

RETICULOCYTE COUNT FROM BUFFY COATS: MEDULAR RECOVERY AFTER BLOOD DONATION

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Abstract: Blood is a special type of connective tissue, associated with several specific functions, consisting of 55% plasma and 45% cellular elements, such as erythrocytes, leukocytes, platelets and a small portion of reticulocytes. of maturation. The importance of blood as an element of life lies in its use in clinical applications, namely, transfusions, differentiated collection of certain blood elements, and also in the specific treatment of certain hematological diseases. Reticulocytes are released into the bloodstream in response to erythrocyte deficits and are a preferred element in the diagnosis of different types of anemias, as an early marker of graft in hematopoietic stem cell transplantation, also allowing the assessment of bone marrow function. Our study aimed to count reticulocytes from buffy coats obtained from blood donors, with subsequent evaluation of the Corrected Reticulocyte Value (VRC) and the Reticulocyte Production Index (IPR), in order to assess the spinal cord response after the donation. In this study, in all samples, a percentage of corrected reticulocytes greater than 1.5% was found, clinically considered as reticulocytosis. Regarding the value of the Reticulocyte Production Index, which is the most representative value of the medullary activity, it was found that the majority of donors (84.6%) presented an IPR value ≥ 3 and 15.4% of the donors showed an IPR ≤ 2 , with a percentage of reticulocytes $>1.5\%$ ($r=0.946$ $p<0.01$), which denotes an increased response of the organism to the donation. The determination of the erythrocyte/reticulocyte ratio from a donor buffy coat can be a way of evaluating and monitoring bone marrow recovery after donation.

Keywords: Human buffy coat, reticulocytes, VRC, IPR.

INTRODUCTION

Blood is a vital bodily fluid, consisting of a fluid part, plasma and a solid part, blood cells. Plasma represents approximately 55% of the total volume, with a composition rich in water and in several proteins. (Arosa, 2012).

Blood cells represent about 45% of total blood, which in an individual corresponds to approximately 7% of their body weight (Fragata, 2014). Cellular elements present in the blood include erythrocytes ($5 \times 10^6/\text{mm}^3$), platelets ($3 \times 10^5/\text{mm}^3$) and leukocytes (7×10^3). The latter are, in circulation, the only cells that have a nucleus and cytoplasm, since platelets are cytoplasmic fragments of bone marrow cells, megakaryocytes and erythrocytes lose their nucleus before entering circulation (Fragata, 2014).

Hematopoiesis is the process at the origin of blood. This is characterized by being a set of events that involves the self-maintenance of the undifferentiated pool of the pluripotent hematopoietic cell, the ability to originate the precursor cells of myeloid and lymphoid lineages, and also the proliferation and differentiation function of migrating cells. into the bloodstream (Hoffbrand, A. V. and Pettit, 2000; Zago, 2013). Another important process is erythropoiesis, which, consisting of the formation of erythrocytes, occurs in the bone marrow, by the differentiation of cells of the erythroid lineage (Hoffbrand, A.V. and Pettit, 2000; Jelkmann, 2011; Oliveira and Aparecido, 2019).

The first cell that can be identified as belonging to the erythroid lineage is the proerythroblast, followed, after several stages of development and maturation, by the premature erythroblast, the mature erythroblast, the normoblast, the reticulocyte and, finally, the erythrocyte (Hoffbrand, 2000).

The normal erythrocyte has an average life span of approximately 120 days and dies by

aging. This event is caused by the fact that in the mature erythrocyte stage it is an anucleated cell, incapable of promoting the renewal of its previously stored enzymes. With this, it becomes unable to promote the synthesis of new proteins. After this time, they are removed from the bloodstream by macrophages of the reticulo-endothelial system of the spleen, and most of their components are absorbed by phagocytosis (Bernard et al, 1979; Melo, 2014).

In peripheral blood, erythrocytes are the most abundant cells and, when in the blood there is a decrease in the ability to transport oxygen, due to a decrease in the amount of erythrocytes in circulation, this implies a decrease in the concentration of hemoglobin and the result is the onset of anemia. If, on the contrary, there is an increase in the concentration of erythrocytes, which usually corresponds to an increase in hemoglobin levels, there is the appearance of polycythemia or polyglobulia (Bernard et al, 1979; Cotran, 2000; Zago, 2013).

Reticulocytes are young erythrocytes that have RNA remnants of ribosomal origin, that is, they are erythrocytes that have a more or less dense reticulum and that were released by the bone marrow into the blood stream at a young age. Reticulocytes are classified into four categories (groups I to IV) according to their degree of maturation. In group I we find the most immature, characterized by a greater amount of granules or dense reticular mass, and in group IV, the most mature, characterized by the presence of few RNA granules and being, mostly, the most found circulating in the peripheral blood (Bain, 2007; Saraiva, 2009).

The reticulocyte count is of paramount importance in cases of anemia, since it allows checking whether the anemia is of a medullary or extramedullary cause. Thus, and in order to express more accurately

the production of erythrocytes by the bone marrow, it is important that after determining the percentage of reticulocytes, its absolute value is determined, taking into account the absolute value of erythrocytes (Bain, 2007; Melo, 2014). According to Bain (2007) it is possible to obtain a more significant result from the value of reticulocytes than from their percentage. This can be obtained by determining the reticulocyte production index (RPI), which corrects the reticulocyte value for the degree of anemia. Also, in the case of blood donations, the IPR can be determined after a blood donation.

Our objective consisted in the analysis, from human buffy coats, of cellular reference parameters, such as hematocrit, erythrocyte count, reticulocyte count and IPR. To obtain this index, it was necessary to determine the number of corrected reticulocytes. With this study, it was intended to evaluate several cellular parameters, at the blood level of erythrocytes and reticulocytes, which allowed us to have an analysis of the quality of these cells and the way in which the bone marrow processes the demand for new cells in the blood donor after gift.

MATERIAL AND METHODS

BUFFY COATS

In this study, blood cells were isolated from discarded buffy coats (BC), potentially usable in research, obtained from the Blood Bank of Centro Hospitalar Universitário São João (CHUSJ) from voluntary blood donors. Blood donors were not identifiable and there was no interaction with living individuals or knowledge of personally identifiable information. Therefore, this work is not classified as human research under the HHS Humans Regulations (45 CFR Part 46). The ethics committee of the Instituto Universitário de Ciências da Saúde was aware of and approved the protocol.

Thirteen buffy coats were collected and, the day after harvest, they were transported and stored following a protocol of good practices. The transport time and storage method were noted and identified with a code for internal registration in the institution's/IUCS laboratory. After the previous procedures were recorded: date of collection, gender of the donor, blood group, hemoglobin and volume of the buffy coat. Then, the parameters were analyzed: reticulocyte count, corrected reticulocyte value and reticulocyte production index.

SMEAR RETICULOCYTE STAINING AND COUNT

For staining the reticulocytes, the methodology based on RNA precipitation by the cationic dye (SIGMA-ALDRICH) was used and performed according to the supplier. Briefly, in an eppendorf tube, two drops of reticulocyte dye were added to three drops of properly homogenized buffy coat blood and proceeded to homogenization. After standing for 10 minutes at room temperature (18-26°C), a drop of the mixture was placed on the right end of the slide and the smear was performed (Bain, 2007). The slides were placed at room temperature for at least 15 minutes and, after drying, the reticulocytes were counted (2 per buffy coat).

METHOD OF COUNTING AND DETERMINING THE NUMBER OF RETICULOCYTES

A reticulocyte, according to the classification of the National Committee for Clinical Laboratory Standards (NCCLS), is any unnuclated erythrocyte that contains two or more blue-stained particles that correspond to ribosomal RNA (Bain, 2007).

The smears were evaluated for the presence or absence of reticulocytes, using an optical microscope, with an immersion objective

that provided a magnification of 1000x and a smear area was randomly selected where the red blood cells were separated, but not overlapping. 1000 erythrocytes were counted including reticulocytes. The presence of clusters of filaments or reticulum of randomly arranged inside the reticulocytes was observed (Bain, 2007).

The proportion of reticulocytes was calculated using the following formula: (Bain, 2007).

$$\text{Reticulocyte count (\%)} = \frac{\text{Total number of reticulocytes}}{10}$$

Reticulocyte reference values for adults range from 0.5% to 1.5%.

CALCULATION OF CORRECTED RETICULOCYTES (RC) AND RETICULOCYTE PRODUCTION INDEX (IPR)

Due to the variability of reticulocytes, it is necessary to correct the observed reticulocyte count, taking into account a hematocrit considered as reference or normal, which will be equal to 45% (Bain, 2007).

$$\text{Corrected reticulocyte count (\%)} = \frac{\text{R (\%)} \times \text{Hct (\%)}}{45\%}$$

R (%) = Percentage of reticulocytes found for the individual under study

Hct (%) = Hematocrit, in percentage, calculated for the same individual

The value of corrected reticulocytes for a given individual was established based on that individual's reticulocyte count and its hematocrit in relation to a hematocrit considered as a reference.

The reticulocyte production index is calculated by dividing the corrected reticulocytes by the mean time of reticulocyte maturation at the peripheral blood level (Figure 1), for the respective degree of anemia (Bain, 2007).

$$\text{Reticulocyte production index} = \frac{\text{Corrected reticulocytes (\%)}}{\text{Maturation time (days)}}$$

The expected maturation time in relation to hematocrit values:

A reticulocyte production index greater than or equal to 3 (IPR \geq 3) is considered a normal index, while a value less than 2 (IPR $<$ 2) is below the normal index.

STATISTICAL ANALYSIS

For the statistical treatment of the data, we used the Statistical Software Statistical Package for Social Sciences [SPSS], version 27.0 of Windows 2007, creating a base where all the data collected were introduced and analyzed. The processing of data and the presentation of the results was carried out in accordance with the research hypotheses put forward by us. In this sense, throughout our data analysis we used different non-parametric techniques, due to the fact that the data do not follow a normal distribution and the number of samples is less than 30. Thus, to verify if the RPI is dependent on sex, Fisher's exact test was used; to verify the association between the percentage of reticulocytes and the RPI, the Spearman correlation coefficient was calculated. The interpretation of the results obtained is based on the guidelines transmitted by Pestana and Gageiro (2008), through which r values lower than 0.2 indicate the existence of very low or very weak associations; from 0.2 to 0.39, low or weak; 0.4 to 0.69, moderate; 0.7 to 0.89, high; and greater than 0.9 are considered very high, with the value 1 indicating a perfect correlation.

Regarding the statistical interpretation of the results, significance levels of 5% ($p < 0.05$), 1% ($p < 0.01$), and 0% ($p < 0.001$) will be used, thus allowing us to guarantee that the themselves, will not be the product of chance.

RESULTS

STUDY POPULATION AND CLINICAL EVALUATIONS

In this study, 13 samples of buffy coats from blood from voluntary donors were randomly selected on the day following collection. In these, the following reticulocyte parameters were evaluated: percentage of reticulocytes, value of corrected reticulocytes (VRC) and reticulocyte production index (IPR).

The sample consisted of eight male BC ($n=8$) and five female BC ($n=5$), corresponding to 61.5% and 38.5%, respectively. Both sexes showed a result greater than 1.5% for the reticulocyte count. It was also verified that all the results are above the values considered as reference, that is, between 0.5% and 1.5%.

CALCULATION OF CORRECTED RETICULOCYTE VALUE (VRC) AND RETICULOCYTE PRODUCTION INDEX (IPR)

Figure 2 shows the reticulocyte production rates of the 13 donors. It was observed that the majority ($n=8$), ie 61.5%, who are male, had an RPI \geq 3. In females ($n=5$), 3 of the donors, ie 23.1%, had an RPI \geq 3 and 15.4%, the corresponding to ($n=2$) had an RPI \leq 2. When analyzing the statistical results, Fisher's exact test showed that there is no statistically significant relationship between the IPR domain and gender (Table 1).

The IPR value was also related to the percentage of reticulocytes (Figure 3). It was found that the majority of donors ($n=11$), that is, about 84.6% had an RPI value \geq 3. However, only 15.4% ($n=2$) of the donors had an RPI \leq 2, namely 2.25 and 2.33 and where the percentage of reticulocytes is $>$ 1.5%.

When analyzing Figure 4, we can see that, in most donors, there is no significant difference between the results of the two counts, that is, between the percentage of reticulocytes counted and the IPR. However,

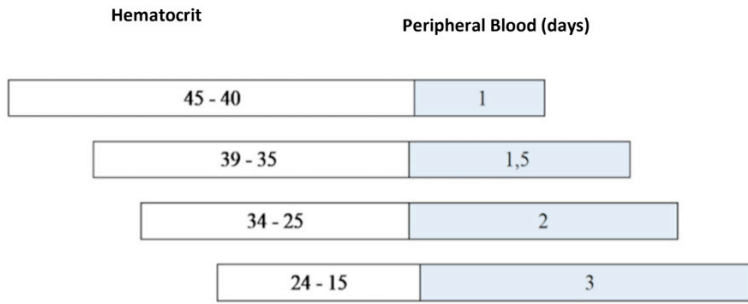


Figure 1: Reticulocyte maturation time in days

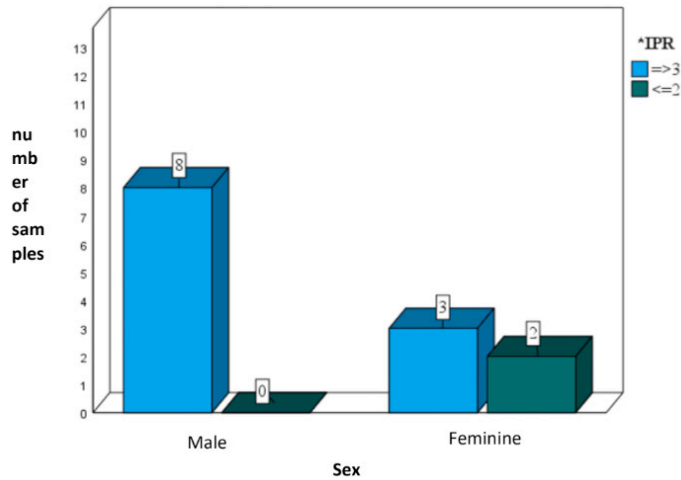


Figure 2: Results of the reticulocyte production index according to sex.

*IPR - Reticulocyte production index

	Value	Sig.	N
Pearson's chi-square	3,782 ^a		13
Fisher's Exact Test		,128	

Table 1: Pearson's Chi-square test between the IPR and sex domains

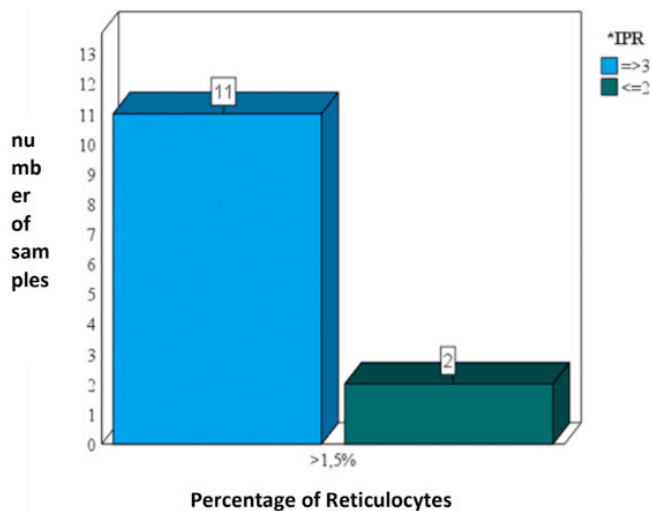


Figure 3: IPR results and percentage of reticulocytes

*IPR - Reticulocyte production index

in samples 6 and 7 there was a considerable difference. Spearman's correlation coefficient shows a very strong and statistically significant positive relationship between the two domains ($r= 0.946$ $p<0.01$), (Table 2).

Regarding the clinical significance of the RPI, it was observed in Figure 5 that 84.6% ($n=11$) of the donors presented an increased response to the request of the organism/bone marrow as a result and 15.4% ($n=2$) presented a response considered adequate ($=2$). However, in all of them there was a percentage of reticulocytes $>1.5\%$, clinically considered as reticulocytosis. It was also found that there were no donors with an RPI value lower than 2 (<2), whose clinical significance is suggestive of lack of bone marrow stimulation, in the sense of replacing the amount of reticulocytes in the bloodstream sufficient to compensate of the erythrocyte loss observed by the blood donation. However, when only the percentage of reticulocytes is determined, percentages between 0.5 and 1.5% are considered as a reference value.

In Figure 6, given that in order to determine the IPR value it is necessary to determine the value of the corrected reticulocytes for the individual/donor, the representation between the IPR and the VRC results was schematized. A greater number of donors ($n=11$) was visible, ie 84.6% with an RPI value $\Rightarrow 3$ and, simultaneously, with a VRC greater than 1.5%. However, it was found that only one donor ($n=1$) had a VRC of 2.25, which corresponds to an IPR value ≤ 2 . One donor ($n=1$) was also observed with a VRC equal to 3.49, with its IPR equal to 2.33 and also considered as ≤ 2 . This value was found taking into account the reticulocyte maturation time which, for its hematocrit value is equal to 1.5 (Table 1). The value of 2.33 is found by dividing the VRC value by this maturation time. Thus, it justifies the fact that, in Figure 6, only one VRC value of 2.25 and two IPR values ≤ 2 are presented.

The statistical results demonstrate that there is a positive, very strong and statistically significant relationship between the domains, the IPR and the VRC ($r=0.946$; $p<0.01$), (Table 3). Which means that a high VRC result is usually related to a high IPR value. This taking into account the individual value of %Hct (hematocrit) and the maturation time of the donor's erythrocyte.

DISCUSSION

Blood cells of all lineages are constantly renewed from a hematopoietic stem cell in the bone marrow, undergo proliferation and differentiation before being released into peripheral blood (Riley et al., 2001). In particular, erythrocytes are released into the bloodstream after a period of maturation in the bone marrow. During this phase, the passage from orthochromatic erythroblasts, due to loss of the nucleus, to reticulocytes or immature erythrocytes is verified. These mature gradually over three days in the bone marrow and, on average, and partially over one day in the peripheral blood (Brugnara, 2000; Riley et al., 2001).

The peripheral blood reticulocyte count is an important data to verify the functional integrity of the bone marrow. This count is an auxiliary diagnostic tool that makes it possible to verify whether, in the presence of anemia, it is regenerative or non-regenerative. In anemic patients and in the presence of a good bone marrow response, reticulocytosis is observed, that is, an increase in the percentage of circulating reticulocytes, in a value higher than the value considered as a reference, from 1.0 to 1.5%, with 3% as the upper limit of normal (Riley et al., 2001). If, on the contrary, there is ineffective erythropoiesis, due to inadequate production of erythrocytes, we observe a decrease in this percentage in circulation, called reticulocytopenia (de Gois et al., 2019).

Traditional counting of reticulocytes by

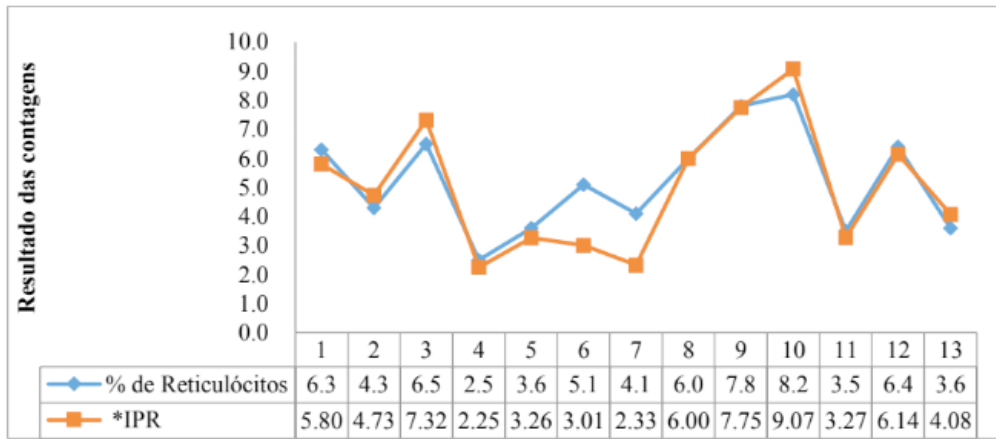


Figure 4: Results of the percentage of reticulocytes and IPR
*IPR - Reticulocyte production index

Spearman's Rho	IPR	Correlation Coefficient	% of Reticulocytes
			.946
		Sig.	0.01
		N	13

Table 2: Spearman's rho test between IPR domains and percentage of reticulocytes

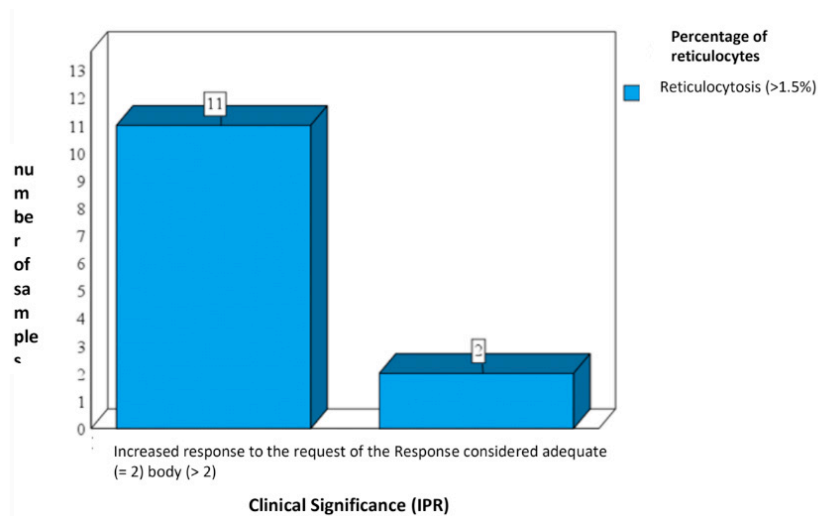


Figure 5: Clinical significance of IPR (Reticulocyte Production Index) and Reticulocyte Percentage

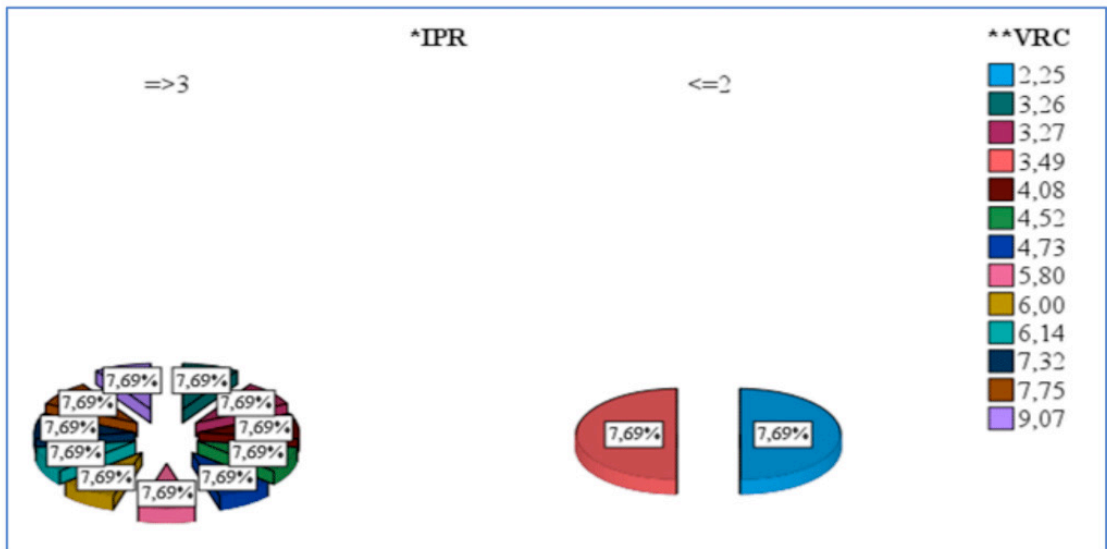


Figure 6: Result of IPR and VRC.

*IPR – Reticulocyte production index; **VRC - Value of corrected reticulocytes

<u>Spearman's Rho</u>	IPR	<u>Correlation Coefficient</u>	corrected reticulocyte values .946
		Sig.	0,01
		N	13

Table 3: Spearman's rho test between IPR domains and corrected reticulocyte values

supravital staining under a microscope has been considered the reference method since 1940, with low cost and simple technical execution (Pinto & Ifr, 2008; de Gois et al., 2019). However, this method being more time consuming, compared to automated methods in obtaining the result, also presents a high coefficient of variation (João et al., 2008; de Gois et al., 2019). It is known that there are several factors that can compromise the accuracy of reticulocyte counts, such as sample collection, transport and storage, smear quality, and inter-observer variability for reticulocyte identification (Riley et al., 2001,). de Gois et al., 2019). Also, and according to Bain (2007), the counts must be performed up to 6 hours after collection, although they are stable for 24 hours at room temperature when EDTA is used as an anticoagulant, or for several days at 4 °C. These requirements are in line with those established in our study, as all BC were stored/evaluated under the same conditions. Furthermore, to minimize some of these factors, several smears were performed for the same donor, having chosen at least the two best ones at the end. The counts were also always performed on two slides, counting 500 erythrocytes in each one (in total 1000 erythrocytes) and, of these, count how many are reticulocytes.

Although studies on reticulocyte counts from human buffy coats are scarce, or little reported in the literature (Bøhn et al, 2019; Salam et al, 2012), it is possible to draw some conclusions regarding the counts obtained from our sample. Thus, the reticulocyte count was determined and when Fisher's exact statistical test was applied ($p=0.128$), it was clear that there is no relationship between this parameter and the sex of the donors.

Hoffmann et al. (2012) found results identical to those of our study, where no statistically significant difference was found between sex and reticulocyte parameters.

However, some studies focused on reticulocyte counts showed differences in values in relation to sex, namely, in the study by Riley et al., (2001) there was a difference in absolute reticulocyte counts in relation to sex ($p<0.005$), but globally this relationship has not been universally confirmed (Koepeke & Koepeke, 1986; Riley et al., 2001; Bain, 2007). It must be noted that more recent scientific works, in which the counts are performed by automated methods, have not shown differences between the sexes (Bain, 2007).

Regarding the clinical significance of the percentage of reticulocytes, it was found that 100% of randomly chosen donors ($n=13$) have an increased value ($>1.5\%$) of circulating reticulocytes in the blood, that is, with reticulocytosis and that it usually occurs in individuals with blood loss, anemia or in donors who have not had time to recover. According to Piva et al. (2015), in their study on the clinical utility of reticulocyte parameters, highlights that the reticulocyte count reflects the erythropoietic activity of the bone marrow, being useful in the diagnosis of anemia and in the monitoring of the marrow response to therapy. Also, Ceylan et al., (2007) in a study that analyzed the percentage of reticulocytes, among other parameters, and in different types of donors, such as healthy donors, donors with iron deficiency (IDA), with vitamin B12 deficiency and with beta-thalassemia minor (TM), reinforced the importance of the clinical utility of the reticulocyte parameter in the diagnosis of ADI and TM. This study, with a sensitivity and specificity of 85.4% and 97.1% respectively, proved to be indicative of iron deficiency.

In our study, when relating the percentage of reticulocytes with the IPR, an essential parameter for bone marrow evaluation, it was found that 84.6% had an IPR value ≥ 3 and 15.4% ($n=2$) had a IPR ≤ 2 , namely 2.25 and 2.33, but where the percentage of

reticulocytes is $>1.5\%$. This result highlights the importance of determining the RPI, and it can be understood, in this context, that these donors have a bone marrow stimulation that will not be very efficient or even that they have a slight deficit in erythropoiesis. Furthermore, there was a statistically significant, very strong positive relationship (Spearman's $\rho=0.946$), with a significance level of 1%, indicating that when the percentage of reticulocytes increases, there is also a tendency for the RPI to increase, value determined for each individual. This is because the IPR value for a given individual is found after correcting the percentage of reticulocytes for their hematocrit, having as a reference a normal hematocrit (45%) and also taking into account the reticulocyte maturation time in the bloodstream and which varies according to the hematocrit value (Bain, 2007; Pinto & Ifr, 2008; Piva et al., 2015; Parodi et al., 2020).

In our study, it was found that, when comparing the RPI for values greater than or equal to 3 ($RPI \geq 3$) with the VRC, 84.6% of the donors ($n=11$) were in this range of RPI values and that, simultaneously, have a VRC greater than 1.5. Only two donors, who, despite having CRV values greater than 1.5%, showed IPR values = 2, which may suggest an insufficient spinal cord response, since an IPR greater than or equal to 3 is considered normal, while an index of less than 2 is considered to be below normal (National Committee for Clinical Laboratory Standards (NCCLS 1985)). Likewise, Hoffmann et al., (2012) in their study on "Extended reference intervals for erythrocyte and reticulocyte parameters", emphasizes that reticulocyte parameters have proved to be of great clinical utility in the differential diagnosis of anemia and, also, in monitoring therapy for these cases.

Regarding the statistical results, they demonstrate that there is a positive, very strong and statistically significant relationship

between the domains, the value of the RPI and the VRC ($r=0.946$ $p<0.01$). Which means that a high VRC value is related to a high IPR value, with a significance level of 1%.

Through our study, we clearly observed the presence of reticulocytosis in all donors, that is, an increase in the percentage of circulating reticulocytes in peripheral blood. Moreover, in most cases, we found an IPR value equal to or greater than 3, which reflects the good activity of the bone marrow in response to blood donation. Despite the fact that the study was carried out on buffy coats, and not on whole blood from voluntary donors, patients with anemia and/or other clinical conditions, the results obtained support the relevance of this type of analysis. In a future study, with a larger sample, the determination of the erythrocyte/reticulocyte ratio from a donor's buffy coat may allow the evaluation and monitoring of bone marrow recovery after donation.

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