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Leukocyte Platelet-Rich Fibrin (L-PRF), a new biomembrane useful in tissue repair: basic science and literature review

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ABSTRACT

PRF is a second-generation platelet concentrate and can be distinguished in P-PRF (Pure Platelet-rich fibrin) and L-PRF (Leukocyte and Platelet-rich fibrin). In vivo, during the platelet clot formation, platelets are bound to β-integrin fibrin and the clot retracts itself. The PRF clot forms a solid fibrin matrix, displaying a complex tridimensional architecture. Measuring the levels of PDGF-BB, TGF-1β and IGF-1, in the PRF matrix, it was revealed that slow fibrin polymerization during PRF elaboration leads to the intrinsic platelet formation of cytokines and glycan chains inside the fibrin mesh. The PRF proximal part (the part closer to the red clot) contains platelets and leukocytes, found to be massively enclosed inside the fibrin net. Platelet localization inside PRF was examined through immunostaining and scanning electron microscope (SEM). Compared to the abrupt basal compression of PRF membrane (G-PRF), preservation of plasma level, of 3D fibrin, and of platelets was found to be more intact in PRF membrane preparations obtained through metallic compression system (PRF-C) (L-PRF Wound Box). The pure platelet-rich fibrin (P-PRF) and the leukocyte and platelet-rich fibrin (PRF-L) are biomaterials with solid fibrin or biomaterials containing leukocytes. No platelet concentration related problems were found in PRF, as all platelets in the drawn blood sample are activated and integrated inside the fibrin matrix of the clot. Approximately 97% of platelets and more than 50% of leukocytes are concentrated inside the PRF clot, which displays a specific tridimensional distribution.
Almost all platelets (>97%) are absent inside test-tubes of groups tested after PRF membrane extraction.
Factors freed by platelets contained in 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).

Keywords: *blood derivatives, growth factors, leucocyte and platelets rich fibrin, L-PRF wound box, stem cells.*

1. INTRODUCTION

Recent scientific evidence suggests that platelets could play a new role in tissue repair and in vascular remodeling, other than being active actors in inflammatory and immune responses. Platelets release biologically active proteins and other substances, able to influence a series of processes, favoring cell intake, growth and morphogenesis. These substances are released or exposed on the surface of activated platelets. The platelet capability of releasing substances inside a clot makes the clot itself a natural autologous source of growth factors and cytokines, which can be therapeutically used to accelerate and speed up physiological healing processes. Many of these substances are gathered and stored in platelets α-granules, easily identified with Scanning electron microscope (SEM) and with immunofluorescence staining.

Thin fibers contained in HPC (Human Platelet Concentrate) could be related to the high initial concentration of platelets in HPC (3-5 x 10^{11} platelets/l), where local procoagulant activity could even be improved through the start of pro-thrombotic stimuli amplification, and it leads to an almost explosive thrombin production, thus causing an increase in fibrinogenesis on the surface of platelets, which in turn leads to fibrin formation and polymerization [1].

Adhesive proteins are rather abundant on the fibrin reticulum: Fibrinogen (Fg), Fibronectin (Fn), Vitronectin (Vn), Thrombospondin-1 (TSP-1). Fn concurs to wound healing and promotes the mitogenic activity of Platelet Derived Growth Factor (PDGF). Among the platelet-stored Growth factors (GF), essential for wound healing, we count PDGF, in particular, PDGF–AB and PDGF–C (prevalent isoforms in platelets); other factors include the Vascular-endothelial growth factor (VEGF), essentially VEGF-A, Transforming growth factor β1(TGF-β1), basic 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). Members of TGF-β family are prominent in wound healing and scar formation. Holding a preponderant role in healing, platelets are a rich source of cytokines and chemokines. An example is RANTES, a chemokine deposited on the inflamed endothelium thanks to a P-selectin-platelet-dependent mechanism.

In general, these factors chemotactically recruit and activate stem cells, as well as induce their mitogenesis and differentiation. Platelets are also a potential source of matrix metalloproteinases (MMPs) (MMP-2, MMP-9, ADAM-10, ADAM-17, ADAMTS-13), as well as tissue inhibitors of

metalloproteinases (TIMP 1-4). MMPs are found in α-granules and in cytoplasmic membrane vesicles as well.

Fibrinogen could improve wound scarring, increasing both cell proliferation and migration; it assembles with Fn in fibrils, without taking into account fibrin formation. Wound healing outcomes are influenced by fibrin structure (fiber thickness, branching points number, clot porosity and permeability) where the wound is present [2].

It appears clear that platelet-rich fibrin clots can be considered a bioactive reservoir. An elevated hematocrit or a low platelet count could be a limiting factor, and further research is needed to establish an optimal platelet count for the material to be used in procedures. Beside secreting proteins, platelets are able to release low molecular weight diffusible compounds, and a great number of microparticles, which carry proteins like TF or IL-1, that is pro-thrombotic substances. Therefore, application in patients displaying hereditary or acquired thrombotic risk factors (including arterial hypertension, Factor V Leiden mutation). Contemporary administration of anti-platelet drugs could theoretically limit the efficacy of the treatment. Aspirin reduces platelet secretion and its administration should therefore be avoided in the days immediately preceding autologous L-PRF preparation since it inhibits cyclooxygenase enzymes (COX).

PRF® (Platelet-rich fibrin) is a new generation platelet concentrate, obtained through autologous peripheral blood centrifugation, without any biological agent addition. It includes, besides platelets, the fibrin matrix polymer, leukocytes, cytokines and stem cells. It is distinguished in P-PRF (Pure Platelet-rich fibrin) and L-PRF (Leukocyte and platelet-rich fibrin) (Figure 1). Another platelet concentrate, PRP, has a transitory effect on wound healing; furthermore, bovine-derived thrombin employed in its formation increases the coagulopathy risk, not seen with PRF® instead. With its (PRF®) use, a natural coagulation process takes place, allowing for an easy leukocyte and PRF® (L-PRF®) collection inside the clot.

Fibrin gels are recommended as scaffolds, to be employed in tissue engineering in many respects. The most prominent reason is its compatibility with fibrin cell life, which differs based on its various and numerous components and the processes involved in scaffold formation. In vivo, at the end of the fibrin clot formation, platelets bind the fibrin to β-integrin and the clot contracts. The clot contracts also along the wound margins, giving rise to tension forces and directing the new temporary matrix.

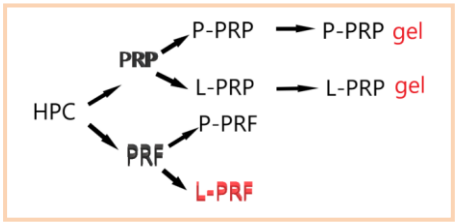

Figure.1 Different types of human platelet concentrates (HPCs): PRP (Platelet-rich plasma); PRF (Platelet-rich fibrin); P-PRP (Pure Platelet-rich plasma); L-PRP (Leukocyte and Platelet-rich plasma); P-PRF (Pure Platelet-rich fibrin); L-PRF (Leukocyte and Platelet-rich fibrin).

The PRF clot is created by a natural polymerization process, during centrifugation, and its natural fibrin architecture seems to be responsible for a slow GF and matrix glycoproteins release (≥7 days). PRF clots are directly used to fill cavities in plastic and general surgery interventions. Even though platelets' GFs cover an essential role in PRF biology, fibrin architecture, leukocyte-contained substances and stem cell presence are three key parameters. Platelets and leukocytes distribution inside fibrin clots is highlighted through blood count, photon microscopy and SEM analyses (Figure 2).

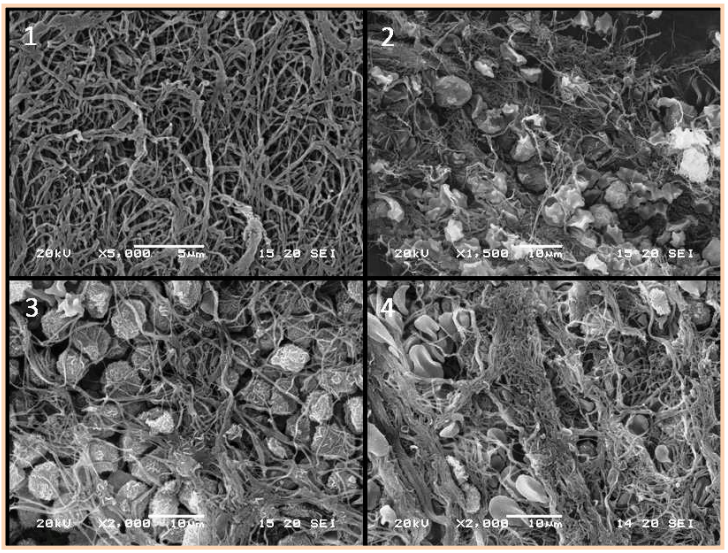

Figure.2. SEM picture displaying: **1** the fibrin-rich layer (5.000 x magnification); **2** a zone of enriched platelets with various degree of activation (.1.500 x magnification); **3** buffy coat with numerous leukocytes and **4** the red blood cell base (2.000 x magnification).

An adequate PRF® preparation method should properly separate platelets from erythrocytes and concentrate them without damaging or lysing platelets themselves. Growth factors contained inside α-granules are not active during secretion, but get activated through fusion with platelet membrane. Consequently, if platelets are damaged during PRF production, they will not secrete bioactive growth factors anymore. Platelets are, as a matter of fact, particularly labile, and sensible to every kind of stressful event during their processing and application; for this very same reason, GFs concentration could be influenced by handling during blood processing events. It is then decisive the type of centrifugation applied, which needs to respect several demands and characteristics, including an initial Low Start, a high-frequency centrifugation as middle phase and a closing Low Stop [3], and it must take place at a set temperature and for a specific time interval (Crisci et al. 2015)[4, 5].

Here, we review the spectrum of platelet-rich blood derivatives, discuss their current applications in tissue engineering and regenerative medicine, reflect on their effect on stem cells, and highlight current translational challenges.

2. EXPERIMENTAL

2.1. L-PRF processing. PRF® production protocol is very simple: blood is immediately centrifuged within 2 minutes from withdrawal, following the subsequent steps: 30" of acceleration, 2' at 2700 rpm, 4' at 2400 rpm, 3' at 3000 rpm, and 36" of deceleration and arrest. The resultant product is made of three layers: PPP (Platelet-poor plasma at the top), PRF (central clot), Red Blood Cells (RBCs) at the bottom (Figure 3). Resulting PRF clots are gathered and red blood cells are removed with the aid of scissors, without macroscopical damage at PRF structure expense.

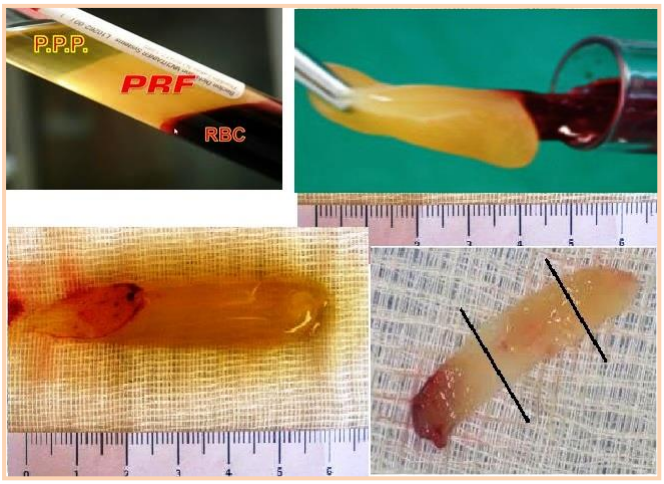

Figure 3. Different phases of PRF preparation (see text).

Fibrinogen is initially concentrated in the middle and superior portion of the test tube, that is in between red blood cells (RBCs) at the bottom and the Platelet-poor plasma (PPP) at the top. Clot compression by means of a compression system (L-PRF box) significantly stimulates cell proliferation and neovascularization [6].

Quantifying the levels of PDGF-BB, TGF-β1 and IGF-1 in PPP and in PRF, analyses showed that slow fibrin polymerization during PRF processing leads to the intrinsic production of cytokines and glycan chains by platelets inside the fibrin mesh. Analyzing three pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), one of the anti-inflammatory cytokines (IL-4) and the angiogenesis promoter (VEGF), we saw that PRF could be a central junction in immune regulation, with the capability to control inflammation. PRF, differently from other platelet concentrates, could be able to progressively release cytokines during fibrin matrix remodeling.

Platelet cytokines and leukocytes are an important factor in determining the effects and role of this biomaterial, but, both the fibrin matrix and its contained elements are responsible for PRF therapeutical enhancement (Tab.1). Cytokines are immediately used in wound healing. The mechanism involved in PRF formation is the concentration of fibrinogen at the top of the test tube, which combines with circulating thrombin, due to the centrifugation process, essential in fibrin formation. PRF central portions contain platelets, massively encased inside the fibrin mesh. The therapeutical success of this kind of technique is entirely dependent on the time interval between blood sample withdrawal and its centrifugation, which should be carried on in the shortest interval possible, as well as on processing temperature and the type of test tube employed.

Blood samples should be withdrawn from patients that have no previous history of aspirin or other anticoagulant drug administration up to two weeks prior the procedure.

Dohan Ehrenfest et al.(2012) [7] detected a slower GF release in PRF compared to PRP, and they observed improved healing capabilities with the employment of PRF. Also, it was demonstrated that cells are able to migrate inside the fibrin network. A slow polymerization procedure imparts a physiological architecture to the PRF® membrane, particularly favorable to sustain healing processes.

In addition to standard formulations, PRF can also be obtained in an injectable form (I-PRF). I-PRF is obtained by producing a PRF membrane, which subsequently is compressed between metallic sheets. Advantageously, this injectable material can coagulate immediately post-injection to form a biomaterial as well as be combined with any biomaterial of choice for non-covalent incorporation [8].

2.2. PRF effects in tissue engineering. Platelet localization inside the PRF gel was examined through immunostaining and with the aid of Scanning Electron Microscope (Figures 2, 4) taken from Kobayashi et al. 2012 [6].

Su and Burnouf [9] demonstrated that a copious amount of growth factors was discarded when pressing took place. Hence, pressing processes could influence the efficacy and clinical quality of PRF preparations, to be used as graft material.

Platelet-derived mediators induce and regulate fibroblasts' late action, and leukocytes' recruitment, neutrophils first, followed by macrophages, consequently eliminating dead cells and cellular debris. Moreover, factors derived from platelets induce and control proliferation and migration of other types of cells, which are critically involved in tissue repair, like smooth muscle cells (SMCs) and mesenchymal stem cells (MSCs).

Activated platelets release a whole range of chemokines and promote adult stem cells' absorption, adhesion and proliferation, including progenitor CD-34 positive cells, MSCs, SMC progenitors and endothelial progenitors. The multipotent nature of these cells and their capability to increase vascular tissue repair, due to paracrine mechanisms, makes them good candidates as therapeutical vehicles to be employed in regenerative medicine fields. Moreover, tissue damages themselves are able to generate strong chemoattractant signals, affecting stem cells, and providing their regenerative action basis.

Platelets regulate adult stem cells recruitment toward damaged cells and could therefore, constitute an essential mechanism for regenerative cellular processes. Activated platelets release HGF and have been linked to MSCs passage through endothelial cells, lining human arteries. Human mesenchymal stem cells' proliferation (hMSCs) is proportional to platelet concentration inside PRF concentrates.

Table 1. Leukocytes, RBC and Platelets number in whole blood (control group) and red clot after PRF membrane collecting (test group) (from Crisci et al. 2015).

	Leukocytes/µl		RBC/µl		Platelets/µl	
	Mean	*Range*	*Mean*	*Range*	*Mean*	***Range***
Controll	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**

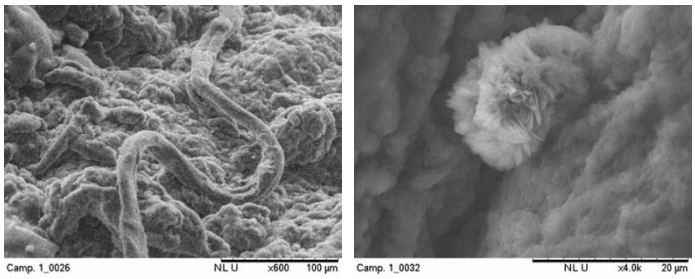

Figure 4. Microscopy picture at S.E.M. Hitachi Tabletop Microscope TM3000 of L-PRF.
On the left: Fibrin mesh (600x magnification); on the right: activated platelet (4000x magnification)

Among tested growth factors, PRF contained PDGF constitutes the major portion, and stimulates, significantly, cell proliferation and neovascularization. An important PRF characteristic is the resulting fibrin gel, shown to be denser than the gel prepared with thrombin addition (PRP).

Thus, the establishment of a standard protocol for PRF preparation was necessary, satisfying the following criteria:

1) Platelet-contained growth factors should be preserved to stimulate surrounding host cells;
2) Platelets should be stored inside the fibrin mesh with minimal damage or activation;
3) The tridimensional fibrin mesh must be used as a scaffold by surrounding host cells.

The PRF was subdivided into 3 regions, of equal length (Fig.3) and platelet presence in each region was observed through S.E.M., through Optic Microscope in horse-derived preparations (Crisci e al.2017)[10-12].

Region 1 is the region closest to the red clot, and shows a conspicuous number of platelets aggregates, displaying some lymphocytes and other white blood cells. Platelet count is reduced as the distance from the red clot is increased. Inside region 2 (central region), we observe fibrin fibers (primary and secondary fibers) and some platelets. Inside region 3, the fibrin mesh is extremely evident, while the platelet count is low (Figure 5).

We identified some anti-CD41 antibody positive cells, through immunocytochemistry, and, as a matter of fact, in L-PRF, on one side of the membrane, many CD41-positive platelets were gathered, and some platelets could be found inside the membrane. On the membrane's opposite side, only a few platelets were observed. The discovery explained in Kobayashi et al.[6] studies are constituted by the fact that platelets are not equally distributed inside and on the surface of the PRF clot, even if the clot is considered as a gel with uniform platelet concentration. Therefore, in a clinical setting where platelet-derived growth factors are expected and desired, the red clot-adjacent region must be used, being richer in platelets.

Basing our actions on the assumption that PRF-retained serum could contain elevated GFs levels, released by platelets, which are more or less active during centrifugation phases, we didn't try to squeeze all the plasma with a complete compression of PRF clots. The C-PRF tendency to contain higher growth factors levels (PRF compressed through metallic compression system), compared to G-PRF (PRF compressed with gauze), could be traced back to platelet-derived GFs (PDGF-AA, PDGF-AB, PDGF-BB).

This obtained result could be due to fibrin, since the fibrin mesh could directly absorb GFs or could entrap serum albumin or heparin, hence indirectly retaining GFs. It is almost impossible counting and regulating the platelet count in PRF preparations before clinical usage. Therefore, the clinically most effective protocol to check result quality is using the PRF region closest to RBC clot.

Cell migration was performed through the employment of MSCs (mesenchymal stem cells), derived from human bone marrow, and human umbilical vein endothelial cells (HUVECs) [13]. MSCs migrated principally at day 3 for L-PRF preparations. A higher migration rate was observed for L-PRF compared to L-PRP at day 3, day 7 and day 14. HUVECs migration also reached its peak at day 3, day 7 and day 14 for PRF preparations.

2.3. Preclinical Studies. Fibrin is a useful substrate for bioengineering purposes, and it is one of the most popular hydrogels in tissue engineering and regenerative medicine. Grafted cells require specific signals from the extracellular matrix to survive.

L-PRF is a preparation that contains leukocytes, and with a high density of the fibrin mesh. These products exist as activated gels, and cannot be injected or used as traditional fibrin glue. However, due to their strong fibrin matrix, they could be handled as a solid material, for purposed applications, achieving interesting results in reconstructive surgery; even so, these applications are still in an experimental phase, since we still lack a necessary protocol for the usage of clots in various, specific surgical procedures.

Fibrin generates a temporary matrix at the graft site, but its fibers have no direction or tension, and it has a low count of associated growth factors. Fibrin gels, instead, show a regular, reticular disposition of its pores, with short, thin fibers, non completely acellular; through SEM, platelets and leukocytes were observed (Figure 5).

Studies carried out on horse models showed that platelet-rich fibrin gels (PRFG) display big, dense fibers, which are casually oriented (with a mean diameter of 117.7±10.53 nm). Fibrin gel fibers are smaller (56.8±5.11 nm). Platelet incorporation inside the fibrin gel results in structural alterations and an increased growth factors' concentration, hence it could improve cell grafts' performances inside the scaffold after transplantation.

In accordance with their growth factor storage, platelet directly contributes to tissue growth, development and repair. PDGF and TGF-β1 are the most abundant growth factors, contained inside platelets' α-granules and they get released inside the extracellular space, after platelet activation. These cell factors orchestrate proliferation, cell differentiation, matrix production, angiogenesis, and wound contraction; growth factors supplementation is able to improve transplanted cell survival and differentiation, in a number of materials and tissues treated [14].

Platelet addition to the fibrin gel (PRFG) is related to an increase in fibers' diameter and the reduction of porous areas; they also increase the fibrin gel rigidity. Measured fiber diameter could be associated with the lower platelet concentration (100 x 10^3 platelets/μl), which is approximately closer to systemic platelet concentration under standard, normal conditions. One possible explanation could be that a lower platelet count would cause decreases in tension applied to fibers.

Porous areas and porous percentage are important structural indices for every biological scaffold. Bigger pores favor internal growth and cell proliferation, while smaller pores favor cell adhesion, thanks to a bigger surface.

Under a clinical point of view, L-PRF shows excellent handling properties: single L-PRF clots can be turned into membranes of fitting thickness and dimensions, thanks to the new "*L-PRF Wound Box*" (Figure 6); merging two or more membranes is useful to create a bioactive membrane of bigger dimensions, to cover and form bigger grafts. L-PRF membrane can also be cut and tailored. Being flexible enough, it adapts to different anatomical areas.

Legally, a physician, is authorized to perform endovenous injections, is also authorized for blood withdrawal. Furthermore, as stated by the European Community regulations, hence with a law judged valid throughout Europe, L-PRF is not considered a hematological derivate, since there are no added substances, but it is included under autologous cell grafting.

Since the wound healing process achieved with this technique is not by primary or secondary intention, it is defined as "*wound healing by modified secondary intention*" [15].

L-PRF family adapts itself to the requirements of the various surgical intervention. Just like clots and membranes, L-PRF has shape and volume easily combined with the great bulk of surgical techniques, as a filling method and healing biomaterial apposition, or as protecting membranes for wound healing. Furthermore, it is easy to prepare, also in great quantities, and very cheap, making it particularly apt for everyday clinical practice. It was successfully used by AA, especially in the treatment of cutaneous diabetic ulcers, also with osteomyelitis presence [16].

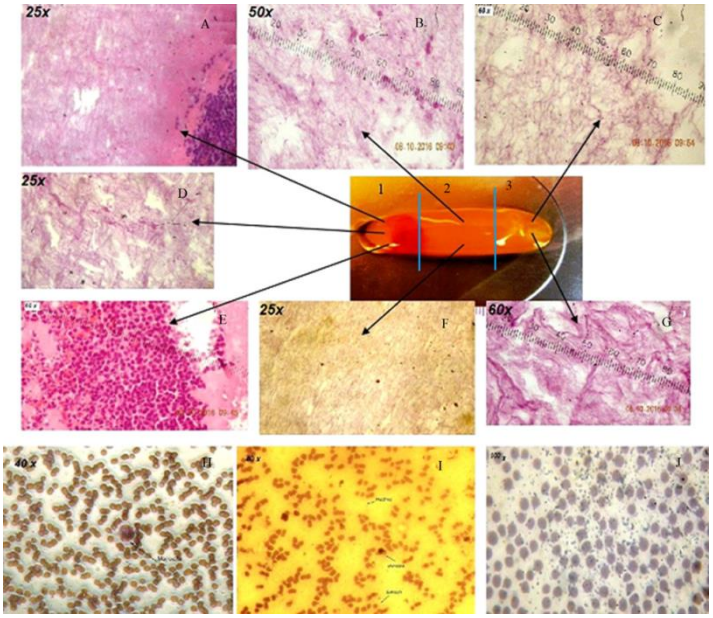

Figure 5. The three C-PRF regions, and O.M. platelet distribution observation on membrane surface. C-PRF was subdivided in three regions: Region 1 adjacent to the red clot (RBC), Region 2 is the central part and Region 3 is the distal part from the red clot. Platelet distribution was observed in region 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) 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). Reprinted from an open access source [11].

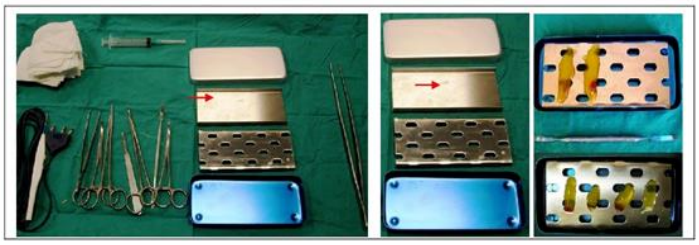

Figure 6. L-PRF Wound Box.

2.4. L-PRF *in Vitro*. In vitro, L-PRF membrane and P-PRP (Pure Platelet-rich plasma)(PRFG-endorest) gel components were compared, through the evaluation of the slow growth factors and matrix molecules' release.

These two gel families were seeded on a growth medium for 7 days, and the slow versions of 3 key growth factors (TGF-β1, PDGF-AB, VEGF) and 3 coagulation proteins and 3 matrix proteins (TSP-1[Thrombospondin 1]), (Fn) e (Vitronectin) were experimentally quantified for seven times (at 20', 1h, 4h, 24h, 72h, 120h, 168h)(Figures 7, 8, 9)(Crisci A.et al.2015)[4-5].

These studies showed that products display two very different profiles: L-PRF membrane stayed solid and intact after at least 7 days and releases continuously a big quantity of growth factors, a significative portion of which is produced inside the membrane. On the contrary, clear P-PRP releases the major

portion of growth factors within the first hours and it is completely dissolved after 3 days (Figures 7-9).

Leukocytes found in L-PRF are not only inflammatory cells, as they show anti-nocireceptor effects as well, through the release of different chemokines, anti-inflammatory cytokines (IL-4, IL-10, IL-13) and peptidic opioids β-endorphins, dimorphin-A etc.), hence they could promote a clinically relevant inhibition of pathological pain (centrifugation process could delicately activate, or stimulate in a pathological way, the inflammatory state, or destroy leukocytes altogether). Many other types of cell are present in these preparations. The leukocyte count is an important parameter: lymphocyte populations are very different and do not have the same impact as monocytes and granulocytes do. Furthermore, many other cells, like circulating stem cells, could be found in a platelet concentrate in a non-unimportant manner [13].

A certain quantity of GFs was observed in the serum released by PRF (PRFR) and in the supernatant serum (SS), right after PRF formation. An additional GF release from PRF was found up to 300' after preparation (5h). SS contained quantities are ≅ 7 ng/ml (PDGF-AB), 9.5 ng/ml (TGF-β1), 0.1 ng/ml (VEGF) and constitute a good index of clot-contained GFs' basal levels inside PRFR.

True PRF is always autologous and not homologous. One example of this misconception is the use of lyophilized platelets from donors. Homologous platelets cannot be used and cannot secrete bioactive GF. Homologous platelet is antigenic due to their abundance of cell membranes.

PRF and SS contain great quantities of GFs, and do not need to be discarded as they could be useful in patient treatment [9]. The initial PRF exudate (rich of growth factors and serum proteins) is collected in the container and PRF membranes are stored in a serum-humidified environment. This is an effective method from a biological point of view. A new device, that we have tested in L-PRF clots and membranes preparation and procedure standardization, is *L-PRF Wound Box* (Crisci A. et al. 2017)[10-12]. It is an instrument where PRF clots can be turned into membranes. L-PRF clot contains almost all of the platelets and more than 50% of white blood cells coming from the initially drawn blood (Tab.1); furthermore, it shows a strong fibrin architecture and a particular tridimensional distribution of platelets and leukocytes. This device permits clot preservation in a humid, sterile environment for 1 hour, and it allows an increase in total growth factor release. Mean PDGF-AB produced quantities are significantly higher in every experimental instant (Figure 8) and TGF-β1 (Figure 8) and VEGF (Figure 9) are significantly higher during the first 4 hours. Numerous studies have confirmed the gradual release of PDGF and TGF for 28 days from PRF formation [16]. A possible explanation is that PRF polymerizes with a 3D structure progressively, slowly and naturally during centrifugation, and this helps to entrap cytokines released by platelets with the fibrin polymer. In addition, using the PRF Box, the clot compression into the membrane is carried out through a light compression, slow and homogeneous, and the resulting membrane always remains homogeneously wet and soaked by serum. This delicate method avoids extraction and loss of a significant GF quantity, and it is particularly obvious with PDGF-AB, because this growth factor is only released by platelets.

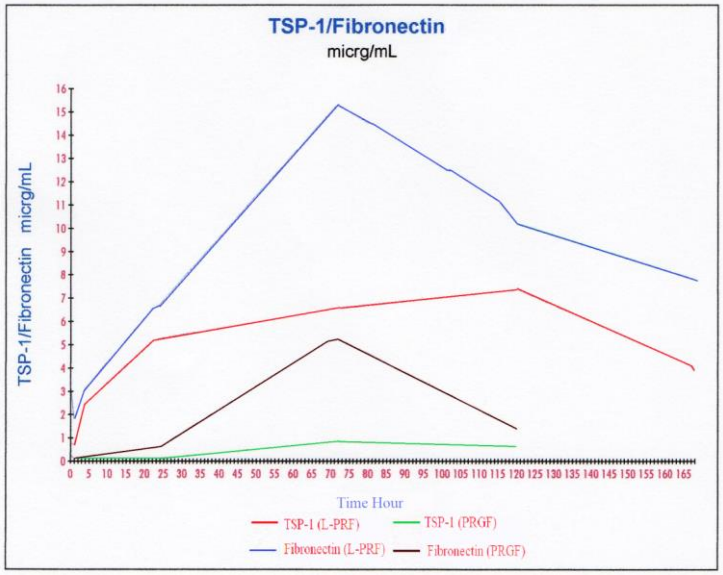

Figure 7. TSP-1 and Fibronectin variations in time. L-PRF comparison with PRGF (Plasma rich in growth factors = P-PRP).

On the contrary, it does not influence other intrinsic growth factors that are released slowly in elevated quantities for several days. The released quantities of VEGF and TGF-β1 are massively produced by leukocytes.

A non-standardized blood withdrawal procedure, slow and inadequate, leads to a small, PRF-like fibrin mass, with unstable fibrin polymerization (with consequent poor mechanical properties), and to an unknown, not-reproducible growth factor. In addition, it is very difficult to divide these small fibrin masses at the RBC base, resulting in a heavy red blood cells mass in the product [9].

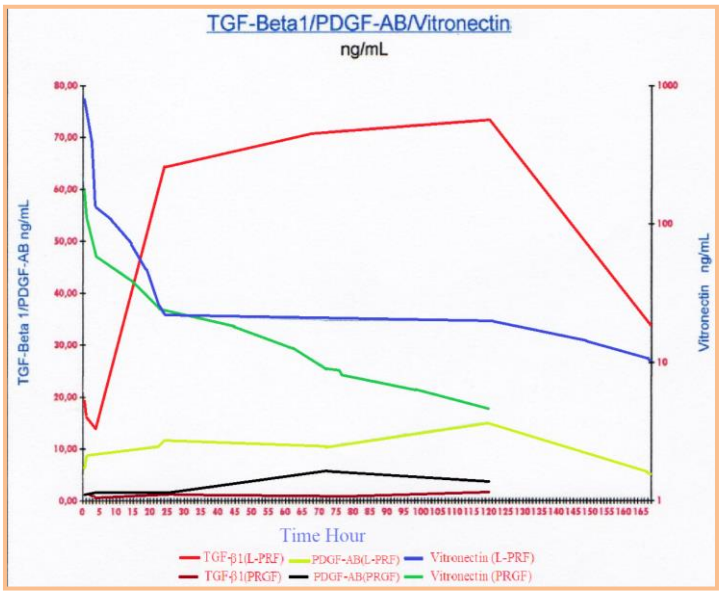

Figure 8. TGF-β1/PDGF-AB and Vitronectin variations in time. L-PRF comparison with PRGF.

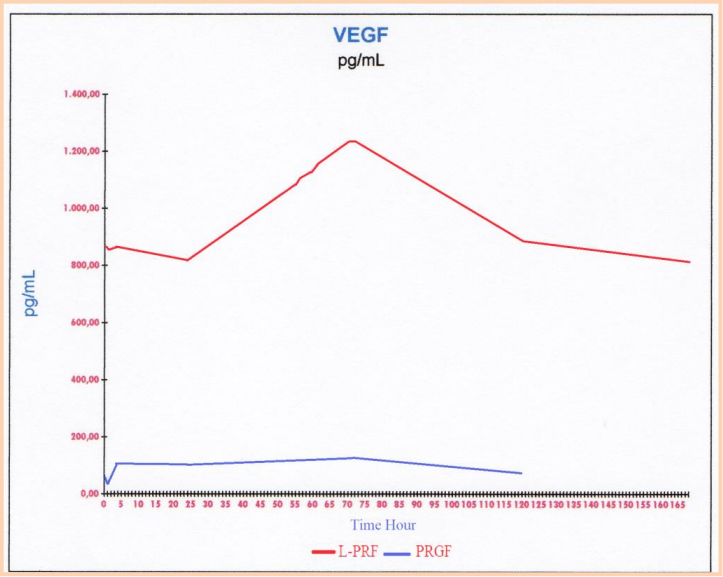

Figure.9 VEGF variations in time. L-PRF comparison with PRGF

2.5. PRF limitations. Platelets, fibrin, and leukocytes naturally act synergistically to promote wound healing and tissue regeneration, and the amplification of this coagulation/regeneration effect on a surgical site or wound is the aim of surgically oriented platelet concentrate employment. According to POSEIDO classification, all products of this category are gathered under the general name of human platelet concentrate (HPC), whichever may their form or cell content be. In addition, it is important to highlight the key role of leukocytes and their influence, as well as fibrin architecture, concurring to the potential clinical or experimental effects of these products, and it is also important to emphasize how each different product is referring to a precise biological distinctive composition.

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

1) Since PRF is an autologous product, an increased requirement for the biomaterial availability is difficultly achieved. Therefore, its use in surgical procedures must be tightly controlled.

2) PRF contains circulating immune cells, as well as antigenic molecules that prevent its use as allogenic material; an increased risk for the transmission of infectious diseases is also to be taken into account.

At this point, among the different parameters that were not included in this type of classification, we recognize platelet concentration, leukocyte concentration and the proportional amount of the different leukocyte types. Platelet concentration-related problems are non-existent, as all platelets included in the blood sample are activated and integrated inside the clot's fibrin matrix. Concerning the leukocytes' count and concentration, their influence should be studied with particular care, as their presence or absence could explain the conflicting results we observed (Tab.2) [11].

A significant correlation between platelet counts and TGF-β1 (p=0.005) and PDGF-BB (P=0.04) was found. TGF-β1 is quantitatively increased in slow release PRF, compared to fast release PRF (Figures 7, 8). WBCs, entrapped inside the PRF matrix, were shown to be the main source of this TGF-β1 supplemental release, and these WBCs keep secreting this GF inside the PRF clot for several days.

PDGF-BB is almost exclusively contained inside platelets' α-granules, and is released when they are activated, hence explaining why its production is at maximum levels in activated PRP and PRF, while it is reduced in slow release forms. It is possible that hematological levels cannot be used to forecast the immediate or slow release of GF inside PRF compounds [14].

Dohan Ehrenfest et al.[18] studies aimed to determine cell composition and tridimensional organization of this autologous biomaterial, and the utility of different test tubes (dry glass, coated glass, plastic), all of them without gel. Almost 97% of platelets and more than 50% of leukocytes were concentrated inside the PRF clot, demonstrating a specific tridimensional distribution. Platelet and fibrin are present in big quantities inside the first few millimeters of the membrane, past the RBCs region boundaries. There is no discernible difference in PRF architecture using different types of test tubes.

2.6. Platelets and leukocytes analysis. Almost all platelets (>97%) were absent inside test tubes of the group analyzed after the PRF membrane extraction. In the test groups, the leukocyte level significantly dropped compared to the control group ($p<0.01$) and more than half of the leukocytes seemed to have disappeared (Tab.2).

Missing platelets and leukocytes are trapped inside PRF matrix when we use the scissor based collection method. The absence of differences between the two test groups ($p>0,05$) seems to indicate that clot compression does not influence the possible release of cell bodies trapped inside the fibrin matrix.

In test groups, the lymphocyte concentration is significantly lower, while granulocyte concentration is significantly higher ($p<0.01$) compared to control group (Tab.3). This would signify that lymphocyte were trapped inside the PRF matrix more than other types of leukocytes. Lastly, mean platelet volume (MPV) is noticeably reduced between test groups and control groups ($p<0.01$); it went down from μm^3 (range:8-11 μm^3) in whole blood to 4.7μm^3 (range: 4,5-5,8 μm^3) in test groups. This phenomenon could be due to plasma osmolarity increases inside test tubes after coagulation cascade activation.

2.7. Blood elements distribution analysis. Histomorphometric analysis was performed using an optical microscope with 100x total magnification. A 10 mm eyepiece, with 100 divisions reticulum, was used to measure the coverage percentage of the total length with at least one cell layer, in each section.

With hematoxylin-eosin staining, the fibrin matrix appears homogeneous, in a pinkish color, while platelet aggregates are in deep blue/violet. RBCs and leukocytes' cytoplasm are deep pink and not easily distinguished. Leukocytes' nucleus is stained in blue with hematoxylin and it is not easily distinguished from platelet aggregates. With Masson trichrome staining (modified by Godman), platelet aggregates are still seen in deep blue, but RBCs are easily distinguished since they are stained in red.

Leukocytes are still difficultly highlighted inside platelets aggregates, however, the boundary line between RBCs and platelet aggregates/leukocytes is very clear. The boundary line between RBCs, leukocytes and platelet aggregates is not distinguishable.

PRF clot observation through SEM at low magnification (15x) demonstrated that the clot shows a central concavity, which is a fixation artefact. In the red portion of PRF clot, clots display RBCs inside the fibrin mesh. RBCs are normal, but the fibrin mesh is premature. At the boundary between the red and yellow parts of the clot (buffy coat area), the SEM examination showed leukocytes, appearing as spherical structures with an irregular surface. Of these, the major part is small (6–8 μm diameter) and hence they could be essentially lymphocytes. Platelet aggregates are evident along fibrin filaments. Over the "buffy coat" area, we distinguish two different areas: the first area is made of thick fibrin filaments and few sparse RBCs, possible due to contamination; fibrin seems to be mature; the second area is made of that strand of condensed platelets, observed at the optic microscope, characterized by platelet and fibrin in dense, big masses, due to ample aggregation and coagulation processes. This aggregate is made of a thick, solid meshwork, and platelets seem to be highly activated during the PRF preparation protocol.

At higher magnifications, fibrin is clearly organized in parallel fibers, very thick and dense. In this organization, it is impossible to distinguish the contained cellular elements.

The highest platelet and leukocyte density were found in the first millimeter of the yellow clot, at the border with the red clot. Platelet and leukocyte distribution become lower as we move from the clot edge, and we didn't found any platelet or leukocyte beyond the first half of the yellow clot. Within the first 2 mm over the boundary between the red and yellow clots, platelets and leukocytes distribution is homogeneous enough over the whole length of the clot. Over this boundary, as we move further from the yellow/red boundary, more and more platelets (and leukocytes) are grouped in specific areas, or central or centrifugal platelet concentration. These zones show a high platelet/leukocyte density, inside a cell-free matrix.

This type of architecture is similar in all clots, independently of the patient, test tubes and compression method. Platelet count showed clearly that there is almost no platelet inside the RBCs layer, in PPP and in the exudate after the PRF clot compression. Therefore, the major part of platelets coming from the whole blood sample is gathered inside PRF membranes. Leukocyte count confirmed that more than half of the leukocytes is trapped inside the PRF membrane, and small lymphocytes seem to be attracted in a selective manner, as confirmed by SEM observations (Tabb.1, 2). These leukocytes do not seem to be damaged during PRF preparation. This result has a huge clinical impact as the leukocyte numbers implanted inside the membrane is substantial, and small lymphocytes are particularly efficient in inflammatory reactions' regulation. Furthermore, thanks to L-PRF cell composition, this biomaterial must be handled carefully, to maintain the cellular content viable and stable.

Therefore, the most useful portion, under a surgical point of view, is the whitish intermediate layer. Hence, it is necessary to preserve a small RBCs layer at the PRF clot boundary, that contains the bulk of platelets and leukocytes. The procedure must be carried out with scissors, and it is operator-dependent, requiring an accurate PRF architecture knowledge. Light compression of the fibrin matrix makes it so that fibrin filaments are condensed and they stick to each other. When PRF membranes are used in surgical procedures, their resorption is slow, and it helps the fibrin matrix remodeling to scar tissue.

Table 2. Count of erythrocytes, plateles and WBC on L-PRF membranes derived from clots at 0' compared 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*		χ^2	
RBC	21,6012	0.0028%	193,966	0.0025%	P=0.000	S	P=0.266	NA
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.

Table 3. Leukocyte formula stabilized in whole blood (control group) and red clot after PRF membrane collecting (test group) (by Crisci et al. 2015).

	Whole blood (%)		Group 1 (%)		Group 2 (%)	
Cell type	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**
Linfocytes	37.7	35.1-39.2	17.5	15.0-20.4	24.8	**21.4-28.0**
Monocytes	71.1	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%)**

3. CONCLUSIONS

Concluding this essay, we can affirm that, to achieve a standard procedure for PRF preparation as graft material for tissue regeneration purposes, we suggest the employment of PRF membrane's region with the highest possible platelet enrichment and, moreover, we suggest to avoid squeezing all of the PRF clot plasma. Hence, it is advisable to compress the clot with a compression device (*L-PRF Wound Box*). It's difficult, therefore, to control precisely the human-derived materials' quality, like PRF preparations, but is it important to apply the highest possible quality-control check on PRF preparations before their clinical application.

Currently, their delivery relies on poorly controlled bulk release. As consequence, prolonged treatments require multiple treatments e.g. numerous injections. This results in strongly fluctuating growth factor concentrations, which impairs clinical predictability. Biomaterials can act as controlled release devices, which will allow for sustained or even on-demand delivery of these growth factor cocktails. In addition, it can be envisioned that biomaterials can covalently bind specific growth factors to locally retain high levels of these molecules.

Further clinical, histological and statistical studies are required to understand the benefits of this new platelet concentration technique. However, we cannot sweep aside the fact that, when obtained from an autologous blood sample, produced PRF is scarce and only a limited volume can be used. This is a limitation for systematic PRF usage in General Surgery interventions. Even if the potential applications of PRF are ample, an accurate knowledge of the biomaterial is necessary, including information on its biology, efficacy and limits, to optimize its usage in everyday clinical practice.

Cell migration plays a crucial role in the healing process. MSCs represent a cell pool, able to reconstruct the damaged tissue, and

endothelial cells contribute to angiogenesis. Migration models induced by the supernatant of platelet concentrates' culture do not differ between the two types of cells.

The stronger MSCs and HUVECs migration were observed as a reply to L-PRF. All of the above signifies that L-PRF could be useful as a healing biomaterial, and as a natural anti-hemorrhagic agent to be used at surgical sites.

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