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ABSTRACT

Purpose: We investigated whether hepcidin and erythroferrone (ERFE) could complement the Athlete Biological Passport (ABP) in indirectly detecting a 130 mL packed red blood cells (RBCs) autologous blood transfusion. Endurance performance was evaluated.

Methods: Forty-eight healthy men (n=24) and women (n=24) participated. Baseline samples were collected weekly followed by randomization to a blood transfusion (BT, n=24) or control group (CON, n=24). Only the BT group donated 450 mL whole blood from which 130 mL RBCs was reinfused four weeks later. Blood samples were collected 3, 7, 14, 21 and 28 days after donation, and 3, 6, and 24 hours and 2, 3, and 6 days following reinfusion. In the CON group samples were collected with the same frequency. Endurance performance was evaluated by a 650-kCal time trial (n=13) before and one and six days after reinfusion.

Results: A time×treatment effect existed (P<0.05) for hepcidin and ERFE. Hepcidin was increased (P<0.01) ~110 and 89% six and 24 hours after reinfusion. Using an individual approach (99% specificity, e.g. allowing 1:100 false-positive), sensitivities, i.e. true positives, of 30% and 61% was found for hepcidin and ERFE, respectively. For the ABP, the most sensitive marker was Off-hr score ([Hb] (g/L) – 60×√RET%) (P<0.05) with a maximal sensitivity of ~58% and ~9% following donation and reinfusion, respectively. Combining the findings for hepcidin, ERFE and the ABP yielded a sensitivity across all time-points of 83% following reinfusion in BT. Endurance performance increased 24 hours (+6.4%, P<0.01) and six days following reinfusion (+5.8%, P<0.01).

Conclusion: Hepcidin and ERFE may serve as biomarkers in an anti-doping context following an ergogenic, small-volume blood transfusion.

Keywords: Transfusion; biomarker; anti-doping; sex-specific; micro-dosing.
INTRODUCTION

Autologous blood transfusion (ABT), i.e. withdrawing and later reinfusing your own blood, is known to increase performance (1) but the detection of ABT remains a challenge. Presently, the World Anti-Doping Agency (WADA) applies the Athlete Biological Passport (ABP) for indirect detection of ABT, which is an individual, longitudinal adaptive threshold approach based on fluctuations in hemoglobin concentration ([Hb]), reticulocyte percentage (RET%), Off-hr score (Off-hr; [Hb] (g/L) $- 60 \times \sqrt{\text{RET}}$) (2) and the multifactorial score Abnormal Blood Profile Score (ABPS) (3). Following reinfusion of larger doses of 450-1,350 mL, the ABP approach can reveal up to 80% of true positive cases (i.e. 80% sensitivity) with no false positives (i.e. 100% specificity) (4, 5). Nonetheless, blood doping prevalence, including doping with erythropoiesis-stimulating agents, may have been as high as 18% and 15% in the 2011 and 2013 World Athletics World Championships (6). One potential challenge is the suspected reinfusion of volumes smaller than 150 mL packed red blood cells (RBCs) referred to as micro-dosing (7), which seems to be ergogenic (8). Smaller reinfusions are likely to cause smaller ergogenic effects as well as hematological fluctuations, the latter consequently reducing the ABP sensitivity. This calls for the discovery of novel biomarkers with improved sensitivity and high specificity.

Despite being influenced by various confounders such as iron intake (9), circadian fluctuations (10) and exercise (11, 12), valuable biomarker additions may include the central governors of iron homeostasis hepcidin and erythroferrone (ERFE). Hepcidin occludes, internalizes and degrades the iron transporter ferroportin, which reduces the absorption of dietary iron and iron release from macrophages (13). Hepcidin has proven to be a sensitive biomarker for blood reinfusion of ~280 mL packed RBCs (14). Furthermore, when systemic erythropoietin levels rise, the erythroblasts increase the synthesis and release of ERFE, which suppresses hepatic hepcidin production (15). Consequently,
ERFE increase after withdrawal of ~450-900 mL blood (16, 17). In addition, even small alterations in erythropoietic homeostasis induced by six injections of 20 IU recombinant human erythropoietin (rHuEPO) per kg body weight increase ERFE levels during treatment while decreasing below baseline levels upon treatment cessation (18). Accordingly, hepcidin and ERFE are hypothesized to be valuable additions to the panel of biomarkers for indirect detection of ABT. However, it remains unknown whether they are sensitive to blood reinfusion in small volumes (<150 mL). Thus, the present study evaluated the sensitivity and specificity of hepcidin and ERFE for detecting small-volume of ABT benchmarked against the ABP.

Another important aspect for biomarker sensitivity, i.e. the fraction of true positive outliers, and specificity, i.e. the fraction of true negatives, is potential sex-differences (19, 20). Male elite athletes have higher [Hb] compared to females (21), whereas RET% is higher in female athletes (22). In addition, the menstrual cycle in female athletes can cause fluctuations of [Hb] and RET% (23, 24), although this is not a uniform finding (25). Likewise, there may be sex differences in levels or variation in biomarkers for iron homeostasis due to a greater need for intestinal iron uptake or mobilization of iron stores in females. Indeed, recent data suggests that up to 79% of female elite athletes routinely supplement with iron for presumed iron deficiency (26). Consequently, if females display larger natural variations in biomarkers relevant for the ABP they may have an increased risk of false-positive findings due to normal variation or causes the adaptive model to calculate wider thresholds of the ABP, thus reducing the chance of detecting dishonest athletes. Accordingly, it is of high relevance to investigate whether males and females exhibit similar fluctuations to account for potential sex-differences within the ABP. The potential differences may also explain why the blood doping prevalence is estimated to be higher in female (~22%, 95% confidence interval [16-28%]) than male (~15%, 95% confidence interval [9-20%]) athletes in certain competitions (6).
Finally, the importance of investigating whether a small-volume ABT is detectable by existing or novel methods is highlighted by a ~5% improved cycling time trial performance following reinfusion of ~135 mL RBCs (8). To date no other study has confirmed these initial findings or investigated whether the effect is present for more than 24 hours. Thus, a final aim of the present study was to confirm whether a small-volume ABT improves cycling time-trial performance 24 h and 6 days after reinfusion.

Thus, here we investigated the hypotheses that an ABT of ~130 ml packed RBCs: (1) up- and downregulate hepcidin and ERFE levels, respectively; (2) is indirectly detectable by the ABP biomarkers; (3) is detectable by the ABP with a higher sensitivity in females than males, but a compromised specificity exist in females due to larger fluctuations in relevant ABP biomarkers; and (4) increase mean power output in a preloaded cycling time-trial.
METHODS
Forty-eight well-trained males (n=24) and females (n=24) participated in the study (Table 1), which was approved the local ethics committee of Copenhagen, Denmark (H-17024876), registered on www.clinicaltrials.gov (NCT04514978) and performed in accordance with the Declaration of Helsinki. All subjects were low-altitude residents and had not been exposed to altitudes higher than 1,000 m or donated blood for at least three months prior to the study. Subjects were instructed not to travel to high altitudes or donate blood for other purposes during the study period. All subjects were informed both orally and in writing of potential risks and discomforts associated with participation before a written consent was obtained from each participant. Menstrual cycle or hormonal contraceptives was not controlled for in the present study.

Design
The study used a counter-balanced, randomized placebo-controlled design (Fig. 1). A randomization algorithm (http://www.randomizer.org) assigned subjects to a blood transfusion (BT) or control (CON) group. All subjects completed an incremental maximal cycle ergometer test to determine maximal oxygen uptake (\(\dot{V}O_2\text{peak}\)), and no differences were evident between groups in exercise capacity or anthropometric data prior to the intervention (table 1). Following a baseline period of either two (12 males, 12 females) or eight (12 males, 12 females) weeks with weekly blood sampling, the BT group donated 450 mL whole blood with venous blood samples obtained 3, 7, 14, 21, and 28 days later. After 28 days, a reinfusion of ~130 mL of stored autologous packed RBCs was performed in the BT group with blood samples collected 3, 6 and 24 hours as well as 2, 3 and 6 days after reinfusion. These time points were selected to enable investigation of the acute effects of a small-volume reinfusion. No intervention was applied to the CON group, but blood was sampled at the same time points. All subjects received 80 mg iron (ferric sulphate) (Tardyferon, Pierre Fabre Pharma
GmbH, Germany) each day following donation and reinfusion to ensure sufficient iron stores for the expected increase in erythropoiesis. Adherence was ascertained by asking weekly whether the subject had ingested the tablets and amounted to an adherence of 97.9%. Gastrointestinal adverse effects were reported by six participants, who were advised to ingest the tablets only every other day, and for two subjects every third day.

In a BT (n=7) and CON (n=6) subgroup, the intervention was double-blinded, to appropriately investigate the performance enhancing effect of a small-volume blood transfusion. Subgroup subjects wore a blindfold and noise-cancelling headphones during the donation of 450 mL whole blood (BT group) or sham-donation (CON group) as well as during reinfusion of ~130 mL stored autologous packed RBCs (BT group) or sham-reinfusion (CON group). Non-blinded personnel not involved in the exercise testing conducted the collection and processing. The subgroup’s mean ± standard deviation (SD) age, weight and \( \dot{\text{V}}\text{O}_{2}\text{peak} \) were 29 ± 8 years, 71 ± 14 kg, and 58 ± 6 ml O\(_2\)×kg\(^{-1}\)×min\(^{-1}\) for CON and 28 ± 4 years, 75 ± 7 kg and 61 ± 7 ml O\(_2\)×kg\(^{-1}\)×min\(^{-1}\) for BT, respectively.

Phlebotomy and autologous blood transfusion

Subjects donated 450 mL whole blood collected into a bag containing 66.5 ml citrate phosphate dextrose solution (Fresinus Kabi, Germany). Within 6 hours, the whole-blood units were separated into RBCs, plasma and buffy coat by hard-spin centrifugation (RCF 4700 g, 9 min. 22°C, Hettich Roto Silenta 630, Hettich GmbH, Germany) followed by separation on a CompoMat G5 (Fresenius-Kabi, Germany). RBCs were leukoreduced and stored at +4°C in saline-adenine-glucose-mannitol (SAG-M) solution for the longest approved period of 28 days by the Danish National University Hospital (Rigshospitalet) to allow maximal possible recovery of the lost blood volume.

The ABT was performed four weeks following phlebotomy at 08:10 ± 00:57 a.m. The subjects rested in a semi-recumbent position, and an 18 G catheter was inserted into an antecubital vein. The weight
of the blood bag was measured before and after the reinfusion, and RBC infusion volume was
determined as the weight of the reinfusion volume divided by a 1.06 g/ml density of RBCs in SAG-
M. The BT group received 128 ± 6 ml packed RBCs obtained from the phlebotomy. No intervention
was applied in the CON group if subjects were not a part of the performance subgroup. Subjects in
the performance subgroup received ~100 ml of saline in a sham-reinfusion. To determine the
hemoglobin concentration in the blood bag, a blood sample (~1 ml) was collected from the blood bag
and analyzed using an ABL 800 blood gas analyzer (Radiometer, Brønshøj, Denmark). The total
amount of transfused hemoglobin was determined by multiplying the infused volume by the
hemoglobin concentration in the blood bag. The calculated amount of transfused hemoglobin was 23
± 3 g.

Blood sampling

All venous blood samples were collected as scheduled and according to WADA guidelines (27),
except for one female participant who had no baseline samples collected and was excluded from the
ABP analysis. Briefly, subjects refrained from physical exercise two hours before and rested for 10
min in a seated position before blood sample collection. The tourniquet was released when the blood
started to flow and was applied for no more than 30 sec. Upon appropriate filling, vacutainers (2x
EDTA and 1x lithium-heparin (Li-He), BD Vacutainer®, Becton Dickinson, NJ, USA) were gently
inverted at least three to five times to homogenize the blood. One EDTA and the Li-He vacutainer
were immediately stored at 4°C and delivered for analysis within 2 h of collection at the Danish
National University Hospital (Rigshospitalet), which follows the European standard quality control
in medical laboratories (DS/EN ISO 15189). The EDTA sample was analyzed for [Hb], RBC count,
mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular
hemoglobin concentration (MCHC), hematocrit (Hct), and reticulocyte percentage (RET%) within 2
h of delivery using a Sysmex XN-2100 (Sysmex, Kobe, Japan). The Li-He sample was analyzed for plasma iron, ferritin, transferrin and transferrin saturation (TSAT) via sandwich electrochemiluminescence immunoassay on a Cobas 8000 (Roche Diagnostics, Rotkreuz, Switzerland). The remaining EDTA vacutainer was centrifuged for 10 min at 5200 g and plasma was stored at -80°C for later determination of hepcidin and ERFE.

**Quantification of hepcidin and ERFE**

Plasma hepcidin and ERFE were determined for 20 females and 20 males in samples collected during baseline and one, two, and four weeks after donation as well as six and 24 hours and three and six days after reinfusion. Of these, 12 males and 12 females completed the 8-week baseline period, and 8 males and 8 females completed the two-week baseline. Hepcidin was determined using a competitive enzyme-linked immunosorbent assay according to the manufacturer’s guidelines (Intrinsic LifeScience, La Jolla, CA, USA) with a coefficient of variation (CV) of 8.4 ± 2.1%. ERFE was quantified using a sandwich ELISA protocol, which have a CV of 6.8 ± 0.8% (16).

**Athlete Biological Passport**

To evaluate the sensitivity and specificity of the ABP, all data were analyzed using the hematological module of the Anti-Doping Administration & Management System (ADAMS) training platform developed by WADA (28). The following variables were imported into the software for each participant: [Hb], Hct, RBC count, MCH, MCV, MCHC and RET%. Upper and lower thresholds were generated for [Hb], Off-hr, RET%, and the ABPS (3). These thresholds are initially based on population-derived data, and adapt subsequently to an individuals’ previous samples based on a Bayesian algorithm (3). All baseline samples, i.e. two (n=23, males=12, females=11) or eight (n=24, males=12, females=12) were entered in the ABP software to individualize the calculated thresholds.
together with one specific time point (e.g. six hours after reinfusion). If the hematological data for the specific time point exceeded the calculated lower or upper intra-individual thresholds corresponding to a specificity of 99% (i.e. accepting 1:100 false positive outcomes) or exceeded a probability of 99 % for an atypical longitudinal profile, this time point was categorized as an outlier.

If a subject exceeded the thresholds for more than one ABP biomarker at a specific time point (e.g. both RET% and [Hb] six hours after reinfusion), this would only count as one outlier for the specific subject. The sensitivity and specificity for [Hb], Off-hr, RET% and ABPS were investigated independently and in combination to determine whether the biomarkers have an additive effect, as some individuals may cross the thresholds for [Hb] while others cross for Off-hr. Specificity was calculated as the percent of samples not exceeding one or more of the calculated thresholds in the baseline samples collected from all subjects as well as all samples collected from participants in the CON group. Sensitivity was calculated for each time point as the percent of samples from the BT group exceeding the calculated thresholds for the passport biomarkers.

**Evaluation of exercise performance**

Blinded personnel performed all exercise tests of the BT and CON subgroups. All exercise tests began with a standardized warm-up (males: 5 min @ 90 W and 150 W, females: 5 min @ 75 W and 125 W) on a cycle ergometer. A period of 3-5 min separated the warm-up and the test. To evaluate whether the blinding procedure was successful, subjects indicated whether they believed to be in the BT or the CON group after the final performance test.

**Maximal aerobic exercise capacity**

Peak aerobic power ($W_{\text{peak}}$) and $\dot{V}O_2\text{peak}$ were determined on an electronically-braked bicycle (Monark 839E, Varberg, Sweden) before initiation of the baseline period. The highest 30 s average $\dot{V}O_2\text{peak}$ was determined from expired breath-by-breath O$_2$ and CO$_2$ fractions and ventilation (Quark
b2, Cosmed, Rome, Italy) during exhaustive incremental cycling (starting at 150 W for males, 125 W for females followed by increments of 25 W × min⁻¹). \( W_{\text{peak}} \) was calculated as \( W_{\text{compl}} + 25 \times (t/60) \) (\( W_{\text{compl}} \), last completed workload [W]; \( t \), time at last workload before exhaustion (s)).

**Time-trial performance**

Endurance performance was determined in a 60 min preloaded 650 kcal time-trial on an electronically-braked bicycle (Monark 839E, Varberg, Sweden) using commercially available software (Monark Testing Software version 1.0.15.0, Monark, Varberg, Sweden). The preload was performed at 60% of the intensity eliciting \( \dot{V}O_2\text{peak} \). Carbohydrate drinks were provided during the preload as previously described (29). Next, the time-trial was initiated by applying a random load of 15 to 20 Newtons (N) by the blinded personnel. The subjects freely selected pedaling cadence, and were able to continuously adjust the workload through verbal communication with test personnel in steps of 1 N. Once the subject reached 650 kcal, the test was terminated. Capillary blood samples were collected prior, halfway and at the end of the preload and halfway and at the end of the time trial. The primary outcome measures were mean power output (MPO) and time to completion.

The subgroups were familiarized once with a preloaded time-trial protocol prior to phlebotomy. The preloaded time-trial was repeated 2-3 days before reinfusion, i.e. 25-26 days after phlebotomy, as well as 24h and 6 days after reinfusion (Fig. 1). The subjects were blinded to the completed work, elapsed time and heart rate but received verbal feedback on the total energy production at every 50 kcal completed, at every 10 kcal completed for the final 100 kcal, and at each 1 kcal during the final 20 kcal. Furthermore, the subjects were verbally encouraged during the trial.
Statistics

SPSS were used for statistical analyses (IBM SPSS Statistics, version 26.0.0) with the level of significance set at P<0.05. GraphPad Prism version 9.0.0 (San Diego, USA) was used for figure preparation. Hepcidin, ERFE, and ferritin data were log-transformed before analysis to obtain a normal distribution and variance homogeneity and presented as back-transformed values. The data are presented as pointwise means [lower limit; upper limit] with lower and upper limit being the limits in 95% confidence interval unless otherwise stated. Differences in anthropometric characteristics were evaluated using an unpaired t-test. A linear mixed model for repeated measures (30) was used to assess the effect on hematological and iron variables with fixed effects of time, treatment (BT vs CON), sex (male vs. female), and their interactions. Participant number was used to identify repeated measures and to define a random factor. The effect of blood reinfusion on performance measures was analyzed with an identical approach, but without sex and interactions with sex as fixed effects, since only three females participated in this part of the study. To investigate whether biomarkers of the Athlete Biological Passport differed in sensitivity, a generalized linear mixed model with time, sex and biomarker ([Hb], Off-hr, RET% or ABPS) as fixed factors, and subject and subject × time as random factors was used for the binary variable (‘1’ and ‘0’ indicating surpassed threshold or not, respectively), thus indicating whether biomarkers of the Athlete Biological Passport surpassed the threshold or not. Subsequently, time × marker was added as fixed factor to determine if any temporal effects were evident. Significant interaction effects were followed by a Sidak-adjusted pairwise comparison.

To systematically determine the sensitivity of hepcidin and ERFE as potential biomarkers, the individual relative changes were analyzed in comparison with a pooled baseline, i.e. the mean of the two or eight baseline samples. Differences from the mean baseline to all time points was calculated for each individual in both groups, and the mean ± 2.58 SD (i.e. >99% specificity) of the changes
were calculated for the CON group and for the baseline period in the BT group, thus allowing for natural fluctuations.

The CV of the time-trial test was calculated by dividing the SD of the differences in the CON group between pre-reinfusion and the post-reinfusion results ($\sigma$) both 24 hours and 6 days after the reinfusion, respectively, by the grand mean ($\bar{X}$) and dividing the quotient by $\sqrt{2}$: $CV = (\sigma / \bar{X}) / \sqrt{2}$.

The effectiveness of blinding in each treatment group was evaluated by the Bang blinding index (BI) (31). The Bang BI ranges from -1 to 1, where -1 indicates that all subjects guessed the incorrect treatment, 0 indicates that all subjects randomly guessed, and 1 indicates that all subjects guessed the correct treatment. The treatment blinding was interpreted as being maintained if CI included the null value.
RESULTS

Iron biomarkers (Figure 2)

Two subjects had continuous ERFE levels below the detection limit and were excluded from the analysis. During baseline, hepcidin and ERFE did not change significantly between groups or sex. There was a time × treatment effect (P<0.05) for both biomarkers. Hepcidin was attenuated (P<0.05) at all time points following donation, whereas ERFE was increased (P<0.05) only 7 and 14 days after donation (Fig. 2) compared to CON. Within the BT group, hepcidin increased 110% [26; 212] and 89% [12; 191] (P<0.05) 6 and 24 hours after reinfusion compared to immediately before reinfusion, while the numerical decrease of ERFE by 40% [-83; 109] six hours after reinfusion did not reach significance. Figure 3 summarizes ferritin, iron, transferrin and TSAT in both groups for male and females.

Following baseline, the relative changes in hepcidin and ERFE levels across all time points in the BT group caused six (30%) and 11 (61%) of the participants to exceed the 99% threshold. However, the thresholds were also exceeded five times in four participants for hepcidin and seven times by six individuals for ERFE during baseline or in the CON group (Fig. 2), providing a specificity of 98.6% and 97.6%, respectively, across all time points.

Sex-specific differences

No sex-specific differences were found for hepcidin, ERFE, TSAT or iron, whereas a time × treatment × sex interaction effect existed for ferritin (P<0.05). Specifically, ferritin was lower (P<0.05) in females compared to males in both groups at all time points.

Hematology (Figure 4 and supplementary table 1)

A time × treatment interaction existed for [Hb], RBC count, Hct, RET%, and Off-hr (all P<0.001) as well as MCV (P<0.01), whereas no significant interactions were found for MCH and MCHC (Fig. 4
The donation decreased (P<0.05) [Hb] compared to CON at all post-donation time points in males, whereas the [Hb] in females was similar to baseline values and CON two weeks after donation. Within the BT group, the [Hb] increased (P<0.001) by 3.8% [2.6; 4.9] three hours after reinfusion compared to immediately before reinfusion. The increase was sustained up to six days after reinfusion, where [Hb] was 4.0% [2.4; 5.7] higher (P<0.001). A peak increase for RET% of ~61 and 49% (P<0.001) was found for females and males 7-14 days after donation, respectively. Following reinfusion, RET% was attenuated (P<0.05) in females but not males six days after when compared to CON. Following donation, Off-hr reached its nadir seven days after donation (P<0.001) when comparing groups. After reinfusion, an increase (P<0.05) of ~14% in Off-hr within the BT group was found when compared to immediately before reinfusion. This increase (P<0.05) was sustained at the subsequent time points.

Sex-specific differences

In general, [Hb] was ~12% higher (P<0.05) in males compared to females but the time × treatment × sex interaction was insignificant. While no difference was found in [Hb] between BT and CON following reinfusion in males, [Hb] increased (P<0.05) in females three hours after reinfusion by 4.4% [2.5; 6.3] compared to CON. The increase (P<0.05) was maintained for six days (Fig. 4).

Detection by the Athlete Biological Passport (Figure 5 and Table 2)

A main effect of ABP biomarker sensitivity existed (P<0.001). Specifically, Off-hr was more sensitive than [Hb] (P<0.05) and ABPS (P<0.05) but not RET%. When investigating if any temporal effects were evident, no interaction effect of time × biomarker was present for the sensitivity of the ABP biomarkers.

The sensitivity for each biomarker and their combinations is presented in Figure 5. Briefly, the Off-hr peak sensitivity was 58% (14/24 subjects) seven days after donation, whereas peak sensitivity for
[Hb], RET% and ABPS was 17% (4/24 subjects), 38% (9/24 subjects) and 13% (3/24 subjects), respectively.

After reinfusion, the most sensitive biomarker was ABPS at 8% (2/24 subjects) three days after reinfusion. The other markers sensitivity ranged from 0-4% (0-1/24 subjects).

Across any time point, the passport identified nine (38%), 15 (63%), 11 (46%) and five (21%) of the 24 participants in the BT group with an outlier for [Hb], Off-hr, RET% and ABPS, respectively.

When combining [Hb] and Off-hr, the passport identified 15 unique participants (63%), whereas 21 unique participants (88%) was identified with an outlier across any time point when combining all markers. When only analyzing time points after blood reinfusion using both the single biomarkers and their combinations, seven participants (~29%) were identified with an outlier (Fig. 5). A specificity <99% was evident when including ABPS for both male and females and for females alone (Table 2).

**Sex-specific differences**

No differences in sensitivity were found between males and females, but numerically differences were evident (Fig. 5). After reinfusion, four of the six participants with an outlier were females.

**Time-trial performance (supplementary table 2 and 3)**

The CV% for MPO was 0.8% and 1.4% for the time-trial 24 h and 6 days after reinfusion, respectively, when compared to before reinfusion and 2.3 and 2.5%, respectively, when compared to before donation. The Bang BI was 0.14 [-0.43; 0.71] for the BT group and 0.00 [-0.58; 0.58] for the CON group, demonstrating that the blinding was maintained. Temperature (23±1ºC) and humidity (29±9%) were not different between trials. Performance and blood metabolites are summarized in supplementary table 2 and 3. A time × treatment interaction for both MPO (P<0.01) and time to completion (P<0.01) was found. The BT group improved MPO 6.4% [3.8; 9.0] (P<0.01) and 5.6%
[3.3; 7.9] (P<0.01) as well as time to completion by 5.8% [2.8; 8.8] (P<0.01) and 4.9% [2.5; 7.3] (P<0.05) 24 hours and six days after reinfusion, respectively, compared to before reinfusion. No changes were found in the CON group or when comparing performance before donation and before reinfusion in either group.
DISCUSSION

Our main finding was that a small-volume ABT of ~130 mL packed RBCs did not induce changes in systemic levels of hepcidin and ERFE in a group of males and females. However, both hormones showed potential as individual biomarkers for identifying a small-volume ABT as they enabled identification of 30% (hepcidin) and 61% (ERFE), respectively, of the transfused participants at a 98% (hepcidin) and 99% (ERFE) specificity. In comparison, the benchmark ABP had a sensitivity of 29% across all time points following reinfusion at 99% specificity when combining all the ABP biomarkers. No sex-specific differences in sensitivity were found. When combining the findings for hepcidin, ERFE and the ABP (i.e. flagged by at least one biomarker), a maximal sensitivity of 83% (20/24 subjects) across all time points following reinfusion was found. Finally, we verified that reinfusion of 130 mL RBC is performance enhancing. Our results demonstrate the continuing need for studies to explore potential biomarkers or detection matrices aiming at ABT detection.

Iron biomarkers

This is the first study investigating the effect of a small-volume blood transfusion on the iron regulatory hormones ERFE and hepcidin. We demonstrated that the reinfusion of ~130 mL packed RBCs does not induce fluctuations in either hormone that is different from a control group. This was despite a clear interaction effect, indicating that this is driven by the donation-induced changes. Within the BT group, reinfusion increased hepcidin numerically by ~50% six hours later. In contrast, hepcidin increased 7-fold 12 h after reinfusion of ~280 mL RBC with the increase being sustained for 24 hours (14). Similarly, a clear increase in ERFE was evident after the 450 mL blood donation as observed previously (16, 17). Accordingly, the small reinfusion volume may explain the unaltered hepcidin and ERFE levels. In addition, differences in quantification methods for hepcidin (Intrinsic LifeScience vs. LC-HRMS) (14) and ERFE exist, with the latter providing different detection limits.
(16, 17). However, even small erythropoietic changes can affect ERFE (12, 18, 32), why other mechanisms also may contribute.

An inherent challenge of biomarker discovery is natural variations. We observed marked inter- and intra-individual differences for hepcidin and ERFE, corresponding with observations of up to 10-fold natural occurring variations in ERFE (33). Oral iron supplementation may affect hepcidin (9), but as an intravenous iron injection does not change ERFE levels (17), iron supplementation appears unlikely to affect ERFE in the present study. Another influential factor for both hormones is exercise (11, 12), which may complicate the interpretation of fluctuations in elite athletes. The present study collected blood samples >2 hours after any physical exercise followed by 10 min of seating to minimize the potential influence (34). Previous results suggest even higher variation of hepcidin in iron-replete females (range: 8.2-199.7 ng/mL) (35) than observed in the present study and it appears unlike that further precautions can be taken in a real-world scenario.

Nevertheless, in an individual approach accounting for baseline variation, hepcidin and ERFE had a sensitivity of 30 and 61%, respectively (Fig. 2E-H) although with a specificity <99%. To achieve >99% specificity, thresholds calculated as the mean ± 3.1 and 3.2 SD for ERFE and hepcidin, respectively, was necessary. Only 1/6 and 1/11 transfused subjects identified by hepcidin and ERFE, respectively, were also identified by the ABP, indicating that both hormones provide additional value to existing methods. Indeed, combining hepcidin, ERFE and the ABP yielded a sensitivity across all time points of 83% (20/24 subjects) following reinfusion compared to 29% (7/24) for the ABP. Using an identical approach, we recently found a sensitivity of 30 and 40% in rHuEPO-treated subjects for hepcidin and ERFE, respectively (32). Finally, there was no apparent association between the numbers of baseline samples (two vs. eight) on the subsequent sensitivity for ERFE, where 6/11 flagged subjects had a long baseline period. For hepcidin, 5/6 flagged subjects had a long baseline period.
We did not find any changes in ferritin or transferrin in contrast to other studies (36, 37), which likely relates to the 2-4 fold larger reinfusion volumes applied previously (14, 36, 37). Additionally, ferritin is only expected to decrease in ~15% of iron-replete individuals donating ~450 mL whole blood (38). Thus, the present data indicates that in young healthy and iron supplemented individuals, ferritin and transferrin are not sensitive biomarkers for a small-volume blood transfusion. Interestingly, iron and TSAT levels increased in both males and females three and six hours after reinfusion (Fig. 3). Using the individual approach, iron had a sensitivity of 17% with a 99% specificity (data not shown), which is inferior to the 93% sensitivity obtained up to one day after reinfusion of 280 mL packed RBCs (39). The difference may be explained by difference in volume but especially also differences in iron the levels of EDTA and lithium-heparin plasma (14, 39). Other promising methods to aid in the detection of ABT may include microparticles of blood aging (40), extracellular vesicles generated by the RBCs during storage (41) or urine metabolomics (42).

**Effect on hematological markers**

Although a time × treatment effect existed for [Hb], no specific time points were different between groups in the post hoc analysis. However, applying the simplest possible comparison, i.e. an unpaired t-test, on changes from immediately before to three hours after reinfusion, a higher (P<0.001) [Hb] of 0.5 g/dL in the BT group compared to CON was found, which is in line with a 4% increase within the BT group and previous reports (8). Importantly, the increase was sustained for up to 6 days. We observed a lower RET% in females in the BT group compared to CON six days after reinfusion, with no differences in males (Fig. 4D). This may partially be due to the reticulocytes’ maturation process (43) as other studies observe a decreased RET% 6-15 days after reinfusion with the nadir occurring at day nine (14). Thus, it is likely that the follow-up period of 6 days did not allow us to observe the full response in RET% in males. In addition, the observed differences may also be explained by a potentially superior recovery of hemoglobin mass in females following donation, indicated by the
slightly higher numeric increase in RET% as well as restored [Hb] levels, thus providing a more pronounced negative feedback loop upon reinfusion.

**Athlete Biological Passport**

This is the first study investigating the sensitivity and specificity of the ABP using a likely real-world small-volume scenario. The present study demonstrates that the ABP can identify ~29% (7/24 subjects) of the subjects reinfused with ~130 mL packed RBCs across all time points with a peak sensitivity of ~13% three days after reinfusion, whereas ~58% (14/24 subjects) were identified by the ABP following a 450 mL whole blood donation. The duration of the baseline (two vs. eight samples) did not influence sensitivity.

The Off-hr was more sensitive than [Hb] and ABPS but not RET%, although a specific time point for the difference was not identified (Fig. 3). Off-hr was designed to be sensitive in periods with a high [Hb] and low RET% or vice versa (2). Indeed, the overall highest sensitivity (58%; 14/24 subjects) for Off-hr was obtained one week after donation, where the largest de- and increase in [Hb] and RET%, respectively, was evident. However, after reinfusion the Off-hr sensitivity ranged between 0-4% (0-1/24 subjects), as only minor in- and decreases of [Hb] and RET% occurred. Reinfusion of 450 and 1,350 mL provide an Off-hr sensitivity of 9% and 32%, respectively (5), which is likely the result of a larger acute increase in [Hb] due to higher hemoglobin mass reinfusion (~23g vs ~47-134g).

Interestingly, combining RET% with [Hb] and Off-hr did not improve the ABP sensitivity, which contrast to our findings that inclusion of RET% improve detection of rHuEPO misuse (44, 45). However, combining all four passport variables elicited the highest sensitivity, where ABPS was the most sensitive after reinfusion at 8% (2/24). To our knowledge, only two studies have investigated ABPS as a sensitive biomarker for blood manipulation (45, 46). Including ABPS improved detection
of 900 IU rHuEPO treatment when compared to [Hb] and Off-hr (46) and a case-study reported that
only the ABPS approached the ABP calculated thresholds in an athlete misusing a HIF stabilizer FG-
4592 though not exceeding them (47). Despite being the most sensitive biomarker yielding a modest
maximal sensitivity, ABPS also had a concurrent reduced specificity (Table 2). When included alone
or in combination with [Hb], RET% and Off-hr, the calculated specificity was below the 99% required
by WADA (28), why additional research into the utility of the ABPS is warranted.

Sex-specific changes

Contrary to our hypothesis, the ABP sensitivity did not differ between sexes. It is well-known that
men have higher Hct, [Hb], and blood volume than women (43, 48). Accordingly, donation of 450
mL whole blood constitutes a 2-3 % larger acute reduction in blood volume in females compared to
males (49, 50). Despite, the larger relative decrease in blood volume in females the ABP biomarkers
does not appear affected in a sex-specific way. Likewise, the menstrual cycle in females with and
without hormonal contraception does not appear to affect interpretations of the ABP (23, 24).

Endurance performance

As hypothesized, we found an increased time trial performance of ~5-6% 24 hours and six days after
reinfusion (supplemental table 2), which is in accordance with our previous results using a similar
reinfusion volume (8). One proposed mechanism responsible for the increase in performance
following the reinfusion is the acute magnitude of change in [Hb] and an increased arterial-venous
O₂ difference, as blood volume and maximal cardiac output appear to remain constant after ABT (1).
Assuming a cardiac output of 24 L/min, a stroke volume of 150 mL based on findings from a similar
cohort (52), a partial pressure of O₂ of 100 mmHg, an oxygen saturation of 97%, an unchanged mixed
venous O₂ content (1), and a mean heart rate as observed during the time trial of 165 bpm
(supplemental table 2), the observed acute mean increase in [Hb] of 0.5 g/dL would theoretically
augment V̇O₂ by ~174 mL O₂ corresponding to ~14 W (53). This aligns with the present study, where
an absolute increase in performance of 14 W was found. However, it should be noted that only ~15
30% of the performance variation was explained by changes in [Hb] when interpreting individual
data (supplemental figure 1), which may be due to the low subject number (n=7 in the BT group) or
fluctuations of other determining variables. Indeed, oxidative phosphorylation capacity of the vastus
lateralis, submaximal blood lactate concentrations, and maximal leg oxygenation account for 78% of
the variation in a time trial performance (54). Finally, it should be noted that the long-term
fluctuations in [Hb] does not appear to determine performance fluctuations. For instance, [Hb] was
not fully recovered four weeks following donation in the present study, yet the aerobic performance
was restored in both males and females. Surprisingly, only 14 days is reported to be sufficient to
recover aerobic performance following a 450 mL blood donation (51). The mechanism responsible
for the rapid aerobic performance recovery remain unknown.

Limitations

One limitation is the frequent blood sample collection, which provides an atypical high temporal
resolution when compared to a real-life testing regime and potentially improved the detection
possibilities in the present study. Another limitation is that the reinfusion occurred 28 days after
donation, although full recovery of total hemoglobin mass following a blood donation takes ~36 days
(55). Hence, the reinfusion was likely a combination of replacing the lost cells and adding more..
Furthermore, we did not monitor exercise training or menstrual cycle which may affect the ABP
results (56, 57), although recent evidence suggests otherwise concerning menstruation (23, 24). In
addition, we did not control for inflammatory markers or standardized the collection time of the day,
which can influence hepcidin concentrations (10). As the majority of samples were collected between
8 AM and 12 noon, where circadian fluctuations are low (10), diurnal variation is likely negligible.
The comparison of the adaptive model of the ABP with fixed hepcidin and ERFE cutoffs may have
inherent limitations as the latter preclude thresholds to adapt over time. However, with additional data
from future studies, it may be feasible to develop a similar adaptive model for hepcidin and ERFE.
CONCLUSION AND PERSPECTIVES

We demonstrate that the ABP is able to detect ~60% of all blood withdrawals with >99% specificity, whereas a performance enhancing, small-volume reinfusion of ~130 mL RBCs is detected in ~29% of the BT participants, across all time points. Hepcidin and ERFE flagged 30% and 61% across all time points with a specificity of ~98 and 99%, respectively. Combing the ABP and iron markers increased the sensitivity following reinfusion to 83%. Thus, hepcidin and ERFE may be valuable additions for detection of ABT.

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CONFLICT OF INTEREST

ABA and TCB were funded by WADA, and JB by Partnership for Clean Competition. ABA, JB, TCB, HS, HS, GJ, NHS, PIJ and NBN declare no competing financial interests. TG and EN are shareholders of and scientific advisors for Intrinsic LifeSciences and Silarus Therapeutics and consultants for Ionis Pharmaceuticals, Protagonist, Vifor and Disc Medicine. TG is a consultant for Akebia. All authors approved the final manuscript. The results of the present study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation and do not constitute endorsement by ACSM.
FIGURE LEGENDS

Figure 1. Timeline illustrating the procedure of the study. After randomization to either control (CON) group or the autologous blood transfusion (BT) group subjects underwent two or eight weeks of baseline testing followed by a donation of 450 mL whole blood. Phlebotomized blood was stored as packed red blood cells before transfusion of ~130 mL four weeks after donation. Blood samples were collected as described in the “Design” paragraph. Cycle icons depicts the performance tests for the subgroup.

Figure 2. Changes with 95% confidence intervals for hepcidin and erythroferrone (ERFE) in males (panel A and C) and females (panel B and D) as well as individual, relative changes in hepcidin (panel E and F) and ERFE (panel G and H). B1-B8 indicate baseline samples (n=12 for B1-6, n=20 for B7-8). 7D, 14D, and 28D indicate days after donation (illustrated by the grey area in panel A-D; n=20), and 6H, 24H, 3D, and 6D indicate hours (H) or days (D) after blood reinfusion (n=20). Filled squares and circles represents males and females in the blood transfusion (BT) group, respectively, and unfilled squares and circles represents males and females in the control (CON) group, respectively. In panel E-F, the 99% and 99.9% specificity limits are denoted with grey and black dashed lines, respectively. Statistically significant differences: asterisks (*) above the dotted line indicate statistical time × treatment effect. *, **, *** P<0.05, 0.01 and 0.001, respectively, for comparison between BT and CON. Within the BT group: # and ##, P<0.05 and 0.01, respectively, when compared to 7D. § and §§, P<0.05 and 0.01, respectively, when compared to 14D. †, P<0.05, when compared to 28D.

Figure 3. Changes with 95% confidence intervals for iron, ferritin, transferrin, and transferrin saturation (TSAT) in the control (CON) and blood transfusion (BT) group. B1-B8 indicate baseline samples (n=12 for B1-6, n=24 for B7-8). 7D, 14D, and 28D indicate days after donation (illustrated by the grey area in panel A-D; n=24), and 6H, 24H, 3D, and 6D indicate hours (H) or days (D) after blood reinfusion (n=24). Filled squares and circles represents males and females in the BT group,
respectively. Unfilled squares and circles represents males and females in the CON group, respectively. Statistically significant differences: asterisks (*) above the dotted line indicate statistical time × treatment effect: ** P<0.01 for comparison between BT and CON. ♂♂ P<0.01 indicate difference between males and females at all time points.

Figure 4. Changes with 95% confidence intervals in hemoglobin concentration ([Hb]), reticulocyte percentage (RET%) and Off-hr in males (panel A, C, E) and females (panel B, D, F). B1-B8 indicate baseline samples (n=12 for B1-6, n=24 for B7-8), 7D, 14D, and 28D indicate days after donation illustrated by the grey area (n=24), and 6H, 24H, 3D, and 6D indicate hours (H) or days (D) after blood reinfusion (n=24). Filled squares and circles represents males and females in the blood transfusion (BT) group, respectively. Unfilled squares and circles represents males and females in the control (CON) group, respectively. Statistically significant differences: asterisks (*) above the dotted line indicate statistical time × treatment effect: *, **, *** P<0.05, 0.01 and 0.001, respectively, for comparison between BT and CON. Within the BT group: ### and #### P<0.01 and P<0.001, respectively, when compared to 3D. ###, P<0.001 when compared to 7D. §§§, P<0.001 when compared to 14D. ‡, ‡‡, and ‡‡‡, P<0.05, 0.01 and 0.001, respectively, when compared to 21D. †† and †††, P<0.01 and 0.001, respectively, when compared to 28D.

Figure 5. Obtained count of outliers in the Athlete Biological Passport for hemoglobin concentration ([Hb]), Off-hr, reticulocyte percentage (RET%) and Abnormal Blood Profile Score (ABPS) in the complete BT group (panel A), for males alone (panel C) and females alone (panel E). In addition, the count of outliers for [Hb] and Off-hr combined, [Hb], Off-hr and RET% combined and [Hb], Off-hr, RET% and ABPS combined, i.e. how many participants exceeded at least one biomarker threshold, is presented for the complete BT group (panel B), for males alone (panel D) and females alone (panel F). The dotted line indicate the number of participants included, and the vertical solid bar blood reinfusion.


Supplemental Data.docx
FIGURES

Fig. 1
Fig. 5
Table 1. Mean ± SD values for pulmonary data and exercise capacity. BT: blood transfusion group, CON: control group, $\dot{V}O_{2\text{peak}}$: peak oxygen uptake, rel. $\dot{V}O_{2\text{peak}}$: peak oxygen uptake relative to bodyweight, HR$_{\text{max}}$: maximal heart rate, BPM: beats per minute, $W_{\text{max}}$: maximal workload.

<table>
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<th>Parameter</th>
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<th>Male (n=24)</th>
<th>Female (n=24)</th>
<th>BT (n=24)</th>
<th>Male (n=12)</th>
<th>Female (n=12)</th>
<th>CON (n=24)</th>
<th>Male (n=12)</th>
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<td>Height (cm)</td>
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<td>Weight (kg)</td>
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<td>$\dot{V}O_{2\text{peak}}$ (mL)</td>
<td>3841 ± 752</td>
<td>4432 ± 493</td>
<td>3249 ± 427</td>
<td>3928 ± 718</td>
<td>4504 ± 477</td>
<td>3391 ± 419</td>
<td>3734 ± 785</td>
<td>4361 ± 519</td>
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<td>Rel. $\dot{V}O_{2\text{peak}}$ (mL×kg$^{-1}$×min$^{-1}$)</td>
<td>56 ± 7</td>
<td>60 ± 5</td>
<td>52 ± 5</td>
<td>56 ± 6</td>
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<td>52 ± 6</td>
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<td>HR$_{\text{max}}$ (BPM)</td>
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<td>$W_{\text{max}}$ (watt)</td>
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<td>267 ± 32</td>
<td>317 ± 50</td>
<td>346 ± 30</td>
<td>269 ± 32</td>
<td>295 ± 58</td>
<td>324 ± 56</td>
<td>246 ± 29</td>
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Obtained specificity for hemoglobin concentration ([Hb]), Off-hr score, reticulocyte percentage (RET%) and Abnormal Blood Profile Score (ABPS) as well as [Hb] and Off-hr score combined, [Hb], Off-hr score and RET% combined and [Hb], Off-hr score, RET% and ABPS combined (i.e. how many false-positive existed for at least one biomarker threshold). Specificity was calculated as the percent of samples not exceeding one or more of the calculated thresholds values in the baseline samples collected from all subjects (n=47) as well as all samples collected from participants in the CON group divided into male (n=12) and females (n=11). Reduced specificity, i.e. <99%, is denoted with *.

<table>
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<td>Male participants (n=12) - 132 samples</td>
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