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Comparative Cytomorphological Analysis on Liquid PRFs Produced with DUO Fixed Angle Centrifuge and Oscillating Centrifuge

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Abstract:
Background: Liquid PRF is an injectable second-generation platelet concentrate rich in platelets, leukocytes, and fibrinogen obtained by centrifugation of autologous blood. Methods: This study aimed to analyze the cellular and fibrinogen content of various types of liquid PRF (C-PRF liquid, A-PRF liquid, i-PRF, liquid fibrinogen) obtained using a swinging centrifuge with Vacumed FL tubes (code 44909) compared to that obtained with a fixed-angle centrifuge DUO and the original S-PRF Sticky tube. This study found that the average accumulation of thrombocytes was approximately 1.5 times higher than that observed in whole blood. Due to the high concentration of platelets and leukocytes, liquid PRFs contain important growth factors for tissue regeneration. Results: In this definitive study we have highlighted that with the use of the Oscillating centrifuge, the type of Liquid PRF with the highest content of Platelets (133.1% vs 122.9%) is the A-PRF liquid (1300 rpm × 5'), the one with the highest content of Monocytes (142.9% vs 125.0%), with a sufficient content of Lymphocytes (220.0% vs 198.7%), Neutrophil Granulocytes (54.0% vs 58.8%) and Fibrinogen (97.1% vs 104.3%), is the i-PRF (700 rpm × 5 min) obtained with the Vacumed FL tube (code 44909) with statistically insignificant differences compared to those obtained with the S-PRF Sticky test tube, while the content of Fibrinogen present in C-PRF (2500 rpm × 8') is higher (104.0% vs 106.5%) obtained with the with S-PRF Sticky tube with a significant difference (p=0.013). Cellular score was also calculated, finding that the type of liquid PRF with the highest score was i-PRF (700 rpm × 5') extracted with a Vacumed FL tube in a rocking centrifuge (127.17%). Conclusions: Values were significantly higher than those obtained with the Fixed Angle Centrifuge and with the S-PRF Sticky tube. However, when calculating the cellular score, the highest result, equal to 127.2%, was obtained with i-PRF (700 rpm × 5') with Vacumed FL tube and Oscillating Centrifuge.

Keywords: Liquid Platelet-Rich Fibrin, Liquid Fibrinogen, Platelet Concentrate, Platelets, Leukocytes, Low Speed Centrifugation, Tissue Regeneration.

INTRODUCTION

An injectable formulation of platelet-rich fibrin (*i*-PRF) was studied in 2021 by Shao Z. et al. [1] through centrifugation at 700 rpm for 3 min (60 g) without anticoagulants. This liquid scaffold contains large amounts of fibrin, which traps large numbers of platelets, white blood cells, and growth factors and can promote cell proliferation, migration, and matrix secretion (Singh D. et al.

2023). [2] Furthermore, the fibrin network dispersed in the liquid scaffold promotes the stable and sustained release of growth factors for more than two weeks (up to 28 days) (Crisci & Crisci, 2022)[3], in addition to transporting stem cells. The platelets contained within it regulate the recruitment of adult stem cells to damaged cells; This may therefore be an important mechanism in regenerative cellular responses (Crisci et al. 2018 [4], Karimi K, Rockwell H., 2019 [5]). Therefore, this injectable scaffold can be used in regenerative medicine alone or as an adjuvant to other biomaterials.

The concentrated platelets present in i-PRF are responsible for the active secretion of growth factors and the induction of the need, proliferation, and differentiation of various cells involved in the regeneration process[4].

Platelet activation begins immediately upon contact with the wall of the centrifugation tube and leads to the formation of a dense fibrin network. A recent study demonstrated that reducing the relative centrifugal force leads to a significant increase in the total number of platelets and leukocytes and the amount of growth factors, indicating that the low-speed centrifugation concept (LSCC) results in an increased PRF regeneration potential (Castro A.B. et al., 2021[6]).

Because we recover the upper fraction of PRF, horizontal centrifuges are theoretically more useful. Furthermore, they reduce the capacity for cell-cell and cell-internal wall collisions, thus preventing accelerated adhesion and potential cell injury. Indeed, horizontal centrifuges achieve higher speeds in recovering platelets in PRF matrices in the upper layers of the tube (Table I).

In clinical practice, the PRF matrix is separated from the red blood cell fraction (Figure 1). At this stage, operators use scissors or spatulas to more or less invade the PRF matrix region. In this case, simply comparing the cross-sectional diameters, PRFs prepared using horizontal centrifuges are shorter. For example, the cross-sectional area of the PRF matrix prepared using an Intra-Spin centrifuge was 1.83 times larger than that prepared using a horizontal centrifuge. Therefore, platelet loss can theoretically be minimized using a horizontal centrifuge.[7]

In PRF matrices, because platelets are not distributed by density gradients, they are not the most accumulated component around the interface. Therefore, regardless of operator skill, platelets are rarely lost during mechanical separation of the RBC fraction, which is almost eliminated (Figure 1, Table II). However, the superiority of the horizontal centrifuge compared to the fixed-angle centrifuge for PRF preparation has not yet been demonstrated. It is therefore important to know the concentration and quantity of platelets that can be obtained and how the leukocytes are concentrated in a particular liquid preparation.

In this experiment, we addressed the following questions:

- Which protocol yields better results in terms of platelet and fibrinogen collection?
- Which centrifuge, fixed or oscillating, is more effective in producing liquid PRF in terms of platelet and leukocyte content?

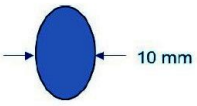
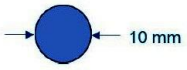
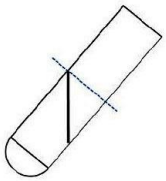
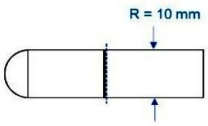
MATERIALS and METHODS

No ethical committee approval was required for this study since no human samples were identified, as previously described. [4] All subjects from which blood samples were drawn agreed to sign the informed consent. Our institutional review board approved the collection of 1 sample of 45 mL or less, and donors provided individual consent. Forty-five subjects in apparent good

health, 23 males and 22 females, aged between 35 and 101 years, were subjected to blood sampling in two phases for a total of 45 ml (5 Vacumed FL tubes × 9 ml) in a first phase and another 45 ml (5 S-PRF tubes × 9 ml) in a second phase.

This study aims to discover and confirm the best tube type and centrifugation speed with a swinging centrifuge (CTL420, CenLee, Beijing, China) to obtain liquid PRF with the highest cell and fibrin content. The main objective is to investigate whether adapting the g-force for the aforementioned modifications on liquid PRF (C-PRF liquid, A-PRF liquid, and *i*-PRF), using a swinging centrifuge and with two types of tube, has any influence on their characteristics in terms of morphology and cellular content, as well as on the content of fibrinogen, a precursor of the fibrin network. A comparison was then performed with liquid PRF produced with a fixed-angle centrifuge DUO (PRF DUO, Process per PRF, Nice, France) according to the procedure recommended by Choukroun J. in 2014[8] and reported in our previous work (Crisci M. et al., 2024).[9]

Table I Angulation of rotors used in centrifuges and cross-sectional areas of the interface between PRF and RBC fractions

	Duo Quattro	Horizontal Type
Angulation (°)	41.3	90.0
Cross-sectional area (mm^2)	151.5π	100.0π
Shape of cross-section		
Images of angulation and cross-section		

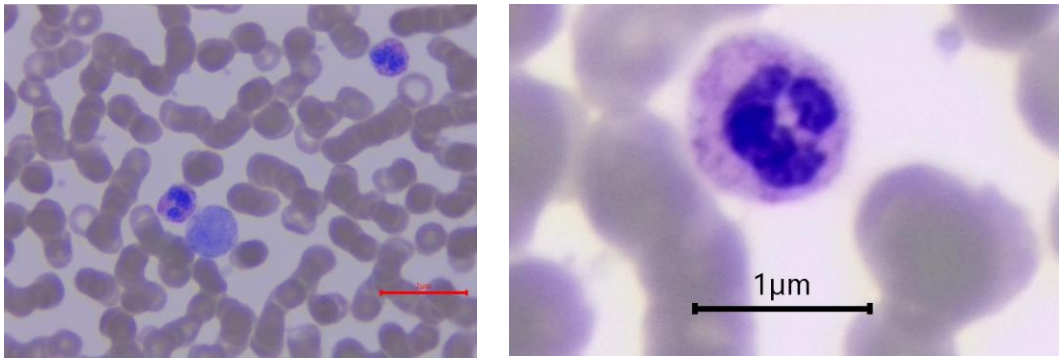


Figure 1: Red Clot from i-PRF 3300 x 3', 40 × and 100 × immersion

Table II: Comparison between the content of erythrocytes present in various types of liquid PRF produced with Horizontal and Fixed Centrifuge. N.B.: The values in red are the lowest; the values in green are the highest.

Centrifuge Horizontal Eritrocytes M/µL Variance (ANOVA) p<0.001			
	Tipe of PRF liquid rpm	Average ± D.S.	Test of Student-Newman-Keus
1	*i*-PRF FL 3300 x 5'	0.057±0.055	1 ▶ 7 >0.05; 1 ▶ 4 <0.05; 1 ▶ 3 >0.05;
2	*i*-PRF S-PRF Sticky 3300 x 5'	0.076±0.046	2 ▶ 4 <0.05; 2 ▶ 7 >0.05;

3	*i*-PRF FL 700 x 5'	0.371±0.47 ⇑	3 ▶8 <0.05; 3 ▶7 <0.05;
4	*i*-PRF S-PRF Sticky 700 x 5'	0.42±0.45 ⇑	4 ▶7 <0.05; 4 ▶6 >0.05;
5	A-PRF FL	0.149±0.13	5 ▶7 >0.05;
6	A-PRF S-PRF Sticky	0.14±0.099	6 ▶7 >0.05;
7	C-PRF Liquid FL	0.015±0.020 ⇓	7 ▶10 >0.05; 7 ▶8 >0.05;
8	C-PRF Liquid S-PRF Sticky	0.018±0.014 ⇓	4 ▶8 <0.05;
9	Fibrinogen FL	0.195±0.199	7 ▶9 >0.05;
10	Fibrinogen S-PRF Sticky	0.297±0.30	

▶= Versus

Centrifuge Fixed Eritrocytes M/µL Variance (ANOVA) p<0.001			
	Tipe of PRF liquid rpm	Average ± D.S.	Test of Student-Newman-Keus
1	*i*-PRF FL 3300 x 5'	0.017±0.011	1 ▶8 <0.05; 1 ▶3 <0.05; 1 ▶7 <0.05;
2	*i*-PRF S-PRF Sticky 3300 x 5'	0.012±0.006 ⇓	2 ▶3 <0.05; 2 ▶8 >0.05;
3	*i*-PRF FL 700 x 5'	0.064±0.061 ⇑	3 ▶9 <0.05; 3 ▶10 <0.05;
4	*i*-PRF S-PRF Sticky 700 x 5'	0.042±0.054	
5	A-PRF FL	0.054±0.058 ⇑	
6	A-PRF S-PRF Sticky	0.048±0.040	6 ▶8 >0.05;
7	C-PRF Liquid FL	0.017±0.019	
8	C-PRF Liquid S-PRF Sticky	0.011±0.007 ⇓	10 ▶8 >0.05; 1 ▶8 >0.05;
9	Fibrinogen FL	0.014±0.01	8 ▶9 >0.05;
10	Fibrinogen S-PRF Sticky	0.021±0.007	

▶= Versus

N.B: For comparison with the values of the Fixed Angle Centrifuge see the work Crisci M. et al., 2024[9]

Centrifuging PRF in the Vacumed FL negative pressure vacuum collection tube made of plastic (PET) (code 44909) and the original S-PRF Sticky tube (recommended by the manufacturing company Process, France) allows the PRF to be prepared in liquid form and used for injection. Liquid PRF can be used in various tissue regeneration applications such as facial aesthetics, intra-articular injections, periulcerative injections, etc.

C-PRF liquid (RCF_{clot}=525 *g*; RCF_{max}=700 *g*; RCF_{min}=280 *g*) sec. Miron [10, 11], or other types of liquid PRF were found after post-centrifugation collection of the resulting fluid, using a sterile tube and an 18 g needle, and can be injected or even solidified into clots and membranes. In this study, fibrin and cell content (neutrophil granulocytes, monocytes, lymphocytes, and platelets) were assessed both with a complete blood count and under an optical microscope at 10-100x magnification with methylene blue and May-Grünwald staining. In general, after centrifugation, an average of approximately 3 ml of PRF was obtained from a tube containing 9 ml of blood, equal to 9.1 g. Some researchers (O'Connell, 2007)[12] are concerned about a possible health risk when using vacuum glass blood collection tubes with silica activators. Therefore, we used only silica-free tubes. Each patient was included in the study after obtaining written consent and was then informed about the procedures.

Inclusion Criteria

No use of anticoagulants or equivalent functional drugs for one month prior to enrollment, and a platelet count in the range of 150,000 to 450,000/µL and a coagulation index determined as normal [prothrombin time (PT) between 11 and 16 seconds]. Hemoglobin concentration >9 mg/dL, serum protein concentration >6 grams/dL, and serum albumin >3 grams/dL. Absolute contraindications for liquid PRF production include platelet dysfunction syndrome, critical thrombocytopenia, hemodynamic instability, and sepsis. Relative contraindications include heavy smokers, drug and alcohol users, patients with chronic liver disease, severe metabolic or systemic disorders, patients with cancer of haematopoietic origin, and patients with low haemoglobin (<10 g/mL) or platelet count (<1.2 × 10^5/µL). Patients taking NSAIDs, prednisolone >20 mg/day, and anticoagulant therapy were also excluded.

Blood Chemistry Procedures

Liquid PRF is a second-generation platelet concentrate, comprising various growth factors, platelets, leukocytes, $CD34^+$ stem cells, and fibrinous matrix (Crisci et al., 2020).[13] For each patient, a quantity of ~1.5 ml of liquid PRF was placed in a tube with 5.4 mg K3E EDTA to perform a complete blood count using a HECO 5 hematology analyzer (Seac Radim Company). The concentration of monocytes, neutrophil granulocytes, lymphocytes, and platelets was assessed (the mean and median values obtained for each type of liquid PRF produced are reported). Fibrinogen was measured by immunoturbidimetric assay with an ACL 3000 (Beckman Instrumentation Laboratory) on a second quantity of ~1.5 ml of liquid PRF.

The authors. They therefore wanted to evaluate the content of fibrinogen, platelets, monocytes, neutrophil granulocytes, and lymphocytes in peripheral blood and, again by performing a complete blood count, in various types of liquid PRF [A-PRF liquid (1300 rpm × 5 min); *i*-PRF (700 rpm × 5 min); *i*-PRF (3300 × 3 min); C-PRF (2500 rpm × 8 min);[7] liquid fibrinogen (2700 rpm × 3 min)][11-14] obtained using two different types of centrifuge tubes for blood collected from patients (Vacumed FL and Verdi S-PRF Sticky tubes) preheated in an incubator at 37°C to simulate body temperature as closely as possible, as already reported in a previous work.[9] The cytological procedures used for light microscopy are reported in the work published in 2024.[9]

Histomorphometric Analysis

Cytometric analysis was performed by the examiner, blinded to the centrifugation technique and type of centrifuge used. The stained slides were examined, and images of three different areas of each section (0.348 mm^2) were taken through a light microscope with an integrated camera, in groups at 10, 20, 40, 60, and 100× magnification (Optika, B-150D-BRPL, Optika S.r.l., Italy). Digital images were stored on a computer. For histomorphometric analysis, AmScope MD 500-CK 5.0 mp imaging software (United Scope, LLC, NNL), Version 2022, was used.

Statistical Measurements and Analysis

Continuous variable data obeying a normal distribution were expressed as "Mean ± Standard Deviation" ($x \pm SD$), "Standard Error," and "Median." After verifying that the data were normally distributed using the Shapiro-Wilk test, the investigator was able to use parametric statistical tests, such as the t-test and the Pearson correlation test. Sample size analysis was not performed, which is a limitation of the study. Regarding the statistical significance of the cytology and fibrinogen measurements, differences between groups were calculated using the Student t-test for repeated measures for parametric variables and the Mann-Whitney signed U-test for non-normally distributed data. Any value of $p \leq 0.05$ was considered statistically significant, $p \leq 0.005$ was highly significant, and $p \geq 0.05$ was considered insignificant. Data were analyzed using version 6.0 of the Santon-Glantz 2007 Statistics for Biomedical Disciplines package.

RESULTS

The procedure was well tolerated in all subjects examined. The mean age of the patients examined was 65.07 ± 14.24 years, S.E.: 2.12; Median: 66 years. No significant differences were found in the baseline hematological comparison of the examined subjects, who had a mean RBC concentration of 4.6×10^6/mL (±1.1; 95% CI) (p=0.34) (range:4-5.5×10^6/mL), WBC of 5.1×10^3/mL (±0.37; 95% CI) (p=0.24) (range:4.5-8.5×10^3/mL) and a mean platelet count of 296.8×10^3/mL (±15.3; 95% CI) (p=0.15) (range:150-400×10^3/mL). The complete blood count performed on the various types of PRF-Liquid generally shows that the IFS (injectable fibrin scaffold) contains >25% white blood cells and >50% platelets compared to whole blood. Specifically, the white blood cells

contained in the liquid scaffolds were almost all lymphocytes, with few neutrophils and few monocytes. The amount of liquid PRF obtained from all PRF-Liquids, both with the S-PRF Sticky and Vacumed FL tubes, is approximately 2.5-3 ml. The tubes used for centrifugation before and after sampling were preheated to more easily obtain PRF-Liquid and also a clot from it, using a 37°C incubator[9], and thus favoring the formation of the fibrin clot by polymerization of fibrinogen under the influence of physiologically available thrombin.

Cytological Study with Optical Microscopy

Both liquid C-PRF obtained at 2500 rpm × 8 min in a Vacumed FL tube and an S-PRF Sticky tube (RCF_{clot}= 525 g; RCF_{max} = 700 g; RCF_{min} = 280 g) showed cellular presence as detected by Methylene Blue and May-Grŭnwald staining at low power (10 ×, 40 × magnification) (Figure S1 [A, B, A′, B′] [C, D, C′, D′] respectively). A conformal fibrin mesh structure was not observed in any of the samples examined.

The red blood cells detected in liquid PRF were highest in i-PRF (700 rpm x 5 min) in both Vacumed FL tubes and S-PRF Sticky tubes, and with both centrifuges. The lowest values were found in liquid C-PRF obtained with S-PRF Sticky tubes with both centrifuges (Table II). Cellular elements of blood origin were identified in varying concentrations depending on the centrifugation tube used (Figure S1).

Higher concentrations of lymphocytes (50.62% vs. 71.39%) and fibrinogen (103.97% vs. 106.5%) compared to whole blood were found in liquid C-PRF obtained with the Vacumed FL tube compared to those with the original S-PRF Sticky tubes (Tables III-IV). Platelets (82.47% vs. 62.98%) were also lower with the use of S-PRF Sticky tubes and are specifically reported in the captions of Figure S1 and the graphs in Figure 2, with statistically insignificant values. The monocyte content, which is particularly interesting for us for use in angiogenesis, is very low in this particular type of liquid PRF, regardless of the type of centrifugation tube used (11.43% vs 8.7%). In particular, mononuclear cells (Monocytes and Lymphocytes) are important because:

- Reduce the inflammatory process through the local production of a large number of cytokines and other specific substances that initiate the tissue healing process.
- stimulate the formation of new blood vessels by releasing angiogenic cytokines and growth factors into the microenvironment of tissue damage;
- stimulate local stem cells and progenitor cells to promote repair processes. [13]

Chronic low-grade inflammation within the vascular wall has been shown to be associated with monocyte infiltration. The maturation of monocytes into macrophages is accompanied by the production of cytokines and growth factors. A large percentage of circulating endothelial progenitor cells (EPCs) are of monocytic origin. Monocytes are therefore largely involved in VEGF-mediated vasa vasorum formation (Jaipersad A. et al. 2014).[15, 16]

Table III: (A) Comparison between Fibrinogen and cell values in C-PRF Liquid 2500 x 8′ in Vacumed FL tube and whole blood obtained with a swinging centrifuge.

Tipe	C-PRF Liquid Vacumed FL (2500 rpm x 8 min)			Blood			*t-test* of Student	Test *U* of Mann-Whitney	Content %
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median			
Monocytes K/µL	0.04±0.07	0.027	0.0	0.35±0.16	0.05	0.35	<0.001	<0.001	11.43%
Plt K/µL	176.9±75.4	20.69	137.5	214.5±32.2	12.6	195.0	0.164	0.326	82.47%
Gran.Neutr.%	13.92±22.42	7.73	18.85	66.1±6.9	2.36	62.9	<0.001	<0.001	21.05%
Lymphocytes %	13.57±21.9	7.56	16.5	26.81±6.5	1.39	28.5	0.083	0.140	50.62%
Fibrinogen mg/dl	471.7±176.1	43.65	427.5	453.7±151.4	47.7	504.5	0.809	0.850	103.97%

(B) Comparison between Fibrinogen values and cells in C-PRF Liquid 2500 x 8′ in Vacumed FL tube obtained with a swinging centrifuge and a fixed centrifuge.

Tipe	Centrifuge Horizontal			Centrifuge Fixed			*t-test* of Student	Test *U* of Mann-Whitney
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median		
Monocytes K/µL	0.04±0.07	0.027	0.0	0.013±0.035	0.009	0.0	0.290	0.758
Plt K/µL	176.9±75.4	20.69	137.5	46.1±37.5	9.68	37.0	<0.001	<0.05
Gran.Neutr.%	13.92±22.42	7.73	18.85	14.87±20.58	5.31	0.0	0.922	0.420
Lymphocytes %	13.57±21.9	7.56	16.5	26.96±32.8	8.48	0.0	0.297	0.597
Fibrinogen mg/dl	471.7±176.1	43.65	427.5	374.8±155.4	40.1	351.0	0.208	0.127

* statistically significant difference p<0.05

Table IV: (A) Comparison between Fibrinogen and cell values in C-PRF Liquid 2500 x 8′ in S-PRF Sticky tube and Whole blood obtained with a swinging centrifuge.

Tipe	C-PRF Liquid S-PRF Sticky (2500 rpm x 8 min)			Blood			*t-test* of Student	Test *U* of Mann-Whitney	Content %
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median			
Monocytes K/µL	0.03±0.07	0.022	0.0	0.37±0.16	0.35±0.16	0.05	<0.001	<0.001	8.57%
Plt K/µL	135.1±66.2	14.18	137.5	214.5±32.2	12.6	195.0	<0.05	<0.05	62.98%
Gran.Neutr.%	25.5±28.2	5.31	18.8	66.1±6.9	2.36	62.9	<0.001	<0.05	38.57%
Lymphocytes %	19.1±22.7	8.48	7.0	26.81±6.5	1.39	28.5	0.315	0.450	71.39%
Fibrinogen mg/dl	483.1±143.8	47.7	449.0	453.7±151.4	47.7	504.5	0.661	0.970	106.5%

B) Comparison between Fibrinogen values and cells in C-PRF Liquid 2500 x 8′ in S-PRF Sticky tube obtained with a swinging centrifuge and a fixed centrifuge.

Tipe	Centrifuge Horizontal			Centrifuge Fixed			t-test of Student	Test U of Mann-Whitney
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median		
Monocytes K/µL	0.03±0.07	0.022	0	0.04±0.13	0.04	0	0.833	0.54
Plt K/µL	135.1±66.2	14.18	137.5	68.0±44.8	14.18	58	<0.05	<0.05
Gran.Neutr.%	25.5±28.2	5.31	18.8	5.78±12.1	3.82	0	0.057	0.76
Lymphocytes %	19.1±22.7	8.48	7	19.1±30.7	9.72	0	1	0.76
Fibrinogen mg/dl	483.1±143.8	47.7	449	228.5±252.3	79.8	164	<0.05	<0.05

(C) Comparison between Fibrinogen and cell values in C-PRF Liquid 2500 x 8′ in Vacumed FL tube and S-PRF Sticky obtained with a swinging centrifuge.

Tipe	C-PRF Liquid Vacumed FL (2500 rpm x 8 min)			C-PRF Liquid S-PRF Sticky (2500 rpm x 8min)			t-test of Student	Test U of Mann-Whitney	correlation
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median			r p
Monocytes K/µL	0.04±0.07	0.027	0	0.03±0.07	0.022	0	0.753	0.76	0.491 0.143
Plt K/µL	176.9±75.4	20.69	137.5	135.1±66.2	14.18	137.5	0.204	0.212	0.773 <0.05
Gran.Neutr.%	13.92±22.42	7.73	18.85	25.5±28.2	5.31	18.8	0.323	0.323	0.224 0.514
Lymphocytes %	13.57±21.9	7.56	16.5	19.1±22.7	8.48	7	0.586	0.569	0.479 0.154
Fibrinogen mg/dl	471.7±176.1	43.65	427.5	483.1±143.8	47.7	449	0.876	1	0.624 0.054

* statistically significant difference, p<0.05

Advanced-PRF liquid (1300 rpm x 5 min) was obtained in a Vacumed FL tube and an S-PRF Sticky tube (RCF_{clot} = 142 g; RCF_{max} = 189 g; RCF_{min} = 66 g). Cellular presence was detected, as evidenced by Methylene Blue and May-Grŭnwald staining at low power (10 ×, 40 × magnification) in Figure S2 (A, B, A', B' respectively), using the S-PRF Sticky tube. Even with the use of the Vacumed FL tube and a mixed Methylene Blue/May-Grŭnwald staining (Figure S2), a conformed fibrin network was not observed, while blood-derived cellular elements were identified in various concentrations.

Higher concentrations of Platelets (122.89% vs 133.07%), Monocytes (44.74% vs 52.63%), Lymphocytes (123.40% vs 162.82%), and Fibrinogen (75.73% vs 77.84%) compared to whole blood were found in A-PRF liquid obtained with the Vacumed FL tube compared to those with the original S-PRF Sticky tubes (Tables V-VI), resulting lower with the use of S-PRF Sticky tubes and are reported in detail in the captions of Figure S2 (A, B, A′, B′), while those with the Vacumed FL tube are reported in the captions of Figure S2 (C, D, C′, D′) and the graphs in Figure 2, with statistically non-significant values.

In the *i*-PRF 700 rpm × 5 min obtained in the Vacumed FL tube and the S-PRF Sticky tube (RCF_{clot} = 38 g; RCF_{max} = 55 g; RCF_{min} = 22 g), a cellular presence was detected as reported in Figure S3 (A,

B, A', B'), respectively, with the use of an S-PRF Sticky tube (Table VII). Even in this case, a well-formed fibrin network structure was not observed in any of the examined samples, except for some thin filaments.

Even when using the Vacumed FL tube with Methylene Blue and May-Grŭnwald staining (Figure S3 [C, D, C', D']), respectively) (ingr. 10, 40 ×), no conformal fibrin network was observed, while cellular elements of blood origin were identified in different concentrations reported in Table VIII. Higher concentrations of Platelets (122.23% vs 91.74%), Monocytes (142.86% vs 125.00%) and Lymphocytes (220.01% vs 199.73%) compared to whole blood were found in *i*-PRF liquid (700 rpm × 5') obtained with the Vacumed FL tube compared to those with the original S-PRF Sticky tubes (Tables VI-VII), while Fibrinogen (97.09% vs 104.35%) was higher with the use of S-PRF Sticky tubes and are specifically reported in the captions of Figure S3 and the graphs in Figure 2, with values that were not statistically significant.

The concentration of monocytes present in *i*-PRF 700 rpm x 5' was particularly important for the possible use in vascular regeneration, 15 obtained with the Vacumed FL tube (142.86%) compared to the S-PRF Sticky (125.00%). In vivo, monocytes differentiate into ECs (Endothelial Cells) and are incorporated into blood vessels (Lopes-Coelho F. et al., 2020).[14]

Also, in the *i*-PRF 3300 rpm × 3 min obtained in the Vacumed FL tube and in the S-PRF Sticky tube (RCF_{clot} =765 *g*; RCF_{max} = 1008 *g*; RCF_{min} =403 *g*) in Figure S4 (A, B) and (A', B') respectively, for the Vacumed FL tube. In this case, too, no well-formed fibrin network structure was observed in any of the samples examined, except for a few thin filaments. Even when using the S-PRF Sticky tube with Methylene Blue and May-Grŭnwald staining (Figure S4 [C, D], [C', D'] respectively) (ingr. 10, 40 ×), not even a hint of fibrin network was observed, while blood-derived cellular elements were identified in varying concentrations (Tables IX, X).

Higher concentrations of platelets (125.42% vs 71.08%, p=0.014) and lymphocytes (191.58% vs 170.80%) compared to whole blood were found in i-PRF liquid (3300 rpm × 3') obtained with the original S-PRF Sticky tube compared to the Vacumed FL tubes (Tables IX, X). Fibrinogen (87.7% vs 99.42%), granulocytes, and neutrophils (44.81% vs 56.91%) were found to be higher with the use of Vacumed FL tubes and are specifically reported in the captions of Figure S4 and the graphs in Figure 2.

Monocyte content was identical (74.29%) in both types of tubes. (*p*=1.000). The platelet concentrate, defined as Liquid Fibrinogen 2700 rpm × 3 min (Serafini et al. 2020)[17] obtained in the Vacumed FL tube and the S-PRF Sticky tube (RCF_{clot} = 408 g; RCF max = 653 g; RCF min = 326 g) was also examined, and a cellular presence was found as highlighted in Figure S5 (C, D) and (C', D') respectively, for the Vacumed FL tube. Even with the use of the S-PRF Sticky tube and with a staining with Methylene Blue and May-Grŭnwald (Figure S5 [A, B], [A', B'] respectively) (ingr. 10, 40 ×) no conformed fibrin network was observed, while cellular elements of blood origin were identified present in different concentrations, which are reported in Tables XI, XII. Higher concentrations of Platelets (130.87% vs 89.21%) and Lymphocytes (184.04% vs 185.67%) compared to whole blood were found in liquid Fibrinogen obtained with the original S-PRF Sticky tube compared to the Vacumed FL ones, while Monocytes (105.56% vs 86.11%), Neutrophil Granulocytes (50.15% vs 50.09%) (Tables X-XI) and Fibrinogen (93.44% vs 89.9%) were found to be higher with the use of Vacumed FL tubes and are specifically reported in the captions of Figure S5 and the following graphs in Figure 2, with statistically non-significant differences.

Table V: (A) Comparison between Fibrinogen and cell values in A-PRF Liquid 1300 × 5′ in S-PRF Sticky tube and Whole blood obtained with a swinging centrifuge.

Tipe	A-PRF Liquid S-PRF Sticky (1300 rpm × 5 min)			Sangue			t-test of Student	Test U of Mann-Whitney	Content %
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median			
Monocytes K/µL	0.20±0.33	0.1	0.15	0.38±0.162	0.051	0.4	0.139	<0.05	52.63%
Plt K/µL	264.8±152.7	48.28	261.5	199.0±75.3	23.81	187.5	0.237	0.241	133.07%
Gran.Neutr.%	24.29±24.3	7.69	22.6	67.5±7.13	2.26	69	<0.001	<0.001	35.99%
Lymphocytes %	29.80±29.2	9.24	32	24.1±6.3	1.99	23.5	0.554	0.571	123.40%
Fibrinogen mg/dl	357.3±245.3	77.6	394.5	459.0±167.9	53.12	420	0.294	0.45	77.84%

(B) Comparison between Fibrinogen values and cells in A-PRF Liquid 1300 × 5′ in S-PRF Sticky tube obtained with swinging centrifuge and fixed centrifuge.

Tipe	Centrifuge Horizontal			Centrifuge Fixed			t-test of Student	Test U of Mann-Whitney
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median		
Monocytes K/µL	0.20±0.33	0.1	0.15	0.10±0.15	0.047	0	0.394	0.544
Plt K/µL	264.8±152.7	48.28	261.5	198.8±116.3	36.77	198.5	0.291	0.427
Gran.Neutr.%	24.29±24.3	7.69	22.6	37.06±12.6	3.99	37.4	0.157	0.29
Lymphocytes %	29.80±29.2	9.24	32	50.45±13.8	4.36	43.5	0.058	0.076
Fibrinogen mg/dl	357.3±245.3	77.6	394.5	283.1±136.5	43.2	336	0.414	0.257

* statistically significant difference, p<0.05

Table VI: (A) Comparison between Fibrinogen and cell values in A-PRF Liquid 1300 × 5′ in Vacumed FL tube and Whole blood obtained with a swinging centrifuge.

Tipe	A-PRF Liquid Vacumed FL (1300 rpm × 5 min)			Blood			t-test of Student	Test U of Mann-Whitney	Content %
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median			
Monocytes K/µL	0.17±0.19	0.061	0.1	0.38±0.162	0.051	0.4	<0.05	<0.05	44.74%
Plt K/µL	252.3±120.6	38.14	230	199.0±75.3	23.81	187.5	0.251	0.65	122.89%
Gran.Neutr.%	29.45±20.1	6.34	36.8	67.5±7.13	2.26	69	<0.001	<0.001	43.63%
Lymphocytes %	39.32±25.7	8.14	38.5	24.1±6.3	1.99	23.5	<0.05	<0.05	162.82%
Fibrinogen mg/dl	347.6±315.9	99.9	321	459.0±167.9	53.12	420	0.338	0.345	75.73%

(B) Comparison between Fibrinogen values and cells in A-PRF Liquid 1300 × 5′ in Vacumed FL tube obtained with a swinging centrifuge and a fixed centrifuge.

Tipe	Centrifuge Horizontal			Centrifuge Fixed			t-test of Student	Test U of Mann-Whitney
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median		
Monocytes K/µL	0.17±0.19	0.061	0.1	0.19±0.22	0.058	0.2	0.83	0.956
Plt K/µL	252.3±120.6	38.14	230	226.5±112.4	29.03	239	0.627	0.912
Gran.Neutr.%	29.45±20.1	6.34	36.8	37.06±12.6	3.99	37.4	0.324	0.824
Lymphocytes %	39.32±25.7	8.14	38.5	44.97±23.5	6.08	48.8	0.614	0.488
Fibrinogen mg/dl	347.6±315.9	99.9	321	320.5±133.2	34.4	332	0.805	0.868

(C) Comparison between Fibrinogen and cell values in A-PRF Liquid 1300 × 5′ in Vacumed FL tube and S-PRF Sticky obtained with a swinging centrifuge.

Tipe	A-PRF Liquid Vacumed FL (1300 rpm × 5 min)			A-PRF Liquid S-PRF Sticky (1300 rpm x 5min)			t-test of Student	Test U of Mann-Whitney	correlation
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median			r p
Monocytes K/µL	0.17±0.19	0.061	0.1	0.20±0.33	0.1	0.15	0.781	0.85	0.230 0.503
Plt K/µL	252.3±120.6	38.14	230	264.8±152.7	48.28	261.5	0.841	0.623	0.818 <0.05
Gran.Neutr.%	29.45±20.1	6.34	36.8	24.29±24.3	7.69	22.6	0.611	0.677	0.267 0.438
Lymphocytes %	39.32±25.7	8.14	38.5	29.80±29.2	9.24	32	0.449	0.449	0.345 0.313
Fibrinogen mg/dl	347.6±315.9	99.9	321	357.3±245.3	77.6	394.5	0.94	0.791	0.833 <0.05

* statistically significant difference p<0.05

Table VII: (A) Comparison between Fibrinogen and cell values in *i*-PRF 700 × 5′ in S-PRF Sticky tube and Whole blood obtained with a swinging centrifuge.

Tipe	*i-PRF S-PRF Sticky (700 rpm × 5 min)*			Blood			t-test of Student	Test U of Mann-Whitney	Content %
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median			
Monocytes K/µL	0.35±0.35	0.11	0.36	0.28±0.099	0.031	0.3	0.547	0.85	125.00%
Plt K/µL	195.6±163.9	51.86	171.5	213.2±32.1	10.17	226	0.743	0.678	91.74%
Gran.Neutr.%	42.84±20.9	6.63	48.8	72.8±10.3	3.26	73.75	<0.001	<0.001	58.83%
Lymphocytes %	39.23±20.2	6.38	38.9	19.7±8.9	2.79	18.3	<0.05	<0.05	198.73%
Fibrinogen mg/dl	340.9±97.5	30.8	336	326.7±96.7	30.59	309.5	0.747	0.791	104.35%

(B) Comparison between Fibrinogen Values and cells in *i*-PRF 700 × 5′ in S-PRF Sticky tube obtained with swinging centrifuge and fixed centrifuge.

Tipe	Centrifuge Horizontal			Centrifuge Fixed			t-test of Student	Test U of Mann-Whitney
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median		
Monocytes K/µL	0.35±0.35	0.11	0.36	0.44±0.28	0.089	0.4	0.533	0.472
Plt K/µL	195.6±163.9	51.86	171.5	249.1±153.3	48.47	234.5	0.461	0.385
Gran.Neutr.%	42.84±20.9	6.63	48.8	42.54±11.2	3.54	43.1	0.969	0.734
Lymphocytes %	39.23±20.2	6.38	38.9	51.41±9.9	3.29	50	0.104	0.174
Fibrinogen mg/dl	340.9±97.5	30.8	336	373.3±263.4	83.3	330	0.72	0.791

*d statistically significant difference p<0.05

Table VIII: (A) Comparison between Fibrinogen and cell values in *i*-PRF 700 x 5' in Vacumed FL tube and Whole blood obtained with a swinging centrifuge.

Tipe	*i-PRF Vacumed FL (700 rpm x 5 min)*			Blood			t-test of Student	Test U of Mann-Whitney	Content %
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median			
Monocytes K/µL	0.40±0.343	0.108	0.35	0.28±0.099	0.031	0.3	0.302	0.623	142.86%
Plt K/µL	260.6±154.5	48.84	312.5	213.2±32.1	10.17	226	0.355	0.104	122.23%
Gran.Neutr.%	39.34±21.6	6.85	41	72.8±10.3	3.26	73.75	<0.001	<0.05	54.02%
Lymphocytes %	43.43±22.7	7.18	45.2	19.7±8.9	2.79	18.3	<0.05	<0.05	220.01%
Fibrinogen mg/dl	317.2±113.1	35.8	328.5	326.7±96.7	30.59	309.5	0.842	0.91	97.09%

(B) Comparison between Fibrinogen values and cells in *i*-PRF 700 × 5' in Vacumed FL tube obtained with a swinging centrifuge and a fixed centrifuge.

Tipe	Centrifuge Horizontal			Centrifuge Fixed			*t-test* of Student	Test *U* of Mann-Whitney
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median		
Monocytes K/µL	0.40±0.343	0.108	0.35	0.49±0.23	0.060	0.60	0.500	0.501
Plt K/µL	260.6±154.5	48.84	312.5	247.4±130.8	33.78	253.0	0.839	0.560
Gran.Neutr.%	39.34±21.6	6.85	41.0	45.65±10.9	2.80	44.8	0.420	0.305
Lymphocytes %	43.43±22.7	7.18	45.2	44.94±10.8	2.78	46.3	0.851	0.978
Fibrinogen mg/dl	317.2±113.1	35.8	328.5	300.3±218.4	72.8	330.0	0.830	0.540

(C) Comparison between Fibrinogen values and cells in *i*-PRF 700 × 5' in Vacumed FL tube and S-PRF Sticky obtained with a swinging centrifuge.

Tipe	*i*-PRF Vacumed FL (700 rpm x 5 min)			*i*-PRF S-PRF Sticky (700 rpm x 5min)			*t-test* of Student	Test *U* of Mann-Whitney	correlation	
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median			r	*p*
Monocytes K/µL	0.40±0.343	0.108	0.35	0.35±0.35	0.11	0.36	0.751	0.734	0.385	0.259
Plt K/µL	260.6±154.5	48.84	312.5	195.6±163.9	51.86	171.5	0.374	0.307	0.573	0.082
Gran.Neutr.%	39.34±21.6	6.85	41.0	42.84±20.9	6.63	48.8	0.717	0.571	0.279	<0.05
Lymphocytes %	43.43±22.7	7.18	45.2	39.23±20.2	6.38	38.9	0.667	0.406	-0.064	0.845
Fibrinogen mg/dl	317.2±113.1	35.8	328.5	340.9±97.5	30.8	336.0	0.662	0.791	0.164	0.623

* statistically significant difference p<0.05

Table IX: (A) Comparison between Fibrinogen and cell values in *i*-PRF 3300 × 3' in S-PRF Sticky tube and Whole blood obtained with a swinging centrifuge.

Type	*i*-PRF S-PRF Sticky (3300 rpm × 3 min)			Blood			*t-test* of Student	Test *U* of Mann-Whitney	Restrained %
	Average±D.S.	Standard Error	Median	Average±D.S.	Standard Error	Median			
Monocytes K/µL	0.26±0.158	0.050	0.25	0.35±0.099	0.031	0.30	0.144	0.140	74.29%
Plt K/µL	254.6±108.3	34.24	258.5	203.0±69.97	22.13	197.5	0.222	0.241	125.42%
Neutr.Gran.%	29.72±19.2	6.08	27.95	66.3±7.84	2.48	64.9	<0.001	<0.001	44.81%
Lymphocytes %	51.63±23.6	7.45	55.90	26.95±6.89	2.18	27.45	<0.05	<0.05	191.58%
Fibrinogen mg/dl	333.1±103.2	32.63	402.5	380.0±87.3	27.61	405.0	0.287	0.273	87.7%

(B) Comparison between Fibrinogen Values and cells in i-PRF 3300 × 3' in S-PRF Sticky tube obtained with swinging centrifuge and fixed centrifuge.

Tipe	Centrifuge Horizontal			Centrifuge Fixed			*t-test* of Student	Test *U* of Mann-Whitney
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median		
Monocytes K/µL	0.26±0.158	0.050	0.25	0.03±0.067	0.021	0.00	<0.001	<0.05
Plt K/µL	254.6±108.3	34.24	258.5	100.7±55.2	17.47	99.0	<0.001	<0.001
Gran.Neutr.%	29.72±19.2	6.08	27.95	10.90±13.4	4.25	5.5	<0.05	<0.05
Lymphocytes %	51.63±23.6	7.45	55.90	31.80±34.3	10.85	25.0	0.149	0.290
Fibrinogen mg/dl	333.1±103.2	32.63	402.5	457.4±134.5	42.52	402.0	<0.05	<0.05

* statistically significant difference p<0.05

Table X: (A) Comparison between Fibrinogen and cell values in *i*-PRF 3300 × 3' in Vacumed FL tube and Whole blood obtained with a swinging centrifuge.

Type	*i*-PRF Vacumed FL (3300 rpm × 3 min)			Blood			*t-test* of Student	Test *U* of Mann-Whitney	Restrained %
	Average±D.S.	Standard Error	Median	Average±D.S.	Standard Error	Median			
Monocytes K/µL	0.26±0.165	0.052	0.25	0.35±0.099	0.031	0.40	0.156	0.256	74.29%
Plt K/µL	144.3±68.1	21.5	130.5	203.0±69.97	22.13	197.5	0.073	0.054	71.08%
Neutr.Gran.%	37.5±23.6	7.47	36.2	66.3±7.84	2.48	64.9	<0.05	<0.05	56.91%
Lymphocytes %	46.0±22.9	7.26	53.6	26.95±6.89	2.18	27.45	<0.05	<0.05	170.80%
Fibrinogen mg/dl	377.8±107.1	33.9	373.5	380.0±87.3	27.61	405.0	0.960	0.791	99.42%

(B) Comparison between Fibrinogen values and cells in *i*-PRF 3300 × 3' in Vacumed FL tube obtained with a swinging centrifuge and a fixed centrifuge.

Tipe	Centrifuge Horizontal			Centrifuge Fixed			*t-test* of Student	Test *U* of Mann-Whitney
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median		
Monocytes K/µL	0.26±0.165	0.052	0.25	0.05±0.1	0.027	0.0	<0.05	<0.05

Plt K/µL	144.3±68.1	21.5	130.5	79.9±67.8	17.5	56.0	<0.05	<0.05
Gran.Neutr.%	37.5±23.6	7.47	36.2	13.3±14.6	3.76	10.0	<0.05	<0.05
Lymphocytes %	46.0±22.9	7.26	53.6	51.2±34.7	8.96	60.0	0.697	0.598
Fibrinogen mg/dl	377.8±107.1	33.9	373.5	358.3±184.3	47.6	351.0	0.776	0.560

(C) Comparison between Fibrinogen values and cells in *i*-PRF 3300 × 3′ in Vacumed FL tube and S-PRF Sticky obtained with a swinging centrifuge.

Tipe	*i*-PRF Vacumed FL (3300 rpm x 3 min)			*i*-PRF S-PRF Sticky (3300 rpm x 3min)			*t-test* of Student	Test *U* of Mann-Whitney	correlation	
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median			r	*p*
Monocytes K/µL	0.26±0.165	0.052	0.25	0.26±0.158	0.050	0.25	1.000	0.970	0.658	<0.05
Plt K/µL	144.3±68.1	21.5	130.5	254.6±108.3	34.24	258.5	<0.05	<0.05	0.030	0.919
Gran.Neutr.%	37.5±23.6	7.47	36.2	29.72±19.2	6.08	27.95	0.429	0.496	0.309	0.368
Lymphocytes %	46.0±22.9	7.26	53.6	51.63±23.6	7.45	55.90	0.595	0.650	0.745	<0.05
Fibrinogen mg/dl	377.8±107.1	33.9	373.5	333.1±103.2	32.63	402.5	0.354	0.307	0.358	0.296

* statistically significant difference p<0.05

Table XI: (A) Comparison between Fibrinogen and cell values in Liquid Fibrinogen 2700 × 3′ in S-PRF Sticky tube and Whole Blood obtained with a swinging centrifuge.

Type	Fibrinogen Liquid Sticky (2700 rpm × 3′)			Blood			*t-test* of Student	Test *U* of Mann-Whitney	Restrained %
	Average±D.S.	Standard Error	Median	Average±D.S.	Standard Error	Median			
Monocytes K/µL	0.31±0.233	0.074	0.30	0.36±0.107	0.033	0.40	0.545	0.734	86.11%
Plt K/µL	312.9±100.9	31.89	317.0	239.1±54.4	17.20	231.5	0.057	<0.05	130.87%
Neutr.Gran.%	34.34±19.9	6.30	35.85	68.5±5.1	1.60	67.3	<0.001	<0.05	50.09%
Lymphocytes %	46.66±22.6	7.15	49.00	25.13±5.30	1.68	26.9	<0.05	<0.05	185.67%
Fibrinogen mg/dl	353.8±117.3	37.11	329.0	393.4±129.3	40.88	368.5	0.482	0.473	89.9%

(B) Comparison between Fibrinogen Values and Liquid Fibrinogen Cells 2700 × 3′ in S-PRF Sticky tube obtained with a swinging centrifuge and a fixed centrifuge.

Tipe	Centrifuge Horizontal			Centrifuge Fixed			*t-test* of Student	Test *U* of Mann-Whitney
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median		
Monocytes K/µL	0.31±0.233	0.074	0.30	0.15±0.31	0.097	0.05	0.208	0.075
Plt K/µL	312.9±100.9	31.89	317.0	114.1±52.2	16.5	100.0	<0.001	<0.001
Gran.Neutr.%	34.34±19.9	6.30	35.85	22.70±22.0	6.95	15.0	0.125	0.212
Lymphocytes %	46.66±22.6	7.15	49.00	48.40±31.9	10.12	59.0	0.890	0.791
Fibrinogen mg/dl	353.8±117.3	37.11	329.0	192.9±222.4	70.32	99.0	0.058	0.162

* statistically significant difference p<0.05

Table XII: (A) Comparison between Fibrinogen and cell values in Liquid Fibrinogen 2700 × 3′ in Vacumed FL tube and Whole Blood obtained with a swinging centrifuge.

Type	Fibrinogen Vacumed FL (2700 rpm × 3′)			Blood			*t-test* of Student	Test *U* of Mann-Whitney	Restrained %
	Average±D.S.	Standard Error	Median	Average±D.S.	Standard Error	Median			
Monocytes K/µL	0.38±0.266	0.084	0.35	0.36±0.107	0.033	0.40	0.828	0.970	105.56%
Plt K/µL	213.3±128.6	40.67	243.5	239.1±54.4	17.20	231.5	0.566	0.970	89.21%
Neutr.Gran.%	34.38±17.7	5.60	36.95	68.5±5.1	1.60	67.3	<0.001	<0.001	50.15%
Lymphocytes %	46.25±19.4	6.13	49.55	25.13±5.30	1.68	26.9	<0.05	<0.05	184.04%
Fibrinogen mg/dl	367.6±140.5	44.43	343.5	393.4±129.3	40.88	368.5	0.674	0.791	93.44%

(B) Comparison between Fibrinogen Values and Liquid Fibrinogen Cells 2700 × 3′ in Vacumed FL tube obtained with a swinging centrifuge and a fixed centrifuge.

Tipe	Centrifuge Horizontal			Centrifuge Fixed			*t-test* of Student	Test *U* of Mann-Whitney
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median		
Monocytes K/µL	0.38±0.266	0.084	0.35	0.04±0.063	0.016	0.00	<0.001	<0.05
Plt K/µL	213.3±128.6	40.67	243.5	105.1±74.8	18.7	196.0	<0.05	<0.001
Gran.Neutr.%	34.38±17.7	5.60	36.95	20.79±22.4	5.6	14.0	0.117	<0.05
Lymphocytes %	46.25±19.4	6.13	49.55	61.5±26.5	7.0	71.20	0.129	<0.05
Fibrinogen mg/dl	367.6±140.5	44.43	343.5	248.7±168.3	42.07	302.5	0.075	0.155

(C) Comparison between Fibrinogen and cell values in Liquid Fibrinogen in Vacumed FL and S-PRF Sticky tubes obtained with a swinging centrifuge.

Tipe	Fibrinog. L. Vacumed FL (2700 rpm × 3 min)			Fibrinog. L. S-PRF Sticky (2700 rpm × 3min)			*t-test* of Student	Test *U* of Mann-Whitney	correlation	
	Average±D.S.	Error Standard	Median	Average±D.S.	Error Standard	Median			r	*p*
Monocytes K/µL	0.38±0.266	0.084	0.35	0.31±0.233	0.074	0.30	0.539	0.571	0.200	0.560
Plt K/µL	213.3±128.6	40.67	243.5	312.9±100.9	31.89	317.0	0.070	0.104	0.321	0.349
Gran.Neutr.%	34.38±17.7	5.60	36.95	34.34±19.9	6.30	35.85	0.996	0.850	0.273	0.427
Lymphocytes %	46.25±19.4	6.13	49.55	46.66±22.6	7.15	49.00	0.966	0.940	0.300	0.382
Fibrinogen mg/dl	367.6±140.5	44.43	343.5	353.8±117.3	37.11	329.0	0.814	0.791	0.833	<0.05

* statistically significant difference p<0.05

Finally, a score was calculated, in an innovative way for this study, by summing all cell types and fibrinogen percentages detected in the various types of liquid PRF analyzed using the two types

of centrifuges and tubes (Figure 3) to assess hypothetical functional capacity. The type of liquid PRF with the highest score was found to be *i*-PRF (700 rpm × 5') extracted with a Vacumed FL tube in a rocking centrifuge (127.17%)(Fig. 3).

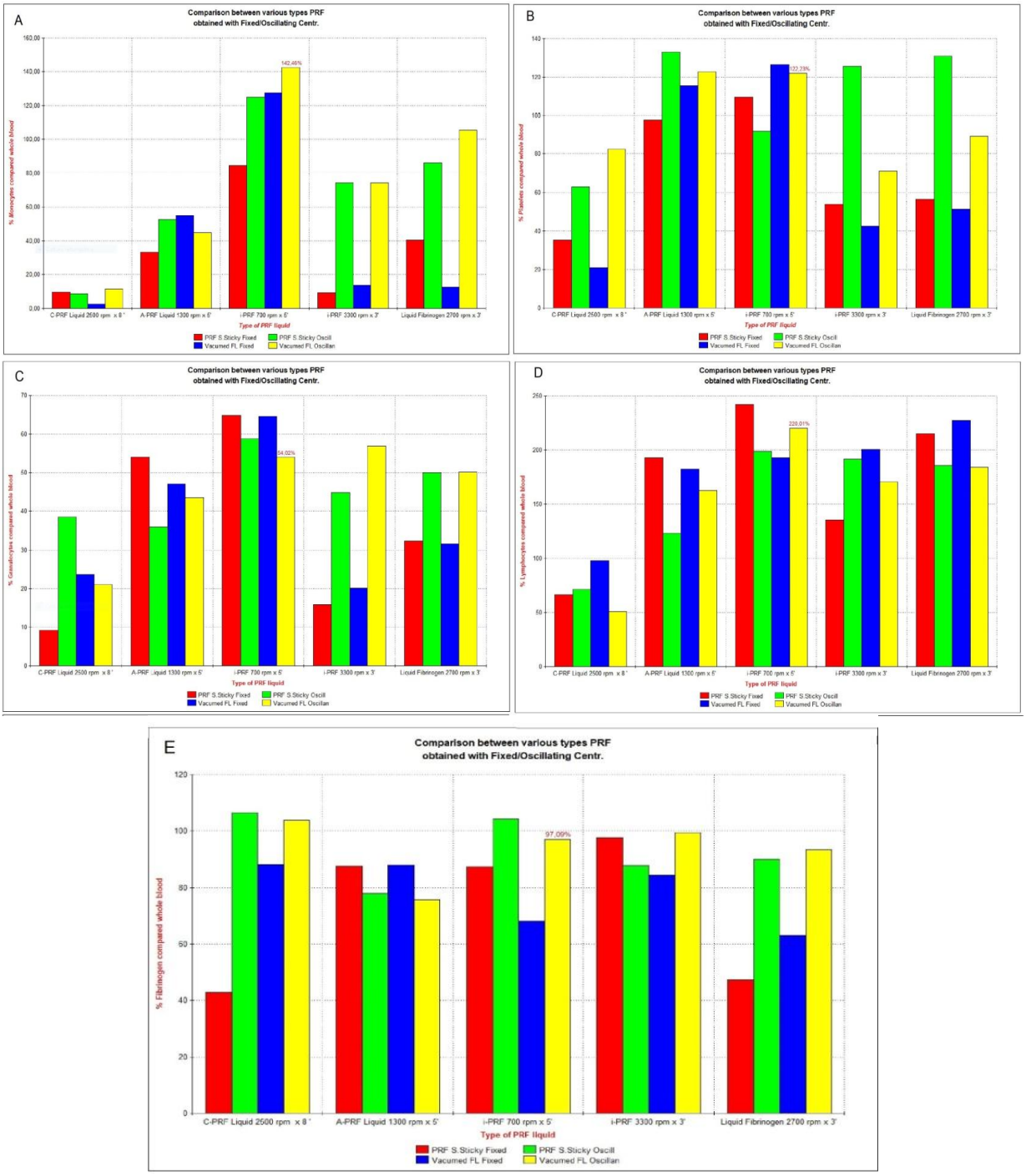


Figure 2: Graphical comparison between the content of Monocytes (A), Platelets (B), Neutrophil Granulocytes (C), Lymphocytes (D) and Fibrinogen (E) in the various types of Liquid PRF produced with the original S-PRF Sticky tube and Vacumed FL in absolute values (mg/dl) and in relative percentage in relation to the initial content in whole blood. The concentrations of platelets and other cells within the PRF samples acquired based on the volumes applied and the centrifugation protocol. No statistical analysis was applied.

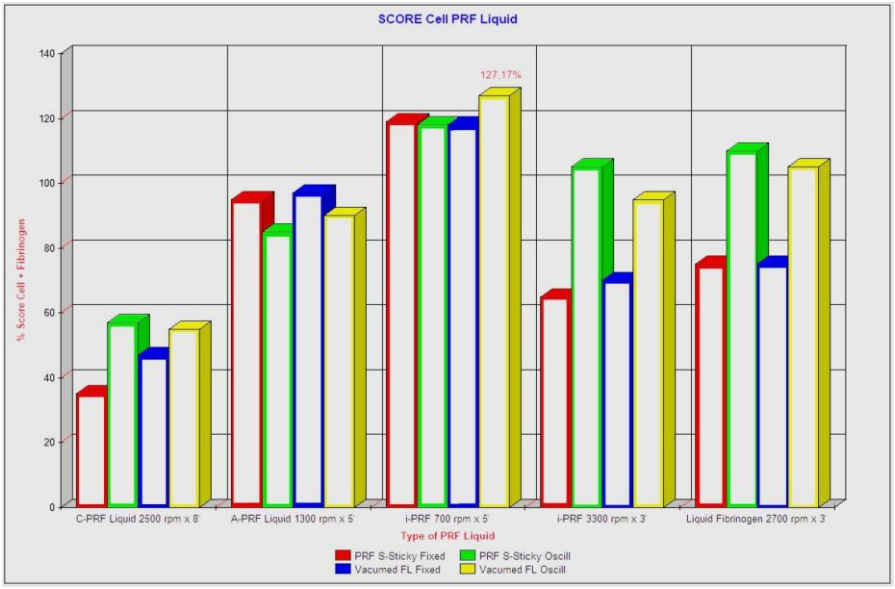


Figure 3: Total cell concentrations within the acquired PRF samples based on the applied volumes and centrifugation protocol (Score).

DISCUSSION

The topical use of platelet-rich fibrin can play an important role in initiating the healing process of chronic wounds. This treatment is now called Natural Guided Regeneration Therapy (NGR-T) because it uses only the patient's biological components without adding anything else.

The efficiency of different protocols in preparing liquid PRF was analyzed, compared using two types of centrifuges, and a small number of samples were used for each protocol. PRF generation is a centrifugation-dependent process. [18-20]

These protocols differed in their relative centrifugal force (RCF), as follows: High RCF (700 g - 2500 rpm), Medium RCF (189 g - 1300 rpm), and Low RCF (55 g - 700 rpm) centrifugation protocols. These results showed a reproducible pattern in both groups tested (Tilting Centrifuge vs. Fixed Angle Centrifuge) and indicated comparable cell concentrations in both groups. Characterization of cellular components is a crucial step to validate the quality of the resulting PRF (Al-Maawi et al. 2024)[21].

Additionally, we performed a qualitative analysis (cytological analysis using light microscopy) combined with a quantitative analysis (cell counting with a hemocytometer and fibrinogen assay) of blood components for PRF production.

In general, these studies focused on the cellular content of the liquid PRF analyzed, but did not analyze the bioactivity of the produced PRF. We focused on the biological composition by analyzing the concentration of leukocytes and platelets, as well as fibrinogen, studying its distribution. In this regard, further research is needed to better understand the regenerative effects and further analyze the chemical components of this specific PRF.

These studies demonstrate that, compared to previous studies on PRF membranes, which reported a maximum apparent platelet/leukocyte density within the first millimeter of the yellow layer, immediately adjacent to the red blood clot fraction, these studies highlighted the importance of having a thorough understanding of the liquid PRF design to achieve optimal

clinical results when using this material. Therefore, in the production of liquid PRFs, it is not essential to maintain a minimal layer of red clot in the final part of the PRF to obtain the highest number of platelets and leukocytes, as they are evenly distributed throughout the liquid PRF. Platelet distribution in PRF matrices is strongly influenced by centrifugal force, rotor type, and PRF production protocols. Different clinical situations require different types of applications. Therefore, this blood-derived product can be individually adjusted and prepared according to specific clinical requirements as liquid and solid PRF matrices. Liquid PRF can be used to biologize biomaterials, such as bone substitutes and xenogeneic collagen membranes, or it can be infiltrated into periulcerative sites.

Because this study was initiated as a pilot study without funding, it was limited to 45 participants. To minimize the risk of bias due to gender differences in this small number of volunteers, this initial study included subjects of both sexes with and without peripheral vascular disease. 9 mL of whole blood was centrifuged in plastic tubes (*i*-PRF S-PRF Sticky tubes, Process per PRF™, Nice, France) to obtain Liquid PRF and Vacumed FL PET tubes. The swing-bucket centrifuge (CTL420, CenLee, Beijing, China) used had a right-angle, no brake, and a 110 mm rotor size according to the protocol (90° rotor angle, 75 mm radius at the center of the tube, 100 mm at the maximum, and 35 mm at the minimum). The importance of determining the amount of fibrinogen present in each fraction and the yield of clottable components present is useful in predicting the ability of liquid PRF to also generate PRF membranes.

A mean platelet accumulation of almost 1.5 times greater was found in liquid PRF compared to whole blood samples. These results are significant because a higher platelet yield with a higher plasma volume has greater clinical significance, and a higher platelet concentration alone is meaningless. [22, 23] This concept led us to use a tube incubator, which allowed us to obtain a larger amount of liquid PRF (>3.5 ml) in numerous cases. The various types of liquid PRF, including liquid fibrinogen, present a high concentration of leukocytes and platelets. [24] The combination of activated platelets in PRF and fibrinogen results in a mass production of fibrin. More than 80% of the platelets and 72% of the leukocytes of the initial blood sample are present in PRF. The same is true for liquid fibrinogen with 88% and 70% respectively. The exudate produced after the collection of liquid PRF showed a low cellular content with 2.5% platelets and 0.9% leukocytes (Crisci et al., 2018, 2019, 2022, 2023).[24-27]

In this study, we highlighted that the type of liquid PRF with the highest content of platelets, monocytes, lymphocytes, and neutrophil granulocytes, with a sufficient fibrinogen content, is *i*-PRF (700 rpm × 5 min). In particular, the content of platelets, monocytes, and neutrophil granulocytes was significant in *i*-PRF (700 rpm × 5') obtained with the Vacumed FL tube (code 44909), with statistically insignificant differences compared to whole blood (Fig. 2), while the content of lymphocytes and fibrinogen was higher with i-PRF (700 rpm × 5') extracted with the PRF-S-Sticky tube. This means that it is possible to choose the type of liquid PRF to produce based on the type of cells that one wishes to use in greater quantities. The remaining *i*-PRF (700 rpm × 5 min) was produced with a Vacumed FL tube, the type of PRF based on the calculated score most useful in the clinic.

CONCLUSIONS

Our study sought to standardize the preparation procedure for Liquid PRF, which, while still being an easy and low-cost technique, does not require specialized equipment and offers a certain consistency in production in terms of macroscopic, microscopic, and cytological characteristics.

To the authors' knowledge, no study to date has investigated the cellular and fibrinogen content compared with the various types of Liquid PRF produced to date using an oscillating centrifuge. In summary, this injectable fibrin scaffold was produced through one-step centrifugation. This method is relatively simple to apply and produces a simple-to-use platelet concentrate in a liquid formulation. The liquid structure contains white blood cells and platelets that can support the release of growth factors. Therefore, this scaffold can be used as a therapeutic agent alone or in combination with other biomaterials to promote tissue regeneration. In fixed-angle devices, cells are pushed against the wall of the tube, and in this process, the (larger) red blood cells trap the platelets and drag them into the red zone. In horizontal centrifuges, this phenomenon does not exist; therefore, there is a clear separation of cells based on their mass. Therefore, the aim was to compare the cellular content of liquid PRF produced with an oscillating centrifuge compared to a fixed-angle centrifuge. However, further research is needed to further optimize the use of PRF as a supportive tool for cell therapy.

Furthermore, in future clinical studies, we plan to use the Liquid PRF we have evaluated to contain the highest concentration of Platelets and Monocytes (*i*-PRF 700 rpm × 5 min produced with Vacumed FL tube) (code 44909) (122.23% and 142.86% respectively higher than whole blood) to inject it subcutaneously at the edges of an ulcerated wound in the quantity of 1 ml every 2 cm to stimulate tissue regeneration[28] and neoangiogenesis (Husakova et al. 2022).[29] Autologous cell therapy represents an innovative therapy for critical ischemia of non-revascularizable limbs. The main benefits of cell therapy are the induction of therapeutic angiogenesis with the formation of collaterals that lead to increased blood flow in the ischemic limb and tissue regeneration in non-healing trophic skin lesions. Autologous cell therapy is more effective than conventional treatment for non-revascularizable critical limb ischemia (NO-CLI) [29]. Peripheral Blood Mononuclear Cells (PBMNC) used in autologous cell therapy are a heterogeneous population composed of $CD34^-$ cells, lymphocytes, and monocytes, and $CD34^+$ hematopoietic stem cells and EPCs, although present in low concentrations. [28, 30, 31]

However, we cannot overlook the fact that, when obtained from an autologous blood sample, the liquid PRF produced is scanty (~3 ml per 9 ml of blood drawn) and can only be used in a limited volume.

Author Contributions

Conceptualization: C.M., S.C. and C.A.; Methodology: Flag.Fab.; Formal analysis: L.G. and Fel.Fed.; Writing—Preparation of original draft: C.A.; All authors have read and accepted the published version of the manuscript.

Disclosures

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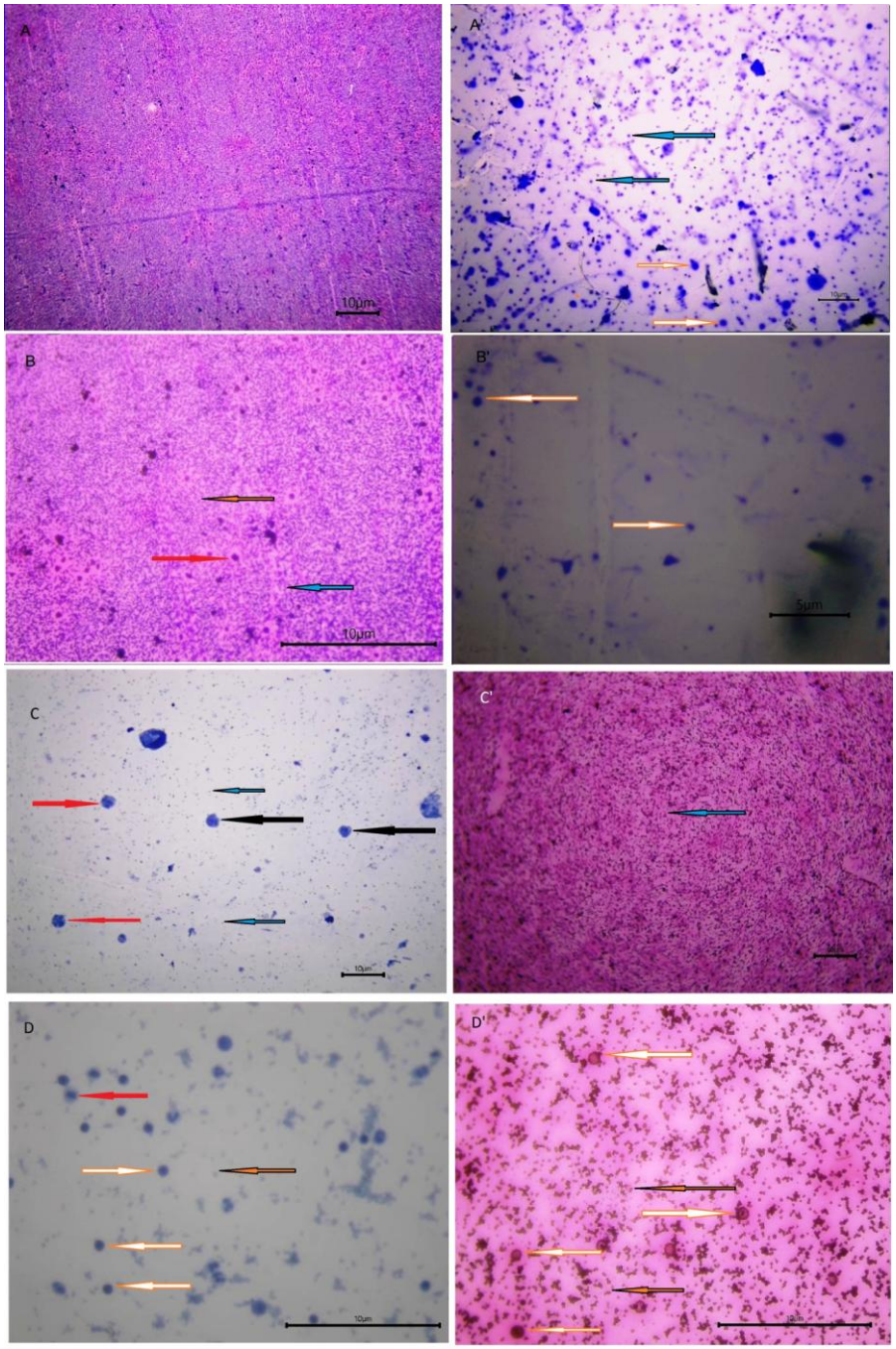

Figure S1 A) C-PRF liquid 2500 rpm × 8 min (S-PRF Sticky tube), col. May-Grŭnwald, magnification 10 ×, Many erythrocytes are highlighted, many leukocytes are present; B) C-PRF liquid 2500 rpm × 8 min (S-PRF Sticky tube) col. May-Grŭnwald, magnification 40 ×, Many platelets and few red blood cells and monocytes are highlighted; A') C-PRF liquid 2500 rpm × 8 min (S-PRF Sticky tube) col. Methylene Blue magnification 10 ×, Leukocytes (many lymphocytes) and few platelets are highlighted; B') C-PRF liquid 2500 rpm × 8 min (S-PRF Sticky tube) col. Methylene Blue magnification 40 x×, Many platelets are highlighted, few lymphocytes; C) C-PRF liquid 2500 rpm × 8 min (Vacumed FL tube) col. Methylene Blue, 10 x magnification, Some granulocytes and monocytes and many platelets are highlighted; D) C-PRF liquid 2500 rpm × 8 min (Vacumed FL tube) col. Methylene Blue, 40 x magnification, Only many platelets and granulocytes and few monocytes are highlighted; C') C-PRF liquid 2500 rpm x 8 min (Vacumed FL tube) col. May-Grŭnwald, 10 x magnification, Some granulocytes and lymphocytes and many erythrocytes are highlighted; D') C-PRF liquid 2500 rpm × 8 min (Vacumed FL tube) col. May-Grŭnwald enlargement 40 ×, Many erythrocytes, few platelets and many lymphocytes are highlighted (A, B, A', C, D, C', D' scale bar 10 μm; B' scale bar 5 μm);

Legend:

Granulocytes Neutrophil
Platelets
Lymphocytes
Eritrocytes
Monocytes

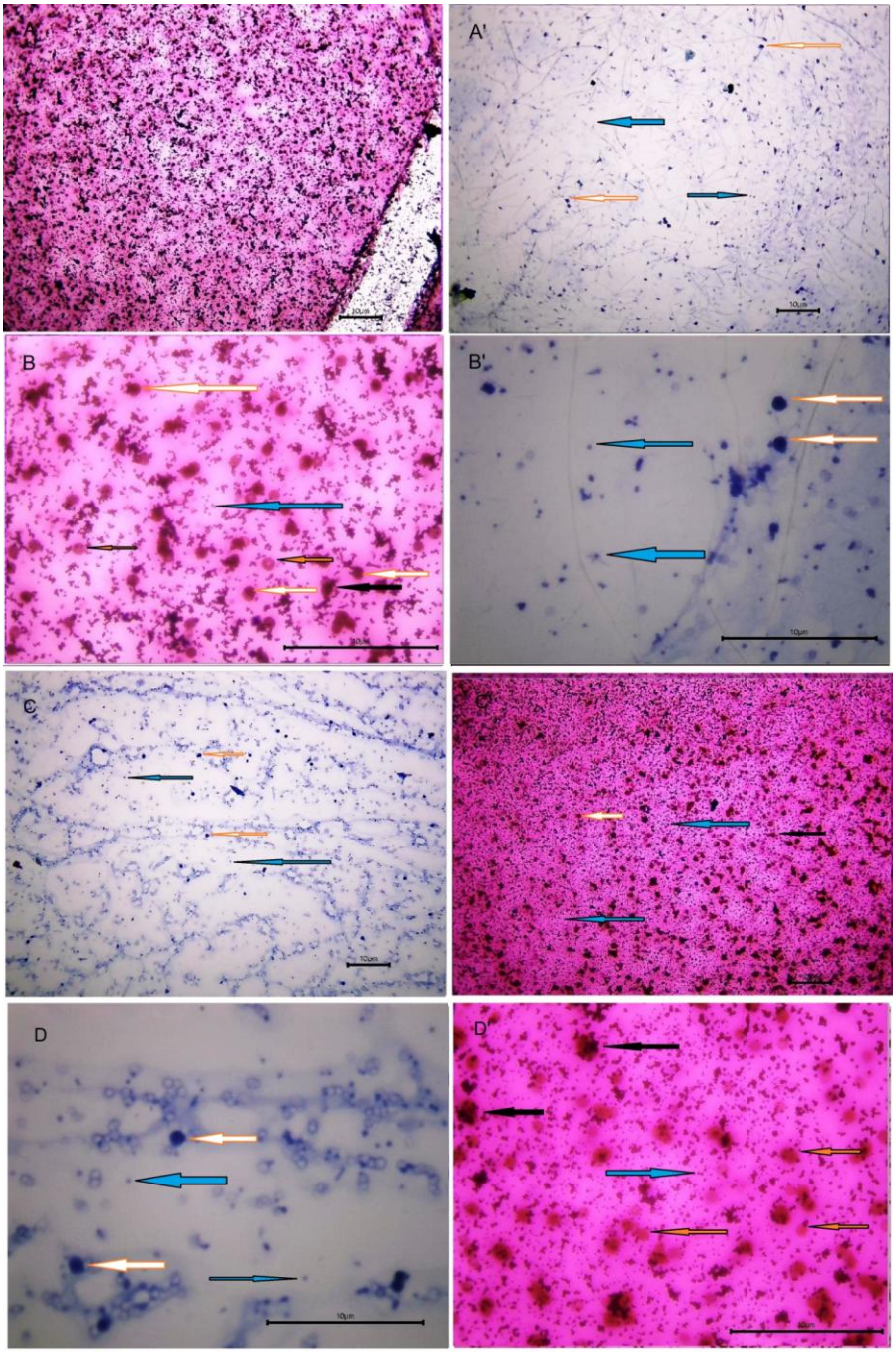

Figure S2 A) A-PRF liquid 1300 rpm × 5 min (Green S-PRF sticky) magnification 10 ×, Many platelets and lymphocytes are highlighted (May-Grŭnwald) (scale bar 10 μm); B) A-PRF liquid 1300 rpm × 5 min (Green S-PRF sticky) magnification 40 ×, Many platelets, many lymphocytes, some granulocytes and many erythrocytes are highlighted (May-Grŭnwald) (scale bar 10 μm); A') A-PRF liquid 1300 rpm × 5 min (Green S-PRF sticky) magnification 40 ×, Many platelets and lymphocytes are highlighted (Methylene Blue); B') A-PRF liquid 1300 rpm × 5 min (Green S-PRF sticky) 40 × magnification, Many platelets and many lymphocytes are highlighted (Methylene Blue); C) A-PRF liquid 1300 rpm × 5 min (Vacumed FL) 10 × magnification, (Methylene Blue), Many platelets and many lymphocytes are highlighted (Vacumed FL); D) A-PRF liquid 1300 rpm × 5 min (Vacumed FL) 40 × magnification, (Methylene Blue), Many platelets, many lymphocytes are highlighted (Methylene Blue

staining); C') A-PRF liquid 1300 rpm × 5 min (Vacumed FL) 10 x magnification, Many platelets, many erythrocytes and many lymphocytes are highlighted (May-Grŭnwald); D') A-PRF liquid 1300 rpm × 5 min (Vacumed FL) ingr.40; Erythrocytes (May-Grŭnwald staining), platelets and neutrophil granulocytes are highlighted (in all scale bar 10 μm).

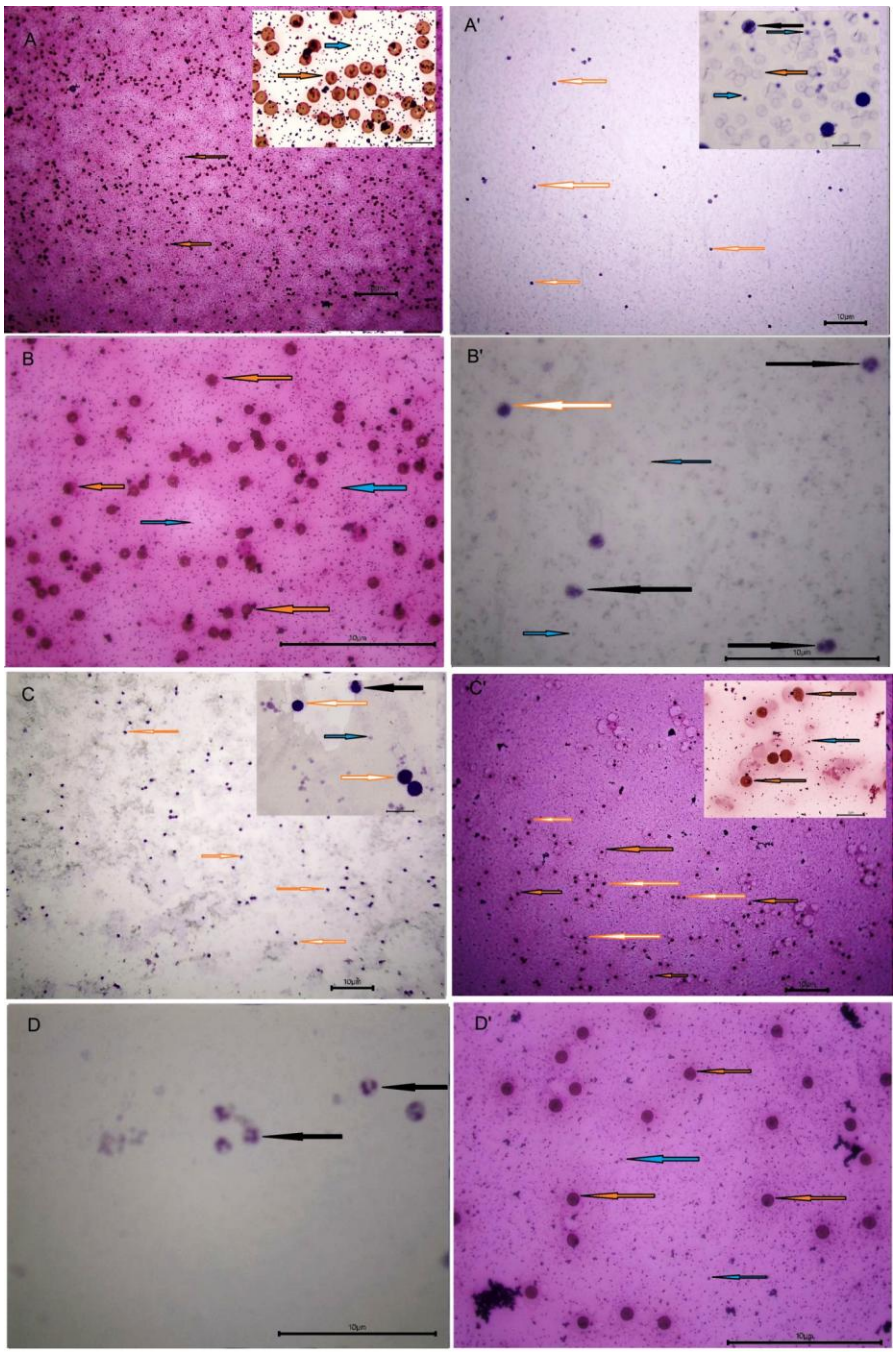

Figure S3 A) *i*-PRF 700 rpm × 5 min (S-Sticky tube) 10 × magnification, many erythrocytes are highlighted (May-Grŭnwald) (scale bar 10 μm); In the box, 100× immersion magnification, May-Grŭnwald staining, scale bar 2 μm, many erythrocytes and many platelets are highlighted; B) *i*-PRF 700 rpm × 5 min (S-Sticky tube) 40x magnification, many erythrocytes and many platelets are highlighted (May-Grŭnwald) (scale bar 10 μm); A') *i*-PRF 700 rpm × 5 min (S-Sticky) 10 × magnification, many neutrophil granulocytes are highlighted (Methylene Blue) (scale bar 10 μm); In the 100 × magnification box, Methylene Blue staining, scale bar 2 μm, many platelets and neutrophil granulocytes with abundant erythrocytes are highlighted; B') *i*-PRF 700 rpm× 5 min (S-Sticky) 40 x magnification, lymphocytes, granulocytes (Methylene Blue) (scale bar 10 μm) and many platelets are highlighted; C) *i*-PRF 700 rpm x 5 min (Vacumed FL) 40 × magnification, many lymphocytes are highlighted (Methylene Blue) (scale bar 10 μm); In the 100 × magnification box, Methylene Blue staining, scale bar 2 μm, many platelets are highlighted (Methylene Blue) (scale bar 10 μm). 100 ×, Methylene Blue staining,

scale bar 2 μm, Many platelets, neutrophil granulocytes and lymphocytes are highlighted; D) *i*-PRF 700 rpm × 5 min (Vacumed FL) 40 x magnification, Many neutrophil granulocytes are highlighted (Methylene Blue staining) (scale bar 10 μm); C') *i*-PRF 700 rpm × 5 min (Vacumed FL) 10 × magnification, Lymphocytes and many erythrocytes are highlighted (May-Grŭnwald) (scale bar 10 μm); In the inset, 100 × magnification, May-Grŭnwald staining, scale bar 2 μm; Erythrocytes and platelets are highlighted; D') *i*-PRF 700 rpm × 5 min (Vacumed FL) 40 × magnification; Many platelets and abundant erythrocytes are highlighted (May-Grŭnwald staining) (scale bar 10 μm);

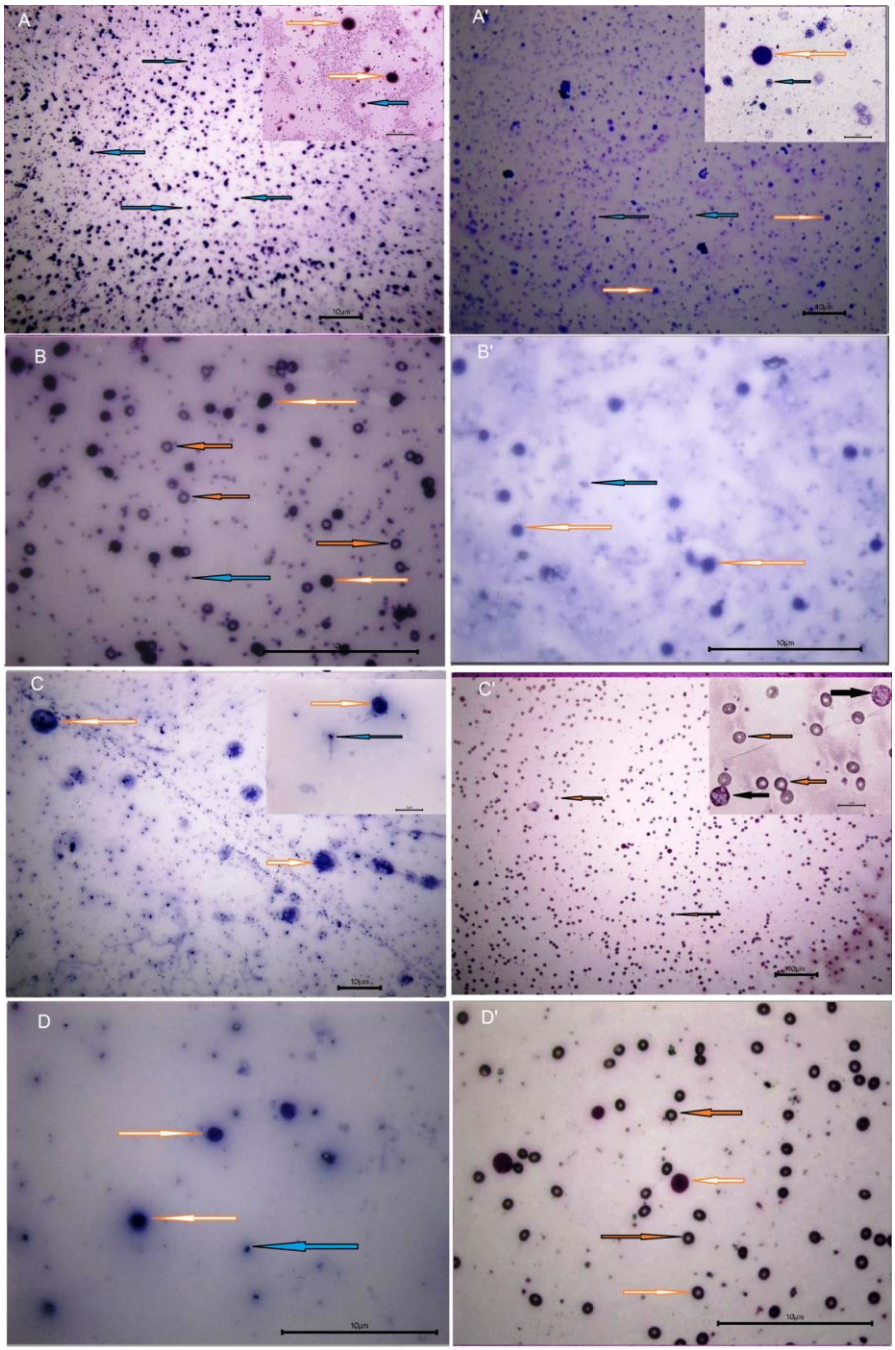

Figure S4 A) *i*-PRF 3300 rpm × 3 min (S-Sticky tube) 10× magnification, many platelets are highlighted (May-Grŭnwald) (scale bar 10 μm); In the box, 100× immersion magnification, May-Grŭnwald staining, scale bar 2 μm, two lymphocytes and many platelets are highlighted; B) *i*-PRF 3300 rpm × 3 min (S-Sticky tube) 40x magnification, many erythrocytes and many platelets with many lymphocytes are highlighted (May-Grŭnwald) (scale bar 10 μm); A') *i*-PRF 3300 rpm × 3 min (S-Sticky) 10x magnification, many platelets and many lymphocytes are highlighted (Methylene Blue) (scale bar 10 μm); In the box, 100× immersion magnification, Methylene Blue staining, scale bar 2 μm, many platelets and one lymphocyte are highlighted; B') *i*-PRF 3300 rpm × 3 min (S-Sticky) 40x magnification, Lymphocytes are highlighted (Methylene Blue) (scale bar 10 μm) and

many platelets; C) i-PRF 3300 rpm × 3 min (Vacumed FL) 40× magnification, many lymphocytes and many platelets are highlighted (Methylene Blue) (scale bar 10 μm); In the box, 100× immersion magnification, Methylene Blue staining, scale bar 2 μm, many platelets and one lymphocyte are highlighted; 100 x immersion, Methylene Blue staining, scale bar 2 μm, Platelets and one lymphocyte are highlighted; D) *i*-PRF 3300 rpm × 3 min (Vacumed FL) 40 × magnification, Many platelets and many lymphocytes are highlighted (Methylene Blue staining) (scale bar 10 μm); C') *i*-PRF 3300 rpm × 3 min (Vacumed FL) 10 × magnification, Lymphocytes and many red blood cells are highlighted (May-Grŭnwald) (scale bar 10 μm); In the inset 100 × immersion magnification, May-Grŭnwald staining, scale bar 2 μm; Red blood cells, one lymphocyte and one neutrophil granulocyte are highlighted; D') *i*-PRF 3300 rpm × 3 min (Vacumed FL) magnification 40 ×; Many platelets and abundant erythrocytes are highlighted with some lymphocytes (May-Grŭnwald staining) (scale bar 10 μm);

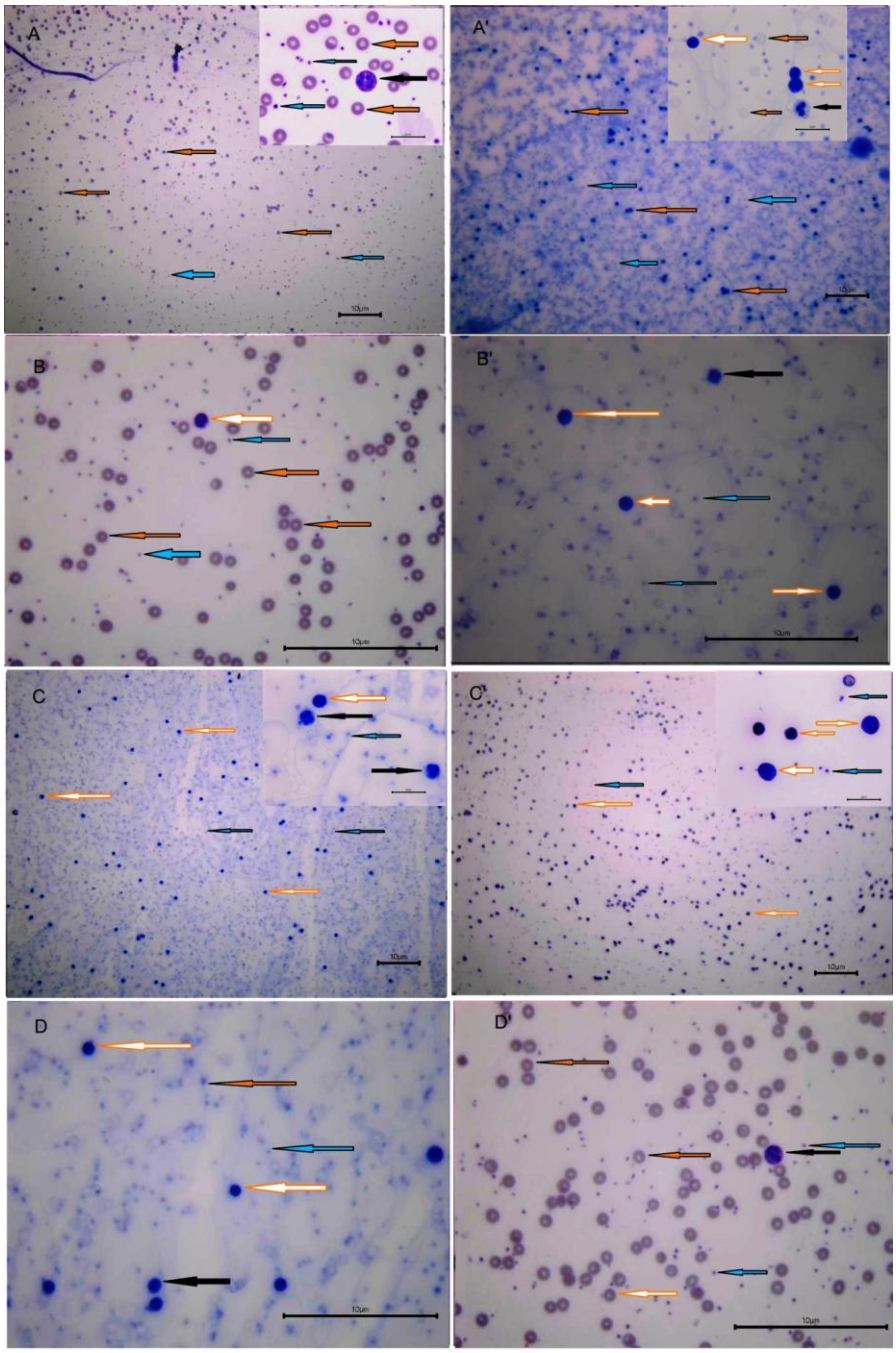

Figure S5 A) Fibrinogen 2700 rpm × 3 min (S-Sticky tube) 10× magnification, many platelets and many erythrocytes are highlighted (May-Grŭnwald) (scale bar 10 μm); In the box, 100× immersion magnification, May-Grŭnwald staining, scale bar 2 μm, erythrocytes, many platelets and a neutrophil granulocyte are

highlighted; B) Fibrinogen 2700 rpm × 3 min (S-Sticky tube) 40× magnification, many erythrocytes and many platelets with many lymphocytes are highlighted (May-Grŭnwald) (scale bar 10 μm); A') Fibrinogen 2700 rpm × 3 min (S-Sticky) 10× magnification, many platelets and lymphocytes are highlighted (Methylene Blue) (scale bar 10 μm); In the box, 100× immersion magnification, Methylene Blue staining, scale bar 2 μm, many erythrocytes, lymphocytes and a neutrophil granulocyte are highlighted; B') Fibrinogen 2700 rpm × 3 min (S-Sticky) 40× magnification, lymphocytes are highlighted (Methylene Blue) (scale bar 10 μm), many platelets and a neutrophil; C) Fibrinogen 2700 rpm × 3 min (Vacumed FL) 40 × magnification, many lymphocytes and many platelets are highlighted (Methylene Blue) (scale bar 10 μm); In the 100 × immersion magnification box, Methylene Blue staining, scale bar 2 μm, platelets, one lymphocyte and two neutrophil granulocytes are highlighted; D) Fibrinogen 2700 rpm × 3 min (Vacumed FL) 40 × magnification, many platelets, erythrocytes and many lymphocytes are highlighted (Methylene Blue staining) (scale bar 10 μm); C' Fibrinogen 2700 rpm × 3 min (Vacumed FL) 10× magnification, Lymphocytes and platelets are highlighted (May-Grŭnwald) (scale bar 10 μm); In the box, 100× immersion magnification, May-Grŭnwald staining, scale bar 2 μm; Erythrocytes, a Lymphocyte and a Neutrophil Granulocyte are highlighted; D') Fibrinogen 2700 rpm × 3 min (Vacumed FL) 40x magnification; Many platelets and abundant erythrocytes are highlighted with a granulocyte (May-Grŭnwald staining) (scale bar 10 μm);