Evaluation of serum markers for improved detection of autologous blood transfusions

Autologous blood transfusion is used by athletes to enhance performance by increasing oxygen transport to muscles. It is the only form of blood doping that is currently not detectable using a direct method. Autologous blood transfusions are detected by an indirect method which monitors the longitudinal profile of hemoglobin concentration (Hb), reticulocyte percent (Ret%), and the Off-score, defined as Hb -60√Ret%.[1] Following blood transfusion, Hb is increased and is followed by a long-term decrease in Ret%, caused by negative feedback inhibition of erythropoiesis. This indirect method allows detection of blood transfusion for up to two weeks and can detect all forms of blood doping, including abuse of recombinant erythropoietin (rhEPO), erythropoiesis stimulating agents (ESAs), and allogeneic transfusions.[2-4] Unfortunately, the success of this method has caused athletes to adopt more complex blood doping regimens to evade detection.[5] Additionally, the current method is not fully able to distinguish between blood doping and training at altitude or with simulated hypoxia, which is not a banned practice.[6] The study herein was therefore designed to identify new serum markers that may improve detection of banned blood doping practices. The primary outcome of the study was defined as a significant change in hepcidin, ferritin, soluble transferrin receptor (sTFR) or serum iron. (*clinicaltrials.gov Identifier: 02684747*).

The measurement of serum markers of erythropoiesis and iron homeostasis may improve detection.[7] Following blood transfusion, older red blood cells are absorbed by macrophages where the hemoglobin iron is recycled and released into the circulation. Thus, increases in serum iron, hepcidin, and ferritin have been observed after blood transfusion.[8,9] However, the observed increases in these markers was dependent upon the blood storage time. Blood stored for less than 5-6 weeks showed little to no increase in serum iron, hepcidin, and ferritin.[8] sTFR was previously used for anti-doping detection of rhEPO abuse and has demonstrated significant increases upon blood withdrawal.[1,2] Conversely, decreases in sTFR concentrations should follow the negative feedback inhibition of erythropoiesis which occurs after blood transfusion. Thus, the use of these serum markers for detection of autologous blood transfusion warrants further analysis.

In the study herein, a randomized, single-blind, placebo concurrent, autologous transfusion clinical trial was performed. Serum markers were measured after transfusion of blood or saline control in 34 healthy volunteers, 20 males and 14 females, between the ages of 18 – 40 years. Seventeen participants, 10 males and 7 females, were included in each group (blood or saline). After blood withdrawal, 6 females became anemic (Hb < 12 g/dL) and/or iron deficient (ferritin < 25 ng/ml) and were not included in the analysis. A flow diagram following the participants throughout the study is shown in the *Online Supplementary Figure S1*. The study was performed as previously described;[10] in summary, three baseline values were collected prior to blood withdrawal followed by a 21-day recovery and blood storage time. Blood, one unit, was leukodepleted and stored as approximately 250 ml of packed red cells at 4°C for 21 days. Transfusion of only one unit of blood was tested, as this is more difficult to detect using the current indirect method. A further description of the methods is provided in the *Online Supplementary Material.*

Figure 1 displays the change in hepcidin, serum iron, ferritin and sTFR concentrations from baseline after blood or saline transfusion. After blood transfusion, hepcidin concentrations increased significantly relative to saline on day 1 (P=0.006), followed by further increases on day 13 (P=0.028) and day 20 (P=0.010) before returning to pre-transfusion levels. Hepcidin concentrations in the saline group remained at pre-transfusion levels, and did not recover for the duration of the study, at five weeks post-transfusion. There was no significant change in serum iron concentrations (Figure 1B). Ferritin concentrations increased after blood transfusion and peaked at day 20 (Figure 1C). However, the change was not significantly different from saline transfusion. sTFR concentration decreased after day 2 and was significantly different from saline on day 6 (P=0.024), day 20 (P=0.003), day 27 (P=0.0003) and day 34 (P=0.007), (Figure 1D). After saline transfusion, sTFR concentration did not recover from blood withdrawal for the duration of the study. The concentrations and post-transfusion response of each serum marker and Hb, Ret%, OFF-score, transferrin saturation (TSAT) and interleukin-6 (IL-6) are listed in the *Online Supplementary Table S1* (blood group) and *Online Supplementary Table S2* (saline group). The observed changes in Hb, Ret% and OFF-score are consistent with previous studies.[2,9] No statistically significant changes were observed in IL-6 concentrations, suggesting that increases in hepcidin were not due to inflammation (*Online Supplementary Table S1* and *Table S2*).

Previous studies have reported the within day/within subject variation of hepcidin is 30.3% and the within subject variation over five days is between 26.8% – 66.2%.[11,12] Thus, it may be difficult to set appropriate individual threshold limits to detect changes in hepcidin due to blood doping. To further test this limitation, individual threshold limits were set to three times the standard deviation of the individual baseline measurements (n=3) and compared to the post-transfusion response. After blood transfusion, 6 out of 10 males and 2 out of 4 females would exceed an individual threshold on at least one or more days post-transfusion (Table 1). After saline transfusion, 3 out of 10 males and no females would exceed an individual threshold limit, which may reflect inadequate estimation of the baseline average and standard deviation. While the response of Hb, Ret%, and OFF-score is consistent with previous studies, the response of hepcidin and serum iron differ significantly from a recent study. In the study by Leuenberger *et al.*, hepcidin was significantly elevated from 12 hours to 1-day, post-transfusion, with increases of 7-fold and 4-fold, respectively, and serum iron was significantly elevated from 3 hours to 1-day post-transfusion.[9] In the current study, only a small increase in hepcidin and no increase in serum iron were observed on day 1 post-transfusion. It is possible that a larger response may have been detected if shorter time points from 3-12 hours post-transfusion were tested. However, the major cause of incongruence between the two studies may be the difference in blood storage times. In the current study, blood was stored for 21 days, while in the Leuenberger *et al.* study, blood was stored for 36 days. Previous studies have demonstrated that increases in serum iron and hepcidin are observed after transfusion of blood that was stored for 5-6 weeks, but not observed after transfusion of blood stored for shorter time periods.[8,13] Transfusion of older blood results in lower post-transfusion recovery of red blood cells and may be associated with increased health risks.[14] Due to the potential risks, several healthcare systems have limit-

Table 1. Number of participants exceeding an individual hepcidin threshold level.

	D(-1)	D1	D2	D3	D4	D5	D6	D13	D20	D27	D34
Blood	3M	3M	1M	1M	3M	4M	2M	4M	3M	3M	3M
		1F	1F	1F	1F		1F	2F	1F		1F
Saline	1M										2M

Individual threshold level was calculated as the individual mean plus 3 standard deviations after blood or saline transfusion, n =14 for each group. D(-1) represents pre-transfusion. M: males; F: females.

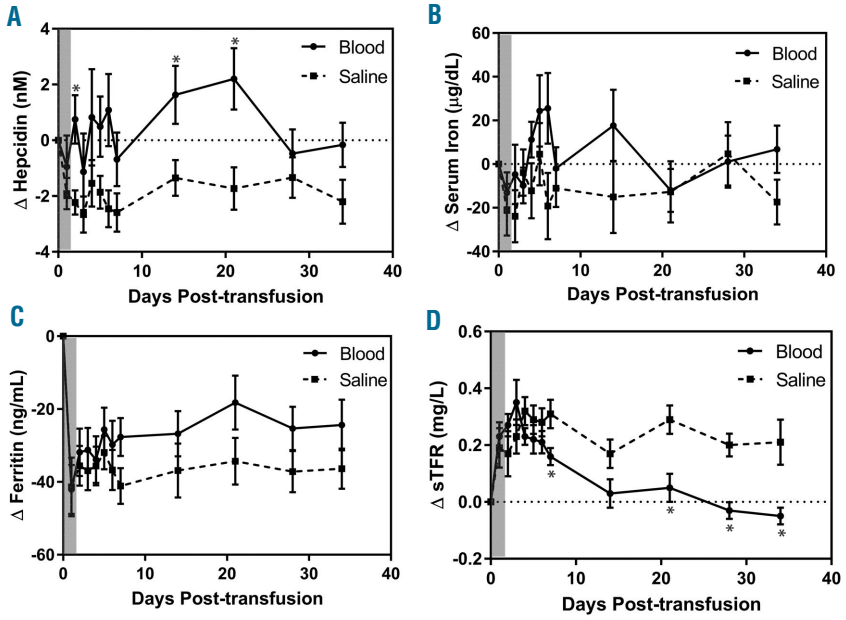

Figure 1. Response to blood transfusion. Change in A) hepcidin, B) serum iron, C) ferritin, and D) sTFR from baseline, n=14 for each group. Shaded area at Day -1 represents the pre-transfusion timepoint. Error bars are SEM. Statistical significance was determined using an unpaired, two-tailed, *t*-test to compare blood to saline values and * indicates a significant difference from saline, $P \leq 0.05$. sTFR: soluble transferrin receptor.

ed the blood storage time to less than is required.[14] Within the context of anti-doping, it is not known how long cheating athletes store blood prior to transfusion, but it likely varies significantly.

Both the Leuenberger *et al*. study and the present study demonstrate significant changes in hepcidin after blood transfusion. In the current study, the largest increases in hepcidin concentration were observed between days 13-20 post-transfusion, which coincided with a peak in ferritin concentration and a decrease in markers of erythropoiesis, sTFR and Ret%. A similar response was reported previously in patients with β-thalassemia major, where an increase in hepcidin was observed at 4-8 days post-transfusion which correlated with increased ferritin and decreased erythropoiesis.[15] Likewise, the Leuenberger *et al*. study reported an increase in ferritin and a decrease in Ret% on days 3-15 post-transfusion. This change corresponded to an approximately 1 nM increase (2-fold) in hepcidin from days 3-15, although it was not statistically significant when comparing absolute values of hepcidin which have large inter-individual variation. Additional markers of erythropoiesis which may decrease after blood transfusion, such as erythroferrone, may also improve detection of autologous blood transfusion. Unfortunately, there is currently no commercially available assay for erythroferrone that has been validated with clinically relevant human samples.

Limitations of the study design should be noted. Specifically, the saline transfusion control was subjected to blood withdrawal in an identical fashion as the blood transfusion group and therefore is not the same as an untreated control. Statistically significant differences observed between the blood and saline group may not be observed when blood transfusion is compared to normal baseline variation of a healthy, non-cheating athlete. However, for the data shown in *Online Supplementary Table S1* and *Table S2*, statistical comparisons for each serum marker were performed relative to pre-transfusion values. Significant differences were observed for ferritin, sTFR, Hb, Ret%, and the OFF-score.

In summary, this report has demonstrated that hepcidin, ferritin and sTFR change significantly upon blood withdrawal and blood transfusion. Ferritin and sTFR demonstrate prolonged changes after blood withdrawal, which may be used to target additional testing for the anticipated blood transfusion phase. After blood transfusion, hepcidin demonstrated a significant short-term increase at day 1, but demonstrated a larger increase 2-3 weeks later, which coincides with the inhibition of erythropoiesis. The measurement of these serum markers may result in better detection of autologous blood transfusions.

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