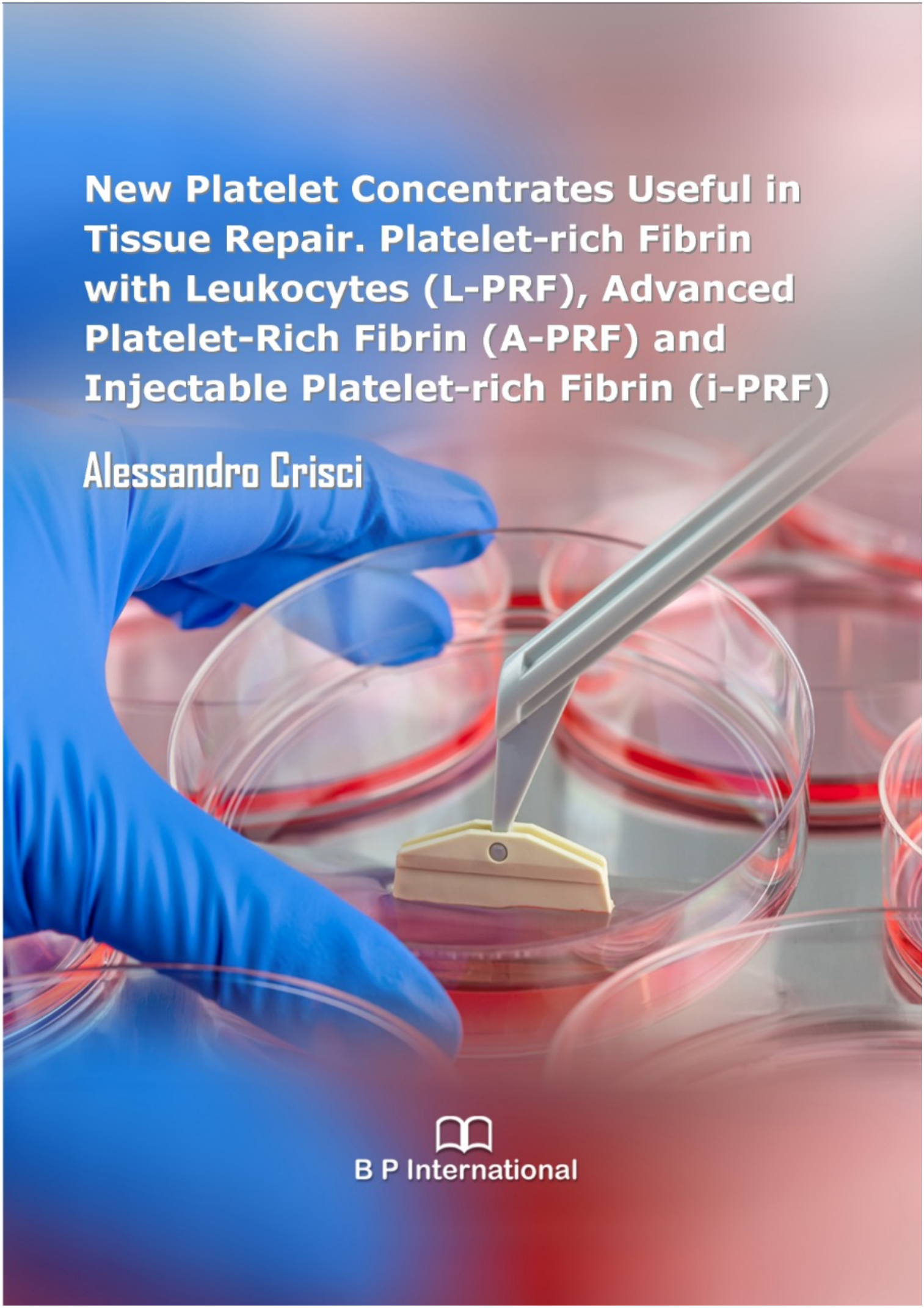
New Platelet Concentrates Useful in Tissue Repair. Platelet-rich Fibrin with Leukocytes (L-PRF), Advanced Platelet-Rich Fibrin (A-PRF) and Injectable Platelet-rich Fibrin (i-PRF)

Alessandro Crisci



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PREFACE

It was with great pleasure that I accepted the invitation of Dr. Crisci to present this text on the regenerative therapy of lesions, inviting someone to present their own work is always one of the greatest acts of affection and esteem and therefore first of all my thanks for this honor.

It is a text that is characterized by ease of reading, completeness of presentation of the techniques of diagnosis and treatment, order in the different expositions. It is clear from the index that this is a text written by a clinician for clinicians.

There is no lack of evaluation of the latest techniques, research bases and new therapeutic concepts, some of which are still in the experimental phase all aimed at practical application, with the purpose of helping patients.

Initiatives such as this are among the best answers that, those working in the field, can provide.

A vademecum available for doctors, specialists and not; are given the tools to operate at 360 degrees on the problematic centered around the so-called regenerative therapy. The reading is simple but rich in content, combining well the high science with clinical practice, leads to simplify the work of those who approach as a new operator and offers insights to those who are familiar with the practice of this art.

Sharing one's knowledge, one's experience is, I believe, one of the most meritorious of the most meritorious works that can be done. I wish the readers a fruitful reading and the Authors the right and deserved success.

Ad Majora
Prof. Mario Capunzo
Professor of Hygiene University of Salerno

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ABSTRACT

Growing multidisciplinary field of tissue engineering aims to regenerate, improve or replace predictably damaged or missing tissues for a variety of conditions caused by trauma, disease and old age. To ensure that tissue engineering methods are widely applicable in the clinical setting, it is necessary to modify them in such a way that they are readily available and relatively easy to use in daily clinical routine. Therefore, the steps between preparation and application must be minimized and optimized to make them realistic implementation. General objective of developing platelet concentrates of natural origin, can be produced *close* to the patient and accelerate the implantation process, being financially realistic for the patient and the health system. Fibrin rich in platelets and leukocytes (PRF) and its derivatives (*L*-PRF, A-PRF, *i*-PRF) have been used in a wide variety of medical fields for soft tissue regeneration. Almost all platelets (> 97%) are absent inside test-tubes in groups tested after PRF membrane extraction.
Growth Factors freed by platelets contained in derivatives of *L*-PRF induce and control the proliferation and migration of other cell types, involved in tissue repair, like smooth cell muscles (SMCs) and mesenchymal stem cells (MSCs).
In conclusion, the results of this work highlight the positive effects of PRF on wound healing after

Keywords: Blood derivatives; growth factors; leucocyte and platelets-rich fibrin; advanced platelets-rich fibrin; L-PRF wound box; stem cells; statistical method.

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Chapter 1
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Involvement of Leukocyte Platelet-Rich Fibrin (L-PRF) as Second-generation Platelet Concentrates in Tissue Repair and Bone Recovery

Alessandro Crisci[1,2*]

DOI: 10.9734/bpi/mono/978-93-91473-15-0

1.1 INTRODUCTION

1.1.1 Leukocyte-platelet Rich Fibrin (L-PRF)

Leukocyte-platelet rich fibrin (L-PRF) is a second-generation platelet concentrate, which is a 3-D autogenously mix of Platelet Rich Fibrin got from the patient's claim blood and clinically used to quicken tissue healing and bone recovery. L-PRF has preferences over PRP and PRGF (Platelet-Rich Plasma and platelet derived growth factor) by having a solid fibrin structure and not requiring any biochemical alteration through bovine thrombin or anticoagulants. As a result of its natural fibrin system properties, growth elements can keep their action for a generally longer period and advance tissue. Platelets could assume another role in tissue fix and in vascular remodeling, other than being a dynamic role in the inflammatory and immune response. A significant number of these substances are accumulated and put away in platelets - granules effectively related to a Scanning electron microscope (SEM) and with immunofluorescence staining. Thin fibers contained in HPC (Human Platelet Concentrate) could be identified with the high initial centralization of platelets in HPC. Glue proteins are somewhat plentiful on the fibrin reticulum: Fibrinogen (Fg), Fibronectin (Fn), Vitronectin (Vn), Thrombospondin-1 (TSP-1)[1].

Fibronectin (Fn) agrees to wound healing and advances the mitogenic migration of the Platelet-Derived Growth Factor (PDGF). Among the platelet-put away Growth factors (GF), basic for wound healing, we count PDGF, specifically PDGF–AB and PDGF–C (predominant isoforms in platelets); different elements incorporate the Vascular-endothelial growth factor (VEGF), basically VEGF-A, Transforming growth factor 1(TGF-1), essential Fibroblast growth factor (bFGF), of the FGF-2 family; Epidermal growth factor (EGF), Hepatocyte growth factor (HGF) and Insulin-like growth factor-1(IGF-1). Individuals from TGF-family are noticeable in wound mending and scar arrangement. Holding a dominant role in healing, platelets are a rich wellspring of cytokines and chemokines. A model is RANTES, a chemokine stored on the kindled endothelium because of a P-selectin-platelet-subordinate instrument. Platelets are the wellspring of tissue inhibitors of metalloproteinases (TIMP 1-4) that are likewise found in the granules and vesicles of the cytoplasmic layer. Metalloproteinases (MMP) present to a group of enzymes whose fundamental capacity is the debasement of extracellular network proteins, for example, collagen, fibronectin, elastin, and proteoglycan protein [2].

The platelet-rich fibrin clots can be viewed as a bioactive repository. An elevated hematocrit or a low platelet count could be a restricting component, and further look into is expected to set up an ideal platelet mean the material to be utilized in methodology. The PRF coagulation is made by a characteristic polymerization process, during centrifugation, and its common fibrin design is by all accounts answerable for a moderate GF and network glycoproteins release (≥7 days). PRF clots are straightforwardly used to fill pits in plastic and general medical procedure mediations. Although platelets GFs spread a fundamental role in PRF science, fibrin design, leukocyte-contained substances, and stem cell nearness are three key parameters significant.

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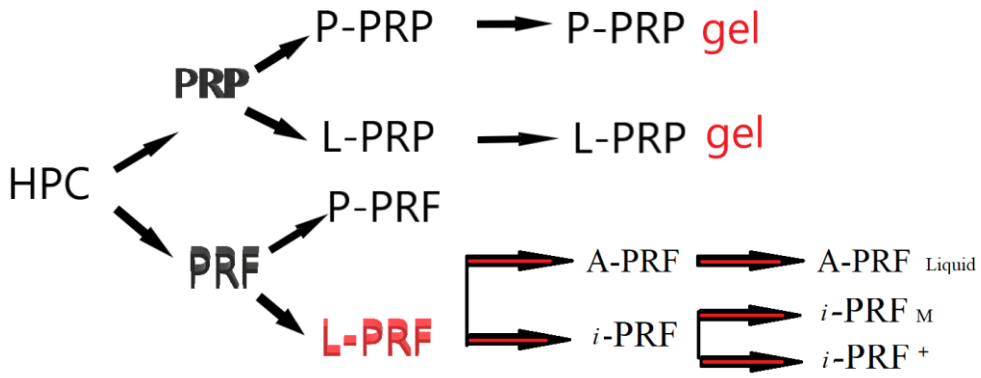


Fig. 1.1. Different types of human platelet concentrates (HPCs): PRP (Platelet-rich plasma); PRF (Platelet-rich fibrin); P-PRP (Pure Platelet-rich plasma); L-PRP (Leukocyte and Platelet-rich plasma); P-PRF (Pure Platelet-rich fibrin); L-PRF (Leukocyte and Platelet-rich fibrin); i-PRF (Injectable Platelet-rich fibrin); A-PRF (Advanced Platelet-rich fibrin);

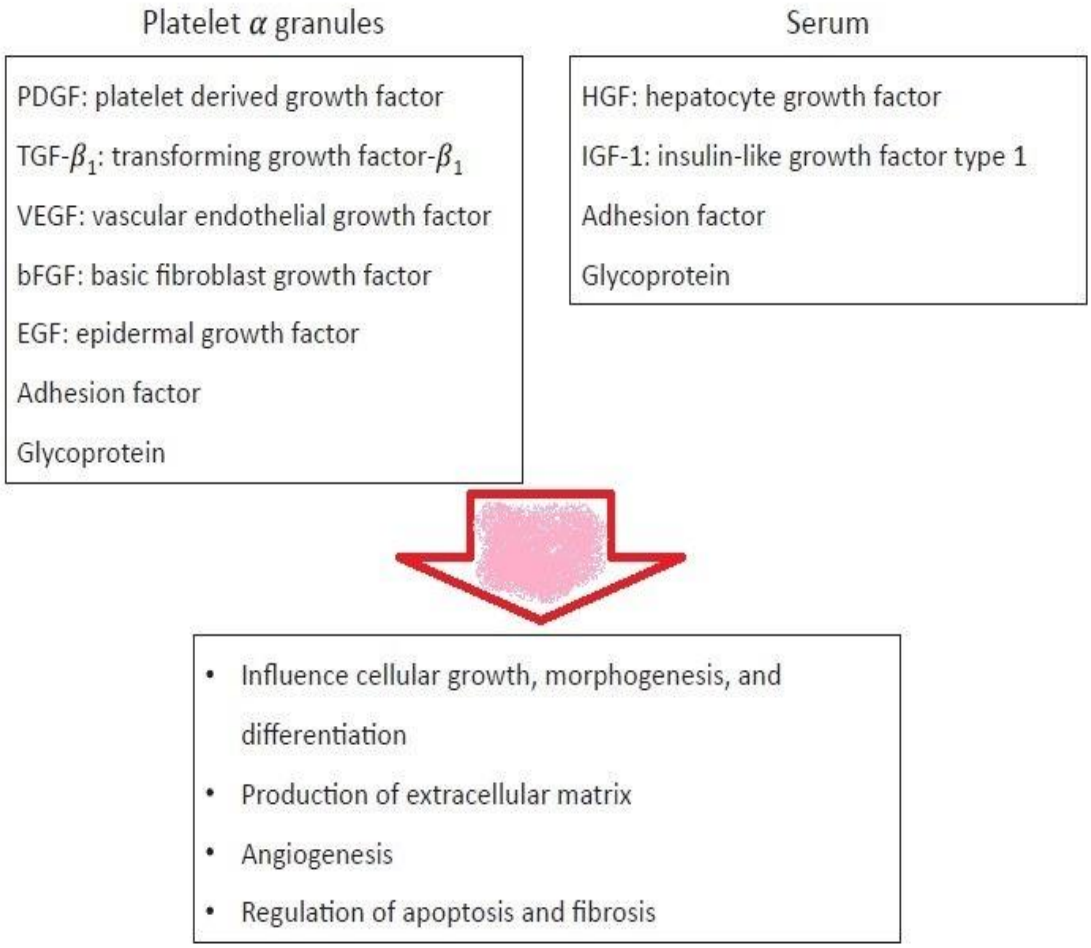


Fig. 1.2. Functions of platelets in regenerative medicine

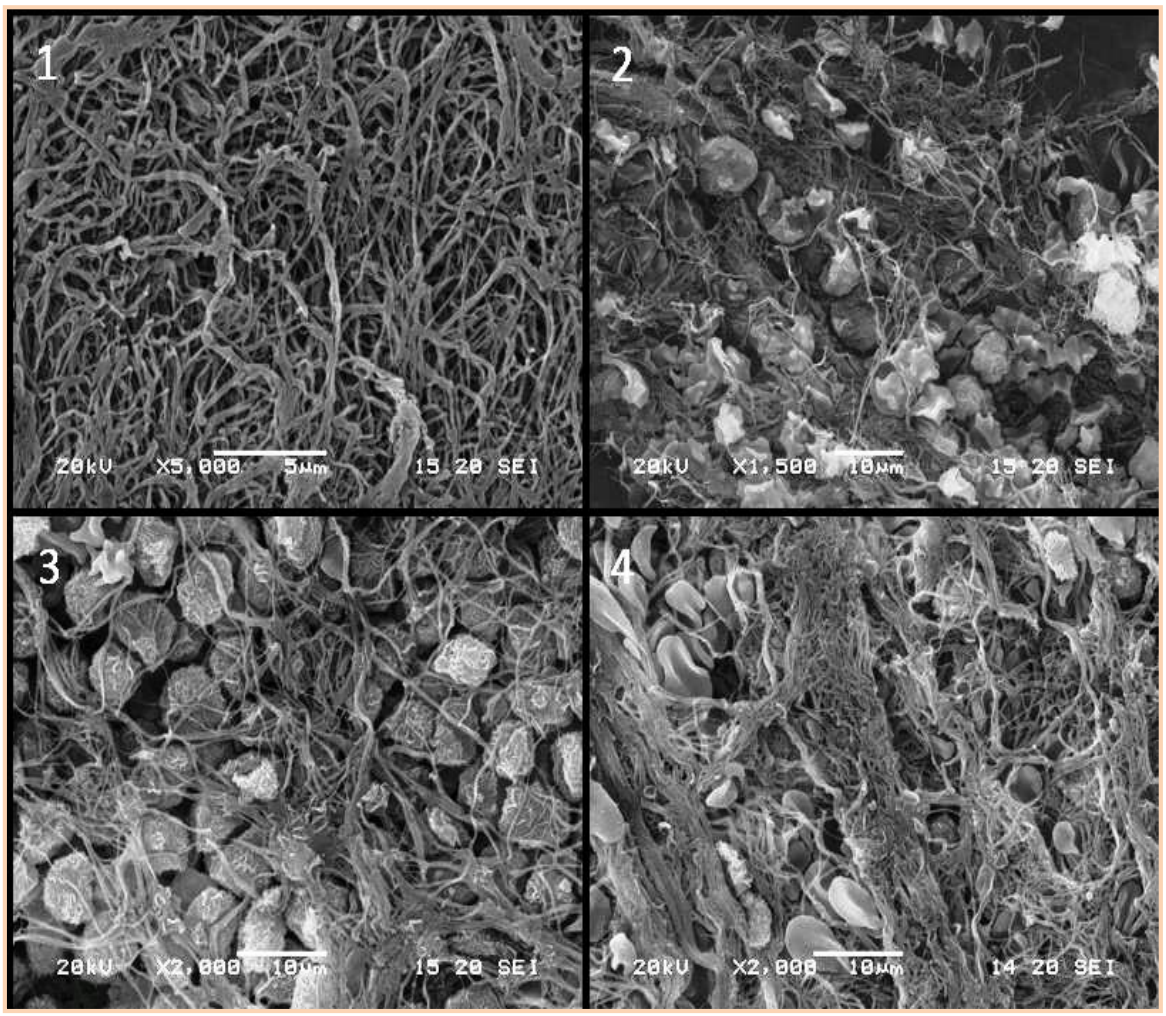


Fig. 1.3. SEM picture displaying: 1 the fibrin-rich layer (5.000 x magnification); 2 a zone of enriched platelets with various degrees of activation (.1.500 x magnification); 3 buffy coat with numerous leukocytes and 4 the red blood cell base (2.000 x magnification)

1.2 MATERIALS AND TECHNIQUES

1.2.1 L-PRF Preparing

In PRF production convention, according to Sacco's initial technique, the blood is promptly centrifuged by 2mins of withdrawal, following the ensuing advances: 30" of increasing speed, 2' at 2700 rpm, 4' at 2400 rpm, 3' at 3000 rpm, and 36" of deceleration and capture. The outcome product is made of three layers: PPP (Platelet-poor plasma at the top), PRF (central clot), and Red Blood Cells (RBCs) at the base. Coming about PRF clots are accumulated and red platelets are evacuated with the guide of scissors, without macroscopical damage at PRF structure cost. Fibrinogen is at first concentrated in the center and a superior bit of the test tube, which is in the middle of red blood cells (RBCs) at the base and the Platelet-poor plasma (PPP) at the top. Clot pressure by methods for a pressure framework (L-PRF box) essentially invigorates cell multiplication and neovascularization. PRF central parts contain platelets, enormously encased inside the fibrin work. Remedial achievement of this sort of strategy is altogether reliant on the time interim between blood test withdrawal and its centrifugation, which ought to be carried on in the briefest interim conceivable, just as on preparing temperature and the kind of test tube utilized [3-5].

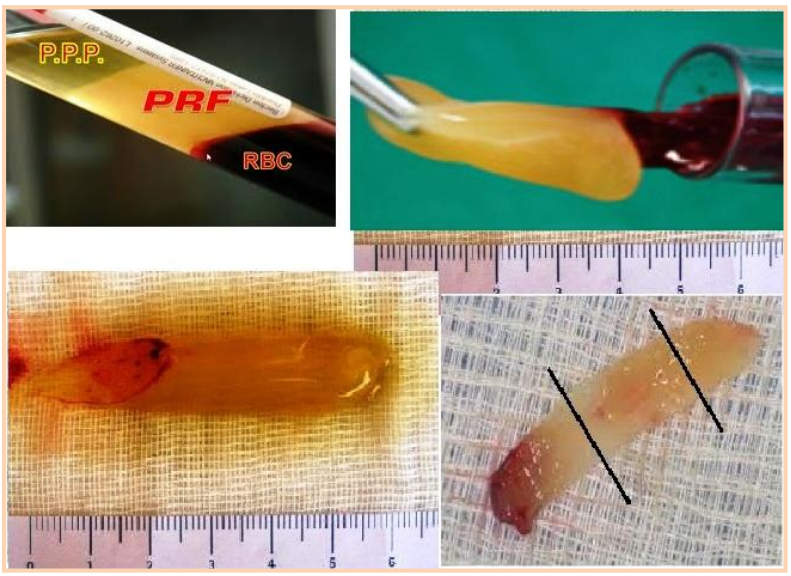


Fig. 1.4. Different phases of PRF preparation

Table 1.1. Leukocytes, RBC and Platelets number in whole blood (control group) and red clot after PRF membrane collecting (test group)

	Leukocytes/μl		**RBC/μl**		**Platelets/μl**	
	Mean	***Range***	***Mean***	***Range***	***Mean***	***Range***
Control	6.900	6.100-7.800	5.19 (10^6)	5.01-5.52 (10^6)	2.66 (10^5)	2.18-3.09 (10^5)
Group 1	3.500	3.000-3.800	5.89 (10^6)	5.75-6.08 (10^6)	6.000	4.000-8000
Group 2	3.600	3.300-4.000	5.84 (10^6)	5.78-5.91 (10^6)	7.000	6.000-9000

1.2.2 PRF Impacts in Tissue Designing

Platelet confinement inside the PRF gel was inspected through immunostaining and with the guide of Scanning Electron Microscope. Also, factors got from platelets initiate and control expansion and migration of different kinds of cells, which are associated with tissue fix, similar to smooth muscle cells (SMCs) and mesenchymal immature microcells (MSCs). Actuated platelets release an entire scope of chemokines and advance grown-up foundational microcells' assimilation, grip, and expansion, including forebear CD34 positive cells, MSCs, SMC begetters and endothelial ancestors. Platelets manage grown-up immature microcells enlistment toward damaged cells and could consequently establish a basic instrument for regenerative cell forms. Enacted platelets release HGF and have been connected to MSCs entry through endothelial cells, lining human supply routes. Human mesenchymal stem cells' expansion (hMSCs) is corresponding to platelet concentration inside PRF concentrates [6].

Along these lines, the foundation of a standard convention for PRF preparation was vital, fulfilling the accompanying criteria:

- Platelet-contained growth factors ought to be protected to animate encompassing host cells;
- Platelets ought to be put away inside the fibrin work with negligible damage or enactment;
- The tridimensional fibrin work must be utilized as a framework by encompassing host cells.

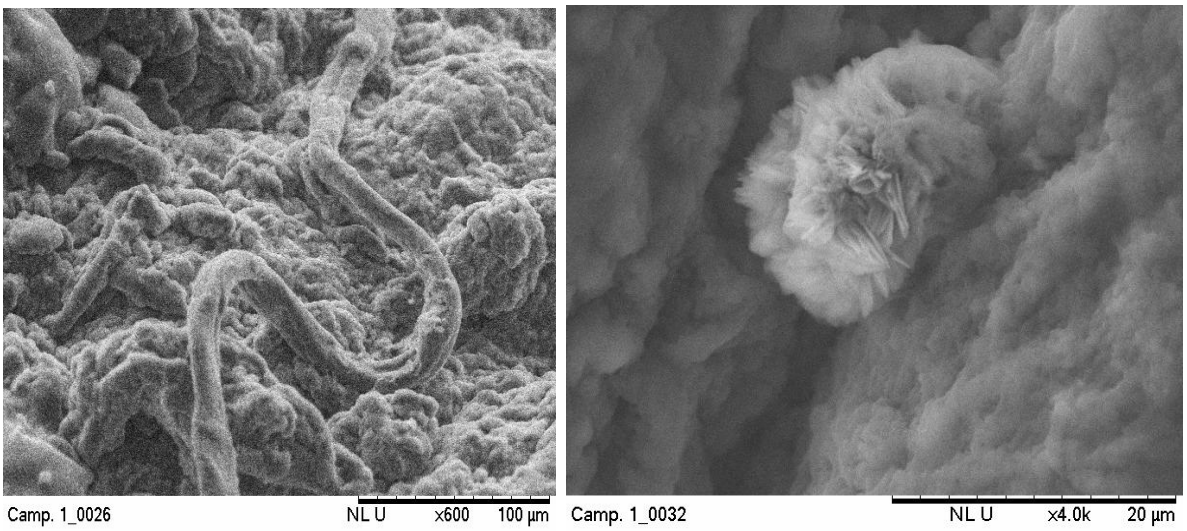


**Fig. 1.5. Microscopy picture at S.E.M. Hitachi Tabletop Microscope TM3000 of L-PRF.
On the left: Fibrin mesh (600x magnification);
On the right: activated platelet (4000x magnification)**

The PRF-held serum could contain raised GFs levels, released by platelets, which are pretty much dynamic during centrifugation stages; we didn't attempt to crush all the plasma with a total pressure of PRF clots. The C-PRF propensity to contain higher growth factors levels (PRF packed through metallic pressure framework), contrasted with G (PRF compacted with cloth), could be followed back to platelet-inferred GFs (PDGF-AA, PDGF-AB, PDGF-BB) [7].

The results could be because of fibrin, since the fibrin work could legitimately ingest GFs or could entangle serum egg whites or heparin, consequently by implication holding GFs. It is practically unimaginable for counting and managing the platelet include in PRF preparation before clinical utilization. A higher relocation rate was watched for L-PRF contrasted with L-PRP on day 3, day 7 and day 14. HUVECs relocation likewise arrived at its top on day 3, day 7 and day 14 for PRF preparation [8].

1.2.3 Preclinical Studies

For bioengineering purposes, Fibrin is a valuable substrate and it is a well-known hydrogel in tissue building and regenerative drug. L-PRF is a preparation that contains leukocytes, and with a high thickness of the fibrin work. These products exist as initiated gels, and can't be infused or utilized as customary fibrin stick. Fibrin creates a brief framework at the join site, however, its fibers have no heading or pressure, and it has a low check of related growth factors. PDGF and TGF-β1 are the most plentiful growth factors, contained inside platelets' α-granules and they get released inside the extracellular space, after platelet actuation [9].

L-PRF shows amazing handling with properties, single L-PRF clots can be transformed into films of fitting thickness and measurements, on account of the new "L-PRF Wound Box" (Fig. 1.7)[10-12]; blending at least two layers is valuable to make a bioactive film of greater measurements, to cover and frame greater unions.

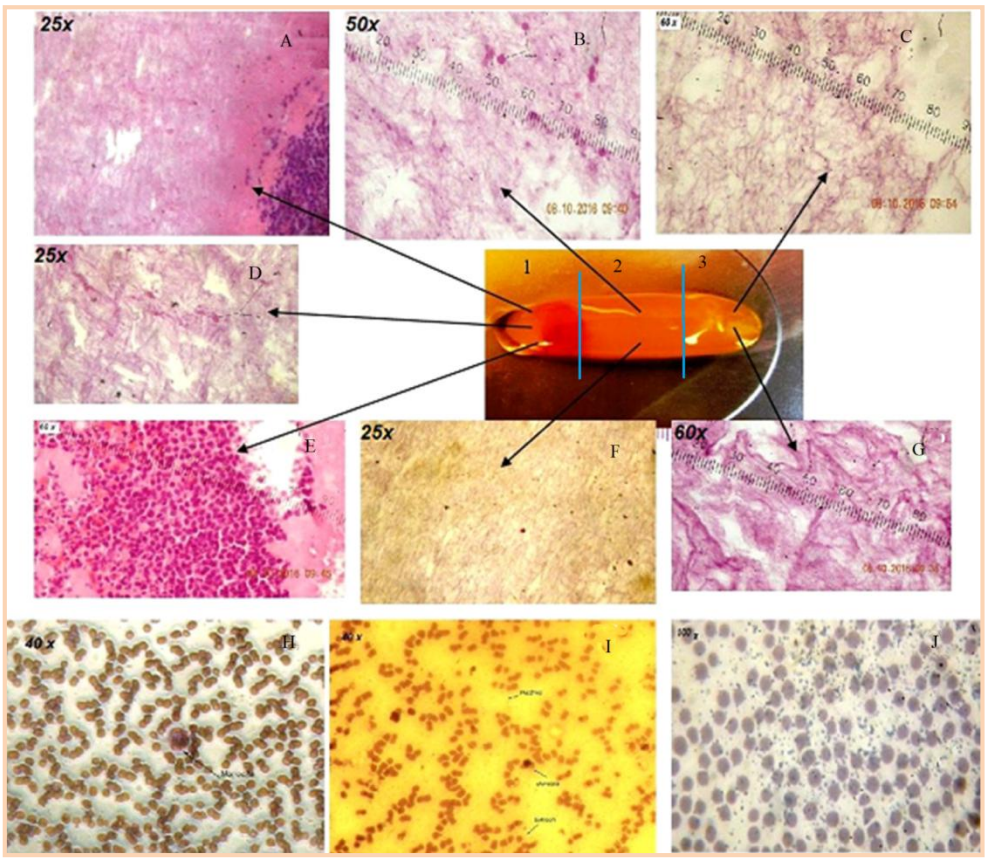


Fig. 1.6. The three C-PRF regions, and O.M. platelet distribution observation on the membrane surface. C-PRF was subdivided into three regions: Region 1 adjacent to the red clot (RBC), Region 2 is the central part and Region 3 is the distal part from the red clot. Platelet distribution was observed in region 1 (A-D-E), region 2 (B-F) and region 3 (C-G). Platelets are at higher concentration in region 1 and at lower concentration in region 3. (from Crisci A. Et al. 2017)[10] Membrane L-PRF 0 min after compression (hematoxylin-eosin staining). (A) III proximal 25× white blood cell-pattern fibrin; (B) medium-III 60× erythrocytes pattern fibrin; (C) III distal 60× pattern fibrin; (D) III proximal 25× erythrocytes-fibrin; (E) III proximal 60× fibrin on the right, the center lymphocytes, erythrocytes, and granulocytes neutrophils on the left; (F) average III 25× pattern of fibrin; (G) III distal 60× pattern fibrin; (H) smear of red clot 40× presence of monocyte in a carpet of red cells; (I) red clot smear 40× presence of red blood cells, monocytes and platelets; (J) red clot smear 100× presence of platelets in a carpet of red cells (staining: May-Grŭnwald-Giemsa)[11]

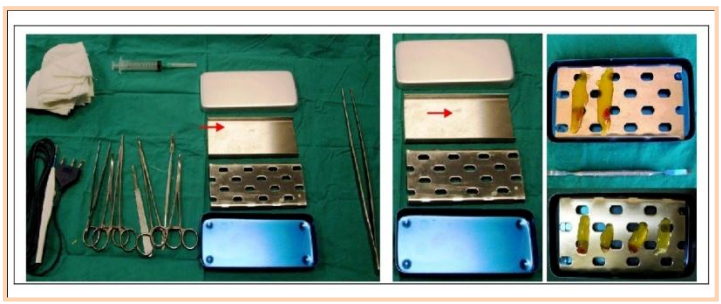


Fig. 1.7. L-PRF Wound Box

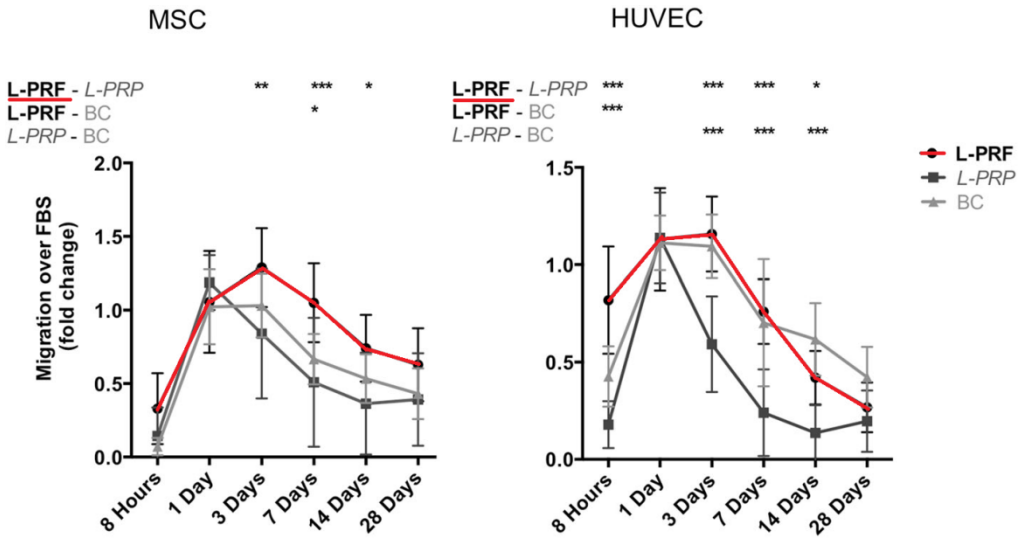


Fig. 1.8. Migration of MSC and HUVEC is shown in response to factors released by L-PRF, L-PRP and blood clot (BC). Migration of MSC and HUVEC was assessed in Boyden chambers with media collected after 8 hours and 1, 3, 7, 14 and 28 days of L-PRP, L-PRF and blood clot compared with soils containing FBS to 10% and expressed how to turn

*Data are presented as mean ± SD from a triple of 11 samples. Statistical evaluation was performed using the repeated two-way ANOVA and the Bonferroni post hoc test. Significant differences for the migration of MSC and HUVEC between platelet concentrates at different time points are indicated: * p <0.05, ** p <0.01, *** p <0.001.*

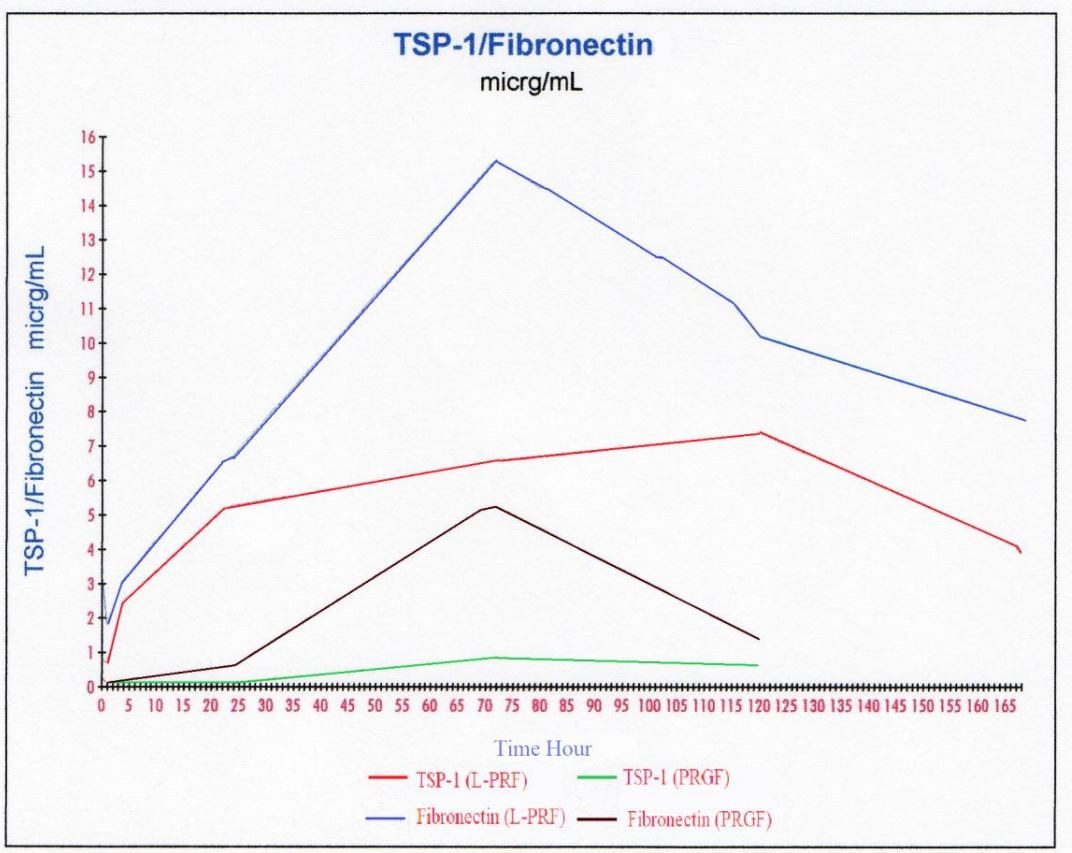


Fig. 1.9. TSP-1 and Fibronectin variations in time.
L-PRF comparison with PRGF (Plasma rich in growth factors = P-PRP)

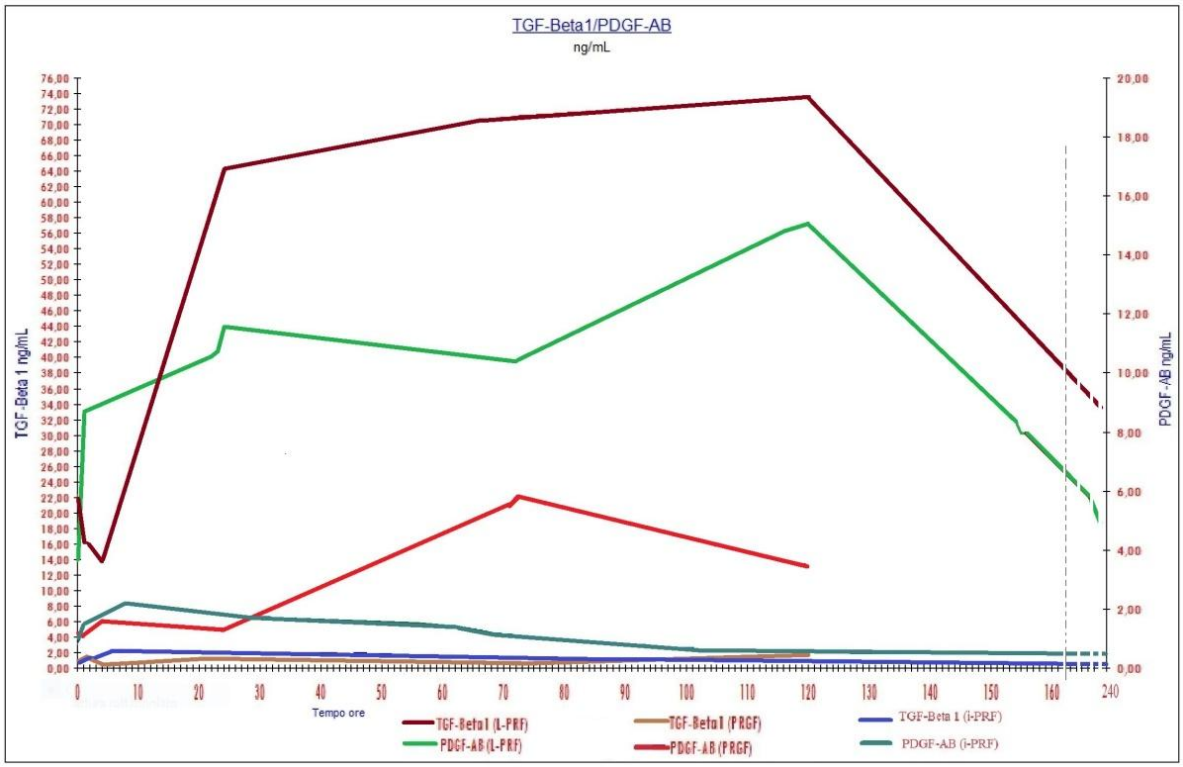


Fig. 1.10. TGF-β1/PDGF-AB variations in time. L-PRF comparison with PRGF and i-PRF

1.2.4 L-PRF *in vitro*

The in-vitro, L-PRF layer, and P-PRP (Pure Platelet-rich plasma)(PRFG-Interest) gel parts were thought about, through the assessment of the moderate growth elements and matrix molecules release. These two gel families were seeded on a growth mechanism for 7 days, and the moderate renditions of 3 key growth factors (TGF-β1, PDGF-AB, VEGF) and 3 coagulation proteins and 3 matrix proteins. Leukocytes found in L-PRF are not just incendiary cells, as they show hostile to nociceptor impacts too, through the arrival of various chemokines, calming cytokines. Genuine PRF is constantly autologous and not homologous. The underlying PRF exudates (rich of growth variables and serum proteins) are gathered in the holder and PRF files are put away in a serum-humidified condition. This is a compelling technique under a natural perspective.

Another device that we have tried in L-PRF clots and layers readiness and strategy institutionalization is L-PRF Wound Box in this the PRF coagulations can be transformed into films. This device grants clot conservation in a moist, sterile condition for 60 minutes, and it permits expansion incomplete growth factor release. By utilizing the PRF Box, the coagulation pressure into the layer is helped out through a light pressure, slow and homogeneous, and the subsequent film consistently remains homogeneously wet and drenched by serum.

A non-institutionalized blood withdrawal system, slow and lacking, prompts a little, PRF-like fibrin mass, with insecure fibrin polymerization (with resulting poor mechanical properties), and to an obscure, not-reproducible growth factor [9-13].

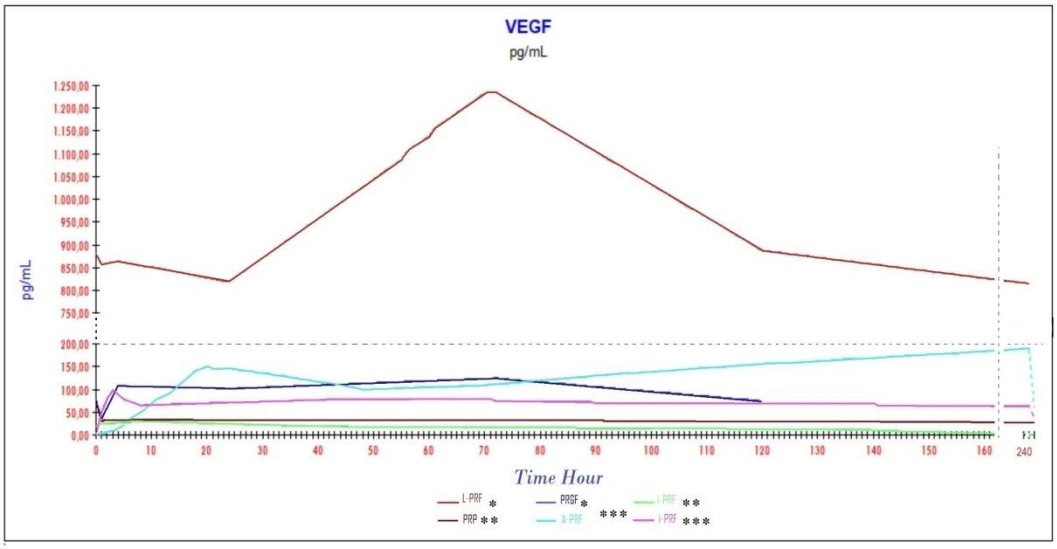
1.2.5 PRF Limitations

Platelets, fibrin and leukocytes normally act synergistically to advance injury healing and tissue recovery, and the intensification of this coagulation/recovery impact on a careful site or wound is the point of precisely arranged platelet concentrate work.

Limitations that were found in the clinical setting and utilization of these products include:

- Since PRF is an autologous product, an expanding necessity for the biomaterial accessibility is troublesomely accomplished. Subsequently, its utilization in surgeries must be firmly controlled.
- PRF contains circulating immune cells, just as antigenic molecules that prevent its utilization as allogenic material; an expanded hazard for the transmission of irresistible maladies is additionally to be considered.

Platelet fixation related issues are non-existent, as all platelets remembered for the blood test are actuated and coordinated inside the coagulation's fibrin matrix. TGF-β1 is quantitatively expanded in moderate release PRF, contrasted with quick-release PRF. There is no noticeable distinction in PRF engineering utilizing various sorts of test tubes [14, 15].



Variazioni di VEGF nel tempo. Confronto tra L-PRF, PRGF, i-PRF, PRP, A-PRF.

** da Dohan Ehrenfest e al. 2012 ; ** da Miron e al. 2017 ; *** da Al-Maawi e al. 2018 ;*

Fig. 1.11. VEGF variations in time. L-PRF comparison with PRGF and *i*-PRF

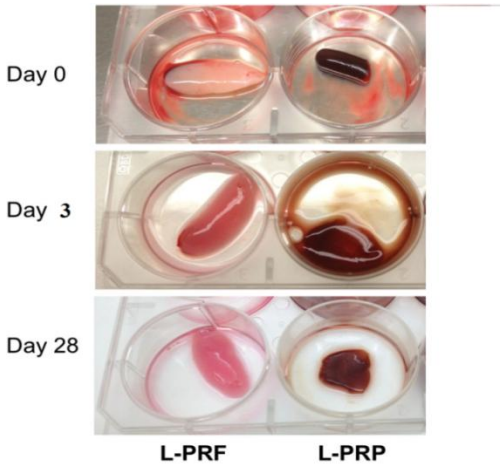


Fig. 1.12. Appearance of each concentrate at the time of preparation (day 0), after day 3 and day 28

Table 1.2. Count of erythrocytes, platelets and WBC on L-PRF membranes derived from clots at 0' compated with those derived from clots at 60' with significance tests (from Crisci et al. 2017)[12]

Type	Membrane 0 min		Membrane 60 min		Between Membrane L-PRF 0-60 min			
	No./mL	%	No./mL	%	*t*-test*		$\chi 2$	
RBC	21,6012	0.0028%	193,966	0.0025%	P=0.000	S	P=0.266	NS
WBC	5,111.15	99.24%	5,036.86	97,80%	P=0.007	NS	P=0.993	NS
PLT	105,801	99.00%	97153	91.00%	P=0.002	S	P=1.000	NS

χ^2 processing performed on two comparisons. Hypothetical content of RBC, WBC, PLT in the L-PRF membranes at 0 and 60 min with significance tests. p> 0.05 =+0.5% no significant difference; p <0.01 = -1% significant difference

1.2.6 Platelets and Leukocytes Investigation

Almost all platelets (>97%) were missing inside test containers of the gathering examined after the PRF layer extraction. Nonattendance of contrasts between the two test gatherings (p>0.05) appears to show that coagulation pressure doesn't impact the conceivable arrival of cell bodies caught inside the fibrin framework. In test gatherings, the lymphocyte concentration is altogether lower, while granulocyte fixation is higher (p<0.01) contrasted with a control gathering. In conclusion, mean platelet volume (MPV) is discernibly diminished between test gatherings and control gatherings (p<0.01); it went down from 9 μm^3 (range: 8-11 μm^3) in entire blood to 4.7 μm^3 (range: 4.5-5.8 μm^3) in test gatherings. This wonder could be because of plasma osmolarity increments inside test tubes after the coagulation course actuation.

Table 1.3. Leukocyte formula stabilized in whole blood (control group) and red clot after PRF membrane collecting (test group)

Cell type	Whole blood (%)		Group 1(%)		Group 2 (%)	
	Mean	Range	Mean	Range	Mean	Range
Neutrophils	51.8	49.7-53.2	72.1	66.1-77.1	66.4	60.9-71.4
Eosinophils	2.9	2.3-3.1	6.1	3.4-8.8	5.1	3.9-6.1
Basophils	0.5	0.3-0.8	0.1	0.0-0.3	0.4	0.1-0.9
Lymphocytes	37.7	35.1-39.2	17.5	15.0-20.4	24.8	21.4-28.0
Monocytes	7.1	6.8-7.6	4.2	1.1-7.6	3.3	2.5-5.0
Total (Mean)/µl		**6.900 (100%)**		**3.500 (100%)**		**3.600 (100%)**

1.2.7 Blood Elements Distribution Investigation

Histomorphometric examination was performed utilizing an optical magnifying lens with 100x all out amplification. A 10 mm eyepiece, with 100 divisions reticulum, was utilized to quantify the inclusion level of the absolute length within any event one cell layer, in each area. With Masson trichrome staining (adjusted by Godman) Fig. 1.13, platelet totals are still found in dark blue, however, RBCs are effectively-recognized, since they are recolored in red. In this association, it is difficult to recognize the contained cell elements. The most important platelet and leukocyte thickness were found in the main millimeter of the yellow coagulation, at the fringe with the red coagulation. This outcome has a gigantic clinical effect as the leukocyte numbers embedded inside the film is considerable, and little lymphocytes are especially proficient in inflammatory responses' guideline. Along these lines, the most helpful segment, under a careful perspective, is the whitish middle of the road layer. Thus, it is important to save a little RBCs layer at the PRF coagulation limit, which contains the heft of platelets and leukocytes [16-18].

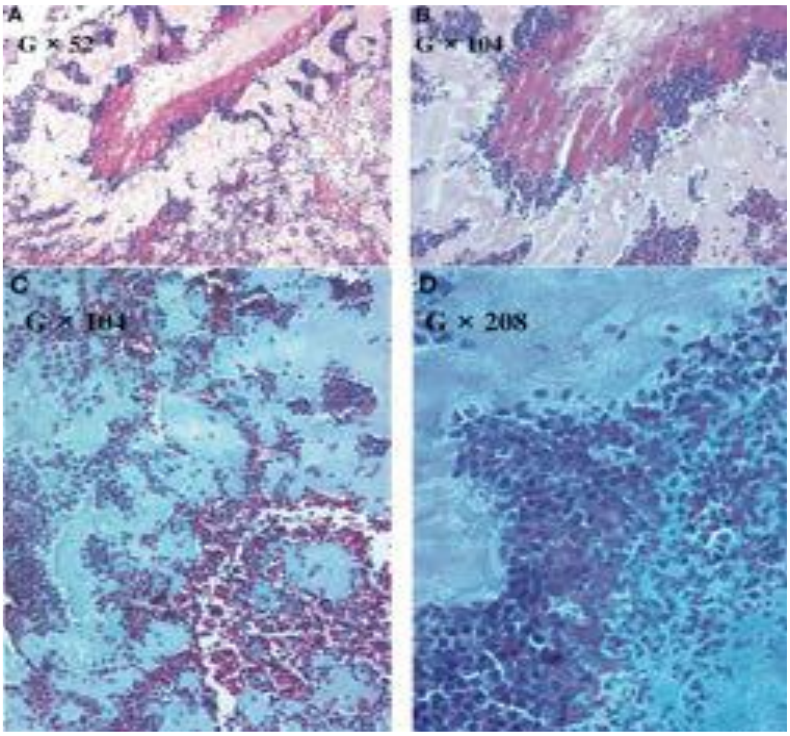


Fig. 1.13. Light microscopic analysis of PRF clots. A and B) Hemalaun-eosin staining is not sufficient to distinguish the various cell types trapped in the fibrin matrix. C and D) Using Masson's trichrome staining, it is possible to easily distinguish platelet aggregates separately from leukocytes (dark blue dark) from the Red blood cells (red). Magnification (G)

1.3 CONCLUSION

As indicated by the examination, the work of PRF membranes region with the most important conceivable platelet enhancement is to accomplish a standard system for PRF preparation as graft material for tissue recovery purposes. It is hard to control the human-inferred materials' quality, as PRF preparation, and essential to apply the most important conceivable quality-control keep an eye on PRF preparation before their clinical application. Biomaterials can go about as controlled release devices, which will take into account supported or even on-request conveyance of these growth factors mixed drinks. Further clinical, histological and statistical investigations are required to comprehend the advantages of this new platelet concentration method. Notwithstanding, we can't clear aside the way that, when acquired from an autologous blood test, produced PRF is rare and just a constrained volume can be utilized and it is a restriction for methodical PRF utilization in General Surgery mediations. As the Migration models prompted by the supernatant of platelet concentrate culture don't contrast between the two sorts of cells. The more grounded MSCs and HUVECs migration was seen as an answer to L-PRF. The entirety of the above means that L-PRF could be helpful as a healing biomaterial, and as a characteristic against of hemorrhagic agent to be utilized at medical and surgical sites.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Chapter 2
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Determination of Tissue Regeneration by Platelet-Rich Concentrates (L-PRF, PRP) Involve in Control of Apoptosis

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2.1 INTRODUCTION

The human body is more than equipped for recovering and healing itself when damage is from a more minor perspective. Platelet Rich Fibrin (PRF), sometimes alluded to as Leucocyte-PRF and pure-PRF is the most recent, cutting-edge cycle on a shockingly entrenched restorative strategy. Cell life fibrin is one of the most important and not quite the same as characteristic segments which improves the arrangement of the new extracellular grid. Platelet Rich Fibrin is derived from a small quantity of a patient's blood which is centrifuged and prepared to isolate red platelets from platelets and leukocytes which structure the premise of a complex fibrin matrix membrane. At the point when a PRF matrix layer is set over a wound, the platelets are enacted discharging a few regenerative proteins with true helpful potential. Platelet Rich Fibrin (PRF) treatment is triple and benefits include, the regenerative proteins and growth factors secreted by actuated platelets quicken the body's regular healing process. The PRF clot frames a powerful fibrin network with a mind-boggling 3-dimensional design. Evaluating the presence of platelet growth factors of platelet-rich fibrin the enzymes attached to immunosorbent analyzes the polymerization of fibrin while processing the PRF and enables the components of platelets. With the help of progenitor cells, the platelets about 97% and leukocytes are transferred in the clot indicating a particular 3-dimensional circulation and in cell apoptosis reduction and survival which are experimentally significant. In tissue repair, the platelet components are inadequately comprehended, which are featured in this survey. The PRP with that of PRF's production and the central points are compared which include; No control of biochemical blood component; Simplified and less expensive production; no information on Use of anticoagulants and bovine-like thrombin; Positive healing on account of a moderate polymerization: More proficient cell relocation and multiplication; PRF goodly affect insusceptible framework; PRF helps hemostasis [1]. The growth factors secreted by L-PRF platelets, especially TGF-1β, instigate and control the multiplication and movement of different kinds of cells associated with tissue repair, for example, smooth muscle cells (SMCs) and mesenchymal stem cells (MSCs). PRF is a characteristic fibrin-based biomaterial arranged from anticoagulant blood collect with no counterfeit biochemical adjustment that permits acquiring fibrin films enhanced with platelets and growth factors. Evidence from the writing recommends the potential role of PRF in recovery and tissue designing. Angiogenesis, the same number of other physiologic procedures, relies upon a harmony among stimulatory and inhibitory sign. On account of incitement, for example, in tumor angiogenesis, and unnecessary, illness development of vascular structures prompts the arrangement of precarious, regularly in blood cells which are immature [2,3].

2.2 TISSUE REPAIR BY PLATELETS

The tissue damage prompts the arrival of flagging particles that trigger the enrollment and actuation of inflammatory cells. By accompanying immune response and empowering tissue rebuilding shields the organism from attacking pathogens manages the evacuation of cell sections, and damaged tissue.

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The inflammatory procedure through an expansive scope of layer receptors and dissolvable go-betweens, which are discharged upon the impact of platelet enactment. The hindrances of tissue breakdown during damage likewise empower the intrusion of microorganisms and might incite tissue diseases. Consequently, tissue healing is the most significant step by the regulation and commencement of immune response against attacking pathogens.

Thrombocytopenia treatment, or draining occasions and to reestablish homeostasis as rule platelets are utilized. The healing of the wound is indistinctly associated with disturbance and requires a fine combination of coinciding by controlling cell movement, the affiliation and redesigning of the extracellular structure, neovascularization [2], cell growth, division, and angiogenesis. The platelets are on a very basic level engaged with the repair and recovery of damaged tissues and the protection of organ work shown clinically and by exploratory [3]. During tissue wounds, for instance, wounds brought about by wound or human tissue ischemia, for example, in myocardial dead tissue or brain stroke, the clot framework and the immune reactions are initiated early, beginning the wound healing process [4]. The platelet-rich plasma and PRF has expanded dynamically in wound healing where Platelets secretes various growth factors like cytokines, chemokines, hepatocyte growth factor (HGF) and PRP treatment has been stretched out to delicate tissue expansion by joining PRP with fat uniting and some positive clinical outcomes have been accounted for, preliminaries supporting the utilization of PRP joined with fat joins in delicate tissue increase stay constrained. Since just a couple of essential examinations and preclinical investigations on animals have been led. In patients with interminable liver ailment, platelet transfusion improved particular parameters of liver capacity, albeit unfavorable occasions identified with platelet transfusion were watched. Platelets secrete the variables which control the multiplication and movement of different sorts of cells associated with tissue repair, for example, SMCs and MSCs. the recombinant growth factors which are secreted by Autologous platelets help in the healing of lower appendages of chronic diabetic ulcers. The invitro conducts a layer of Platelet Rich Plasma pure were looked at through the assessment of the moderate arrival of growth components and matrix molecules. The key components of growth factors (TGF-β1, VEGF, PDGF-AB) and 3 clot proteins thrombospondin-1, (Fn) (Vitronectin) were evaluated tentatively multiple times.

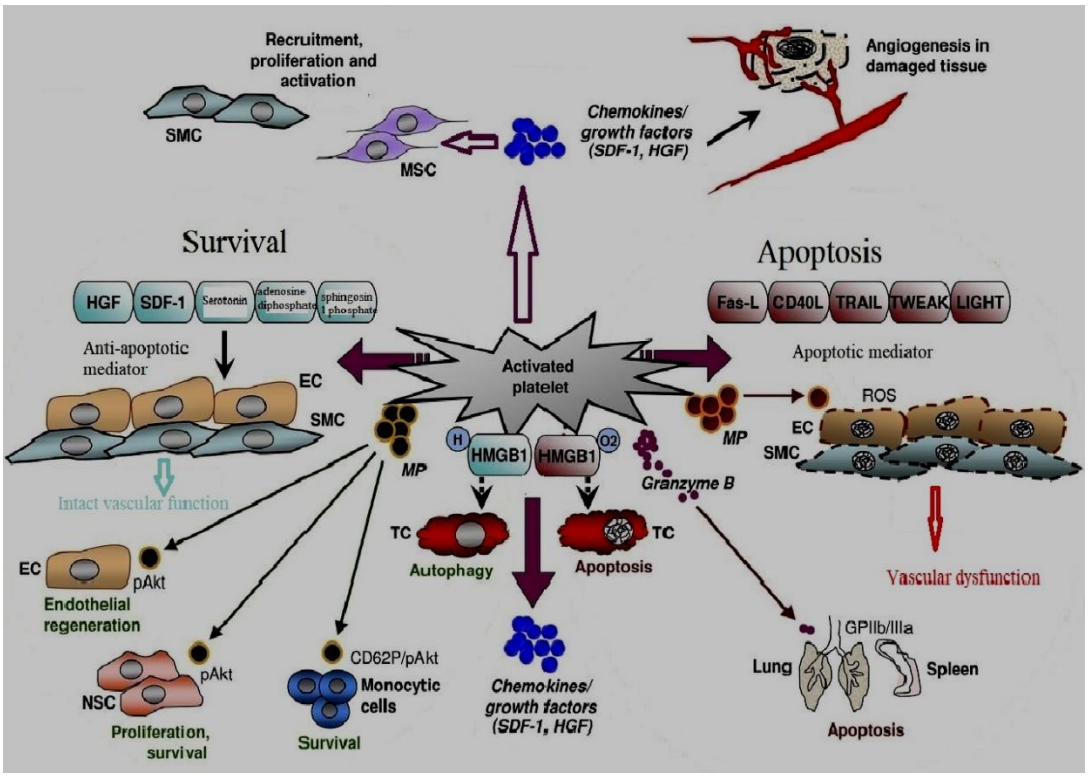

Fig. 2.1. Mechanisms regulating tissue repair mediated by platelets

In treating the osteomyelitis, diabetic foot ulcer the L-PRF and platelet gel are used and permitting healing from this serious infection and it is another progression. To improve the continuance of transplanted fat the PRP has ascended as the new lattice. By twofold turn centrifugation, the PRP derived from whole blood which contains diverse growth components and bond particles in the α-granules and is progressively secure and recombinant growth factors or stem cells are more practical in clinical treatment than other medicines which are convincing in bone recuperation, wound mending, and improvement of musculoskeletal injuries. In the field of hepatic pathophysiology, a clear understanding of the platelet components and the controls with respect to tissue fix was made. Thrombocytopenia and lessened platelet activity in mice fundamentally revoked cell extension in the liver of mice; thrombopoietin-instigated thrombocytosis to cause a strong platelet assortment in the sinusoids of the liver and the acknowledgment of hepatocyte duplication not long after hepatectomy. Some evidence shows that platelets expect a key role in choosing tissue balance among the damage of tissue repair.

2.3 PLATELET CELLS AND PROGENITOR CELLS RECOVERY AND ITS INTERACTION

The Activated platelets secrete chemokines and advance the expansion of adult stem cells. The intramuscular, intracoronary, or intravenous MSC transplantation has been done to myocardial infarction, where cells move to the damaged heart and essentially reestablishes cardiovascular capacity. Besides injury of the tissue produces stem cells a solid chemo attractive sign, giving the premise to their regenerative action. Platelets control the enlistment of grown-up stem cells to injured cells. Along these lines, it can be a significant measurement in regenerative cell reactions. The growth factor known as HGF is delivered after the myocardial infarction and with the properties of antiapoptotic, immunosuppressive action and proangiogenetic, it applies cardioprotective properties. After trial myocardial infarction, the organization of SDF1-GPVI [5] applied huge cardioprotective impacts, advancing CXCR4 positive movement of progenitors from the bone marrow, reinforcing endothelial separation of the safeguarding cell survival, and uncovering proangiogenic impacts. Alongside HGF [6], another significant go-between engaged with stem cell dealing, after myocardial infarction and actuate enrollment of progenitor bone marrow into bones after intravenous infusion of cells through intravenous into a mouse model where SDF-1, is likewise managed. In the studies of clinical trials, progenitor cells of CD34-positive have fundamentally answered to be engaged with the healing of myocardial and recovery, adding to the conservation of cardiovascular capacity. CD34-positive enacted platelets that secrete SDF-1 backings the enlistment of progenitor cells into blood vessel thrombus and the cells isolate through invivo endothelial progenitor cells [7-10].

2.4 THE CELL SURVIVAL AND APOPTOSIS BY ADMINISTRATIVE PLATELETS: TISSUE RECOVERY SYSTEMS

Apoptosis and cell survival are balanced and controlled by Platelets, which is significant for the destiny of tissue injury. The significant pathway of extracellular apoptosis includes the individuals from the tumor necrosis factor (TNF) which are the dead cells that control apoptosis. In mice, the platelets with sepsis instigated by apoptosis and the mice experienced through a system autonomous of platelet components by CD34-positive. In the central nervous system (CNS), the phosphorylation of Akt caused by platelet microparticles, related to cell expansion, and survival and neuro cell isolation and Akt-intervened have improved endothelial recovery [11-17]. Necrotic cells secrete HMGB1, a molecular protein latently during injury of tissue or effectively emitted by natural immune cells, which distinguished as a notice signal that initiates an insusceptible reaction and directs death of the cell and survival, as showed for diseased cells, contingent upon HMGB1 redox. Endogenously HMGB1 present in platelet, and is sent out to the area of cell surface after enactment, and provides HMGB1 a contender for managing platelet mortality/survival balance. The kind of local circulation and the power of articulation of the surface receptors and the target cells characterize the consequence of the apoptosis and against the elements of platelets [18-25].

2.5 CONCLUSION

As per the studies, the platelets control complex tissue repair components. As the cells can fall to pieces through the initiation of cell death apoptosis, to which just particular withdrawal cells, for

example, those of the heart muscle and sensory tissue. From the embryonic advancement, Apoptosis is a programmed cell death together with necrosis and assumes a key role in transformative and controls all presence of the organism beginning. Antiapoptotic mechanisms can be performed by platelet mechanisms, moving equalization to cell survival and tissue repair and secrete mediators with antiapoptotic action supporting the sign of cell survival. This directs the enrollment of adult undeveloped cells into cell injury. Hence it is a significant system in regenerative cell reactions. Platelets are additionally secreted after HGF actuation and have been depicted to advance the take-up of MSC into conduit endothelial cells of humans. The focal points offered by L-PRF against L-PRP containing TGF-β1secretions generally in higher measures, a supported, long term arrival of growth factors analyzed, and more grounded enlistment of cell relocation. Other exploratory and clinical examinations must be directed to give a superior comprehension of the opposite talk in midst of the platelets which help in controlling the healing of tissue, described by procedures, for example, the enlistment of regenerative cells and the regulation of cell death or its survival. The utilization of organic materials and blood divisions is likewise a lawful issue with respect to logical tests, utilization of items that are not for the blood transfusion coordination, patients, research centers, hospitals, and manufactured industries. Despite the fact that from a lawful perspective a specialist being approved for i.v infusion is approved likewise to the duty. Moreover, by guideline Community, at that point with the law of legitimate the included substances are not present in L-PRF, it's anything but a blood subsidiary however falls inside the instance of re-joining cells. These new bits of knowledge will assist us with discovering better helpful alternative platelets that depend to encourage the recovery and fix of tissue and organ injury. Platelets secrete cytokines, growth factors, and chemokines, for example, HGF and SDF-1which control the recruiting, multiplication, and different cells that are predominantly engaged with wound healing. Platelets appear to be a focal point of the system neutralizing tissue breakdown and pathogen intrusion and their role in tissue rebuilding healing from the acceptance of apoptosis and the enrollment of progenitor cells to the support of vascular uprightness. An expansive scope of fundamental research, translational methodologies, and clinical examinations are as yet required to improve our comprehension about the basic systems regarding how platelets adjust the movement of and the recovery from infections, so as to utilize this information for focused treatment. In redox state the threat signal is HMGB1 which been sent to the cell surface from platelets for the initiation directs cell death and damage within the cell in tumor cells contingent. Platelets likewise manage angiogenesis in damaged tissues.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Chapter 3
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Evaluation of PRF Second-generation Concentrates in Cutaneous Wound Surgery of the Foot

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3.1 INTRODUCTION

3.1.1 Platelet Rich-fibrin (PRF)

Platelet rich-fibrin (PRF) is a second-generation platelet concentration, which involves in quick wound healing mechanism by means of influencing angiogenesis, immunity, and epithelial expansion. The leukocytic cytokines and fibrin complex interactions play an important role in the regeneration. The wound could be chronic if it doesn't heal in a precise and auspicious way, or if the healing procedure doesn't have structural integrity [1-3]. Chronic wound healing happens through similar procedures of an intense wound, yet for this situation, more granulation tissue generally shapes, with exorbitant fibrosis that prompts scar constriction and capacity loss [4,5]. Wound healing includes a mind-boggling course of occasions, requested and complex, including numerous sorts of cells, which are driven by the arrival of soluble middle people and flag ready to impact these circling cells and cause them to home back to harmed tissues. Platelets demonstrated themselves to be significant cells directing hemostasis stages through the vascular impediment and encouraging fibrin clot growth. It is realized that they are additionally answerable for enactment and arrival of significant biomolecules, including explicit platelet proteins, growth factors, including Platelet-inferred growth factors (PDGF), clot factors, bond atoms, cytokines and angiogenic factors, ready to invigorate multiplication and actuation of cells engaged with wound healing forms, among which fibroblasts, neutrophils, macrophages, and stem cells are incorporated. Despite the diffuse use of Human platelet concentrates (HPC), like platelet-rich plasma (PRP), one of the revealed downsides is the utilization of anti clot factors that can postpone ordinary wound healing stages. Macrophages are additional cells gotten from myeloid ancestry and are viewed as one of the cell types associated with growth factor emission during wound healing, including Transforming growth factor β (TGF-β), PDGF and Vascular-Endothelial growth factor (VEGF). These cells, together with neutrophils and platelets, are fundamental players in wound healing and, in relationship with their emitted growth factors; they can encourage tissue recovery, the arrangement of new blood vessels (angiogenesis) and disease prevention (antimicrobial activity)[6,7].

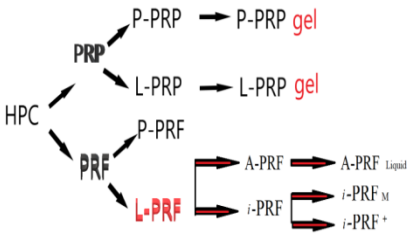

Fig. 3.1. Platelets concentrate (HPC)

Fig. 3.2. Clinical Centrifuge PRF-DUO

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A-PRF+	A-PRF+	1300 rpm, 8min
i-PRF	I-PRF	700 rpm, 3min
i-PRF M	I-PRF *M*	700 rpm, 4min
i-PRF+	I-PRF+	700 rpm, 5min
A-PRF Liquid	A-PRF Liquid	1300 rpm, 5min
Custom	Custom	1300 rpm, 3min
Manual	Manual	FREE SETTINGS

Fig. 3.3. Types of platelet concentrate obtainable by means of Clinical Centrifuge PRF-DUO

3.2 L-PRF AND ITS DERIVATIVES IN CHRONIC FOOT WOUND HEALING

3.2.1 L-PRF

In the longitudinal area of L-PRF clot, created observing the standard centrifugation convention (30" of increasing speed, 2' at 2700 rpm, 4' at 2400 rpm, 3' at 3000 rpm and 36" of deceleration and capture) according to Sacco's technique a thick clot of fibrin with an insignificant between inter-fibrous space is found. Cells are seen along with the entire clot; regardless of whether they are lessened in the distal PRF clot partitions [4].

3.2.2 Advanced-PRF

PRF clots shaped with the Advanced-PRF (A-PRF) centrifugation convention, in its A-PRF+ (1300 rpm, 8 minutes) and A-PRF Liquid (1300 rpm, 5 minutes) variations signs, showed a more liberated structure, with an expanded between stringy space and a higher tally of cells inside the fibrin rich clot [8-10].

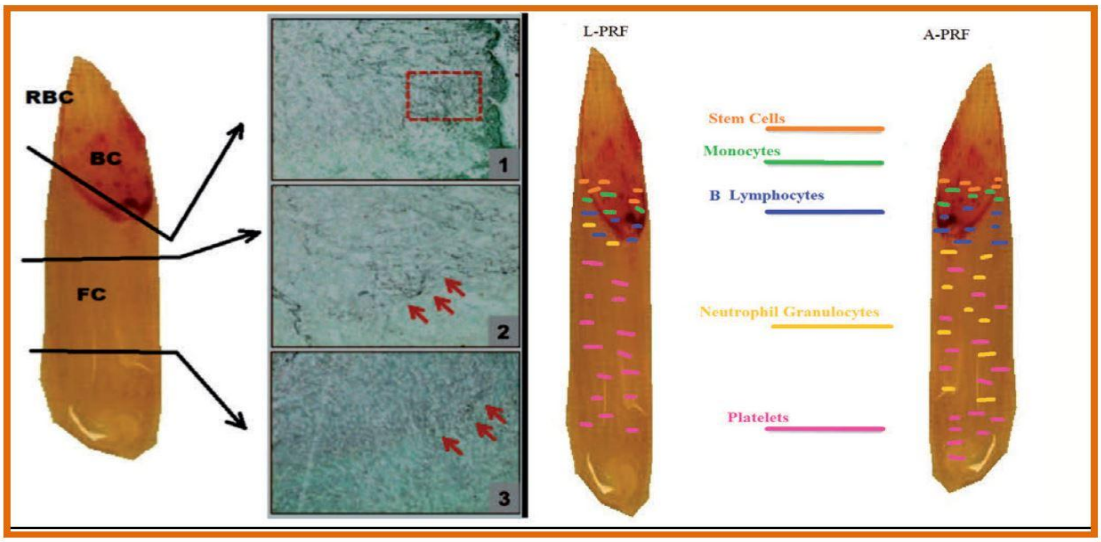

Fig. 3.4. Advanced-PRF (A-PRF) total scan of a fibrin clot along its longitudinal axis (Masson-Goldner staining). RBC represents the fraction of red blood cells. The buffy coat (BC) is the transformation zone between the fraction of RBC and the fibrin clot and FC represents the fibrin clot. The three bars within the scan and the arrows show the first floors of the respective areas. The red arrows mark cells that are trapped inside the fibrin network [11]

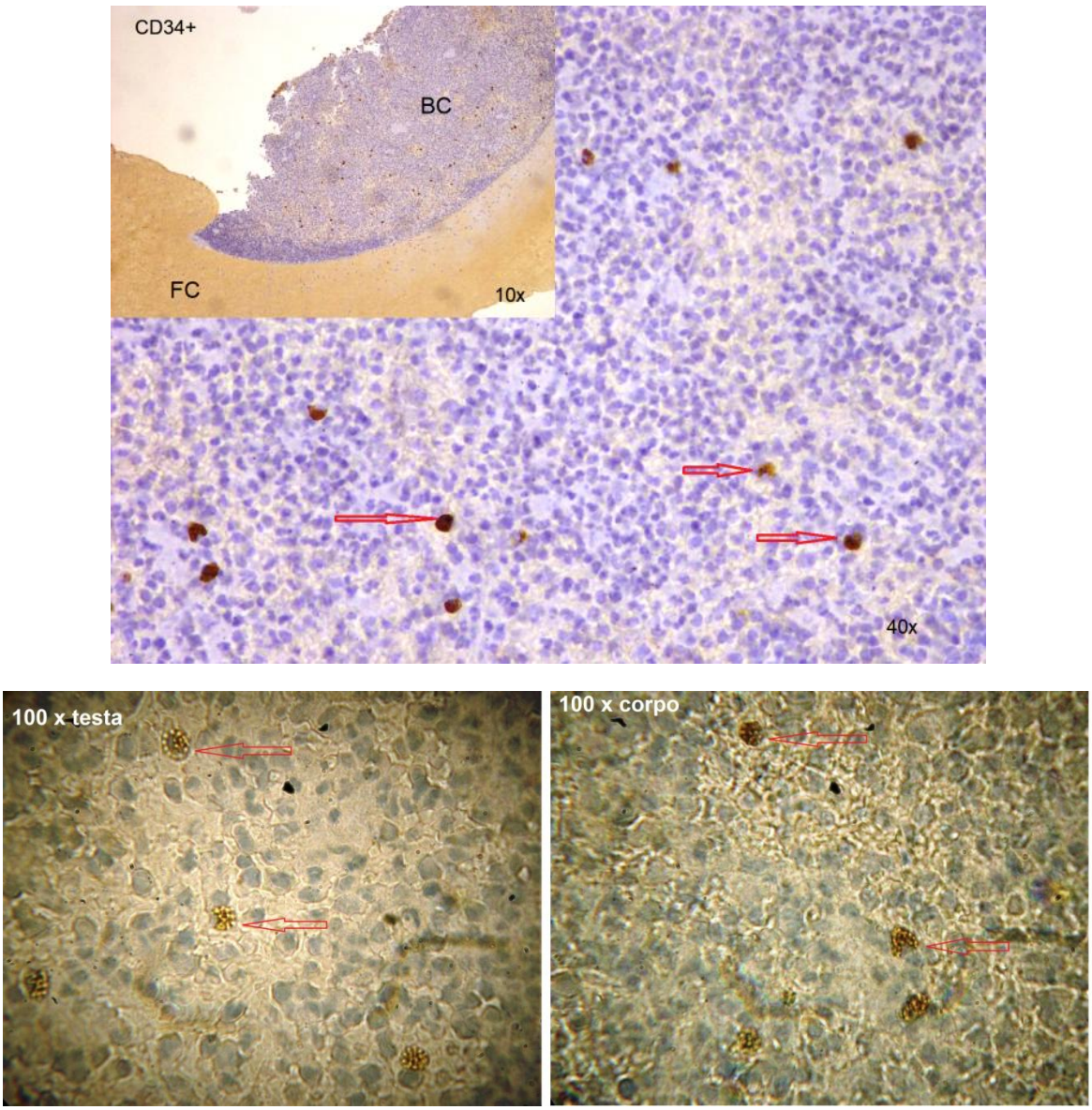

Fig. 3.5. CD34+ stem cells (⇨) found in the middle (body) of horse A-PRF self-compressed membrane.
Below difference in CD34+ cell content of the head and tail of the membrane at ingr. 100 x immersion

3.2.3 Injectable PRF Plan (I-PRF)

Growth of a PRF injectable arrangement (named I-PRF) [11,12] (centrifuged at 700 rpm [60g] for 3 minutes) and its derivatives I-PRF M (700 rpm for 4 minutes) and I-PRF+ (700 rpm for 5 minutes) (Fig. 3.3) was sought after with the expect to convey a platelet concentrate to doctor which demonstrated simple to use in a fluid detailing, to be utilized alone or effectively joined with different biomaterials. Exploiting an increasingly slow centrifugation speed, it is conceivable to watch an expanded tally of regenerative cells with an expanded grouping of growth factors, contrasted with other PRF details got at higher centrifugation speed. This sort of concentrate can possibly improve angiogenesis by communicating the enzymatic network metalloproteinase-9. Hence, the neutrophilic incorporation in the PRF layer, with the utilization of A-PRF, may be considered if angiogenesis is one of the points.

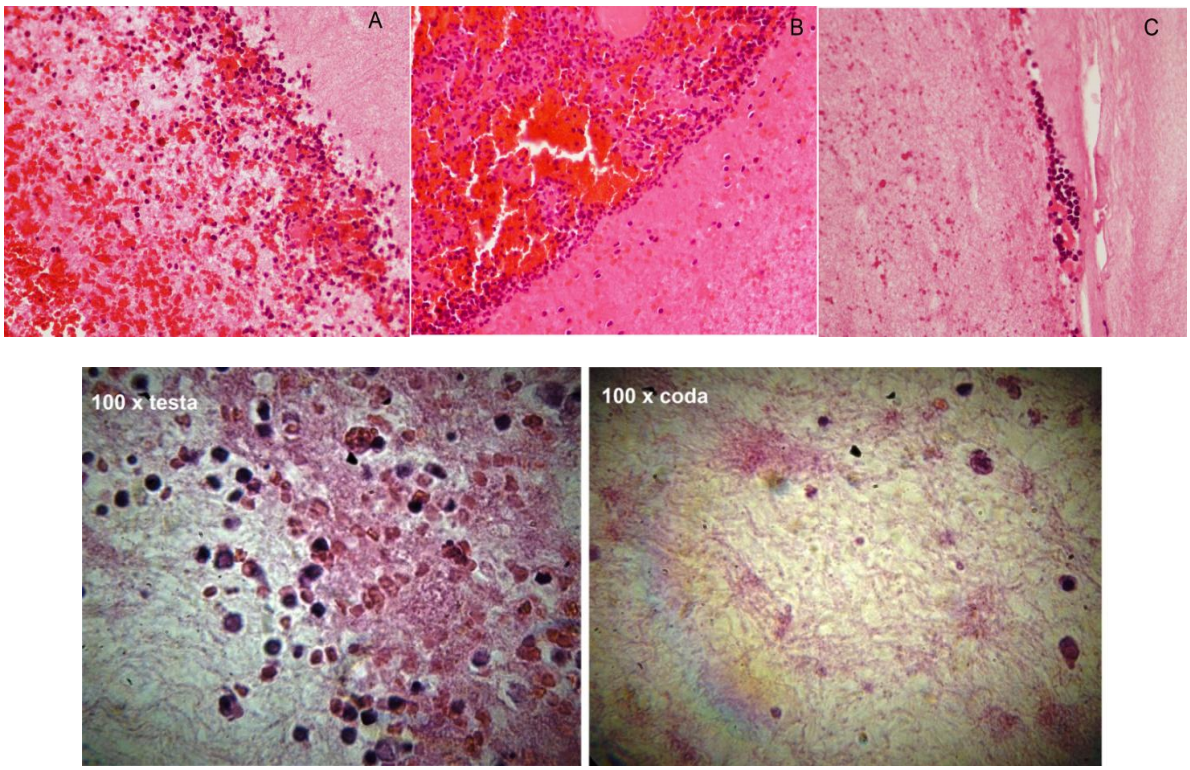

Fig. 3.6. Head (A), Body (B) and Tail (C) of self-compressed membrane of Horse A-PRF (ingr.40x Hematoxylin-Eosin). There are abundant Leukocytes (neutrophils) together with Erythrocytes distributed in the fibrin lattice in A and B. In C presence of abundant reticulum and scarce cells. Below difference in cellular content of the head and tail of the membrane at ingr. 100 x immersion

3.3 PRF OF VARIOUS TYPES AND EFFECTS ON GROWTH FACTORS RELEASE

It was for some time saw that PRF secretes a progression of growth factors for the microenvironment. The TGF-β has wide viability of more than 30 components, known as fibrogenic agents, with TGF-β1 being the most depicted in writing. It is a known trigger of different kinds of cell expansion, including osteoblasts, and it comprises the most dominant fibrogenesis specialist among all cytokines. It assumes a conspicuous role in grid atom combination, similar to collagen-1, and fibronectin, both from osteoblasts and fibroblasts [13]. VEGF is the most dominant growth factor in tissue angiogenesis. It effectively affects the tissue redesigning and VEGF consolidation by its own in different bony biomaterials showed an expansion in the novel growth of bone, in this way calling attention to the quick and amazing impacts of VEGF. Insulin-like growth factors (IGF) is a positive controller of expansion and separation for most of mesenchymal cell types, going about as cell assurance agents. As indicated by certain investigations, the PRF properties, which may add to its anti of calming/antimicrobial exercises [14,15].

3.4 IMPACTS OF PRF ON CUTANEOUS WOUND HEALING OF FOOT AND IN VIVO ANGIOGENESIS

The tissue growth factors impact and specifically PRF and its subordinates have been specially considered concerning delicate tissue wound healing and angiogenesis in different creature models. In numerous restorative methodologies, PRF utilizations were essentially joined to accomplish fruitful administration of leg ulcers, recently demonstrated hard to heal, including the diabetic foot ulcers, venous ulcers and arteriopathy ulcers of lower appendages. In this examination, the point was to institutionalize the utilization of L-PRF in patients with osteomyelitis, to utilize this second-age platelet concentrate, encouraging healing forms [16, 17]. Layers, together with the fluid determined by Wound

L-PRF Box pressure, were embedded in the cutaneous sore, up deep down, after careful debridement. All patients demonstrated energy to the Probe-to-Bone test, and Nuclear Magnetic Resonance indicated cortico-periosteal thickening and additionally osteolysis foci of the cortico-spongious, neighboring the ulcer. Gram-positive microscopic cells were found in our patients in 52% of cases.

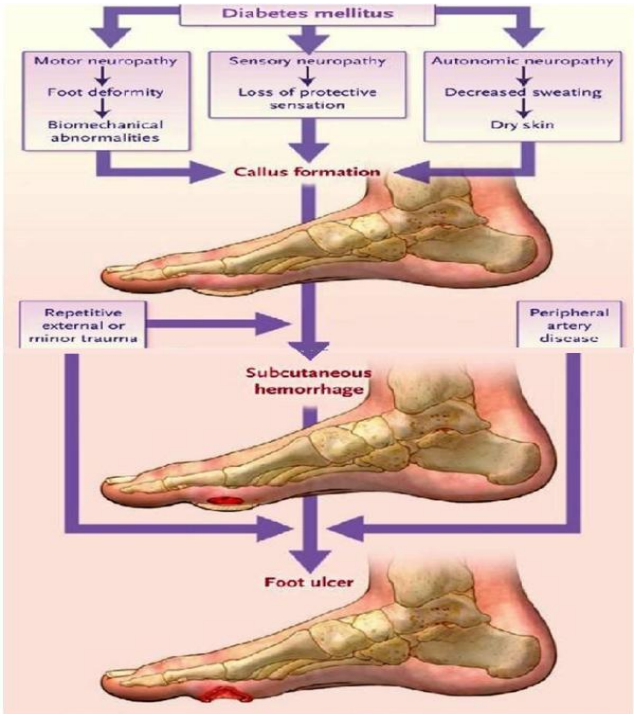

Fig. 3.7. Diabetic foot cutaneous ulcers pathogenesis

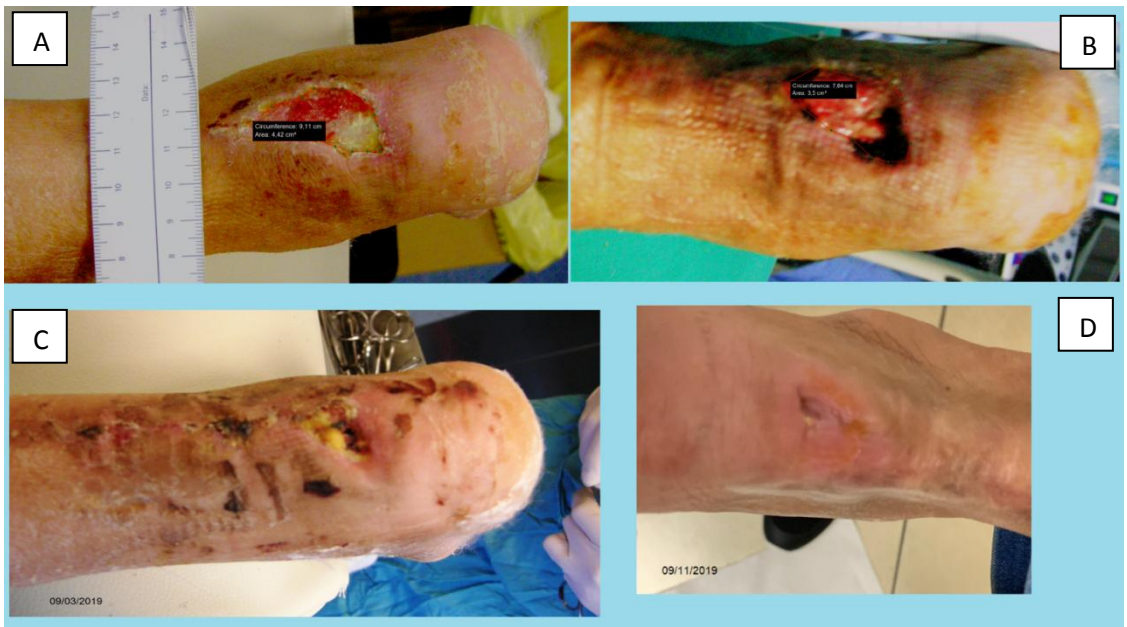

Fig. 3.8. Post-surgical dehiscence following Achilles' tendon reconstruction (A) treated with L-PRF (B) after seven days from therapy (C) and complete healing (D)

3.5 DISCUSSION

Regenerative properties of L-PRF and its derivatives (A-PRF, I-PRF) (Fig. 3.1) as a careful adjuvant got an eminent consideration as far back as the material was presented in the principal long stretches of the thousand years. Then again, there is no reasonable proof to clarify the antimicrobial capability of this biomaterial, which contrasts both fundamentally and naturally from other HPC structures. A-PRF as a cell grid seeded on fibrin, which contains different platelets, including platelets, lymphocytes (B and T)[17,18], monocytes, stem cells and neutrophilic granulocytes, which can secrete a progression of growth factors. Every one of these distinctions may have a significative result on the separate calming and antimicrobial exercises. These outcomes are steady with those got from past investigations, assessing the antimicrobial properties of other HPC arrangements. Since A-PRF shows antimicrobial properties, there is the need to set up if this movement is fundamentally higher than an entire blood clot. Future research is expected to investigate the antimicrobial range of A-PRF and all L-PRF derivatives, and to send out the likelihood that it may go about as substrate to encourage the growth of explicit living beings [19-23].

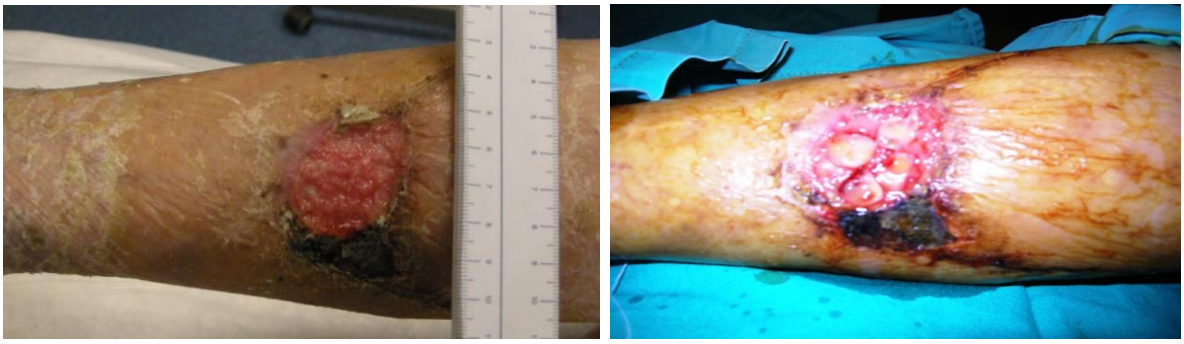

Fig. 3.9. L-PRF clots, used on a cutaneous leg wound

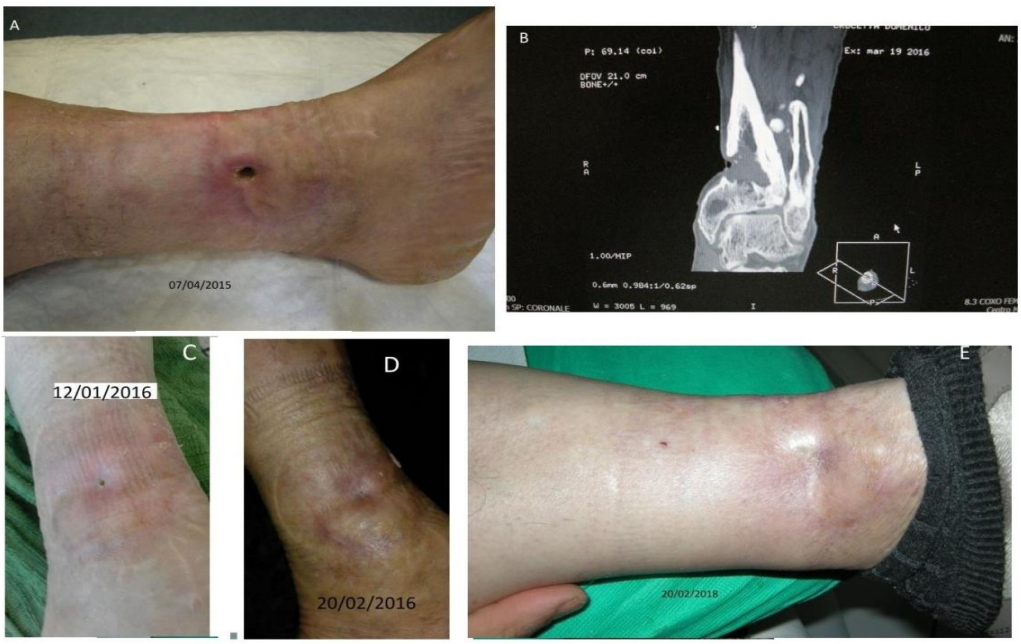

Fig. 3.10. Use of Leukocyte Platelet (L-PRF) Rich Fibrin in diabetic foot ulcer with osteomyelitis. (A,C,D,E) Different moments of the wound healing, stable after two years; (B) NMR of the patient with the bone lesion [16]

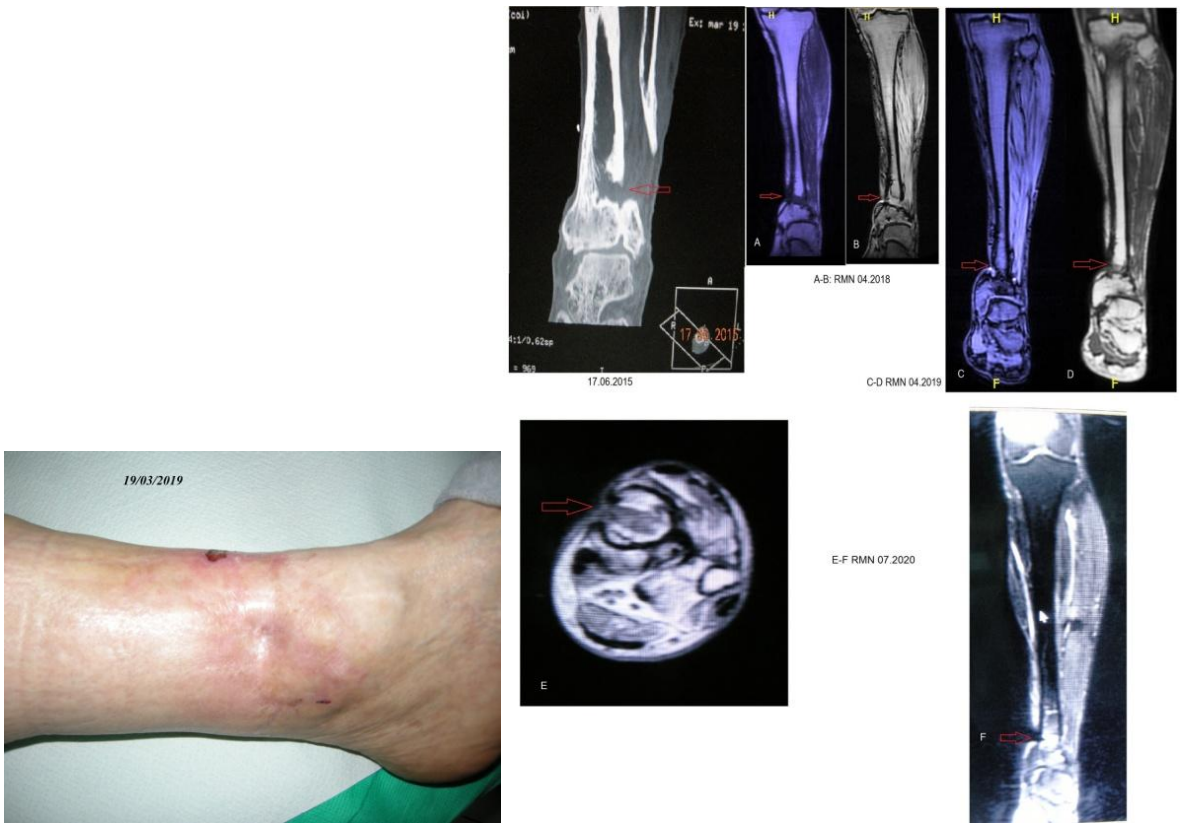

Fig. 3.11. Same patient 5 years after L-PRF therapy. NRM finding demonstrates bone regeneration of the lesion (from Crisci et al.2018)[16].

Specifically, for the specialist, recollect that Staphylococcus Aureus is one of the primary drivers of procured nosocomial contaminations, diseases corresponded to inside restorative gadgets and diseases of careful injuries. Significative research is these days centered around elective S. Aureus contamination medicines, to bring down the danger of choosing anti-infection safe strains. Candida albicans are the most habitually contagious species confined in the microbiome. Invulnerable reaction impedance may enable these artful growths to give contamination. A-PRF has a higher ability to continually restrain C. Albican's growth, contrasted with an entire blood clot. In addition, C. Albicans is less helpless to antimicrobial parts of platelets and saw how antimicrobial peptides of human platelets were more dominant against microscopic cells than parasites. A-PRF shows a more noteworthy potential repressing Streptococcus mutans contrasted with common blood clots. Nonetheless, since no other HPC was tried against this life form, the restraint component and of its clinical potential requires extra examinations [22-25].

Despite the fact that the consequences of different examinations recommend that A-PRF shows an antimicrobial movement, a few impediments are available. In the first case, the in vitro assessment doesn't emulate a clinical circumstance where A-PRF would be utilized in a situation, encompassed by tissues that respond to a careful occasion. In this situation, A-PRF can associate with a few cells and cytokines, engaged with wound healing procedures and it can adjust the underlying insusceptible reaction and the healing stages [26,27]. The fibrin lattice framed in their PRF form in old patients was all the more conventionally sorted out contrasted with fibrin framework shaped in more youthful patients. The greatness of this disclosure still should be resolved. Cell types, number of cells and plasma part fixation contrast inside each clot and between single clots, each example circle can't be indistinguishable from another. One of the issues to be evaluated is that there is still no real way to decide whether they tried material is bactericidal or bacteriostatic. In this contention, our investigation bunch is working at the exact instant. Downsides put in a safe spot, the plate dispersion technique demonstrated enough to exhibit that A-PRF, similar to all other L-PRF derivatives, shows antimicrobial movement [28, 29].

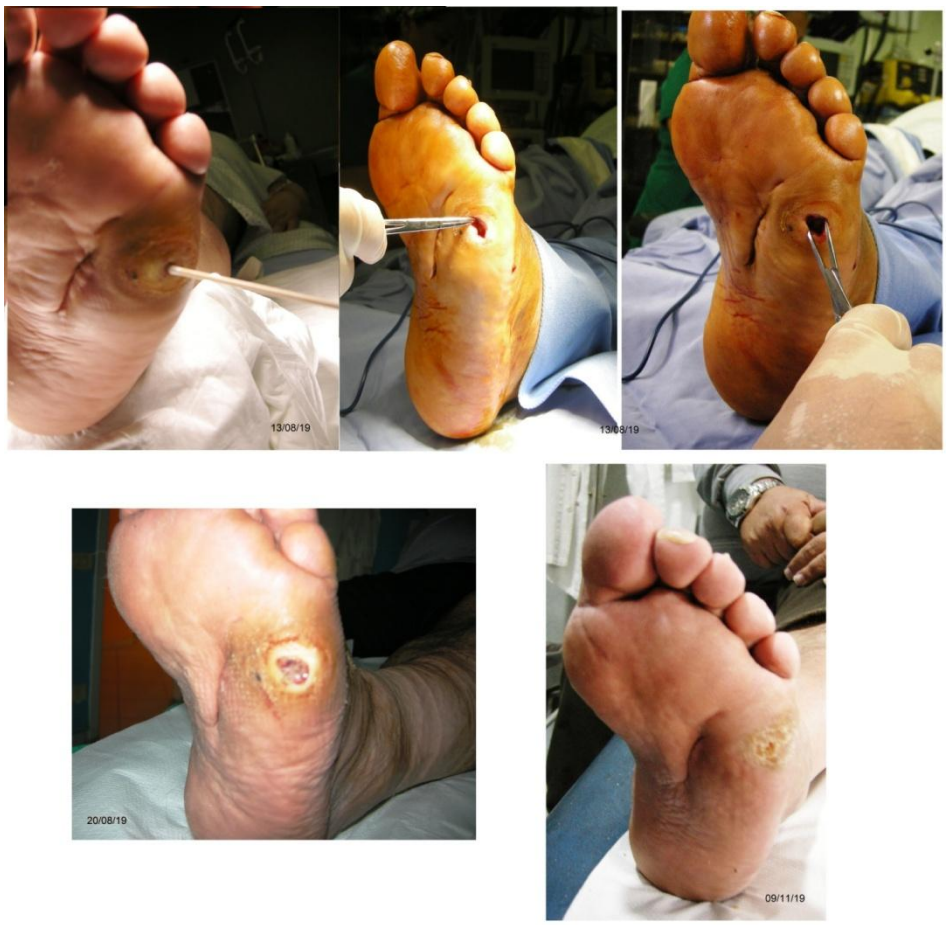

Fig. 3.12. Diabetic Ulcer patient with osteomyelitis for 2 years. Outcome after a single application of A-PRF as therapy.

3.6 CONCLUSION

According to the investigation, the Platelet rich-fibrin (PRF) is a second-generation platelet concentration, which involves in quick wound healing mechanism using influencing angiogenesis, immunity, and epithelial proliferation. Under a tissue designing perspective, it is fascinating to bring up that no examination venture concentrated on PRF quality, unbending nature, and versatility, despite its clinical utilization. The utilization of PRF shows a positive edge over customary medicines during the time spent on the recovery of sick tissue as they secrete different polypeptide development factors. In spite of the evidence of the clinical favorable position of these arrangements, proof of their gainful impacts is as yet deficient. Thus there is a necessity for further studies of their broad use. Thus, an intriguing future possibility is to more readily describe its biomaterial properties, and future research should concentrate on those elements that may improve further its qualities, for its different biomedical applications. Insignificance that future looks into, focusing on PRF use as co-adjuvant in delicate tissue regenerative treatments would configuration proper investigations, with the necessary controls, to additionally assess the regenerative capability of PRF in delicate tissue twisted healing, specifically concerning foot wound healing. The utilization of A-PRF in clinical practice demonstrated an extraordinary potential to improve healing and to improve careful results, since it fills in as an autologous framework, ready to have cells and bioactive mixes. Be that as it may, the antimicrobial capability of this material was illustrated, and it very well may be a significant property, which adds to clinically learned quickened and non-entangled healing occasions. Aftereffects of this amendment bring up that A-PRF appears, in any case, an antimicrobial movement against S. Aureus, S. mutans, Enterococcus faecalis and C. Albicans. Besides, range and power as antimicrobial agents are far not

exactly those of a built-up careful antimicrobial (explicit anti-microbial). It is vital, in this manner, future examinations that would include A-PRF and its subordinates to determine the whole range of its antimicrobial action in vitro, its interest in vivo, and the impact of patient's qualities on its organic movement. Additionally, the clinical potential as an organization vehicle for neighborhood sedates in contaminated destinations ought to be investigated. Future examinations should build quiet changeability and the example measurements for all HPC based investigations. Further clinical, histological and measurable investigations are required to appreciate the benefits of this new procedure. In any case, it's wouldn't be attainable to disregard that, once got from autologous blood tests, L-PRF and its derivatives have a diminished volume, and just a constrained amount can be utilized. This confines the methodical utilization of PRF in more prominent cutaneous sores. Regardless of whether there are abundant potential utilizations of PRF, profound information on this biomaterial working is required, just as information on its science, viability and cutoff points, to all the more likely enhance its utilization in ordinary practice.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Chapter 4
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Quantification of Platelets and Leucocytes in Blood Pletelet Concentrates

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4.1 INTRODUCTION

The multidisciplinary field of tissue engineering aims to repair, regenerate or restore damaged tissues in a predictable manner.

Developed by Choukroun (2001), PRF (platelet-rich fibrin) has a simple, fast, cheap and free access technique that results in the formation of a fibrin clot rich in platelets and trapped leukocytes. L-PRF differs from other forms of Platelet Concentrates (HPCs) because its production protocol exploits the coagulation properties inherent in whole blood without the need for biochemical changes. Therefore, it denies the use of additives such as anticoagulants, thrombin, calcium chloride or synthetic preservatives. The original PRF protocol requires venous blood to be taken from the patient and deposited in 10 ml dry glass tubes. The PRF clot is intrinsically charged with platelets, leukocytes and growth factors. These growth factors begin to free themselves from the PRF matrix within 5-10 minutes of clot formation and continue to be released for 60-100 hours [1]. Schär et al. [2] showed a constant release of growth factors and active stem cells for a period of 28 days. This indicates that the PRF clot is a reservoir of inactive and active cells capable of interacting with the cells and the molecules native to the site in which it is applied. A-PRF ™ (advanced PRF) has a relatively recent development based on a hypothesis which states that reducing the relative centrifugation force (force G), by reducing the centrifugation rate, would increase the number of leukocytes (in particular neutrophils and macrophages) within the PRF matrix [3,4].

Choukroun [5] states that A-PRF ™ was developed primarily in an attempt to include monocytes within the fibrin network because these cells play an essential role in bone growth, of vessels and in the production of two chemokines, that of the vascular endothelial growth factor (VEGF) and that of the bone morphogenic protein (BMP-2). Ghanaati et al. [3] studied this concept and found that A-PRF ™ contains significantly more neutrophil granulocytes and their wider distribution in the fibrin clot than L-PRF. The group also found that, histologically, platelet distribution is more homogeneous throughout the A-PRF ™ clot than the L-PRF clot. It has also been shown that A-PRF ™ releases significantly greater total amounts of growth factors than L-PRF prepared at 2700 rpm (325G) for 12 minutes [4]. However, the A-PRF ™ processing protocol seems to have evolved within the literature. Previous studies show a centrifugation protocol of 1500 rpm (252G) for 14 minutes [3,4], while more recent studies have used a spin speed of 1300 rpm (189G) for 14 minutes for the production of A-PRF ™ and 1300 rpm (189G) for 8 minutes to produce A-PRF +. The current A-PRF ™ processing protocol involves the use of a new pre-programmed centrifuge (PRF DUO, PROCESS © for PRF, Nice, France), a standardized blood collection kit and a patented 10 ml vacuum tube of glass. The DUO centrifuge is able to produce the classic L-PRF, A-PRF ™ and lastly i-PRF ™ (injectable PRF ™).

Among most clinicians who study tissue regenerative therapy, it has generally been accepted that platelets are highly concentrated in buffy coat and are hardly present in other nearby fractions, particularly in the red blood cell (RBC) fraction, after fractionation through centrifugation.

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This misunderstanding does not apply to the evaluation of the efficacy of platelet concentrations in liquid samples, which can be quantified with a simple blood count, but is especially extended to the evaluation of platelet counts in self-compressed platelet concentrates [(A-PRF, L-PRF and concentrated growth factors (CSFG)]. Because platelets have no nuclei, their count cannot be determined through DNA content. Therefore, to determine the platelet count in fibrin clots, a calculation is applied with the "subtraction method" (Dohan Ehrenfest, DM et al. [6] Crisci A. et al. [7]) or "simulation" [8] (Fig. 4.1).

According to the subtraction method, the platelet count contained in fibrin clots is carried out by subtracting the number of platelets contained in the clot exudate (Surface post-compression), in the supernatant serum (PPP) and in the red blood cell fraction (i.e. the thrombus red) from those present in the initial whole blood sample. However, this method does not consider the possibility of the presence of platelets in the RBC fraction or the possible loss and damage of platelets during processing for cell counting.

To facilitate the carrying out of individual quality inspections, Kitamura Y. et al. [9] developed a method to directly determine the platelet count in an insoluble PRF matrix using the tissue-plasminogen activator digestion procedure (t-PA Alteplase) (GRTPA®; Mitsubishi Tanabe Pharma Corp., Osaka, Japan) (Fig. 4.2). However, with this method, it is difficult to evaluate individual PRF matrices in a timely manner in a clinical setting. On the contrary, however, an effective standardization of preparation and practical application while not guaranteeing the quality of the individual PRF matrices is expected to effectively minimize variability and maximize efficacy in the same blood samples, consistent with other types of protocols of platelet concentration. The main advantages of the method proposed by Kitamura et al. [9] they are: (1) high precision, (2) simple procedure, (3) non-technical skill and (4) no limitation to the types of fibrin matrix, while the main disadvantages are: (1) long completion times, (2) reagent cost (t-PA), (3) incubator requirements and (4) additional tubes required for growth factor analysis.

Therefore, in this study the authors, starting from the results obtained in the work of Kitamura, wanted to elaborate a simpler and inexpensive system to calculate the precise number of platelets and leukocytes present in the PRF, compared to that present in whole blood, starting from either the subtraction method that is from a simple blood count.

Leukocytes (WBC)		Dohan e al.(2010)	%	Crisci e al. (J. Unexplored Med. Data 2017)*	%	Watanabe e al. (MDPI 2017) A-PRF	%	Kitamura e al. (2018)	%
	WHOLE BLOODSHED	69 x 10^2/µl	100,00	51,5 x 10^2/µl	100,00	45,0 x 10^2/µl	100,00	36,22 x 10^2/µl	100,00
	RED CLOT	35 x 10^2/µl	50,7	0,085 x 10^2/µl	0,16	1,0 x 10^2/µl	2,22	n.r.	n.r.
	PRF	33,7 x 10^2/µl	48,8	51,1 x 10^2/µl	99,24	580,0 x 10^2/µl	?	26,73 x 10^2/µl	73,8
	SERUM OVER (PPP) CLOT	n.r.	n.r.	0,0002 x 10^2/µl	0,00	n.r.	n.r.	n.r.	n.r.
	SURFACE post compression	n.r.	n.r.	0,3 x 10^2/µl	0,58	1,5 x 10^2/µl	3,33	n.r.	n.r.
Red Blood Cells (RBC)									
	WHOLE BLOODSHED	5,19 x 10^6/µl	100,00	7,65x 10^6/µl	100,00				
	RED CLOT	5,8 x 10^6/µl	111,7	7,39 x 10^6/µl	96,6				
	PRF	0 x 10^6/µl	0,0	0,216 x 10^6/µl	0,0028				
	SERUM OVER (PPP) CLOT	n.r.	n.r.	0,002 x 10^6/µl	0,00				
	SURFACE post compression	n.r.	n.r.	0,013 x 10^6/µl	0,16				
Platelets (PLT)									
	WHOLE BLOODSHED	2,66 x 10^5/µl (266.000)	100,00	1,06 x 10^5/µl (106.780)	100,00	0,25 x 10^5/µl (25.000)	100,00	1,524 x 10^5/µl (152.400)	100,00
	RED CLOT	0,06 x 10^5/µl (6.000)	0,02	0,005 x 10^5/µl (500)	0,47	0,00 x 10^5/µl (000)	0,0	n.r.	n.r.
	PRF	2,6 x 10^5/µl (260.000)	97,7	1,05 x 10^5/µl (105.801)	99,00	3,2 x 10^5/µl (320.000)	?	1,27 x 10^5/µl (127.000)	83,3
	SERUM OVER (PPP) CLOT	n.r.	n.r.	0,0002 x 10^5/µl (2)	0,002	n.r.	n.r.	n.r.	n.r.
	SURFACE post compression	n.r.	n.r.	0,0048 x 10^5/µl (479)	0,45	0,00 x 10^5/µl (000)	0,0	n.r.	n.r.
Methods Used		Subtraction methods				Simulation method		Method for t-PA counting	

n.r.= not reported; *= values in the horse;

Fig. 4.1. Comparison of platelet, leukocyte and erythrocyte counts with various methods

4.2 MATERIALS AND METHODS

Starting from the study by Kitamura Y. et al. [9] (Fig. 4.2), the A.A. they wanted:

- Evaluates the percentage deviation between the t-PA count and the PLT and WBC values obtained with the "subtraction method", for which adding or subtracting the calculated percentage difference, to the value obtained with the subtraction method, we hypothesize to obtain the value derived with the t-PA method.

The procedure for counting with "subtraction method" is performed as described by Watanabe et al. [8] according to the equation:

> *PLT/WBC in A-PRF and L-PRF = PLT/WBC in whole blood - [(PLT/WBC in the red clot) - (PLT/WBC in serum over the PRF clot) - (PLT / WBC in the supernatant after compression of the coagulant PRF)]*

- Evaluate the percentage deviation between the t-PA count and the PLT and WBC values obtained with a blood count (WB), for which adding or subtracting the calculated percentage difference, to the value obtained with a blood count, it is possible to have the value obtained with t-PA method.

The basic blood count is performed using test tubes with EDTA K3E 5.4 mg (VacuMed) and conducted with a Cell Dyn 3500 R (Abbott) Cell Counter.

The validity and precision of the measurements was evaluated through the coefficient of variation, considering the values valid if it is <2% on at least three measurements.

The zero hypothesis ($H_{\varnothing}$) formulated is that there is no statistically significant difference between the values measured with the t-PA method and the values with the "subtraction method" ± X% and between the values measured with the t-PA method and the measured values with the "blood count method" (WB) ± X%, both for PLT and WBC.

Formally $H_{\varnothing}$: t-PA = Met.Sottr. (PLT) (WBC) ± X%; t-PA = W.B. (PLT) (WBC) ± X%;

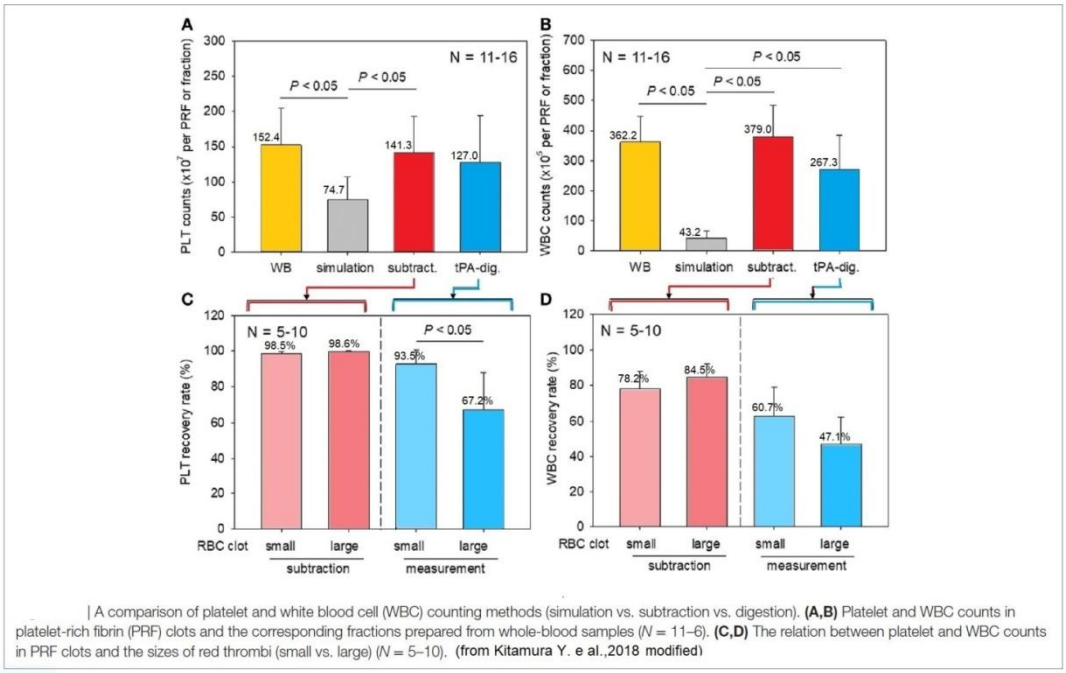
The relationship between the variables was evaluated by calculating the Pearson-Bravis Correlation Coefficient between t-PA and Met.Sottr. ± X% and between t-PA and W.B. ± X%, both for PLT and WBC, in the PRF.

The degree of agreement observed in the Correlation Coefficient was generally classified as: moderate if the coefficient r is between 0.65 and 0.8, good if the value is between 0.8 and 0.9 and optimal if it is> 0.9. A 95% confidence interval is used as a range of statistical significance.

To verify the zero hypothesis ($H_{\varnothing}$) the criterion Variance test (ANOVA) between t-PA and Met.Sottr is used. ± X% and between t-PA and W.B. ± X% for PLT and WBC in L-PRF and in other types of Platelet Concentrate (PC), then two other significance tests were used: the parametric Student's t-test and the Chi-square (χ^2).

The raw data detected with the t-PA counting method were provided by the Niigata University working group, Japan. From these we proceeded to the Statistical Analysis and to the Comparisons with the "method by subtraction" and with the "method by haemochrome" [9].

This calculation will justify statistically using Met.Sottr. ± X% and W.B. ± X% instead of t-PA in the evaluation of platelet and leukocyte counts in L-PRF and in various types of solid HPC, but with differences that is statistically significant between them ($p \leq 0.000$).



| A comparison of platelet and white blood cell (WBC) counting methods (simulation vs. subtraction vs. digestion). **(A,B)** Platelet and WBC counts in platelet-rich fibrin (PRF) clots and the corresponding fractions prepared from whole-blood samples (*N* = 11–6). **(C,D)** The relation between platelet and WBC counts in PRF clots and the sizes of red thrombi (small vs. large) (*N* = 5–10). (from Kitamura Y. e al.,2018 modified)

Fig. 4.2.

4.2.1 Statistical Analysis

The results were calculated as Mean ± Standard Deviation (SD) and evaluated for significant differences at any time with the one-way variance analysis (ANOVA), the Student parametric test and the χ^2, using the Statistics software for Disciplines Biomedicals by Santon A. Glatz Ed. 2007 Version 6.0.

The differences were considered with p values lower than 0.05 (*) as significant and p values lower than 0.01 (**), 0.001 (***) and (p <0.0001 ****) as highly significant.

We also calculated the significance with the Wilcoxon and Friedman tests when there were results with Low Correlation to the Pearson-Bravis test.

4.3 RESULTS

The data provided by the Division of Oral Bioengineering, Institute of Medicine and Dentistry, Niigata University, Japan, are reported in Tables 4.1 and 4.2 and were statistically analyzed. The percentage deviation between the values obtained from the blood count (W.B.) and those of the same patient obtained with t-PA digestion was calculated. The value obtained for PLT is an average percentage deviation equal to -10.85 ± 26.01%. Since the results of the t-PA values for some were higher than the value of W.B., these values (in red) were excluded from a second calculation for the percentage deviation, obtaining an average deviation of -27.30 ± 15.82% (?).

The value obtained for WBC is an average percentage deviation of -22.74 ± 30.73%. Since, also in this case, for some values of t-PA higher than the value of W.B., these values (in red) have been excluded from the calculation of the percentage deviation obtaining an average deviation of -38.82 ± 16.03% (?).

We carried out the comparison between t-PA and W.B.-10.85% and W.B.-27.3% for PLT and between t-PA and W.B.-27.74% and W.B.-38.82% for WBC, carrying out Pearson-Bravis Correlation Coefficients.

Table 4.1. Method for counting with t-PA digestion. Prior to coagulation, PLT and WBC counts were detected directly (with blood count). After coagulation, PLT and WBC counts were determined with t-PA digestion. The second calculation derived from the hemochrome (W.B.) was performed by eliminating the values in red that are positive values with respect to W.B. (Data derived from the study by Kitamura Y et al., 2018)

PLT conta	**W.B. ematico**	**t-PA-digerito**	**% di deviazione**	**W.B.-10,85±26.01%**	**W.B. -27,30±15.82%**	**WBC conta**	**W.B. ematico**	**t-PA-digerito**	**% di deviazione**	**W.B.-22,74±30.73%**	**W.B. -38,82±16.03%**
x10^7/CGF	157.48	108.60	31.04	140.39	114.48	x10^5/CGF	355.60	136.50	61.61	274.73	217.45
	89.27	107.10	16.65	79.58			331.80	242.60	26.88	256.35	202.89
	100.80	91.10	9.62	89.86	73.28		400.00	314.00	21.50	309.04	244.60
	116.80	90.20	22.77	104.12	84.91		376.00	310.00	17.55	290.49	229.92
	261.63	132.80	49.24	233.24	190.20		583.20	312.00	46.50	450.58	356.62
	152.60	81.70	46.46	136.04	110.94		287.00	184.00	35.89	221.73	175.50
	141.75	126.20	10.97	126.37	103.05		249.80	112.20	55.08	192.99	152.75
	190.76	217.80	12.42	170.06			342.00	431.80	20.80	264.22	
	197.34	251.10	21.41	175.92			343.20	451.50	23.99	265.15	
	96.60	76.30	21.01	86.11	70.23		378.00	205.00	45.77	292.04	231.14
	171.00	217.10	21.23	152.45			337.50	401.30	15.90	260.75	
media	152.37	136.36	-10.85	135.83	106.73	media	362.19	281.90	-22.74	279.82	
t-test Student I.C.95%		p>0.05 da -35 a -67.01 p=0.52		p>0.05 da -49.3 a -48.3 p=0.98	p>0.05 da -43.9 a -32.4 p=0.75	t-test Student I.C.95%		p>0.05 da -2.9 a -163.6 p=0.057		p>0.05 da -81.8 a -85.9 p=0.96	p>0.05 da -76.0 a -77.4 p=0.985
χ^2		85.54 p=0.000		81.25 p=1.000	18.44 p=0.005	χ^2		261.08 p=0.000		231.40 p=0.000	53.49 p=0.000
ANOVA				4.128 P=0.97	115.4 P=0.75	ANOVA				23.69 P=0.96	1.84 P=0.98

Comparing the t-PA method and the blood count -% deviation for PLT, we have obtained that by subtracting 10.85% from the values of W.B. we have: t-Student test = 0.98 (Statistically Not Significant Difference) (NS); χ^2 = 1.000; ANOVA: 4.128 p = 0.97 (NS); Correlation Coefficient: r = 0.573; p = 0.065; which means Low Concordance between the values with Difference St.N.S.) *.

By subtracting 27.3% from W.B.: t-Student = 0.75 (NS); χ^2 = 0.005 (Statistically Significant Difference) (S); ANOVA: 115.4 p = 0.75 (NS);

Correlation Coefficient: r = 0.745; p = 0.054; which means Moderate Concordance between values (N.S.) *.

However, if we assume a significance of 0.001 ***, the difference values between t-PA and W.B.-27.3% are all statistically not significant. Therefore we can use the value of W.B. of PLT -27.3% to obtain the count values for t-PA with sufficient statistical appropriateness.

Comparing the t-PA method and the blood count -% deviation for WBC, we have obtained that by subtracting 22.74% from the WB values: t-Student = 0.96 (NS); χ^2 = 0.000 (S); ANOVA: 23.69 p = 0.96 (NS); Correlation Coefficient: r = 0.269; p = 0.423; which means Low Concordance between values (NS) *.

Instead, subtracting 38.82% from WB values we have: t-Student = 0.98 (NS); χ^2 = 0.000 (NS); ANOVA: 1.84 p = 0.98 (NS);

Correlation Coefficient: r = 0.695; p = 0.055; which means Moderate Concordance between values (NS) *.

If we assume a significance of 0.05 *, the difference values between t-PA and W.B.-38.82% are not Significant with a Moderate Concordance. Therefore we can use the value of W.B. of WBC -38.82% to obtain the count values for t-PA with sufficient statistical appropriateness.

The Wilcoxon pair examination between t-PA and W.B.-38.82% is W = -2.0 p> 0.054 (NS); Friedman test p = 1,000 (NS);

Other tests for t-PA, W.B. counts were subsequently performed on the same patients (Kitamura et al. 2018) [9] and with the Subtraction Method, which are reported in Tables 4.2A and B.

Table 4.2. Count of Platelets and Leukocytes with "Method of counting with t-PA digestion" and "with Method of Subtraction". Before coagulation, the counts of PLT (A) and WBC (B) were detected directly (with blood count). After coagulation, PLT and WBC counts were determined by t-PA digestion and the Subtraction Method according to Watanabe et al. (2017) and Crisci e al (2017). The second calculation was performed by eliminating the values in red that are positive values compared to W.B. (Data derived from the study by Kitamura Y et al., 2018)

A

PLT count	W.B. blood	t-PA-digested	Method Subtract	% deviation between W.B. and Sottr..	W.B. -20,43± 21.44%	W.B. -25,09± 20.42%	% deviation between t-PA and Sottr.	Met.Sub. -19,69± 21.88%	Met.Sub. -24,31±21.02%
x10^7/CGF	155.00	126,20	154.50	18.58	123.33	116.11	18.32	124.07	116.90
	132.50	108.60	130.90	18.04	105.40	99.25	17.04	105.10	99.07
	117.80	107.10	112.40	9.08	93.73	88.24	4.72	90.26	85.07
	228.40	217.10	227.90	4.95	173.30	171.09	4.74	183.03	172.49
	245.60	217.80	245.60	11.32	195.42	183.97	11.32	196.72	185.89
	248.50	251.10	247.60	1.04	197.73		1.39	198.32	
	126.70	132.80	125.50	4.59	100.81		5.50	100.52	
	104.60	91.10	103.00	12.91	83.23	78.35	11.55	82.50	77.96
	100.70	81.70	100.00	18.87	80.12	75.44	18.30	80.10	75.69
	108.20	34.10	108.20	68.48	86.09	81.05	68.48	86.66	81.89
	136.60	76.30	136.60	44.14	108.69	102.33	44.14	109.41	103.39
	144.90	80.40	144.90	44.51	115.29	108.54	44.51	116.06	109.67

PLT count	**W.B. blood**	**t-PA-digested**	**Method Subtract**	**% deviation between W.B. and Sottr..**	**W.B. -20,43± 21.44%**	**W.B. -25,09± 20.42%**	**% deviation between t-PA and Sottr.**	**Met.Sub. -19,69± 21.88%**	**Met.Sub. -24,31±21.02%**
mean	154.13	127.03	153.09	-20.43	121.93	110.44	-19.69	122.73	110.80
t-test Student		p>0.05	p>0.05	p>0.05	p>0.05	p>0.05		p>0.05	p>0.05
I.C.95%		da -24.7 a -78.9 p=0.29	da -45.6 a -47.7 p=0.96	da -78.0 a -25.9 p=0.31	da -42.4 a -42.6 p=0.826	da -43.4 a -50.6 p=0.874		da -43.7 a -52.4 p=0.85	da -44.1 a -50.5 p=0.88
χ^2		54.14 p=0.000	0.118 p=1.000	55.29 p=0.000	51.345 p=0.000	38.731 p=0.000		50.972 p=0.000	40.024 p=0.000
ANOVA					155.9 P=0.83	64.91 P=0.87		110.7 P=0.85	52.4 P=0.89

B

WBC count	**W.B. blood**	**t-PA-digested**	**Method Subtract**	**% deviation between W.B. and Sottr.**	**W.B. -45,9±16.67%**	**% deviation between t-PA and Sottr.**	**Met.Sub. -34,35±16.67%**
x10^5/CGF	350.0	112.20	251.20	67.94	189.35	55.33	164.91
	426.3	136.50	272.30	67.98	230.60	49.87	178.76
	380.0	242.60	271.00	36.16	205.58	10.48	177.91
	667.3	401.30	575.50	39.86	361.00	30.27	377.81
	616.0	431.80	559.70	29.90	333.25	22.85	367.44
	592.8	451.50	489.60	23.84	320.70	7.78	321.42
	563.2	312.00	458.80	44.60	304.69	32.00	301.20
	410.8	314.00	389.60	23.56	222.24	19.40	255.77
	420.8	184.00	319.30	56.27	227.65	42.37	209.62
	385.4	126.50	334.30	67.18	208.50	62.16	219.46
	469.2	205.00	404.40	56.31	253.84	49.31	265.48
	462.0	290.40	417.20	37.14	157.11	30.39	273.89
mean	478.65	267.32	395.24	-45.90	251.21	-34.35	259.47
t-test Student		p>0.05	p>0.05		p>0.05	p>0.05	p>0.05
I.C.95%		fro -306.3 a -116.4 p=0.00	da -174.5 a -7.659 p=0.071		da -64.8 a -97.0 p=0.68	da 30.73 a -225.1 p=0.012	da -75.6 a -91.3 p=0.84
χ^2		165.82 p=0.000	28.343 p=0.003		163.44 p=0.000	110.824 p=0.000	93.09 p=0.000
ANOVA					3344.0 P=0.59		1413.0 P=0.73

Comparing the t-PA method and the blood count -% deviation for PLT, we obtained that by subtracting 20.43 ± 21.44% from WB values we find: t-Student = 0.83 (NS); χ^2 = 0.000 (S); ANOVA: 155.9 p = 0.83 (NS);

Correlation Coefficient: r = 0.92; p = 0.000; which means Excellent Concordance (S) **** (Table 4.2A).

By comparing, instead, the t-PA method and the Subtraction Method -% of deviation for PLT, we have obtained that reducing by 19.69 ± 21.88% the values obtained with Subtraction Met. We have: t-Student = 0.85 (NS); χ^2 = 0.000 (S); ANOVA: 110.7 p = 0.85 (NS); Correlation Coefficient: r = 0.92; p = 0.000; which means an Excellent Concordance (S) **** (Table 4.2A) (Figs. 4.3, 4.4). For the calculation of PLT the differences between t-PA and W.B.-20.43% and t-PA and Met.Sottr.-19.69% are the same. In all cases the t-test and ANOVA demonstrate statistically non-significant differences, while χ^2 shows statistically significant differences together with the correlation coefficient. However, if we assume a significance of 0.0001 **** the difference values are not significant with an Excellent Concordance.

Since values of t-PA were found for some higher than the value of W.B., these values (in red) were excluded from the calculation of the percentage deviation obtaining an average deviation of -25.09 ± 20.42%.

Comparing the t-PA method and the blood count -% deviation for PLT, we have obtained that by subtracting the 25.09 ± 20.42% to the WB values: t-Student = 0.874 (NS); χ^2 = 0.000 (S); ANOVA: 64.91 p = 0.87 (NS); Correlation Coefficient: r = 0.94; p = 0.000; which means Excellent Concordance (S) **** (Table 4.2A).

Comparing, instead, the t-PA method and the Subtraction Method -% of deviation for PLT, we have obtained that by reducing by 24.31 ± 21.02% to the values of the Subtraction Method: t-Student = 0.88 (NS); χ^2 = 0.000 (S); ANOVA: 52.4 p = 0.89 (NS); Correlation Coefficient: r = 0.91; p = 0.000; which means Excellent Concordance (S) **** (Table 4.2A). For the calculation of PLT the differences between t-PA and W.B.-25.09% and t-PA and Met.Sottr.-24.31% are the same. In all cases the t-test and ANOVA demonstrate statistically non-significant differences, while χ^2 shows statistically significant differences together with the correlation coefficient. However, if we assume a significance of 0.0001 **** the difference values are not significant with an Excellent Concordance.

Furthermore, comparing the t-PA method and the blood count -% deviation for WBC, we have obtained that by subtracting 45.9 ± 16.67% from the WB values: t-Student = 0.68 (NS); χ^2 = 0.000 (S); ANOVA: 3344.0 p = 0.59 (NS); Correlation Coefficient: r = 0.73; p = 0.007; which means Moderate Concordance (S) *** (Table 4.2B).

Comparing the t-PA method and the Subtraction Method -% deviation for WBC, we have obtained that by reducing by 34.35 ± 16.67% to the values of the Subtraction Method: t-Student = 0.84 (NS); χ^2 = 0.000 (S); ANOVA: 1413.0 p = 0.73 (NS); Correlation Coefficient: r = 0.88; p = 0.000; which means Good Concordance (S) **** (Tables 4.2B, 3) (Figs. 4.3, 4.4). For the calculation of WBC the differences between t-PA and W.B.-45.9% and t-PA and Met.Sottr.-34.3% are the same. In all cases the t-test and ANOVA demonstrate statistically non-significant differences, while χ^2 shows statistically significant differences together with the correlation coefficient. However, if we assume a significance of 0.001 *** the difference values between t-PA and WB-45.9% for WBC are not Significant with a Moderate Concordance. If, instead, we assume a significance of 0.0001 **** the values of difference between t-PA and subtraction-34.35% for WBC are Not Significant with a Good Concordance.

For this comparison the Wilcoxon pair examination between t-PA and W.B.-45.9% was W = 16.0 p> 0.052 (NS); Friedman test p = 0.564 (NS);

Subsequently we added the values obtained in Tables 4.1, 4.2A and 4.2B exclusively for the comparison values between t-PA and W.B. and the results obtained would seem to be the most valid Statistically (t-PA and WB-15.12 ± 24.87% for PLT and between t-PA and WB-34.12 ± 28.2% for WBC (Table 4.3).

Comparing the t-PA method and the blood count -% deviation for PLT (Table 4.3), we obtained that by subtracting 15.12% from the WB values: t-Student = 0.93 (NS); χ^2 = 0.000; (S); ANOVA: 10.45 p = 0.953 (NS) *; Correlation Coefficient: r = 0.766; p = 0.000, with Good Concordance between the values (S) (Figs. 4.3, 4.4). The t-test and ANOVA demonstrate statistically non-significant differences, while χ^2 demonstrates statistically significant differences together with the correlation coefficient. If we assume a significance of 0.0001 **** the difference values between t-PA and W.B.-15.12% are all not Significant. This means that we can use the WB value of PLT - 15.12% to obtain the count values for t-PA with sufficient statistical accuracy.

Comparing the t-PA method and the blood count -% deviation for WBC (Table 4.3), we obtained that by subtracting 34.12% from the WB values: t-Student = 0.88 (NS); χ^2 = 0.000; (S); ANOVA: 217.6 p = 0.88 (NS) *; Correlation Coefficient: r = 0.47; p = 0.022; which means Poor Concordance between values (S). The t-test and ANOVA and the Correlation Coefficient demonstrate statistically non-significant differences, while χ^2 demonstrates statistically significant differences. If we assume a significance of 0.0001 **** the difference values between t-PA and W.B.-34.12% are all not Significant. Since the comparison between the t-PA and WB-34.12% methods for WBC has a Low Correlation Coefficient (r = 0.47; p = 0.022) with a Statistically Significant Difference (Figs. 4.3, 4.4), we proceeded to calculate the significance with Wilcoxon tests and Friedman between the values of t-PA and WB-34.12%, WB-45.9% and WB-38.82%.

Based on the comparison with the Wilcoxon and Friedman tests, the calculation of W.B.-34.12% for WBC is considered valid.

From this we can deduce that we can use the WB value of WBC- 34.12% to obtain the count values for t-PA with sufficient statistical accuracy (Table 4.4, 4.5).

Table 4.3. Comparison between method for counting with t-PA and CBC digestion for WBC and PLT

WBC count x10^5/CGF					PLT count x10^7/CGF			
t-PA-digested	**W.B. blood**	**W.B-t-PA**	**W.B. -34,12±28.2%**		**t-PA-digested**	**W.B. blood**	**W.B-t-PA**	**W.B. -15,12±24.87%**
136.50	355.60	61.61	234.27		108.60	157.48	31.04	133.67
242.60	331.80	26.88	218.59		107.10	89.27	-19.97	75.77
314.00	400.00	21.50	263.52		91.10	100.80	9.62	85.56
310.00	376.00	17.55	247.71		90.20	116.80	22.77	99.14
312.00	583.20	46.50	384.21		132.80	261.63	49.24	222.07
184.00	287.00	35.89	189.08		81.70	152.60	46.46	129.53
112.20	249.80	55.08	164.57		126.20	141.75	10.97	120.32
431.80	342.00	-26.26	225.31		217.80	190.76	-14.17	161.92
451.50	343.20	-31.56	226.10		251.10	197.34	-27.24	167.50
205.00	378.00	45.77	249.03		76.30	96.60	21.01	81.99
401.30	337.50	-18.90	222.35		217.10	171.00	-26.96	145.14
112.20	350.00	67.94	230.58		126.20	155.00	18.58	131.56
136.50	426.30	67.98	280.85		108.60	132.50	18.04	112.47
242.60	380.00	36.16	250.34		107.10	117.80	9.08	99.99
401.30	667.30	39.86	439.62		217.10	228.40	4.95	193.87
431.80	616.00	29.90	405.82		217.80	245.60	11.32	208.47
451.50	592.80	23.84	390.54		251.10	248.50	-1.05	210.93
312.00	563.20	44.60	371.04		132.80	126.70	-4.81	107.54
314.00	410.80	23.56	270.64		91.10	104.60	12.91	88.78
184.00	420.80	56.27	277.22		81.70	100.70	18.87	85.47
126.50	385.40	67.18	253.90		34.10	108.20	68.48	91.84
205.00	469.20	56.31	309.11		76.30	136.60	44.14	115.95
290.40	462.00	37.14	304.37		80.40	144.90	44.51	122.99
274.29	422.95	34.12	278.64	Mean	131.49	153.28	15.12	130.11
115.31	110.75	28.20	72.96	±D.S	63.45	52.26	24.87	44.36

Table 4. Significance Tests. Confidence Interval:95% for PLT; Note: Values shown in gray are those that are statistically significant.

Statistically Significant Differences					* Second Measurement			
	t-test	***χ2***	**Interval Confidence at 95%**		**ANOVA**			**D.S.**
per W.B. e t-PA	p>0,05	85,54	p=0,000	da -35 a -67,01	p=0,52			
per t-PA e W.B.-10,85%	p>0,05	81,25	p=1,000	da -49,35 a -48,28	p=0,98	4.128	p=0,97	±26,01%
per t-PA e W.B.-27,30%	p>0,05	18,44	p=0,005	da -43,91 a -32,42	p=0,005	115,4	p=0,75	±15,82%
per W.B. e t-PA*	p>0,05	54,14	p=0,000	da -24,71 a-78,91	p=0,29			
per t-PA e W.B.-20,43%	p>0,05	51,34	p=0,000	da -42,42 a-52,61	p=0,83	155,9	p=0,83	±21,44%
per t-PA e W.B.-25,09%	p>0,05	38,73	p=0,000	da -43,4 a-50,61	p=0,87	64,91	p=0,87	±20,42%
per t-PA e Sottr.-19,66%	p>0,05	50,97	p=0,000	da -43,76 a-52,36	p=0,85	110,7	p=0,85	±21,88%
per t-PA e Sottr.-24,31%	p>0,05	40,02	p=0,000	da -44,06 a-50,54	p=0,85	52,4	p=0,89	±21,02%
per W.B. e Sottr.	p>0,05	0,118	p=1,000	da -45,63 a-47,69	p=0,85			
per t-PA e Sottr.	p>0,05	40,02	p=0,000	da -78,05 a -25,91	p=0,31			
Per t-PA e W.B.-15,12%	p>0,05	133,01	p=0,000	da -31,14 a -33,92	p=0,93	10,45	p=0,953	±24,87%

Table 5 Significance Tests. Confidence Interval:95% for WBC; Note: Values shown in gray are those that are statistically significant.

Statistically Significant Differences						* Second Measurement		
	t-test	$\chi 2$		Interval Confidence at 95%		ANOVA		D.S.
per W.B. e t-PA	p>0,05	261,08	p=0,000	da -2,99 a-163,6	p=0,057			
per t-PA e W.B.-22,74%	p>0,05	231,40	p=0,000	da -81,79 a-85,94	p=0,98	23,69	p=0,96	±30,73%
per t-PA e W.B.-38,82%	p>0,05	53,49	p=0,000	da -76,01 a -77,37	p=0,005	1,84	p=0,98	±16,03%
per W.B. e t-PA*	p>0,05	165,82	p=0,000	da -306,3 a-116,4	p=0,29			
per t-PA e W.B.-45,90%	p>0,05	163,44	p=0,000	da -64,81 a -97,03	p=0,00	3344,0	p=0,59	±16,67%
per t-PA e Sottr.-34,35%	p>0,05	93,09	p=0,000	da -75,61 a -91,3	p=0,87	1413,0	p=0,73	±16,67%
per W.B. e Sottr.	p>0,05	28,343	p=0,003	da -174,5 a -7,659	p=0,071			
per t-PA e Sottr.	p>0,05	110,82	p=0,000	da -30,73 a -225,1	p=0,012			
Per t-PA e W.B.-34,12%	p>0,05	439,73	p=0,000	da -61,69 a -52,99	p=0,88	217,6	p=0,88	±28,20%

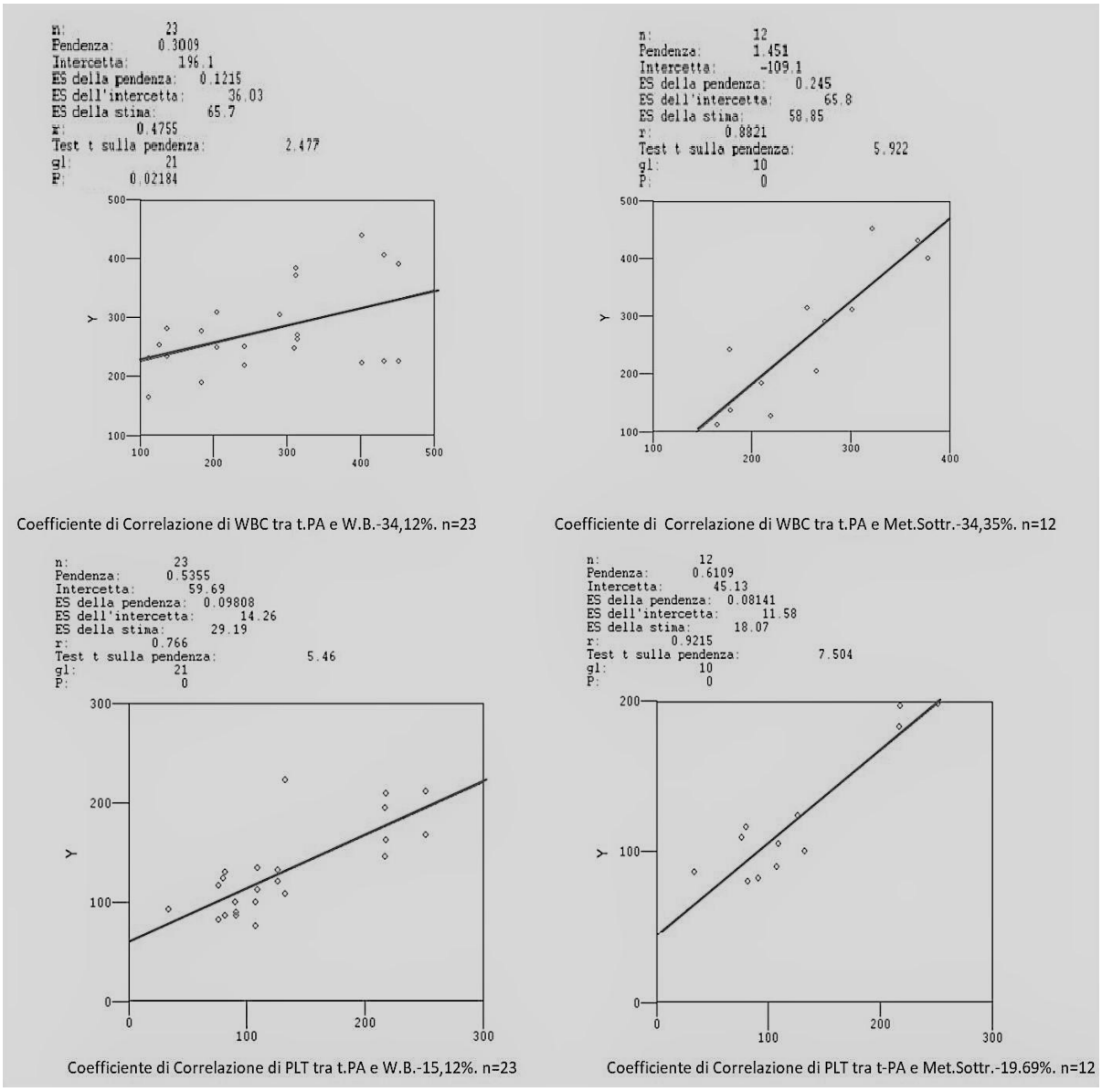


Fig. 3. PLT and WBC Correlation Coefficient between t-PA, W.B. and Subtraction Method

Leukocytes (WBC)		N°	%		N°	%		N°	%	
	WHOLE BLOODSHED	36,22 x 10^2/µl (3.622/mm^3)	100,00	Diff.% **-34,12%** t-test=0.88* χ^2=0.000 ANOVA=0.88* Coeff.Corr. r=0.47 p=0.022	47,86 x 10^2/µl (4.786/mm^3)	100,00	Diff.% **-34,35%** t-test=0.84* χ^2=0.000 ANOVA=0.73* Coeff.Corr. r=0.88 p=0.000	36,22 x 10^2/µl (3.622/mm^3)	100,00	Difference between PRF's with count for simulating and count from t-PA
	RED CLOT	n.r.	n.r.		n.r.	n.r.		n.r.	n.r.	
	PRF	26,73 x 10^2/µl (2.673/mm^3)	-26.2%		37,90x10^2/µl (3.790/mm^3)	-20,8%		4,32x 10^2/µl (432/mm^3)	-88,1%	
	SERUM OVER (PPP) CLOT	n.r.	n.r.		n.r.	n.r.		n.r.	n.r.	
	SURFACE post compression	n.r.	n.r.		n.r.	n.r.		n.r.	n.r.	
Red Blood Cells (RBC)							+29,47%			-83,83%
	WHOLE BLOODSHED	n.r.	n.r.		n.r.	n.r.		n.r.	n.r.	
	RED CLOT	n.r.	n.r.		n.r.	n.r.		n.r.	n.r.	
	PRF	n.r.	n.r.		n.r.	n.r.		n.r.	n.r.	
	SERUM OVER (PPP) CLOT	n.r.	n.r.		n.r.	n.r.		n.r.	n.r.	
	SURFACE post compression	n.r.	n.r.		n.r.	n.r.		n.r.	n.r.	
Platelets (PLT)										
	WHOLE BLOODSHED	1,524 x 10^5/µl (152.400/mm^3)	100,00	Diff.% **-15,12%** t-test=0.93* χ^2=0.000 ANOVA=0.95* Coeff.Corr. r=0.77 p=0.000	1,541 x 10^5/µl (154.100/mm^3)	100,00	Diff.% **-19,69%** t-test=0.85* χ^2=0.000 ANOVA=0.85* Coeff.Corr. r=0.92 p=0.000	1,524 x 10^5/µl (152.400/mm^3)	100,00	Difference between PRF's with count for simulating and count from t-PA
	RED CLOT	n.r.	n.r.		n.r.	n.r.		n.r.	n.r.	
	PRF	1,27 x 10^5/µl (127.000/mm^3)	-16,7%		1,413 x 10^5/µl (141.300/mm^3)	-8,3%		0,747 x 10^5/µl (74.700/mm^3)	-50,9%	
	SERUM OVER (PPP) CLOT	n.r.	n.r.		n.r.	n.r.		n.r.	n.r.	
	SURFACE post compression	n.r.	n.r.		n.r.	n.r.		n.r.	n.r.	
Methods Used		Method for t-PA counting			Subtraction methods		+10,12%	Simulation method		-41,2%

A value of P less than 0.05 was considered a Statistically Significant Difference; *p>0.05 Statistically Not Significant Difference;
n.r.= not reported;

Fig. 4. Results obtained between t-PA, Subtraction and Simulation counting methods

4. DISCUSSION

Platelet concentrates are safe, reliable and cost-effective means of accelerating tissue healing and improving tissue repair efficiency after injury.

The platelets, fibrin and leukocytes contained in them act naturally in synergy to promote wound healing and tissue regeneration. The concept of platelet concentrates for surgical use is to multiply this coagulation / regeneration effect on a surgical site or wound.

Despite the clinical use of PRF in the last 15 years, no research to date has managed to quantify the number of platelets and leukocytes present in this and other platelet concentrates in a direct, simple and effective way, in order to relate it to their regenerative potential. The presence of leukocytes also has a great impact on wound healing biology, not only because of their additional release of growth factors and their implications for antibacterial immune defense but also because it is the key regulators that control wound healing through the local regulation of growth factors. Future basic research should focus specifically on the contribution of these cells to specific cell knock-down/knock-in systems to determine the functional roles of each cell type in the wound healing process when PRF is used. Thus, in theory, the concept of developing new modified PRF protocols to further increase the number of white blood cells would lead, in principle, to increase wound repair. Nevertheless, a better understanding of the individual roles of the various cells found in PRF could prove to be an important discovery for the development of these technologies, which leads to modern changes in their protocols and to further increase their regenerative potential.

The number of platelets is a quantity, but it has been accepted as one of the main indexes to guarantee the quality of the platelets presents [10]. However, up until current studies, there is lack of a method for the accurate determination of platelet counts in gel types.

Platelet concentrates, including PRF and A-PRF, have been used, in fact, for regenerative procedures in various fields of medicine, including dentistry, reconstructive plastic surgery and dermatology, to provide supraphysiological concentrations of autologous growth factors directly to guest fabrics. These growth factors have been shown to be chemotactic for various cell types, including monocytes, fibroblasts, endothelial cells, stem cells and fibroblasts, creating tissue microenvironments and directly influencing the proliferation and differentiation of progenitor cells.

The beneficial effect of PRF membranes in complex wound healing can be explained by the high concentration of platelets and leukocytes along with long-term release of specific growth factors.

It is clear that platelet-rich fibrin clots form a bioactive reservoir. Therefore, a high hematocrit or a low number of platelets may be a limiting factor and this is why further research is needed to establish the optimal number of platelets for their clinical use. The cytokines produced by platelets and leukocytes are therefore an important part in the role of this biomaterial, but both the fibrin matrix and the elements contained in it are responsible for the real therapeutic enhancement of the PRF. Until now it was almost impossible to count and regulate the number of platelets in PRF preparations before their clinical use, so as to put it in relation to the result for each patient. Therefore, the most clinically effective way to control the quality of the results was to use the PRF region closest to the RBC clot.

Activated platelets release a full range of chemokines and promote the uptake, adhesion and proliferation of adult stem cells, including CD34-positive progenitor cells, MSCs, SMC progenitors, and endothelial progenitors. The leukocytes present in L-PRF are not only inflammatory cells, as they also present anti-nociceptive effects through different chemokines, anti-inflammatory cytokines (IL-4, IL-10, IL-13) and opioid peptides (β-endorphins, dimorphine -A etc.) and therefore can promote a clinically relevant inhibition also of pathological pain. The released amounts of VEGF and TGF-β1 are produced massively by leukocytes. A significant correlation between platelet number and release of TGF-β1 ($p = 0.005$) and PDGF-BB ($p = 0.04$) was detected.

Wend et al. [11] showed that a decrease in PRF centrifugation speed leads to a greater number of leukocytes, lymphocytes, neutrophils, monocytes and platelets in it, compared to the higher PRF

generated at RCF (Applied centrifugal force). Thus, the amount of growth factor and cytokine release from PRF matrices increases with decreasing RCF forces. The pro-angiogenic effect of PRF is also significantly greater when a low-RCF PRF is used compared to the PRF with high RCF and can be related to the presence of a large number of trapped extracellular neutrophils and neutrophils (NET) that are released.

The limitations, however, found in the clinical use are therefore:

1) Due to the fact that PRF is an autologous product, the availability of this biomaterial in greater quantity is difficult. Therefore, its use in surgical procedures must be well controlled.
2) The PRF possesses circulating immune cells and antigenic molecules that prevent its use as an allogeneic material, therefore, there is an increased risk of transmission of infectious agents.
3) The number of cells (platelets, leukocytes and stem cells) contained can be very variable.

At this point of our knowledge, among the important parameters to be taken into account we therefore have: the concentration of platelets, the concentration of leukocytes and the proportion between the various types of leukocytes. Regarding the concentration of platelets, leukocytes and their formula, their influence on the clinical effect of second-generation platelet concentrates, has yet to be studied carefully, as their more or less abundant presence can explain the contradictory results that were observed in the published works.

5. CONCLUSIONS

In this study it will be possible to deduce that, subtracting the value of the Platelet count by the subtraction method from the Emocrome value by 34.35%, and by 19.69% the value of the Leucocyte count by subtraction Method and by 34.12% value of the blood count, the value obtained with the method of digestion from t-PA will be obtained with a much simpler system. From this study it will therefore be possible to validate a simple and inexpensive system for calculating the precise number of platelets and leukocytes present in platelet concentrates. The present study has shown that it is indeed possible to quantify the number of cells in biomaterials, a complex cellular system due to the presence of platelets, leukocytes, stem cells etc., using a clinical method that can be applied quickly (max 15 minutes) and safely [12].

With this, it will be possible to evaluate the minimum/maximum level of platelets and leukocytes useful for having a clinical result in the use of Second Generation Platelet Concentrates.

Other experimental and clinical studies must be conducted to provide a better understanding of the cross-talk between the number of platelets, leukocytes and the mechanisms that control tissue repair, characterized by processes such as the recruitment of cells with regenerative potential and the regulation of apoptosis/cell survival.

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Chapter 5
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The Platelet-rich Fibrin Optimized with the Concept of Low Centrifugation Rate (LSCC): Growth Factor Release, Biocompatibility and Cellular Response

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5.1 INTRODUCTION

Centrifugation is a technique widely used to separate a biological mixture within a liquid phase. The principles of this technique are based on the use of centrifugal force, which is a force much more intense than gravity. During centrifugation, different forces interact and affect moving particles within the liquid, including centrifugal force, gravity force, and cell drag force. This process results in a migration of particles that depends on their size, density and mass. Because centrifugation depends on the weight and density of cells, a higher RCF (Relative Centrifugation Force) may be the reason for a sedimentation of the majority of platelets in the lower portion of the clot according to the formula of their density and size, as observed in L-PRF. The decrease in RCF allows platelets to separate from red blood cells and become evenly distributed within the fibrin network. The effectiveness of PRF clots with low platelet count and their uneven distribution may have less influence on clinical outcomes than clots with a uniformly distributed and enhanced platelet number because the applied clot may have uneven biological activity and thus reduced release of growth factors.

PRF-based matrices include various inflammatory cell matrices, such as platelets and leukocytes, in combination with various plasma proteins embedded in a fibrin network (Fig. 5.1) [1]. The components of PRF-based matrices are known to play an important role during the wound healing process. Platelets are the first cells found in the site of an injury. In addition to their role within hemostasis, platelets have an inflammatory potential, including the recruitment of additional inflammatory cells, such as neutrophils and macrophages, and the promotion of angiogenesis and tissue repair [2, 3]. In this context, platelets are able to express a number of biologically active signaling molecules and growth factors, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and the transforming growth factor beta (TGF-β). These growth factors are essential for vascularisation and the formation of new tissues [4]. In addition, platelets contain granules with cytokines, chemokines and other inflammatory mediators that are released after platelet aggregation to improve hemostasis and activate and recruit cells at the site of inflammation [5]. A systematic study conducted by Choukroun and Ghanaati (2017) demonstrated the influence of the reduction of relative centrifugation force (RCF), on the number of leukocytes and platelets, as well as their role in the release of growth factors in fluid PRF-based matrices (i-PRF) following the concept of low centrifugation rate (LSCC), this indicates that the reduction of the RCF value increases the number of cells and the release of growth factors within PRF-based matrices [6]. The distribution of platelets in PRF-based matrices, identified with immunohistochemical staining with CD-61 antibodies, was conducted by El Bagdadi and al (2017) [8] (Fig. 5.2) to determine the platelet distribution in cross-sections of three PRF-based matrices. Platelet distribution was evaluated in relation to the location in the clot. Platelets form accumulations within all three clots. The L-PRF, which was prepared with a high RCF (2400 rpm, 708 g, for 12 min) showed a different distribution pattern based on location. The upper (C) and central (B) portions of the clot showed only a few platelets, while most of the platelets were distributed at the bottom (A) of the L-PRF (Fig. 5.1).

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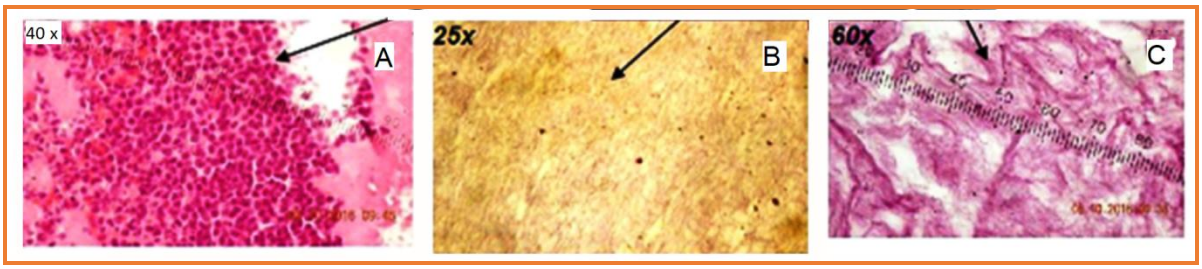

Fig. 5.1. Membrane L-PRF 0 min after compression (hematoxylin-eosin staining). (A) III proximal 60× fibrin on the right, the center lymphocytes, erythrocytes, and granulocytes neutrophils on the left; (B) average III 25× pattern of fibrin; (C) III distal 60× pattern fibrin (Crisci A. et al. 2017)[7]

In contrast, A-PRF+, which is prepared with a reduced RCF (1300 rpm, 208 g, for 8 min), has a different distribution scheme. The platelets are scattered all over the clot. A-PRF+ with a reduced RCF and reduced centrifugation time displayed a uniform platelet distribution pattern within the clot (Figs. 5.2, 5.3).

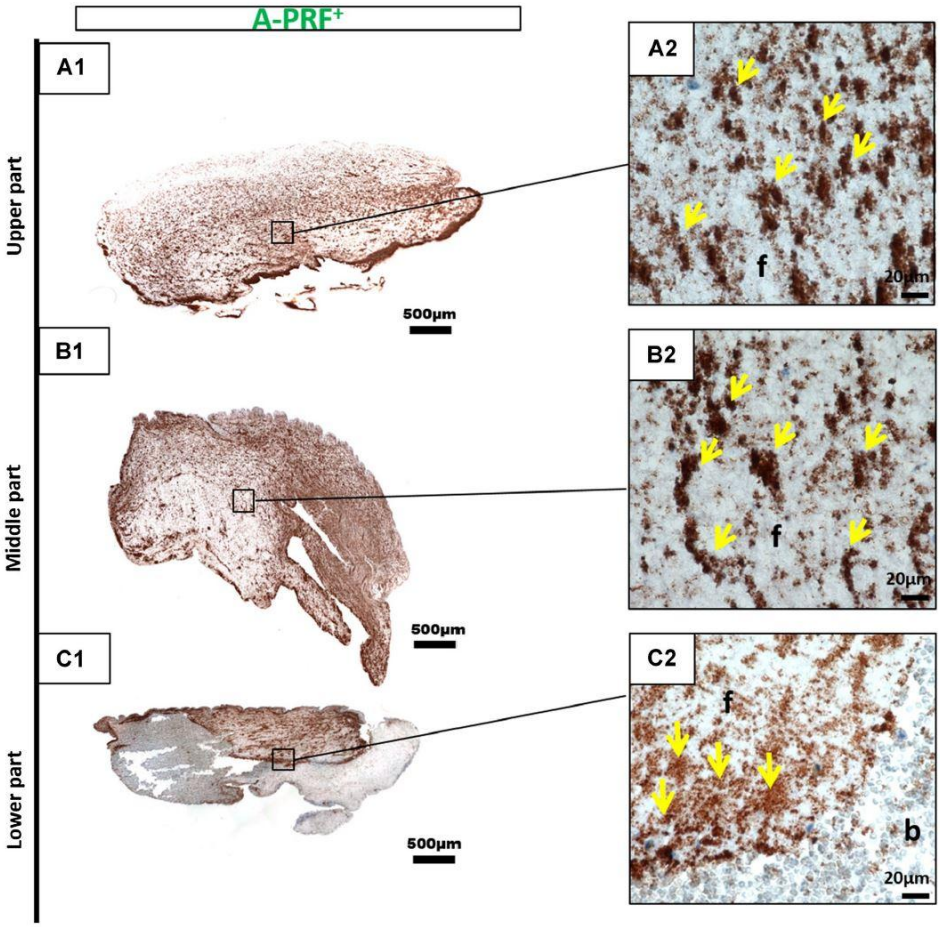

Fig. 5.2. CD-61 immunohistochemical analysis of A-PRF+ according to the different regions. a1, a2 upper portion; b1, b2 middle portion; c1, c2 lower portion (a1, b1, c1 total scan sections; ×100 magnification, *scale bar* 500 µm). a2, b2, c2 Show the distribution pattern of platelets (*yellow arrows*) in higher magnification (*f* fibrin; *b* buffy coat; ×400 magnification; *scale bar* 20 µm) (El Bagdadi et al. modified 2017)[8]

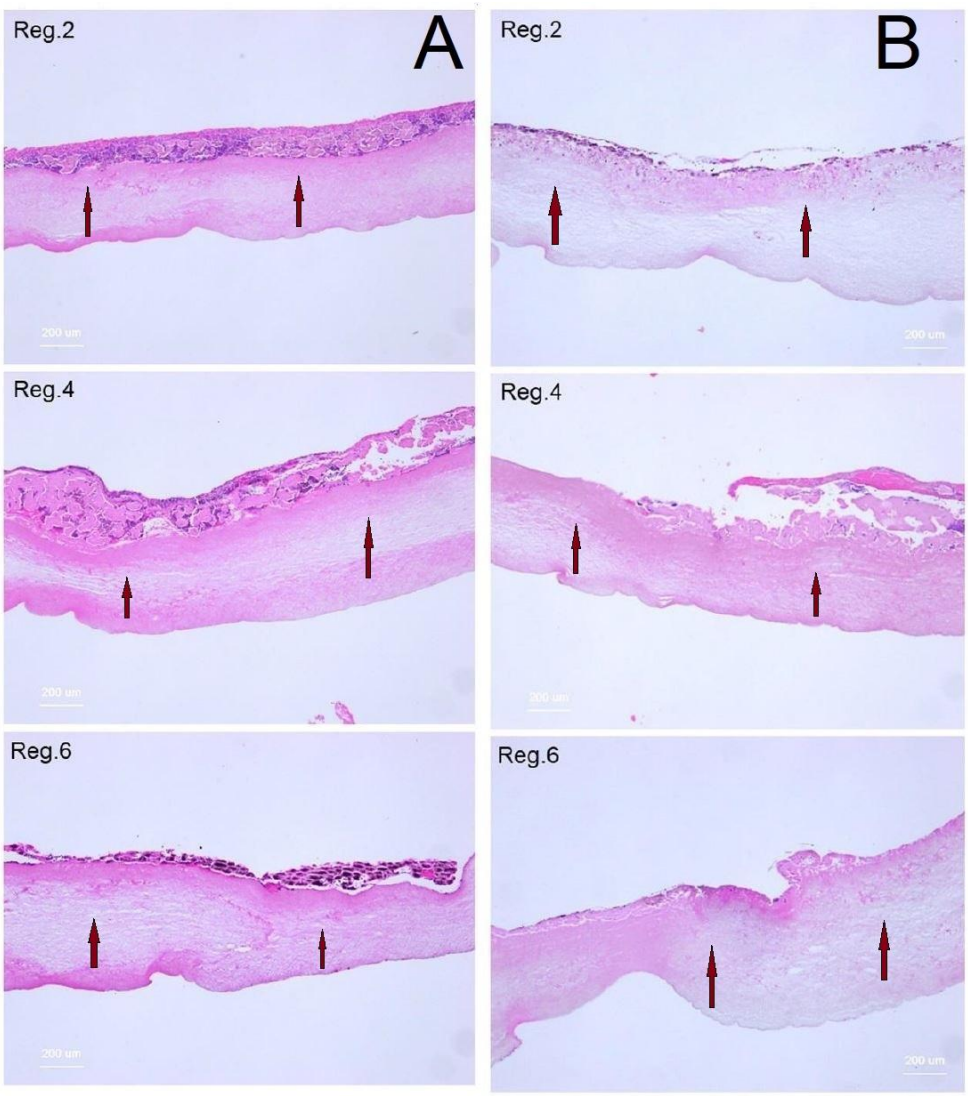

Fig. 5.3. Platelet distribution in PRF matrix prepared using glass tubes for A-PRF+ by low centrifugation (A: 700 rpm for 14 minutes) and high speed (B: 30 s, acceleration; 2 min, 692 g; 4 min, 547 g; 4 min, 592 g; 3 min, 855 g; 36 s, deceleration). Sections of regions (Reg.) 2, 4 and 6 are shown, colored with HE. Arrows indicate the direction of the centrifugal force. The upper edges of the PRF membrane, to which blood cells and whey proteins are attached, represent the region facing the inner wall of the tube (Tsujino T. et al. modified 2019)[9]

The results of the work of El Bagdadi and al. revealed a continuous release of the growth factors VEGF, TGF-β1 and EGF over the study time (Fig. 5.4) and statistically significant differences were demonstrated between the preparation protocols of L-PRF and A-PRF+. One of the most potent stimulants of angiogenesis is the vascular endothelial growth factor (VEGF). A-PRF+ releases much more VEGF than L-PRF on day 7 (Fig. 5.4). These results are probably related to the specific structure of fibrin and the cell distribution model of A-PRF+.

VEGF plays a key role in wound healing and tissue regeneration by promoting vascularization and new blood vessel formation [10]. The enhanced release of VEGF by A-PRF + could lead to greater benefits in regeneration and vascularization and thus provide a nutrient supply to support wound healing and improve the biomaterial-driven regeneration model. TGF-β1 release in A-PRF + indicates maximum release values on day 7, and they are significantly higher when comparing A-PRF + and L-PRF.

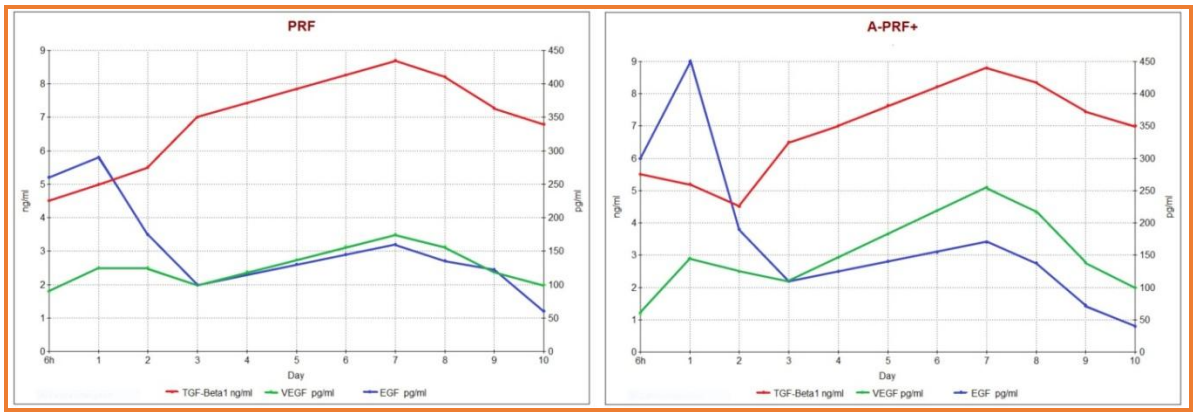

Fig. 5.4. Statistical analysis of the growth factor releases by time points as the mean ± standard deviation for PRF and A-PRF+. VEGF, TGF-β1 release, EGF release,

TGF-β1 is essential for wound healing [11]. In fact, chronic wounds have been observed to have a lower expression of TGF-β receptors. Thus, PRF matrices with higher TGF-β1 release, as is the case with A-PRF +, could have a greater influence on wound healing as a catalyst for wound repair steps. Furthermore, this growth factor is known to stimulate fibroblast migration, improve collagen synthesis and promote angiogenesis [12]. All these characteristics are essential in the biomaterial-based regeneration process.

As a result, PRF-based matrices as an additional autologous addition of inflammatory cells and growth factors could be promising in the field of guided tissue and bone regeneration (GTR and GBR)where biomaterials should provide scaffolding and support regeneration in the area of the defect.

The EGF release is generally higher in the A-PRF+ group than in the L-PRF. Statistically very significant differences were detected when comparing A-PRF+ with L-PRF after 24 hours. EGF has been described as a cell growth promoter, increasing the migration of keratinocytes, inhibiting apoptosis under hypoxic conditions [13], and stimulating support for re-epitelization and skin healing. In addition, EGF supports the process of healing chronic wounds [14], the non-healing of chronic wounds and ulcers, which are, for example, observed in diabetic patients, are due to the lack of the necessary growth factors capable of maintaining the healing process [15].

5.2 DISCUSSION

These patient groups can benefit from the application of PRF matrices as an autologous drug delivery system. In addition, immunohistochemistry indicates a uniform distribution scheme of platelets in all regions of the clot in the case of A-PRF+ (Fig. 5.2), while in the case of L-PRF, most platelets are located at the bottom of the clot (Fig. 5.1). These results are, according to El Bagdadi and al., related to LSCC (low centrifugation rate level), indicating that in reducing the centrifugation rate, the applied RCF increases the number of inflammatory cells and platelets, and the release of growth factors from L-PRF matrices [1].

These observations highlight the influence of RCF reduction, namely from L-PRF (708 g) to A-PRF+ (208 g) on platelet distribution, correlating as previously demonstrated the automated cell count indicating a significantly higher number of platelets in PRF matrices prepared with a low RCF than with a high RCF application. A previous ex vivo immunohistochemistry study by Ghaanati and al.(2014) demonstrated the distribution model in PRF and A-PRF, which included, in addition to platelets, a wide range of inflammatory cells that physiologically exist within peripheral blood, such as leukocytes, including neutrophils, monocytes and lymphocytes [1].

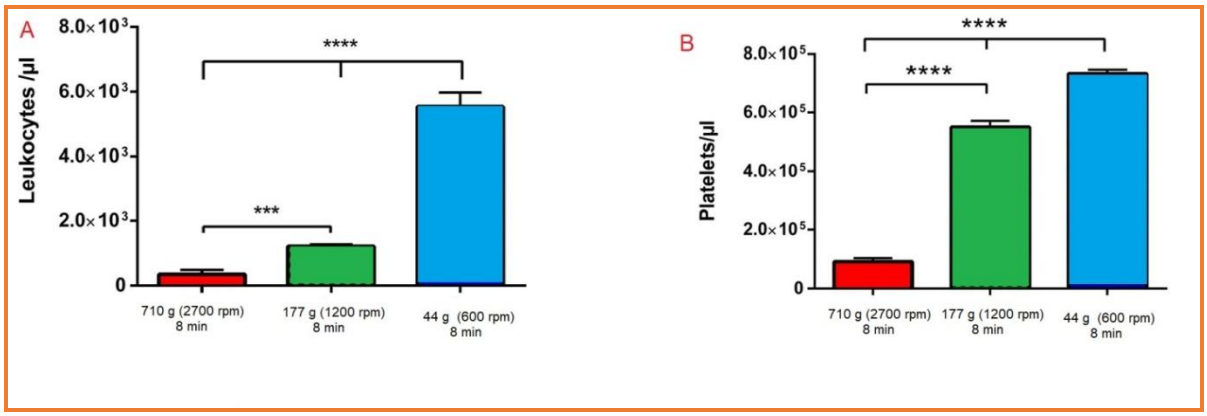

Fig. 5.5. A Number of leukocytes within the different experimental PRF-based matrices (i-PRF). B Number of platelets within the different experimental PRF-based matrices (i-PRF) (from Choukroun and Ghanaati modified, 2017)[6]

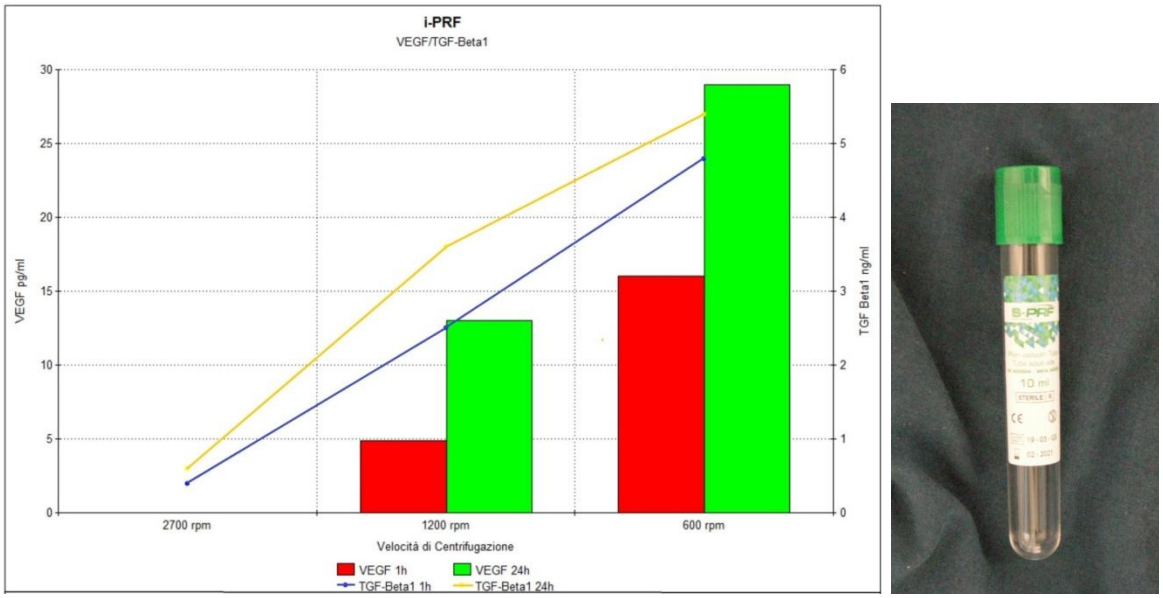

Fig. 5.6. VEGF and TGF-β1 concentration within the different experimental PRF-based matrices (*i*-PRF) 1 h after clotting and concentration within the different experimental PRF-based matrices 24 h after clotting (from Choukroun and Ghanaati modified, 2017)[6]. Right. Test tube for the production of *i*-PRF

These cells, in particular platelets and neutrophil granulocytes, contribute to neoangiogenesis and the liberation of VEGF [16]. In addition, platelets are the primary cells secreting EGF and TGF-β1 [17]; therefore, the large presence within PRF-based matrices is a possible explanation for the release of observed growth factors. These cells are essential for wound healing and tissue regeneration [18]. In the El Bagdadi et al. study (2017)[8], release kinetics showed a higher release of the growth factor over the study time and at maximum day 7 for VEGF and TGF-β1, as well as an increase of the release of the growth factor to 24 hours in the case of EGF (Fig. 5.4). Based on the release of growth factors and the kinetics demonstrated here, it can be assumed that the release pattern of growth factors within the various PRF-based matrices is an active release from living cells within the different PRF clots, that most likely experienced apoptosis during the study period, if the 10 days reflect the reduction in the release of the growth factor compared to day 7 in all groups and for all growth factors. In addition, leukocytes and platelet interaction via cell cross-talk have been described in bone regeneration.

In this context, the high regeneration potential of advanced PRF-based matrices could be useful in various types of clinical applications, such as improved regeneration in biomaterial models in terms of GTR and GBR. In addition, autologous biologization biomaterials that use PRF-based matrices can improve the regeneration model in large defects, soft and bone tissues to catalyze wound healing and regeneration. The study performed by El Bagdadi et al.[8] shows that the application of LSCC, decreasing the RCF results in a significant higher release of VEGF, TGF-β1 and EGF. In particular, the 10-day accumulated release of TGF-β1 and EGF, the ratio of RCF reduction to release of growth factors (Fig. 5.4).

Thus, the A-PRF+ membranes, displayed comparable results that were significantly higher than the L-PRF membranes, which was prepared with an RCF more than three times higher. These comments underline the fact that the application of the LSCC method is useful for modifying and optimising solid Prfs. The various PRF release profiles assessed on the basis of PRF matrices may also be a consequence of the different type of growth factor that binds affinity with fibrin. It has been shown that growth factors, such as VEGF, have a high affinity to bind to fibrinogen and fibrin so that these factors are released in a sustained manner. This information is reflected in the current results, showing the release of VEGF greatly increased on day 7 in the case of A-PRF+. Conversely, the EGF is released in high concentration at a very early point of time (24 hours). An explanation of this observation may be the low binding affinity of EGF to fibrin and fibrinogen [19]. The structure of PRF-based matrices may be another consideration. A-PRF+, in fact, shows a more porous structure than L-PRF [1]. It is possible that a more porous structure, as in A-PRF+, is one of the reasons for a higher release of the growth factor [1].

Thus, it remains debated whether the release of growth factors is related to the specific physical properties of the fibrinic network or to inflammatory cells and platelets included, or a combination of both.

The clots of PRF, in the study of El Bagdadi, were not compressed or manipulated, but nevertheless produced the large amount of growth factors.

Using specific experimental centrifugation protocols, which were systematic modifications of the described first PRF, Choukroun and Ghanaati (2017) generated fluid PRF-based matrices (i-PRF) with different platelet and leukocyte proportions. In addition, a relationship between the reduction of RCF and the release of growth factors has been demonstrated here. Current data provide new information on the potential of the centrifugation process to generate different total amounts of cells and different levels of release of growth factors within the same blood volume, only in relation to the amount of exposure to specific Rcfs.

Three experimental protocols were established with a constant centrifugation time (8 min) to focus on the impact of RCF on various ranges, including high, medium or low in the production of an injectable i-PRF (Figs. 5.5, 5.6). The adaptation of the RCF spectrum (710-44 g) based on the first PRF protocol described [7], Choukroun and Ghanaati performed a systematic reduction of the RCF with a four-fold reduction of the RCF for each protocol (I-III) as a result of the gradual halving of revolutions/min. Automated cell counts by flow cytometry were performed in the Choukroun and Ghanaati study to determine the total number of leukocytes and platelets. The results showed that decreasing RCF up to 16 times less than the first PRF described, led to a significant increase in the number of leukocytes and platelets within the number of turns used to generate PRF matrices. The first I-protocol (710 g, 2400 rpm) showed the lowest detectable number of leukocytes and platelets in the samples tested (Fig. 5.4). It is interesting to note that the reduction of RCF in protocol-II (177 g, 1200 rpm) led to a significantly higher result of the number of leukocytes and platelets than the protocol with 710 g. In addition, the second phase of reduction of RCF towards protocol-III (44 g, 600 rpm) revealed a further significant increase in the total number of leukocytes and platelets, with the highest value compared to all other groups. These data underline that RCF reductions contribute to a clear and significant increase in the number of leukocytes and platelets in the lower RCF ranges (177-44 g) (Fig. 5.5).

The Choukroun and Ghanaati results on i-PRF suggest that the mass, size and density range of leukocytes and platelets require a low RCF, which is enough to separate them from the rest of the blood components, while not causing aggregation at the bottom of the test tube used (Fig. 5.6). Leukocytes are also known to be involved in communication between mesenchymal cell precursors regarding bone formation [13, 20]. Consequently, without leukocytes, sophisticated cell-cell communication for tissue regeneration is not possible. In addition, platelets are known to host powerful growth factors including platelet-derived growth factors (PDGF) for tissue regeneration such as vascular endothelial growth factor (VEGF) and beta-transforming growth factor (TGF-ß) [21, 22], which can only be released after platelet aggregation [23]. In addition, platelets are not the only actors in tissue regeneration, but require the presence of leukocytes for better performance in their capacities towards tissue regeneration [24]. The reduction of the RCF applied results, therefore, in a clear tendency to the concentration of growth factors (Fig. 5.6). Thus, determined growth factors such as VEGF and TGF-β1 were lower in the protocol at 710 g, which was prepared within the high range of RCF. However, the reduction of RCF to the mid-range of RCF led to a significant increase in the concentrations of VEGF and TGF-β1 in the 177 g protocol. A further reduction of RCF showed at the highest level of VEGF and TGF-β1 in the 44 g protocol within the lower spectrum of RCF. This observation has been highlighted both 1 and 24 hours (Fig. 5.6). These results are correlated with the results obtained by increasing the number of platelets and leukocytes. In this context, the increase in the concentration of the growth factor within the medium and low RCF ranges is probably linked to the increase in the number of platelets and leukocytes, as these cells are important sources of growth factors. In addition, it may be that applying a high RCF not only results in fewer platelets and leukocytes, but also influences the ability of these cells to release growth factors. As a result, the improvement in the concentrations of growth factors within fluid PRF-based matrices reflects the improvement in the regenerative capacity of liquid PRF-based matrices as a reservoir of autologous growth factors. VEGF is one of the most important signaling molecules for neoangiogenesis, which is highly required during wound healing. In addition, TGF-β1 contributes to tissue regeneration, to the recruitment of keratinocytes, especially in the early stages of wound healing [25].

5.3 CONCLUSIONS

Today there is no data indicating how many leukocytes or platelets within PRF-based matrices are sufficient to have the best possible physiological condition that can be generated an optimal condition for wound healing or a basis for success of soft tissue and bone regeneration. The results of the studies of El Bagdadi and al. and Choukroun and Ghaanati, however, show, that the reduction of RCF could be a clinical application to adapt the amount of leukocytes within the blood concentrates derived from the PRF according to the requirement. On the basis of current data, the reduction of RCF in PRF matrices enriched with leukocytes and platelets and the release of enhanced growth factors has been hypothesized the so-called LSCC, which can also be used to generate fluid PRF matrices, enriched with cells and peripheral blood plasma-proteins as an autologous source for intelligent tissue regeneration in complex tissue engineering. In a clinical environment, there is a need to increase the potential regeneration of bone replacement materials or biomembran. This could be achieved by adding autologous tissue to liquid systems for engineering. Consequently, the plastic present in the tubes (i-PRF tubes) and the reduction of RCF is allowed for the generation of an injectable liquid PRF matrix (i-PRF) without the use of anticoagulants. This i-PRF, prepared according to LSCC, is highly enriched with platelets, leukocytes and growth factors, and could provide a significant benefit in the regeneration process. Recently, it has been demonstrated that the addition of isolated monocytes from peripheral blood alone contributes to increased in vivo vascularisation of synthetic bone in substitute materials [26]. Consequently, looking at the PRF as a complex "physiological" system, which can be generated with a centrifugation at a time, it is assumed that this system can be enriched with monocytes and all other substances and cells. This system could then be able to contribute to a better wound healing condition with increased vascularization, while remaining within the specific niche for the cell. These data point out that fluid PRF-based matrices, obtained with reduced RCF, can be used to make biomaterials functional by an autologous source, namely peripheral blood concentrates to promote tissue regeneration. El Bagdadi's ex vivo work (2017) has shown that controlling RCF within solid PRF-based matrices (A-PRF+) increases cell distribution and increases the number of neutrophil granulocytes, as a subgroup of leukocytes within the advanced PRF clot [11]. However, further studies are needed to show the role of RCF reduction on cell number and

release of growth factors in solid PRF matrices. The blood composition is specific and individual according to a particular donor. However, donor-related values within the different PRF-based matrices are approximately similar in all groups with regard to the distribution of platelets and leukocytes. These results indicate that first of all PRF-based matrices are reproducible systems and individually applicable regardless of the characteristics of the donor. Secondly, the results also determine the reproducibility of LSCC in various types of blood samples when comparing different donors. Data show that LSCC can be applied for the enrichment of blood concentrates with platelets, leukocytes and growth factors. The combination of leukocytes and platelets plays an important role in tissue regeneration [27]. Thus, the ability to control the content of cells within the blood-derived fluid based on PRF matrices, changing only the centrifugation settings, such as the number of turns, i.e., changing the exposure to a specific RCF, could serve as a valuable step to have a widespread and clinically applicable cell-level tissue engineering medicine. In this way, using the RCF as a tool to control the number of cells included, it could be used to adapt the preparation protocol to the specific needs of individual patients according to clinical indications.

Based on current research, LSCC helps to show that the number of different blood components responsible for tissue regeneration, namely platelets, leukocytes and growth factors, can be selectively enriched by the application of a clinically applicable system through a single parameter within the centrifugation process. Considering the complexity of isolation of cells and their cultivation in sterile laboratories, the PRF system embodies a relatively simple tool to influence the number of these cells. In this context, further in vivo systematic clinical studies assessing the benefits of this system, i.e., by improving autologous regeneration capacity, are necessary to assess the correlation between cell enrichment and the improvement of tissue regeneration potential and wound healing. Controlled clinical trials are essential to assess the potential for regeneration of A-PRF+ and i-PRF and to establish the extent to which a homogeneous distribution of platelets and a greater release of growth factors in addition to the porous structure will help improve wound healing. In addition, potential regeneration of PRF-based matrices may also be related to the age of the donor. Therefore, it may be that, since the age of donors increases, less growth factor is released and vice versa. In addition, studies are needed to demonstrate the extent to which the higher levels or lower RCF ranges are compared to the currently investigated spectrum, may have on any additional benefits regarding cells and accumulation of growth factors within the fluid matrices of PRF. Thus, RCF reduction can be used as an "instrument" to generate fluid matrices based on PRF enriched with leukocytes and platelets.

This phenomenon is of high scientific level and clinical relevance, as leukocytes are one of the main engines of soft tissue and bone regeneration, contributing to the release of angiogenic and lymphogenic factors responsible for cellular cross-talk in the tissue regeneration process.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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To my Father,
Who could not enjoy my professional progress

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