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Short communication

Transcriptomic biomarkers of altered erythropoiesis to detect autologous blood transfusion

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Summary

Autologous blood transfusion is a powerful means to improve performance and remains one of the most challenging method to detect. Recent investigations have identified three candidate reticulocytes genes whose expression was significantly influenced by blood transfusion. Using quantitative reverse transcription polymerase chain reaction as alternative quantitative method, the present study supports that delta-aminolevulinate synthase 2 (*ALAS2),* carbonic anhydrase *(CA1)* and solute carrier family 4 member 1 (*SLC4A1)* genes are down-regulated post-transfusion. The expression of these genes exhibited stronger correlation with immature reticulocyte fraction than with reticulocytes percentage. Moreover, the repression of reticulocytes' gene expression was more pronounced than the diminution of immature reticulocyte fraction and reticulocyte percentage following blood transfusion. It suggests that the three candidate genes are reliable predictors of bone marrow's response to blood transfusion and that they represent potential biomarkers for the detection of this method prohibited in sports.

Keywords: Transcriptomics; Transfusion; IRF

Introduction

Blood manipulations are strictly prohibited by the World Anti-Doping Agency (WADA) although they remain a significant problem in sports. Autologous blood transfusions (ABT) stand as a particular challenge for sports authorities due to the lack of direct detection method. ABT is currently indirectly detected with the hematological module of the Athlete Biological Passport (ABP) [1]. It consists in a longitudinal monitoring of various hematological parameters (hemoglobin level (Hb) and reticulocytes percentage (Ret%)) to identify suspicious patterns of altered erythropoiesis.

Indeed, blood manipulations leave a characteristic fingerprint on the athlete's physiology [2, 3]. For instance, blood reinfusion suppresses erythropoiesis with a subsequent decrease of erythropoietin (EPO) concentration through a negative feedback system [4, 5]. It leads to a diminution of the release of immature red blood cells (RBCs) from the bone marrow, and thus a decline of Ret% and immature reticulocytes fraction (IRF).

Based on that assumption, Salamin et al. demonstrated by a pilot study that the expression of genes involved in biological processes related to reticulocytes decreases following ABT using digital multiplex mRNA profiling [6]. These genes seemed particularly more sensitive to ABT than Ret%.

The primary aim of our study was to confirm the results of this pilot study using additional volunteers (fifteen) and time points (eight), and quantitative reverse transcription polymerase chain reaction (RT-qPCR) as alternative quantitation method. Secondly, it was demonstrated that the expression of those genes was better correlated with IRF than with Ret%.

Material and methods

Study design

Fifteen healthy male volunteers (age range, 20-35 years; body mass index, 18-30), that were eligible for blood donation according to national regulations, were included in the study approved by the human research ethics committee of the Canton de Vaud (Switzerland; Protocol 06/14). Details regarding the clinical trial were previously described (NCT02423135) [4, 7]. Briefly, during the control phase, all volunteers were infused with 500 mL of saline solution (NaCl 0.9%, B.Braun, Crissier Switzerland). Fourteen days later, all volunteers donated one full bag of blood (approximately 500 mL) using international standard methods of transfusion. Blood was drawn into Anticoagulant Citrate Phosphate Dextrose Solution, conditioned and concentrated in RBC for an approximate volume of 280 mL. The procedure to perform RBC concentration was described in Leuenberger et al. [7]. The concentrated RBCs were stored at ~4°C in a solution containing saline, adenine, dextrose and mannitol (ADSOL preservation solution, Fenwal Laboratories, Lake Zurich, IL) until re-infusion 36 days later. Blood samples were obtained using standard procedures from the antecubital vein. Blood was drawn into EDTA tubes (K2EDTA, 7.2 mg, 4 mL, BD Vacutainer, Plymouth, UK) and Tempus Blood RNA tubes (Life Technologies, Carlsbad, CA, USA) at 1 and 4 days before (baseline) and 1, 2, 3, 6, 9, and 15 days after saline infusion or re-infusion.

Hematological and gene expression analyses

The whole blood from the EDTA samples was analyzed for blood variables using a fully automated hematology analyzer (Sysmex XN 2000, Sysmex, Norderstedt, Germany).

Total RNA was extracted from blood stabilized in Tempus Blood RNA Tubes using Tempus Spin RNA isolation kit (Life Technologies, Carlsbad, CA, USA). RNA yield was assessed

using a Qubit® 3.0 fluorometer (Life Technologies, Carlsbad, CA, USA). RNA was aliquoted and stored at -20°C until further analysis.

The amount of 200 ng of extracted RNA was first reverse-transcribed into cDNA using Roche Transcriptor Universal cDNA Master kit (Roche Diagnostics). The resulting cDNA was diluted ten folds and 4 µl of the diluted cDNA was used in a 10 µl-PCR amplification reactions using specific primers of target genes (*ALAS2, CA1, SLC4A1*). *TBP, MRFAP1, ACTR10* genes were selected as housekeeping genes for expression normalization from publicly available database. These genes were selected due to their well expression and stability in whole blood (mean 27 Ct). PCR AMPLIFICATION WAS PERFORMED USING A ROCHE LIGHTCYCLER 480 REAL-TIME PCR SYSTEM. RAW DATA AND NORMALIZATION WERE PERFORMED WITH LIGHTCYCLER 480 SOFTWARE (RELEASE 1.5.0). ALL PRIMERS SEQUENCES ARE AVAILABLE ON THE QIAGEN WEBSITE (HTTPS://WWW.QIAGEN.COM/CH/SHOP/PCR/RT2-QPCR-PRIMER-ASSAYS).

Statistical analyses

Normalized data were first log2-transformed and a one-way ANOVA followed by *post hoc* pairwise comparisons (Tukey's Honestly Significant Difference) were used to test differences between time points during each phase. Correlations were calculated with Pearson's correlation method. Statistical analyses were performed using R software.

Results and discussion

Based on the blood transcriptional signature of recombinant human EPO (rHuEPO) [8], Salamin et al. demonstrated that delta-aminolevulinate synthase 2 (*ALAS2*), carbonic anhydrase (*CA1*), and solute carrier family 4 member 1 (*SLC4A1*) were down-regulated after reinfusion of donated blood [6]. Using a different method of quantitation (RT-qPCR), we confirmed the results of this pilot study with additional subjects (n=15 vs 7) and time points tested (t=8 vs 5) (Fig. 1A). The expression of the three target genes exhibited a significant maximum decrease 9 days ($P<0.05$) after blood transfusion. The diminution of the number of copies was also significant at 15 days for *ALAS2* and *SLC4A1,* and at 6 days for *SLC4A1*. These results corroborate the pilot study and certify that the decrease of gene expression actually begins 6 days after the reinfusion of one's own blood. Because ABT could be performed few days (1-2 days) before an anti-doping control, other biomarkers such as circulating miRNAs could complement this detection strategy [3]. After saline infusion, the number of transcripts of the three genes did not vary substantially and remained constant throughout the time points (Fig. 1B). Finally, the expression of the housekeeping genes (*TBP*, *MRFAP1, ACTR10*) used for normalization demonstrated no variation and was uniform among the subjects (data not shown).

Interestingly, the results obtained with the RT-qPCR correlated with those of Nanostring nCounter technology (*ALAS2*, r=0.81; *CA1*, r=0.88; *SLC4A1*, r=0.7) (Fig. 2) suggesting that both medium-throughput mRNA abundance measurement technologies are robust and sensitive for quantitation of reticulocytes' mRNA. While RT-qPCR analysis is less expensive, can profile smaller subset of specific genes such as candidate gene validation and is considered as the gold standard for nucleic acid quantification, Nanostring technology does

not necessitate reverse transcription and has the ability to profile directly Tempus tubes lysed whole blood without any purification steps [9].

The reinfusion of one's own blood triggered a decline of the erythropoietic activity with a diminution of Ret% being maximal at 9 days [4]. ABT also produced a similar, although non-significant, decrease in IRF with a comparable maximal decrease at 9 days (Fig. 3). The measure of this parameter exhibited some variability for several volunteers which could be rationalized by the absence of normalization in comparison with gene expression. However, the variability of these individuals did not influence the overall trend after autologous transfusion. Both hematological parameters fluctuate in a similar manner to that of the three candidate genes investigated.

Depending on the maturation stage, reticulocytes contains variable amounts of RNA. They are thus divided into low- (LFR), middle- (MFR), and high-fluorescence reticulocytes (HFR) populations, of which the HFR fraction includes the most immature reticulocytes [10]. Although not fully exploited in anti-doping context, IRF appeared as a particular sensitive indicator of bone marrow function and of erythropoietic status in clinic as it includes a count of the most immature reticulocytes [11, 12]. Pearson's correlation between the genes, Ret%, and IRF was calculated, and the level of significance determined. A stronger correlation between IRF and *ALAS2, CA1,* and *SLC4A1* (Fig. 3) was discovered than for Ret% and these three genes (Fig. S1). This can be easily explained by the more abundant RNA content in IRF compared to Ret%, which influence greatly the gene expression analysis.

Although the gene expression significantly correlates with IRF, the magnitude of the changes in the number of transcripts was more significant than that of IRF or Ret%. It suggests that in response to ABT, the copies remaining in immature RBCs reflect more substantially the bone marrow activity than variations of IRF or Ret%. Indeed, *ALAS2*, *CA1,* and *SLC4A1* genes all code for proteins implicated in reticulocyte and erythrocyte metabolism and are thus specific

to these sorts of cells and to erythropoiesis [13-15]. It suggests that transcriptomic biomarkers might be promising complements to hematological parameters of the ABP in a longitudinal monitoring for the detection of transfusion. Moreover, as demonstrated by Durussel et al., these genes might also serve as biomarkers of rHuEPO administration [8].

However, to ensure the specificity of the genes' response to ABT and a possible integration into the adaptive model of the ABP, the impact of intrinsic and extrinsic factors such as altitude exposure or physical exercise must be fully characterized. Because the genes' functions are closely related to reticulocytes' metabolism, the influence of those confounding factors on IRF measurement should also be valuable for the transcriptomic biomarkers.

In conclusion, our results confirm and consolidate that autologous blood transfusion triggers a down-regulation of genes implicated in reticulocytes life cycle. The decrease of *ALAS2*, *CA1*, and *SLC4A1* expression correlated better with IRF than with Ret%. This stronger correlation could be explained by the higher amount of RNA contained in IRF compared to Ret%. The fluctuations of the number of transcripts seemed also more important than that of IRF or Ret%, suggesting that combined with those markers, they can improve the sensitivity of ABT detection.

Conflict of interests

The authors declare that they have no competing of interest relevant to the manuscript submitted to *Drug Testing and Analysis.*

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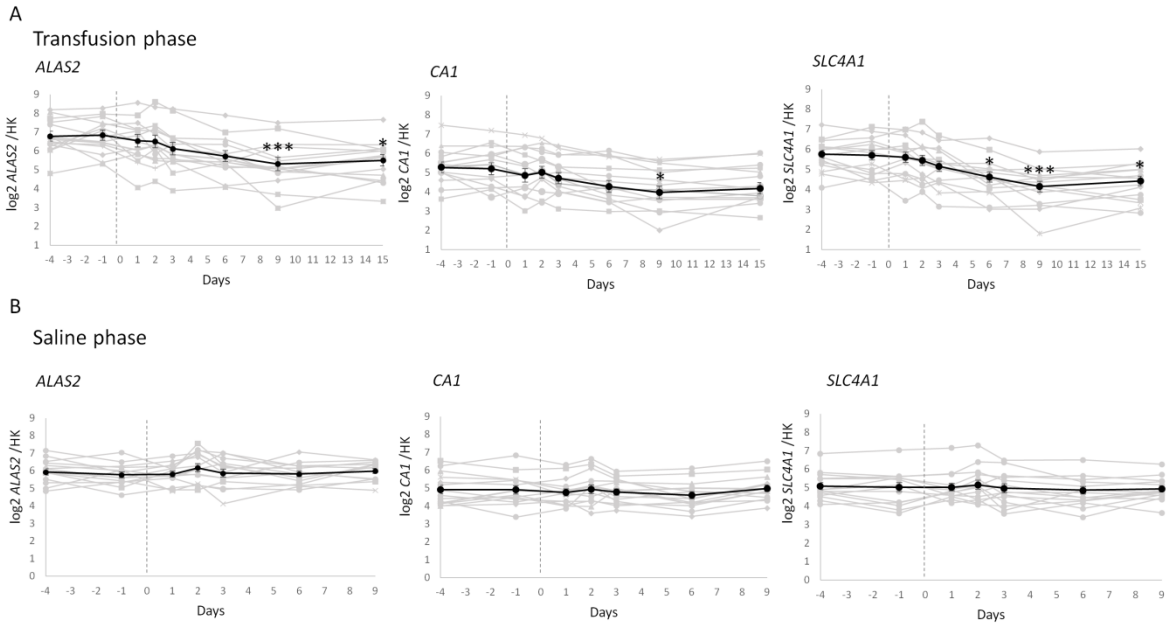

Figure 1. A: Transfusion phase. Relative expression of *ALAS2, CA1,* and *SLC4A1* before (D-4 and D-1) and 1, 2, 3, 6, 9, 15 days after autologous blood transfusion (n=15). Dashed line indicates blood transfusion. Data were normalized to the corresponding levels of housekeeping genes (HK: *TBP, MRFAP1, ACTR10*). The *Y* axis represents log2-transformed relative expression of the genes. Grey lines indicate individual monitoring of gene expression and black lines indicate mean (±SE) of the 15 independent subjects. *$P \leq 0.05$; *** $P \leq 0.001$, statistically significant difference compared with baseline values (mean of the data from 1 and 4 days pre-transfusion). **B: Saline phase.** Relative expression of *ALAS2, CA1,* and *SLC4A1* before (D-4 and D-1) and 1, 2, 3, 6, and 9 days after saline infusion (n=14). Dashed line indicates saline infusion. Data were normalized to the corresponding levels of housekeeping genes (HK: *TBP, MRFAP1, ACTR10*). The *Y* axis represents log2-transformed relative expression of the genes. Grey lines indicate individual monitoring of gene expression and black lines indicate mean (±SE) of the 14 independent subjects. No statistically significant difference was observed between time points.

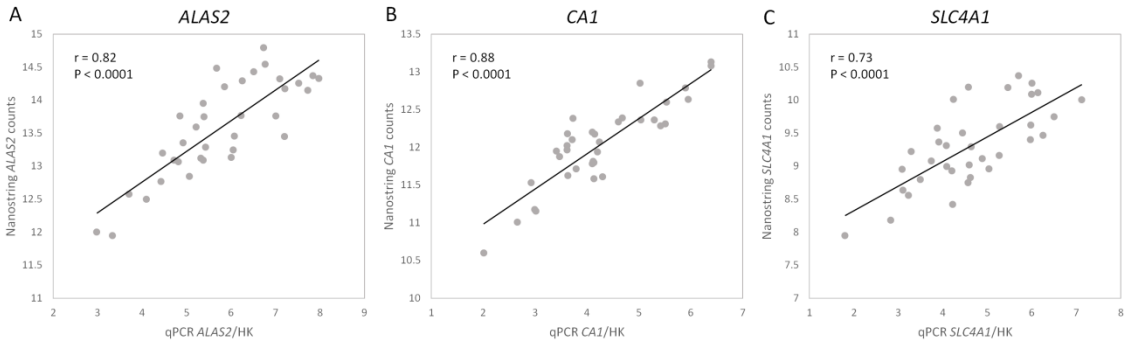

Figure 2. Correlation between Nanostring nCounter system and RT-qPCR results after autologous blood transfusion. A: *ALAS2.* Pearson correlation of Nanostring and RT-qPCR results (Pearson r=0.81; P<0.0001; n=35). *X* axis represents the log2-relative expression of *ALAS2* (RT-qPCR) and *Y* axis represents the log2-normalized mRNA counts of the gene (Nanostring). **B: *CA1.*** Pearson correlation of Nanostring and RT-qPCR results (Pearson r=0.88; P<0.0001; n=35). *X* axis represents the log2-relative expression of *CA1*(RT-qPCR) and *Y* axis represents the log2-normalized mRNA counts of the gene (Nanostring). **C: *SLC4A1.*** Pearson correlation analysis of Nanostring and RT-qPCR results (Pearson r=0.7; P<0.0001; n=35). *X* axis represents the log2-relative expression of *SLC4A1*(RT-qPCR) and *Y* axis represents the log2-normalized mRNA counts of the gene (Nanostring).

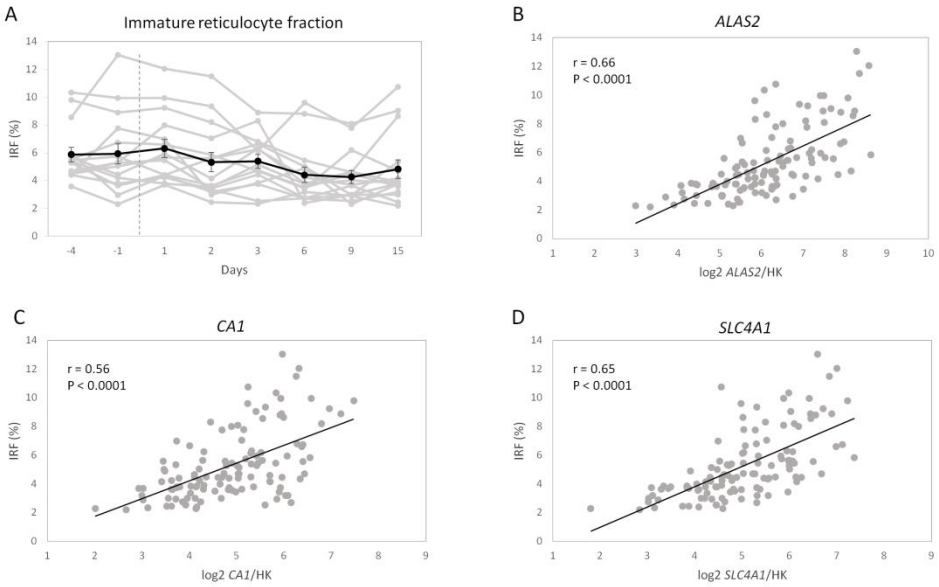

Figure 3. A: Impact of transfusion on IRF. Longitudinal monitoring of IRF before (D-4 and D-1) and 1, 2, 3, 6, 9, 15 days after autologous blood transfusion (n=15). Dashed line indicates blood transfusion. Grey lines indicate individual monitoring of IRF and black line indicates mean (±SE) of the 15 independent subjects. **B: Relationship of IRF and *ALAS2* after ABT. C: Relationship of IRF and *CA1* after ABT**. **D: Relationship of IRF and *SLC4A1* after ABT.** Line represents linear regression of data. *X* axis represents relative expression of the gene and *Y* axis represents IRF (%).