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TESI di DOTTORATO

"**White cell and platelet content affect the release of bioactive factors in different blood-derived scaffolds**"

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1. Introduction

Platelet concentrates for surgical use are innovative tools of regenerative medicine and are widely used for clinical and surgical applications that require tissue regeneration [Bielecki et al., 2012; Burnouf et al., 2013]. Their potential beneficial outcomes, including hard and soft tissue regeneration [Marx et al., 1998], local haemostasis [Sammartino et al., 2011] and acceleration of wound healing [Picard et al., 2015], make them suitable, in different medical fields, as therapeutic options [Del Corso et al., 2012; Dohan et al., 2014; Leo et al., 2015]. The driving concept is to collect and gather the most active components from the blood sample, like platelets, fibrin and eventually leukocytes and to prepare them in a clinically usable form [Dohan et al., 2013].

1.1 Definition and history

Platelet concentrates for surgical use are autogenous products prepared through the centrifugation of a blood sample of the patient [Dohan et al., 2013]. These preparations can be solutions or gels and can be injected or placed in a surgical site, on a wound or in an injured area, in order to regenerate the damaged tissues [Bielecki & Dohan, 2012].

In transfusion medicine, a specific platelet concentrate was originally named PRP (Platelet Rich Plasma) to describe a blood component that contains high levels of platelets prepared by using a centrifuge to separate the platelet-rich fraction from the whole blood and used for the treatment and prevention of hemorrhage in thrombocytopenic patients [Borzini et al., 2012]. Platelet-rich plasma is defined as the volume of the plasma fraction from autologous blood having platelet concentration above baseline (200.000 platelets/µl) [Mazzucco et al., 2009]. In particular, for example, advantageous biological effects on bone regeneration seem to occur when PRP with a platelet concentration of approximately 1.000.000/µl is used [Weibrich et al., 2004].

The first topical application of platelet gel, called "platelet-fibrinogen-thrombin mixtures", was that used by Rosenthal for the corneal adhesion [Rosenthal et al., 1975] and to seal perforating corneal wounds in the rabbits [Rosenthal et al., 1978]. Then it was used, with the name "platelet-fibrinogen-thrombin adhesives", to reduce the hemorrhage related to microvascular anastomosis in a rat model [Pearl et al., 1977] and to repair the cerebrospinal fistulas in dogs [Silverberg et al., 1977].

Several years after, these products were considered not only as fibrin tissue adhesive but having direct healing properties. In fact, Knighton and collaborators (1986) used platelet concentrates containing such as "platelet

derived wound healing factor" (PDWHF) for the treatment of chronic non-healing cutaneous ulcers.

Finally, in 1998, Marx reintroduced the concept and the term PRP to define an autologous source of platelet-derived growth factor and transforming growth factor beta and used it in maxillofacial bone reconstruction [Marx et al., 1998]. Few years later, a second generation of platelet concentrates was introduced such as PRF (Platelet Rich Fibrin) by Choukroun and started to replace the use of PRP in oral and maxillofacial surgery [Dohan et al., 2006].

1.2 Classification and techniques

Several techniques for platelet concentrates are available. However, their applications have been confusing because each method leads to a different product with different biology and potential uses [Dohan et al., 2012a].
Three main sets of parameters are necessary for a clear classification of platelet concentrates.
The first set of parameters relates to the used preparation kits and centrifuges. The size of the centrifuge, the duration of the procedure and the cost of the device and kits are significant factors considering the repetitive use of these techniques in daily surgical practice.

The second type of parameters relates to the content of the concentrate. The efficiency in collecting platelets and leucocytes and their preservation during the entire process defines the basic pharmacological relevance of the product and indicates its potential applications.

The third set of parameters relates to the fibrin network that supports the platelets and leucocytes concentrate during its application. The density of the fibrin network and the fibrin polymerization process may affect the biomechanical properties of the concentrate.

Using these sets of parameters, the available methods can be classified in four main categories, depending on the pharmacological and material characteristics of the obtained product: pure PRP (P-PRP), leucocyte-rich PRP (L-PRP), pure PRF (P-PRF) and leucocyte-rich PRF (L-PRF) [Dohan et al., 2009a], as described in the following table.

Table. Classification of the main available methods of production of platelet concentrates, in the 4 main families of products [Dohan et al., 2009a].

Platelet Concentrate Class and terminology		Methods of production (generic name, detailed appellation when existing, company, city, country)
P-PRP (Pure Platelet- Rich Plasma), before activation (**P-PRP gel** after activation)	**AP**	- Cell separator PRP (experimental) - Vivostat PRF (Vivolution, Alleroed, Denmark)
	MP	- PRGF/Endoret (Preparation or Plasma Rich in Growth Factors, BTI BioTechnology Institute, Vitoria, Spain) - E-PRP (Eye Platelet-rich Plasma) (experimental) - Nahita PRP (Nahita, Navarra, Spain)
L-PRP (Leukocyte- and Platelet-Rich Plasma), before activation (**L-PRP gel**, after activation)	**AP**	- PCCS PRP (Platelet Concentrate Collection System, Palm Beach Gardens, FL, USA) - SmartPReP PRP (Harvest Corp, Plymouth, MA, USA) - Magellan PRP (Magellan APS (Autologous Platelet Separator), Medtronic, Minneapolis, MN, USA) - Angel PRP (Angel Whole Blood Processing System (AWBPS), Sorin Group, Mirandola, Italy) - GPS PRP (Gravitational Platelet Separation System, Biomet Biologic, Warsaw, IN, USA)
	MP	- Friadent PRP (Friadent-Schütze, Vienna, Austria) - Curasan PRP (Curasan, Kleinostheim, Germany) - Regen PRP (Regen Laboratory, Mollens, Switzerland) - Plateltex PRP (Plateltex, Prague, Czech Republic) - Ace PRP (Surgical Supply and Surgical Science Systems, Brockton, MA, USA)
P-PRF (Pure Platelet- Rich	**MP**	Fibrinet PRFM (Cascade Medical, Wayne, NJ, USA)
L-PRF (Leukocyte- and Platelet-Rich Fibrin)	**MP**	- Intra-Spin L-PRF (Intra-Lock, Boca Raton, FL, USA) - Titanium-prepared PRF (experimental)

AP: Automated Procedures; MP: Manual Procedures

Pure Platelet-Rich Plasma (P-PRP) – or Leukocyte-Poor Platelet-Rich Plasma products are preparations without leukocytes and with a low density fibrin network after activation. Per definition, all the products of this family can be used as liquid solutions or in an activated gel form. Many methods of preparation exist, particularly using cell separators (continuous flow plasmapheresis) from hematology laboratory. One largely advertised method of P-PRP is known under the commercial name PRGF (Plasma Rich in Growth Factors) and was tested in many clinical situations. This manual procedure was invented by Anitua in 1999 [Anitua, 1999] and is currently marked by BTI, BioTecnology Institute (Vitoria, Spain). After a soft centrifugation of blood with anticoagulant, four layers appear in the tube: the superficial plasma suspension is often called PPGF (Plasma Poor in Growth Factors), the intermediate plasma suspension is called PRGF (Plasma Rich in Growth Factors), the whitish layer below the PRGF is called "buffy coat", and the red blood cells are finally gathered at the bottom of the tube. The PPGF and the PRGF are collected by pipetting the plasma solution above the red blood cell base. The specificity of this technique is that the buffy coat layer (between the red blood cells base and the acellular plasma) is not harvested in order to avoid the collection of leukocytes.

Another technique of P-PRP is known under the commercial name Vivostat PRF (Platelet-Rich Fibrin, Vivostat A/S, Alleroed, Denmark), what can be a source of confusion as this technique is not a PRF following the terminology, but clearly a P-PRP product [Dohan et al., 2009a].

Leukocyte-and Platelet-Rich Plasma (L-PRP) products are preparations with leukocytes and with a low-density fibrin network after activation. Per definition, like the P-PRP, all the products of this family can be used as liquid solutions or in an activated gel form. It can therefore be injected (for example in sports medicine) or placed during gelling on a skin wound or suture (similar to the use of fibrin glues) [Dohan et al., 2009a].

It is in this family that the largest number of commercial or experimental systems exists with many interesting results in general surgery, orthopedic and sports medicine. Particularly many automated protocols have been developed in the last years, requiring the use of specific kits that allow minimum handling of the blood samples, for example Harvest Smart-PreP (Harvest Technologies, Plymouth, MA, USA) and Biomet GPS III (Biomet Inc., Warsaw, IN, USA). Other kits with more handling also exist, such as Plateltex (Prague, Czech Republic) or RegenPRP-Kit (RegenLab, Le Mont-sur-Lausanne, Switzerland).

Pure Platelet-Rich Fibrin (P-PRF) – or Leukocyte-Poor Platelet-Rich Fibrin – are preparations without leukocytes and with a high-density fibrin network. Per definition, these products only exist in a strongly activated gel form, and cannot be injected or used like traditional fibrin glues. There is only one product in this family, commercially known as Fibrinet PRFM (Platelet-Rich Fibrin Matrix, Cascade Medical, Wayne, NJ, USA, also marketed for orthopedic applications by Vertical Spine, Marconi Road Wall, NJ, USA). The main inconvenient of this technique remains its high cost and relative complexity in comparison to the other forms of PRF available, the L-PRF (Leukocyte- and Platelet-Rich Fibrin) [Dohan et al., 2009a].

Leukocyte- and Platelet-Rich Fibrin (L-PRF) products are preparations with leukocytes and with a high-density fibrin network. Per definition, these products only exist in a strongly activated gel form, and cannot be injected or used like traditional fibrin glues. One largely advertised method of L-PRF is called Choukroun's L-PRF. This technique was invented in 2001 by Choukroun [Choukroun et al., 2001] and adequate kits and centrifuge are marketed by Process (Nice, France). This technique is often considered as a second generation platelet concentrate technology.

To summarize briefly, all available PRP techniques have some points in common: blood is collected with anticoagulant just before or during surgery and it is immediately processed by centrifugation. The time for platelet concentrate preparation is variable but it is always completed within an hour. A first centrifugation step is designed to separate the blood into three layers, red blood cells (RBCs) are found at the bottom, acellular plasma (PPP, platelet-poor plasma) is in the supernatant level and a "buffy coat" layer appears in between, in which platelets are concentrated. The next steps vary among the numerous protocols but are an attempt to discard both the RBC layer and the PPP layers to collect only the "buffy coat" stratum. Finally, the obtained platelet concentrate is applied to the surgical site with a syringe, together with thrombin and/or calcium chloride (or similar factors) to trigger platelet activation and fibrin polymerization.

Although the methods for platelet gel preparation are similar, there are different performances concerning growth factor recovery and the kinetics of its release from the gel [Mazzucco et al., 2009].

In Choukroun's PRF (platelet-rich fibrin) the blood is collected without any anticoagulant and immediately centrifuged. Then a natural coagulation process then occurs and allows for the easy collection of a leucocyte- and platelet-rich

fibrin (L-PRF) clot, without the need for any biochemical modification of the blood since no anticoagulants, thrombin or calcium chloride are required.

1.3 Tridimensional structure

Although platelet and leucocyte play an important role in the biology of platelet concentrates, the fibrin architecture which support them as well as the biomechanical properties of the final fibrin network are essential factors [Clark, 2001]. Fibrin matrix is considered an efficient matrix for mesenchymal stem cells transplantation [Bensaid et al., 2003] and for tissue engineering [Whelan et al., 2014].

The formation of the fibrin matrix during platelet concentrate activation and the leucocyte concentration are obviously key parameters of the growth factors release [Dohan et al., 2009b].

The fibrin polymerization process is based on fibrinogen and thrombin concentrations. Fibrinogen is activated by thrombin, which initiates polymerization into fibrin. In PRP concentrates, these kinetics depend on the pro-coagulant enzyme used to induce the gel formation [Mazzucco et al., 2009]. Fibronectin and vitronectin are two key cell adhesion and migration proteins and they are also other components of the architecture of the fibrin clot [Dohan et al., 2009b].

The type of used tube (dry glass or glass-coated plastic tubes) and the compression process of the clot (forcible or soft) did not seem to influence the architecture of PRF but these two parameters could influence the growth-factor content and the matrix properties of the product [Dohan et al., 2010b]. However, the fibrin fibrillae can be assembled in two different biochemical architectures: either via condensed tetramolecular or bilateral junctions or via connected trimolecular or equilateral junctions [Mosesson et al., 2001]. Bilateral junctions are provoked by a drastic activation and polymerization, for example with high thrombin concentrations, that leads to a dense network of monofibres similar to a fibrin glue, which is not particularly favorable to cytokine enmeshment and cellular migration. On the contrary, a slow physiological fibrin polymerization yields a higher percentage of equilateral junctions, which allow the establishment of a flexible fibrin network with multifibre assembly that is capable of supporting cytokine enmeshment and cellular migration.

In typical P-PRP and L-PRP preparations, the fibrin network is immature and consists mainly of fibrillae with a small diameter. This fibrin network supports platelet application during surgery but dissolves quickly like a fibrin glue. In P-PRF and L-PRF preparations, fibrin clot is thick and constitute a resistant matrix that can be considered as a fibrin biomaterial [Dohan et al., 2009a].

2. The biological behavior

2.1 Tissue repair and regeneration

Healing of an acute wound occurs through 3 sequential and partially overlapping phases: the fibrin clot, the recruitment of inflammatory cells to the wound site and the reepithelialization [Martin, 1997].

The clot forms immediately after wounding and consists of platelets embedded in a mesh of crosslinked fibrin fibers derived by thrombin cleavage of fibrinogen, together with smaller amounts of plasma fibronectin, vitronectin, and thrombospondin. The clot acts as a physical barrier against microbial infections; it is a reservoir of growth factors and cytokines, and provides a favorable environment for cell migration. Neutrophils normally begin arriving at the wound site within minutes of injury, followed by monocytes and lymphocytes. The proliferation phase then starts with the migration and proliferation of keratinocytes followed by the proliferation of fibroblasts. Angiogenesis and nerve sprouting occur later. After a few days, the remodeling phase and reepithelialization occur. The entire process is regulated by various growth factors and cytokines [Werner & Grose, 2003].

Thus, many cell types, including blood cells, fibroblasts and endothelial cells participate to the final healing process and each cell type may specifically affect

the function of the other cell types, both by cell-cell and cell-matrix contacts and by producing and releasing soluble factors [Singer & Clark, 1999]. The extracellular matrix provides structural and functional support to the cells and tissues, and it consists of several molecules such as collagen, proteoglycans, heparin sulfate, chondroitin sulfate, hyaluronic acid, elastin, fibronectin, and laminin. A few plasma-derived proteins such as fibrin, thrombospondin, and fibronectin act as provisional ECM (extracellular matrix). Soluble factors include cytokines, chemokines, hormones, nucleotides, electrolytes, free ions, and growth factors [Mazzucco et al., 2010].

Normal wound repair includes a vigorous angiogenic response that delivers nutrients and inflammatory cells to injured tissue [Nissen et al., 1998]. The mediators of wound angiogenesis include numerous soluble factors that have been identified in various wound models [Lynch & Ahsan, 2014; Förster et al., 2016,].

2.2 The release of cytokines and growth factors by different platelet concentrates

The complex mechanisms regulating tissue repair and regeneration is still incomplete and unfortunately, neither clinical outcomes nor biochemical

mechanisms are sustained by a comprehensive literature on the role of growth factors in accelerating tissue repair [Mazzucco et al., 2010].

During normal tissue repair in vivo, platelets release high concentrations of biologically active proteins, such as growth factors (GFs), and other substances [Reed 2004]. Primary factors associated with platelets include Platelet Derived Growth Factor (PDGF) and Transforming Growth Factor β (TGF-β), which have been mostly involved in cell proliferation, chemotaxis and extracellular matrix production/angiogenesis [Lubkowska et al., 2012; Burnouf et al., 2013]. Other GFs discharged from the platelets are Fibroblast Growth Factors (FGF) 1 and 2 and Vascular Endothelial Growth Factor (VEGF) which play critical roles in the hemostasis, proliferative and remodeling phases of wound healing [Intini, 2009; Lacci et al., 2010; Scarano et al., 2016]. Platelets degranulation also leads to the release of cytokine and chemokines, such as CCL5, IL-1β, IL-8 and MIP-1α, which contribute to the healing process [Rozman & Bolta, 2007].

Platelets represent a known source of cytokines and growth factors involved in wound healing and tissue repair [Demidova-Rice et al., 2012, Murphy et al., 2012]. Many platelet-derived factors are considered important players in wound healing processes. In particular, beside their known functions in hemostasis and clot formation, platelet granules contain growth factors, including platelet-

derived growth factor (PDGF), transforming growth factor β (TGF-β), IGF-1, involved in cell proliferation and differentiation [Prakash &Thakur, 2011].

The bioavailability of growth factors in tissue healing depends on the amount of growth factors stored in platelets but a portion of these is lost during platelet manipulation [Mazzucco et al, 2009].

Leucocytes are also a significant source of cytokines and growth factors which may synergistically interact with those released by platelets [Keen, 2008].

The main growth factors involved in the tissue repair and their functions are as the follow:

- *Platelet-derived growth factor* (PDGF): mitogen for fibroblasts, smooth muscle cells and glia cells; chemotactic [Alvarez et al., 2008; Andrae et al., 2008]; stimulates bone DNA synthesis [Canalis, 1981]; promote periodontal tissue regeneration of tooth-supporting osseous defects [Sarment et al., 2006] and in both Class II furcations and interproximal intrabony defects [Nevins et al., 2003].

- *Vascular endothelial growth factor* (VEGF): Mitogen for vascular endothelial cells; angiogenic; chemoattractant for monocytes; induces the synthesis of the metalloproteinase, interstitial collagenase;

differentiation of adipocytes [Nissen et al., 1998; Gale & Yancopoulos, 1999; Tarsitano et al., 2006]

- *Epidermal growth factor* (EGF): Mitogen; proliferation; differentiation; angiogenesis [Seeger & Paller, 2015].

- *Transforming Growth Factor β* (TGF-β): Growth inhibitor for normal and transformed epithelial cells, endothelial cells, fibroblasts, neuronal cells, lymphoid cells; angiogenesis; induces the synthesis of bone matrix and ECM [Lamora et al., 2016].

- *Insulin like growth factor-1* (IGF-1): Mitogen; differentiation; cell proliferation and matrix synthesis by chondrocytes and osteoblasts [Trippel, 1998; Conover, 2000].

- *Fibroblast growth factor* (FGF): Mitogen; differentiation; angiogenesis [Beenken & Mohammadi, 2009].

It was proven that L-PRF membranes slowly release significant amounts of some growth factors (TGFβ-1, PDGF-AB, VEGF) and thrombospondin-1 (TSP-1), a matrix protein, during at least 7 days. In particular, the PDGF-AB initial quantities are similar to the final released amounts after 7 days, but the released amounts of TGFβ-1 and VEGF after 7 days are more than 6 times higher than the initial content [Dohan et al., 2009b].

A recent study compared the effect of two different platelet concentrates (PRP and PRF) on the release of cytokines and growth factors [Passaretti et al., 2014].

The amount of several inflammatory cytokines such as IL-6, IL-8, IL-10, IFN-γ, MIP-1a, MIP-1b, TNFα, was found higher in the PRF compared to PRP. At variance, the levels of RANTES were 3-fold higher in PRP compared to PRF. Concerning the concentration of growth factors, the levels of FGF were detectable in small amounts compared to the other growth factors both in PRF and in PRP. VEGF and TGFb1 levels were 11- and 2.6-fold higher, respectively, in PRF compared to PRP. Instead, the amount of PDGF was 2-fold lower in PRF compared to PRP.

Interestingly, while PRP released significantly higher amounts of PDGF and RANTES, the amount of several cytokines, typically involved in wound healing and re-vascularization, was more abundant in PRF. It is conceivable that the enrichment of platelets obtained in the standard procedure for PRP is responsible for the higher release of PDGF. Also, RANTES, a proinflammatory chemokine, is very abundant in platelets [Gleissner et al., 2008]. On the other hand, the clot obtained by the PRF procedure is most likely enriched in white blood cells, which may represent major producers of inflammatory cytokines (IL-6, IL-8, IL-10, IFN-γ, MIP-1a, MIP-1b, TNFα) and of pro-angiogenic

factors (VEGF and TGFb1). These differences may recommend the use of one or the other method, based on the specific goal to achieve.

2.3 The role of the leucocytes and the platelets in inflammation and infection

The inclusion of the leukocytes within platelet biomaterials has always been a matter of debate. White blood cells are mainly considered to contain and produce inflammatory cytokines. Nevertheless, they play an important role as immune modulators [van Dooren et al., 2013].

Some Authors claim the elimination of the leucocytes from the surgical platelet concentrates [Anitua et al., 2007]. However, the leucocytes, when present in moderate quantities in platelet concentrates, are essential actors in wound healing [Martin & Leibovich, 2005]. Moreover, some Authors have suggested that the inclusion of the leucocytes in the fibrin mesh may help to improve the stability of the scaffold while increasing the antimicrobial potential of the formulation [Dohan et al., 2009a]. Leukocytes are not only inflammatory cells, but they also present anti-nociceptive effects through different chemokines, anti-inflammatory cytokines (IL-4, IL-10 and IL-13) and opioid peptides (β-endorphin, metenkephalin, and dynorphin-A), and can therefore promote a clinically relevant inhibition of pathological pain [Dohan et al., 2014].

They clean up the surgical site, regulate the expression of inflammatory and wound-healing chemokines, and secrete angiogenic factors such as VEGF.

Viable neutrophilic polymorphonuclear leukocytes or neutrophils play an important role in the innate immune defense against infections [Everts et al., 2006].

Furthermore, it has been demonstrated that platelets also have an antimicrobial efficacy, as they contain multiple antimicrobial peptides, such as platelet factor 4 (PF-4), RANTES, connective tissue activating peptide 3 (CTAP-3), platelet basic protein, thymosin β-4 (Tβ-4), fibrinopeptide B (FP-B), and fibrinopeptide A (FP-A) [Tang et al., 2002]. In addition to their capacity to release prestored mediators, stimulated platelets are able to generate eicosanoids for regulation of hemostasis and acute inflammation [Klinger & Jelkmann, 2002].

For these reasons these biological blood product, also have antimicrobial properties.

The antimicrobial effect of PLG (platelet-leukocyte gel) against Staphilococcus aureus has demonstrated to be limited to the first hours after application. Although a proportional reduction of bacteria of approximately 99% compared to the control could be maintained up to 8 h, the number of bacteria started to increase again after 4 h [Moojien et al., 2008].

Incorporating the patient's leucocytes into the PRGF formulation, that do not contain leucocytes, all formulations had a strong bacteriostatic effect, especially in the first 4 h after application, and no differences in the bacterial inhibitory effect were found between the formulations, showing that an additional leucocyte dose does not significantly improve the antimicrobial properties of PRGF [Anitua et al., 2012].

2.4 The biological effect on cells

Several studies have assessed the biologic response of oral cells to PRP [Kanno et al., 2005; Graziani et al., 2006]. Most of them evaluated the capacity of these preparations to induce cell proliferation and bone differentiation. Okuda and collaborators demonstrated that PRP stimulated osteoblastic DNA synthesis and cell division and DNA synthesis in gingival fibroblasts and periodontal ligament cells [Okuda et al., 2003].

On human gingival fibroblasts (GFs), PRP modulated relevant mechanisms potentially involved in wound repair, including cell adhesion, cell migration, and myofibroblastic differentiation in GFs [Caceres et al., 2008].

Although the effect of PRP on proliferation was dose-dependent, increasing PRP concentrations did not result in increasing proliferation [Graziani et al., 2006].

A recent study compared the effect of two different platelet concentrates (PRP and PRF) on the ability to induce the growth of primary cultures of human fibroblasts and vascular endothelial cells (HUVEC) [Passaretti et al., 2014]. PRP gel and PRF induced proliferation of both cell types. However, PRP was significantly more effective than PRF in inducing fibroblast growth. At variance, PRF effect on proliferation of endothelial cells was slightly higher than that of PRP, suggesting a prevalent pro-angiogenic function.

All previous studies on human bone mesenchymal stem cells BMSC cultures in the presence of PRP have demonstrated contradictory results. The platelet concentrate generates a strong proliferation, but inhibits osteogenic differentiation [Arpornmaeklong et al., 2004; Gruber et al., 2004].

In the same way, PRP supplemented MSC numbers compared to control but retained a similar capacity to differentiate towards the osteogenic, chondrogenic and adipogenic lineage [Vogel et al., 2006].

On the contrary, Graziani and collaborators demonstrated that the increasing of PRP concentrations led to increased production of osteocalcin (OCN) and decreased production of osteoprotegerin (OPG) by the osteoblast harvested from oral sites, indicating that PRP is an effective stimulator of osteoblast [Graziani et al., 2006].

On the other hand, in vitro effects of Choukroun's PRF on human bone mesenchymal stem cells (BMSC), harvested in the oral cavity, showed that the number of BMSC in culture was significantly higher compared to the control. Thus, it was able to significantly stimulate BMSC proliferation in vitro. Moreover, the osteoblastic differentiation analysis of the BMSC, performed by the assessment of the ALP activities per cell and the number of mineralization nodules in culture, was significantly higher compared to the values of the respective control [Dohan et al., 2010a].

PRP was shown to have an angiogenic effect on human umbilical vein endothelial cells (HUVECs) in co-culture with human dermal fibroblast cells (NHDFs) in vitro. PRP with gelatin hydrogel granules significantly enlarged the area containing newly formed capillaries and promoted the microvascular network in murine subcutaneous tissue in vivo, representing a potential therapeutic combination for the treatment of ischaemic disorders [Kakudo et al., 2015].

PRGF was able to activate neuronal progenitor cells, enhancing hippocampal neurogenesis, and to reduce Aβ-induced neurodegeneration in a mouse model of Alzheimer's disease [Anitua et al., 2013].

3. Clinical applications of platelet concentrates

- Reconstructive and implant surgery

In bone graft, implant and reconstructive surgery, the literature is particularly dense about the use of the various forms of PRP subfamilies and PRF [Simonpieri et al., 2012]. To understand if these preparations can improve or accelerate bone healing and remodeling is still very complex, and the analysis of the literature does not give a clear answer [Simonpieri et al., 2012]. The first application of topical use of platelet concentrates in oral surgical started about 20 years ago [Whitman et al., 1997; Marx et al., 1998]. Adding platelet-rich plasma to grafts, it was evidenced a higher radiographic maturation rate than that of grafts without platelet-rich plasma and also a greater bone density in grafts in which platelet-rich plasma was added than in grafts in which platelet-rich plasma was not added [Marx et al., 1998].

In vitro cell data are quite contradictory with PRP. As a whole, PRPs are considered to increase the proliferation of osteoblasts in various cell models [Kanno et al., 2005; Graziani et al., 2006], even if the contrary was also proven [Cenni et al., 2005],

Many in vivo data were collected in various animal models with various PRPs alone or in association with various bone biomaterials. Some Authors concluded that PRP gels have no impact on bone regeneration alone or in

association with bone graft [Broggini et al., 2011; Torres et al., 2008]. On the contrary, other Authors concluded that these PRP gels stimulate significantly bone healing in association with bone graft.

In a recent systematic review, randomized controlled clinical trials comparing a group receiving PRP as an adjunctive material in the sinus lift technique with a control group without PRP, were included [Pocaterra et al., 2016]. Of the studies identified, only one reported a significant difference in bone augmentation in favor of the adjunctive use of PRP [Torres et al., 2009]. The results of the meta-analysis seem to indicate that PRP does not provide additional benefits in bone formation or improve the implant survival rate. However, given the low number of included studies and the high risk of bias of them, no clinical recommendation can be made.

In implant surgery PRF membranes was used to cover the head of the implants, and thus act as a fibrin bandage between the allograft and the gingival tissue [Simonpieri et al., 2009a]. Moreover, the use of PRF led to a substantial thickness of keratinized gingival tissue around the implants, playing a significant role on the stability of the grafted bone surface [Simonpieri et al., 2009b] and on the final result of prosthodontic rehabilitation improving the aesthetic integration [Saadoun & Touati, 2007].

PRF seems to reduce postoperative pain and edemas, and to limit even minor infectious phenomena. Thus, the control of inflammation and especially the risk of sepsis within a bone graft seems yet another advantage to use PRF during bone grafting [Simonpieri et al., 2009b].

- <u>Prevention of hemorrhagic complications after dental extraction</u>

Dental extractions in heart surgery patients treated with artificial mechanical heart valves under anticoagulant oral therapy can be difficult as these patients present a significant risk for postoperative hemorrhagic complications [Scully & Wolff, 2002].

PRP gel placed into residual alveolar bone after extraction without heparin administration after suspension of oral anticoagulant drugs reduced the bleeding after dental extractions [Della Valle et al., 2003].

At the same way, the application of L-PRF clots significantly reduced the bleeding after dental extractions without the suspension of the continuous oral anticoagulant therapy in heart surgery patients [Sammartino et al., 2011]. The advantage of this concentrate is that PRF has plastic and soft tissue–adhesive properties and it is much cheaper than fibrin glues and most PRP available kits [Sammartino et al., 2011].

- Periodontology

The effect of platelet concentrates in periodontology is controversial.

PRP was found effective in inducing and accelerating bone regeneration for the treatment of periodontal defects at the distal root of the mandibular second molar after surgical extraction of a mesioangular deeply impacted mandibular third molar compared with the controls [Sammartino et al., 2005]. In the same clinical application, comparing PRP alone versus PRP and resorbable membrane, clinical results can be considered similar, but from a histologic point of view, the association showed earlier signs of bone maturation but not a higher grade of bone regeneration [Sammartino et al., 2009].

Comparing intra-bony defects treated either with autologous PRF or a conventional open flap debridement alone, there was greater reduction in probing depth (PD), more clinical attachment level (CAL) gain and greater intra-bony defect fill at sites treated with PRF than the open flap debridement alone [Thorat et al., 2011].

More recently PRF was compared to demineralized freeze-dried bone allograft (DFDBA) in intrabony defects, showing no significant difference between materials in CAL gain as well as in bone fill after 6 months of healing [Chadwick et al., 2016].

On the other hand, the addition of PRF did not improve the clinical and radiographic outcomes in the treatment of intrabony defects (IBDs) in chronic periodontitis patients with enamel matrix derivative (EMD) and EMD + platelet-rich fibrin [Aydemir Turkal et al., 2016].

In the gingival recession treatment, Jancovic and collaborators showed that the use of PRF membranes provided acceptable clinical results, followed by enhanced wound healing and decreased subjective patient discomfort compared to connective tissue graft (CTG). However, a greater gain in keratinized tissue width was obtained in the CTG group respect to PRF group [Jancovic et al., 2012].

- Orthopedic and sports medicine

There is a very large debate in sports medicine on the selection of the adequate technique, particularly concerning the exact cell content of the injectable platelet suspensions [Dohan et al., 2014; Mishra et al., 2012].

Preclinical and human cell culture studies support the use of PRP for the treatment of tendon injuries and disorders [Kajikawa et al., 2008], ligament injuries and muscle injuries [Sanchez & Anitua, 2009].

Moreover, it is thought that PRP can stimulate chondral anabolism, reduce catabolic processes, and may improve overall joint homeostasis reducing synovial membrane hyperplasia in osteoarthritis [Kon et al., 2009].

- Plastic surgery and dermatology

A beneficial influence of PRP on wound healing was described thanks to the improved proliferation of endothelial cells and vascularization and the stimulating effects on formation of granulation tissue [Sommeling et al., 2013]. Not only did more wounds heal when platelet gels were used for chronic ulcers, but also the time to healing and the hospital stay were notably shorter, leading to a decrease of sickness-related morbidity and health costs [Mazzucco et al., 2004; Saad Setta et al., 2011].

More recently, interest has been increasing in the application of PRP in dermatology, for example, in skin rejuvenating effects [Sommeling et al., 2013] and as a new therapy for androgenetic alopecia (AGA), showing an increased hair density when comparing with the control side [Alves & Grimalt, 2016], clinical improvement in the mean number of hair and in the number of hair follicles 2 weeks after the PRP treatment [Gentile et al., 2015].

4. White cell and platelet content affects the release of bioactive factors in different blood-derived scaffolds

4.1 Introduction

Several technical procedures have been developed to obtain different platelet concentrates with variable yield of platelets and cellular components [Dohan et al., 2012a; Cieslik-Bielecka et al., 2012]. Consistently, they also differ for the qualitative and quantitative release of GFs, cytokines and chemokines and may find different indications [Rozman & Bolta, 2007; Passaretti et al., 2014; D'Esposito et al., 2015]. For instance, Plasma Rich in Growth Factors (PRGF) provides supra-physiological concentrations of growth factors at the injury environment, that could be used therapeutically to accelerate natural healing [Anitua et al., 2009]. PRGF does not contain leucocytes and requires a specific centrifugation to separate the whole blood into two or three fractions, from the poorest in GFs (the upper fraction) to the richest in GFs (the lower fraction) [Anitua et al., 2012; Anitua et al., 2015]. Additionally, in a new protocol PRGF is obtained from the whole plasma column with a reduction in the amount of anticoagulant and activator [Anitua et al., 2016]. Continuous attempts are made to develop new, easy-to-use platelet-derived biomaterials. Leucocyte-Platelet Rich Fibrin (L-PRF) is one such product specifically developed for use in oral and maxillofacial surgery and tissue healing [Dohan et

al., 2016; Marenzi et al., 2015]. This procedure requires blood collection without anticoagulant and immediate centrifugation for the formation of a fibrin clot, which includes not only platelets but also leucocytes [Sammartino et al., 2011; Marenzi et al., 2015]. Leucocytes are also a significant source of cytokines and GFs which may synergistically interact with those released by platelets. Together with many cell types, including blood cells, fibroblasts and endothelial cells, leucocytes participate to the final healing process and, with an intricate crosstalk, each cell type may specifically affect the function of the other cell types [Passaretti et al., 2014; D'Esposito et al., 2015]. Most recently, a modified L-PRF protocol has emerged. A-PRF procedure requires glass tubes, a decreased velocity and an increased time of centrifugation compared to original L-PRF. The modification of the original protocol has been carried out to further improve tissue regeneration [Ghanaati et al., 2014]. However, little is known about the leucocyte composition and the factors released by A-PRF. Therefore, the aim of this study was to gain insight into the leucocyte subpopulations of L- and A-PRF and investigate cytokines and growth factors released by these biomaterials, in comparison with PRGF.

4.2 Methods

4.2.1 Subject characteristics and biomaterial preparation

For the preparation of L-PRF, A-PRF and PRGF, blood collection was carried out on nine healthy volunteer donors (M/F: 4/5; age: 26,4± 1.6). All were non-smokers, non-obese (BMI range: 20.4-26.3) and with no history of recent medication neither disease correlated with the coagulation process. Platelet, red blood cell, and leucocyte counts were determined in anticoagulated blood from the donors. Informed consent was obtained from every subject before blood drawing. The procedure was approved by the ethical committee of the University of Naples (prot. n° 148_2015).

Four tubes of blood without anticoagulant were obtained from the antecubital vein of each volunteer to obtain two L-PRF and two A-PRF clots.

L-PRF clots were prepared from 9-ml of peripheral blood collected in plastic tubes (Intra-Spin by Intra-Lock, Boca-Raton, USA) without anticoagulant and immediately centrifuged for 12-min at 700 g using a dedicated centrifuge (PC-02, PROCESS for PRF, Nice, France), as recommended by the manufacturer. For a better comparison of the results, A-PRF clots were obtained by 9-ml of whole blood, instead of commonly used 10-ml, from a single 14-min step of centrifugation at 100 g using glass tubes (Process, Nice, France) according to manufacturer's instruction.

PRGF preparations were obtained as described by Anitua et al. 2012. Briefly, blood trough antecubital vein puncture was collected into 9-ml tubes with 3.8% sodium citrate and was separated in a centrifuge (BTI System IV, Victoria, Spain) at 580 g for 8-min at room temperature. After centrifugation, PPGF (Plasma Poor in Growth Factors) was discarded and the remaining plasma was drawn off in three consecutive fractions. The first fraction (F1) made up about 2 ml located at the top of the tube, F2 formed the next 1 ml and the F3 the bottom ml just above the red cell layer. Each fraction was gently poured in a sterile glass container and was activated by adding 10% calcium chloride and waiting a dense polymerization at 37°C for 15 minutes.

4.2.2 Sample preparation

For the immunophenotypic analysis of cells isolated from PRF, a single-cell leukocyte suspension was prepared by pressing the clots through a 120 μm nylon screen mesh. Then, cell suspensions were centrifuged (300 × g for 5 minutes, 4°C) and washed twice with PBS. To remove erythrocytes, cell pellets were resuspended in 1 ml of ACK lysing buffer (Lonza, Basel, Switzerland), incubated for 10 minutes on ice and washed twice with PBS.

For the evaluation of platelet preparation release, L- and A-PRF clots and PRGF gels were incubated for 24 hours in serum-free Dulbecco modified Eagle

medium (DMEM)-F12 (1:1) containing 0.25% BSA. Then, conditioned media were collected and centrifuged at 14000 x g to remove cellular debris and analyzed for cytokine and growth factor content, as described below.

4.2.3 Flow cytometry

Single-cell leukocyte suspensions were stained (20 minutes, 4°C) in PBS + 10% human AB serum (Lonza, Basel, Switzerland) + 0.05% NaN3 (Staining buffer, SB). The following antibodies were used: anti-CD45 APC (clone 5B1, dilution 1:20), anti-CD25 PE (clone 4E3, dilution 1:20), anti-CD3 PerCP (clone BW264/56, dilution 1:10), anti-CD14 FITC (clone TÜK4, dilution 1:20) (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD4 FITC (clone RPA-T4, dilution 1:10), anti-CD4 PE-Cy7 (clone SK3, dilution 1:40), anti-CD8 FITC (clone SK1, dilution 1:20), anti-CD45RA FITC (clone L48, dilution 1:20), anti-CD45RO PE (clone UCHL1, dilution 1:20), anti-HLA-DR PE (clone L243, dilution 1:20), anti-CD56 FITC (clone NCAM16.2, dilution 1:20) (BD Biosciences, Franklin Lakes, NJ, USA). Samples were acquired on a BD LSRFortessa and analyzed using FACSDiva Software (BD Biosciences, Franklin Lakes, NJ, USA). Data are expressed as percentage of positive cells and median fluorescence intensity (MFI) of positive cells.

4.2.4. Determination of cytokines and growth factors released by platelet derivatives

Conditioned media collected from L-PRF, A-PRF and F1, F2 and F3 gels were screened for the concentration of IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, Eotaxin, IFNγ, MIP-1α, MIP-1β, CCL5, TNFα, PDGF and VEGF using the Bioplex Multiplex human cytokine and growth factor assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

4.2.5 Statistical analysis

Data were analyzed with GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA) by unpaired two-tailed t-test and one way Anova followed by Sidak's multiple comparison tests. p value of <0.05 was considered statistically significant.

4.3 Results

4.3.1 Analysis of leukocyte subpopulations isolated from L-PRF and A-PRF

We selected nine blood donors whose mean leukocyte count was 5.64±0.5 x10^3/µl (Neutrophils 57.4±3.3 %; Lymphocytes 33.75±2.8 %; Monocytes 6.7±0.8 %; Eosinophils 1.17±0.3 %; Basophils 0.87±0.3 %). The mean platelet count in the whole blood samples was 234.5±29.6 x10^3/µl, while red blood cells were 4.8±0.2 x106/µl. Next, we performed an immunophenotypic analysis of leukocytes isolated from PRF. The majority of leukocytes (CD45+ cells) isolated from PRF were CD3+ T cells, with a prevalence of CD4+ over CD8+ cells. We also found small percentages of CD56+ NK cells and CD14+ monocytes (Fig. 1A). Since CD4+ T cells represented the most frequent cell subset among CD45+ leukocytes, we sought to characterize their phenotype. CD4+ T cells isolated from PRF comprised both naïve (CD45RA+) and memory (CD45RO+) cells, with a prevalence of the former over the latter. When we assessed the expression of the activation markers CD25 and HLA-DR we found that only a small percentage of CD4+ T cells expressed CD25, while no expression of HLA-DR could be observed (Fig. 1B). Importantly, there was no statistically significant difference between L-PRF and A-PRF in terms of

frequency on each leukocyte subpopulation and expression of CD25 on CD4+
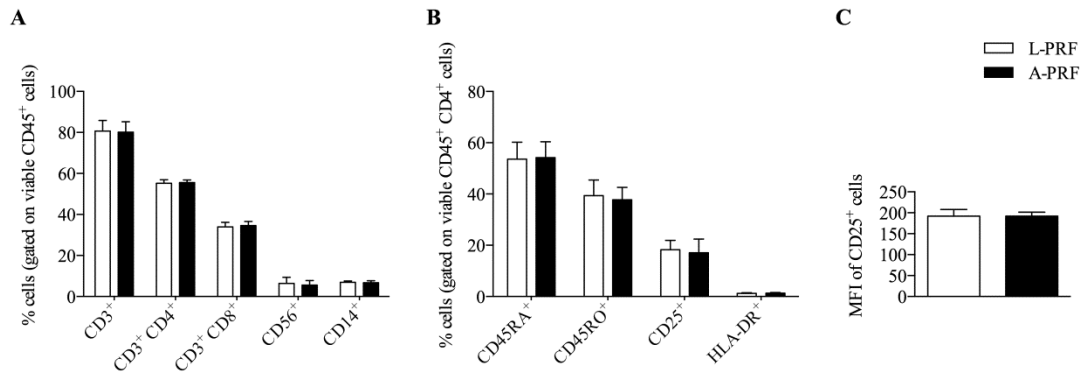
T cells (Fig. 1C).

Figure 1. *Analysis of leukocyte subpopulations and activation markers in cells isolated from L-PRF (white bars) and A-PRF (black bars).* (A) *The percentages of the following subpopulations were assessed among total leukocytes (viable CD45+ cells): T lymphocytes (CD3+), CD4+ T lymphocytes (CD3+ CD4+), CD8+ T lymphocytes (CD3+ CD8+), NK cells (CD56+), CD14+ monocytes (CD14+).* (B) *The percentages of the following subpopulations and of cells expressing activation markers were assessed among CD4+ T lymphocytes (viable CD45+ FSclo SSclo CD4+ cells): naïve (CD45RA+) and memory (CD45RO+) cells, CD25+ and HLA-DR+ (activation markers) cells.* (C) *The expression of CD25 was quantified on viable CD45+ FSclo SSclo CD4+ CD25+ cells as MFI.*

4.3.2 Comparison of L-PRF and A-PRF released factors

We have compared the release of cytokines/chemokines and growth factors by

original L-PRF and modified A-PRF clots, incubated in serum-free medium for

24 hours. Detectable amount of IL-4, IL-6, IL-8, IL-17, Eotaxin, IFNγ, MIP-1α,

MIP-1β, CCL5, TNFα, PDGF and VEGF were released by both platelet concentrates. In contrast, IL-2, IL-10 and bFGF were not detected. Analysis of cytokine and chemokine release revealed that A-PRF secreted Eotaxin and CCL5 at higher levels (increased by 3- and 1.6-fold, respectively) compared to L-PRF (Table 1). Moreover, A-PRF clots released a higher amount of IFNγ, albeit without reaching a statistical significance (p= 0.057). In contrast, no differences were noted for the concentrations of IL-4, IL-6, IL-8, IL-17, MIP-1α and MIP-1β released from the two platelet derivatives (Table 1). Next, we analyzed PDGF and VEGF secretion, which were, respectively, 3 and 1.7-fold increased in A-PRF compared to L-PRF (Table 1).

	L-PRF (pg/ml)	A-PRF (pg/ml)
IL-2	ND	ND
IL-4	1.16 ± 0.26	0.96 ± 0.23
IL-6	1320.91 ± 477.00	2164.62 ± 713.65
IL-8	6562.96 ± 931.91	7316.73 ± 471.68
IL-10	ND	ND
IL-17	9.17 ± 5.05	2.42 ± 0.56
Eotaxin	4.24 ± 1.49	12.02 ± 1.15 **
IFNγ	30.05 ± 14.75	79.89 ± 17.71
MIP-1α	36.84 ± 7.27	75.35 ± 26.43
MIP-1β	97.73 ± 21.57	117.09 ± 10.11
CCL5	117.08 ± 15.92	191.19 ± 20.27 *
TNFα	15.33 ± 3.63	5.87 ± 1.99
bFGF	ND	ND
PDGF	22.74 ± 7.90	69.96 ± 10.53 **
VEGF	10.16 ± 1.91	17.93 ± 2.06 *

Table 1: Cytokines, Chemokines and Growth Factors released by L-PRF and A-PRF. L-PRF and A-PRFs clots were incubated with serum-free DMEM-F12 (1:1). After 24 hours, the media were collected and tested by using the Bioplex multiplex cytokine, chemokines and growth factors assay kit as described in "Methods" section. Data are shown as mean ± S.E.M. * denotes statistically differences (*p<0.05; **p<0.01).

4.3.3 Platelet-derived factors from PRGF scaffold

Next, the factors released by the three different PRGF fractions were determined. PRGF gels (F1, F2, F3) were separately collected and activated with calcium chloride. The different fibrin scaffolds were incubated in culture medium for 24 h to obtain CM. As expected, platelet-derived factors were detected in the medium of PRGF fibrin scaffolds. In contrast, no traces of IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, IFNγ, MIP-1α, MIP-1β, TNFα, bFGF and VEGF were found (data not shown). Interestingly, we have observed a significant increase of CCL5 release by F2 and F3 compared to F1 fraction (1.8- and 2.2- fold increase respectively) (Table 2). In addition, PDGF content slightly increased in F3 fraction. No differences were noted for the concentration of Eotaxin in F1 and F2, while in F3 it was not detectable (Table 2). Finally, PDGF release did not significantly change in F3-PRGF compared to both L-PRF and A-PRF (Tables 1 and 2). At variance, CCL5 delivery from F3-PRGF gels was 3 and 2.5 fold higher than that from L-PRF and A-PRF, respectively (Figure 2).

	PRGF-F1 (pg/ml)	PRGF-F2 (pg/ml)	PRGF-F3 (pg/ml)
Eotaxin	5.36 ± 2.54	1.1 ± 0.13	ND
CCL5	175.86 ± 17.29	322.83 ± 48.71 *	387.48 ± 63.74 *
PDGF	33.74 ± 8.24	27.34 ± 10.58	49.85 ± 18.15

Table 2: Cytokines and growth factors released by PRGF. PRGF gels were incubated with serum-free DMEM-F12 (1:1) for 24 hours; the media were collected and tested by using the Bioplex multiplex cytokines and growth factors assay kit as described in "Methods" section. Data are shown as mean ± S.E.M. * denotes statistically differences of F2 or F3 versus F1 (*$p<0.05$).
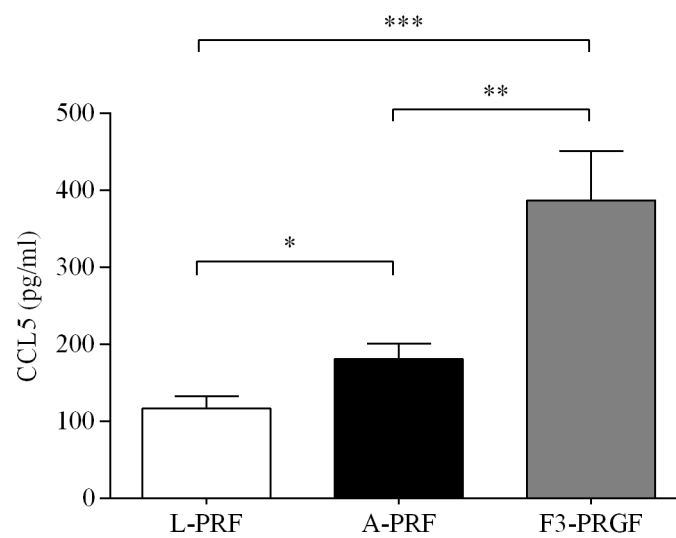

Figure 2. CCL5 release by L-PRF (white bars), A-PRF (black bars) and PRGF-F3 (grey bars). Platelet-based biomaterials were incubated in serum-free DMEM-F12 (1:1). After 24 hours, the media were collected and tested by using the Bioplex multiplex assay kit as described in "Methods" section. Data are shown as mean ± S.E.M. * denotes statistically differences (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

4.4 Discussion

A large variety of platelet concentrates for surgical use is commercially available and includes a number of unclear products. Under similar names, more than ten different autologous glues or biomaterials are available. The goal of this research field consists in the use of blood-derived biomaterials to create different therapeutic formulations that adapt to the needs of various biomedical applications, including orthopedic and maxillofacial surgery, sports medicine, bone reconstruction, tissue engineering, cosmetic and dental implant surgery [Sammartino et al., 2011].

The tissue regeneration or repair process requires harmonious reaction of various types of cells, including immune response cells (neutrophils, macrophages, lymphocytes), epithelial cells, fibroblasts, stem cells. The PRF scaffolds represent a good source of all components necessary for the healing process releasing a large variety of factors [Anitua et al., 2012; Passaretti et al., 2014]. Our current data show that A-PRF and L-PRF composition reflects that of peripheral blood in terms of frequency on each leukocyte subpopulation. Both kinds of clots contain living white blood cells, and, possibly, circulating progenitor cells, entrapped in a high-density fibrin network, which also includes platelets.

It is now accepted that platelets have a major role in cell communication, inflammatory and healing responses [Weyric & Zimmerman, 2004; Anitua et al., 2013; van Dooren et al., 2013]. Platelets are able to synthesize and contain granules with high levels of bioactive molecules, promoting recruitment, growth and morphogenesis of cells [Reed, 2004]. The inclusion of leukocytes within platelet biomaterials has always been a matter of debate. At variance with platelets, white blood cells are mainly considered to contain and produce inflammatory cytokines. Nevertheless, they play an important role as immune modulators [van Dooren et al., 2013]. We have compared in vitro behavior of both A-PRF and L-PRF, as platelet/leukocyte scaffolds, through the evaluation of the release of growth factors, cytokines and chemokines. Moreover, we have investigated the release properties of a leukocyte free scaffold derived from a platelet enrichment, the PRGF.

Our data show that A-PRF releases VEGF at higher levels compared to L-PRF, while in the different fractions of PRGF the growth factor is undetectable, possibly due to the sensitivity of the assay. However, we cannot exclude the release of this GF by PRGF. Indeed, Anitua et al. have described the release of VEGF, although at lower concentration compared to other GFs [Anitua et al., 2015]. VEGF is largely produced by leukocytes [Loffredo et al., 2014] and is crucially important for the promotion of angiogenesis during tissue repair.

Indeed, reduced expression and rapid proteolytic degradation of VEGF are considered partially responsible for poor wound healing, as for example in diabetic wounds, because these effects result in poor angiogenesis during granulation tissue formation [Saaristo et al., 2006]. Thus, the addition of VEGF alone or in combination with other growth factors may represent a significant step in medical treatment of non-healing wounds and other ischemic processes [Bao et al., 2009].

The most abundant factor released by platelets is PDGF, mostly involved in cell proliferation, chemotaxis and extracellular matrix production/angiogenesis [Lubkowska et al., 2012; Burnouf et al., 2013]. It has been described that PDGF and other GFs can be released up to 7 days by PRGF [Anitua et al., 2015]. In agreement with other reports [Kobayashi et al., 2016], we have shown that the release pattern of PDGF was higher in A-PRF compared to L-PRF. Moreover, our data demonstrate that all the PRGF fractions release PDGF in a similar fashion with that of A-PRF. The finding that A-PRF releases a greater amount of PDGF compared to L-PRF suggests that the reservoir property of A-PRF fibrin network could enhance platelet content compared to L-PRF; however, this hypothesis requires further investigation.

Consistently with the absence of leukocytes in PRGF, we showed an almost complete absence of proinflammatory cytokines in the gel fractions. However,

all PRGF fractions deliver CCL5, a chemokine largely produced by both platelets and leukocytes [Marques et al., 2013]. We have reported that this chemokine is secreted at greater amount in the fractions F2 and F3. In addition, CCL5 content in the F3 fraction is higher compared to both L-PRF and A-PRF. A-PRF releases greater amount of both CCL5 and Eotaxin compared to L-PRF. CCL5 and Eotaxin chemokines are small secreted proteins highly involved in chemotaxis and angiogenesis. For instance, Eotaxin (also known as CCL11), induces the formation of blood vessels and stimulates the chemotataxis of human microvascular endothelial cells [Salcedo et al., 2001]. Whereas, CCL5, previously called RANTES, promotes angiogenesis both in vivo and in vitro mainly increasing the expression of VEGF [Suffee et al., 2012]. CCL5 is released at higher levels also by Platelet-Rich Plasma gels [Galliera et al., 2012; Passaretti et al., 2014; D'Esposito et al., 2015; Jalowiec et al., 2016]. The finding that platelet derivatives secrete CCL5 is particularly interesting for tissue engineering strategies because the locally release of this chemokine may intensify cell mobilization, enhance vascularization, and therefore improve the healing process [Jalowiec et al., 2016].

The mechanism responsible for the differences between L and A-PRF has not been completely explained, but it may involve activation of the cells embedded in the clot, following condensation and mechanical stress. A-PRF is,

in fact, a variation of the original L-PRF based on lower centrifugation speed and longer centrifugation time [Ghanaati et al., 2014]. Thus, changing the original protocol could modify the secretory properties of PRF, which is key for final therapeutic outcomes. Thus, we have reported that L- and A-PRF entrap the same content of viable leukocytes and release a similar amount of inflammatory cytokines. However, A-PRF released higher amount of chemotactic and pro angiogenic molecules, like VEGF, PDGF, CCL5 and Eotaxin compared to L-PRF. This finding suggests the use of A-PRF in the clinical fields that require tissue repair and new vessel formation. It should be noticed that all our observations on PRFs are still investigational and refer to "in vitro" models, not necessarily intended for use in clinical applications,

On the other side, PRGF does not contain leukocytes and inflammatory cytokines but only platelet-derived factors, which has been reported to improve the homogeneity of the product [Weibrich et al., 2004]. PRGF gels may be useful when it is required a local haemostasis with an initial level of tissue repair. Platelet gels increase the proliferation of various cell models [Passaretti et al., 2014; Weibrich et al., 2004; D'Esposito et al., 2015] and may be considered as transitory pharmaceutical adjuvants [Del Corso et al., 2012].

Developing therapeutic autologous formulations that control the dose of growth factors and their local release into injured tissue is critical to achieving a successful outcome [Anitua et al., 2007].

4.5 Conclusion

Biomaterials can act as controlled release devices which will allow for sustained or even on demand delivery of these growth factor cocktails. Further investigations are needed to consider our experimental approach for a specific clinical use.

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