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Detection of autologous blood transfusions using a novel dried blood spot method

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In doping control laboratories, autologous blood transfusions are currently detected using an indirect method that monitors changes in an athlete's hemoglobin concentration [Hb] and reticulocyte percent (Ret%) over time. The method is limited by the need for a phlebotomist to collect venous blood and the limited blood stability which requires temperature-controlled shipment and analysis within 72 hours. These limitations significantly reduce the number of samples collected from each athlete and thus the utility of the method. We have recently developed a method to measure immature reticulocytes (IRC) and red blood cells (RBC) in dried blood spots, which could replace the current venous blood method. In the DBS method, cell-specific proteins are digested with trypsin and measured by mass spectrometry. Two proteins, CD71 and Band3, are measured to count IRC and RBC, respectively. The method was tested in an autologous transfusion study consisting of 15 subjects who received blood and 11 subjects who received saline. After transfusion, the average CD71/Band3 ratio in the blood group was statistically different from the saline group at days 5, 6, 13, and 20. The average CD71/Band3 ratio decreased to a minimum of 61 ± 8% of baseline, while Ret% decreased to 75 ± 5% of baseline. Based on experimentally defined criteria, the CD71/Band3 ratio could detect 7 out of 10 blood transfusion subjects, while Ret% could detect 3 out of 10. Thus, the DBS method could improve detection of autologous transfusion and allow increased sample collection.

Keywords: dried blood spot; mass spectrometry; autologous transfusion; blood doping; immature reticulocytes

Introduction

Use of autologous blood transfusions by athletes is a dangerous practice. Transfusion of blood into otherwise healthy individuals can elevate hematocrit well above normal levels resulting in increased blood viscosity, increased risk of blood clotting, and risk of deep vein thrombosis.[1,2] This method and all forms of blood transfusions are banned by the World Anti-doping Agency (WADA).[3] Autologous blood transfusions, as well as abuse of recombinant human erythropoietin (rhEPO) and other erythropoiesis stimulating agents (ESAs), are often detectable using the hematological module of the athlete biological passport (ABP). The ABP is an indirect method designed to detect blood doping practices through longitudinal monitoring of hemoglobin concentration [Hb], reticulocyte% (Ret%), and the OFF-score, defined as [Hb] – 60 x √Ret%.[4,5] The OFF-score method was originally designed to detect abuse of rhEPO and ESAs, but later was demonstrated to successfully detect autologous blood transfusions.[6] In that report, the authors demonstrated that autologous blood transfusions could be detected by a blinded investigator when 10 blood collections were obtained over a 42-week period. Unfortunately, the number of blood samples collected in practice is significantly less due to several limitations. Sample collection is limited, in part, by the need to schedule a phlebotomist for venous blood collection, which limits collection of unannounced out-of-competition samples. Additionally, blood samples have limited stability and must be shipped in temperature-controlled containers and analyzed within a maximum of 72 hours.[7] Finally, blood collections are often needed in remote locations where shipping times will exceed required limits.

Despite these limitations, the ABP method has been successful in the field,[8,9] which has caused athletes to modify their blood doping practices to evade detection based on the current model. Thus, it is necessary to improve the sensitivity of the ABP method to detect more subtle blood manipulation practices. Recently, a few reports have described potential methods to improve detection of blood transfusion by measurement of specific microRNAs in plasma or mRNAs in blood cells.[10–12] One way to improve the sensitivity of the current method is to measure immature reticulocytes (IRC), rather than total reticulocytes. IRC represent the small group of cells first to differentiate in response to an erythropoietic stimulus including treatment with rhEPO.[13–15] The immature reticulocyte fraction (IRF%) is defined as the percent of immature reticulocytes relative to the total reticulocyte number, which is approximately 3-14% in male athletes.[16] Unfortunately, measurement of this small cell fraction on the current, WADA-approved, Sysmex XT

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2000i does not provide sufficient precision for use in the ABP passport.[17] One recent autologous transfusion study, reported by Salamin *et al.*, has demonstrated that specific reticulocyte mRNA transcripts, which are enriched in the IRF% fraction, are significantly decreased after blood transfusion.[11]

We have recently reported a dried blood spot (DBS) method to address the shipping limitations, improve the sample stability, and improve the sensitivity of the ABP passport.[18] In this method blood spots are washed in a series of buffers to remove soluble proteins while leaving membrane proteins in the spot. After washing, the remaining membrane proteins are digested with trypsin and the released peptides are measured by mass spectrometry. We applied this method to the measurement of cell-specific membrane proteins, CD71 and Band3, to count IRC and RBC in DBS, respectively. CD71 is the membrane-bound form of the transferrin receptor. It is highly expressed on immature reticulocytes where it is responsible for iron transport into the cells for hemoglobin synthesis.[19] It's expression decreases during reticulocyte maturation and is absent in mature reticulocytes and RBC.[20,21] Band3 is an integral membrane protein also known as anion exchanger 1 or solute carrier family 4 member 1 (SLC4A1). In blood, Band3 is only expressed in RBC and reticulocytes and thus represents the total erythroid population. It is expressed at high levels in RBC where it interacts with cytoskeletal proteins to stabilize the RBC membrane and prevent hemolysis.[22] It is also responsible for the export of bicarbonate ion, which is produced from tissue carbon dioxide that diffuses into RBC.[23] The concentration of CD71 and Band3 in DBS has been shown to correlate well with cell numbers for IRCs and RBC.[18] While the current ABP method measures Ret%, the DBS method measures IRC/RBC%. This is measured by calculation of the CD71/Band3 ratio. Collection of blood onto DBS cards significantly increases blood stability and simplifies shipment, thus allowing an increased number of samples to be collected. Additionally, DBS samples are designed for use with finger-prick or capillary blood, which does not require a phlebotomist. Finally, measurement of IRC rather than total reticulocytes may improve the sensitivity of the method. In this report, the DBS method was tested in an autologous transfusion study for comparison to the current ABP method.

Experimental

Reagents and materials

High purity acetonitrile, 95% reagent alcohol, 12 x 75 mm polypropylene tubes, siliconized, polypropylene deep-well 96-well plates, sodium phosphate dibasic, and Tris were obtained from VWR (Radnor, PA, USA). Thermomixers were obtained from Eppendorf (Hauppage, NY, USA). Mass spectrometry grade formic acid, Whatman DMPK-C cards, sodium chloride, sodium carbonate, sodium acetate, potassium phosphate monobasic, PMSF (phenylmethanesulfonyl fluoride), and Roche Protease Inhibitors with EDTA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade trypsin was obtained from Promega (Madison, WI, USA). Recombinant soluble transferrin receptor (sTFR) was purchased from KeraFAST (Boston, MA, USA). Rat blood with K_2EDTA, was obtained from Bioreclamation (Hicksville, NY, USA). Stable isotope-labeled peptide internal standards were obtained from AnaSpec (Fremont, CA, USA). The peptides were purified to ≥95% purity and the peptide content was determined by amino acid analysis.

Autologous study design

The study included 34 subjects, 20 males and 14 females, and a subset of these subjects, 12 males and 14 females, were selected for dried blood spot sample collection and are described in this report. Healthy, active subjects aged 18 to 40 years, were recruited for participation. Because a banned, performance-enhancing method was used in this study, subjects were excluded if they participated in competitive sporting events or if they were a member of a registered testing pool for any international federations, national anti-doping organizations, or professional sporting organizations. All participants provided written, informed consent prior to study commencement. Approval for this study (US Clinical Trial NCT02684747) was granted by the University of Utah Institutional Review Board (IRB_00083533). All blood donation and transfusion-related procedures were closely monitored by an on-site, trained medical staff.

An outline of the autologous study design and collection time points is shown in Figure 1. Venous blood samples were collected in K_2EDTA tubes at the time points indicated. Blood parameters were measured on the Sysmex XT 2000i including: [Hb], hematocrit, RBC, WBC, platelets, Ret%, Ret#, and IRF%. Subjects were randomly assigned to the blood or saline transfusion group. Following three baseline collections, each subject in the study, regardless of cohort, donated one unit of blood (~475 mL) according to the Associated Regional and University Pathologists (ARUP) standard operating procedures. Briefly, blood was collected into a storage bag containing citrate, phosphate, dextrose, and adenine and immediately refrigerated. Shortly thereafter, the blood was filtered to remove leukocytes and centrifuged to remove plasma, leaving only the packed red blood cell component (~275 mL), which was stored between 1-6°C for 21 days prior to transfusion. Twenty-one days following blood donation, subjects received a transfusion of either their packed RBCs (blood group) or an equal volume of saline (placebo group). They were blinded to their group using a curtain to hide the contents of the transfusion bags. Infusions were carried out at a constant flow rate, and depending on the individual, took approximately 60-180 minutes to complete.

DBS sample spotting

Whatman DMPK-C cards were pre-treated with 1.5X Roche Protease inhibitor cocktail with EDTA (ethylenediaminetetraacetic acid), 20 µl/spot and allowed to dry for 1 hour. Cards were then pre-treated with 10 mM PMSF (phenylmethanesulfonyl fluoride) in isopropanol, 20 µl/spot, and allowed to dry for 20 min. After measurement on the Sysmex XT 2000i, venous blood was pipetted onto pre-treated DBS cards, 20 µl/spot and dried for 3 hours. Each sample was spotted in duplicate. After drying, cards were stored in sealable plastic bags with desiccant and the bags were stored in a desiccator cabinet. It should be noted that venous blood was spotted onto DBS cards for this study, but capillary blood would be used for real doping control samples.

DBS sample extraction

DBS spots were extracted as previously described.[18] Each sample was extracted in duplicate. DBS spots, 20 µl, were quartered, excised, and transferred to 12 x 75 mm polypropylene tubes. The spots were washed in a series of 3 buffers designed to remove soluble proteins and matrix interference while leaving membrane proteins on the spot. Each was step was performed with 3 ml of buffer/tube, at 10°C for 30 min/ wash, 1400 rpm shaking in a thermomixer.

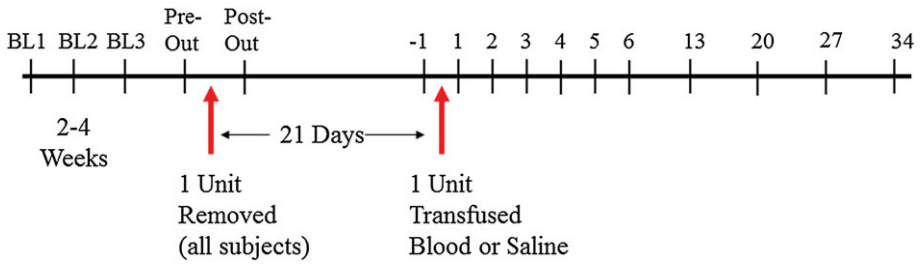

Figure 1. Autologous blood transfusion study design. Three baseline (BL) blood samples were collected prior to removal of 1 unit of blood. One blood sample was collected immediately prior to blood withdrawal (pre-out) and 2–3 days post-withdrawal (post-out). After 21 days, one blood sample was collected immediately prior to transfusion (-1) and every day post-transfusion for 1 week (1–6). Blood samples were then collected once a week on days 13, 20, 27, and 34 post-transfusion

The first buffer was phosphate buffered saline, pH 7.4 containing 5.3 mM sodium phosphate, 1.5 mM potassium phosphate, 137 mM sodium chloride, and 1 mM PMSF, followed by a wash in 100 mM Na_2CO_3 containing 1 mM PMSF, followed by a wash in 95% reagent alcohol containing 50 mM sodium acetate, pH 5. After each wash step, the buffer was removed by vacuum suction. After washing, the spots were equilibrated in 50 mM Tris, pH 8, 3 ml/tube at 10°C for 15 min. The buffer was removed by vacuum suction and the spots were transferred to a polypropylene deep-well 96-well plate. The internal standards were ^{13}C,^{15}N-stable isotope-labeled peptide internal standards with trypsin-cleavable extensions listed in Table 1. The peptide internal standards, 20 µl, were added to each spot at a concentration of 50 nM and 0.5 nM for Band 3 and CD71, respectively. The spots were digested in 100 µl of 50 mM Tris, pH 8 containing 2 µg of sequencing grade trypsin/spot at 60°C for 2 hours, 1400 rpm shaking. After digestion, formic acid was added to a final concentration of 0.2% and the digestion supernatant was transferred to a new 96-well plate for peptide quantification.

Calibration curves for CD71 and band 3

For calculation of the CD71 concentration in DBS samples, each batch was extracted with a 3-point calibration curve. The curve consisted of rat blood DBS spots fortified with recombinant human sTFR, which is the extracellular, soluble domain of CD71. Recombinant sTFR was added after washing and just prior to digestion at 1.25 nM, 2.5 nM and 5.0 nM. Rat blood was chosen as the matrix substitute because the sequence of rat CD71 differs from human CD71. Each batch was also extracted with a 3-point DBS hematocrit curve made from human blood, at 35%, 45% and 55% hematocrit. The RBC numbers in the curve were measured on the Sysmex XT 2000i prior to spotting. The curve was then used for calculation of the RBC number from the band3 peak area ratios in the samples. The CD71/Band3 ratio was calculated from the concentration of CD71 divided by the RBC number calculated from Band3. For comparison, the IRC/RBC% was calculated from the Sysmex IRF% x Ret%.

Liquid chromatography-mass spectrometry

Liquid chromatography was performed on a Dionex UltiMate 3000 using a Waters BEH C18 pre-column, 2.1 x 5 mm, and a Waters BEH C18 column, 2.1 x 100 mm, 1.7 µm, maintained at 50°C. Solvent A was 0.1% formic acid and Solvent B was acetonitrile. Sample injection volume was 15 µl and the peptides were separated on a gradient from 5% B to 40% B over 18 min, followed by 40% to 90% B over 3 min, a hold at 90% B for 3 min, 90% B to 5% for 2 min, followed by re-equilibration at 5% B for 4 min. The total run time was 30 min with at a flow rate of 400 µl/min.

Peptides were quantified using high resolution accurate mass spectrometry on a Thermo Fisher QExactive Plus. The instrument was set to 3.5 kV spray voltage, 350°C capillary temperature, using nitrogen gas as the source and collision gas. Data was acquired in parallel reaction monitoring mode (PRM) with resolution of 70,000 full width at half maximum, 200 ms maximum IT, 2.0 *m/z* isolation width and 4X multiplexing. Peptide *m/z* values and collision energies are listed in Table1. Peak areas were measured from the extracted ion chromatograms for the product ions with 20 ppm mass extraction window. Peak area ratios were calculated from the peak area of the peptide and the corresponding internal standard.

Statistical analysis

Graphs are presented as percent of baseline, where the baseline value represents the mean of 1-3 measurements taken 2-3 weeks prior to blood withdrawal. Error bars represent the standard error of the mean (SEM). Statistical significance was determined using unpaired, two-tailed t-test. P values ≤0.05 were considered significant. Data analysis was performed using Excel with Data Analysis add-in.

Results and discussion

The outline of the blood withdrawal and transfusion study is shown in Figure 1. While several previous studies have performed transfusions of 2-3 units of blood,[24,25] this study along with other recent

Table 1. Peptides and internal standards

Protein	Peptide sequence	Final IS Conc. (nM)	Parent/ product *m/z*	IS parent/ product *m/z*	Charge state	NCE (eV)
CD71	RATS**R** LTTD*FGNAE**K** TDRFV	0.5	548.3/ 881.3999	553.3/ 891.4266	2+	16
Band 3	GILE**K** IPPDSEATL*VLVG**R** ADFLE	50	489.6/ 444.2929	491.7/ 450.3053	3+	20

*Indicates position of the ^{13}C, ^{15}N-labeled amino acid; **Bold** indicates the trypsin cleavage sites; NCE = normalized collision energy.

studies performed transfusion of 1 unit of blood to mimic more subtle blood manipulation tactics currently used by cheating athletes.[26] The study was limited to a 21-day recovery and blood storage period, as mandated by the local institutional review board. Thus, the effects of blood withdrawal were still observed at the time of transfusion in some subjects, as discussed below and shown in Table 2. The number of male and female subjects in each group is listed in Table 2. To our knowledge, this is the first autologous transfusion study to include female subjects for the analysis of ABP blood parameters. Data describing the response of females to autologous transfusion is needed to improve detection using the ABP passport method. Female athletes often suffer from iron deficiency (ferritin <25 ng/ml) and iron deficient anemia (hemoglobin <12 ng/ml) and this was observed in 6 females during the study. These subjects responded differently to blood withdrawal/transfusion and were analyzed as a separate group described below and listed in Table 2.

The DBS method measures the ratio of two cell-specific proteins, CD71 and Band3, to measure immature reticulocyte% rather than Ret%. Figure 2 shows the average response, expressed as % of baseline, for Ret% and CD71/Band3 ratio in 10 subjects who received blood transfusion and 8 subjects who received saline transfusion. The data in Figure 2A demonstrates a decrease in Ret% that is statistically different from saline transfusion on day 4, 5, 6, 13 and 20 post-transfusion. Ret% reached a minimum, on average, of 75 ± 5% of baseline on day 20 post-transfusion, which is consistent with previous reports.[26] In comparison, Figure 2B shows the average % change observed using the new DBS method. As shown, the CD71/Band3 ratio demonstrates a decrease that is statistically different from saline transfusion on day 5, 6 13, and 20 post-transfusion. CD71/Band3 ratio reached a minimum, on average, of 61 ± 8% on day 13 post-transfusion. Figure 2C shows a comparison of the Ret% and CD71/Band3 response after blood transfusion. The data demonstrate that the CD71/Band3 ratio shows a larger % decrease than Ret%. For both methods, the average response to blood transfusion was similar for males and females as shown in Supplemental Figure S-1. Additionally, for both methods, the saline group remained elevated over baseline for at least 4 weeks after blood withdrawal representing recovery-stimulated erythropoiesis, Figure 2A/2B.

The data in Figure 2 compares the average response of 10 blood transfusion subjects, however, the magnitude and duration of the response is variable between individuals. Therefore, the DBS method was evaluated by comparison of the number of subjects that would produce an abnormal profile indicating a positive test. Since the normal baseline variation of CD71 and Band3 has not been determined, a threshold value of ≤80% of baseline for 3 consecutive weeks was set based upon the existing data. These criteria may be more appropriate for use with the DBS matrix, which allows increased testing frequency. However, the suggested criteria are solely for method comparison purposes. Using the experimental criteria, Table 3 compares the number of subjects with a positive

Table 2. Transfusion subjects

Treatment	Subject #	Anemic* subject #	Poor response**	Total subjects
Blood	7 M, 3 F	3 F	1 M, 1 F	15
Saline	4 M, 4 F	3 F	N/A	11

*Subjects whose [Hb] dropped to <12 g/dL after blood withdrawal and/or ferritin dropped to <25 ng/ml.

**Subjects whose [Hb] did not increase >2% after blood transfusion; M: male; F: female.

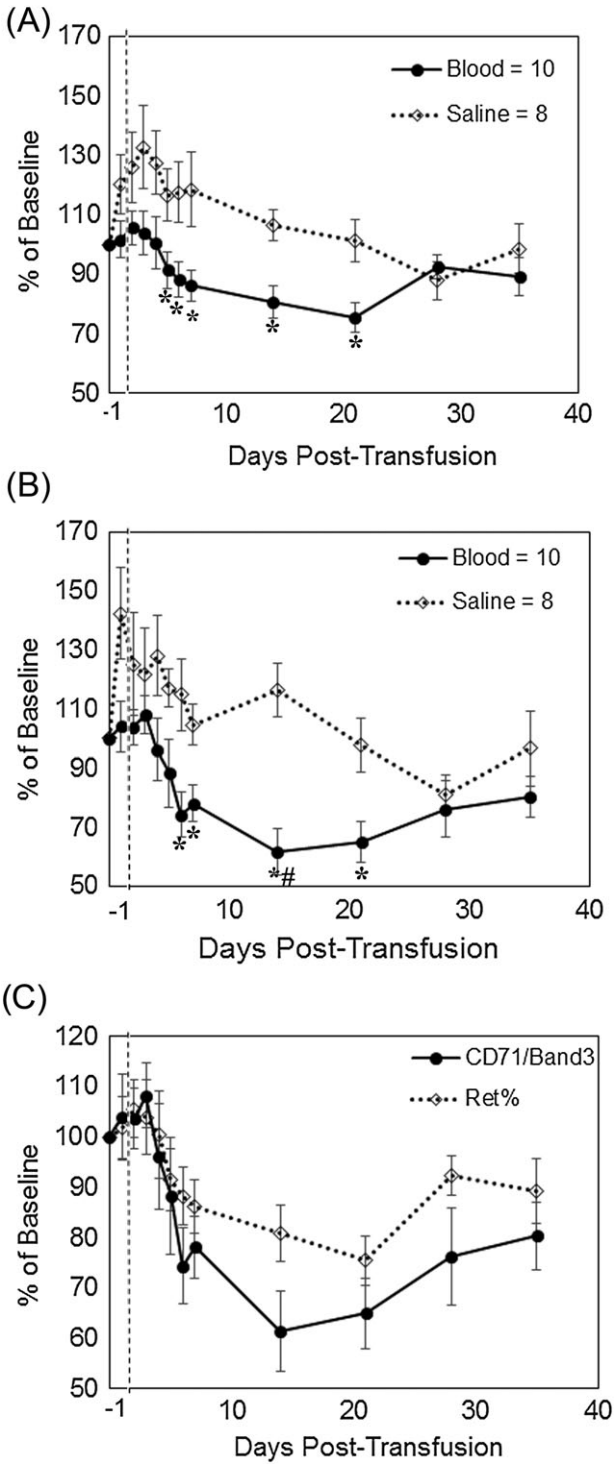

Figure 2. Average response to blood transfusion. Data is expressed as % of baseline, which was calculated as the mean of 1–3 measurements taken prior to blood withdrawal and set to 100%. (A) Reticulocyte% after blood and saline transfusion, (B) CD71/Band3 ratio after blood and saline transfusion, (C) comparison of CD71/Band3 ratio and reticulocyte% after blood transfusion. Error bars represent SEM. Statistically significant difference from saline group, *p ≤ 0.05, #p ≤ 0.001

Table 3. Subjects with a "positive" test

	CD71/Band3*	Ret%*	Off score**
Blood, N = 10	7	3	1
Saline, N = 8	0	0	0

*Positive test defined as 3 consecutive weeks with values ≤80% of baseline.

**Positive test defined as a breach in the individual threshold values with a 99% significance.

test using Ret%, CD71/Band3 ratio, or the OFF-score with a 99% significance level for the threshold. Using OFF-score data alone, 1 subject breached the threshold level which could result in a positive test. Using Ret%, 3 subjects would have a positive test, which supports a previous report suggesting that analysis of Ret% in addition to OFF-score could improve detection of EPO abuse.[27] Finally, using the CD71/Band3 ratio, 7 subjects would have a positive test, suggesting that the DBS method may improve the sensitivity of the test for autologous blood transfusions. Graphs displaying the response of each subject to blood or saline transfusion for Ret% and CD71/Band3 ratio are shown in Supplemental Figure S-2 in the Supporting Information.

Immature reticulocytes

The enhanced response of the CD71/Band3 ratio is likely caused, in part, by the enhanced response of the immature reticulocyte population, which is first to respond to erythropoietic signals.[13] Immature reticulocyte data is reported on the Sysmex XT 2000i as IRF%, which is defined as the percent of immature reticulocytes relative to total reticulocytes. The IRF% has demonstrated utility for the detection of anemias and erythropoietic activity.[28,29] For the DBS method, it was previously demonstrated that CD71 correlates with IRC number and this value is normalized to Band3 which correlates with RBC number.[18] Thus, the CD71/Band3 ratio represents the percent of immature reticulocytes relative to RBC (IRC/RBC%), which differs from the Sysmex value, IRF%, which is relative to total reticulocytes. Additionally, automated blood analyzers such as the Sysmex, count immature reticulocytes based on their RNA content, while the DBS method measures CD71 protein. Therefore, while the two values correlate well they are not identical.

A comparison of the three values, IRC/RBC%, IRF% and CD71/Band3 ratio, in response to transfusion is shown in Figure 3. After blood transfusion, IRC/RBC% decreases to a minimum of 69 ± 9% at 20 days post-transfusion. The decrease is statistically different from saline transfusion on day 5, 6, 13 and 20 post-transfusion, Figure 3A. In comparison, IRF% was decreased to a minimum of 86 ± 7% at 5 days post-transfusion. However, this decrease was not statistically different from saline transfusion at any time point, Figure 3B. The graph in Figure 3C demonstrates that CD71/Band3 ratio showed the largest % decrease when compared to IRC/RBC% and IRF% after transfusion. Differences observed between CD71/Band3 ratio and IRC/RBC% are likely caused by differences in CD71 protein regulation relative to the RNA content of the IRCs. The data support the previous results from Salamin *et al.* who reported that IRF% had a very small response to blood transfusion, while specific mRNA transcripts enriched in IRCs had a much greater response.[11]

Anemic subjects

Since iron deficient anemia is a condition often experienced by female athletes, it was important to determine how this condition

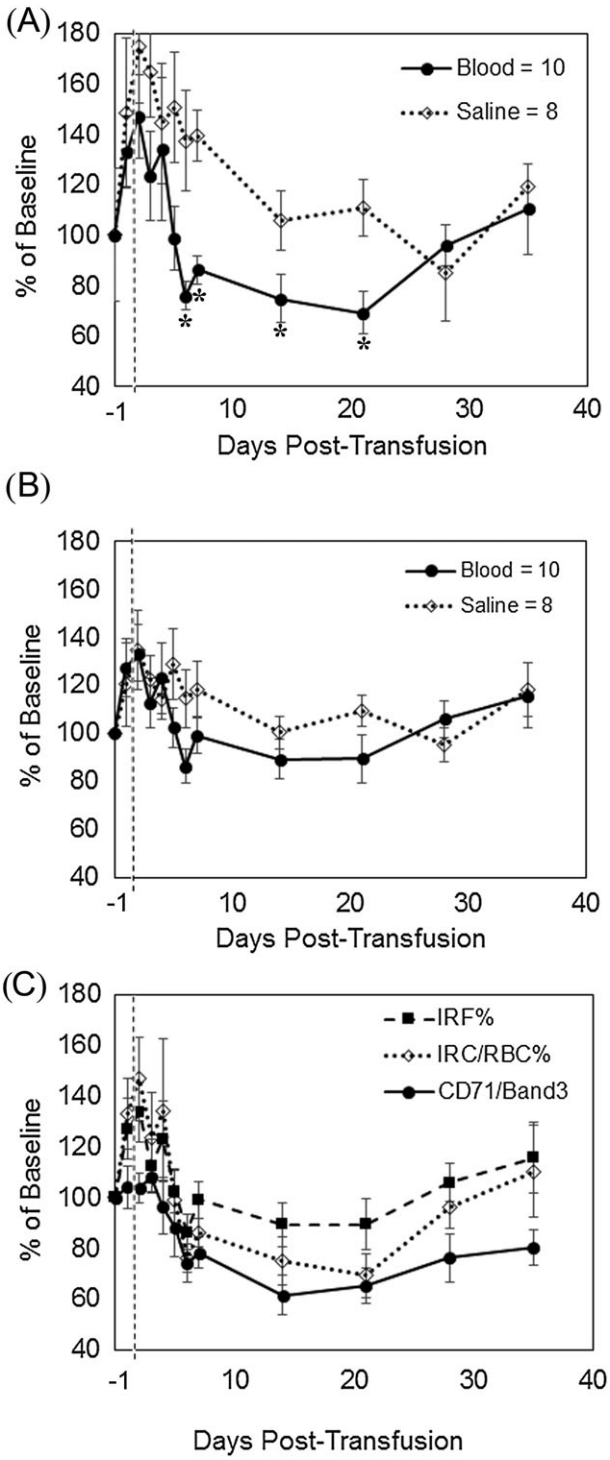

Figure 3. Response of immature reticulocytes. Data is expressed as % of baseline, which was calculated as the mean of 1-3 measurements taken prior to blood withdrawal and set to 100%. (A) IRC/RBC% after blood and saline transfusion, (B) IRF% after blood and saline transfusion, (C) comparison of IRF%, IRC/RBC% and CD71/Band3 ratio after blood transfusion. Error bars represent SEM. Statistically significant difference from saline group, * $p \leq 0.05$

could affect potential blood parameters.[30–32] As shown in Table 1, 6 females were classified as anemic and/or iron deficient. In these subjects, removal of 1 unit of blood resulted in a decrease in hemoglobin concentration and/or ferritin concentration below normal levels, < 12 g/dL and <25 ng/ml, respectively. These subjects displayed a different response after blood withdrawal and blood transfusion, Figure 4. After blood withdrawal from normal subjects, the response of Ret% and CD71/Band3 was similar at a maximum of 115 ± 5% and 117 ± 10%, respectively, Figure 4A. The IRC/RBC% increased to a maximum of 148 ± 13% at 2-3 days post-out and then decreased to 138 ± 14% at 21 days post blood withdrawal (pre-transfusion). In contrast, the anemic subjects displayed a large increase in CD71/Band3 ratio that continued to increase to 21 days post blood withdrawal to 192 ± 29%, Figure 4B. The IRC/RBC% continued to increase in a similar manner to 166 ± 17%. The Ret% response remained similar to normal subjects and increased to 112 ± 10%. The enhanced IRC response in anemic subjects is consistent with the suggested measurement of these cells for the diagnosis and monitoring of anemias.[28,29] The difference in CD71/Band3 response between normal and anemic subjects is a result of CD71 mRNA regulation by iron. CD71, ALAS2, and ferritin (H and L) mRNA sequences contain iron response elements that regulate mRNA levels based on the iron content of the cell.[33,34] Thus, it is important to monitor [Hb] to identify athletes who may have anemia when interpreting results. Hemoglobin concentration is currently monitored as part of the OFF-score equation and is measured in DBS by colorimetric assay.[35,36]

Since the IRC/RBC% and the CD71/Band3 ratio continued to increase to day -1 prior to transfusion, it is clear that the anemic subjects had not recovered from blood withdrawal and therefore, did not show feedback inhibition after blood transfusion, Figure 4C. Finally, 1 male and 1 female did not respond to blood transfusion with an increase in hemoglobin concentration of greater than 2% or subsequent feedback inhibition, Table 1. This may be due to their failure to recover to 95% of the baseline hemoglobin concentration prior blood transfusion.

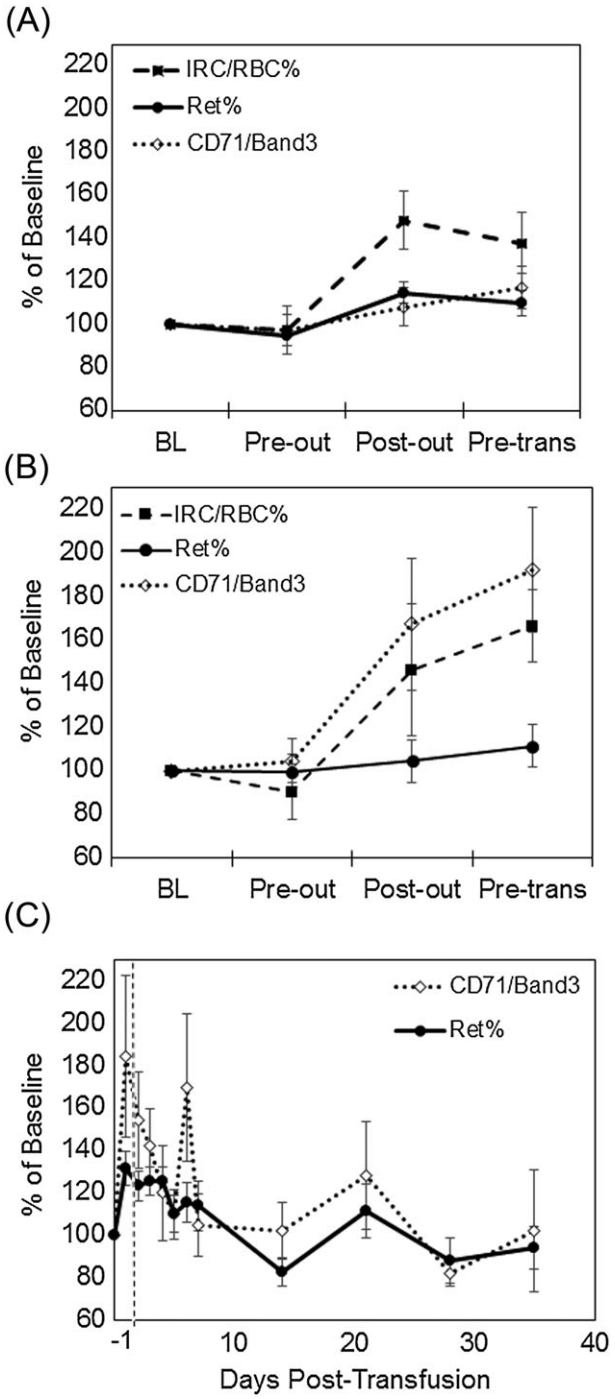

Figure 4. Comparison of anemic and normal subjects**.** Data is expressed as % of baseline, which was calculated as the mean of 1–3 measurements taken prior to blood withdrawal and set to 100%. (A) Response to blood withdrawal in normal subjects, N = 18, (B) response to blood withdrawal in anemic subjects, N = 6, (C) response to blood transfusion in anemic subjects, N = 3. Error bars represent SEM. BL = baseline, pre-out = sample collected immediately before blood withdrawal, post-out = sample collected 2–3 days after blood withdrawal, pre-trans = sample collected just prior to transfusion

Conclusion

In summary, we previously validated a method to measure CD71 and Band3 proteins in DBS.[18] The CD71/Band3 ratio reflects the IRC/RBC% in blood, which is more sensitive to changes in erythropoiesis than Ret%. In this report, the method was tested in an autologous transfusion study of 26 subjects. While the response of the CD71/Band3 ratio was similar to Ret%, the magnitude of the % decrease from baseline was larger for the CD71/Band3 ratio. Using the criteria of a decrease ≤80% for 3 consecutive weeks, the CD71/Band3 ratio would detect 7 out of 10 subjects receiving blood transfusion, while Ret% would detect 3 subjects. Using the established OFF-score calculation, 1 subject would be detected with a breach in the threshold when set to 99% specificity. This response for CD71/Band3 was observed after transfusion of 1 unit of blood, which is often more difficult to detect using existing methods.

The DBS experiments described here were performed on venous blood, but are intended for use with capillary blood collected in the field. However, further work is needed to define an anti-doping protocol for capillary blood collection and the best source of capillary blood, finger-prick or alternative site, before a comparison with venous blood can be performed. Additionally, CD71 is the primary marker used to count immature reticulocytes by flow cytometry, however, low levels of CD71 may be induced upon activation of CD4+/CD8+ T cells and B cells. While serum transferrin receptor concentration is not affected by infection,[37] it is unclear if CD71

in DBS could be elevated during an active infection and this will require further testing. The DBS method has also been validated to measure WBC through measurement of CD45. If necessary, WBC counts could be monitored to rule out the presence of infection. Further testing is also needed to determine the longitudinal stability of the CD71/Band3 ratio, as well as the response to altitude, intense exercise, and rhEPO micro-dosing methods.

When combined with established methods for hemoglobin measurement in DBS, this method could replace the existing ABP method to monitor longitudinal blood parameters. The DBS method may allow increased sample collection and improved sensitivity over the current method. It may be combined with the recently developed dried plasma spot (DPS) cards to allow measurement of additional biomarkers in DPS. The new cards contain a membrane filter to collect blood cells on the top while allowing plasma to flow through for collection on a second membrane below.[38] Finally, measurement of additional biomarkers of blood transfusion, protein or RNA-based, may be included with the DBS or DPS matrix to further enhance the sensitivity.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.