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REVIEW ARTICLE

Indirect biomarkers of blood doping: A systematic review

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Abstract

The detection of blood doping represents a current major issue in sports and an ongoing challenge for antidoping research. Initially focusing on direct detection methods to identify a banned substance or its metabolites, the antidoping effort has been progressively complemented by indirect approaches. The longitudinal and individual monitoring of specific biomarkers aims to identify nonphysiological variations that may be related to doping practices. From this perspective, the identification of markers sensitive to erythropoiesis alteration is key in the screening of blood doping. The current Athlete Biological Passport implemented since 2009 is composed of 14 variables (including two primary markers, i.e., hemoglobin concentration and OFF score) for the hematological module to be used for indirect detection of blood doping. Nevertheless, research has continually proposed and investigated new markers sensitive to an alteration of the erythropoietic cascade and specific to blood doping. If multiple early markers have been identified (at the transcriptomic level) or developed directly in a diagnostics' kit (at a proteomic level), other target variables at the end of the erythropoietic process (linked with the red blood cell functions) may strengthen the hematological module in the future. Therefore, this review aims to provide a global systematic overview of the biomarkers considered to date in the indirect investigation of blood doping.

KEYWORDS
antidoping, biomarkers, blood doping, blood transfusion, EPO

1 | INTRODUCTION

Blood doping is principally targeted to artificially increase the convective transport of oxygen throughout the body,[1] as a direct means to rapidly improve aerobic performance. Blood transfusions and recombinant human erythropoietin (rhEPO) misuse are the prohibited practices historically identified in endurance sports.[2] Additional blood-related practices were subsequently diverted from their medical purposes, such as hypoxia-inducible factor stabilizers, hemoglobin substitutes such as hemoglobin-based oxygen carriers and perfluorocarbon emulsions (i.e., synthetic blood),[3,4] or synthetic allosteric modifier of hemoglobin such as Efaproxiral.[5] Despite ongoing debates about the effect of specific practices on performance enhancement (e.g., microdoses),[6] the benefits of blood doping in athletes through an improved oxygen-carrying capacity provided by the artificial increase of red cell mass have been underlined in recent research.[7,8]

By targeting specific xenobiotics in athletes' fluids, direct detection methods have historically been used in antidoping for more than

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50 years.[2] Banned by the International Olympic Committee following the 1984 Olympic Games despite no available detection method,[9] homologous blood transfusions (involving blood exchange between two compatible individuals) are currently detectable by flow cytometry.[10] Despite a potential risk of false negatives due to a similar panel of surface antigens (although further reduced with the inclusion of additional minor blood group antigens), this method provides a detection up to 50 days post-transfusion of donor blood cells in the receiver.[11] Providing greater accuracy, an expansion of the antigen panel and a better selection of red blood cell (RBC) gating area has been suggested to decrease the number of false negative results.[12] However, no method has been implemented to date for the direct detection of autologous blood transfusions (ABT) (where the individual stores his own blood to have it later reinfused).[13] Moreover, although a robust electrophoresis technique is currently applied to discriminate rhEPO from endogenous EPO,[14] the required time for analysis and costs besides the growing similarity between electric charges of EPO isoforms only guarantee the evaluation of a limited number of samples.[15] Nevertheless, the overall increase in financial and time costs due to further analyses for indirect detection may be significant for antidoping laboratories and needs to be considered when evaluating the implementation of additional markers.

To cope with these limitations, indirect approaches were gradually adopted to complement the traditional direct detection strategy.[16,17] Erythropoiesis is a dynamic and tightly regulated mechanism of RBC production,[18] and understanding its pathways with the role of oxygen sensing was a paramount advance outlined recently with a Nobel prize.[19] From a laboratory perspective, it may provide a precious platform for biomarker detection related to blood doping. Overall, indirect detection of blood doping shall involve sensitive and specific biomarkers with the ability to discriminate an upregulation or downregulation of the erythropoietic function due to doping from physiological variability.[20] Initially applied using a "no-start" rule based on a cut-off level of hematocrit (Hct)[21] or hemoglobin concentration ([Hb]),[22] indirect detection later evolved through the introduction of the Athlete Biological Passport (ABP) by using individual, longitudinal, and adaptative monitoring of altered hematological variables.[23] Since then, the antidoping research aimed to identify new valid biomarkers for longitudinal monitoring to improve the indirect detection approach.[24] This review, therefore, aimed to provide a global systematic overview of the biomarkers investigated to date for the indirect screening of blood doping and shed light on novel perspectives to further deter blood doping practices.

A systematic search for peer-reviewed publications was performed in November 2022 using PubMed and Web of Science databases following PRISMA Statement guidelines[25] (Figure 1). The following inclusion criteria were applied to the abstracts: English language, original article, human subjects, sports doping-related, and targeting indirect detection. Finally, based on full texts, articles seeking to test the stability of existing biomarkers, define reference ranges, or develop new direct detection methods were removed to determine the final selection of articles ($n = 63$) to be included in this review.

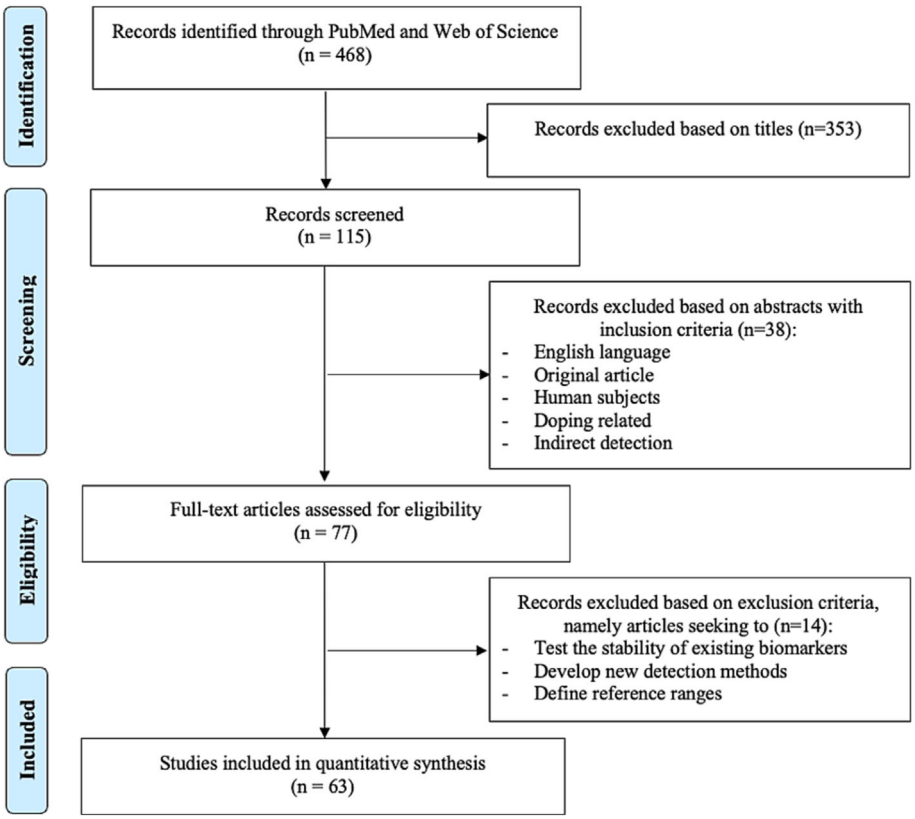

FIGURE 1 "PRISMA" flow diagram (Moher et al., 2009).

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2 | DISCUSSION

This literature review highlights a wide range of biomarkers investigated for the indirect detection of blood doping presented with a chronologic perspective in the physiological cascade of erythropoiesis. Regulating RBC production, erythropoiesis is hence the key metabolic pathway. Involved at an early stage of the biological process, the *transcriptomic* markers will be a key stage of the erythropoietic chain impacted by doping practices (Table 1). Following the *-omics* stages, the metabolism of specific proteins will subsequently be altered (Table 2), to underpin the importance of various markers related to iron metabolism (Table 3), before multiple metabolites are finally influenced (Table 4). After 2 weeks of maturation in which the hematopoietic stem cells will follow different stages of division and differentiation, the young RBC will subsequently result in more commonly investigated hematological variables (Table 5). Finally, some (physical and/or mechanical) properties will be impacted in hematological cells during their life span (Table 6), before being removed from the bloodstream by the macrophages after approximately 100–120 days. The successive phases investigated have been summarized in Figure 2.

3 | TRANSCRIPTOMIC ANALYSES

The *-omics* strategies aim to identify and characterize the structure and function of a biological system by analyzing target molecules.[89] Initially used in a medical setting to investigate specific issues at a cellular level, this approach was later suggested in an antidoping context with *transcriptomic*, *proteomic*, or *metabolomic* investigations for innovative biomarkers discovery.[90] Based on a homeostatic principle, this approach aims to explore unique biological dynamics and molecular signatures associated with doping practices.[91]

Regrouping post-transcriptional regulations,[90] the transcriptome is sensitive to environmental fluctuations. Therefore, providing a powerful representation of cellular activity,[13] transcriptomic biomarkers

TABLE 1 Transcriptomic biomarkers of blood doping.

Transcriptomics ($n = 13$)							
Nb	**Title**	**Doping**	**Subjects**	**Matrices**	**Method of analysis**	**Main biomarker**	**Protocols**
1	Loria et al. [26]	rhEPO	21	Blood	PCR	ALAS2	rhEPO 40 IU/kg 8× over 20 days during the first phase and 8× 13 IU/kg over 12 days during the second phase
2	Salamin et al. [27]	ABT	15	Blood	PCR	ALAS2 + CA1 + SLC4A1 genes	ABT 500 mL
3	Salamin et al. [28]	ABT	7	Blood	PCR	ALAS2 + CA1 + SLC4A1 genes	ABT 500 mL
4	Wang et al. [29]	rhEPO	39	Blood	PCR	BCL2L1 + CSDA	rhEPO 20–40 IU/kg twice a week for 7 weeks
5	Varlet-Marie et al. [30]	rhEPO	10	Blood	PCR	HBB + FTL + OAZ genes	rhEPO 50 IU/kg 3× a week for 4 weeks and 20 IU/kg 3× a week for 2 weeks
6	Varlet-Marie et al. [31]	rhEPO	14	Blood	SAGE and PCR	Multiple genes	rhEPO 0.72 µg/kg every week for 4 weeks
7	Durussel et al. [32]	rhEPO	38	Blood	PCR	Transcriptional biomarkers	rhEPO 50 IU/kg every second day for 4 weeks
8	Pottgiesser et al. [33]	rhEPO	6	Blood	PCR	Transcriptional biomarkers	ABT one or two units of packed red cells
9	Leuenberger et al. [34]	rhEPO	6	Blood	PCR	microRNA	rhEPO 200 µg single dose
10	Leuenberger et al. [35]	ABT	20	Blood	PCR	microRNA	ABT 500 mL
11	Haberberger et al. [36]	ABT	12	Blood	PCR	microRNA	Erythrocyte concentrates storage
12	Gasparello et al. [37]	ABT	24	Blood	PCR	microRNA	ABT 450 mL
13	Mussack et al. [38]	ABT	30	Blood	PCR	microRNA	ABT 500 mL

Abbreviations: ABT, autologous blood transfusions; ALAS2, 5′-aminolevulinate synthase 2; BCL2L1, BCL2-like 1 protein; CA1, carbonic anhydrase; CSDA, gene name so no complete name; FTL, ferritin-light chain; HBB, hemoglobin-β; OAZ, ornithine decarboxylase antizyme; PCR, polymerase chain reaction; rhEPO, recombinant human erythropoietin; SAGE, serial analysis of gene expression; SLC4A1, solute carrier family 4 member 1.

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TABLE 2 Proteomic biomarkers of blood doping.

Proteomic ($n = 6$)							
Nb	**Title**	**Doping**	**Subjects**	**Matrices**	**Method of analysis**	**Main biomarker**	**Protocols**
14	Cox et al. [39]	rhEPO	20	Blood	MS	CD71 + FECH + Band3	rhEPO 40 IU/kg 8× over 3 weeks + 900 IU 6× over 2 weeks
15	Marrocco et al. [40]	ABT	4	Blood	Electrophoresis	Peroxiredoxin 2	Erythrocyte concentrates storage
16	Nikolovski et al. [41]	ABT	2	Blood	LC–MS	Red blood cell membrane proteome	Whole blood storage
17	Al-Thani et al. [42]	ABT	6	Blood	LC–MS	Red blood cell membrane proteome	Whole blood storage
18	Cox et al. [43]	ABT	26	Blood	LC–MS	Reticulocytes proteomes	ABT 475 mL
19	Chang et al. [44]	rhEPO	20	Blood	Flow cytometry	Red blood cell surface markers	rhEPO 1500 IU daily for 4 weeks

Abbreviations: ABT, autologous blood transfusion; FECH, ferrochelatase; LC–MS, liquid chromatography–mass spectrometry; MS, mass spectrometry; rhEPO, recombinant human erythropoietin.

could be of interest for the detection of artificial stimulation of erythropoiesis as initial biological indicators or subsequently affected by negative feedback due to doping practices. As a pioneering approach, erythroid gene markers were investigated after rhEPO injection.[30] Using real-time-polymerase chain reaction quantification, results showed an increased expression of several transcriptomes, mainly related to the ornithine decarboxylase antizyme gene due to its function in erythroid differentiation. These results were later supported by considering a larger number of genes susceptible to being impacted by erythropoiesis-stimulating agents (ESA)[31] and ABT.[33] This field was further investigated through an untargeted approach, highlighting the alteration of 34 transcripts related to RBC functional properties.[32,92] Finally, a set of 15 relevant genes impacted during both ON and OFF phases up to 4 weeks after microdoses have been suggested,[29] among which BCL2-like 1 protein and CSDA demonstrated a sensitivity (i.e., true positive rate or type II errors) and specificity (i.e., true negative rate or type I errors) of 93%, respectively. Involved in the erythrocyte membrane structure and RBC interactome networks, respectively, a combination of RBC metabolism biomarkers seems therefore particularly promising. Subsequently, reticulocyte-related mRNAs expression for 5′-aminolevulinate synthase 2 (ALAS2), carbonic anhydrase (CA), and solute carrier family 4 member 1 were identified as promising candidates following a 500-mL blood reinfusion due to their involvement in heme synthesis (ALAS2) and oxygen transport (CA and solute carrier family 4 member 1).[27,28] The reliability of these three candidate genes for the detection of rhEPO has been later confirmed,[26,93] supporting the interest in biomarkers related to immature RBC metabolism to detect multiple forms of blood doping without being significantly affected by iron injection.[94] Moreover, the possibility to quantify ALAS2 levels in dried blood spots,[95] an increasingly used matrix in antidoping for individual sample collection in athletes, makes it a promising candidate protein to be further investigated.[96,97]

Using a similar transcriptomic approach, circulating microRNAs (miRNAs) were also investigated as potential antidoping biomarkers. Small noncoding RNA involved in gene regulation, miRNA regulates a wide diversity of biological pathways through the suppression of mRNAs.[35] From this perspective, circulating miRNAs were investigated to detect ESA abuse in plasma samples[34] where a large increase in miR-144 was notably observed after third-generation rhEPO ("Continuous erythropoietin receptor activator") injection. Detectable up to 27 days after continuous erythropoietin receptor activator intake samples,[34] these results demonstrated the considerable potential of this specific class of transcriptomic markers.[98] Despite still limited knowledge, the impact of exercise training on the expression profile of circulating miRNAs has recently been highlighted,[99] demonstrating distinctive adaptive responses depending on the type of training.[100] In addition, exposure to altitude hypoxic environments also is associated with multiple RBC-related miRNA profiles alteration.[101] Therefore, a thorough understanding of the biomarkers' variability among athletes is crucial to determine the specificity of these variables.

An alteration of multiple circulating miRNA was observed following long-term blood storage.[36] Based on these findings, a set of 28 miRNA was successfully created for blood transfusion screening. Furthermore, additional miRNAs have been identified for being upregulated following blood reinfusion, including markers related to the erythropoiesis[37] or bound to lung and liver tissues.[102] Highly stable and less impacted by environmental factors compared with classical hematological variables (i.e., [Hb]),[35] these results highlighted the sensitivity of miRNA biomarkers for the detection of blood manipulation.[34,98,103]

Nevertheless, a recent study comparing the effectiveness of miRNA to ABP biomarkers in the monitoring of ABT tempered the absolute effectiveness of the transcriptomic approach.[38] Indeed, despite an interesting response of miRNA markers following blood transfusion, the detection window did not appear to be significantly

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TABLE 3 Ironomics biomarkers of blood doping.

Ironomics ($n = 15$)							
Nb	**Title**	**Doping**	**Subjects**	**Matrices**	**Method of analysis**	**Main biomarker**	**Protocols**
20	Gareau et al. [45]	rhEPO	24	Blood	EIA	Soluble transferrin receptor	rhEPO 50 to 100 U/kg once or twice a week
21	Berglund et al. [46]	ABT	12	Blood	Blood cell differential analysis	Hemoglobin + erytrhopoietin	ABT 450 mL
22	Robach et al. [47]	rhEPO	39	Blood	EIA and MS	Erythroferrone and hepcidin	rhEPO 20 IU/kg or 50 IU/kg 6 times every second day
23	Ramirez Cuevas et al. [48]	rhEPO/ ABT	18	Blood	ELISA	Erythroferrone	rhEPO 5000 IU on Days 1, 3, and 5 or ABT 450 mL
24	Magnani et al. [49]	rhEPO	18	Blood	RT-PCR	Ferritin + soluble transferrin receptor	rhEPO 30 IU/kg at 4-day intervals from Days 0 to 10 or 200 IU/kg at 4-day intervals from Days 0 to 28
25	Christensen et al. [50]	rhEPO	36	Blood	LC–MS	Haptoglobin	rhEPO 40 μg 1× per week for 3 weeks, followed by 20 μg for 7 weeks (10 weeks)
26	Christensen et al. [51]	rhEPO	8	Blood	MS	Haptoglobin + transferrin + hemopexin + albumin	rhEPO 5000 IU every second day for 16 days
27	Leuenberger et al. [52]	rhEPO	109	Blood	LC–MS	Hepcidin	rhEPO 5000 IU on Days 1, 3, and 5
28	Leuenberger et al. [53]	ABT	37	Blood	LC–MS	Hepcidin	ABT 500 mL
29	Andersen et al. [54]	ABT	48	Blood	ELISA	Hepcidin + erythroferrone	ABT 450 mL and reinfusion of 130 mL of packed red blood cells
30	Lainé et al. [55]	rhEPO	14	Blood	ELISA	Hepcidin + iron	rhEPO single dose 50 IU/kg
31	Leuenberger et al. [56]	ABT	20	Blood	Colorimetry	Iron	ABT 500 mL
32	Audran et al. [57]	rhEPO	9	Blood	IRMA	Soluble transferrin receptor	rhEPO 50 IU/kg every day for 26 days
33	Birkeland et al. [58]	rhEPO	20	Blood	IRMA	Soluble transferrin receptor	rhEPO 5000 IU every 3 days for 30 days
34	Nissen-Lie et al. [59]	rhEPO	8	Urine	IEF	Soluble transferrin receptor	rhEPO 5000 IU 3× a week for 30 days

Abbreviations: ABT, autologous blood transfusion; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; IEF, isoelectric focusing; IRMA, immunoradiometric assay; LC–MS, liquid chromatography–mass spectrometry; MS, mass spectrometry; rhEPO, recombinant human erythropoietin; RT-PCR, real-time polymerase chain reaction.

longer in comparison with traditional markers. Therefore, if the heterogeneity in individual responses seems to be the major limitation when using a single marker,[37] a combination of multiple markers in a mathematical model completed with existing variables would certainly be the most promising approach.[102]

4 | PROTEOMIC ANALYSES

The following step in the genetic signaling cascade is represented by the proteome including all proteins translated and synthesized in the organism.[90] In an antidoping perspective, it is for example known that blood storage (i.e., for later reinfusion) impacts RBC membrane proteins.[104] A proteomic analysis, therefore, provides a great opportunity to identify markers reflecting the storage period of blood transfusion.[13] Multiple proteins located in the RBC transmembrane and cytoskeleton were indeed significantly altered after blood storage.[41] Likewise, markers associated with oxidative stress[40] and neocytolysis[44] were further suggested as indicators of ABT.

More recently, 14 proteins involved in RBC energy metabolism and membrane vesiculation confirmed the potential of membrane proteins in the development of new biomarkers, although the in vivo

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TABLE 4 Metabolomic biomarkers of blood doping.

Metabolomic ($n = 8$)							
Nb	**Title**	**Doping**	**Subjects**	**Matrices**	**Method of analysis**	**Main biomarker**	**Protocols**
35	Appolonova et al. [60]	rhEPO	2	Urine	LC–MS	ADMA + DDAH + NOS	rhEPO 2000 IU daily for 10 days
36	Bejder et al. [61]	ABT	12	Urine	LC–MS	DEHP + (iso)caproic acid glucuronide	ABT 900 mL
37	Monfort et al. [62]	ABT	226	Urine	LC–MS	DEHP metabolites	ABT 500 mL
38	Al-Nesf et al. [63]	ABT	66	Blood	LC–MS	Serum metabolites	ABT 450 mL
39	Varlet-Marie et al. [64]	ABT	34	Blood	GC–MS	DEHP	ABT 450 mL
40	Monfort et al. [65]	BT	221	Urine	LC–MS	DEHP metabolites	Blood transfusion for medical reasons
41	Leuenberger et al. [66]	ABT	15	Urine	LC–MS	DEHP metabolites	ABT 500 mL
42	Solymos et al. [67]	ABT	578	Urine	LC–MS	DEHP metabolites	Blood transfusion for medical reasons

Abbreviations: ABT, autologous blood transfusion; ADMA, asymmetrical dimethylarginine; BT, blood transfusion; DDAH, dimethyl-arginine dimethylaminohydrolase; DEHP, di(2-ethlyhexyl) phthalate; GC–MS, gas chromatography–mass spectrometry; LC–MS, liquid chromatography–mass spectrometry; NOS, nitric oxide synthase; rhEPO, recombinant human erythropoietin.

clearance kinetics remains to be evaluated.[42] Thus, the principal issue through this strategy is related to the small proportion of reinfused blood compared with the total blood volume. A unit of reinfused blood does not represent more than 5% of the recipient's blood volume for instance[105] with a nonnegligible part of RBC removed from the bloodstream in the first 24 h (5–10%),[104] hence shortening the detection window and reducing the sensitivity. In addition, immature reticulocyte proteins showed a greater response to rhEPO administration compared with the current ABP biomarkers: With a level below 75% of the baseline during 4 weeks following rhEPO injection when monitored as a ratio (below 75% of baseline), CD71 and ferrochelatase thus seem particularly promising and need further investigation.[39] In addition, using a validated liquid chromatography–mass spectrometry quantification method in dried blood spots,[106] cell-specific membrane proteins (CD71 and Band3) were altered up to 20 days post-transfusion.[43] Providing a specificity of ~70%,[43] these results are of significant interest for early erythropoietin stimulation screening.

5 | IRON METABOLISM

Erythropoiesis and iron metabolism are two interdependent processes that continuously influence all stages of the hematopoietic system.[107] Principal iron consumer in humans, the erythropoietic activity will decrease the hepcidin transcription through erythroferrone (ERFE) suppression.[108] This will result in a stimulation of iron absorption and delivery during erythropoiesis stress, essential to the subsequent production of new RBC. Consequently, because of the direct dependence of iron metabolism on the erythropoietic process,[109] longitudinal monitoring of specific iron-related biomarkers could offer a relevant way to better target doping practices.

Most of the iron in the human body is incorporated in circulating erythrocytes as the heme compound of hemoglobin. Iron is found in the ferrous (Fe^{2+}) state in hemoglobin molecules and therefore plays a vital role in the mere existence of RBC.[107] Rapidly considered for blood doping detection,[45,110] iron levels seem to largely increase within hours after blood reinfusion.[56] Therefore, quantifiable in ethylenediaminetetraacetic acid (EDTA) samples,[56] iron levels can be easily measured by using the same blood sample and are frequently used as additional information to the routine biomarkers in case of suspicious cases. However, iron injections may have a major impact on the individual iron level. Therefore, because iron supplementation is frequently recommended for endurance athletes[111] or during altitude training camp,[112] this remains a major limitation to ferritin implementation for longitudinal monitoring. In addition, a higher prevalence of hemochromatosis, a hereditary disease affecting the iron metabolism, has been observed in professional endurance athletes.[113,114] Due to the homeostatic iron regulator gene mutation, these genetic characteristics could therefore influence the iron longitudinal monitoring of the concerned athletes.

Known as a useful indicator of iron stores,[53] circulating ferritin decreased after blood withdrawal,[48,53] and tends to increase after reinfusion[46] but with lower amplitude.[53] In addition, the decrease in serum ferritin seems to be progressive with rhEPO treatment[47] although not always observed with microdoses.[49] However, similarly to iron, circulating ferritin can be significantly affected by repeated iron injections[48] and accentuated day-to-day variability

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TABLE 5 Biomarkers of blood doping related to the hematological variables.

Hematological variables ($n = 14$)							
Nb	**Title**	**Doping**	**Subjects**	**Matrices**	**Method of analysis**	**Main biomarker**	**Protocols (dose + frequency + duration)**
43	Pottgiesser et al. [68]	ABT	21	Blood	CO rebreathing	Hemoglobin mass	Multiple (3×) ABT 500 mL
44	Parisotto et al. [69]	rhEPO	73	Blood	Flow cytometry	Hematological variables + soluble transferrin receptor	rhEPO 50 IU/kg 3× per week for 25 days
45	Bejder et al. [70]	rhEPO	16	Blood	Flow cytometry	Reticulocyte percentage	rhEPO 65 IU/kg every second day for 14 days + 390 IU/kg on three consecutive days
46	Lamberti et al. [71]	ABT	24	Blood	LC–MS	Glycated hemoglobin-HbA1c/ HbPI + hematological variables	ABT 450 mL
47	Casoni et al. [72]	rhEPO	20	Blood	Flow cytometry	Hematological variables	rhEPO 30 IU/kg every day for 30 to 45 days
48	Parisotto et al. [73]	rhEPO	202	Blood	Flow cytometry	Hematological variables	rhEPO 300 IU/kg on Days 1, 4, 7, and 10 + 600 IU/kg on Days 1 and 10
49	Mørkeberg et al. [74]	ABT	44	Blood	Flow cytometry	Hematological variables + hemoglobin mass	Multiple (1–3×) ABT 450 mL
50	Mørkeberg et al. [75]	ABT	16	Blood	Flow cytometry	Hematological variables + RBCHb:RetHb ratio	ABT 450 mL
51	Parisotto et al. [76]	rhEPO	27	Blood	Flow cytometry	Hematological variables + soluble transferrin receptor	rhEPO 50 IU/kg 3× per week for 25 days
52	Gore et al. [77]	rhEPO	57	Blood	Flow cytometry	Hematological variables + soluble transferrin receptor	rhEPO 3× per week for 8 weeks: the first 3 weeks at a dosage of 50 IU/kg and the next 5 weeks at 20 IU/kg
53	Damsgaard et al. [78]	ABT	10	Blood	Flow cytometry	Hematological variables + soluble transferrin receptor	ABT 20% of the subjects' blood volume
54	Pottgiesser et al. [79]	ABT	10	Blood	CO rebreathing	Hemoglobin mass	ABT one or two unit of packed red cells
55	Jeppesen et al. [80]	rhEPO	39	Blood	Flow cytometry	Immature reticulocytes	rhEPO 20 IU/kg every second day for 3 weeks
56	Berglund et al. [81]	ABT	12	Blood	Hemoglobinometer	Hematological variables	ABT 1350 mL

Abbreviations: ABT, autologous blood transfusion; LC–MS, liquid chromatography–mass spectrometry; rhEPO, recombinant human erythropoietin.

has been observed in female athletes compared with the population standards.[115] Furthermore, as a positive acute phase protein (i.e., proteins increasing in response to inflammation), serum ferritin concentration may be modulated in various conditions without changes in iron storage.[116] Therefore, in addition to mild infections [117] exercise-induced inflammation-like reactions may impact ferritin levels,[118] increasing the longitudinal variability in an athletic population.

Key parameter in iron metabolism, the soluble transferrin receptor (sTfr) is a protein in charge of the transferrin-bound iron absorption[119] First observed with hemodialyzed patients[45] increased sTfr level following rhEPO injection was later registered in athletes[49,57–59] despite the observed disparity between ethnicity[120] and presumed shorter detection window than rhEPO effects on endurance performance.[58] Thus, in combination with other markers (e.g., Hct), sTfr has been considered in the first generation of blood biomarkers to detect rhEPO abuse in athletes.[69,76] In contrast to ferritin, sTfr levels are not affected by inflammatory reactions and physical exercise.[116] However, a gradual increase of sTfr level is usually observed during altitude training,[121] although the kinetics seem to be at some point different from the EPO level.[122]

Involved in the early stage of the iron metabolism system, ERFE is also known to play an important role in the erythropoietic process.[123] Dependent on EPO release from the kidney, ERFE level is consequently increased in the erythroblasts of the bone marrow.[124] Therefore, ERFE tends to increase after various types of ESA injections[48] including microdoses,[47] despite a large interindividual variability.[52] Regarding blood manipulation, an increase in ERFE is generally observed during the blood withdrawal phase,[54] followed by a decrease during the reinfusion phase. However, the known impact of

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TABLE 6 Biomarkers of blood doping related to the red blood cell properties.

Red blood cells properties ($n = 7$)							
Nb	**Title**	**Doping**	**Subjects**	**Matrices**	**Method of analysis**	**Main biomarker**	**Protocols**
57	Robinson et al. [82]	rhEPO	475	Blood	Photometry	Erythrocyte aspartate aminotransferase activity	rhEPO 2000–18,000 IU per week
58	Voss et al. [83]	ABT	6	Blood	Flow cytometry	Red blood cell extracellular vesicles	ABT 450 mL
59	Voss et al. [84]	ABT	8	Blood	Flow cytometry	Red blood cell microparticles	Whole blood storage
60	Bizjak et al. [85]	ABT	6	Blood	Ektacytometry + viscometry	Red blood cells rheology	ABT 500 mL
61	Grau et al. [86]	ABT	8	Blood	Ektacytometry + viscometry	Red blood cells rheology	ABT 500 mL
62	Donati et al. [87]	ABT	n.a.	Blood	Flow cytometry	Red blood cells size + density	Whole blood storage
63	Harrison et al. [88]	ABT	4	Blood	Electrophoresis	Red blood cells size + distribution	Whole blood storage

Abbreviations: ABT, autologous blood transfusion; rhEPO, recombinant human erythropoietin.

multiple confounding factors such as altitude exposure[47,125] or physical exercise[126] on ERFE basal level mainly limits the relevance of this marker for antidoping purposes. In addition, the reliability and standardization of analytical measurement remain a major issue with ERFE, especially with high endogenous levels[127]

By inhibiting the ferroportin in charge of iron transport, hepcidin is another central regulator of iron metabolism.[109] If hepcidin tends to decrease after multiple rhEPO administrations,[47,52] an early increase in serum concentration has been noticed after a single intravenous injection,[55] and the opposite relationship was observed during ABT scenario until 1 day after blood reinfusion[53] due to a suppression of hepcidin during increased erythropoietic activity periods. Because of the interindividual variations,[53] a longitudinal follow-up complementing the ABP seems to be the most relevant approach.[52] In addition, hepcidin was shown not to be impacted by altitude training[125] and would yield a relevant discriminative power in an antidoping context with athletes prone to use hypoxic exposures. Finally, other interesting serum proteomic isoforms were investigated for ESAs screening,[50,51] such as haptoglobin, serotransferrin, transferrin, or hemopexin isoforms, and would deserve further investigation.

The investigation of multiple markers from iron metabolism demonstrated a high potential of the iron metabolism in the development of new biomarkers, both for ABT screening [13] and rhEPO abuse.[128] The robustness of current analytical methods for markers such as hepcidin by liquid chromatography-high resolution mass spectrometry[129] makes them biomarkers serious candidates for longitudinal monitoring allowing a more robust interpretation of abnormal hematological variations.[52] Based on these findings, a recent study tested hepcidin and ERFE as additional markers of the ABP following low-volume ABT.[54] The ability to detect true positives following blood reinfusion was improved by 83%. In addition, the confirmed correlation between hepcidin, ferritin, and ERFE[52] supports the potential interest of a multiple marker combination similar to the current OFF score. However, the nonspecificity of these iron-related markers to blood doping practices must be carefully considered. Consequently, the examination of these additional markers should therefore be conducted with a holistic approach considering other biomarkers.

6 | METABOLOMIC ANALYSES

Final stage in the *-omics* cascade, a metabolomic approach seems to offer particularly promising perspectives for complementary biomarkers of doping abuse.[130] Metabolomics analysis seeks to identify a set of specific metabolites by using targeted approaches or a wide range of metabolites through untargeted metabolomics protocols in biological samples.[131] A targeted metabolomic approach was initially applied in the context of rhEPO abuse.[60] Thereafter, investigations were mainly related to identify ABT after exogenous blood storage.[65] Providing a longer shelf life for blood bags intended for reinfusion, di-(2-ethylhexyl)phthalate (DEHP) is a plasticizer commonly applied during blood storage to preserve the flexibility of the plastic bag.[132] Therefore, an initial study investigated the presence of DEHP metabolites using liquid chromatography–tandem mass spectrometry in patients' urine receiving a blood transfusion, observing detection windows up to 48 h post-reinfusion[65] and demonstrating good longitudinal stability.[133] By using a validated ultraperformance liquid chromatography–tandem mass spectrometry quantification method,[67,134] these results were later confirmed in healthy moderately trained individuals,[62] even with bags claimed to be plasticizer-free,[66] where 5cx-MEPP and 2cx-MMHP emerged as the most interesting markers because of their extended half-life.[13] In addition, DEHP was also detected in blood samples using gas chromatography–mass spectrometry, although the very short detection window limits its interest.[64] However, the

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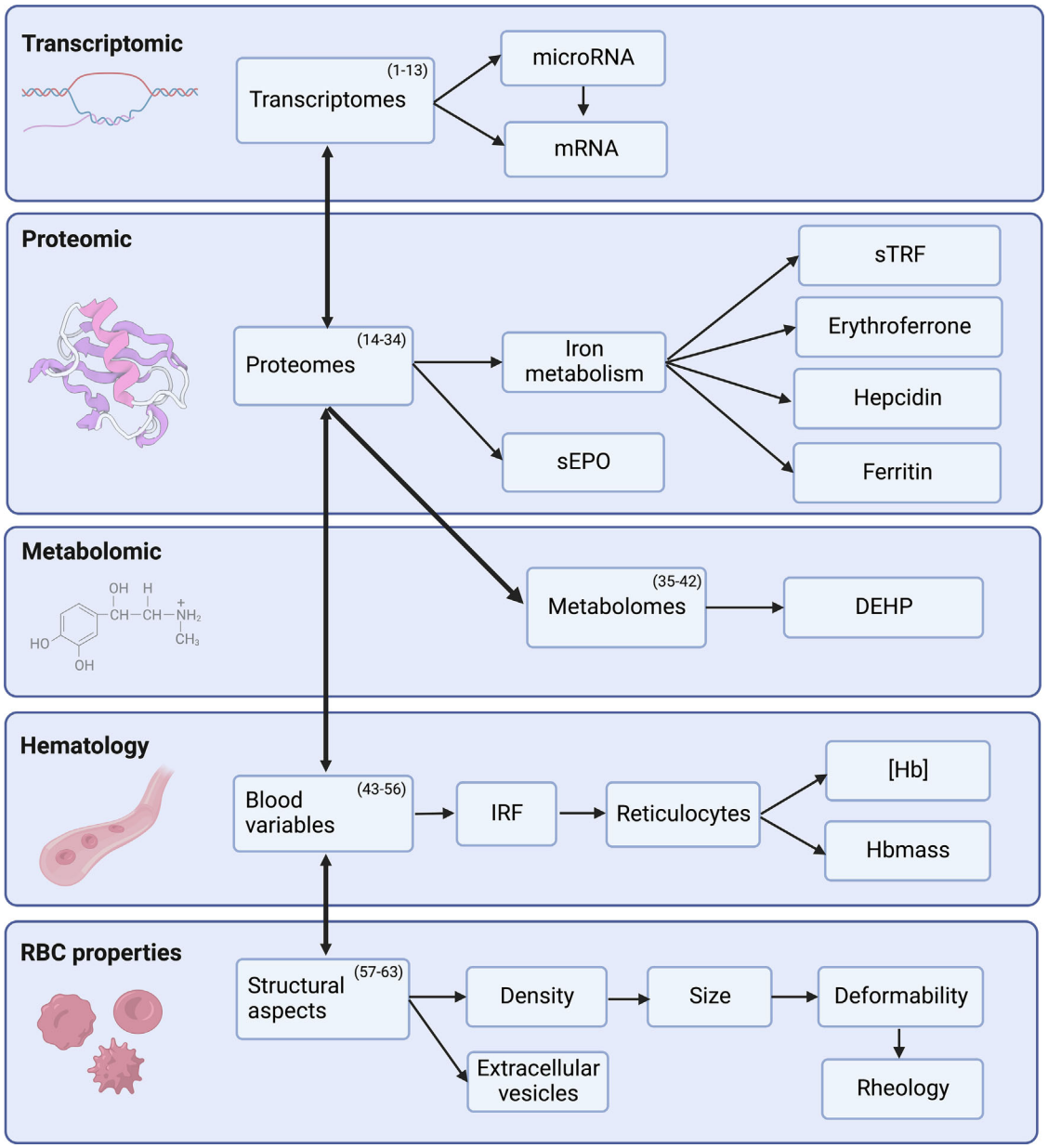

FIGURE 2 Investigated biomarkers of blood doping.

contamination risk should not be excluded, and various sources of external intake mainly related to contaminated foods[135] or medical procedures involving plastic materials experience[136] have been observed, justifying the caution observed by sports authorities when evaluating DEHP results.[66]

Presently, explorative metabolomics protocols seem to be the most promising approach to identify indirect markers of doping abuse.[137] Nevertheless, untargeted metabolomics-based strategies were only suggested very recently in the lack of prior spectral databases and compound libraries for the identification of targeted marker candidates. Focusing on urine samples, a first study suggested several metabolites from plasticizers as the most relevant markers after blood reinfusion.[61] On the other hand, a second investigation reported 11 serum and 8 urinary metabolites altered at different time points after blood donation and up to 7 days post-reinfusion.[63] Although plasticizer markers tend to be rapidly eliminated, nine other serum and urine metabolites showed a detection window beyond 7 days, making their monitoring particularly promising for ABT screening. Therefore, based on these promising results, metabolomics will definitely be the field of forthcoming research in the near future,[137] although external influences can be sources of disturbances during a longitudinal follow-up (e.g., intense exercise or nutritional supplements).[138,139]

7 | HEMATOLOGICAL VARIABLES

Conclusively, hematological variables represented a direct snapshot of an athlete's hematological status and erythropoietic processes. It

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obviously supports the use of full blood counts as robust variables sensitive to blood doping and with known (and explainable) confounders.[140] The influence of blood transfusions and rhEPO on hematological values and exercise performance has been identified for a long time[58,141,142] with widespread use of transfusions as ergogenic aids before their prohibition. A range of hematological biomarkers of altered erythropoiesis was first proposed for the detection of ABT in cross-country skiers.[81] Analyzed by flow cytometry, [Hb] and Hct were mainly identified as sensitive markers of ESA intake with a significant increase after rhEPO treatment.[69,72,76,77,143] Following blood transfusion, a drop is usually observed after blood withdrawal, followed by an opposite kinetic after reinfusion.[74,75,78] Because antidoping controls were originally limited to competition day, hematological markers had the central benefit of being immediately analyzed, making it possible to test the athletes before the race.[144] In contrast, any abnormal variation in these markers would require longitudinal monitoring to track atypical changes. In combination with other markers sensitive to altered erythropoiesis, this thinking historically then led to the development and implementation of the ABP in the late 2000s.[21]

Commonly used as a marker of the erythropoiesis status,[145] reticulocytes have been deeply investigated for antidoping purposes. Increasing during rhEPO supplementation phase, the reticulocytes percentage (Ret%) is usually downregulated upon rhEPO cessation.[72,73,77] Currently implemented as a secondary marker in the ABP, a study supported the relevance of using the Ret% as a standalone variable with a twofold higher sensitivity during rhEPO supplementation.[70] In addition, despite the reported impact of altitude training in elite athletes on the immature reticulocyte fraction level,[146] recent results demonstrated the sensibility of immature reticulocyte fraction in the detection of low doses of rhEPO at sea level and altitude.[80] Therefore, despite an expected increase in false positives profile caused by external confounders, these outcomes highlight the great interest of Ret% parameters in future biomarkers development. Moreover, a dose-dependent increase of the mean corpuscular volume following rhEPO treatment in both mice and humans was reported.[147] By regulating the number and speed of cell divisions as well as the duration of terminal differentiation through the signaling of the erythropoietin receptor,[147] mean corpuscular volume, as a biomarker of the ABP and included in the calculation of the Abnormal Blood Profile Score,[148] could be promising as an individual marker also because it appears to be reasonably stable during altitude training.[149] Finally, RBCHb/RetHb ratio[75] and hemoglobin types (glycated hemoglobin-HbA1c and HbPI)[71] seem to be promising parameters to detect ABT reinfusion by suggesting alternative erythrocyte parameters and require complementary examinations.

By combining multiple variables, applications of statistical classification techniques were subsequently investigated to increase the specificity of single markers.[150,151] For instance, the Abnormal Blood Profile Score or the ON/OFF scores have been specially designed to identify the early days and cessation phases of rhEPO intake.[77] Included in the ABP, the 14 current variables of the hematological module remain the only biomarkers actually used for the indirect detection of blood doping.[152] However, although numerous studies confirmed the sensitivity of the ABP to multiple blood doping protocols,[153–155] the hematological module of the ABP has shown its limitation to identify rhEPO microdoses[156] and direct detection opportunities remain possible.[14] In addition, consistently adjusted by the body, plasma volume (PV) plays a key role in the variability of many markers of the ABP,[157] especially on [Hb] and the resulting OFF score. Consequently, multiple confounding factors (e.g., prolonged physical exercise) have demonstrated a significant effect on ABP variables mainly due to their effect on PV,[158] making the interpretation of individual profiles particularly challenging.[140] In this context, a multiparametric approach using protein blood biomarkers has been suggested to remove the influence of PV on the ABP,[159,160] namely, transferrin, creatinine, calcium, platelets, low-density lipoproteins, albumin, and total proteins. Applied to endurance athletes' profiles[161,162] and more recently in active women taking oral contraception,[163] this approach may definitely strengthen the interpretation of the ABP by providing the correction of concentration-based variables.

To cope with these limitations, total hemoglobin mass (Hbmass) was investigated, showing a high sensitivity following various transfusion protocols.[164,165] Measured by the CO-rebreathing method,[79] the natural stability of Hbmass is an essential asset for longitudinal monitoring,[68] in particular when this marker is combined in multiple ratios.[74] Furthermore, an accurate assessment of PV is provided using the same CO-rebreathing technique. Therefore, the absolute measurement of PV could be of particular interest in the evaluation of the ABP variables measured in concentration (e.g., [Hb]), thereby emphasizing the impact of potential confounding factors. However, due to additional influences such as altitude training or periods of reduced training, the stability seems to be lower in elite athletes.[166] In addition, the necessary collaboration of the athlete,[167] as well as the toxic nature of the carbon monoxide currently used during the measurement protocol,[105] remains a major limitation to its implementation in the routine antidoping field.

8 | RBCs PROPERTIES

Finally, other forms of biomarkers were investigated by targeting the mechanical properties of RBC. Among the morphological characteristics, a difference in the relative size distribution of RBC between fresh and stored blood was identified by using capillary electrophoresis.[88] Following the same approach, several potential biomarkers such as erythrocytes size, density, and microparticles have shown interest in the screening of ABT by using flow cytometry in stored blood samples.[87] Besides, the aspartate aminotransferase activity has been suggested to estimate the RBC rejuvenation triggered by rhEPO intakes.[82] However, chronic hypoxia exposure showed a very similar pattern, thereby limiting the interest of this enzyme as a blood doping marker.

Induced by an alteration of multiple membrane proteins, RBC-microparticles (MP) release is a well-known consequence of prolonged blood storage.[168] By using a simple storage procedure (whole blood

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storage at 4°C), findings revealed a large RBC-MP increase after 14 days of blood storage.[84] However, subsequent results showed an important interindividual clearance variability after reinfusion,[83] suggesting a longitudinal approach of RBC-MP as potential additional evidence during ABP interpretation. Defining nanometric-size extracellular vesicles released by cells, exosomes play a role in multiple physiological and pathological mechanisms.[169] Also known to be released by RBCs during blood storage,[170] exosomes could therefore be highly relevant markers for the screening of blood transfusion. Nevertheless, the impact of disease conditions and other environmental stressors on these extracellular vesicles[171] needs to be attentively considered.

In addition, RBC rheological parameters were monitored after blood reinfusion to understand the in vivo impact of RBC cryopreservation (storage at −80°C): Despite no significant influences with low volumes (~4% of total blood volume),[85] results highlighted a shift in the age distribution of RBC subpopulations as well as a deformability alteration after a reinfusion representing 7.7% of the total blood volume.[86] Nevertheless, the clearance of these markers once the blood is reinjected has not always been investigated.[40,42,84,87] Crucial in an antidoping context, further studies are needed to determine the detection window of such markers and thus evaluate their suitability for ABT detection.

Therefore, going from the beginning to the end of erythropoiesis metabolism, current studies have investigated different steps of RBC production. The hematological variables are the most investigated markers so far. Nevertheless, if several markers such as reticulocyte-related mRNA (e.g., ALAS) or storage metabolites (e.g., DEHP) are emerging as robust markers, these new biomarkers are still in the investigation phase. Therefore, more research seems to be required before considering these markers as complements to the current variables of the ABP. Finally, alternative models to estimate early erythropoietic stimulation for clinical purposes[172] provide a glimpse of future innovative advances applicable to an antidoping context and ultimately improve the fight against doping.

9 | CONCLUSION

Multiple approaches are being investigated for the confirmation of new biomarkers relevant to the detection of blood doping. Because it enhances endurance performance through an improved convective oxygen transport, stimulation of the erythropoiesis was primarily targeted. However, due to hormonal negative feedback occurring after altered erythropoietic homeostasis, a drop in RBC production can similarly be used as an indication of possible doping practices.

In addition to the hematological variables currently used by ABP, the application of *-omics* strategies for antidoping purposes seems to be particularly promising in the identification of new markers related to rhEPO or ABT protocols.[91] Providing a complementary source of information,[173] a multi-biomarker combination approach using algorithms equivalent to the OFF score currently used by the ABP may be the most powerful approach to increase the detection window.[13] If the transcriptomic approach seems to be the most studied to date, other *-omics* approaches are currently undergoing promising research for the detection of blood doping. In addition, alternative approaches related to the RBC properties have been investigated and particularly for the screening of ABT. Focusing on hemorheological parameters or membrane lesions after various storage procedures, results confirmed the interest in RBC morphological characteristics for longitudinal or confirmation procedures and should be further investigated with reinfusion protocols.

Nevertheless, the complexity of the genetic material and the high interindividual variability remain crucial questions that need to be investigated.[90] In conclusion, this review highlights multiple confounding factors that could impact these newly suggested biomarkers. Therefore, like the current ABP variables, these new biomarkers would require extensive examinations to determine the influence of common intrinsic and extrinsic factors before considering their implementation in the field.[27] Furthermore, the large interindividual variability reported by several results indicates that an individual approach as currently apply with the ABP is the necessary approach to distinctly target doping practices. Consequently, the future of indirect detection will probably require the implementation of complementary variables to the existing model, providing higher sensitivity and specificity related to blood doping.

AUTHOR CONTRIBUTIONS

BK, RF, and JS conceived the project and designed the review. BK, JS, and RF contributed to the acquisition and analysis of data. BK, RF, JS, FD, and FB interpreted the results. BK wrote the first draft of the manuscript. BK, JS, RF, FD, and FB contributed to revising the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors do not have any conflict of interest or competing interests to disclose.

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