Research article

Analysis of the Leukocytes in peripheral blood and Leukocyte- and Platelet-Rich Plasma (L-PRP) in rats: A flow cytometry study

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Abstract

Background and objectives. Platelet concentrates for surgical use were often tested as surgical adjuvants in the literature, as a source of platelet growth factors to stimulate healing. Many products are often regrouped under the generic and inaccurate term of Platelet-Rich Plasma (PRP). However, what is tested in many studies is usually a combination of platelets and leukocytes (accurately termed Leukocyte- and Platelet-Rich Plasma – L-PRP). The quantity and impact of leukocytes in these preparations were not yet accurately investigated. In this article, the characteristics of white blood cells in a L-PRP obtained from rats were investigated, in order to point out the main actors and some of the mechanisms that may influence the properties of the platelet concentrates.

Materials and Methods. Blood and platelet concentrate samples were obtained from 64 healthy Wistar rats and leukocyte phenotypes were identified using flow cytometry after labeling leukocytes for CD3, CD4, CD8, CD11bc, CD18, CD25, CD27, CD28, CD45R, CD45RA, CD80, CD90, CD106 (VCAM-1), CD161a and TCRab, TCRgd, RT1B with fluorochrome-conjugated antibodies.

Results. The results have shown that the tested L-PRP contained substantial amounts of leukocytes of many different kinds, particularly T lymphocytes, B lymphocytes, NK cells, monocytes, granulocytes and eosinophils.

Discussion and Conclusion. To highlight the various ways in which these cells can influence their environment will help to better understand the complex interactions of the PRPs with the tissues. This identification of the exact cell content and the understanding of this complex cell equation are important steps towards using these blood concentrates in the best possible way, as a reliable therapeutic option to promote better healing, particularly in infected surgical or wound sites.

Keywords. Blood platelets, infection, leukocytes, platelet-rich plasma, wound healing.

1. Introduction

Platelet concentrates for surgical use represent a relatively novel inductive therapy that could be valuable to accelerate and improve healing processes **[1-3]**. The general concept of these technologies is to concentrate the platelets and their many growth factors, to inject them to stimulate healing and hopefully to promote tissue regeneration. All these techniques are using centrifugation of whole blood, in order to reach high concentrations of platelets and growth factors **[2,4]**. Many products are often regrouped under the generic and inaccurate term of Platelet-Rich Plasma (PRP)**[5]**. Another family is termed PRF (Platelet-Rich Fibrin), when the platelet concentrate was designed and only exists under a strongly polymerized fibrin gel form **[6,7]**.

However, in the vast literature on these technologies, it is often neglected that many platelet concentrate technologies collect also a significant amount of leukocytes **[8,9]**. These cells have a strong direct impact on healing and also produce many molecules including large amounts of growth factors **[10]**. For this reason, a more accurate terminology was proposed and 4 families of products were suggested **[5,6,11,12]**. Two of these 4 main families of platelet concentrates contain higher concentration of leukocytes compared to the amounts of these cells found in peripheral blood: these are termed Leukocyte and Platelet-Rich Plasma (L-PRP) and Leukocyte and Platelet-Rich Fibrin (L-PRF) **[6]**. In fact, these two families with leukocytes are the most frequently used platelet concentrates in many fields of medicine **[3,6,13-15]**. There is still a limited number of studies concerning the detailed composition of leukocytes and their role in these products, and these investigations were mostly done on L-PRF **[7,16]**. Despite a wide spectrum of available diagnostic techniques, we have not found animal and clinical studies identifying extensively the characteristics of white blood cells (WBC) in the various types of L-PRP. Therefore, the objective of this study was to investigate the detailed characteristics of white blood cells in a L-PRP obtained from rats, in order to point out the main actors and some of the mechanisms that may influence the properties of the platelet concentrates.

2. Materials and methods

2.1. Preparation of L-PRP

The study group consisted of 64 healthy male Wistar rats. The Silesian Medical University Bioethics Committee approval was obtained. The rats were anaesthetized with Ketamin (10 mg/kg) after Diazepam (0.1 mg/kg) premedication. For the study, 3.5±0.1 ml of whole blood were collected directly from the heart into a syringe containing 0.7 ml of sodium citrate 105 mmol/l. 4.0±0.1 ml were drawn into a sterile tube and centrifuged for 10 minutes at 1000 RPM (Janetzki K23, Berlin, Germany). This resulted in blood separation into its three basic components: red blood cells, L-PRP sometimes referred to as “buffy coat”, and leukocyte- platelet-poor plasma (L-PPP). Subsequently, L-PPP and L-PRP were removed into 5 ml syringes and centrifuged for 10 minutes at 3000 RPM. After centrifugation, supernatant was removed and 600±50µl L-PRP was obtained. Next, samples with whole blood and L-PRP were examined.

2.2. Cell preparation and flow-cytometric analysis

Blood and L-PRP samples were processed under standardized and optimized conditions within less than 4 hours after collection. The antibody set was designed to identify all major leukocyte populations as well as different lymphocyte subpopulations. For this

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purpose, sample aliquots were stained with 3-to-5 fluorochrome-conjugated mouse anti-rat monoclonal antibodies (Becton Dickinson, San Diego, CA, USA; Invitrogen, Carlsbad, CA, USA; Serotec, Raleigh, NC, USA), as presented in the **Table** (CD3, CD4, CD8, CD11bc, CD18, CD25, CD27, CD28, CD45R, CD45RA, CD80, CD90, CD106, CD161a and TCRab, TCRgd, RT1B). After the staining step, erythrocyte lysis and fixation was performed with FACSLyse Solution (Becton Dickinson). Subsequently, the sample was washed with CellWash solution (Becton Dickinson) and finally resuspended in FACSFlow solution (Becton Dickinson). Acquisition of data **(Figures 1 to 3)** was performed with the use of FACSCanto II flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The data were acquired and analyzed with Diva software (Becton Dickinson).

3. Results

The **Table** shows the results of platelet and leukocyte measurements in blood and L-PRP. In the whole blood, the mean platelet number was 456 ± 78 SD × 10^9/l and the mean leukocyte number 8.501 ± 2.412 SD × 10^9/l. In L-PRP, platelet counts were increased by a mean of 335% (310% to 380%) and leukocyte counts by 350% (320% to 380%) in comparison to the whole blood reference values, showing a strong concentration of both platelets and leukocyte populations in L-PRP. The Mean Fluorescence Intensity (MFI) showed that vascular cell adhesion molecule 1 (VCAM-1/CD106) decreased from 222 to 84 after centrifugation. In the L-PRP tested in this study and in this animal model, the concentrations of most other leukocytes populations were increasing in similar proportions, and the proportions of the various populations (percentages of final leukocyte formula) were relatively similar to the whole blood references. However, granulocytes and monocytes proportions had a tendency to increase during the L-PRP processing, while lymphocytes (T and B) proportions had a tendency to decrease, even if the differences did not appear clearly significant.

4. Discussion

In the literature, most authors focus on the impact of platelet concentration and platelet growth factors amounts **[17-19]**. The presence of the latter in PRPs is believed to accelerate the wound healing process **[14]** and therefore the role of platelet derived growth factors was investigated and discussed in priority in many publications **[2]**. However, most authors were in fact using platelet separation systems collecting also leukocytes **[10,13,20,21]**. The mean leukocyte concentration increase observed in L-PRP was 5 to 8-fold compared to baseline level. In our study, a lower concentration of WBC (3.5-fold on average) was observed, because of the use of a 2-steps harvesting procedure.

Despite the strong potential of leukocytes in the anti-infectious defense and healing process in an injury site (particularly through their antimicrobial molecules and growth factors), some authors still wish to exclude leukocytes from blood concentrate to obtain pure platelet-rich plasma (P-PRP) without leukocytes **[6,22]**. The exact influence of the leukocytes on the mechanism of L-PRP action is obviously very complex and remains to be investigated extensively; its supporters claim that their influence on the inflammatory state is beneficial, while opponents – on the other hand – claim their negative effect in the form of solid enzymes release **[2,12]**. At the time being it is a known fact that leukocytes, and especially neutrophils, are a rich source of not only the natural antibacterial proteins **[23]**, but especially of the growth factors **[10]**.

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		Blood	L-PRP
Platelets (G/L)		**456**	**1530**
WBC (G/L)		**8.5**	**29.75**
Lymphocytes T (CD3+) [%]		**37.81**	**32.62**
[%]	Lymphocytes T CD8+	26.49	24.26
	Lymphocytes T CD4+	70.56	72.81
	Lymphocytes T CD4+CD8+	1.37	1.92
	Lymphocytes T CD4-CD8-	1.58	1.01
	Lymphocytes T TCRab+	96.76	96.6
	Lymphocytes T TCRgd+	1.71	1.63
	Lymphocytes T CD27+	94.65	95.16
	Lymphocytes T CD28+	97.93	98.64
	Lymphocytes T CD8+CD27+	25.05	17.98
	Lymphocytes T CD8+CD28+	24.72	17.88
	Activated lymphocytes T RT1B+	5.05	5
	Activated lymphocytes T CD25+	3.73	3.35
Lymphocytes B (CD45RA+) [%]		**11.6**	**8.78**
[%]	Lymphocytes B CD90+CD45R-	14.61	19.98
	Lymphocytes B CD90+CD45R+	20.42	20.96
	Lymphocytes B CD90-CD45R-	5.57	9.13
	Lymphocytes B CD90-CD45R+	59.4	49.94
Lymphocytes NK (CD161a+) [%]		**1.71**	**1.81**
Monocytes (gran-/CD18+) [%]		**1.3**	**3.33**
[%]	Monocytes CD11bc+	100	100
Granulocytes (gran+/CD18+) [%]		**34.48**	**37.64**
[%]	Granulocytes CD11bc+	100	100
Eosinophils [%]		**2.27**	**1.96**
CD106 median		**222**	**84**

Table. Mean values of selected blood/L-PRP cells.

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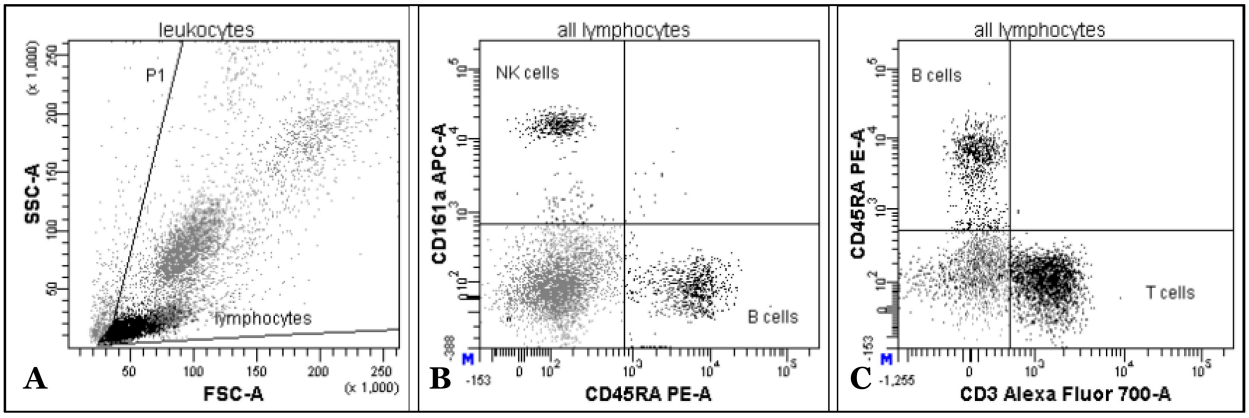

Figure 1. A-C. Rat leukocytes in forward- (FSC) and side scatter (SSC) representation. Main populations of lymphocytes are gated based on their lineage-specific markers: T-cells (CD3), B-cells (CD45RA), and NK cells (CD161a).

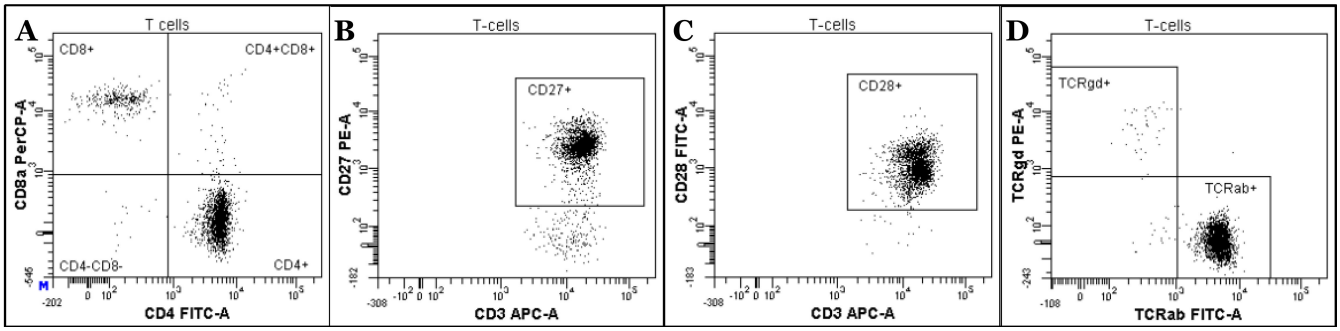

Figure 2. A-D. Main subpopulations of T-cells. Majority of rat T-cells are CD27 and CD28-positive and T-cell surface receptor (TCR) is mostly formed of alpha and beta subunits.

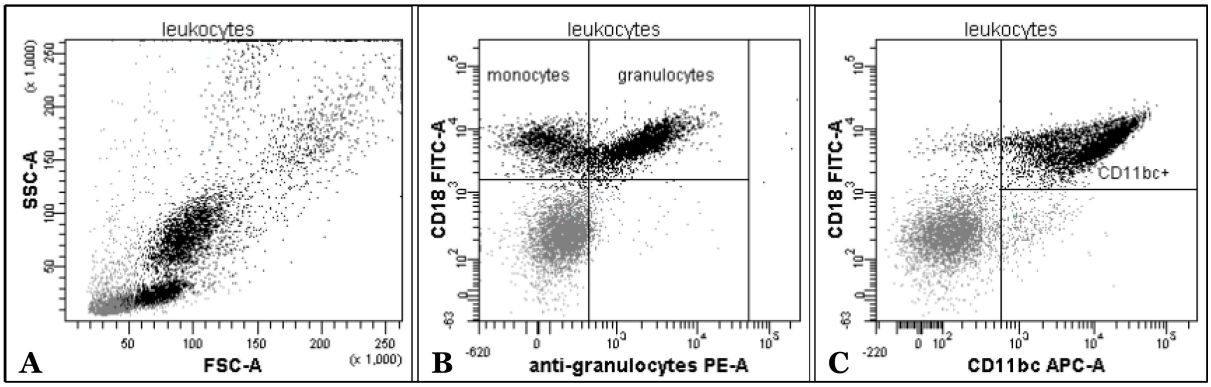

Figure 3. A-C. Monocytes and granulocytes, separated on the FSC-SSC plot and with the use of anti-granulocyte antibody cocktail. Both monocytes and granulocytes are CD11bc and CD18 positive, which form the surface integrin complex.

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To reach plasma with a minimal leukocyte count, the g-force and time of centrifugation need to be decreased. As a consequence, the platelet concentration is lower in comparison to baseline levels and so are, in consequence, the growth factors levels **[24]**. Only in the studies by Aspenberg and Virchenko, where L-PRP was irradiated with 25 Gy to inactivate the WBCs, truly pure platelet concentrate could be obtained **[25]**. However such experimental method is clearly not practical in daily use. Consequently, most PRPs used in the literature have in fact a significant quantity of leukocytes. However, most authors do not mention leukocyte concentration even though they use standard separation systems, and they focus mostly on platelets and growth factors **[6]**. This situation is a major source of confusion and bias in the PRP literature and may explain the many controversies and mixed clinical results obtained with these products in the literature **[5,14,15]**. To clarify this confusion, a terminology was proposed in 2009 **[6]**, and then reinforced in following consensus articles **[5,11,12]**, in order to separate the many different products following at least 2 parameters, the fibrin density and the leukocyte content. For the PRPs, 2 main families were defined: the L-PRPs have a significant quantity of leukocytes, while the Pure PRP (P-PRP) have no or only traces of leukocytes. This first classification was designed to highlight the issue, and it is expected to be extended in the future, when the exact function and impact of the various possible concentrations and proportions of leukocytes will be clarified **[12]**.

Leukocytes are the cells of the immune system defending the body against both infectious diseases and foreign materials **[10]**. According to their appearance under light microscope, there are two broad categories of lymphocytes, namely the large granular lymphocytes and the small lymphocytes. Functionally distinct subsets of lymphocytes correlate with their appearance. Most, but not all large granular lymphocytes are more commonly known as the Natural Killer cells (NK cells). The small lymphocytes are the T-cells and B-cells. Lymphocytes play an important and integral role in the body's defenses. T-cells and B-cells are the major cellular components of the adaptive immune response. T-cells are involved in cell-mediated immunity whereas B-cells are primarily responsible for humoral immunity (relating to antibodies). The function of T-cells and B-cells is to recognize specific “non-self” antigens, during a process known as antigen presentation. Once they have identified an invader, the cells generate specific responses that are tailored to maximally eliminate specific pathogens or pathogen infected cells. B-cells respond to pathogens by producing large quantities of antibodies which then neutralize foreign objects like bacteria and viruses. In response to pathogens, some T-cells, called helper T-cells produce cytokines that direct the immune response whilst other T-cells, called cytotoxic T-cells, produce toxic granules that induce the death of pathogen infected cells. Following activation, B-cells and T-cells leave a lasting legacy of the antigens they have encountered, in the form of memory cells **[10,23,26]**. Finally, B and T lymphocytes have very strong regulatory functions during the inflammatory process, and investigating their role in these complex mechanisms is a very intensive path of research **[27-30]**.

NK cells are a part of innate immune system and play a major role in defending the host from both tumors and virally infected cells. NK cells distinguish infected cells and tumors from normal and uninfected cells by recognizing alterations in levels of a surface molecule called MHC (major histocompatibility complex) class I. NK cells are activated in response to a family of cytokines called interferons. Activated NK cells release cytotoxic (cell-killing) granules, which then destroy the altered cells **[10,23]**. They were named "natural killers" because of the initial notion that they do not require prior activation in order to kill cells which are missing MHC class I.

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Antibacterial properties of L-PRP were investigated in the literature **[31,32]**. However, until now no one can explain the exact mechanism that inhibits bacteria growth, despite several authors have tried. Cieslik-Bielecka et al. described various mechanisms, which can cause microbicidal effects **[23]**. They focused on antibacterial peptides (HDP – Host Defense Peptides), which are produced by the macrophages, epithelial cells, as well as neutrophils and thrombocytes, and are one of the important elements shaping the human natural immunity. Neutrophils are the most common cells with strong phagocytic properties. They constitute the first line of antibacterial defense. The cytoplasm of the neutrophil granulocytes contains numerous granules. The most important ones are primary (azurophil) granules connected to the process of intracellular bacteria destruction and containing numerous bactericidal factors, including defensins, cathelicidins, serprocidins, Bactericidal/permeability-increasing protein (BPI) of gram-negative bacteria, myeloperoxidase and cytoplasmic calprotectin. The secondary (specific) granules are rich in antibacterial proteins such as lysozyme, collagenase, gelatinase, lactoferrin, phospholipase A2, transcobalamin-1 and membrane proteins **[26]**. Neutrophils release enzymes which destroy some specific components of the damaged tissues (to allow cell migration, tissue cleaning and finally tissue reconstruction), and first of all damage microbes, especially bacteria. Neutrophils circulating in the blood become activated by chemotactic factors and this constitutes a signal for the merging of the secretory granules with the superficial membrane. The process of phagocytosis starts by surrounding the organism by pseudopodia, and then closing it inside a phagosome, which undergoes a merge with granules, mainly the primary. The granules release their content exposing the microbe to the activity of a strong mixture of antibacterial proteins. The destruction of the phagocytosed bacteria takes place with the participation of oxygen species, or without oxygen with the use of lactoferrin or lysozyme **[23]**.

Moojen et al. confirmed L-PRP antimicrobial activity against Staphylococcus Aureus **[32]**. Bielecki et al. also used L-PRP in infected bone non unions with good outcomes **[31,33]**. It was justifiable to conclude that L-PRP gel is an inductive biomaterial, which might possess local antimicrobial activity. Some authors have also reported decrease of infections after L-PRP usage in orthopaedic and cardiac surgery **[34]**. Yuan et al. reported a case of infection after intramedullary nailing, which has been a serious problem in orthopaedic surgery **[35]**. In this case, many kinds of treatments had been previously applied, but were not effective. During the operation, they observed a great deal of canal or sinus in most of the callus, and dead bone could be seen from the radiographs. However, it was difficult to remove all of these dead tissues, because if these were removed, the femur would fracture again. Furthermore, in the latter period, the patient refused any open operation under anesthesia. That is why they attempted to use L-PRP to treat the patient, as there were no alternatives to be chosen. To their surprise, the wound healed after L-PRP application.

In Khalafi's study, the L-PRP group had one incidence of sternal infection (0.18%) compared to 11 cases (1.98%) in the control group **[34]**. There were 3 cases (0.53%) of notable drainage from the sternum in the L-PRP compared to 30 cases (5.39%) in the control group. For the leg vein harvest site, the L-PRP group had no reported infections and 61 (10.89%) incidences of excessive drainage, compared to 3 (0.66%) surgical site infections and 212 (48.4%) cases of excessive leg drainage in the control group. Following propensity scoring, they concluded that L-PRP application reduced the odds of chest wound infection by 93%, chest drainage by 96%, and leg wound drainage by 88%.

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VCAM-1 with others adhesion molecules have been found to be significantly increased during viral and bacterial infection **[36]**. However, in our study there was a slight decrease in percentage of CD106-positive leukocytes in L-PRP samples as compared to peripheral blood samples. This may suggest that these rare cells are getting lost during additional centrifugation stages. For other cells, it is interesting to point out that the proportions of the various leukocyte types remain quite stable between blood and final concentrate in the specific L-PRP tested in this study. In another family of platelet concentrates termed L-PRF (Leukocyte- and Platelet-Rich Fibrin), which only exists under a strongly polymerized activated form **[37-39]**, the leukocyte formula of the L-PRF clot is very different from the normal blood composition, with a higher proportion of lymphocytes and a lower proportion of monocytes and granulocytes **[7]**. This composition was advocated to explain the very strong effects in vitro of L-PRF on the bone cell proliferation and differentiation **[40,41]**, and this parameter needs to be evaluated carefully in all PRP and PRF available on the market.

The clinical studies with L-PRP mentioned above illustrate that the associated antibacterial effects of L-PRP (and of L-PRF) play an important role in positive clinical outcomes in many clinical applications. What is also shown is that the characteristics of different L-PRPs are not identical, because their leukocyte content and formula can vary. This aspect is rarely discussed in the literature and more studies are needed in this area. The presence of leukocytes in L-PRP may also influence growth factors levels, as it was already well shown with L-PRF **[4,16,42,43]**. The relative influence of platelets and leukocytes on growth factor levels in PRPs requires further investigation. As it was proven with L-PRF, leukocytes also influence the proliferation and differentiation pathways of many cell types in culture, not only with mediators but also directly **[10,40,41]**. The role of WBC as regulation turntables is essential to fully understand the complex biology of the L-PRP/L-PRF.

5. Conclusion

Until now, the detailed characteristics of white blood cells in L-PRP in rats have never been published in the international literature, and very little is known about the leukocytes populations in most L-PRP available on the market and tested in the literature. Considering the many possible ways in which various leukocyte populations can influence the properties of L-PRP, this study confirms the need to analyze the pattern of white blood cells in blood and L-PRP, before starting to investigate the effects of a platelet concentrate.

Leukocytes have obviously a major impact on the properties and biological activity of platelet concentrates. The ways in which these cells influence the intrinsic biology of L-PRP/L-PRF include their immune and antimicrobial potential as well as their key-role in wound healing processes. That is why the presence of leukocytes in PRPs cannot be neglected and requires further investigation, as this field of research opens new perspectives and possibilities in many clinical situations.

Disclosure of interests

The authors have no conflict of interest to report.

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Author Contributions

All authors participated to the technical design and organization of the study, the treatment of data and to the elaboration of the manuscript. ACB, PP, LS and AS were in charge of the collection of the samples and raw data.

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