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Autologous Blood Transfusion in Sports: Emerging Biomarkers

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ABSTRACT

Despite being prohibited by the World Anti-Doping Agency, blood doping through erythropoietin injection or blood transfusion is frequently used by athletes to increase oxygen delivery to muscles and enhance performance. In contrast with allogeneic blood transfusion and erythropoietic stimulants, there is presently no direct method of detection for autologous blood transfusion (ABT) doping. Blood reinfusion is currently monitored with individual follow-up of hematological variables via the athlete biological passport, which requires further improvement. Microdosage is undetectable, and suspicious profiles in athletes are often attributed to exposure to altitude, heat stress, or illness. Additional indirect biomarkers may increase the sensitivity and specificity of the longitudinal approach. The emergence of "-omics" strategies provides new opportunities to discover biomarkers for the indirect detection of ABT. With the development of direct quantitative methods, transcriptomics based on microRNA or messenger RNA expression is a promising approach. Because blood donation and blood reinfusion alter iron metabolism, quantification of proteins involved in metal metabolism, such as hepcidin, may be applied in an "ironomics" strategy to improve the detection of ABT. As red blood cell (RBC) storage triggers changes in membrane proteins, proteomic methods have the potential to identify the presence of stored RBCs in blood. Alternatively, urine matrix can be used for the quantification of the plasticizer di(2-ethyhexyl)phthalate and its metabolites that originate from blood storage bags, suggesting recent blood transfusion, and have an important degree of sensitivity and specificity. This review proposes that various indirect biomarkers should be applied in combination with mathematical approaches for longitudinal monitoring aimed at improving ABT detection.

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Increasing oxygen delivery capacity to exercising muscles to enhance aerobic performance is a well-established concept. Blood manipulation may be used by athletes pursuing a rapid increase in red blood cells (RBCs) and hemoglobin. According to the World Anti-Doping Agency, this approach comprises the reintroduction of any quantity of blood, RBC products, artificial enhancement of oxygen delivery, and intravascular manipulation of blood [1]. These methods are covered by blood transfusion and the administration of recombinant human erythropoietin (rHuEPO) and are the most common means used by athletes attempting to manipulate their blood. After its global commercialization

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between 1987 and 1989, widespread abuse of rHuEPO in athletes during the 1990s/2000s was observed and attributed to its easy access and its tremendous impact on performance. Although the drug was banned by sporting authorities, there was no detection method available at that time. A detection method based on isoelectric focusing was developed in 2000 [2,3]. Since then, there has been an increase in the use of blood transfusions in athletes. In 2006, "Operacion Puerto" revealed the presence of refrigerated blood bags from professional athletes associated with sophisticated calendars of blood reinfusion, suggesting that blood transfusion is still abused by athletes in an attempt to boost performance [4].

Flow cytometry can be used to detect allogeneic transfusion by detecting variations in blood group antigens [5,6]. Nevertheless, a group reported that the number of occurrences of two individuals sharing an identical phenotype in the same sport was 5 times higher than the theoretical probability [7]. Autologous blood transfusion (ABT), reinfusion of own blood or red cell concentrates, is, however, undetectable using this approach. The absence of direct detection of ABT is an important challenge facing antidoping laboratories in the fight against doping.

Autologous blood transfusion is currently monitored using indirect markers in a longitudinal profile via the hematological module of the athlete biological passport (ABP) [8]. Although the introduction of the ABP was a breakthrough toward the detection of blood transfusion and blood doping, the transition to microdose regimens of rHuEPO and blood transfusion has raised questions concerning the efficiency of the hematological module [9,10]. The addition of new indirect biomarkers in the ABP would improve the detection of blood transfusion and blood manipulation. As blood doping alters erythropoiesis, it may cause variations at the transcriptomic level, which may be more sensitive than classical hematological parameters. Iron metabolism is involved in the production of RBC and hemoglobin, and quantification of proteins involved in iron regulation may offer a valid alternative for the detection of ABT. Red blood cell storage results in changes in the membrane or shape of RBCs, which may indicate blood transfusion. Finally, urinalysis may indicate the presence of plasticizers leaked from blood storage bags, suggesting recent transfusion.

The ABP and Hematological Markers

Implementation of the hematological module of the ABP by the International Cycling Union in 2008 was a small revolution in the antidoping world. Rather than the direct detection of the prohibited substance, this new paradigm aimed to investigate the effects of doping methods on metabolism [11]. The ABP relies on an individual and longitudinal monitoring of specific biomarkers of doping. This new approach offers the great advantage of being independent of the marketing of new pharmaceutical doping drugs [12]. Moreover, the longitudinal follow-up of athletes can be used to suspend those from competition due to doping and can be a powerful tool to establish targeted testing of suspicious profiles [13].

Blood withdrawal and reinfusion cause characteristic alterations in several markers of erythropoiesis, leaving a characteristic fingerprint on the biology of the athlete [14] (Table 1). This concept is the underlying basis of the ABP in detecting blood doping. Furthermore, instead of setting population-based cutoffs such as 50% hematocrit, individual references are defined by an adaptive model [15]. Biomarkers of erythropoiesis (hemoglobin concentration [Hb] and reticulocytes percentage [Ret%]) are monitored over time and analyzed using a mathematical model based on Bayesian inference that considers previous values and identifies patterns of blood manipulation [16]. A suspicious case can be reported if a particular value lies outside the defined range.

Although the ABP met a certain success, blood doping remains omnipresent among cheating athletes. This analytical tool must thus be continuously refined and correlated with the introduction of new markers of altered erythropoiesis. In addition to standard blood parameters (Hb and Ret%), the total mass of hemoglobin (Hb_{mass}) appeared as a sensitive indicator of blood transfusion and was evaluated as a marker of the adaptive model in a longitudinal blinded study [17]. A new score (Hb_{mr}) that included Hb_{mass} and Ret% was introduced and demonstrated superior sensitivity in detecting the highest dosage of blood transfused, but 0% when 1 U was reinfused [18]. Moreover, Hb_{mass} measurement can detect ABT performed with frozen RBCs [19]. The potential of Hb_{mass} for the detection of rHuEPO misuse was also assessed in another study [20]. The main advantage of this variable over other parameters is its independence of plasma fluctuations and lower variability [17,21]. However, the primary drawback of this promising parameter is that the measurement of Hb_{mass} is based on the carbon monoxide (CO) rebreathing method [22]. CO is toxic and may reduce exercise capacity. Furthermore, the CO rebreathing method requires athletes to fully cooperate, which is unlikely in cheating athletes [23]. Therefore, research is focused on the indirect modeling of Hb_{mass} from indirect markers.

Transcriptomics

The genome represents the genetic material of an organism and is organized in genes. Each gene codes for a protein that is first transcribed into RNA. The transcriptome is the set of all RNA transcripts and also includes noncoding RNA. In contrast to the genome, which is invariable, the transcriptome is subject to environmental variations. Doping substances or methods have been recognized to influence messenger RNA expression. The apparition of high-throughput techniques such as microarray or polymerase chain reaction has allowed an easier application of transcriptomics and offered a promising alternative in the research of biomarker for the detection of blood transfusion.

Through these tools, and based on the hypothesis that exposure of cell detritus originating from stored blood induces a cellular and molecular immune response, a pilot study demonstrated that blood reinfusion altered the expression profile of T lymphocytes [24]. At 72 and 96 hours posttransfusion, the expression of more than 700 genes was altered, particularly in genes coding for proteins regulating surface receptor endocytosis, the Toll-like receptor pathway and the adaptive immune response. The aforementioned study had several limitations including a limited number of subjects. Furthermore, the approach used is susceptible to false-positive results caused by infection or hemolysis [25].

Reticulocytes still retain quantities of functional residual nucleic acid material, even after expelling their nucleus [26,27]. Blood doping influences the production of immature RBC and may alter their gene expression. Consistent with this idea, Varlet-Marie et al [28] identified and confirmed 95 genes that were differentially expressed after administration of high and microdoses of rHuEPO using serial analysis of gene expression and quantitative real-time polymerase chain reaction. Recently, Durussel et al [29] reported the whole-blood transcriptional signature of rHuEPO in 2 distinct and independent groups composed of endurance-trained Caucasian males at sea level and Kenyan endurance runners at moderate altitude who received rHuEPO injections for 4 weeks. On the basis of this study, our laboratory demonstrated that ABT altered the expression of genes whose functions are related to RBC metabolism using digital multiplexed gene expression. Interestingly, the variations in the number of transcripts were more significant than those of the percentage of reticulocytes (unpublished results). This promising approach has the potential to be a powerful complement and appears more sensitive to small variations than classic hematological biomarkers. Athletes often combine ABT with rHuEPO injections to avoid fluctuations and are thereby undetectable with the ABP.

As previously mentioned, the transcriptome also includes noncoding RNAs. MicroRNAs (miRNAs) play a crucial role in gene expression regulation. Cell-free miRNAs are detectable in blood plasma or serum and can be used as specific and sensitive markers of various pathophysiological processes. The ability of circulating miRNAs to serve as biomarkers of ABT was investigated by Leuenberger et al [30]. Blood reinfusion triggered a distinct change in the pattern of circulating miRNAs whose

Table 1
Variations of hematological and iron-related parameters after various methods of blood manipulations

	Hb	Ret%	HCT	s-EPO	Free iron	Ferritin	sTfR	Hepcidin	ERFE
rHuEPO	↗ [25,35,69]	↗ [35]	↗ [35]	↗ [35,36]	↘ [36,70]	↘ [36,70]	↗ [35,36]	↘ [36,70][a]	↗ [46]
Iron	↔/↗ [71–77]	↗ [78]	↔ [73,75,79]	↔ [b]	↔/↗ [71,75]	↗ [41,71–76,80,81]	↘ [79]	↗ [41,71]	↘ [c]
Iron + rHuEPO	↗ [82,83]	↗ [82,84]	↗ [82,84]	↗ [82,84]	↔ [d]	↘/↗ [82-84][e]	↗ [82,84]	↔ [b]	↗/↔ [c]
ABT (reinfusion)	↗ [19,25,37,85,86]	↘ [37,85,86]	↗ [85]	↔/↘ [32,37,85]	↗ [32,44,85]	↗ [32,85]	↘ [37,85]	↗ [32]	↘ [87]
ABT (withdrawal)	↘ [14,23,85,86,88,89]	↗ [14,85,86]	↘ [37]	↗ [85]	↘ [85,90]	↘ [85,88,91]	↗ [85]	↘ [88,89,91]	↗ [46]
ABT (withdrawal) + iron	↘/↗ [37,76,92]	↗ [37]	↘ [37]	↗ [37]	↔ [b]	↔/↗ [41,76,92]	↔ [b]	↔/↗ [41,76,92]	↔ [c]

Abbreviations: HCT, hematocrit; s-EPO, serum erythropoietin.
[a] Hepcidin levels significantly increased 4 hours after rHuEPO injection and significantly decreased after 12 and 24 hours.
[b] Unknown.
[c] The variations in ERFE are based on the preliminary results from our laboratory.
[d] Personal communication.
[e] Plasma ferritin levels significantly increased in the intravenous iron + EPO group and significantly decreased in the EPO + oral iron group.

origin was related to pulmonary and liver tissues. The observed changes were detectable up to 3 days posttransfusion. Interestingly, these miRNAs could be combined with erythropoietin (EPO) concentration in a mathematical model to enhance the efficiency of ABT detection. These results provide the basis for using a combination of transcriptomic and hematological biomarkers in longitudinal measurements to detect ABT.

Ironomics

Iron plays a key role in hemoglobin synthesis and in erythrocyte production. Iron metabolism is tightly regulated, and iron-related variables are therefore likely to be influenced by blood manipulation (Table 1). Transferrin is the main protein involved in the transport of iron in the circulation, and serum transferrin is a common biomarker of iron-related disorders [31]. When blood is transfused, the concentration of iron tends to increase and is associated with a rapid increase of transferrin saturation [32,33]. Iron required for the synthesis of hemoglobin is taken up by erythroblasts through the transferring receptor (TfR). Measurement of soluble TfR (sTfR) is proportional to the total mass of TfR cellular expression. This parameter is an interesting candidate for blood doping because it reflects the erythropoietic activity of the body [34]. Increased erythropoiesis caused by rHuEPO injections or blood withdrawal results in an increase in sTfR [35,36], and a decrease in erythropoietic activity, which is characteristic in blood transfusion, implicates a decrease in sTfR [37] (Table 1). On the other side, ferritin is an iron storage protein and is inversely correlated with transferrin. Small quantities of ferritin are also present in human serum and can serve as a potential biomarker of ABT and, in particular, blood withdrawal [32,38].

Hepcidin, a hepatic peptide that regulates the availability of iron to erythropoiesis by adaptation of iron absorption and recirculation [39], has been investigated as a new potential marker of blood transfusion [32]. Blood transfusion significantly increased hepcidin concentrations at 12 hours and 1 day postreinfusion by 7- and 4-fold, respectively, with no increase in inflammatory markers (C-reactive protein and white blood cells). This 25–amino acid peptide hormone is easily quantified in human plasma or serum using liquid chromatography combined with high-resolution mass spectrometry (LC-MS/MS), a technique implemented in most accredited laboratories [40]. Furthermore, hepcidin is correlated with ferritin and may serve as a marker of the iron repletion required for erythropoiesis [41].

Storage of RBC promotes the accumulation of so-called storage lesions that reduce their survival posttransfusion [42]. After transfusion, these impaired RBC are digested, and their iron content is released in blood, exhausting the iron binding capacity of plasma transferring receptor and inducing a transient increase of free iron [39,43]. Measurement of iron in serum or EDTA-plasma has thus been proposed as a rapid screening method for the detection of blood transfusion [32,44,45]. Recently, the newly discovered protein erythroferrone (ERFE), which is involved in iron metabolism and erythropoiesis, has emerged as an interesting candidate biomarker of blood manipulation [46]. Erythroferrone is produced by erythroblasts upon hypoxia or EPO stimulation and suppresses hepcidin expression; however, ERFE functions were exclusively investigated in a mouse model, and comparative studies in humans are currently underway in our laboratory.

Because all of these players in iron metabolism demonstrate high interindividual variation, individual follow-up of these variables in an "ironomics-based" approach appears to be an appropriate strategy in detecting blood manipulation. Nevertheless, before implementation of such a follow-up, the effects of confounding factors such as high-altitude training, physical exercise, or iron injections on the measurements of iron parameters should be explored. Differences in iron metabolism between men and women should also be well defined before any potential implementation of an ironomic passport.

Plasticizers in Urine Matrix

In 2010, quantification of the plasticizer molecule di(2-ethyhexyl)phthalate (DEHP) and its metabolites in urine was proposed as an indirect method for the detection of ABT [47]. Blood bags used in the storage of RBCs are composed of polyvinyl chloride, to which softening substances such as DEHP are added. Long-term storage of blood in these bags leads to a diffusion of the phthalates into stored blood, which are subsequently transfused with blood. In the human body, DEHP is hydrolyzed to mono(2-ethylhexyl)phthalate (MEHP) and is subsequently oxidized and glucuronidated to mono-(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP), mono-(2-ethyl-5-oxohexyl)phthalate (5oxo-MEHP), mono-(2-ethyl-5-carboxypentyl)phthalate (5-cx-MEPP), and mono-(2-carboxymethylhexyl)phthalate (2cx-MMHP) (Fig 1A), which are all markers of DEHP exposure [48]. Various studies have demonstrated the detection of these substances in urine for a short period of time after transfusion using LC-MS/MS [47,49,50]. The metabolites 5cx-MEPP and 2cx-MMHP are of particular interest due to their long half-life [48]. Although most metabolites are detectable several hours after transfusion (12 hours), these two molecules extend the detection window for up to 1 to 2 days [47,50].

Measurement of DEHP metabolites as a screening method for blood transfusion is the first approach to use urine matrix, which can be easily collected at antidoping controls. In contrast to flow cytometry, the LC-MS/MS–based method is cost-efficient and less time-consuming [23]. Furthermore, because plasticizers are exogenous compounds, the

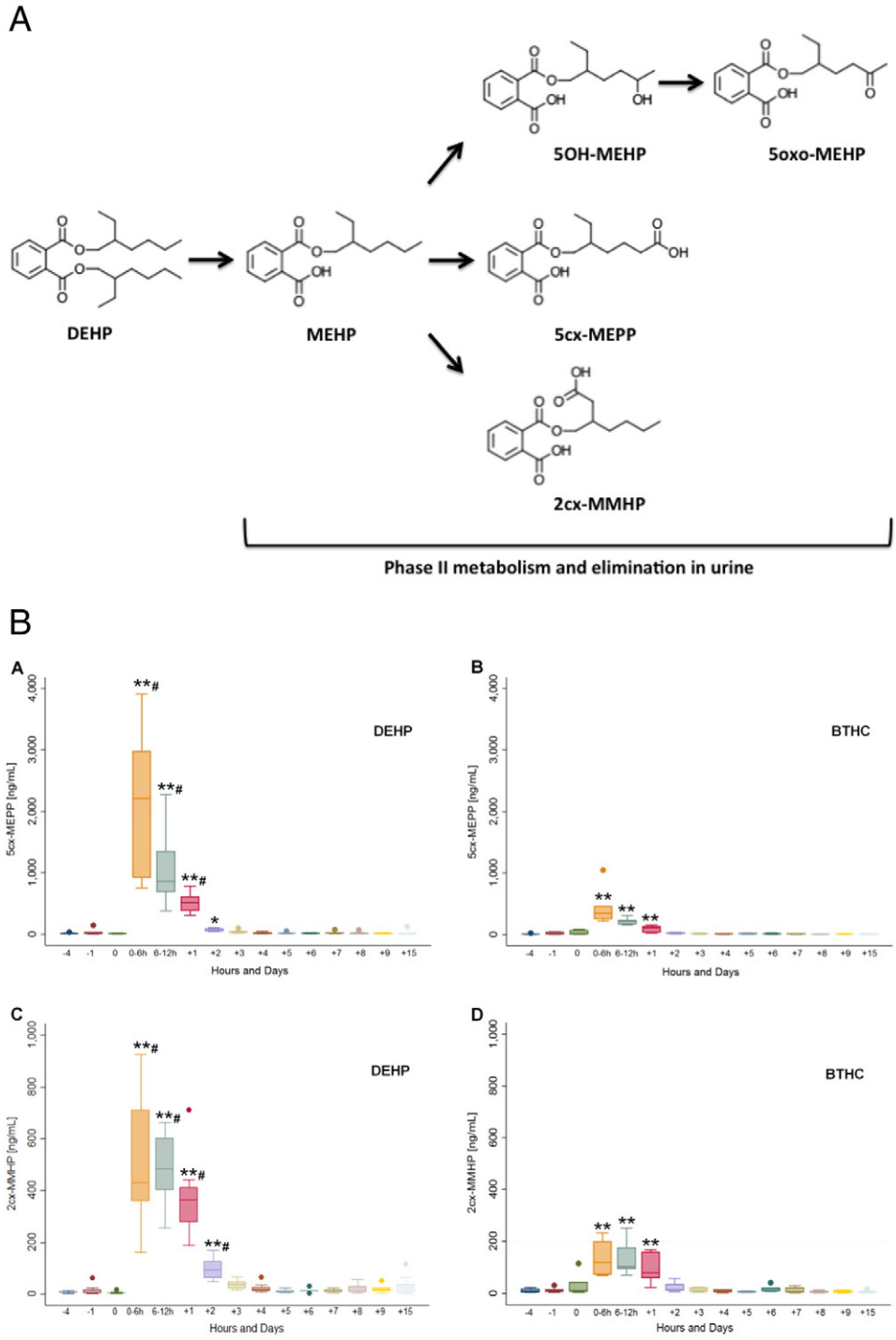

Fig 1. A, Di(2-ethyhexyl)phthalate metabolism. B, Di(2-ethyhexyl)phthalate metabolite concentrations before and after transfusion: 5cx-MEPP and 2cx-MMHP. Data were log transformed, and all concentrations were adjusted for specific gravity. 5cx-MEPP and 2cx-MMHP concentrations after transfusion with autologous blood stored in blood bags containing DEHP (A,C) or BTHC (B,D) are depicted in the box plots. The collection time point is indicated on the x-axis. $^{*}P < .05$; $^{**}P < .01$ compared to baseline (mean of the −4-, −1-, and, 0-hour data). $^{\#}P < .05$ compared with BTHC results at the same time point [50].

qualitative "dilute-and-shoot" screening approach can be applied and easily implemented in accredited laboratories [51–53].

Nevertheless, the main drawback of this detection method is that phthalates are ubiquitous and can be found in urine after occupational or dietary exposures [54]. Consequently, different studies have investigated normal daily exposure to plasticizers to define a threshold for each metabolite in athletes [50,51,55,56]. Using a control phase with saline infusion, Leuenberger et al [50] investigated the specificity of urinary DEHP quantification for the detection of blood transfusion. With the exception of 1 volunteer, metabolite concentrations were low, and the mean and median values of the 5 metabolites were similar to concentrations reported in a Spanish cohort [49], suggesting that contamination of DEHP from environmental exposure is uncommon.

With the recent commercialization of polyvinyl chloride blood bags containing *n*-butyryl-tri-(*n*-hexyl)-citrate (BTHC) instead of DEHP, athletes may prefer to use these plasticizer-free blood bags to avoid detection of DEHP metabolites after recent blood transfusion [57]. Leuenberger et al [50] measured considerable levels of urinary DEHP

metabolites up to 1 day after blood transfusion with BTHC blood bags (Fig 1B). The two long-term metabolites 5cx-MEPP and 2cx-MMHP were particularly sensitive for the detection of ABT with BTHC bags. Levels of DEHP were high in BTHC bags (6.6%), tubing of the transfusion kit (25.2%), and the white blood cell filter (22.3%). This suggests that urinary DEHP quantification is still effective even in cases where an athlete may shift to using labeled DEHP-free blood bags.

Longitudinal studies of plasticizers similar to those of the ABP appear to be an appropriate approach instead of setting absolute thresholds. This follow-up would indicate the usual degree of exposure of each athlete and provide additional evidence of suspicion when a sudden elevated value is found. Because blood transfusions are likely to occur shortly before important cycling events such as the Olympics or World Championships, a strategy implicating urine collection before and after the race with the quantification of plasticizers appears to be of practical relevance. Finally, athletes often justify an abnormal profile with a specific condition, such as fluid loss caused by gastroenteritis [10]. The addition of data from urinary phthalates to the adaptive model would, therefore, provide additional evidence of blood transfusion and decrease the probability that the observed variations are caused by natural processes or specific conditions. It has been proposed that urinary DEHP metabolites should be measured for each urinary antidoping analyses and that confirmatory analyses should be performed for each suspicious sample [50].

Study of Storage Lesions Using Proteomics

The storage of RBCs triggers a series of biochemical and biomechanical changes in the cell, reducing subsequent in vivo survival and function [58]. These alterations include 2,3-diphosphoglycerate and adenosine triphosphate depletion, morphologic changes (crenation and spicule formation), accumulation of extracellular potassium, loss of membrane phospholipid, protein oxidation, and lipid peroxidation [58–60]. These changes may cause negative effects on transfusion, and their identification is imperative. Although hemolysis is a simple parameter to monitor, it does not reflect the profound molecular changes that affect RBC during storage [60]. Concerning the notion of "omics"-based methods [61], proteomics has been proposed as a potential tool to assess and identify RBC changes at the protein level during storage [62]. Alterations of RBC due to storage based on proteomics have been suggested as an indication of the use of blood transfusion for doping control purposes [63].

Using 2-dimensional electrophoresis gel and isobaric tags for relative and absolute quantification, Nikolovski et al [63] observed changes in 2 main protein groups composed of proteins decreasing and increasing during 42 days of storage. These 2 complexes included proteins that were primarily located in the cytoskeleton such as spectrin β, band 4.2, anykrin-1, tropomodulin-1, β adducing, band 4.9 (dematin), tropomyosin, and transmembrane proteins (glycophorin C, aquaporin-1, and band 3). Oxidative degradation has been observed to be prevalent in band 4.2, to a minor extent in bands 4.1 and 3, and in spectrin after 7 days of storage [59]. Extending RBC storage to 14 days resulted in the identification of new fragments from β-actin, glyceraldehydes-3-phosphate dehydrogenase, band 4.9, and ankyrin. Peroxiredoxin 2 was also identified as a candidate biomarker of oxidative stress of stored RBC [64,65]. This antioxidant protein is normally located in the cytosol of RBC of healthy volunteers. During storage, Prdx2 migrates to the RBC membrane and may be used as a biomarker of blood transfusion if the blood bags have been stored for a sufficient period of time. The protein content of RBC-derived microvesicles may also reveal blood that has been stored for a certain period. A recent study highlighted differences between calcium-stimulated and storage-induced RBC-derived microvesicles and identified few differences in terms of lipid raft proteins, notably stomatin and flotillin-2 [66]. However, the detection window of these potential biomarkers has not been investigated yet.

Nevertheless, such phenomena are unlikely to occur when blood is stored under anaerobic conditions such as helium [59,67]. Furthermore, these hypotheses should be verified with individuals subjected to blood transfusion to determine the potential of proteomic-based methods to detect blood transfusion in athletes. Given that the reinfused blood constitutes only 4% (when 1 U is reinfused) of the circulating pool of RBCs and that approximately 20% of transfused RBCs are removed within the first 24 hours, changes based on proteomic methods will be difficult to detect [21].

Combination of Biomarkers

The hematological module of the ABP relies on a combination of specific hematological parameters including [Hb], Ret%, OFF-score ($[Hb] - 60 \times \sqrt{Ret\%}$), and the abnormal blood profile score. On the basis of this model, future research using mathematical methods and algorithms should be conducted on possible combinations of the emerging markers mentioned in this review, which often demonstrate good sensitivity and poor specificity. Different biological sources such as urine, plasma, or whole blood will provide complementary data and increase the specificity and the discriminative performance of the ABP. The combination of longitudinal biomarkers improves diagnostic accuracy compared with use of a single marker and provides a powerful tool in personalized medicine [68].

The various emerging biomarkers of blood transfusion react differently and have a diverse range of detection windows (Fig 2). Markers that are measured several hours posttransfusion generally vary with important amplitude, whereas parameters that react days after blood reinfusion have weaker variations. Combining short-term and long-term biomarkers would thus improve the general detection window and the specificity-sensitivity relationship. Therefore, the combination of omics-based technologies with classic hematological variables will allow the identification of the biological fingerprint of blood transfusion (allogenic/autologous) and blood doping in general. Finally, this strategy will provide a powerful tool for targeting and intelligent testing strategies [13,61].

Conclusion

Since the apparition of a direct detection method for rHuEPO, there is increasing evidence that athletes have returned to blood transfusions to artificially increase oxygen delivery to exercising muscle. Although homologous blood transfusion can be effectively detected by flow cytometry, there is no validated methodology currently available for the detection of ABT. The introduction of the hematological module of the ABP in 2008 based on longitudinal monitoring of blood-based parameters has provided an instrument for the detection of ABT and blood doping in general. This approach allows for the detection of both autologous and homologous blood transfusion and represents an advantage of being independent from the doping technique or the marketing of new pharmaceutical drugs.

This promising tool reaches its limits in situations where the athlete justifies abnormal blood values with specific conditions such as a recent stay at high altitude. Additional data would therefore be required to reveal whether an athlete has undertaken blood transfusion. Omics technologies provide a multitude of promising markers that may be used in an individual subject-based model and have the potential to significantly improve the ABP approach. Furthermore, urine DEHP metabolites offer a powerful complement to blood-based biomarkers in case of doubtful results. This strategy would be easily implemented in doping control laboratories. Furthermore, plasticizers may be integrated into the ABP.

Conflict of interest

The authors have disclosed no conflict of interest.

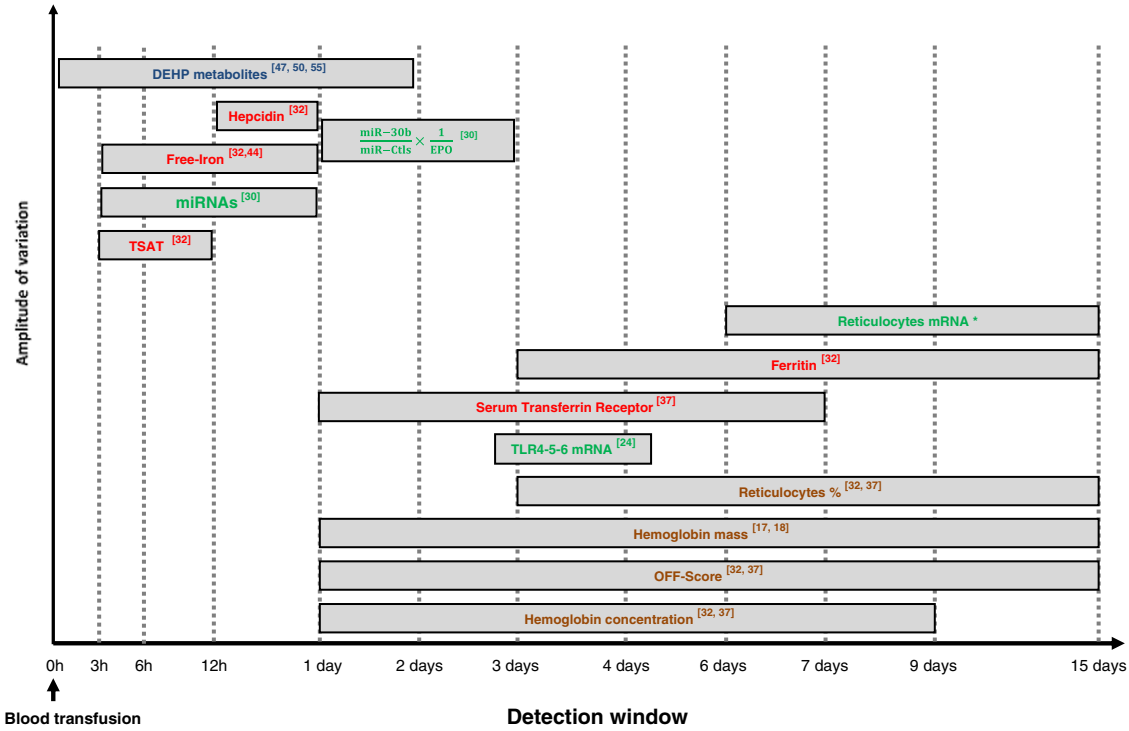

Fig 2. Detection window of emerging and implemented biomarkers of autologous blood transfusion. Biomarkers are classified according to their amplitude of variations and their detection window after blood reinfusion. Plasticizers are indicated in blue; iron metabolism, hematological, and transcriptomic variables are indicated in red, brown, and green, respectively. *Unpublished data. Abbreviations: mRNA, messenger RNA; TLR, Toll-like receptor; TSAT, transferrin saturation.

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