CHAPTER 2

Characterization of Plasma Rich in Growth Factors (PRGF): Components and Formulations

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SUMMARY

Platelet-rich Plasma (PRP) is a set of autologous platelet products used to reduce pain and speed up recovery from injury while maintaining the tissue function. Its basic rationale is to mimic yet enhance the natural processes of healing by bringing to the injury site a set of molecules that will accelerate functional recovery, and even regenerate the tissue. In the array of PRP-products, Plasma Rich in Growth Factors (PRGF)-Endoret is a pioneering autologous regenerative technology with multiple therapeutic potentials. It can be produced in at least four different formulations, depending on coagulation and degree and type of activation. PRGF-Endoret technology is safe and versatile, and has a wide range of applications.

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1. POTENTIAL OF PLASMA RICH IN GROWTH FACTORS (PRGF-ENDORET): MIMICKING THE NATURAL HEALING PROCESS

The increasing number of musculoskeletal injuries has produced a concurrent stimulus in both the number and the effectiveness of different treatments of these lesions, especially in the search for minimally invasive procedures or adjuvants[1-3]. One of these cutting-edge technologies is Plasma Rich in Growth Factors (PRGF-Endoret)1. This biological treatment mimics the natural pathways of wound healing[4] by driving to the injury site the whole protein array of PRGF that is involved in the repair of damaged tissues. In this way, all the bioactive molecules (including growth factors and other proteins) necessary for tissue repair are efficiently and locally released.

The tissue repair process occurs naturally in a staged fashion[5] and includes removal of dead cells, proliferation, migration of cells to the injury site, production of new vascular structures, and other events. The organization of all these elements influences healing in a given injury, preventing fibrotic elements that cause loss of functional capacity in that tissue[6,7] i Growth factors play an important role coordinating the whole process in an orchestrated fashion in all tissues of the musculoskeletal system, including muscle[8], tendon[9], bone[10,11], and cartilage[12]. Growth factors act on other tissues as well, including skin[13], oral soft tissue[14,15], and cornea[16] among others.

PRGF-Endoret technology mimics the natural healing mechanisms, but with two special features: avoiding loss of functionality (fibrous tissue) and shortening healing times. This is achieved in part by adjusting the PRGF-Endoret formulation and dosage to the type of tissue and injury.

PRGF-Endoret therapy accelerates and improves tissue healing by local delivery of autologous bioactive molecules and hence, contributing a first line provisional scaffold[1]. This autologous therapeutic toolbox consists of platelets as both reservoir and vehicle of a large repertoire of proteins[17,18.] Recently, a proteomic dissection of PRGF scaffold was performed[19]. In this research, the authors studied those proteins that remained most closely bound to the fibrin network and that were therefore retained by the mesh itself, rather than being released into the supernatant. The high-throughput proteomic techniques used in this characterization allowed us to produce a catalogue of these proteins and subsequently to classify them into families on the basis of their function and gene ontology. The results of this process showed that the fibrin network is enriched in proteins specifically involved in tissue regeneration and wound healing. Interestingly, there was found to be an enrichment in certain lipoproteins, which are involved in regenerative processes, particularly by delaying degradation (fibrinolysis) of the fibrin network, thereby extending the controlled release of other molecules. Similarly, an important family of proteins involved in the acute phase reaction was found to be present. These proteins form the first line of defence in the immune system[19].

In the last decade, several systems have been developed to produce a biologically active product, both commercially and in-house, but they differ in the presence of white blood cells, growth factors concentration, and architecture of fibrin scaffold[20-24]. The different PRP commercial systems can be certified for various medical applications, but the therapeutic outcome will depend on the type of platelet-rich plasma used and the dosage employed. Establishing a proper classification of PRPs and identifying the biological differences among them is absolutely necessary to understand some of the controversial results obtained with these types of technologies so far[25].

One of the most relevant and controversial issues is the presence of leukocytes in the platelet-rich plasma. In order to distinctly define the PRGF technology, and thus be able to compare other PRPs, PRGF can be categorized according to three of the most cited classifications that have been proposed for PRPs. The first and most widely used[26] classifies PRGF as pure-PRP (P-PRP) since it does

not contain WBC. The PRGF is classified as type 4-B (Minimal WBCs, activated with $CaCl_2$, and platelet concentration below 5x) as has been proposed[27] for sports medicine classification. Finally, PRGF would fit in the P2-x-Bβ category (platelet count greater than baseline levels to 750,000 platelets/µL, exogenous activation with $CaCl_2$, with WBC -and specifically neutrophils- below to baseline levels) according to the PAW (platelets, activation and WBC) classification[28].

2. UNDERSTANDING THE PROPERTIES OF PLATELET-RICH PLASMA PRODUCTS

Several key biological mediators are present in a PRP. The more studied growth factors contained in platelet-rich plasma that are important during tissue repair include IGF-I (Insulin-like Growth Factor type I), TGF-β1 (Transforming Growth Factor β type 1), PDGF (Platelet Derived Growth Factor), HGF (Hepatocyte Growth Factor), VEGF (Vascular Endothelial Growth Factor), EGF (Epithelial Growth Factor) and bFGF (basic Fibroblastic Growth Factor) among others (Table 1)[29,30]. Some of them (IGF-I and HGF) are plasmatic proteins, and their concentration does not depend on the platelet enrichment. However, most of the growth factors are indeed platelet proteins, both synthesized and adsorbed, and thus their quantity does depend on the platelet concentration. To understand the properties of platelet-rich plasma products, it is necessary to detail the different roles of molecules that it contains:

- IGF-I: This protein circulates in plasma as a complex with binding proteins (IGFBP). This determines the bioavailability and regulates the interaction between this IGF-I and its receptor[31,32]. IGF-I is involved in keratinocyte migration and wound healing[33,34], stimulates bone matrix formation and maintenance[35] by promoting preosteoblast proliferation[36,37], and also is involved in striated muscle myogenesis[38]. Furthermore, knockout mice for IGF-IR in muscle exhibited impaired muscle regeneration and deficient myoblast differentiation[39]. Recently, It has been observed that IGF-1 promotes tissue repair of skeletal muscle without scar tissue formation by increasing fibre size and muscle size hypertrophy[40]. Also, and related to this, IGF-1 is considered a potent enhancer of tissue regeneration, and its overexpression in muscle injury leads to hastened resolution of the inflammatory phase[41].

- TGF-β1: The role of TGF- β family proteins in wound healing has been recently reviewed[42]. TGF-β has different effects, depending on the tissue and the cell type[6]. The release and posterior bioactivation of latent TGF-β contributes to the early cellular reparative responses, such as migration of cells and neovascularization and angiogenesis[43] into the wound area. In bone, TGF-β1 induces osteogenic differentiation of mesenchymal cells of the bone marrow, upregulating osteoblast differentiation markers[44]. TGF- β plays a crucial role in maintaining homoeostasis of both articular cartilage and subchondral bone[45].

- PDGF: This growth factor is a mitogen and chemotactic factor for all cells of mesenchymal origin[46]. It is important in the repair of joint tissue, including cartilage and meniscus[47,48]. Bone is also a target of PDGF, influencing its metabolism and acting in repair mechanisms[49,50], including the recruitment of pericytes to stabilize new blood vessels[51].

- HGF: Also called scatter factor, it regulates cell growth, migration and morphogenesis[52] and plays an important role in wound-healing through an epithelial-mesenchymal interaction[53]. HGF modulates central inflammatory and immune events that are common to many diseases and organ systems[54]. The antifibrotic effect of HGF has been shown in various tissues[55,56], through induction of Smad[7], and thus regulates the myofibroblast phenotype, allowing the initial contraction of the wound, but gradually making the myofibroblast disappear[57].

- VEGF: This growth factor is a key mediator in wound healing[58] and the main inducer of angiogenesis since it stimulates chemotaxis and proliferation of endothelial cells[59]. This protein is crucial in the sprouting of new capillaries from preexisting vasculature, mainly initiated by hypoxia in ischemic tissue[60]. Also, VEGF is involved in the regulation of many organ homeostases, such as brain, heart, kidney, and liver[61], and its role may be crucial in cell-mediated tissue regeneration[62].

- EGF: This protein promotes chemotaxis and mitogenesis in epithelial and mesenchymal cells[63,64] by acting on the regeneration of multiple tissues. It has an important role in skin, cornea, gastrointestinal tract and nervous system[65-69].

- bFGF: This factor, also called FGF-2, is a potent inductor of cell proliferation, angiogenesis and differentiation[70,71]. Its role in the repair process has been observed in several tissues[72], including bone[73-75], tendon[76,77], and periodontal tissue[78-80].

Classification	Protein	Biological effects
Adhesive proteins	Von Willebrand factor (vWF) propeptide, Fibrinogen, Fibronectin, Vitronectin, Thrombospondin 1 (TSP-1), laminin-8 (alpha4- and alpha5- laminin subunits), signal peptide-CUB-EGF domain containing protein 1 (SCUBE 1)	Cell contact interactions, homeostasis and clotting, and extracellular matrix composition
Clotting factors and associated proteins	FactorV/Va, FactorXI-like protein, multimerin, protein S, high-molecular weight kininogen, antithrombin III, tissue factor pathway inhibitor (TFPI)1	Thrombin production and its regulation
Fibrinolytic factors and associated proteins	Plasminogen, Plasminogen activator inhibitor-1 (PAI-1), urokinase plasminogen activator (uPA), alpha2-antiplasmin, histidine-rich glycoprotein, thrombin activatable fibrinolysis inhibitor (TAFI), alpha2-macroglobulin (α2M)	Plasmin production and vascular modelling
Proteases and anti-proteases	Tissue inhibitor of metalloprotease 1–4 (TIMPs 1–4), metalloprotease-1, -2, -4, -9, A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), tumor necrosis factor-alpha-converting enzyme (TACE), protease nexin-2, C1 inhibitor, serpin proteinase inhibitor 8, alpha1-antitrypsin	Angiogenesis, vascular modelling, regulation of coagulation, and regulation of cellular behaviour
Growth factors	PDGF, TGF-beta1 and -beta2, EGF, IGF-1, VEGF (A and C), bFGF (FGF-2), HGF, Bone morphogenetic protein (BMP)-2, -4, -6, connective tissue growth factor (CTGF)	Chemotaxis, cell proliferation and differentiation, and angiogenesis
Chemokines, cytokines and others	Regulated upon Activation - Normal T-cell Expressed, and Secreted (RANTES), Interleukin-8 (IL-8), Macrophage inflammatory protein-1 (MIP-1) alpha, Epithelial Neutrophil-Activating Peptide 78 (ENA-78), Monocyte chemotactic protein-3 (MCP-3), Growth regulated oncogene- alpha (GRO-alpha), angiopoietin-1, IGF-1 binding protein 3 (IGF-BP3), interleukin-6 soluble receptor (IL-6sR), Platelet factor 4 (PF4), beta-thromboglobulin (bTG), platelet basic protein, neutrophil-activating protein-2 (NAP-2), connective tissue-activating peptide III, high-mobility group protein 1 (HMGB1), Fas ligand (FasL), Homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus (HSV) glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes (LIGHT), Tumor necrosis factors (TNF)-related apoptosis-inducing ligand (TRAIL), Stromal cell-derived factor-1 (SDF-1) alpha, endostatin-I, osteonectin-1, bone sialoprotein	Regulation of angiogenesis, vascular modelling, cellular interactions, and bone formation
Anti-microbial proteins	Thrombocidins, defensins	Bactericidal and fungicidal properties
Others	Chondroitin 4-sulfate, albumin, immunoglobulins, disabled-2, semaphorin 3A, Prion protein (PrPC)	
Human adipose-derived stromal cells	PRP releseate after thrombin activation	Increased cell proliferation, ALP activity and mineralization

TABLE 1
Platelet protein classification and their biological role. A set of proteins present in platelets and its physiological role in the regeneration of tissues is shown. Reproduced with permission [126]

Growth factors classically promote several important functions in the regenerative milieu: they are able to stimulate cell proliferation (mitosis), cellular migration (chemotaxis), differentiation (morphogenic effect), angiogenesis, and the combination of several of these effects. These peptides exert the above-mentioned functions in the local environment, close to the site of the application.

However, it is difficult to dissect the contribution of each molecule contained in platelet-rich plasma and examine its effect separately, since many have multiple effects, some of which overlap with others. Also, many molecules are activated in the presence of others, such as TGF-β, which is in a latent state[81] and becomes functional after proteolytic activation or in the presence of other molecules, such as thrombospondin-1[82] or various integrins.

The idea that platelet-rich plasma contains only factors that stimulate angiogenesis and proliferation would be a little simplistic. In fact, another important property of the PRP is the bacteriostatic effect[83]. These antibacterial effects were observed against Staphylococcus aureus and Escherichia coli[84]. Classically, these properties have been shown in leukocyte-enriched platelet-rich plasma. However, recently these antimicrobial properties have been evidenced in PRGF-Endoret[85], which by definition has no white cells. Specifically, PRGF-Endoret has bacteriostatic effect against Staphylococcal strains. Moreover, the addition of leukocytes to the PRGF-Endoret preparation did not yield greater bacteriostatic potential than it already had. This data raise questions about the role that leukocytes may play in a platelet-rich plasma preparation, since they do not improve the bacteriostatic properties but, on the contrary, they might significantly increase the presence of pro-inflammatory molecules.

Platelet-rich products also act as anti-inflammatory mediators by blocking monocyte chemotactic protein-1 (MCP-1), released from monocytes, and lipoxin A4 production[86]. HGF in PRP inhibits NF-kB, a key nuclear factor implicated in inflammatory responses, by activation of its inhibitor (ikBα). In this same study, it was also observed that PRP reduced the chemotaxis of the monocytic line U937[87]. In addition, serotonin, a neurotransmitter and hormone present in platelets, has been reported to directly mediate liver regeneration[88].

3. PRGF-ENDORET: A PIONEERING TECHNOLOGY

For almost two decades our research group has characterized this technology and has studied its therapeutic potential in tissue repair and wound healing[1]. PRGF-Endoret contains a moderated platelet concentration, a two-third fold increase compared to peripheral blood, a dosage shown to induce optimal biological benefit[89]. In fact, lower platelet concentrations can lead to suboptimal effects, whereas higher concentrations might have an inhibitory effect[90]. PRGF-Endoret does not contain leukocytes, and activation is performed only with $CaCl_2$.

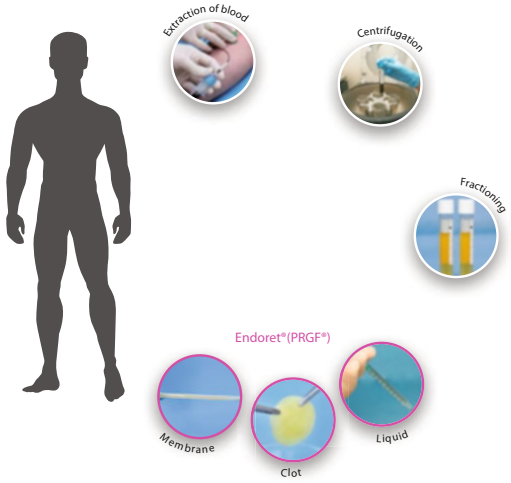

FIG. 1
PRGF-Endoret technology overview. PRGF-Endoret aids in the preparation of different autologous therapeutic formulations from patient's own blood.

The process to produce PRGF-Endoret is easy, fast and reproducible (fig. 1). Blood collection is performed in tubes containing sodium citrate as anticoagulant. Thus, platelets are well preserved. Subsequently, centrifugation is achieved in a specifically designed centrifuge (PRGF System V). The centrifuge has specific parameters to maximize the production of platelets and keep the plasma leukocyte-free. Three typical layers are obtained after centrifugation: (i) a yellowish top layer, the plasma, which contains a gradient of platelets, with maximum concentration of those platelets above the buffy coat; (ii) the leukocyte layer, or buffy coat, is located below the plasma layer; and (iii) the bottom layer, that is the layer containing the red cells. Regarding the plasma volume, it is possible to empirically differentiate between two different fractions, depending on the respective concentration of platelets. The upper fraction will contain a similar number of platelets as peripheral blood whereas the lower fraction will contain 2 to 3-fold the concentration of platelets compared with blood. However, depending on the application, as in the case of PRGF eye drops, it is possible to collect the entire PRGF column without performing two fractions[91]. The basic characteristics of PRGF (whole plasma column) are shown in Table 2.

With the aim of collecting these plasma fractions from PRGF-Endoret technology, we have recently developed an optimized device, the plasma transfer device (PTD2) (fig. 2). The PTD2 is a disposable and sterile aspiration system that allows separating the different fractions obtained after centrifugation. In contrast to the traditional pipetting system, the PTD2 system is faster, avoiding intermediate pipetting steps. In addition, the plasma transfer device does not require maintenance of the pipetting system. Depending on clinical needs, the fractionation can be made in one or two fractions, achieving higher volume - lower concentration of platelets (a single fraction), or lower volume - higher concentration of platelets (two fractions, F1 and F2). After fractionation, PRGF-Endoret can be activated in a controlled way by the addition of $CaCl_2$, providing a clot that mimics its natural structure. Moreover, the coagu-

N=30	Whole blood	PRGF
Leukocytes (x 103/µL)	6.1 ± 1.4	0.3 ± 0.2
Erythrocytes (x 106/µL)	4.78 ± 0.41	0.01 ± 0.01
Platelets (x 103/µL)	235 ± 41	517 ± 107
Leukocyte concentration factor (LCF)	-	0.05 ± 0.03
Platelet concentration factor (PCF)	-	2.2 ± 0.2
Platelet yield (%)	-	66 ± 7

TABLE 2
Summary of the characterization of whole blood and PRGF samples from thirty donors. The values for PRGF correspond to the whole plasma column. Leukocyte, platelet and erythrocyte concentration was measured in whole blood and PRGF. Leukocyte and platelet concentration factor (enrichment as fold increase) relative to the level of peripheral blood (LCF and PCF) and platelet yield (%) are also indicated. Data are expressed as mean ± SD. Reproduced with permission [95].

FIG. 2
The plasma transfer device 2 (PTD2) is a disposable and sterile aspiration system that allows the fractionation of PRGF. The device contains an ergonomic button that allows fine control of the suction flow. The suction is performed by the vacuum contained in the fractionation tube (TF9). The aspiration needle is a blunt needle to prevent accidental stab injuries. In this way, PRGF-Endoret is obtained directly in a fractionation tube, where it can be directly activated with calcium chloride. It is possible to perform the whole procedure without opening the extraction tubes, using an adapter needle.

lation is conducted at a speed that allows control of the whole process. Activation with $CaCl_2$ avoids the use of exogenous bovine thrombin, a source of possible immunological reactions[92-94]. Recently, the PRGF obtaining protocol has been improved[95] in order to reduce both the amount of anticoagulant and activator: the new blood extraction tubes (TB9) contain 400 µL of trisodium citrate as anticoagulant, and the new ratio of PRGF Activator would be 20 µL of calcium chloride / mL PRGF.

Another important feature of the PRGF-Endoret technology, when compared with other platelet-rich plasma systems, is the absence of leukocytes, which categorizes it as a safe and homogeneous, because the values of leukocytes are highly variable between donors[96], and within the same donor are highly dependent on small perturbations of the body homeostasis. In addition, polymorphonuclear neutrophils (PMN) contain molecules designed to kill microorganisms, but can seriously damage the body tissues. For example, PMNs are important producers of matrix metalloproteinases (MMP), mainly MMP-8 and MMP-9, which can hamper the regeneration of damaged tissue. PMNs also produce free radicals, reactive oxygen species and nitrogen, which can destroy not only microorganisms but surrounding cells[97]. Of special concern would be to avoid leukocytes if muscle regeneration is required, as in vivo PMNs increase muscle damage[98] and do not provide extra functionality. Therefore, it is recommended to use leukocyte-free platelet-rich plasma in infiltrations of damaged muscle[99].

4. PRGF-ENDORET TECHNOLOGY: A VERSATILE TOOLBOX WITH MULTIPLE FORMULATIONS

A key point that distinguishes the PRGF-Endoret technology from other platelet-rich plasma products is its versatility. Four different formulations (fig. 3) with therapeutic potential are obtained from the patient's blood, depending on the coagulation and activation degree of the samples. These formulations may be used for different therapeutic purposes:

PRGF-Endoret scaffold. This three-dimensional matrix encloses autologous growth factors, both plasma and platelet proteins. This scaffold can be used in various applications, such as the treatment of ulcers[100,101], wound closure and tissue engineering[102]. The three-dimensional structure of the fibrin mesh (fig. 4) allows cell proliferation, since, as mentioned, it contains factors necessary for growth and migration of cells. In addition, this formulation can be combined with other materials[103], such as autologous bone, demineralized freeze-dried bovine bone, and collagen, among others, fine-tuning the resulting characteristics of the scaffold[102].

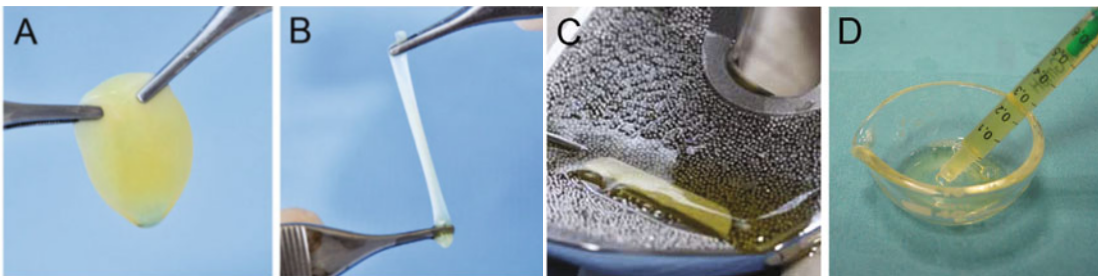

FIG. 3
PRGF-Endoret technology formulations: (A) three-dimensional clot or scaffold, (B) elastic and dense autologous fibrin membrane. (C) liquid formulation activated at the moment and deposited on the implant surface, and (D) the PRGF supernatant, ideal as eye drops or cell culture supplement.

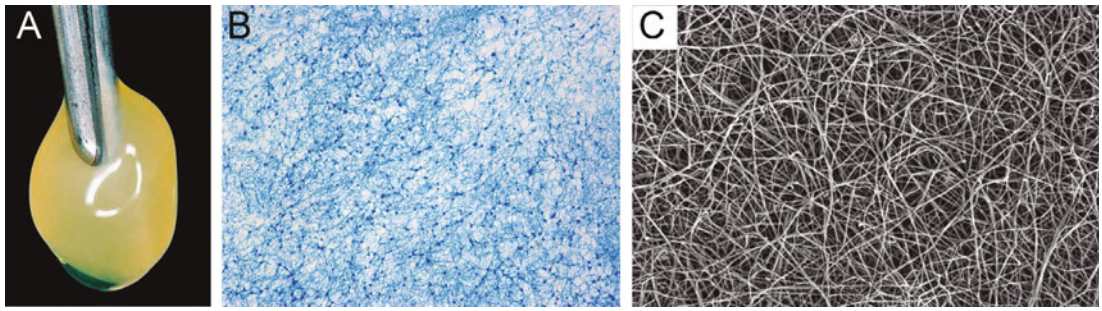

FIG. 4
Three-dimensional structure of PRGF-Endoret clot or scaffold. (A) PRGF scaffold observed with the naked eye. (B) Optical microscopy reveals a 3D network of fibrin with platelet aggregates scattered throughout the network (May-Grunwald-Giemsa staining, original magnification x 400). (C) Closer inspection reveals regular and interconnected intact fibrin strands in a leukocyte-free plasma rich in growth factors (PRGF)-Endoret scaffold (original magnification x 3500). Adapted, with permission,[102].

1. Liquid PRGF-Endoret, activated at the time of use, is used in intra-articular[104-106] and intraosseous[107-109] injections, surgery[110-112], treatment of skin disorders[100,101,113], and implant surface bioactivation by producing a biologically active layer on the titanium surfaces[114,115].

2. The PRGF-Endoret supernatant contains plasma proteins and platelet releasate and can be used as eye drops treatment for dry eye disease[116] and other corneal defects[117,118]. Both in basic research studies and applied areas, this formulation can be used to supplement the cell culture medium[102,119].

3. Autologous fibrin membrane. At the end of the process of coagulation, fibrin scaffold retracts[120]. At that stage, the fibrin membrane can be shaped with tweezers or similar instruments to obtain an elastic, dense and suturable membrane. It is an excellent tool to seal the post-extraction tooth sockets[121-123] and to promote the full epithelialization of other soft tissues[124].

The autologous platelet products have a high therapeutic potential and can be used in various formulations and in various fields of medicine and tissue engineering. At present, there are over forty of these products with different characteristics, in terms of enrichment of platelets, presence of leukocytes, activator type, and final volume among others. This great variability makes it difficult to standardize protocols and compare results. Furthermore, this large variability can engender confusion among clinicians and researchers[125]. It is, therefore, necessary to reach a consensus and better definition of each product. Our research team has spent more than 20 years developing this technology, which makes PRGF-Endoret one of the best characterized autologous platelet-rich plasma, with multiple and growing therapeutic applications, as result of a continuous research translation to the clinical setting.

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