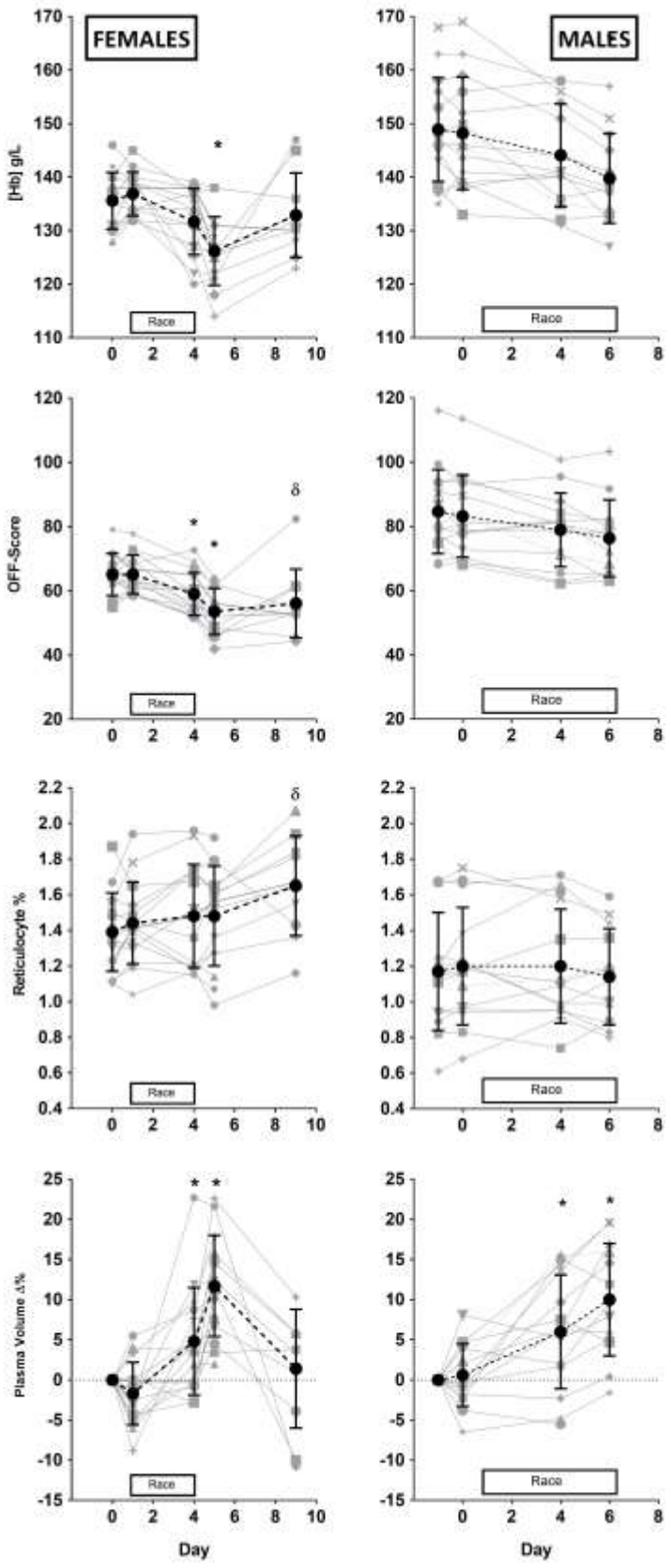


figure 1

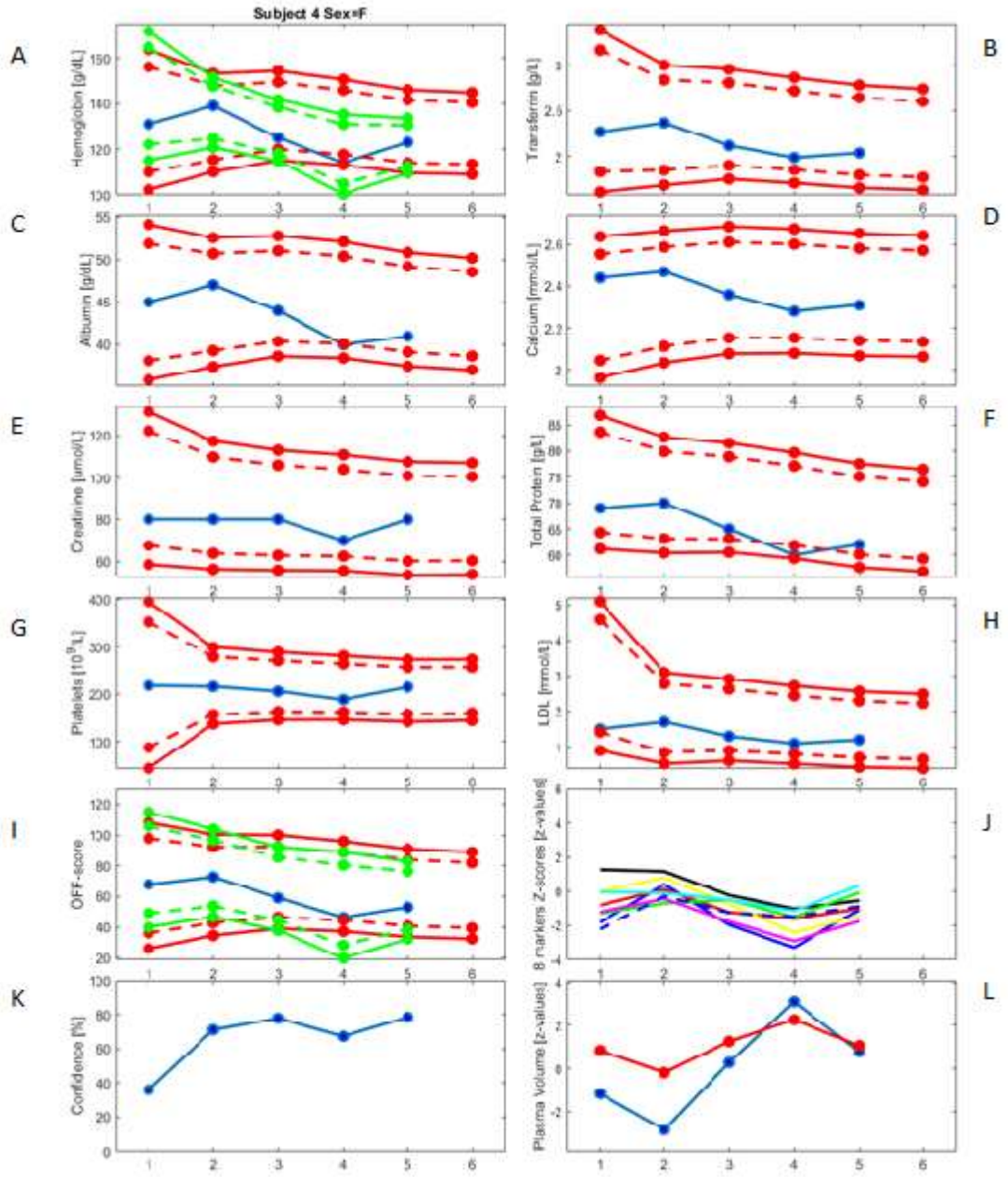


figure 2

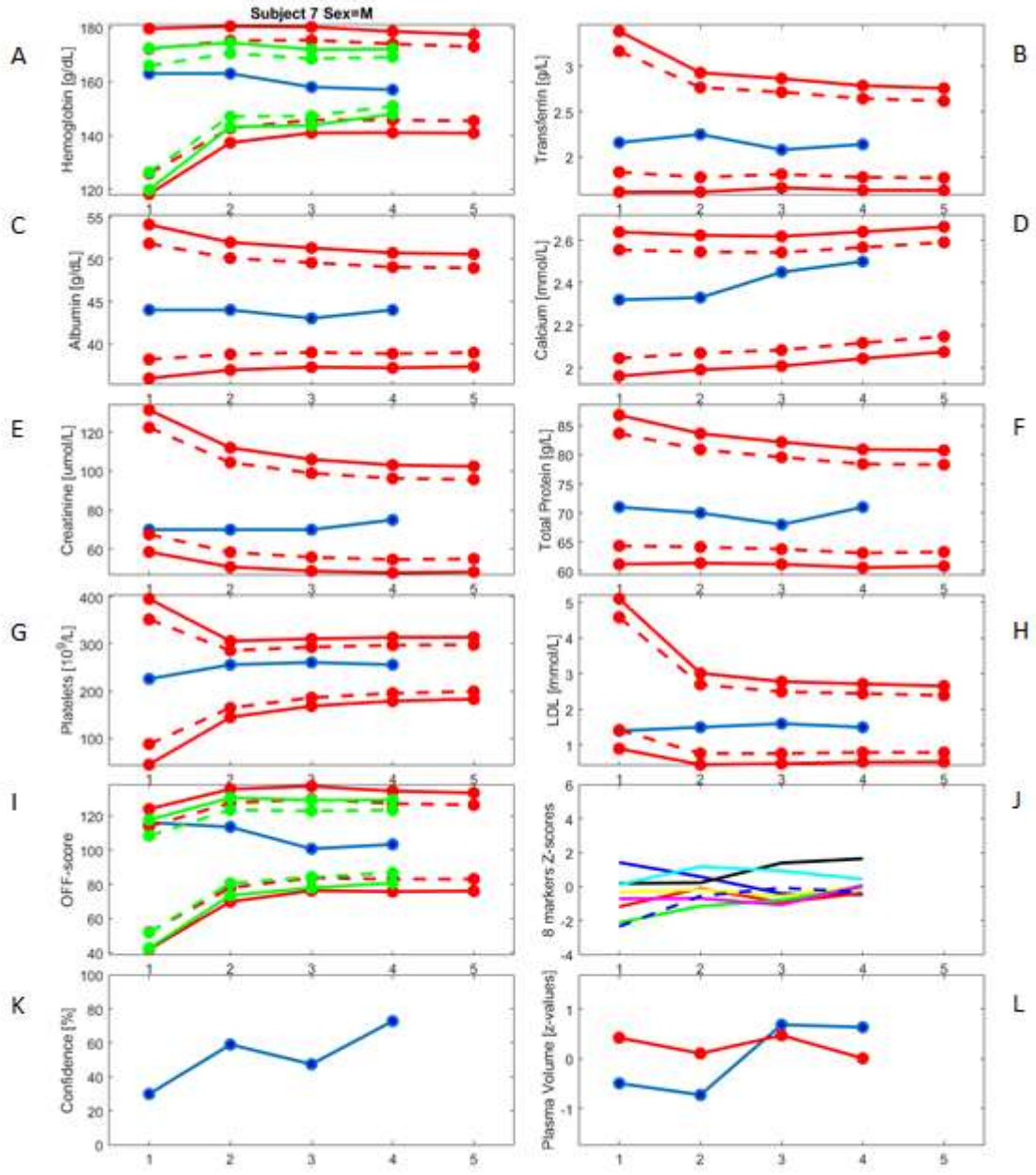


figure 3

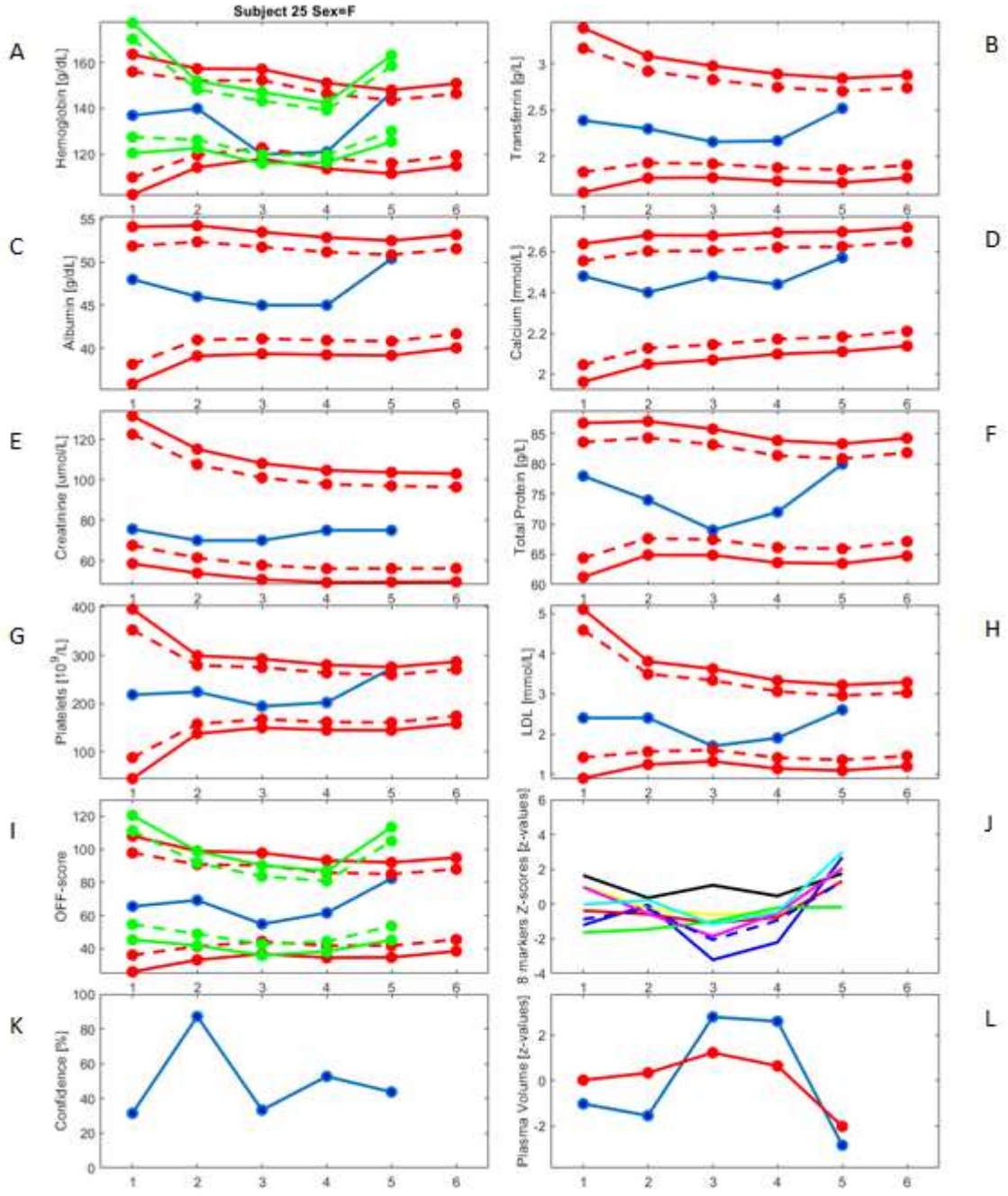


figure 4

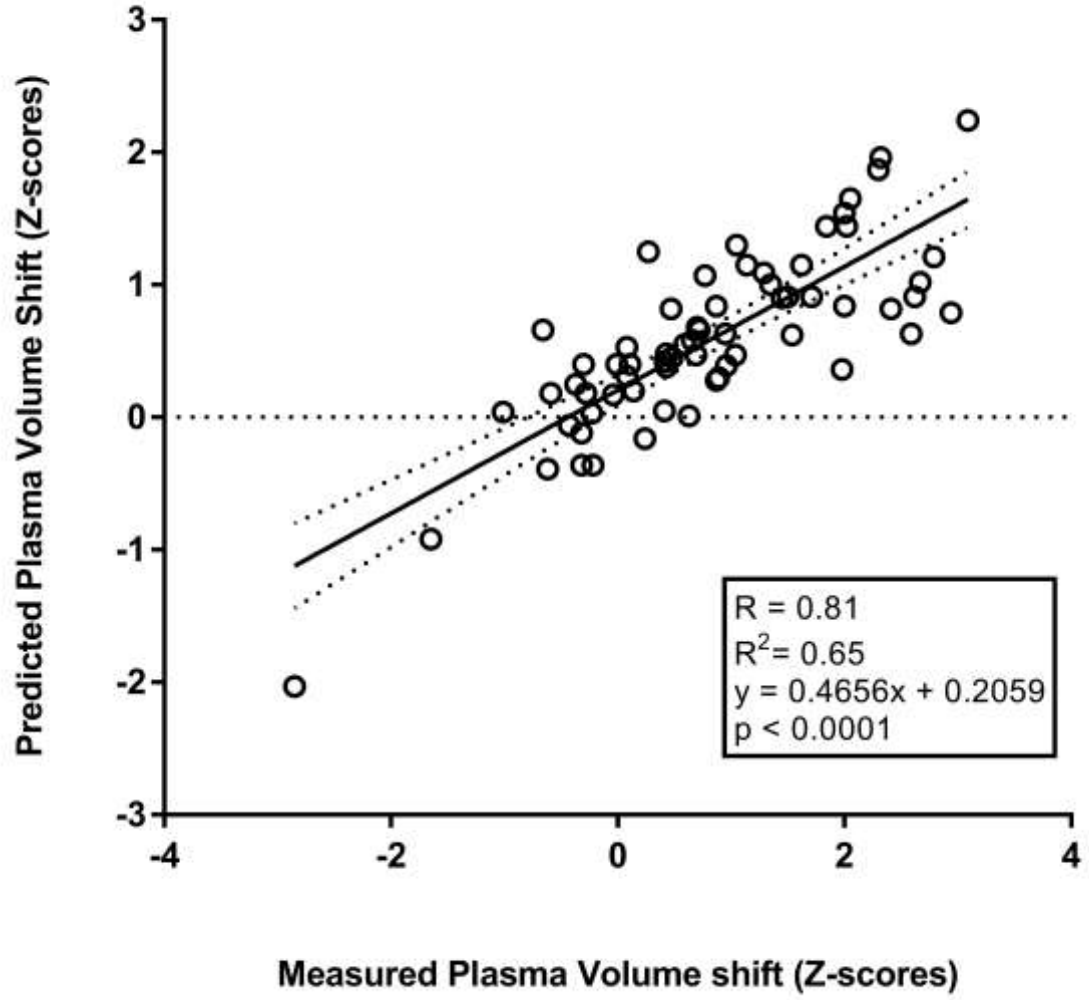


figure 5