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SHORT COMMUNICATION

Impact of low-volume blood withdrawal on hematological biomarkers for the athlete biological passport

B. Krumm | J. J. Saugy | F. Botrè | R. Faiss

REDs, Research & Expertise in anti-Doping sciences, Institute of Sport Sciences, University of Lausanne, Lausanne, Switzerland

Correspondence
R. Faiss, REDs, Research and Expertise in anti-Doping sciences, University of Lausanne, Synathlon, 1015 Lausanne, Switzerland.
Email: raphael.faiss@unil.ch

Abstract

This study investigated the impact of low-volume blood withdrawal on the hematological biomarkers currently considered for anti-doping purposes. After baseline measurement (D − 7), a 140 mL blood withdrawal was completed (D + 0) on 12 healthy volunteers, followed by weekly monitoring for 21 days (D + 7 – 21). Each visit consisted of a full blood count (Sysmex XN-1000) and duplicate blood volume measurements by CO-rebreathing. A significant decrease in total hemoglobin mass (Hbmass) (−2.3%, $p = 0.007$) and red blood cell volume (RBCV) (−2.8%, $p = 0.028$) was reported at D + 7. Despite no atypical passport finding (ATPF) when considering the athlete biological passport adaptive longitudinal model, hemoglobin concentration ([Hb]) increased significantly at D + 21 (+3.8%, $p = 0.031$). Besides, ferritin (FERR) was significantly downregulated at all points following blood withdrawal, with the largest decrease occurring at D + 7 (−26.6%, $p < 0.001$). Regardless of the presumable effect of blood reinfusion on ABP biomarkers, these results illustrate the challenge of monitoring hematological variables for the detection of low-volume blood withdrawal. Finally, this study outlines the sensitivity of FERR to altered erythropoiesis to support the implementation of iron markers as complementary variables for the longitudinal monitoring of blood doping, despite the potential influence of confounding factors (e.g., iron supplementations).

KEYWORDS
athlete biological passport, blood doping, blood transfusion, blood withdrawal, hematology

1 | INTRODUCTION

Blood transfusions are among the most common doping practices used by endurance athletes. By increasing the total hemoglobin mass (Hbmass), the beneficial effect of blood reinfusion on endurance performance has been demonstrated.[1] By exchanging blood between a donor and a recipient, homologous blood transfusions (HBTs) are currently detectable by targeting the surface antigens of the red blood cell (RBC) by flow cytometry.[2] Therefore, through the progressive expansion of the antigen panel, the sensitivity allows the detection of up to 0.2% of the donor cells into the recipient.[3] However, no direct detection method is currently available for the screening of autologous blood transfusions (ABTs), which are currently traceable through the hematological module of the athlete biological passport (ABP) only, although the multiple confounding factors affecting the hematological biomarkers remain a major challenge in the detection of blood doping.[4]

Relying on the indirect monitoring of biomarkers, the ABP was implemented in 2009 to support the original anti-doping approach. The hematological module of the ABP consists of an individual and longitudinal follow-up of hematological parameters and biomarkers responsive to erythropoiesis alteration.[5] Generated using an adaptive Bayesian model, the individual ABP profiles of athletes seek to identify non-physiological variations due to different types of blood doping (e.g., blood transfusion).

The substantial impact of a standard blood donation (~500 mL) on ABP parameters demonstrated the interest of the model with a traditional blood doping scenario,[5] despite a low sensitivity level sometimes observed mainly because of an expansion of the plasma volume (PV) in the period following RBC re-infusion, thus normalizing all hematological variables that constitute the algorithms of the ABP.[6] However, it seems that a low volume of 135 mL of packed RBCs is sufficient to improve cycling time trial performance.[7] This process is not new. Indeed, because a low-volume withdrawal does not result in remarkable detrimental effects on performance in contrast to a larger volume,[8] several projects demonstrated the interest of repeating small-volume withdrawals in order to constitute a blood bank for subsequent reinfusion.[9] Although blood withdrawal is not a prohibited practice by the World Anti-Doping Agency (WADA), repeated low-volume blood withdrawals and reinfusions seem to be a likely modern blood doping scenario: in addition to limiting suspicious fluctuations in the hematological profile, these withdrawals would have a negligible impact on the training of athletes. Therefore, the ability to detect low-volume blood withdrawal becomes particularly relevant in an anti-doping context.

At the same time, there is only scarce evidence on the capacity of the hematological module of the ABP to detect single or multiple withdrawals of smaller volumes of blood, closer to a modern blood doping strategy. Repeated withdrawals not only make the detection of ABTs more difficult but also have the advantage of allowing the athlete to maintain a sustained training regimen. It is hypothesized that the withdrawal of a low volume of blood may result in alterations of the ABP variables that can be detected in blood samples collected closer to the withdrawal. This study investigated the impact of low-volume blood withdrawal on a panel of hematological variables, not limited to those presently included in the hematological module of the ABP.

2 | METHODS

2.1 | Subjects

Twelve healthy active subjects (seven women and five men; 26 ± 6 years, 170 ± 7 cm, 65 ± 7 kg) were recruited in this study and provided their informed written consent. This study was approved by the local ethics committee (CER-VD, Lausanne, Switzerland, #2018-01019) and conducted in accordance with the Declaration of Helsinki.

2.2 | Study design

This research protocol was performed at the end of a 1-year monitoring study that included monthly blood sampling (8 mL collected in BD Vacutainer® tubes K2-EDTA) and duplicated blood volume measurement using a validated CO-rebreathing procedure (OpCo: Detalo Instruments, Birkerod, Denmark). First, shortly after a conventional blood sample, 140 mL of whole blood was withdrawn. This blood removal was immediately followed by a blood volume measurement (D + 0). Afterward, a weekly follow-up over 21 days was performed (D + 7 – 21). Each sample was collected in strict accordance with the WADA ABP Operating Guidelines.[10] In addition, the following information was reported at the beginning of each visit: hypoxic exposure in the last 2 weeks (>1500 m), physical exercise for the last 7 days, and the menstrual cycle phase.

2.3 | Hematological analyses

Hematological parameters were measured by flow cytometry (Sysmex XN-1000, Sysmex Corporation, Japan). As part of the hematological module of the ABP, the following markers were analyzed: hematocrit (Hct), hemoglobin concentration ([Hb]), RBC count, reticulocyte percentage (Ret%) and count (Ret#), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), mean corpuscular volume (MCV), immature reticulocyte fraction (IRF), platelets (PLTs), red cell distribution width (RDW-SD), white blood cells (WBCs), OFF-score (OFFs), and the abnormal blood profile score (ABPS). The OFFs index was calculated as $([Hb] \times 10) - 60 \times \sqrt{Ret\%}$.[10] In addition, ferritin (FERR) and transferrin (TRF) concentrations were additionally measured by chemiluminescence immunoassay technique (Dimension® clinical chemistry system, Siemens AG, Munich, Germany). Individual ABP profiles were generated using the official ABP adaptive software (Anti-Doping Administration and Management System [ADAMS]) provided by the WADA. To provide fully individualized ABP profiles, monthly hematological measurements for the 11 months prior to blood withdrawal were included in the adaptive model of each subject. To comply with the ABP approach considering variations from one point to another, comparisons for hematological and blood volume variables were compared with the last value collected before blood withdrawal, that is, D − 7 for blood volumes and D + 0 for ABP parameters (shortly before withdrawal). However, due to day-to-day biological variation of iron markers, the individual average (baseline) of the last 11 months was selected for post-blood withdrawal comparisons to provide a more accurate representation of the FERR and TRF individual values.

2.4 | Statistical analyses

Data are presented as means and standard deviations (±SD). Distribution normality was assessed by the D'Agostino & Pearson test. Mixed

model repeated measures analyses were run for each parameter to determine whether changes in the dependent (hematological variables) variables differed over time (fixed factor). All statistical analyses were conducted using GraphPad Prism 9 software (https://www.graphpad.com). The null hypothesis was rejected for $p < 0.05$.

3 | RESULTS

Based on Hct and [Hb], 43.2 ± 8 g of hemoglobin were removed with the 140 mL blood withdrawal. Compared with baseline (D + 0), a significant decrease in Hbmass (−2.3%; 726 ± 185 vs. 709 ± 189 g; $p = 0.007$) and RBC volume (RBCV) (−2.8%; 2190 ± 521 vs. 2129 ± 552 mL; $p = 0.028$) was observed at D + 7, with a return to basal value at D + 14 (Figure 1). No atypical passport findings (ATPFs) were generated in the individual ABP profiles. However, a significant increase in [Hb] was observed at D + 21 (+3.8%; 13.3 ± 1.4 vs. 13.8 ± 1.3 g/dL, $p = 0.031$) (Figure 2). No significant variations were observed for the other ABP variables (i.e., OFFs, Ret%, and ABPS), despite a positive trend for Ret% post-withdrawal ($p = 0.067$). Finally, compared with a yearly average, FERR was significantly downregulated at all time points post-withdrawal, with the largest decline at D + 7 (−26.6%; 118.6 ± 90.7 vs. 87.1 ± 76.5 µg/L, $p < 0.001$) (Figure 3).

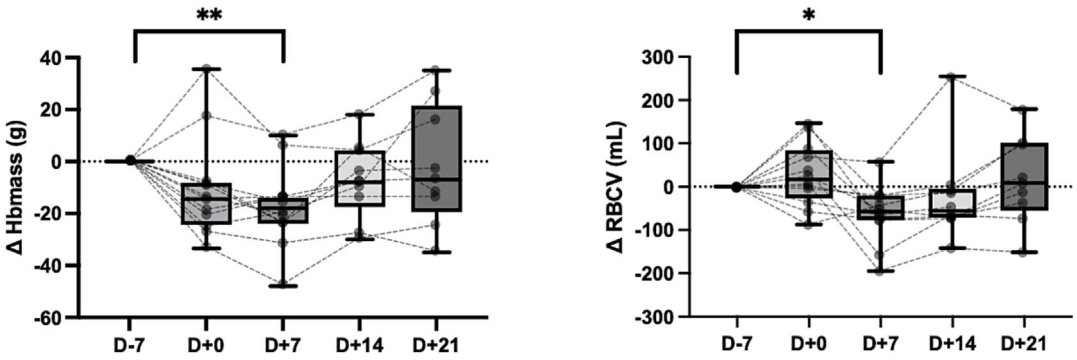

FIGURE 1 Relative variations for total hemoglobin mass (Hbmass) and red blood cell volume (RBCV) before (D − 7) and after blood withdrawal (D + 0 – 21). $^{**}p < 0.01$, and $^{*}p < 0.05$ for the difference between days.

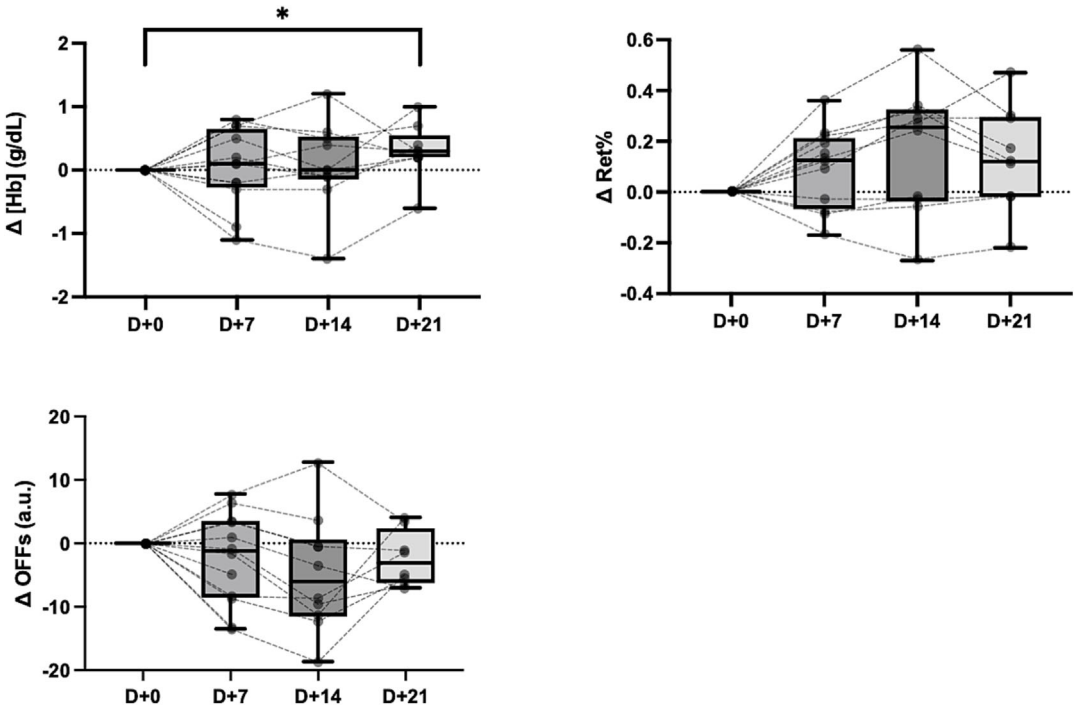

FIGURE 2 Relative variations for hemoglobin concentration ([Hb]), percentage of the reticulocytes (Ret%), and OFF-score (OFFs) before (D – 7) and after blood withdrawal (D + 7 – 21). $^{*}p < 0.05$ for the difference between days.

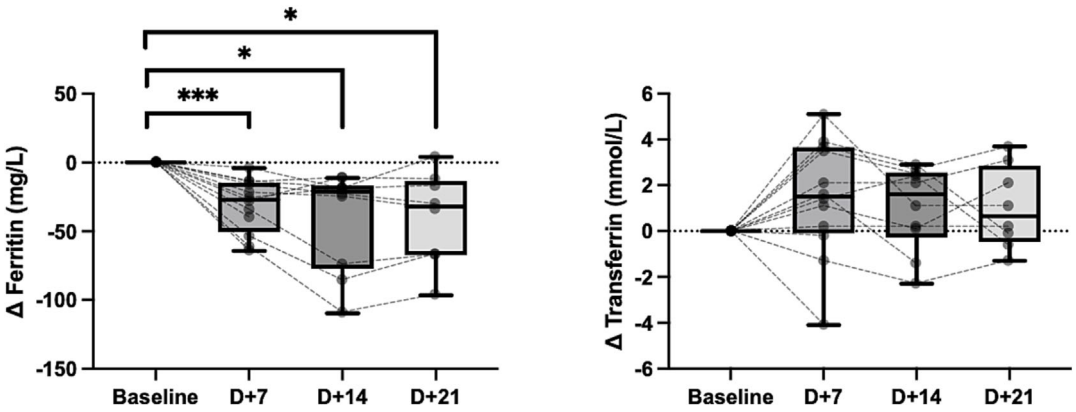

FIGURE 3 Relative variations for ferritin and transferrin after blood withdrawal (D + 7 – 21). ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ for the difference between days.

4 | DISCUSSION

This study reported the impact of low-volume blood withdrawal on hematological and iron biomarkers. No atypical variation was observed in the subjects' passports. Besides [Hb], OFFs did not result in any atypical profile either, although they are known to be an effective algorithm to detect altered erythropoiesis.[11] This is probably explained by the delay between the blood withdrawal and the subsequent visit (7 days). While the ABP may present a few advantages to complement the direct detection of prohibited substances,[5] our results confirm the challenge of indirectly interpreting low-volume blood manipulation in a contemporary blood doping scenario.[12]

Counterintuitively, the decrease of Hbmass and RBCV was greater at D + 7 (−11 ± 19 g) compared with D + 0 (−18 ± 15 g) (Figure 1). Although the typical measurement error usually associated with blood volume measurement by the CO-rebreathing method (1.5%–2%) should not be excluded,[13] this limited decrease at D + 0 can possibly be explained by the spleen contraction following the blood withdrawal. Indeed, the spleen is known as a concentrated reserve of oxygenated RBC (∼100 mL) with a buffer capacity to eject them transiently in circulation in reaction to various physiological stresses.[14] If hypoxic stress caused by repeated apneas has been recently studied,[15] such RBC release seems to occur after hemorrhage in order to allow for a rapid increase in arterial oxygen content.[16] Therefore, because blood volume measurement was performed immediately after withdrawal, a splenic contraction may explain the absence of a significant decrease in Hbmass and RBCV at D + 0.

An increased [Hb] (a primary biomarker of the hematological module in the ABP) was observed at the end of the longitudinal monitoring (D + 21) (Figure 2). This late [Hb] increase can be related to an erythropoietic rebound, which requires several weeks before reticulocytosis becomes noticeable,[17] although transient hemoconcentration induced by a PV shift cannot be excluded and needs to be considered. If serum erythropoietin (EPO) levels increase significantly in the hours following a reduction in oxygen transport capacity (e.g., blood withdrawal), the complete erythropoietic process lasts more than 2 weeks before resulting in mature RBC. In addition, despite the known impact of confounding factors on [Hb] fluctuation due to PV shifts,[4] the assumption of an erythropoietic stimulation leading to [Hb] increase is supported by the positive trend of Ret% observed in the weeks following blood withdrawal (Figure 2).

Besides, FERR, a common indicator of iron stores, was significantly downregulated following blood withdrawal (Figure 3). These results are consistent with previous studies investigating FERR as a marker of blood transfusion.[18,19] Abnormally high FERR values, hence favorably mediating erythropoietic response,[20] were observed in athletes convicted of blood doping (personal communication from anti-doping organizations, unpublished data). Excessively high FERR levels may hence help trigger additional targeted tests to complement the indirect monitoring of any erythropoietic stimulation through ABP profiling. Furthermore, although not significant, the positive trend observed in the TRF level supports the hypothesis of an alteration of iron metabolism following a low-volume blood removal. Nevertheless, the natural biological variability of iron markers needs to be considered. Indeed, if the variation of FERR at D + 7 (−26.6%) seems to be superior to the day-to-day coefficient of variation observed in healthy subjects (14.5%),[21] these values need to be confirmed in an athletic population. In addition, sporadic or regular iron supplementation commonly administered to athletes (especially in endurance sports)[22] remains a crucial limitation of using iron parameters for blood doping screening.[23] Finally, the impact of external factors on the FERR level, such as physical exercise[24] or inflammation,[25] also needs to be taken into consideration when interpreting these markers. Therefore, the concomitant analysis of multiple markers seems to be the most reliable approach to reduce potential interpretation bias.[26] In addition, similarly to other markers of iron status,[27] the large inter-subject variability in FERR and TRF underlines the importance of individual longitudinal monitoring to provide sufficient sensitivity in the interpretation of hematological variations.

Despite significant fluctuations in the hematological biomarkers of the ABP ([Hb]), no criteria to report an "atypical passport finding" (ATPF) were observed in any of the participants. Nevertheless, the hematological response following blood reinfusion was not investigated in the present study and could certainly affect ABP variables

(e.g., Ret% decrease). Therefore, further studies, including a complete ABT protocol, are needed to thoroughly understand the effect of single or multiple withdrawals of low volumes of blood on hematological variables and newly suggested biomarkers.

4.1 | Conclusion

The effects of ABTs performed following multiple low-volume blood withdrawals are particularly difficult to discriminate from physiological effectors (e.g., altitude training), supporting the need for additional markers to complement the ABP. FERR as a marker of altered iron metabolism was thereagainst shown to be sensitive and possibly suitable for outlining low-volume blood transfusions, although the specificity of these markers needs to be further investigated. Finally, our study confirms that even low-volume blood withdrawals are associated with significant hematological changes, while targeted frequent blood samples and additional biomarkers are certainly required to better tackle blood doping strategies using such low-volume transfusions.

4.2 | Limitations

This study includes some important limitations that need to be considered. First, the study includes a small number of subjects ($n = 12$), which requires tempering current findings and does not support a robust analysis of some additional parameters (e.g., male–female comparison). Furthermore, in addition to strict compliance with the ABP Operating Guidelines, additional monitoring of external factors (e.g., hydration status) would be warranted to limit the impact of some confounding factors on the variables under investigation.

AUTHOR CONTRIBUTIONS

Raphael Faiss designed the study. Bastien Krumm, Raphael Faiss, and Jonas Saugy contributed to data collection. Bastien Krumm drafted the first version of the manuscript, and Raphael Faiss, Jonas Saugy, and Francesco Botre revised it critically. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

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.

ORCID

B. Krumm https://orcid.org/0000-0002-9132-606X
R. Faiss https://orcid.org/0000-0001-6029-9516

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