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RESEARCH ARTICLE

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Minor red blood cell antigen phenotyping of athletes sampled in international competitions

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Abstract

Blood transfusion is performed by cheating athletes to rapidly increase oxygen delivery to exercise muscles and enhance their performance. This method is banned by the World Anti-doping Agency (WADA). Heterologous or allogenic blood transfusion happens when blood from a different person is transfused. The method used to detect this type of doping is based on flow cytometry, by identifying variations in blood group minor antigens present on the red blood cells' surface. Transfusion practices have regained interest since the introduction of human recombinant erythropoietin detection method. It has been reported that the number of occurrences of two athletes sharing an identical phenotype in the same sport was five times higher than the theoretical populational probability. The present work describes the prevalence of 10 erythrocytes surface antigens in a population of 261 athletes from all five continents. The matching phenotype per sport is also described.

KEYWORDS
athletes, blood doping, flow cytometry, homologous blood transfusion, red blood cell antigens

1 | INTRODUCTION

The practice of blood boosting consists of prohibited methods performed by cheating athletes to increase blood cell mass, resulting in extra oxygen transport to muscles, thus conferring an unfair performance advantage. One of these methods, prohibited by the World Anti-doping Agency (WADA), is blood transfusion.[1,2] Blood transfusion doping in sports peaked in the 1980s, and its use fell in the following decades because of the commercial introduction of recombinant human erythropoietin (rhEPO).[3] Because of the easy access and its high impact on the athletes' performance, the use of rhEPO was disseminated during the 1990/2000 decades.[4] However, with the development of anti-doping laboratory techniques capable of detecting rhEPO, the use of this hormone tends to decrease considering the risk of adverse analytical findings, and consequently, the use of blood transfusion regained interest among cheating athletes.[3,5] Homologous blood transfusion (HBT) cases were detected during the Tokyo 2020 Olympic Games, demonstrating that HBT has been revisited as doping practice.[6] A blood transfusion may be autologous, when the athlete uses his/her own blood, or homologous, when the

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athlete receives blood from a compatible donor for the ABO and Rh systems.[3] Autologous blood transfusion (ABT) is a complex procedure, requiring withdrawal and proper storage of the athlete's blood, which can be avoided in the misuse of HBT.[7] Every red blood cell (RBC) of an individual has an identical and specific spectrum of blood group antigens under genetic control.[1] HBT detection method is based on the analysis of erythrocyte populations by flow cytometry, utilizing antisera in combination with fluorescent-labeled secondary antibodies, seeking differences in minor antigens of blood groups.[1,7] HBT can be performed using different blood volumes. The infusion of one unit of blood (approximately 450 ml) will dilute the recipient's blood by around 10%.[8] Lower volumes, such as 135 ml were shown to increase athlete's performance.[9] Flow cytometry analysis sensitivity has been estimated to be 0.07%, and this is nowadays the method of choice in anti-doping laboratories around the world since the 2004 Summer Olympic Games in Athens. This approach is a direct method for detecting the presence in an athlete's blood of two different RBC populations, which is obtained by two separate histogram peaks.[1,5,10]

According to the International Society of Blood Transfusion, there are 43 recognized blood group systems containing 345 RBC surface antigens.[11] It makes the combination of identical phenotypes unlikely, making it possible to identify HBT dopers.[1,8] According to a Western European population study, the likelihood of two ABO and Rh compatible blood samples also being compatible for a panel of 12 other groups of blood antigens is less than 0.2%.[8] However, differences can be expected in a population with different genetic backgrounds. A study with a population of 535 athletes demonstrated that, for a panel of 10 antigens, the number of identical sets of antigens was five times greater than the theoretical probability.[7] Different authors and anti-doping laboratories use different panels of erythrocyte antigens to detect mixed populations, adding an extra layer of complexity for those who propose to compare data around the world. Herein, we provide further information regarding HBT analysis in athletes sampled in international competitions. Hence, the study aimed to compile the phenotypes referring to minority antigens of athletes from five continents of different ethnicities.

2 | MATERIAL AND METHODS

2.1 | Blood samples

Samples were collected from athletes for HBT anti-doping purposes according to WADA standards (Guidelines for Sample Collection, 2021 v 1.0). "Confidentiality and reporting of the World Anti-Doping Code" was accomplished since no personal data able to identify athletes were obtained. All the information used in this work came from the testing authorities meaning that all personal information has been de-characterized before samples arrived at the laboratory. Peripheral whole blood was collected in EDTA tubes (BD Vacutainer K2EDTA, REF360057, UK) in the venue and immediately transported to anti-doping laboratory under monitored refrigeration. Analyses were finalized within 30 h of arrival at the laboratory. The laboratories involved in the study have the methods under ISO17025 accreditation.

2.2 | Erythrocyte phenotyping

For HBT, certified phenotyped RBCs (ID-Diacell, Bio-Rad, São Paulo, Brazil) were used as controls.

A volume of 250 µl of homogenized whole blood was washed twice with Cell Stab buffer (BioRad, Diamed, Brazil), centrifuging at 1500*g* for 3 min between each wash step, and the final pellet was resuspended in Cell Stab buffer to a 0.07 × 10^6 RBC concentration. A volume of 25 µl of cell suspension was added to 12 different wells

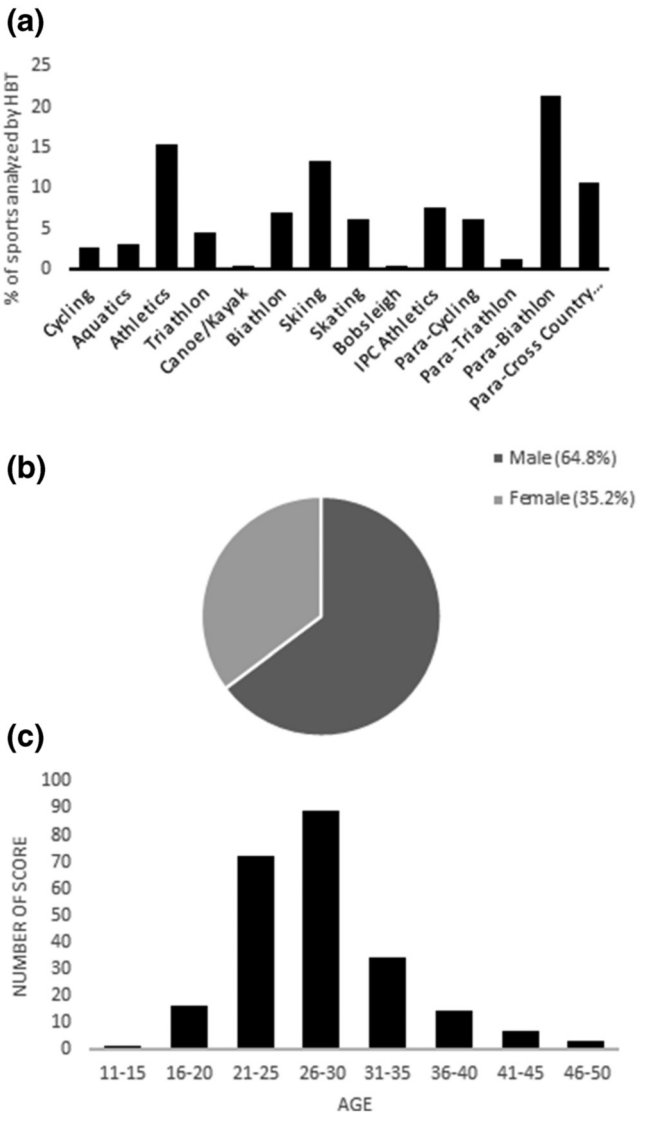

FIGURE 1 General data of athletes analyzed by homologous blood transfusion (HBT). Bar graphs show the type and frequency of sports analyzed by HBT (a). Percentage of male and female athletes analyzed by HBT (b), and distribution of athletes analyzed by HBT by age (c)

in a 96-well round bottom polypropylene plate (Costar, Corning, USA) and washed with 175 µl of flow buffer (BSA 0.1%, Sodium Azide 0.05% in PBS). The final pellet was resuspended with 50 µl of different primary antibodies at their optimal dilutions or PBS and incubated for 30 min at room temperature (RT). RBCs were phenotyped for different surface antigens, C, c, E, Jka, Jkb, and K (IgM, Diaclon, BioRad, Switzerland) and S, s, Fya, and Fyb (IgG, ID-Antigen Profile III, BioRad, Switzerland). After three washings with 200 µl of flow buffer, cells were incubated for 30 min, protected from light, with 50 µl of the respective secondary antibodies: PE-conjugated IgG Gt F (ab′)2 anti-human-IgM or -IgG (Life Technologies, USA) at optimal dilutions. The two PBS wells were incubated with 50 µl of a mixture of the two PE-conjugated secondary antibodies (1:1) or with FITC-conjugated anti-glycophoryn (CD235a) (Beckman-Coulter, France). Cells were washed twice, resuspended with 200 µl of flow buffer, and sent to flow cytometry acquisition (FC-500 ou Gallius, Beckman-Coulter, USA). Laser alignment was checked using Flow Check Fluorospheres beads (6605359, Beckman-Coulter, USA). Non-stained cells were used to adjust cytometer voltage and gain settings. Cells labeled only with the secondary antibodies were used as negative controls. Flow cytometry analyses were carried out using CXP 2.2 software (Beckman-Coulter). 50,000 events were acquired. For analyses, RBCs from samples were first selected by specific Glycophorin-A-labeling, observed as a positive population in a number (Y axis) versus fluorescence intensity (X axis, in log scale) histogram. The main population, excluding any high expressing cell due to agglutination, was backgated to the FSC × SSC scatter plot in linear scale, in which the final gate for RBCs was selected excluding undesired RBCs by size and complexity.

3 | RESULTS

3.1 | Profile of athletes and sports analyzed by HBT

Athletes were from 14 different sports (Figure 1a), and most were from endurance modalities such as athletics, skiing, para-biathlon, and para-cross country skiing, representing 60.9% of the total analyzed. In total, we analyzed 261 samples from 169 male and 92 female international athletes (Figure 1b) by HBT with ages ranging from 15 to 49 years (Figure 1c), in 51 countries from the five continents (Figure 2a,b).

3.2 | Erythrocyte phenotype of athletes

Phenotypes of the minor blood groups were evaluated with a panel of minor surface antigens (**C**, **c**, **E**, **e**, **Fya**, **Fyb**, **JKa**, **JKb**, **K**, **S**, and **s**). The surface antigens have a binary behavior, being either present or not

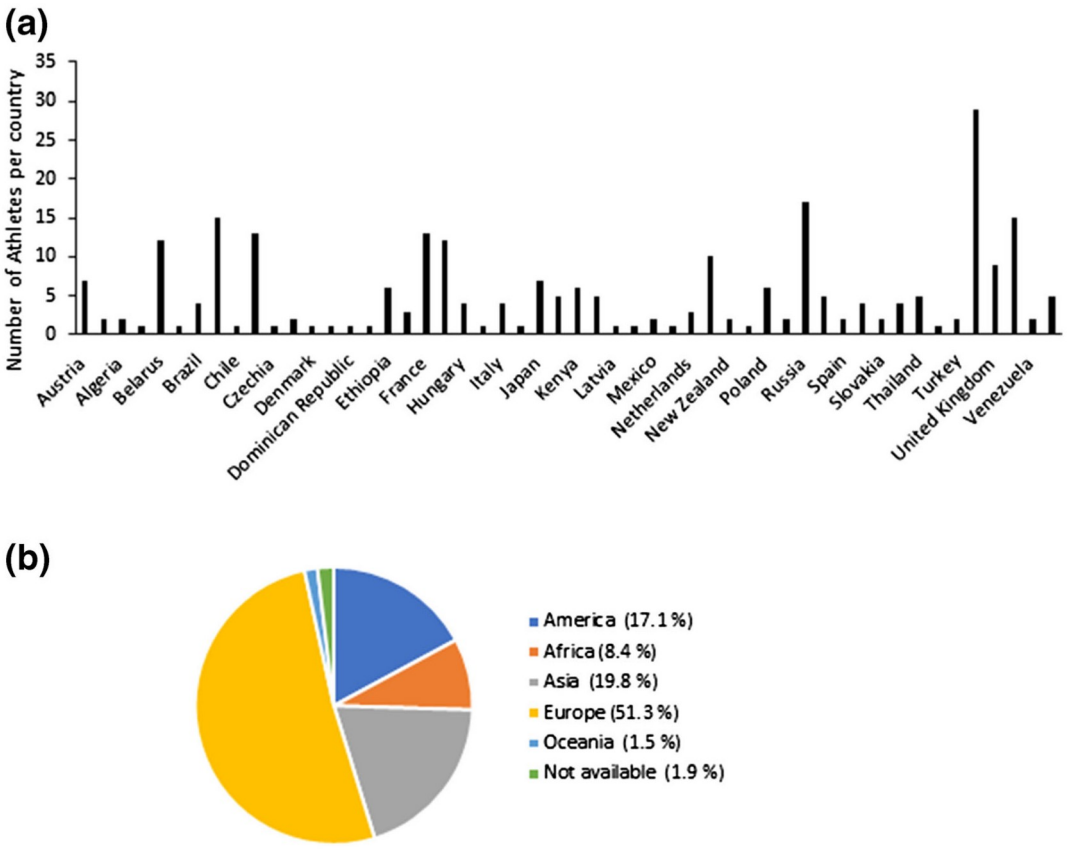

FIGURE 2 Distribution of athletes analyzed by homologous blood transfusion (HBT). Graphs represent the distribution of athletes per country (a) and the percentage of athletes per continent (b) [Colour figure can be viewed at wileyonlinelibrary.com]

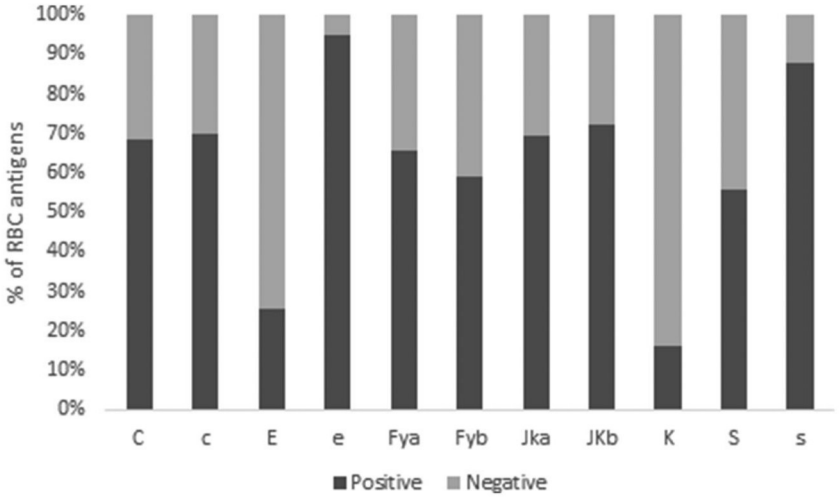

FIGURE 3 Frequency of antigens analyzed in homologous blood transfusion (HBT). Frequency of red blood cell (RBC) minor antigens presented in athletes analyzed by HBT

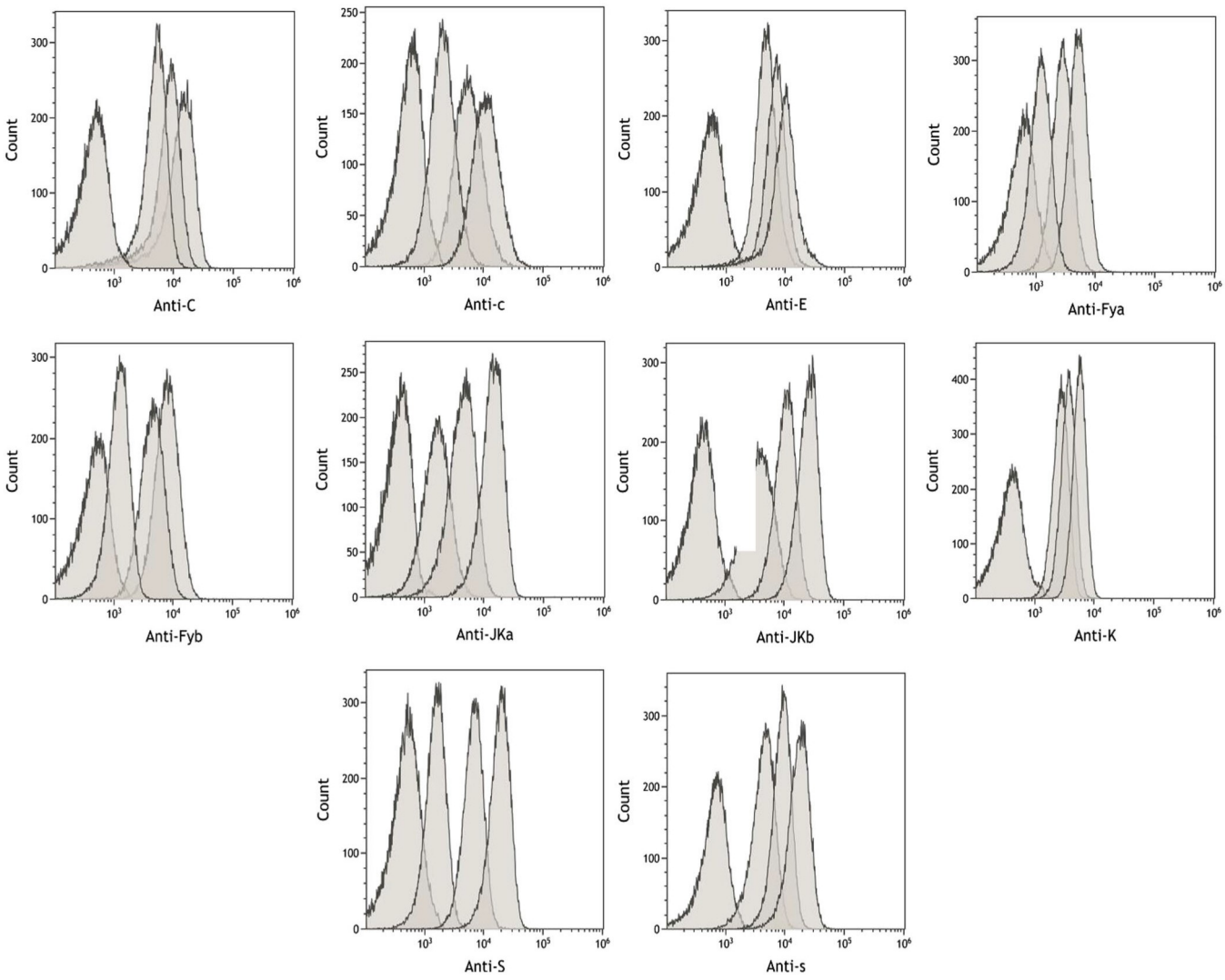

FIGURE 4 Histogram peak variations observed among antigens analyzed. The first peak of each histogram represents a certified red blood cell non-expressing the antigen incubated with the respective antibody. All antibodies were used at optimal dilutions. [Colour figure can be viewed at wileyonlinelibrary.com]

on the erythrocyte membrane. The frequency of positive antigens observed was **C**, 68.5%; **c**, 69.8%; **E**, 25.7%; **Fya**, 65.8%; **Fyb**, 59%; **Jka**, 69.6%; **Jkb**, 72.4%; **K**, 18%; **S**, 55.9%; and **s**, 87.7% (Figure 3).

3.3 | Variations in media intensity fluorescence on histograms peaks

The histograms for each antigen were analyzed separately, and a different pattern of histogram peaks among athletes was observed, showing that the exact positions of the peaks were not identical (Figure 4). Thus, the mean fluorescence intensity (MFI) for each antigen was calculated to understand these results better. Our results demonstrated a variation between the MFIs for each antigen tested, showing that individuals have a different level of antigens expression in their RBCs, which is expected. To ensure that this phenomenon was not an artifact because of laser alignment calibration on flow cytometry, we checked the expression of these antigens in certified RBCs (DiaCell) from the same batch. We found variations in their coefficient of variation (CV); however, in all cases, such variation observed in certified RBCs (DiaCell) was significantly smaller than those observed in athletes. These data strongly suggest that differences observed in athletes are due to individual variations; otherwise, CVs would be similar between samples and certified RBCs. Furthermore, the more significant the difference in CV, the greater chance that the difference in antigen expression is because of individual variations (Table S1).

3.4 | Similar phenotypes shared by sports

Using a panel of 10 antigens, **C**, **c**, **E**, **Fya**, **Fyb**, **Jka**, **Jkb**, **K**, **S**, and **s**, we have identified athletes from the same sport presenting the same RBC phenotype. In triathlon and cycling, a pair of athletes, and in athletics, two pairs and two triads (Table 1) shared the same RBC antigen phenotype. Other samples, analyzed by a panel of eight antigens, **C**, **E**, **e**, **Fya**, **Fyb**, **Jka**, **Jkb**, and **S**, also have demonstrated shared phenotypes among athletes from the same sport: two pairs, one triad, and two quartets of athletes in para-biathlon; two pairs and one triad in biathlon; one pair in skating; three pairs in para-cross country skiing; and two pairs, one quintet, and one ennead in skiing (Table 2).

4 | DISCUSSION

The use of blood transfusion as doping has regained attention among cheating athletes since anti-doping laboratories have developed techniques capable of detecting rhEPO, making techniques to detect HBT and autologous needed. For HBT, flow cytometry is the method of choice, representing the gold standard technique since its first

TABLE 1 Similar phenotypes shared by two or more athletes tested by HBT for the presence or absence of **C**, **c**, **E**, **Fya**, **Fyb**, **Jka**, **Jkb**, **K**, **S**, and **s** antigens are summarized by sport

Sports/number of shared phenotypes	Phenotypes									
Triathlon (2)	C^+	c^+	E^-	Fya^+	Fyb^+	Jka^+	Jkb^+	K^-	S^-	s^+
Cycling (2)	C^+	c^+	E^-	Fya^+	Fyb^-	Jka^-	Jkb^+	K^+	S^+	s^-
Athletics (2)	C^+	c^+	E^-	Fya^-	Fyb^-	Jka^+	Jkb^-	K^-	S^+	s^+
Athletics (3)	C^+	c^-	E^+	Fya^+	Fyb^-	Jka^-	Jkb^+	K^-	S^-	s^+
Athletics (2)	C^-	c^+	E^-	Fya^-	Fyb^+	Jka^+	Jkb^-	K^-	S^-	s^+
Athletics (3)	C^-	c^+	E^-	Fya^-	Fyb^-	Jka^+	Jkb^-	K^-	S^-	s^+

TABLE 2 Similar phenotypes shared by two or more athletes tested by HBT for the presence or absence of **C**, **E**, **e**, **Fya**, **Fyb**, **Jka**, **Jkb**, and **S** antigens are summarized by sport

Sports/number of shared phenotypes	Phenotypes							
Para-biathlon (2)	C^+	E^+	e^+	Fya^+	Fyb^-	Jka^+	Jkb^+	S^-
Skiing (9)/para-biathlon (4)	C^+	E^-	e^+	Fya^+	Fyb^+	Jka^+	Jkb^+	S^+
Biathlon (2)/skating (2)	C^+	E^-	e^+	Fya^+	Fyb^+	Jka^-	Jkb^+	S^-
Skiing (5)/para-cross country skiing (2)/para-biathlon (2)	C^+	E^-	e^+	Fya^+	Fyb^-	Jka^+	Jkb^+	S^-
Para-biathlon (4)	C^+	E^-	e^+	Fya^+	Fyb^-	Jka^+	Jkb^+	S^+
Skiing (2)	C^+	E^-	e^+	Fya^-	Fyb^+	Jka^+	Jkb^-	S^+
Biathlon (3)/para-biathlon (3)/para-cross country skiing (2)	C^+	E^-	e^+	Fya^-	Fyb^+	Jka^+	Jkb^+	S^+
Skiing (2)	C^+	E^-	e^+	Fya^-	Fyb^+	Jka^-	Jkb^+	S^+
Biathlon (2)	C^-	E^+	e^+	Fya^+	Fyb^+	Jka^+	Jkb^+	S^-
Para-cross country skiing (2)	C^-	E^+	e^+	Fya^+	Fyb^+	Jka^+	Jkb^+	S^+

description by Nelson and colleagues.[8] Since then, many modifications of the original protocol have been performed by different labs to increase sensibility and specificity and to decrease time consumption at the bench. Two modifications should be emphasized: the use of phycoerythrin (PE) fluorochrome, instead of fluorescein isothiocyanate (FITC) fluorochrome,[10,12] and the use of 96-well plates for labeling the RBCs, instead of standard flow cytometry tubes.[7] The exchange of FITC to PE was one of the most crucial modifications since PE is one of the brightest fluorochromes and consequently one of the most useful for detecting rare events, which is the case of donor RBCs. Because of it, the use of PE is known to increase sensibility compared with FITC; the sensibility obtained by our group was higher than the one obtained by Giraud and coworkers[5] (data not shown). The exchange of flow cytometry tubes by 96-well plates was important to reduce the time at the bench from at least 6 h to at most 3 h (from the bench up to the acquisition in the flow cytometry).

It is known that many RBC antigens are expressed in approximately 99% of the population, whereas others are almost not expressed (less than 1%), making them undesired candidates for HBT screening.[12]

Herein, we have analyzed the phenotype of the minor blood groups from athletes from the five continents. To the best of our knowledge, this is the first study to compile this range of viability, different ethnicities, and sports modalities. The findings reported in the present study may open the way for further research to assess whether such phenotypes would be associated with genetic characteristics capable of conferring advantages in different types of competition.

Our results demonstrate that the best antigen candidates for HBT are **C**, **c**, **Fya**, **Fyb**, **Jka**, **Jkb**, and **S**, once they have a moderate frequency being expressed from 50% to 70% of the athletes analyzed in our study. It was previously reviewed that -**s** and -**K** antigens are found with a moderate frequency being useful in HBT analysis.[12] However, herein with an international cohort of athletes from different sports, we have demonstrated that -**K** has a minor frequency (16%) and -**s** have a high (87.7%) frequency and, therefore, are not considered suitable antigens.

Even though the procedures have been reviewed and ameliorated throughout the years, histogram peaks are sometimes classified as uncertain, presenting large shoulders, or overlapping peaks. Such uncertainty could be the result of RBC agglutination and nonoptimal incubations, for example. However, such large peaks, often recognized as uncertain peaks, can result from mixed RBC from different donors once we have demonstrated a slight difference in MFI for the same antigen regarding two different donors. Because of this, anti-doping labs should revise the strict criteria that define when a sample is suspicious or positive for HBT. Nowadays, to give a positive result, there is a need for at least two different RBC antigens, two clearly defined and separated peaks, a reason that may justify, together with the reasons explained above, why we have a low number of suspicious or positive HBT reported since 2008. Krotov and coworkers have shown that the frequency of RBC antigen compatibility among athletes belonging to the Russian team was five times higher than the theoretical probability,[7] similar to our findings herein. Our results have demonstrated that when analyzing panels of eight to 10 antigens, the chance of finding similar phenotypes among athletes from the same sports cannot be discarded. HBT is still in use, as shown by the HBT cases detected during the Tokyo Olympic Games in 2021. However, cheating athletes may be selecting their potential donors more carefully. In this way, more flexible criteria such as two overlapping peaks or an unusual histogram profile with a shoulder should be considered suspicious.

Finally, anti-doping labs should turn more flexible the criteria for HBT and increase the investment in new methodologies capable of improving the detection of this illegal practice. It has been recently suggested that expanding the antigen panel, a more accurate gated RBC area, an alternative fluorochrome, and alternative staining protocols depending on the sample may minimize the risk of false negatives.[13]

In this way, we can envisage when samples presenting such unusual profiles in HBT screening will be sent to genetic and genomics screening to search for mixed DNA.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this research.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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