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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