

Influence of blood/anticoagulant ratio and storage temperature on platelet parameters using Cell Dyn Ruby® analyzer

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ABSTRACT

Introduction: The pre-analytical phase directly influences the laboratory result, such as the method of collection, transport, and storage of biological samples. Therefore, the stability of the biological sample is a crucial and determinant aspect of the quality of results in a clinical laboratory. Studies show that some platelet parameters may suffer alterations in the presence of altered blood/anticoagulant ratio, with increased storage time and/or variations in storage temperature, possibly leading to unrepresentative results. This study aimed to investigate the reliability of platelet parameters regarding the effect of the anticoagulant/blood ratio and storage temperature in samples stored for up to 24 hours after collection using the Cell Dyn Ruby® equipment.

Methodology: A total of 351 blood samples were evaluated under different analysis periods: 2, 5, 12, and 24 hours and storage methods: at room temperature (25 °C) and 4 °C, in addition to the analysis of anticoagulant/blood ratio. The Platelet parameters selected were: PLT (total platelet count), MPV (mean platelet volume), PDW (Platelet Distribution Width) and PCT (plateletcrit). The imprecision of the results was evaluated by the CVa (%) within the maximum allowed analytical variation, as well as by the mean difference of the results concerning the baseline sample (2 hours). **Results:** The total platelet count was the only parameter evaluated that showed reproducibility of results in all conditions analyzed. Regarding the other platelet parameters, it could be observed an imprecision of results emitted by Cell Dyn Ruby® after five hours of storage, both at room and refrigerated temperature. **Conclusion:** This study demonstrates that pre-analytical factors, such as storage temperature and storage time, can affect the variability of platelet parameters, which may produce erroneous results. Thus, the correct blood/anticoagulant ratio must be respected to avoid the late processing of the sample.

Palavras-chave: Blood cell count, Platelet count, Anticoagulant.

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INTRODUCTION

The platelet count is a parameter provided in the complete blood count, obtained by different methods: impedance, optical and immunological, depending on the automated analyzer used¹. Currently, in addition to the platelet count, hematology analyzers provide new parameters, such as mean platelet volume (MPV), platelet distribution width (PDW), and plateletcrit (PCT)².

In most laboratories, automated analyzers can process hematological samples efficiently and quickly³. The late analysis of samples is recurrent in laboratory practice once some of those are processed in laboratories far from the collection site⁴. In other situations, processing delay may occur due to workflow interruption, for operational or technical reasons, or when the test needs to be repeated to ratify the result⁵.

The laboratory results are directly influenced by stages of the pre-analytical phase, such as the method of collection, transport, and storage of biological samples⁶. Regarding venous blood collection, the tourniquet must not exceed one minute, and the blood/anticoagulant ratio must be respected, with EDTA being the main anticoagulant used in samples intended for the Blood Count analysis. After collection, the tubes must be gently homogenized by inversion (minimum five times) so that the anticoagulant is properly mixed in the sample and microclots do not appear⁷. Biological samples for carrying out the blood count must be transported respecting biosafety standards, at room temperature (15 to 22 °C) in the shortest possible time⁸. After some time at rest, for the sample to be processed correctly, it must be homogenized for 15 minutes before performing the exam. Whole blood samples stored at 4 °C become viscous and should be allowed to return to room temperature before homogenization⁷.

The higher frequency of errors in laboratory diagnosis is associated with the pre-analytical phase, that is, incorrect procedures during the collection, transport, preparation, or storage of samples⁹. In order to avoid providing inaccurate results, it is essential to straight out the pre-analytical interferences, which can compromise the reliability of the laboratory⁶.

Studies show that some platelet parameters may change in the presence of altered blood/

anticoagulant ratio, with increased storage time or changes in storage temperature, which may lead to unrepresentative results^{4,10,11}. The variability of hematological equipment can influence complete blood count results¹². Therefore, a more comprehensive assessment of the imprecision of the tests on the equipment can be evaluated by the coefficient of variation obtained by the typical error, reckoned with the standard deviation of the individualities of each sample¹³.

Platelet parameters are considered biomarkers of platelet activation related to the morphology and kinetics of platelet proliferation². The evaluation of platelet biomarkers allows for a broader clinical investigation focused on the diagnosis and prognosis of various pathologies¹⁴.

Therefore, it is necessary to standardize the temperature and storage time of the biological sample, in addition to standardizing the anticoagulant/blood ratio during material collection and learning about the analytical variability of the equipment, so that there is a correct interpretation of the parameter's platelets. Therefore, this study targeted to evaluate possible changes in platelet parameters resulting from variants of the pre-analytical phase, such as: the effect of the anticoagulant/blood ratio and storage temperature, in samples stored for up to 24 hours after collection, using Cell Dyn Ruby® equipment.

METHODS

Study design

An observational study was carried out, obtaining data from the routine of the Vitale Clinical Analysis Laboratory located in the city of Irati, Paraná, Brazil. The study was approved by the Research Ethics Committee of the Universidade Estadual de Ponta Grossa under protocol number 4.825.497.

Only the results of complete blood counts with the medical request were analyzed, evaluated anonymously, and presented in an aggregated form, not allowing the individual identification of the participants; therefore, the free and informed consent term was not necessary. This research was conducted following the Helsinki Declaration.

Sampling

The sample size was defined by the hematology analyzer stability validation procedures, requiring at least ten samples. The study included individuals over 18 years of age with normal hematological reference values, free from hematological disorders such as anemia, changes in blood count, leukocytosis, leukopenia, thrombocytosis, and thrombocytopenia. The study exclusion criteria were lipemic, hemolyzed samples, and multiple puncture attempts. In total, 27 biological samples were selected to determine the maximum storage time under different conditions.

Sample collection

Blood samples, for the evaluation of platelet parameters, were collected with sterile needles and syringes, with a maximum tourniquet time of 30 seconds. After collection, the blood was distributed in tubes containing the anticoagulant EDTA and immediately homogenized 5 times by inversion.

Two blood samples were collected per person, as follows:

i) Sample 1 - peripheral venous blood collected in a tube containing anticoagulant (EDTA K3, 4 ml, VACUETTE®, Premium, Greiner Bio-One, Austria). The volume of blood collected: 4 mL.

ii) Sample 2 - peripheral venous blood collected in a tube containing anticoagulant (EDTA K3, 4 ml, VACUETTE®, Premium, Greiner Bio-One, Austria). The volume of blood collected: 2 mL.

First, the collected samples were processed within two hours after collection in the Cell Dyn Ruby® hematology counter (Abbott Core 7 Laboratory, Chicago, USA) after homogenization of 15 minutes at 10 rpm in the HH28 equipment – Digital Homogenizer (Hoffmann Lab, São Paulo, Brazil). The following platelet parameters were obtained from the hematology counter: PLT (total platelet count), MPV (mean platelet volume), PDW (platelet distribution width), and PCT (plateletcrit).

Equipment

The Cell Dyn Ruby® automated hematology analyzer (Abbott Core Laboratory, Chicago, USA) is hydrodynamically focused on aligning cells in single

rows as they pass through the Optical Flow Cell, which is an optically clear quartz chamber. A vertically polarized helium-neon laser is the light source. There are 256 channels of size for each of the parameters, each channel of red blood cell size being equivalent to 1fL and each channel of platelet size being equivalent to 0.137fL. Erythrocyte parameters are calculated using data from 0°, 10°, and 90° sensors, while platelet parameters are calculated using data from 0° and 10° sensors.

Processing

Then, the sample from each patient was homogenized for 15 minutes at 10 rpm and divided into two equal aliquots (aliquots 1 and 2). Aliquots 1 were stored at room temperature (25 °C) and aliquot 2 at 4 °C. Therefore, the samples were divided into four groups for analysis:

i) Room temperature (25 °C), with adequate anticoagulant/blood ratio;

ii) Refrigerated at 4 °C, with adequate anticoagulant/blood ratio;

iii) Room temperature (25 °C), with change in the anticoagulant/blood ratio;

iv) Refrigerated at 4 °C, with alteration of the anticoagulant/blood ratio;

Subsequently, the aliquots were analyzed at different times: 5, 12, and 24 hours, for the parameters evaluated in the study. Before each analysis, homogenization and temperature stabilization of the sample were performed. All analyses used reagents from the same batch, daily internal quality control was performed, and the hematology counter was calibrated when necessary.

Statistics

The normality of continuous variables was analyzed using the Kolmogorov-Smirnov test. Once normality was confirmed, comparisons of the different storage times (5, 12, and 24 hours) concerning the base sample (2 hours) were performed by analysis of variance for repeated measures (ANOVA), and the data were presented by the mean. The imprecision of the results was evaluated by the analytical coefficient of the CVa% variation, obtained by the typical error (TE), which corresponds to the standard deviation of the individual differences between the

base sample (2 hours) and the different times: 5, 12, and 24 hours, for different forms of storage, at room temperature (25 °C) and 4 °C, divided by the square root of 2. The analytical coefficient of variation CVa% was assumed as the ratio between the TE and the mean of all values observed. The reliability of the results was evaluated by the CVa (%) within the maximum allowable analytical variation, 4.6% for platelet counts, 2.2% for the MPV, 1.4% for the PDW, and 6.0% for the PCT¹⁵, as well as the mean difference of the results concerning the base sample (2 hours). Data were analyzed using the SPSS 20® program (IBM Corp., Armonk, NY, USA), and the significance level was considered when $p < 0.05$.

RESULTS

In total, 351 blood counts were evaluated from individuals with an average age of 34 ± 10 years, with platelet counts within the reference values. Table 1 demonstrates the analytical variability of platelet parameters in samples with normal and altered blood/anticoagulant ratios, at different storage temperatures (25 °C and 4 °C) and storage times evaluated in the study (5, 12, and 24 hours after collection), according to the baseline samples (2 hours).

Reproducibility was only shown in the platelet count of results up to 24 hours, regardless of the blood/anticoagulant ratio and storage method (Table 1). The parameters MPV, PDW, and PCT showed imprecision in the results, that is, the CVa were higher than the maximum value allowed for all conditions studied (Table 1).

Figure 1 shows the comparison among the values of the platelet parameters for the samples with normal and altered blood/anticoagulant ratio, stored at room temperature (25 °C) and a temperature at 4 °C, at the different times evaluated in the study, concerning the results baseline (2 hours).

For samples with a normal blood/anticoagulant ratio, the platelet count showed a significant difference only at 4 °C from 24 hours onwards ($p = 0.049$) (Figure 1 A). Meanwhile, for samples with altered blood/anticoagulant ratio, there was a significant difference at 4°C from 5 hours on ($p = 0.017$) and at room temperature from 12 hours on (Figure 1 A).

From 5 hours of storage, mean values of MPV decreased significantly ($p < 0.020$) concerning

samples analyzed within 2 hours after collection for all conditions evaluated in the study (Figure 1 B).

The PDW showed no difference in mean for samples stored at room temperature for both normal and altered blood/anticoagulant ratios (Figure 1 C). However, samples stored at 4 °C showed a significant increase in mean values for samples with normal ($p < 0.033$) and altered ($p < 0.004$) blood/anticoagulant ratios after 5 hours (Figure 1 C).

PCT showed a significant decrease after 5 hours, and in samples kept at room temperature, the normal and altered blood/anticoagulant ratios showed the same mean values (Figure 1 D). Samples kept at 4°C also showed the same mean values, overlapping values on the graph (Figure 1 D).

Figure 1. Comparison of the results of the average values for the samples with normal and altered blood/anticoagulant ratio between the baseline sample (2 hours) and the different storage times 5, 12, and 24 hours for different forms of storage: room temperature (25 °C) and temperature at 4 °C. *Statistically significant difference, $p < 0.05$.

DISCUSSION

The results showed inaccuracy for some platelet parameters emitted by Cell Dyn Ruby® for up to 24 hours, regardless of blood/anticoagulant ratio and storage method.

The total platelet count was the only parameter evaluated that showed reproducibility of results in the two storage modes during the storage period, according to the maximum CVa% allowed for the latest generation hematology analyzers (CVa less than 4.6%).

Any automated method used for platelet counting must be accurate, demonstrate minimal fluctuation in repeated results on the same sample, and present linear results over the entire analytical range¹⁶. The platelet count showed a decrease until the storage time of 12 hours in all storage modes; however, samples refrigerated at 4 °C had increased counts at 24 hours of study. However, the variability of the means was minimal, ranging from 240,000 to 246,000 platelets/mL throughout the analyzed period. These results are in agreement with a study by Tanaka et al. (2014), in which it was found that platelet counts remained stable for up to 48 hours in samples stored at 4 °C or room temperature¹⁷.

Table 1. Analytical variation between baseline samples (2 hours) and storage times (5 to 24 hours) at room temperature and 4 °C, for samples with normal and altered blood/anticoagulant ratio.

Parameters	CVa (%) Maximum allowed	Normal Blood/Anticoagulant Ratio					
		Room temperature			4°C		
		Storage times			Storage times		
		5 hours TE (CV%)	12 hours TE (CV%)	24 hours TE (CV%)	5 hours TE (CV%)	12 hours TE (CV%)	24 hours TE (CV%)
Platelets (10 ³ cell/mL)	4.6	9.31 (3.90)	8.68 (3.65)	9.09 (3.82)	9.72 (4.06)	8.94 (3.75)	10.56 (4.35)
MPV (fL)	2.2	0.36 (3.96)*	0.44 (4.91)*	0.42 (4.75)*	0.45 (4.85)*	0.54 (5.94)*	0.70 (7.61)*
PDW (%)	1.4	0.49 (2.50)*	0.50 (2.54)*	0.55 (2.39)*	0.65 (3.30)*	0.58 (2.95)*	0.72 (3.62)*
PCT (%)	6.0	0.01 (6.31)*	0.01 (6.87)*	0.02 (7.89)*	0.02 (8.66)*	0.02 (7.24)*	0.02 (8.95)*
Altered Blood/Anticoagulant Ratio							
Platelets (10 ³ cell/mL)	4.6	9.28 (3.90)	9.91 (4.19)	10.38 (4.38)	8.97 (3.79)	8.32 (3.52)	8.63 (3.59)
MPV (fL)	2.2	0.43 (4.71)*	0.54 (6.09)*	0.54 (6.09)*	0.40 (4.37)*	0.50 (5.39)*	0.62 (6.74)*
PDW (%)	1.4	0.52 (2.67)*	0.67 (3.42)*	0.67 (3.42)*	0.52 (2.67)*	0.60 (3.03)*	0.61 (3.06)*
PCT (%)	6.0	0.02 (7.21)*	0.02 (7.97)*	0.02 (8.07)*	0.01 (6.63)*	0.01 (6.19)*	0.02 (8.33)*

*Imprecisão, CVa (%) maior que o máximo permitido. CVa, coeficiente de variação analítico. ET, erro típico.

MPV reflects the average volume of circulating platelets and correlates with platelet function and may be more sensitive than platelet count as a biomarker of several pathologies¹⁸. The MPV results showed imprecision of the values, with coefficients of variation greater than 2.2%, in both storage modes, regardless of the blood/anticoagulant ratio and storage time. In addition, there was a decrease in the average values in all analyses throughout the storage time. It should be noted that in the samples stored at room temperature, there was a decrease of approximately 1.0 fL in the average values of VPM in up to 24 hours. The instability of this parameter was also observed in other studies for refrigerated and room temperature samples^{19–21}. The results obtained contradict a study developed by Daves et al. (2015), where a significant increase for MPV could be observed after 3 hours at room temperature and only after 24 hours when refrigerated at 4 °C using the Sysmex XN® analyzer¹⁹. Gunawardena et al. (2017) evaluated Sysmex XS 500i® and observed a significant increase in MPV after 6 hours at room temperature and after 24 hours when refrigerated at 4°C²⁰. Hedberg et al. (2009) used the Abbott CELL-DYN Sapphire® equipment and demonstrated a minimal increase in MPV only between 48 and

72 hours when refrigerated at 4 °C²¹. The works discussed above^{19–21}, although using different hematological analyzers, both used the anticoagulant EDTA K2, while the present study used EDTA K3, and it seems that EDTA K3 tends to present lower values for MPV²².

Another possible explanation for this divergence of information may be related to the methodology used, impedance or optics, to measure this parameter. For example, in the impedance methodology, the MPV tends to increase over time due to the swelling of the platelets caused by the anticoagulant EDTA²³. This methodology is based on the measurement of oscillations of an electric current during the passage of blood cells through a small hole between two electrodes. From this change in current, an electrical impulse proportional to the cell volume is generated. The sum of the pulses provides the value of the platelet cell (PCT), analogous to the hematocrit. From these values, the MPV is obtained by the formula: $MPV = PCT \times PLT^{16}$. According to Bowles et al. (2005), an initial increase of 7.9% and an overall increase of 13.4% can occur over 24 hours²⁴. However, this increase in platelet size may be less than approximately 0.5 fL when the analysis is performed within 2 hours after venipuncture²⁵.

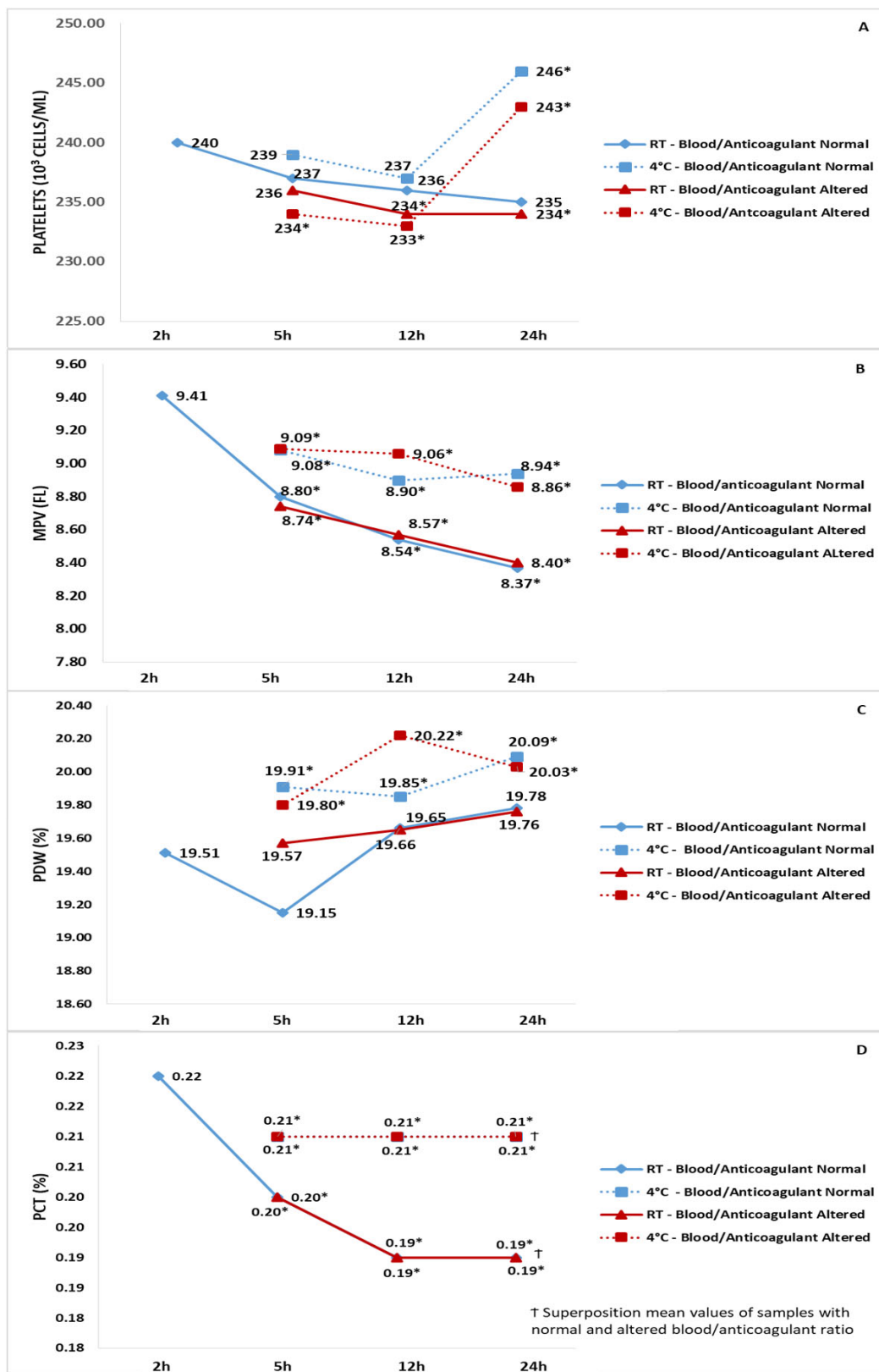


Figure 1. Comparison of the results of the average values for the samples with normal and altered blood/anticoagulant ratio between the baseline sample (2 hours) and the different storage times 5, 12, and 24 hours for different forms of storage: room temperature (25 °C) and temperature at 4 °C. *Statistically significant difference, $p < 0.05$.

However, in the methodology of optical light scattering systems, the MPV tends to decrease, due to a possible dilution of the cytoplasmic content, leading to a decrease in the refractive index²⁶. A possible explanation for the reduction in the refractive index is the exposure to the anticoagulant EDTA, capable of altering the permeability of the cytoplasmic membrane and causing a decrease in optical density over the storage time²⁷. In the present study, the MPV was determined by the average volume of counted platelets obtained from the distribution histogram in the Cell Dyn Ruby® equipment using the optical system, with a consequent decrease in the averages in all the analyses of the study.

Therefore, the MPV can increase when determined by the impedance method and decrease when determined by the optical method, according to the storage time of the biological sample. Furthermore, in some situations, the differences between the two methodologies can often be amplified by a kind of "hypersensitivity" to EDTA, which is accompanied by changes in platelet morphology²⁸.

PCT corresponds to the total volume of platelets in a given volume of blood and can be calculated according to the formula $PCT = PLT \times MPV / 10.000$ ¹⁴, analogous to the hematocrit of the red series of the hemogram. Under physiological conditions, the amount of platelets in the blood is maintained in a steady state by regeneration and elimination¹⁴, and PCT is directly proportional to MPV; PCT results also tended to decrease in all study analyses. However, the variability of the means was minimal, ranging from 0.22% to 0.19%, throughout the analyzed period, being the normal range for PCT considered between the values of 0.22% to 0.24%¹⁴.

The PDW indicates the heterogeneity of platelet volumes, indicating the presence of platelet anisocytosis, obtained at the level of 20% of the platelet size distribution histogram¹⁴. For all samples stored under refrigeration at 4 °C, regardless of the blood/anticoagulant ratio, there was an increase in mean values after 5 hours. However, the variability of the means was minimal, ranging from 19.80% to 20.09%, throughout the analyzed period. The PDW can show marked variability, with reference ranges ranging from 8.3% to 56.6%¹⁴.

CONCLUSION

The results of the present study showed that at both storage temperatures, being, room

temperature and 4 °C, and both anticoagulant/blood ratios (normal/altered), significant changes may occur in some platelet parameters, analyzed in the Cell equipment Dyn Ruby®, starting 5 hours after collection.

Although these parameters are already available by most trailblazing hematology counters, they are still poorly understood and used in medical and laboratory practice. However, knowing the variables that can interfere with the values of platelet indexes is necessary to assure the reliability of the tests.

Therefore, it has been shown that pre-analytical factors, such as storage temperature and storage time, can affect the variability of platelet parameters, which can produce misleading results. On that account, the correct blood/anticoagulant ratio must be respected, and the late processing of the sample must be avoided.

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Authors' contribution:

DCKB, MJW, and MFM contributed to the conception and design of the study. MJW contributed to the data acquisition. DCKB performed the statistical analyses. MJW and DCKB interpreted the results and prepared the manuscript. MFM and DCKB critically evaluated the manuscript. All authors read and approved the final manuscript.

Conflict of interest:

The authors declare no conflict of interest.

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