Prevalence of antibodies to a new histo-blood system: the FORS system

Carlos Jesus[1], Camilla Hesse[2], Clara Rocha[3,4], Nádia Osório[1], Ana Valado[1], Armando Caseiro[1,5], António Gabriel[1], Lola Svensson[2], Ali-Reza Moslemi[6], Wafa Abu Siba[7], Mahmoud A. Srour[7], Cristina Pereira[8], Jorge Tomaz[8], Paulo Teixeira[1,9], Fernando Mendes[1,10,11,12]

[1]Biomedical Sciences Department of ESTESC - Coimbra Health School, Polytechnic Institute of Coimbra, Coimbra, Portugal; [2]Department for Clinical Chemistry and Transfusion Medicine, Institute for Biomedicine, Sahlgrenska Academy, University of Gothenburg, Sweden; [3]Polytechnic Institute of Coimbra, ESTESC-Coimbra Health School, Department Complementary Sciences, Coimbra; [4]INESC Coimbra, Coimbra; [5]I&D Unit "Química-Física Molecular", Faculty of Science and Technology, University of Coimbra, Coimbra, Portugal; [6]Department of Pathology, Institute for Biomedicine, Sahlgrenska Academy, University of Gothenburg, Sweden; [7]Department of Medical Laboratory Sciences, Faculty of Health Professions, Al-Quds University, East Jerusalem, Palestine; [8]Blood and Transfusion Medicine Department, Coimbra Hospital and University Centre (CHUC), Coimbra; [9]Department of Anatomic Pathology, Coimbra Hospital and University Centre (CHUC), Coimbra; [10]Biophysics and Biomathematics Institute, IBILI-Faculty of Medicine, University of Coimbra, Coimbra; [11]CIMAGO, FMUC-Faculty of Medicine, University of Coimbra, Coimbra; [12]CNC.IBILI, University of Coimbra, Coimbra, Portugal

Background. In 1987, three unrelated English families were reported with a putative blood subgroup called A_{pae}. Swedish researchers later found evidence leading to abolishment of the A_{pae} subgroup and establishment instead of the FORS blood group system (System 31 - ISBT, 2012). It is important to know the prevalence of antibodies in order to make the best decisions in transfusion medicine. Cells expressing the Forssman saccharide, such as sheep erythrocytes, are needed to detect the anti-Forssman antibody. The aim of this study was to define the prevalence of human anti-Forssman antibody.

Materials and methods. Plasma samples from 800 individuals were studied. Sheep erythrocytes or Forssman "kodecytes" were mixed with the plasma samples using the tube technique. Plasma from an A_{pae} individual was used as a negative control and monoclonal anti-Forssman antibody (M1/22.25.8HL cell line supernatant) was used as the positive control.

Results. Of the 800 individuals tested, one was negative for the presence of anti-Forssman antibody. We compared the anti-Forssman antibody reaction pattern between genders and found that males have weaker reactions than females, both at room temperature (p=0.026) and at 37 °C (p=0.043). We also investigated the reaction pattern of anti-Forssman antibody in relation to ABO and Rh blood group types without finding any significant differences.

Discussion. Sheep erythrocytes are suitable for searching for human anti-Forssman antibody. The quantity of anti-Forssman antibodies in plasma is higher in females than in males. In the population (n=800) studied here, we found one individual lacking the anti-Forssman antibody. These results contribute to the data already published, confirming that FORS is a rare blood group.

Keywords: FORS blood group, human anti-Forssman, function-spacer-lipids constructs, ABO blood-group prevalence.

Introduction

In 1900, Karl Landsteiner discovered the existence of natural antibodies reacting with the red blood cells (RBC) of other human beings, which revealed the ABO blood group system[1]. The ABO blood group system is of great importance in transfusion/transplantation medicine[1,2]. The research and development of the human antiglobulin test by Coombs and Race led to other blood systems of major clinical significance being discovered[2].

In 1911, Professor John Forssman found that injection of a suspension of kidney tissue from guinea pigs or horses into rabbits led to the production of an antibody capable of haemolysing sheep RBC, whereas injection of a suspension of kidney tissue from cows or rats did not produce antibodies. The antigen on the sheep RBC was named the Forssman (Fs) antigen[3].

Species were divided into Fs-positive (sheep, dogs, horses, etc.) and Fs-negative species (rabbits, pigs, humans, among other)[3]. Although Fs expression was

initially recognised on sheep RBC, this antigen is rarely found on RBC, its expression being restricted only to tissues in many species[3]. Nevertheless, there are species that express the Fs antigen on both RBC and tissues, such as chicken, and others that express the antigen only on RBC, such as sheep[3].

In 1987, members of three unrelated English families were reported to have a putative new subgroup of blood group A, called A_{pae}[4,5]. RBC from the A_{pae} positive family members showed unusual antibody and lectin reaction patterns, with a strong reaction to *Helix pomatia* lectin, a weak reaction to some polyclonal anti-A antibodies and, strangely, no reaction to monoclonal anti-A antibody, thus leading to some controversy[5,6].

According to a recent study, the Fs glycolipid (which had only been found on the RBC of non-primate mammals) was strongly expressed on the RBC from individuals with subgroup A_{pae}[4]. The Swedish group that made this discovery were also able to demonstrate that the Fs glycolipid is transmitted hereditarily[4].

The Fs antigen, like the A group antigen, terminates with an α3-N-acetylgalactosamine and is synthesised by Fs-synthase (encoded by the *GBGT1* gene), which is not usually active in humans[4,7,8] 3-N-acetylgalactosa minyltransferase; EC 2.4.1.88. However, Yamamoto *et al.* found that reversion of two inactivating missense mutations in the human *GBGT1* gene (c.688G>A [p.Gly230Ser] and c.887A>G [p.Gln296Arg]) fully restored glycosyltransferase activity allowing the synthesis of Fs antigen *in vitro*[8]. These glycine and glutamine residues are conserved among functional *GBGT1* genes in Fs-positive species[8].

In brief, Svensson *et al.* provided evidence leading to the conclusion that subgroup A_{pae} should be abolished and a new histo-blood group should be created, the FORS blood group system[4], which, in 2012, was accepted by the International Society of Blood Transfusion as the 31st blood group system. This system could have important implications in transfusion/transplantation medicine.

The Fs antigen is present in several forms of human cancer, including gastric, colon, and lung cancers[9,10]. Ono *et al.* demonstrated the presence of Fs antigen in the cytoplasm of colon goblet cells, especially those in the so-called transitional mucosa adjacent to carcinoma, from 69 of 70 patients with colon cancer[9]. Using chemical and immunological methods of detection it was confirmed that the Fs antigen is absent from healthy tissues of individuals with Fs-positive tumours, making this antigen an interesting candidate for targeted cancer therapy[10].

With regards to transfusion/transplantation medicine, it is important to know the prevalence of an antibody when making decisions. In order to determine the prevalence of the anti-Fs antibody, cross-reactions must be performed between RBC containing the Fs saccharide and plasma samples.

Nowadays, it is possible to modify the membranes of RBC for such studies, or modify a solid phase, using function-spacer-lipid (FSL) constructs (KODE™ technology)[6,11-14]. This technology allows us to modify various biosurfaces with bioactives products, within a couple of hours and with a relative simple protocol, without affecting cell viability and functionality[6,11,12].

The aim of this study was to determine the prevalence of anti-Fs antibodies in plasma samples from humans, thereby contributing data to strengthen the classification of the FORS blood group as a new histo-blood group.

Materials and methods

"Kodecyte" production

"Kodecytes" were produced as described by Frame *et al.*[11]. RBC were incubated with a sugar suspension (FSL - KODE Biotech Limited, Auckland, New Zealand) to modify the RBC membrane. Type O RBC were washed six times with isotonic saline. Next, one part of the O RBC was incubated with two parts of 50 μg/mL Forssman pentasaccharide (FSL-Fs penta) modification solution. This solution was incubated for 2 hours at 37 °C, with occasional mixing, and then transferred for overnight incubation at 4 °C. The modified cells ("kodecytes") were washed six times and suspended in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 [pH=7.4]) to obtain a 0.8% working solution. Each batch of "kodecytes" was tested, weekly, by cross-reacting it with anti-Fs antibody (M1/22.25.8HL cell line supernatant) and 1:100 *Helix pomatia*, as a positive control, and the original O RBC as the negative control, in NaCl and Coombs' gel cards (DiaMed, Cressier, Switzerland).

Sheep red blood cell preparation

Ovis aries ibericus RBC, collected at Coimbra College of Agriculture, were washed six times and suspended in phosphate-buffered saline to make a 0.8% solution. Plasma from an A_{pae} individual was used as a negative control and anti-Fs antibody (M1/22.25.8HL cell line supernatant) was used as the positive control.

Gel card haemagglutination

The gel card haemagglutination tests were prepared by introducing 50 μL of 0.8% "kodecyte" solution or sheep RBC to wells containing NaCl and Coombs' gel cards and then adding 25 μL of each plasma sample to be tested. The gel cards were incubated for 15 minutes at room temperature or 37 °C (ID-Incubator 37 SI, DiaMed). After incubation, the gel cards were centrifuged (ID-Centrifuge 24S, DiaMed) and the results were read.

Tube haemagglutination

All reactions were tested in duplicate, one at room temperature and the other at 37 °C. An aliquot of 100 µL from each plasma sample was pipetted into the respective tubes and 50 µL of 0.8% sheep RBC suspension were added. The tubes were incubated for 1 hour at room temperature or 37 °C (ID-Incubator 37 SI, DiaMed). After the incubation the tubes were gently tapped and the results were read under an agglutinoscope (J.P. Selecta S.A., Barcelona, Spain). Agglutination was graded according to standard blood banking practice where 4+ is the strongest, 3+, 2+ and 1+ are gradually weaker but positive reactions, and 0 is negative.

Samples

We studied a total of 800 plasma samples of which 468 (58.5%) were from Portugal, 206 (25.75%) from Palestine, and the remaining 126 (15,75%) from Sweden (Figure 1A).

Tube haemagglutination was used as the gold-standard method, while gel card haemagglutination was used to confirm results. Informed consent was obtained according to the Helsinki Declaration.

Statistical analysis

The statistical programme IBM SPSS® 246 v.19 (National Opinion Research Center, Chicago, USA) was used for the statistical computations.

We applied the chi-square test to compare anti-Fs antibody reaction patterns according to gender and ABO and Rh blood groups, and the paired samples *t*-test to compare reaction patterns for tests carried out at room temperature and 37 °C for each sample.

Results

The studied population consisted of 437 males (55%) and 363 females (45%), with an average age of 35 years (standard deviation, 15 years) (Figure 1B). Regarding the ABO blood group distribution, 69% of the samples were A Rh positive or O Rh positive. B Rh negative, AB Rh positive and AB Rh negative blood types accounted for 7% of the total samples (Figure 2).

The Portuguese samples came from 257 males (55%) and 211 females (45%), with an average age of 40 years (standard deviation, 14 years) (Figure 3). In this subset of sample, 72% of the samples were A Rh positive or O Rh positive. B Rh negative, AB Rh positive and AB Rh negative blood types accounted for 5% of the Portuguese samples.

Samples from Palestine were donated by 106 males (51%) and 100 females (49%), with an average age of 21 years (standard deviation, 2 years) (Figure 3). Of these samples, 66% were A Rh positive or O Rh positive, while B Rh negative, AB Rh positive and AB Rh negative blood types accounted for 12% of the Palestinian samples analysed.

Finally, the samples from the Swedish population came from 74 males (59%) and 52 females (41%), with an average age of 44 years (standard deviation, 14 years) (Figure 3). Regarding ABO blood group distribution, 64% of the samples were A Rh positive or O Rh positive. The B Rh negative, AB Rh positive and AB Rh negative blood types formed 6% of the Swedish samples.

Regarding the ABO and Rh blood type distribution we did not observe significant differences between the samples from Portugal, Palestine and Sweden.

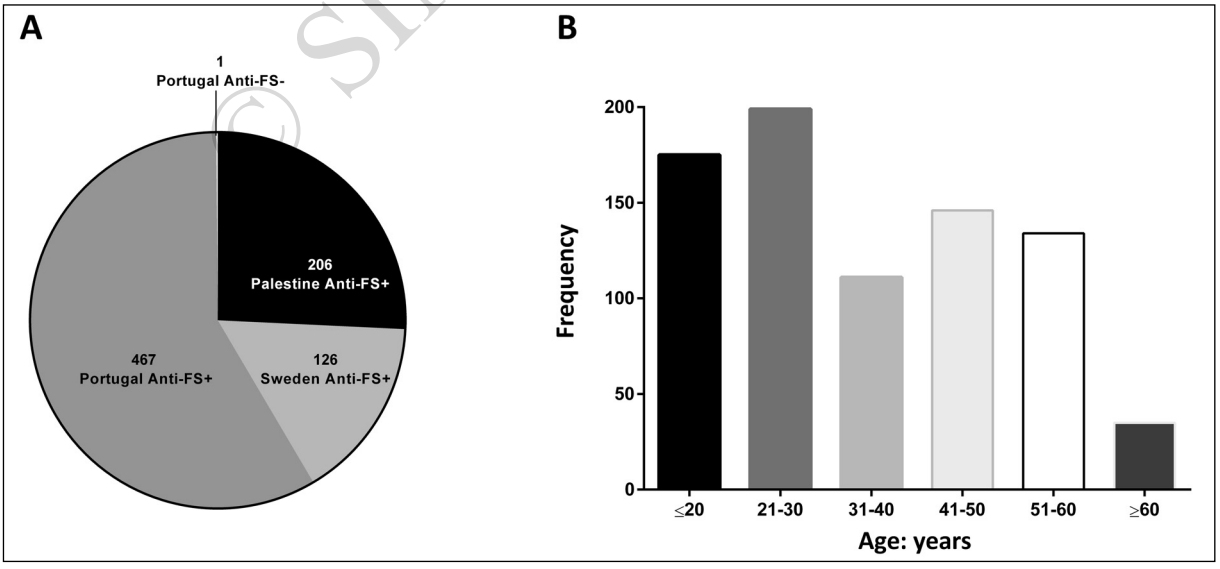

Figure 1 - (A) The distribution of anti-Forssman antibody in the sample studied, divided according to country. (B) The distribution of age of the blood donors' in the sample studied.

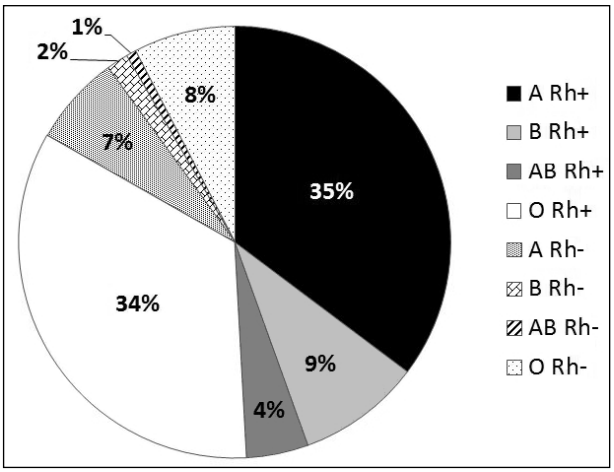

Figure 2 - The distribution of ABO and Rh blood groups in the sample studied.

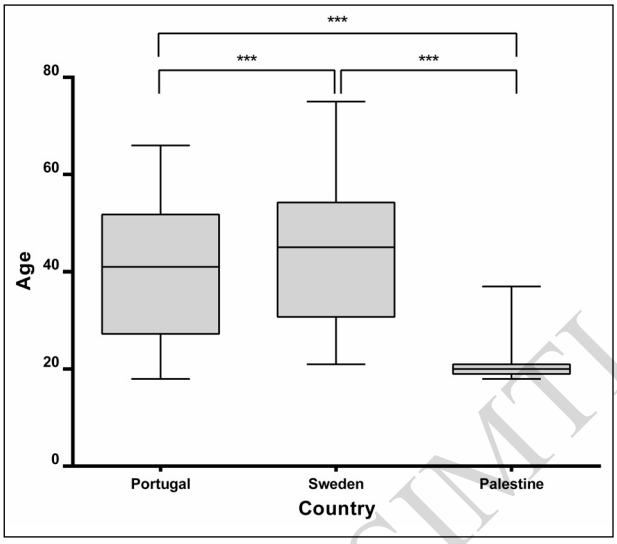

Figure 3 - The distribution of age of the blood donors of the sample studied, divided by country.
Statistical significance is expressed by ***p<0.001.

The prevalence of anti-Fs antibody in the 800 samples studied was 99.88%, with only one sample (Portuguese) negative in both screening tests, namely at room temperature and at 37 °C (Figure 1A).

We first compared the anti-Fs antibody reaction pattern between genders and found that males have weaker reaction (1+ and 2+) than females (3+, 4+ and visible [V]) both at room temperature (p=0.026) and at 37 °C (p=0.043) (Figure 4). We then compared the reaction patterns of anti-Fs antibody according to ABO and Rh blood groups, without finding any significant differences.

In order to characterise the anti-Fs antibody better, we compared the reaction pattern between the screening at room temperature and 37 °C for each sample. No significant differences were found (Figure 5).

Discussion

The prevalences of ABO blood group subtypes in this study were similar to those in previous studies[15,16] included 14,916 (13,691 retrospective and 1,225 prospective). Regarding the age of each population we observed that the cohorts from Sweden and Portugal were older, while in Palestine the maximum age was 37 years, with this being a statistically significant younger population (Figure 4). This is explained by the fact that all the Palestinian samples came from university students.

Svensson *et al.* described the new blood group system, FORS, as rare in humans[4]. Our prevalence data on the anti-Fs antibody confirm that the FORS system is a rare blood group system. However, while the literature describes only three unrelated English families with the FORS system, it is possible that we have found a Portuguese person with this rare blood group, given the individual's lack of anti-Fs antibody.

Nevertheless, there are various possible explanations for the absence of anti-Fs antibody in an individual. First, some individuals may not be able to synthesise anti-Fs antibody, as their immune response against Fs antigen could be under genetic control. Second, anti-Fs antibody might be produced in all people, but could be absorbed onto some normal or cancerous tissues containing the Fs antigen. Alternatively, Fs antigen could be shed from tissues resulting in the formation of anti-Fs-Fs immune complexes[17]. Third, some individuals could have very low anti-Fs antibody titres which could be below the level of detection of the assaying method used.

We found that females had statistically significant more anti-Fs antibodies than had males (Figure 4). However, the reaction pattern of this antibody does not seem to be affected by either ABO or Rh group. Likewise, the different reaction pattern of the anti-Fs antibody at room temperature and at 37 °C was not statistically significant (Figure 5).

There are several possible explanations for the larger amount of anti-Fs antibody in women, including the fact that women, in general, have a stronger immune system and responses than men. This gender diversity is believed to be controlled by differences in the blood levels of gonadal steroid hormones, including the female hormone, oestrogen, which stimulates immune responses, and the male hormone, testosterone, which has an immunosuppressive effect[18].

In this study we have demonstrated and described new strategies for the search of the human anti-Fs Ab, using FLS constructs ("kodecytes") and sheep RBC.

Conclusions

We can conclude that the prevalence of anti-Fs antibody is high in humans, since we found only one negative person in this study, supporting the data already

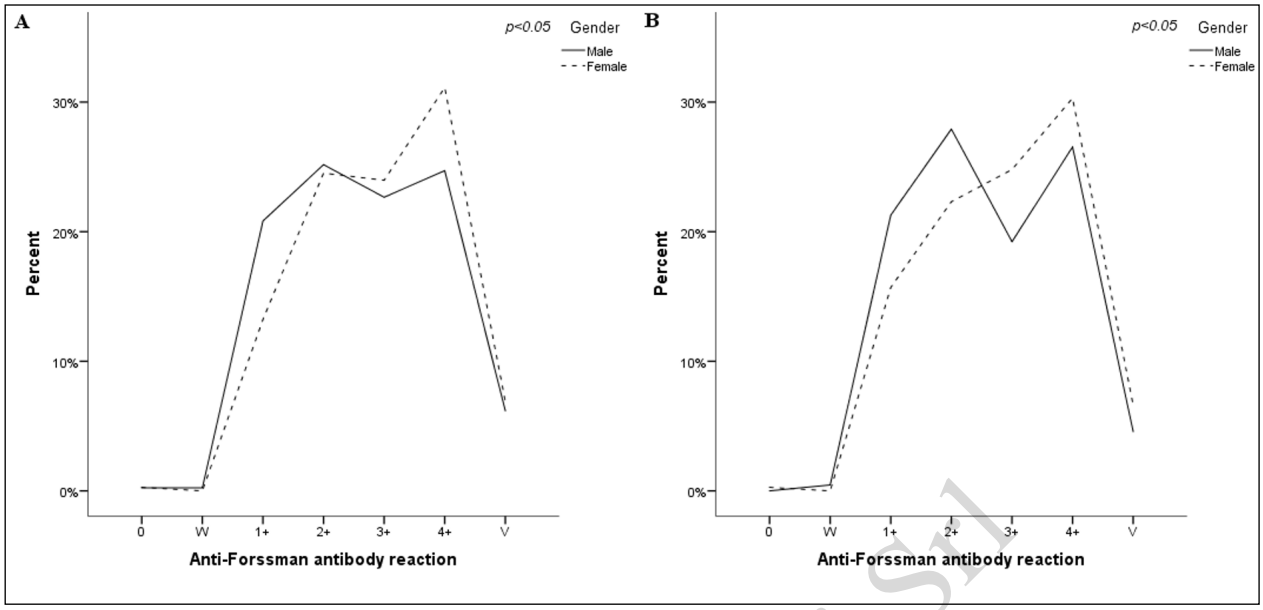

Figure 4 - Comparison of anti-Forssman antibody reactions between genders (male, – continuous line; female, - - dashed line): (A) at room temperature and (B) at 37 °C.

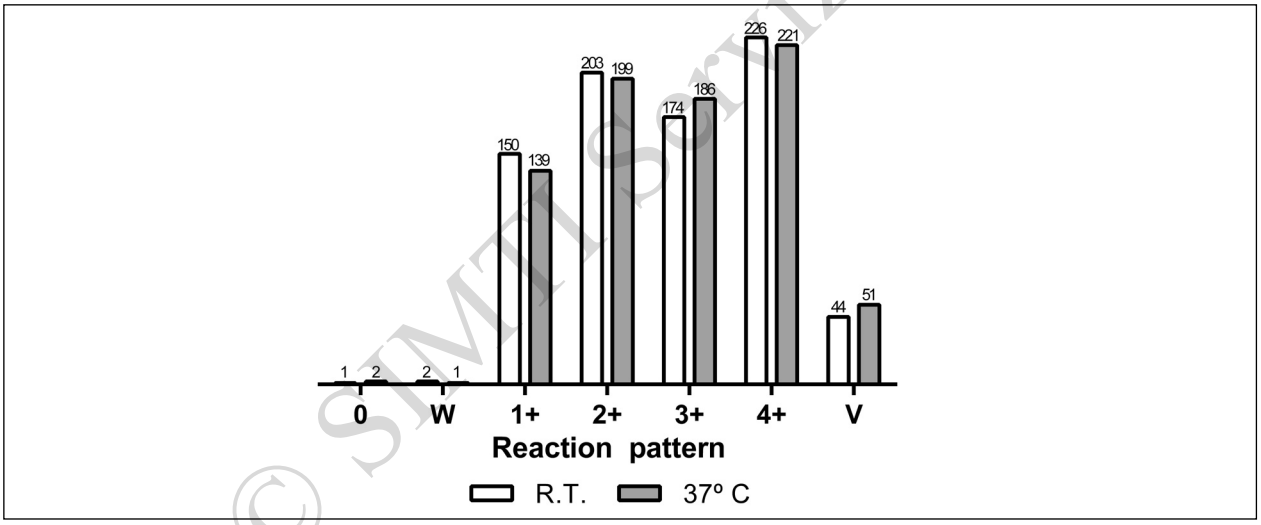

Figure 5 - Comparison of the frequency of anti-Forssman antibody reactions at room temperature (R.T.) and at 37 °C.

published that FORS is a rare blood group system. Anti-Fs antibody is not affected by ABO or Rh blood group and is present at higher concentrations in females than in males. Sheep RBC and "kodecytes" can be used to screen for human anti-Fs.

As future perspectives, it will be interesting to study the RBC of the donor who lacked the anti-Fs antibody and his family in order to clarify whether they are FORS antigen-positive and the impact of these antibodies in transfusion medicine.

Acknowledgements

Our gratitude to Bio-Rad Laboratories for their sponsorship, Coimbra College of Agriculture for providing the sheep blood for our research procedures, the Blood Bank Service at Coimbra Hospital and University Centre for providing the samples from the Portuguese blood donors and to Ricardo Teixo for producing the figures.

Authorship contributions

CJ: main researcher, research design, laboratory procedures, analysis of results from Portuguese, Swedish and Palestinian donors, article writer; CH: research design, donor's selection, analysis of samples from Swedish donors, article writer and critical analysis; CR: research design, statistical analysis and interpretation; NO: critical review, sample separation and analysis;

AV, AC and AG: critical review; LS, A-RM and MAS: research design and critical analysis; WAS: selection of samples, analysis of samples from Palestinian donors, article writer and critical analysis; CP: selection of donors, analysis of samples from Portuguese donors, article writer and critical analysis; JT: Donor's selection, article writer and critical analysis; PT: research design, article writer and critical analysis; FM: principal researcher tutor, research designer, supervision of all laboratory procedures, analysis of results from Portuguese, Swedish and Palestinian donors, article writer and critical analysis.

The Authors declare no conflicts of interest.

References

1) Yamamoto F. Review: ABO blood group system--ABH oligosaccharide antigens, anti-A and anti-B, A and B glycosyltransferases, and ABO genes. Immunohematology 2004; **20**: 3-22.
2) Mollison PL, Engelfriet P. Blood transfusion. Semin Hematol 1999; **36** (4 Suppl 7): 48-58.
3) Forssman J. [The production of high-quality specific sheep's hemolysin without the use of sheep's blood. Contribute to the teaching of heterologous antibody formation.] Biochem Z 1911; **37**: 78-115. [In German.]
4) Svensson L, Hult AK, Stamps R, et al. Forssman expression on human erythrocytes: biochemical and genetic evidence of a new histo-blood group system. Blood 2013; **121**: 1459-68.
5) Stamps R, Sokol RJ, Leach M, et al. A new variant of blood group A. Apae. Transfusion 1987: **27**: 315-8.
6) Barr K, Korchagina E, Popova I, et al. Monoclonal anti-A activity against the FORS1 (Forssman) antigen. Transfusion 2015; **55**: 129-36.
7) Haslam DB, Baenziger JU. Expression cloning of Forssman glycolipid synthetase: a novel member of the histo-blood group ABO gene family. Proc Natl Acad Sci USA 1996; **93**: 10697-702.
8) Yamamoto M, Cid E, Yamamoto F. Molecular genetic basis of the human Forssman glycolipid antigen negativity. Sci Rep 2012; **2**: 975.
9) Ono K, Hattori H, Uemura K, et al. Expression of Forssman antigen in human large intestine. J Histochem Cytochem 1994; **42**: 659-65.
10) Feng J, Hevey R, Ling C-C. Synthesis of a Forssman antigen derivative for use in a conjugate vaccine. Carbohydr Res 2011; **346**: 2650-62.
11) Frame T, Carroll T, Korchagina E, et al. Synthetic glycolipid modification of red blood cell membranes. Transfusion 2007; **47**: 876-82.
12) Heathcote D, Carroll T, Wang J-J, et al. Novel antibody screening cells, MUT+Mur kodecytes, created by attaching peptides onto red blood cells. Transfusion 2010; **50**: 635-41.
13) Williams E, Barr K, Korchagina E, et al. Ultra-fast glyco-coating of non-biological surfaces. Int J Mol Sci 2016; **17**: 118.
14) Korchagina EY, Henry SM. Synthetic glycolipid-like constructs as tools for glycobiology research, diagnostics, and as potential therapeutics. Biochem 2015; **80**: 857-71.
15) Skaik YA, Alhawary AS, Shbair AS, Hamouda BB. Frequency of ABO and Rh(D) blood groups in five governorates in Gaza-Strip. Pak J Med Sci 2007; **23**: 924-7.
16) Duran JA, Chabert T, Rodrigues F, Pestana D. [Distribution of blood groups in the Portuguese population.] AB0 2007; **29**: 5-17. [In Portuguese.]
17) Young WW, Hakomori SI, Levine P. Characterization of anti-Forssman (anti-Fs) antibodies in human sera: their specificity and possible changes in patients with cancer. J Immunol 1979; **123**: 92-6.
18) Kovacs EJ, Messingham KAN. Influence of alcohol and gender on immune response. Alcohol Res Health 2002; **26**: 257-63.

Arrived: 4 May 2016 - Revision accepted: 19 July 2016
Correspondence: Carlos Jesus
Biomedical Laboratory Sciences Department
Coimbra Health School
Rua 5 de Outubro - SM Bispo
3046-854 Coimbra, Portugal
e-mail: carlosgaspardejesus@gmail.com