EDITORIAL

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Platelet rich plasma (PRP) and similar products derived from peripheral blood are now widely used in clinical medicine, and especially in orthopaedics, for supporting the tissue regeneration and reparation after trauma or surgery. The increase of the PRP use and, consequently, of the current scientific literature deserves a specific review of basic, research and clinical aspects of its introduction as an adjuvant therapy in clinical medicine.

Categorization of different phases of PRP preparation, the quality of the final product, the real composition of the product, the application techniques are crucial for obtaining correct and valid data and for judging the efficacy of such a therapy.

We collected in the present Supplement a series of papers properly describing the various aspects of PRP therapy.

The content of platelets collected in the enriched plasma, the lipidomic of these particles, and the anti-inflammatory potential of the released substances from activated particles are described in some papers. In additional articles, the use of PRP in sports medicine, maxillo-facial surgery, and in orthopaedics is reviewed, showing pros and cons of the treatment and the efficacy on some specific inflammatory and(or) degenerative processes. A paper is also devoted to the antimicrobial properties of the procedure, which is a new and very interesting insight for using platelets concentrates.

We hope that this Supplement could be useful for the readers of the Journal and could focus the attention of the academic and professional world on scientific aspects of this widely accepted treatment.

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GROWTH FACTOR CONTENT IN PLATELET RICH PLASMA AND THEIR APPLICABILITY IN MEDICINE

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This paper reviews available reports on the advantages and possibilities of clinical use of platelet-rich plasma preparations, with particular emphasis on platelet growth factors.

Platelets, an important reservoir of growth factors in the body, play an important role in many processes such as coagulation, immune response, angiogenesis and the healing of damaged tissues. Numerous proteins are contained in the α-granules of platelets: platelet-derived growth factor (PDGF), transforming growth factor (TGF), platelet factor interleukin (IL), platelet-derived angiogenesis factor (PDAF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin-like growth factor IGF and fibronectin.

The development of methods and systems for blood and cell sorting (e.g. CAPSS - compact advanced platelet sequestration system Elektromedics 500, PCCS - platelet concentrate collection system Curasan) have made it possible to obtain significant concentrations of platelets (even by 338%) and high concentrations of growth factors, in a form of sterile mass that can be used immediately for clinical purposes. Platelet-rich plasma (PRP; autologous platelet-rich plasma - APRP) are platelet concentrates made of autogenous blood with a high number of platelets in a small volume of plasma. The clinical efficacy of platelet concentrates depends mainly on the number of platelets and the concentration of their growth factors, which act as transmitters in most processes in tissues, particularly in healing where they are responsible for proliferation, differentiation, chemotaxis and tissue morphogenesis. They operate as part of autocrine, paracrine and endocrine mechanisms.

Growth factors derived from centrifuged blood were first used in patients with chronic skin ulcers. The clinical use of PRP for a wide variety of applications has been reported mostly in oral and maxillo-facial surgery, orthopedic surgery, treatment of soft tissue diseases and injuries, treatment of burns, hard-to-heal wounds, tissue engineering and implantology.

Platelet Concentrate Products

For over two decades we have seen a growing interest in blood as a source of substances that could potentially accelerate the healing of tissues and wounds, and the possible use of blood-based products in the complex interactions between cells of connective tissue, epithelial cells, immune cells and blood morphotic elements that accompany healing and regeneration. Current treatment methods use the advantages of tissue engineering (biomimetics) that allow emulation of embryogenetic processes in tissue healing. Proper tissue regeneration requires three components that are completely dependent on one another: media, cells and extracellular matrix components that include growth factors, morphogens,

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adhesins, hormones and vitamins (1). The process of tissue healing is divided into three phases: inflammation, proliferation and remodeling. Blood platelets play a key role in the phase of inflammation: they are responsible for hemostasis,the release of signal particles that affect the developing inflammatory processes, as well as the growth, differentiation and migration of cells taking part in tissue reconstruction. Platelets, the smallest blood cells, with a diameter of 2-4 μm, devoid of a nucleus, discoid-shaped and with megakaryocytes as their stem cells, serve a variety of critical functions including hemostasis (coagulation), inflammation, antimicrobial host defense, angiogenesis, and wound healing, and are subject to daily consumption that reaches 25% inder conditions of physiological equilibrium. Platelet activity is related to their age; young platelets exhibit glycolytic activity and the capacity for biosynthesis of glycogen and proteins, mainly thrombosthenin and glycoprotein.

Once a platelet is exhausted and dies off, the macrophages which arrived in the region via vascular in-growth stimulated by the platelets, assume the function of wound healing regulation by secreting some of the same growth factors (2).

Recent years have seen a growing demand for platelets and their concentrates, associated with the increasing number of cardiac operations, organ transplants and cancer treatment procedures associated with their use.

Platelets are a potential source of therapeutically active proteins such as mitogenic, chemotactic, adhesive, angiogenic and antiangiogenic proteins, and neurothrophic factors. Recently developed procedures can be used to create platelet-rich plasma (PRP), while many techniques are available for collecting platelet concentrates, although each method may lead to a different product with different biology and potential uses. The term PRP firstly relates to platelet concentrates used in transfusion medicine, but nowadays it has started to be used for all platelet concentrates, regardless of the exact composition of the products, resulting in a certain confusion in terms of terminology. There is a lack of clear and uniformly accepted terminology, making a source of confusion in the field. Knowledge and terminology regarding the preparation, differentiation and use of blood products have been greatly enriched and structured thanks to the contributions of the teams under Marx, Anitua and Dohaan Ehrenfest (2-6).

Platelet-rich plasma (PRP) is an autologous concentration of human platelets in a small volume of plasma. During the preparation procedure, centrifuging at varying speeds accelerates the sedimentation of heavier cells such as white blood cells and red blood cells, while platelets (which sediment at a lower rate) remain floating within the plasma fraction at the top of the tubes. Usually 2 spins are used. The first spin (hard spin) separates the platelet poor plasma (PPP) from the red fraction and platelet rich plasma (PRP). The second spin (soft spin) separates the red fraction from the PRP (7).

PRP is prepared in a laboratory, surgical or dental suite from blood collected in the immediate preoperative period. Heterologous or allogenic materials carry the potential risks of infection, immune responses and pathogen transmission and are not appropriate for clinical feasibility. At the time of application, the PRP is combined with an equal volume of sterile saline solution containing 10% calcium chloride (a citrate inhibitor that allows the plasma to coagulate) and 100 U/mL of sterile bovine thrombin (an activator that allows polymerization of the fibrin into an insoluble gel, causing the platelets to degranulate and release the indicated mediators and cytokines) (8).

Very often the term PRP is preferred to autologous platelet gel, plasma-rich growth factors (PRGFs), or autologous platelet concentrate. Pure platelet-rich plasma (P-PRP) and leukocyte- and platelet- rich plasma (L-PRP) are liquid platelet suspensions either with or without leukocytes. PRP gel and L-PRP gel are formed by mixing PRP or L-PRP, derived from centrifuging autologous whole blood, with thrombin, calcium chloride, batroxobin or other activators, which then start to polymerize into a fibrin gel presenting a light and fragile architecture, to be used in various applications with apparent clinical success (9).

PRGF (plasma, rich in growth factors) has a moderated platelet concentration and does not contain leukocytes, with the aim of avoiding the proinflammatory effects of the proteases and acid hydrolases contained in the white blood cells (4).

Pure Platelet-Rich Fibrin (P-PRF) and Leukocyte- and Platelet-Rich Fibrin (LPRF) are solid fibrin biomaterials without or with leukocytes, respectively. They only exist in an activated form, the activation being part of their production process, and present a stronger and more stable fibrin polymerization than the PRP gel families.

P-PRP and L-PRP without activation can be injected as liquid preparations in regenerative medicine treatment, mostly in nonoperative sports medicine. The platelets slowly release their contents during several days to relieve pain and stimulate regeneration of damaged tissues. P-PRP gel, L-PRP gel, P-PRF, and L-PRF are used like fibrin-based solid biomaterials for their hemostatic and adhesive properties. The strong matrix architecture of P-PRF and L-PRF allows for the release of key growth factors and matrix molecules over several weeks in some conditions (5, 10, 11).

Although the definition of platelet rich plasma, proposed by Marx in 2001, mentions 1 million platelets

in one microlitre, measured in the standard 6-mL aliquot, a 4-5 fold increase in the concentration of platelets compared to whole blood (6), in clinical practice the number of platelets in the preparations may even be 14 times greater than in the whole blood (13).

PRP containing approximately 1 million platelets/ μL is recommended as advisable according to in vivo experiments in bone regeneration that showed that an advantageous biological effect was observed with the application of PRP at this platelet concentration (14), while higher concentrations could actually be an inhibitory factor. Additionally Graziani et al. suggest that fibroblasts can be optimally stimulated to increase proliferation at lower platelet concentrations (15). On the contrary, Lui et al. (16) showed that fibroblast proliferation and type I collagen production were also enhanced by increasing platelet concentrations, and that much of the response was pH dependent with the best responses occurring at more acidic pH levels.

Many factors can lead to platelet activation during the preparation of platelet concentrates. Platelets may undergo activation during collection, processing and storage of platelet concentrates (17). The latest study suggests that the concentration of grow factors of platelet concentrates can be retained for at least 6 months in storage at -70°C, and GF of platelet concentrates can be obtained by means of direct freezing, regardless of whether thrombin activation is used. Freeze-thaw procedures are a common method for releasing intracellular GF (18). The use of EDTA anticoagulated samples is potentially more harmful than citrate in the preparation of PRP gel. Although EDTA gave greater yields of platelets. they appeared damaged by the presence of EDTA (19).

Despite many experimentally confirmed advantages resulting from PRP use, it can be noticed that due to the different conditions in each experiment, variable results are unavoidable. This review describes the scientific and mechanistic bases of the biological activities and clinical possibilities of using PRP technology. A literature search was conducted using the MEDLINE/PubMed database for appropriate articles in English language. Databases were searched from 1985 up to 2011. The articles were successively searched by keyword or title using the following keywords: "PRP", "platelet derived growth factor", "platelet-rich plasma", "autologous gel", "platelet gel", and "growth factors".

Growth factors in PRP

Platelet α-granules have been shown to contain mitogenic and chemotactic growth factors (GF) and associated healing molecules in an inactive form, important in wound healing, such as platelet-derived growth factor (PDGF), transforming growth factors β1, β2, β3 (TGF-β1, TGF-β2, TGF-β3), platelet-derived angiogenesis factor (PDAF), insulin-like growth factor 1 (IGF-1), platelet factor 4 (PF-4), epidermal growth factor (EGF), epithelial cell grow factor (ECGF), vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF) and others cytokines. Additionally, plasma fluid also contains a number of biologically active proteins such as growth factor IGF-I and hepatocyte growth factor (HGF). During normal wound healing, trapped platelets become activated and degranulate, resulting in a release of the α-granule content. In this process, the granules fuse to the platelet cell membrane where the protein growth factor is moved to a bioactive state by the addition of histones and carbohydrate side chains to these proteins. The secreted growth factors immediately bind to the external surface of cell membranes in the graft, flap, or wound via transmembrane receptors in mesenchymal stem cells, osteoblasts, fibroblasts, endothelial cells, and epidermal cells (2,6).

They mediate many of the cellular functions including cell migration, proliferation, differentiation, metabolism and apoptosis, as well as cellular cycle (2, 20, 21). Platelets are therefore expected to stimulate damaged tissues and to regulate local inflammatory processes. The effects of these growth factors on cell behavior and on wound healing have been thoroughly studied. Platelet counts and PRP growth factor content are likely to depend on the particular technique used in obtaining the PRP. Platelet activation during preparation of the platelet concentrate can result in early α-granule release and loss of the growth factors during the collection process. It is therefore critical to recognize that each platelet-rich plasma preparation method may differ in regard to platelet number, platelet activation rate and growth factor profile.

Theoretical levels of platelet-derived growth factors in PRP might be expected to depend on the number of platelets involved, but the study by Weibrich et al. did not show a statistically significant correlation between PRP platelet count and growth factor level (22), and important variations in GF concentrations were detected between individuals having very similar platelet counts (23, 24). Martineau et al. observed inter-individual variations in GF concentration, especially evident for VEGF (up to a 900-fold difference between donors), and also did not find a correlation between concentrations of different GF within the same donor (24).

Literature data demonstrates that platelet counts in either whole blood or PRP are not predictors for the resulting growth factor levels in PRP. Scientists concluded that this result was connected with high individual variability in cellular production or storage of cytokines. Several parameters influence the relationship between platelet concentration and growth factor levels, such

as sufficient activation of platelets to initiate α-granule release, white blood cell count, plasma growth factor contamination, and collection of growth factors from the fibrin clot. Fréchette et al. recently demonstrated that the release of GF is significantly regulated by the amount of calcium and thrombin added to the PRPs. The addition of calcium and thrombin to PRPs had different effects on extracellular GF concentration. EGF and Ang-2 levels increased immediately after treatment, IGF-1 decreased slowly, and a delayed increase in IL-1 concentration was observed over time. These patterns were observed for all donors, suggesting that calcium and thrombin regulate GF release, synthesis, and/or degradation in stereotyped patterns that are specific to each growth factor (25).

It has been shown that PDGF, TGFβ1, VEGF and EGF cytokines were all significantly greater in platelet-rich plasma samples than in the whole blood baseline samples. On average, the PDGF-BB isoform increased from 3.3 ± 0.9 ng/ml to 17 ± 8 ng/ml, TGF-β1 increased from 35 ± 8 ng/ml to 120 ± 42 ng/ml, VEGF increased from 155 ± 110 pg/ml to 955 ± 1030 pg/ml, and EGF increased from 129 ± 61 pg/ml to 470 ± 317 pg/ml. Weibrich et al. (22, 26), in their examination of the concentrations of individual grow factors in PRP produced by discontinuous flow separation, showed the presence of three growth factors: PDGF-AB (117.5763.4 ng/ml), TGF-b1 (169.47 84.5 ng/ml) and IGF-I (84.2723.6 ng/ml). PDGFBB (9.977.5 ng/ml) and TGF-b2 (0.470.26 ng/ml) were present only in small amounts. The discontinuous cell separation method for PRP production yielded an approximately 5-fold increase in platelet concentration when compared with the initial platelet concentration in the donor blood. Pearson's correlation coefficient between platelets in PRP and in whole blood in the mentioned study was significant (r=0.77), however the Pearson's correlation coefficients for whole blood platelets, PRP platelets, and growth factor contents demonstrated little relationship between these parameters ($r \leq 0.35$). In addition, they showed a slight decrease of IGF-I in PRP with age.

Fréchette et al. (25) demonstrated that the low level of EGF detected in supernatants from nonactivated PRPs (57 ± 77 pg/mL) prepared with the Platelet Concentrate Collection System (PCCS) increased in response to addition of calcium and thrombin (4.7- to 11-fold; $p < 0.005$) even up to 595 ±195 pg/mL. On the contrary, IGF-1 concentrations in supernatants from non-activated PRPs were significantly higher compared with all other GF and did not increase after the addition of calcium and thrombin (5.5 ± 2.1 ng/Ml). Mean TGFα concentrations were either not detectable or were very close to the detection threshold. In the study conducted by Martineau et al. ELISA assays revealed that bFGF and VEGF were present at low concentrations in platelet concentrate (PC) supernatants prepared using the PC Collection System. bFGF concentrations ranging from 6 to 46 pg/ml in non-activated PC, and that treatment with high calcium and thrombin concentrations contributed to significant and rapidly increases in bFGF levels, being very similar for all of the donors (mean increases: calcium:325%, thrombin:745%), with maximum concentrations detected 1h after activation. Treating PC with low calcium and thrombin concentrations tended to reduce the total amount of bFGF. VEGF were detected in supernatants from non-activated PC at 57.2 pg/ml, while calcium and thrombin concentrations induced a moderate increase (57%) regardless of their concentrations. The amounts of PDGF-BB ranged from 1.7 to 10.6 ng/ml and were evaluated in non-activated PC in the mentioned study with an increase (range 16–50 ng/ml) following calcium and thrombin incubation. Baseline levels of TGF-b1 were within 0.3–5.3 ng/ml, an 84% increase in TGF-b1 concentration was observed in supernatants from PC treated with calcium and thrombin concentrations (24).

Banks et al. (1998) reported similar VEGF concentrations in thrombin-activated PC (range: 38–505 pg/ml) and in calcium-activated PC (range: 28–369 pg/ml) (27).

The potential loss of bioactivity of the growth factors may be concerned with the storage of PRP, and less than 48h of storage would be allowable for the better retention of platelet properties (28, 29).

One should also remember that plasma as a PRP component is a rich source of many proteins and ions of organic and inorganic particles, and that plasma proteins are also known to be critical components in the healing mechanism of connective tissues.

PRP also contains three proteins in blood known to act as cell adhesion molecules for osteoconduction and as a matrix for bone, connective tissue and epithelial migration. These cell adhesion molecules are fibrin itself, fibronectin, and vitronectin (7). When plasma is exposed to thrombin, either by the addition of exogenous thrombin or by coming in contact with tissue thromboplastin (also known as tissue factor), the clotting cascade is initiated and platelets are activated resulting in formation of a fibrin clot providing a provisional scaffold for cell migration as well as a reservoir of growth factors (30). However, research by Landesberg et al. showed that the amounts of TGFβ and PDGF present in the platelet-free plasma were minimal, indicating that almost all of the growth factor/cytokine present in the PRP gel is derived from the platelets (19). Additionally, Peterson et al. documented that the concentrations of VEGF, PF-4, PDGF, TSP-1, and bFGF in platelets of normal human subjects differ significantly from those in plasma. The concentrations of the selected angiogenesis regulators investigated in this

study were higher in platelets than in plasma (31).

Clinical and experimental significance of platelet growth factors

Platelet-rich plasma concentrate is applied in many fields of medicine. The general concept of useful PRP is to concentrate blood platelets through centrifugation of whole blood and to apply them with or without direct activation where their healing potential is needed (3). PRP works via degranulation of the granules in platelets which contain the synthesized and prepackaged growth factors. More than 95% of the presynthesized growth factors are secreted within 1 hour, and degranulation in platelets and secretion of growth factors occurs up to 3-5 days after the combination of the platelet-rich plasma with thrombin and calcium ions; the growth factors remain active for 7-10 days. The PRP growth factors never enter the cell or its nucleus, they are not mutagenic, and have no ability to induce tumor formation (6, 12, 32). Platelets in PRP also play a role in host defense mechanisms at the wound site by producing signaling proteins that attract macrophages (33).

Platelet concentrates containing growth factors are applied in clinical practice in various ways. A lot of studies have been performed to investigate the effect of PRP upon tissue regeneration, however, the results are controversial, often due to differing methods of platelet concentrate preparation. Biology and clinical potential of preparations are dependent on their composition and the way the growth factors, matrix and cells are assembled together.

The first attempts to apply the platelet gel in the treatment of bone defects were made by maxillofacial surgeons, demonstrating higher bone density in histomorphometric studies in patients with bone defects filled with bone marrow mixed with PRP gel, compared to patients who used only bone marrow; a significantly greater percentage of trabecular bone was achieved after the addition of PRP (74% with PRP compared with 55% without PRP) (6). Research by Cieślik-Bielicka et al. on the use of PRP in cysts in the mandible confirmed its osteoinductive properties (34). Using autogenous platelet-rich plasma (PRP) is currently being widely studied in applications for dental implants as a method of accelerating the maturation of bone in the mandible (35). Anuita et al. after putting PRP in the alveolus after extraction of teeth, observed a faster bone regeneration and healing of damaged tissue during surgery on oral mucosa (4), and the clinical advantages of PRP use have been highlighted by Marx (2). Kassolis et al. used PRP with freeze-dried bone allografts in sinus floor elevation and alveolar ridge augmentation with good postoperative results (36). These procedures are applicable for improving the contour of the alveolar ridge in relation to ideal pontic papilla esthetics for fixed partial prosthesis, healthy dentoalveolar complex for periodontal attachment and bone for dental implant placement (37). Growth factors exert regulatory effects on the homeostasis of the periodontal tissues and they also have the ability of modifying the response of periodontal soft and hard tissues during the healing processes after their exogenous application (38). In the field of implant dentistry, the application of PRP in addition to autogenous grafts used for these procedures like sinus lifts, ridge augmentations, etc. will promote and accelerate the osseo-integration process. It seems specially benefitted in maxilla, in cases of previous implant failures in type 4 bone in osteoporotic women (7, 12).

PRP has found clinical applications in fully autogenous bone grafts and composites of autogenous bone grafts with a variety of bone substitutes with as little as 20% autogenous bone (39), has shown improved results in sinus lift augmentation grafting (36, 40, 41), in horizontal and vertical ridge augmentations (39), and in ridge preservation grafting (35).

Franchini et al. used a platelet concentrate in fractures, in pseudoarthrosis, bone reconstructions in hip joint arthroplasty, in fibrous dysplasia and osteomyelitis (13). These preparations are also used with good effect in patients from risk groups that undergo ankle arthrodesis (42) and in reconstructive knee surgeries (43, 44).

In continuing inflammation, or directly after operation and application of PRP, the activation of PDGF, VEGF, TGF-β1 and TGF-β2 result in the improved control of cellular metabolism by reducing the formation of oxygen radicals (45).

PRP is used to treat injuries and chronic tendon and ligament pathologies. Factors released from platelets significantly increased the proliferation of tendon cells and also stimulated them to secrete VEGF and HGF to promote angiogenesis and prevent the formation of scar tissue around the tendon. These processes are closely linked with the tendon's ability to heal (46).

The effectiveness of PRP has been reported both after the surgical repair of ruptured tendons and in various tendinopathies (47, 48).

Hiramatsu et al. (2002) examined the effects of reinfusion of autologous platelet concentrate after open heart surgery in patients with noncyanotic congenital heart disease. Reinfusion of freshly prepared autologous PRP was followed by good aggregation responses and low blood loss. The results of that study suggest that this procedure might be useful in open heart surgery to avoid blood transfusions and minimize the need for homologous blood products (49).

Hammond et al. (2009) in an experimental animal study proved that the use of platelet-rich plasma (PRP) concentrate accelerates the healing of muscle tissue, PRP

Table 1. *Human and animal clinical applications using different kind of platelets-rich preparations. The table described the main results of each study, listed in chronological order.*

Study	Investigated human/animal group	Treatment	Results
Kashima et al. (2000)	8 patients underwent nine Thoracic Aortic Aneurysm surgery	PRP transfusion during operations (PRP gained during operation)	Autologous platelet-rich plasma transfusion was an effective way to reduce homologous blood transfusions in thoracic aortic aneurysm surgery.
Man et al. (2001)	20 patients undergoing cosmetic surgery involving the creation of a surgical flap. The types of surgical procedures: face lifts, breast augmentations, breast reductions, and neck lifts.	For each patient the type of surgical procedure and the average area was different. Only of the capillary bed under the flap in each case was the same/autologous fibrin glue=PPP (Platelet-poor plasma), PRP-gel/ 7,5 cc of PPP and PRP	Autologous fibrin glue and platelet gel in cosmetic surgical procedures, resulted in many advantages included: shorter operating times, the elimination of the need for drains, a reduction in the need for compressive dressings, a reduction in pain and postoperative swelling, and improved wound healing with a shorter recovery time.
Froum et al. (2002)	3 patients with bilateral sinus augmentation procedures prior to the placement of the implants.	Experimental side (PRP) and the control side (non-PRP). All six sinuses in this pilot study were grafted with an organic bovine bone (0.25 to 1 mm cancellous BioOss, Osteohealth)/ 30 mL of PRP/ observation was after 11 months.	Platelet-rich-plasma did not make a significant difference in the production of vital bone in sinuses grafted with BioOss. The use of platelet-rich plasma with grafts consisting of 100% BioOss should be considered only in respect to the improved handling quality (containment) of the particulate graft material that can be achieved through the activation of the platelet-rich plasma with thrombin.
Aghaloo et al (2005)	15 white male rabbits with four 8mm diameter defects in cranium with a trephine bur with copious irrigation	grafted with freeze-dried mineralized bone (FMB) alone, FMB+PRP, freeze-dried demineralized bone (FDDB) alone, and FDDB+PRP/ 1–1.5 cm3 of PRP/ 4 months period	Radiographically, FMB+PRP showed a tendency toward increased bone density over FMB alone, and FDDB+PRP showed a tendency toward increased bone density over FDDB alone. Histomorphometrically, FMB+PRP showed a tendency toward increased bone area over FMB alone at 1 and 4 months, and FDDB+PRP showed a tendency toward increased bone area over FDDB alone, at 1 and 2 months.
Dominiak & Mierzwa-Dudek (2005)	1 patient with multiple complex bone defects of teeth	PRP+ heterogenous bone graft Bio−Oss Spongiosa®/ 5–7 ml of PRP/ 4 months period	After surgery, there was no presence of plaque and gingival bleeding. The average depth of periodontal pockets in four measurement points decreased by 10.6 mm and the average level of the loss of connective tissue attachment decreased by 11.4 mm. A pantomogram showed the reconstruction of the alveolar process bone tissue.
Shayesteh et al. (2005)	8 white rabbits with three identical full thickness bony defects with a round bur (3×6 mm) in the frontal and parietal bones with a distance of approximately 2mm from the sagittal and coronal sutures.	The defects was grafted with Deproteinized Bovine Bone Mineral (DBBM) and PRP+DBBM/ 0.5 ml of PRP/ 12 weeks period	A significant increase in bone formation was seen with the addition of PRP to DBBM at 2, 4 and 8 week intervals. At 12 weeks, the level of bone formation was similar between the two groups. There was also a significant increase in the rate of biodegradation of the DBBM particles with the addition of PRP at 2, 4, 8 and 1 weeks.
Steigmann & Garg (2005)	20 patients who required sinus augmentation with a crestal bone height of approximately 7–9 mm in the posterior maxilla	autologous PRP	PRP alone in appropriate sinuslift cases can produce bone growth
Casati et al. (2006)	10 male adult mongrel dogs with defects in dehiscence-type bone around dental implants. Before implant placement, one dehiscence-type defect (4 mm x5 mm) was bilaterally created on the buccal aspect of the implant osteotomies with a high rotation conic bur with sterile continuous saline solution irrigation. Two 4 mm x8.5 mm machined screw-shaped commercial pure titanium implants were placed on each side of the mandible, and the soft tissues were repositioned and sutured.	PRP gel/ 5 ml of blood were drawn from each dog, this blood was used to obtain separation of basic blood fractions (PRP)/ platelet count of PRP was 460,350 platelets/ml/ 3 months period	Bone-to-implant contact (BIC), bone density (BD) within the limits of implant threads, bone density (BO) and new bone area (NB) in a zone lateral to the implant, corresponding to bone defects, were obtained and measured. No statistically significant differences for any of the investigated parameters when PRP was used. Within the limits of the present study, it was concluded that platelet-rich plasma alone did not enhance bone regeneration for peri-implant defects
Czuryszkiewicz-Cyrana et al. (2006)	26 patients with diagnosed chronic and advanced periodontitis.	PRP, PRP + autogenous bone/ 8.5 ml of blood were drawn from each patient, this blood was used to obtain separation of basic blood fractions (PRP)/ observation after implantation of autogenous bone with	Autogenous bone with added PRP in treatment ofintrabony defects caused by periodontitis have given significant clinical improvement of the periodontal tissues. The combination of PRP and autogenous bone caused the elimination of a convenient environment for subgingival bacterial plaque eliminating periodontitis.

		added PRP was 12 months after surgery	
Kaul et al. (2006)	14 female rabbits with a cylindrical osteochondral defect in the patellar groove of each, with a manual cannulated burr (3.2 mm in diameter)	Lapine articular chondrocytes overexpressing a lacZ or a human FGF-2 gene sequence were encapsulated in alginate and further characterized. The resulting lacZ or FGF-2 spheres were applied to cartilage defects in the knee joints of rabbits/ 14 weeks period.	In vitro, bioactive FGF-2 was secreted, leading to a significant increase in the cell numbers in FGF-2 spheres. In vivo, FGF-2 continued to be expressed for at least 3 weeks without leading to differences in FGF-2 concentrations in the synovial fluid between treatment groups. Histological analysis revealed no adverse pathologic effects on the synovial membrane at any time point. FGF-2 gene transfer enhanced type II collage expression the cell morphology and architecture of the new tissue. Overall articular cartilage repair was significantly improved at both time points in vivo.
Klongnoi et al. (2006)	16 minipigs with extraction of maxillary premolars	Sinus augmentations were performed bilaterally using grafting materials: autogenous bone and Algipores® with or without PRP/ 5ml of PRP was available for each augmentation site/ 12 months period	The grafting materials chosen showed increasing levels of bone-implant-contact (BIC) and newly formed bone throughout the period of observation in both PRP and non-PRP groups. Adding PRP resulted in lower BIC and newly formed bone compared with autogenous bone grafts or Algipores alone. However, a statistical significance was not found. The percentages of the remaining bone substitute in both the PRP and non-PRP groups were closely comparable in all observation periods.
Mishra. & Pavelko (2006)	20 patients with elbow epicondylar pain for 15 months. All patients were considering surgery	injection of PRP (active group, n=15) or bupivacaine (control group, n=5)/ 2 to 3 mL PRP / observation was after 1. 3. 6 months and after 1 year	Eight weeks after the treatment, the PRP patients noted 60% improvement in their visual analog pain scores vs. 16% in control patients. At 6 months, the patients treated with PRP noted 81% improvement in their visual analog pain scores. At final follow-up the platelet-rich plasma patients reported 93% reduction in pain compared with before the treatment.
Sarkar et al. (2006)	16 sheep with critical diaphyseal long bone defect (critical size defect -2.5 cm)	autogenous PRP in a collagen carrier or with collagen alone (controls)/ 3.5 ml of PRP/ treatment extended for 12 weeks	Bone volume, mineral density, mechanical rigidity and histology of the newly formed bone in the defect did not differ significantly between the PRP treated and the control group, and no effect of PRP upon bone formation was observed; PRP could not promote bone regeneration in critical size defects with a low regenerative capacity
Plachokova et al. (2007)	45 rats, in which one cranial defect with a diameter of 6.2 mm	The defects were filled with combination with dense biphasic hydroxyl apatite (HA)/b-tricalcium phosphate (TCP) particles HA/b-TCP particles and HA/b-TCP particles combined with PRP gel/ 150 µl of PRP/ observation was after 1 and 2 weeks	A 6.2mm cranial defect is not a critical-sized defect in rats. Rat PRP had no effect on the early stages of bone healing in addition to an osteoconductive material. Dense HA/b-TCP particles showed a beneficial effect on bone formation already after 1 and 2 weeks of implantation in non-critical-sized cranial defects in rats.
Sánchez et al. (2007)	12 athletes with spontaneous complete rupture of the Achilles tendon	Control group (6 athletes not receiving PRGF), and test group (6 athletes receiving PRGF) PRGF/ 4 ml of PRGF/ 12 months period	Athletes receiving PRGF recovered their range of motion earlier, showed no wound complication, and took less time to take up gentle running and to resume training activities
Tunç et al. (2007)	22 patients with infrabony defects	10 ml of blood were drawn from patients, this blood was used to obtain separation of basic blood fractions (PRP)/PRP + DFDBA (demineralized freeze-dried bone allograft)	The results indicate that DFDBA/PRP combination is more effective than PRP alone for the treatment of infrabony defects, and the amount of CAL gain, PPD reduction, and bone fill increases when the infrabony defect is narrow and deep before DFDBA/PRP combination treatment.
Azzena et al. (2008)	75-year-old woman with painful, adherent scar at the shoulder level	20 ml of blood were drawn from patient, this blood was used to obtain separation of basic blood fractions (PRP)+fat	The results were satisfactory, showing fat survival 1 year after surgery.
Cieslik–Bielecka A. et al. (2008)	PRG on healing of mandibular odontogenic cysts	cases divided into control (no PRG treatment) and experimental (PRG-treated) groups; each participant was followed on a regular basis with clinical examinations, roentgenograms, and dual-energy x-ray absorptiometry (DEXA) examinations/ PRG-gel	Clinical observations showed that oral mucosa healed faster in patients treated with PRG compared with cases where gel was not added. Roentgenograms and DEXA examinations showed considerable enhancement of bone regeneration beginning from the 5th week and continuing during subsequent periods after implantation of PRG in the experimental group compared with the control group.
Gawande & Halli (2008)	20 patients having bilateral mandibular third molar impaction with similar angulations	5 ml of blood were drawn from patients, this blood was used to obtain separation of basic blood fractions (PRP)/ PRP gel	The result of the study shows rapid bone regeneration in the extraction socket treated with PRP when compared with the socket without PRP. Also there was less postoperative discomfort on the PRP treated side.
Simman et al.	48 rats with following	thrombin-activated PRP	Radiographic analysis demonstrated higher callus

(2008)	creation of unilateral open femur fractures	(PRP treated group) and saline (control group)/ 500µl of PRP/ 4 weeks period	to cortex in the PRP group. Three-point load bearing showed increased bone strength following PRP treatment. Fracture histology showed enhanced bone formation in the PRP group. Immunohistochemistry and Western-blotting demonstrated healing-associated changes in transforming growth factor (TGF)-β1 and bone morphogenetic protein (BMP)-2.
Lyras et al. (2009)	40 skeletally white rabbits. Two groups 10 rabbits each (PRP and control group) were used to evaluate mechanical properties and histology after 14 days and two groups ten rabbits each (PRP and control groups) were used to evaluate mechanical properties and histology after 28 days. The 3-mm width of the defect is approximately equal to one-third of the width of the tendon	PRP-gel/ 8 ml of blood were drawn from each dog, this blood was used to obtain separation of basic blood fractions (PRP)/ 28 weeks period	The mechanical properties of the regenerated tendon in the PRP group were significantly improved in relation to the control group. It appears that PRP has a strong effect in the early phase of tendon healing. This effect is probably due to the growth factors that are released from the platelets during activation
Pieri et al. (2009)	8 adult minipigs in which two mandibular premolar teeth were extracted bilaterally; the defects of 3.5 mm diameter and 8 mm depth were created in each root site	The defects were randomly grafted with autogenous mandibular bone, (fluorohydroxyapatite) FHA alone, PRP-FHA, or mesenchymal stem cells MSCs-PRP-FHA/ 16 mL of PRP gel/ observation was after 3 months	MSCs-PRP-FHA-treated sites showed new vital bone between residual grafting particles. PRP-FHA- and FHA-treated sites showed residual particles in a background of marrow soft tissue with a moderate quantity of newly formed bone. Autogenous bone (46.97%) and MSCs-PRP-FHA (45.28%) produced a significantly higher amount of vital bone than PRP-FHA (37.95%), or FHA alone (36.03%). Further, the MSCs-PRP-FHA-treated defects showed a significantly higher percentage of contact between graft particles and newly formed bone compared with PRP-FHA and FHA group (59.23% vs. 48.37% and 46.43%, respectively).
Spyridakis et al. (2009)	52 patients with pilonidal sinus disease who underwent open excision and secondary closure of the surgical wound (n=22) or additional local postoperative infusion of platelet-derived growth factors (n=30) were evaluated. Duration of total wound healing and time to return to normal activities were evaluated.	PRP-gel/ 4 weeks period	Wound-healing rates were much greater for the platelet group. Complete healing of the surgical wound required 24 days for the PRP-gel, more than 30 days for the control group. PRP-gel group returned to their normal activities around the postoperative day 17 vs. control group day 25. The use of platelet-derived growth factors directly to the surgical wound enhances the healing process resulting in faster recovery of patients surgically treated for pilonidal sinus disease.
Szypuła & Kędziora (2009)	10 patients with chronic suppurative osteomyelitis resistant to surgery and antibiotics	PRP/ treatment duration dependent on the case	Use of a platelet rich plasma preparation in cases of posttraumatic suppurative osteomyelitis has a beneficial effect on healing and regeneration of bone.
Arenaz-Búa et al. (2010)	82 patients with bilateral impacted mandibular third molars	PRP, AUTOLOGOUS BONE +PRP, PRP +NOVABONE , PRP + DBX (DBX®-Allogeneic demineralized bone matrix NOVABONE®-synthetic material based on synthetic calcium hydroxyapatite)	Did not observe that the platelet-rich plasma accelerated bone formation in post-extraction sockets. Platelet-rich plasma mixed with other biomaterials facilitates the manipulation of the graft (made of hydroxyapatite, for example) and therefore could be useful as a biological carrier in mandibular bone reconstruction due to its low cost and ready availability.
Filardo et al. (2010)	15 patients affected by chronic jumper's knee, with exercise-associated pain, pain or tenderness on palpation and imaging findings of degenerative changes and control group of 16 patients treated with the physiotherapy	PRP/ 20 ml of PRP/ injections were performed every 15 days, end of the treatment and at 6 months follow-up	Analysis showed a significant improvement in both the score recorded by an individual for their current health-related quality of life state and sport activity; decrease in the pain level on the from basal evaluation to the end of to the end of the PRP injections.
Hartmann et al. (2010)	15 patients, who had suffered an injury of the thoracic or lumbar spine and had undergone an anterior fusion using cages/ 20 patient in control group	Bone graft combined with PRP. A control group made up of 20 patients received a similar treatment, but without the addition of PRP/ 110 ml of blood were drawn from each dog, this blood was used to obtain separation of basic blood fractions (PRP).	In both groups, 40% of the patients had reached a complete fusion in the CT scans. No or minimal fusion was documented in 20% of the PRP group and 30% of the control group. When measuring the density within the newly formed bone mass, both groups showed nearly identical percentages with a density of over 100 Hounsfield units (HU). The share of bone with a density of over +500 HU was 29.33% in the PRP group and 23.57% in the control group. Within the partition of over +100 HU, the absolute density was significantly higher in the PRP group.
Khoshzaban et al. (2010)	30 male rats with the cavity which was drill in the Calvarias 6.2 mm in diameter	Group1:treated by PRP/Bio-Oss; group 2:treated by PRP-Gel /Bio-Oss; group 3:had two cavities; Bone chips mixed with individual blood/ 4-5 ml of blood were drawn	The author suggested that neither PRP nor PRP-Gel could be as beneficial as bone chips. Statistically, in PRP-Gel group, due to the existence of fibrin and thrombin, solid bone bridging at the treated site was indicated.

		from each rat, this blood was used to obtain separation of basic blood fractions (PRP)/ 16 weeks period	
Kon et al. (2010)	100 consecutive patients, affected by chronic degenerative condition of the knee	PRP/ 5 ml of PRP/ 12 months period	The preliminary results indicate that the treatment with PRP injections is safe and has the potential to reduce pain and improve knee function and quality of live in younger patients with low degree of articular degeneration.
Luaces-Rey et al. (2010)	20 patients who underwent secondary bone graft in the alveolar cleft	autogenous bone graft, autogenous bone and platelet-rich plasma (PRP)/ 6 months period	No significant differences were found between both therapeutic groups on bone regeneration.
Lyras et al. (2010)	48 white rabbits with a full thickness defect in the central portion of the patellar tendon	PRP gel was then applied and filled the tendon defect (in the control group, without the application of PRP)/ 2 ml of PRP/ 4 weeks period	The histological examination showed a superior healing process in the PRP vs. control group; the tissue formed in the PRP group was more mature and dense with less elastic fibers remaining. Neovascularisation was significantly higher in the PRP group.
Mallo et al. (2010)	1 patient with shoulder pain in his right dominant extremity. His pain was exacerbated with overhead movement, especially forward elevation above 90^{o}. The patient had pain with passive forward lexion at 100^{o} as well as marked pain with passive forward flexion to 90^{o} combined with internal rotation. He showed no tenderness to palpation over his biceps tendon.	Diagnostic arthroscopy with partial bursectomy, subacromial, and PRGF injection at the conclusion of the surgery/ 3ml of the PRGF was delivered into subacromial space/ 9 months period	The patient achieved active and passive forward flexion from 0^{o} to 160^{o} with external rotation from 0^{o} to 70^{o} at the end of 3 months, he reported persistence and progressive worsening of his preoperative pain. An exuberant synovitis subsequently developed, maybe resulted from the PRGF treatment.
Sun et al (2010)	48 rabbits with osteochondral Defects in the femoropatellar groove were left untreated, treated with autogenous PRP in a polylactic-glycolic acid (PLGA), or with PLGA alone	A loading volume consisting of 20 µl human lyophilized thrombin and 80 µl of PRP mixture was sequentially pipette loaded onto each PLGA scaffold placed in a well of a 24-well plate/ 80 µl of PRP/ 12 weeks period	PRP yielded better osteochondral formation than the empty PLGA scaffold after 12 weeks. PRP incorporated in PLGA could successfully resurface the defect with cartilage and restore the subchondral bone in the rabbit model.
Alpigiani et al. (2011)	1 patient with right hip pain and functional limitation. Of the hip joints which showed osteonecrosis in chondral/ subchondral regions at the superior-external convexity of the right femoral head	Bone Marrow Cells (BMC) added to PRP. Implanted BMC plus PRP in the osteonecrotic region with improvement of pain and mobilization.	Patient walks autonomously, without joint pain and with improved hip movements. BMC plus PRP implantation represents only a palliative care to delay surgical treatment or if it is a valid alternative to traditional orthopedic surgery
Cervellin M. et al. (2011)	Forty young athletes with the indication of ACL reconstruction with patellar tendon grafts	56 ml of blood were drawn from patients, this blood was used to obtain separation of basic blood fractions (PRP)/ PRP gel	In 85% of PRP group patients, the tibial and patellar bone defect was satisfactorily filled by new bony tissue (70% of bone gap filled), whereas this percentage was just 60% in control group patients.
Dalal et al. (2011)	1 patient complained of swelling in upper right front tooth region. There was a single, diffuse swelling with smooth overlying skin	PRP gel+ autologous bone graft/ 10 ml of blood were drawn from patient, this blood was used to obtain separation of basic blood fractions (PRP)/ 3 months period	Promising result with fast and good quality bony regeneration was observed.
Dhollander et al. (2011)	5 patients with focal cartilage defects involving the patella and with clinical symptoms (pain, swelling, locking and ‘‘giving away’’)	autologous matrix-induced chondrogenesis (AMIC)+PRP gel	A clinical improvement became apparent after 24 months of follow-up. The formation of intralesional osteophytes was observed in 3 of the 5 patients during the 2 years of follow-up.
Martins et al. (2011)	22 patients with cancer and bisphosphonate-related osteonecrosis of the jaws	PRP + LPT(laser phototherapy)/ 4ml of PRP/ period 6 months	A significantly higher percentage of patients reached the current state of bisphosphonate-related osteonecrosis of the jaws (BRONJ) without bone exposure (86%) in the PPR plus LPT group than in the pharmacological (0%) and surgical (40%) groups after 1-month follow-up assessment. These results suggest that the association of pharmacological therapy and surgical therapy with PRP plus LPT significantly improves BRONJ healing in oncologic patients.

Orcajo et al. (2011)	1 patient with type 2 diabetes, insulin treated since 2002 with microangiopathy and diabetic Polyneuropathy. The patient had amputations of the 1st, 2nd and 3rd toes of right foot due to persistent ulcers. An infection in the 4th toe caused an ulcer with bone exposed, which finally resulted in amputation of the toe	PRGF/ 5 ml of PRGF in syringe which was connected to a sterile cannula and introduced into the mal perforant ulcer from the dorsum to the sole of the foot/ 10 weeks period	After PRGF treatment the severe mal perforant ulcer completely healed.
Rezaie et al. (2011)	20 white diabetic rabbits with hole in size of 4×5 mm in diameter and depth between lateral and medial condyles of left femur	autologous PRP gel/ 5 ml of blood were drawn from each rabbit, this blood was used to obtain separation of basic blood fractions (PRP)/ observation was after 2 months	PRP provide a rapid regeneration of bone defects in femoral cancellous bone in diabetic rabbits.
Sardari et al. (2011)	Five male, mixed breed dogs under general anesthesia (using acepromazine 0.05 mg/kg IM and ketamine 20 mg/kg IM, followed by halotan) had six full-thickness skin wounds (20×20 mm) created on the back of each dog symmetrically (three wounds in each side)	Total amount of 1,5 ml of PRP given to dogs in 1, 3 and 5 day after wounding.	No significant difference was seen in the percentage of wound contraction, epithelialization, and healing between test and control. There were no significant differences between median of hydroxyproline levels between left and right wounds in dogs treated with dexamethasone. There were no significant differences between median of epithelialization, inflammatory cell infiltration, presence of dermal granulation tissue, fibroblast proliferation, arrangement of fibroblasts, collagen deposition, and collagen bundle formation scores, in the specimens of left and right wounds. The results of the study demonstrated that PRP did not have significant effects to promote cutaneous regeneration and wound healing in dogs treated with dexamethasone at least 16 days after last injection.
Shahabuei et al. (2011)	48 dogs with experimental defects with 5 mm horizontal and 5mm vertical open probing depth in furcation areas of each premolar	test group grafted with Bio-oss+PRP, control group grafted with bio-oss alone, negative control group in which no graft was used/ 3 months period	The bone fill was 61% in the test group, 58 % in the control group, and 39 % in the negative control group. In the control group, the lamellar bone formation was higher and the chronic inflammatory infiltration was lower.
Zhang et al. (2011)	27 white rabbits with the unilateral 15-mm critical-size defects in the distal radial diaphysis	The bone defect was filled with BG (degradable bioactive borate glass) alone, BG combined with autologous PRP or left empty/ 0.8 mL of PRP/ treatment extended for 12 weeks.	At 12 weeks, the volume of the newly formed bone in the BG + PRP animals was significantly higher than in those receiving only BG; PRP improved bone healing in a diaphyseal rabbit model on BG. The combination of PRP and BG may be an effective approach to repair critical defects.

was administered into the injury site in the tibialis anterior muscle (50).

Wright et al. and Sanchez et al. showed that the use of PRP is an effective method of treatment in muscle injuries; the use of PRP accelerated recovery of patients and professional athletes who were given platelet concentrates (51, 52).

Also the first clinical study carried out on humans with PRP, used for the treatment of tennis elbow, provided the basis for consideration of platelet-rich plasma as an alternative to surgical treatment in patients with chronic tendinosis resistant to conservative therapies (53).

Man et al. (2001) used PRP in patients undergoing cosmetic surgery, including face lifts, breast augmentations, breast reductions and neck lifts, and reported that bleeding capillaries were effectively sealed within 3 minutes after application of the platelet gel (PRP) and fibrin glue (PPP) (54).

Recently, there has been research and tests on the use of PRP in order to obtain an easy-to-handle biologic gel that could be mixed with adipocytes. It is postulated that proteins from PRP could stimulate revascularization of the implanted adipocyte rich gel and constitute a three-dimensional matrix that allows for the arrangement of adipocytes into a correct spatial organization (55). Cervelli et al. treated patients affected by facial aging, characterized by atrophy of the subcutaneous tissue and deficits of soft tissue with loss of volume and elasticity with loss of volume and elasticity, with PRP mixed with centrifuged fat tissue (56).

In particular, growth factors regulate cellular events in wound healing, such as proliferation, differentiation, chemotaxis and morphogenesis of tissues and organs, acting in an autocrine, paracrine or endocrine manner. They are deposited in the extracellular matrix and are then released during matrix degradation. Their interaction with

surface receptors on the target cells activates an intracellular signalling pathway that induces transcription of the messenger RNA and proteins needed for the regenerative process. These growth factors, in combination with other transcription factors, then activate a set of genes. The growth factors also induce specific changes at a cellular level. All of these effects are controlled by feedback mechanisms involving binding proteins and other growth factors. Growth factors may also interact with one another, consequently forming a cascade of different signal proteins with multiple pathways, ultimately leading to the activation of gene expression and then protein production. Even in a single cell type, the nature of growth factor action may depend on the context set by other substances present. For example, TGF- stimulates growth of certain fibroblasts in vitro in the presence of PDGF but inhibits their growth if epidermal growth factor is present (21).

Giannobile et al. evaluated the interactions of IGF-I with other growth factors, including PDGF and TGF-b. They demonstrated various degrees of mitogenic activity, and the increase in DNA synthesis receiving IGF-I/TGF-b1 and IGF-I/PDGF-BB treatment was significantly greater than the individual factor (57). Mott et al. examined the effects on proliferation of a combination of IGF-I/TGF-b1, PDGF-BB/TGF-b1 and IGF-I/PDGF-BB, and showed that the proliferation of osteoblasts was enhanced by a combination of growth factors (58).

It is worth noticing that there are also some studies that do not suggest PRP as an effective factor for bone reconstruction (59, 60, 61). From a therapeutic point of view, there are two major processes related to the potential of this technology: the release of proteins and growth factors from platelets that actively stimulate tissue regeneration, and the formation of a three-dimensional fibrin matrix that retains and later releases part of the growth factors, and which also acts as a temporal nesting scaffold for the cells. After activation, platelets release a pool of biologically active proteins and other substances that are able to influence the recruitment, growth and morphogenesis of cells. The creation of a chemotactic gradient would mediate cell recruitment and the onset of the healing process. PRGF causes a fourfold increase in cell growth in vivo 72h after its application, enhances collagen deposition (twofold at 7 days) and while stimulating the proliferation of human gingival fibroblasts (twofold), inhibits keratinocyte growth by 40% (62, 63). Initiation of bone cell growth takes place mainly with the participation of growth factors PDGF, TGF-b and IGF. Their main source is platelet rich plasma (PRP) which is used among others as a component of platelet gel. In order to fully exploit the regenerative properties of PRP, it is combined with osteoconductive material, resulting in a demineralized bone matrix used to regenerate bone defects (64).

PDGF (Platelet-derived growth factor) seems to have numerous positive effects on wound healing including mitogenesis, angiogenesis, activation of macrophages and upregulation of other growth factors. PDGF is a powerful mitogen for fibroblasts and smooth muscle cells and is involved in all three phases of wound healing, including angiogenesis, formation of fibrous tissue, and re-epithelilization (65). PDGF is known to emerge from degranulating platelets at the time of injury. There are four possible isoforms of this thermo-resistant dimeric glycoprotein (molecular weight of approximately 30 kDa) with 3 disulphide bonded polypeptides referred to as A, B and C chains: AA, BB, CC and the heterodimeric AB. However, more recently an additional isomer PDGF-DD has also been verified (66). In lesser quantities, A-A and B-B homodimers exist in human beings with the same activity. Cells susceptible to PDGF action express α (or A-type receptors) and β (or B-type receptors), which are both involved in the transduction of mitogenic stimuli, while the β receptor mediates chemotactic stimulation. PDGF alpha-receptors bind all isoforms with high affinities; however, beta-receptors bind PDGF-BB with high affinity and PDGF-AB with a comparatively lower affinity, but do not bind PDGF-AA with any considerable affinity (67, 68).

Its mechanism is to activate cell membrane receptors on target cells, which in turn are thought to develop high-energy phosphate bonds on internal cytoplasmic signal proteins; the bonds then activate signal proteins to initiate a specific activity within the target cell (6).

All isoforms have proliferative activity on periodontal ligament fibroblasts, the AA and BB isoforms enhance proliferation of bone cells increasing the production of PDGF-AA in osteoblast cultures by an autocrine process. PDGF is also chemotactic for adipocytes, and it promotes collagen type I and protein synthesis, stimulates cell growth, but also changes cell shape and motility. It is the most thoroughly described growth factor in terms of its effects on the periodontium both in vitro and in vivo. The PDGF stimulates mitogenesis of the marrow stem cells and endosteal osteoblasts transferred in the graft to increase their numbers by several orders of magnitude. It also begins an angiogenesis of capillary budding into the graft by inducing endothelial cell mitosis and additionally indirectly, by activating macrophages (69, 70).

TGF-βs (Transforming Growth Factor beta) this superfamily of growth and differentiating factors, including among others, BMP (Bone Morphogenetic Protein), TGF-β1, TGF-β2 (with molecular weights of approximately 25 kDa) are synthesized and found in platelets and macrophages, as well as in some other cell types, and are involved with bone regeneration in

mitogenesis of osteoblast precursors, acting as paracrine growth factors. The paracrine mechanism also causes the aforementioned target cells to release proteins of TGF-β, acting on the cells in the direct vicinity. The activation process is maintained by an autocrine mechanism, involving the effect of growth factors secreted by the cell on its own membrane receptors. When released by platelets or secreted by macrophages, TGF-β exerts its effects on adjacent cells, including fibroblasts, marrow stem cells, endothelial cells, and preosteoblasts. TGF-β stimulates angiogenesis and chondrogenesis, the production of fibronectin, glycosaminoglycans, and collagen in connective tissue. In addition, it inhibits the proliferation of lymphocytes (71). Stimulates monocytes to secrete FGF, PDGF, TNF– α and Interleukin-1. TGF-β initially activates fibroblasts and preosteoblasts to mitose and increase their numbers, as well as promoting their differentiation toward mature functioning osteoblasts. Continued TGF-β secretion influences osteoblasts to lay down bone matrix and the fibroblasts to lay down collagen matrix to support capillary ingrowth (6). Fibroblasts under the influence of TGF-β increase the synthesis of collagen, fibronectin, and integrin. TGF-β also inhibits the enzymes that degrade these proteins (72). Additionally, they inhibit osteoblast formation and the proliferation of epithelial cells in the presence of other growth factors (73). TGF-β therefore represents a mechanism for sustaining a long-term healing and bone regeneration module, and even evolve into a bone remodeling factor over time (6). Repeated injections of TGF-β resulted in ossification by means of endochondral bone formation in the long bones (74). In addition, it promotes angiogenesis and extracellular matrix production. TGF-β chemotactic factor stimulates proliferation and differentiation of mesenchymal stem cells and promotes the synthesis of collagen type I by osteoblasts, and has been reported to be angiogenic (75).

IGF-1 (Insulin-like growth factor; somatomedin-C) is thought to increase the number of osteoblasts and thereby accelerate bone formation, the formation of bone matrix by promoting pre-osteoblast proliferation and stimulates the synthesis of osteocalcin, alkaline phosphatase activity in osteoblastic cells and collagen type I biosynthesis by osteoblasts and prostaglandin E2 in fibroblasts (76). It also stimulates the proliferation and differentiation of mesenchymal stem cells in chondrogenesis, adipogenesis, myogenesis, promotes neuronal differentiation and induces a chemotactic effect on vascular endothelial cells (77, 78). IGF has 2 forms, I and II, each of which has 2 single chain peptides, with respective molecular masses of 7.7 and 7.5 kDa. IGF binds to the same receptors as insulin and is involved in the development of many tissues, including the teeth. Both forms of IGF are potent factors for the survival of hematopoietic cells, fibroblasts and the nervous system. IGF is also an important modulator of cell apoptosis, and in combination with PDGF, can promote bone regeneration. IGF-I may directly stimulate the cells as an autocrine factor, additionally IGF-I transcripts have been isolated from macrophages in wounds, suggesting that this growth factor may also act as a local messenger as a paracrine factor. The autocrine growth-promoting activity is inhibited by IL4 (21). IGF has an effect on intraosseus osteoblasts, intensifying the formation of bone trabeculae in the spongy bone transplant. Increased cell activity at the beginning is the effect of PDGF, TGF-ß and IGF and, to a smaller degree, other factors (79).

EGF (Epidermal growth factor) has a mitogenic and chemotactic effect on fibroblasts and epithelial cells. It was described as an inductor for cell migration and stimulator for the formation of granulation tissue. The cells which synthesize and express receptors for EGF are mainly fibroblasts, pre-osteoblasts and pre-chondrocytes, indicating the participation of this growth factor in physiological processes connected with those cells (80). Menetrey et al. proved that FGF and IGF-1 accelerate the muscle healing process (81).

b-FGF (Basic-fibroblastic growth factor) is a member of the multifunctional fibroblast growth factor family. FGFs are pleiotropic factors that act on various cells including endothelial cells. The biological activity of FGF-2 is mediated through a dual receptor system consisting of four high-affinity, tyrosine kinase receptors and low-affinity, heparan sulfate proteoglycans located at the cell surface. To date, the FGF family consists of 23 members, all of which contain a conserved 120 amino acid core region that contains six identical, interspersed amino acids. The biological effect of FGFs is mediated by four tyrosine kinase FGF receptors, with multiple specificities noted for almost all FGFs (82). Basic fibroblast growth factor (FGF-2), present in platelet α-granules, stimulates and coordinates the mitogenesis of mesenchymal stem cells during growth, maintenance and tissue repair. Such effects were reported for fibroblasts, osteoblasts, chondrocytes, smooth muscle cells and skeletal myoblasts. FGF-2 stimulates chemotactic migration of marrow-derived mesenchymal cells into articular cartilage defects, resulting in a higher cell density. Angiogenesis is also enhanced through endothelial cell stimulation to undergo mitosis and migration (83). Analysis of type II collagen expression suggests that FGF-2 may selectively increase type II collagen content. In contrast, FGF-2 did not lead to an increase in type I collagen production (84).

VEGF (Vascular Endothelial Growth Factor) is essential for many angiogenic processes both in normal and pathological conditions. It is a thermo-resistant growth factor, a protein with a mass of 40-45 kDa, which

induces the chemotaxis and proliferation of endothelial cells to provoke the angiogenesis and hyperpermeability of blood vessels, and can promote healing of chronic wounds and aid in endochondral ossification. In vivo, application of VEGF to the surface of rat molars promoted cementogenesis after reimplantation. It is mitogenic, proapoptotic and promotes the chemotaxis and differentiation of epithelial, renal and glial cells as well as fibroblasts (85). VEGF has been successfully used in the treatment of various vascular diseases (86, 87).

PF4 (Platelet Factor 4) is a chemokine that functions as a negative regulator of angiogenesis and as a powerful inhibitor of endothelial cell proliferation. It is a chemotactant for neutrophils and fibroblasts and is a potent antiheparin agent (88).

Beside the most important GFs, described above, following mediator molecules are released from the platelet following activation: IL-8 (interleukin-8), TNF-α (tumour necrosis factor alpha), CTGR (connective tissue growth factor), GM-CSF (granulocyte macrophage colony stimulating factor), KGF (keratinocyte growth factor), and Ang-2 (angiopoetin).

The results of clinical applications using different kind of rich platelets preparations are presented in Table 1.

The role of platelet growth factors in angiogenesis

In recent years we have seen a growing interest in the process of neovascularization from existing structures (angiogenesis is the formation of new blood vessels from preexisting vasculature), both in relation to therapeutic angiogenesis, neovascularization in ischemic diseases and the search for inhibitors of tumor vasculature. It is a process essential for wound healing, and also in the formation of blood vessels in various disease processes such as atherosclerosis and cancer (89, 90).

In the process of angiogenesis, a key role is played by migrating and proliferating endothelial cells (EC), with paracrine interaction of growth factors the most important stimulating factor. Endothelial cell proliferation is very slow and the length of their turnover rate exceeds 1000 days (91), but it is an easily excitable structure, responding quickly to stimuli which activate it and change its function. Angiogenesis is the result of a complex interplay between growth factors, vascular endothelial cells, extracellular matrix molecules, chemokines and cell signalling molecules. The process of angiogenesis begins with the decomposition of the basement membrane, allowing the migration and proliferation of endothelial cells and their distribution in the extracellular matrix. It was recently demonstrated that platelets, as a cellular system, could induce an angiogenic response and that platelet microparticles affect EC, protecting them from apoptosis and inducing proliferation and formation of tubule-like structures (92, 93).

Among the growth factors two play a crucial role in angiogenesis, i.e. VEGF and bFGF; both are released by platelets and may be applied when using PRGF or PRP. These growth factors induce neovascularisation and chemotaxis of fibroblasts and tenocytes and stimulate fibroblast and tenocyte proliferation and the synthesis of collagen (94, 95).

VEGF has been studied extensively and plays a critical role in both vasculogenesis and angiogenesis (96, 97, 98). The pro-angiogenic effect of VEGF is associated with the presence of receptors for this factor. VEGF binds to tyrosine-kinase receptors, Flt-1 (also known as VEGF receptor 1) and Flk-1 (also known as VEGF receptor 2, KDR as human counterpart), which are present on endothelial macrophages, monocytes and some tumor cells. Lymphatic endothelial cells have different receptors (99, 100). VEGF signalling is modulated by angiopoietin-1 (Ang1) that binds to receptor tyrosine kinases (Tie-2) (101). It has been shown to stimulate vascular bud formation and maintain endothelial cell survival. The action of Ang1 is associated with mesenchymal cells surrounding the endothelium. An excess of Ang1 causes the production of more numerous and finer capillaries (102). Ligand binding to Flk-1/KDR induces autophosphorylation of Flk-1/KDR intracellular tyrosine residues and activates several signaling pathways, leading to cell proliferation, survival, migration, and permeability. VEGF, binding to one of its receptors, promotes endothelial cell proliferation which otherwise shows limited growth potential in the absence of stimulation by this factor. Stimulation of the second receptor on mesodermal stem cells causes the transformation of these cells into endothelial cells (103). Recent evidence suggests that cyclooxygenase-2 (COX-2) modulates angiogenesis by interacting with the VEGF system. Nitric oxide (NO) is a vasodilator and is implicated in VEGF mediated vascular permeability and angiogenesis (104).

bFGF is a powerful mitogenic and chemotactic factor for endothelial and smooth muscle cells. Their action is dependent on interaction with the homeostatic factor – heparin. FGF interacts with heparinsulphate proteoglycans (HSPGs) and transmembrane receptor tyrosine kinase FGFR (FGFR-1, FGFR-2, FGFR-3, FGFR-4). Upon binding with their angiogenic ligands (FGF-1, FGF-2 and FGF-4), FGFRs are autophosphorylated leading to activation of several intracellular signalling pathways leading to the recruitment of Shc, FRS2 and Crk adaptor molecules (105). It is perhaps the interaction with HSPG that makes extracellular matrix (ECM) a key player in the regulation of angiogenesis. Induction of endothelial cell proliferation by FGF leading to angiogenesis, involves not only the activation of the mitogen-activated protein (MAP) kinase pathway, but also sustained activation of

protein kinases C (PKC) and PI3 –kinase (106). Once the path for cell migration has been carved, FGF-1, FGF-2, FGF8b isoform and FGF-10 promote chemotaxis in endothelial cells (107, 108). PI3-kinase plays a pivotal role in mediating EC survival, proliferation, cytoskeleton reorganization, and cell motility, all critically important for vessel growth. PI3-kinase is activated by VEGF and bFGF through VEGF receptor (Flk-1/KDR) on EC upon binding to its ligand (VEGF); FGF receptor-1; and via Src kinase family members (109). The platelet derived endothelial cell growth factor PD-ECGF is a monomer with a weight of 45 kDa, which stimulates the synthesis of DNA in endothelial cells but does not affect the proliferation of these cells (110).

Because platelets are the first cells to appear in wounds and inflammatory foci, PD-ECGF may prepare the vascular bed for changes caused by other growth factors (111, 112). One should remember that PRP may also be a source of factors that inhibit angiogenesis. Produced by platelets, platelet factor 4 cytokine of low molecular weight, inhibits the formation of new blood vessels due to blocking the binding of angiogenic growth factors to membrane proteoglycans. It can inhibit in vitro proliferation and migration of endothelial cells. Activity of endothelial cell mitogens, such as VEGF and bFGF, are sensitive to inhibition by platelet factor 4 (113).

Conclusion

Autogenous platelet-rich plasma (PRP) was first used in the 1990s and is raising interest and controversy among researchers and clinicians. Investigations relating to the use of autogenous material in medicine focus on inter-relationships between factors released during the activation of platelets, plasma factors, potential additive mechanisms, and the search for optimal preparation procedures and dosing of blood products, including PRP.

Additionally at this point is an increased focus on the cellular content, and the direct effects of the leukocytes and platelets themselves, not only on the growth factor synthesis and release, but also on the direct regulation of the inflammatory response, healing process, and anti-infectious activities of these preparations.

These technologies have been popularized over the last decade, particularly in cardiac surgery, in many areas of dentistry such as periodontics, oral implantology and oral and maxillofacial surgery, orthopedics, repositioning skin flaps in plastic surgery, against small diffuse bleedings, a mal perforant ulcer in the foot of a person with diabetes, to speed up the healing process of burns, to promote retinal neurogenesis, and in the treatment of musculoskeletal injuries and disorders but also in sports medicine.

PRP is mainly used in maxillofacial, restorative, and orthopedic surgery, as well as cardiology, implantology and cosmetic surgery. The pro-angiogenic mechanisms that occur with the participation of platelet grow factors are still not fully understood and require further research, both in vitro and in vivo.

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PLATELET LIPIDOMIC

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Lipids account for 16-19% dry platelets' matter and includes 65% phospholipids, 25% neutral lipids and about 8% glycosphingolipids. The cell membrane that surrounds platelets is a bilayer that contains different types phospholipids symmetrically distributed in resting platelets, such as phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine, and sphingomyelin. The collapse of lipid asymmetry is exposure of phosphatidylserine in the external leaflet of the plasma bilayer, where it is known to serve at least two major functions: providing a platform for development of the blood coagulation cascade and presenting the signal that induces phagocytosis of apoptotic cells. During activation, this asymmetrical distribution becomes disrupted, and PS and PE become exposed on the cell surface. The transbilayer movement of phosphatidylserine is responsible for the platelet procoagulant activity. Exposure of phosphatidylserine is a flag for macrophage recognition and clearance from the circulation. Platelets, stored at room temperature for transfusion for more than 5 days, undergo changes collectively known as platelet storage lesions. Thus, the platelet lipid composition and its possible modifications over time are crucial for efficacy of platelet rich plasma therapy. Moreover, a number of substances derived from lipids are contained into platelets. Eicosanoids are lipid signaling mediators generated by the action of lipoxygenase and include prostaglandins, thromboxane A2, 12-hydroxyeicosatetraenoic acid. Isoprostanes have a chemical structure similar to this of prostanoids, but are differently produced into the particle, and are ligands for prostaglandins receptors, exhibiting biological activity like thromboxane A2. Endocannabinoids are derivatives from arachidonic acid which could reduce local pain. Phospholipids growth factors (sphingolipids, lysophosphatidic acid, platelet-activating factor) are involved in tissue regenerating process. Finally, a warning concerning the atherogenic role of platelets, although it should not be exerted in a local therapy, is mentioned. The lipid content of plaletets must be taken into account when these particles are concentrated and used for a local therapy, while the different categories of lipid derivatives could improve or affect the quality of the product

Lipids account for 16-19% dry platelets' matter includes 65% phospholipids, 25% neutral lipids and about 8% glycosphingolipids. The cell membrane that surrounds platelets is a bilayer that contains different types phospholipids symmetrically distributed in resting platelets, such as phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine, and sphingomyelin. Uncharged phospholipids, such as phosphatidylcholine and sphingomyelin, are mainly present in the outer leaflet of the bilayer, whereas the inner leaflet contains negatively charged aminophospholipids PS and PE [1]. During activation, this asymmetrical distribution becomes disrupted, and PS and PE become exposed on the cell surface [2]. ATP-dependent directional lipid transport is the primary mechanism for generation and maintenance of lipid asymmetry. The transport is catalyzed by an enzyme called the aminophospholipid translocase, a P-type ATPase that specifically and rapidly

Key words: platelets, phospholipids, arachidonic acid derivatives, sphingolipids

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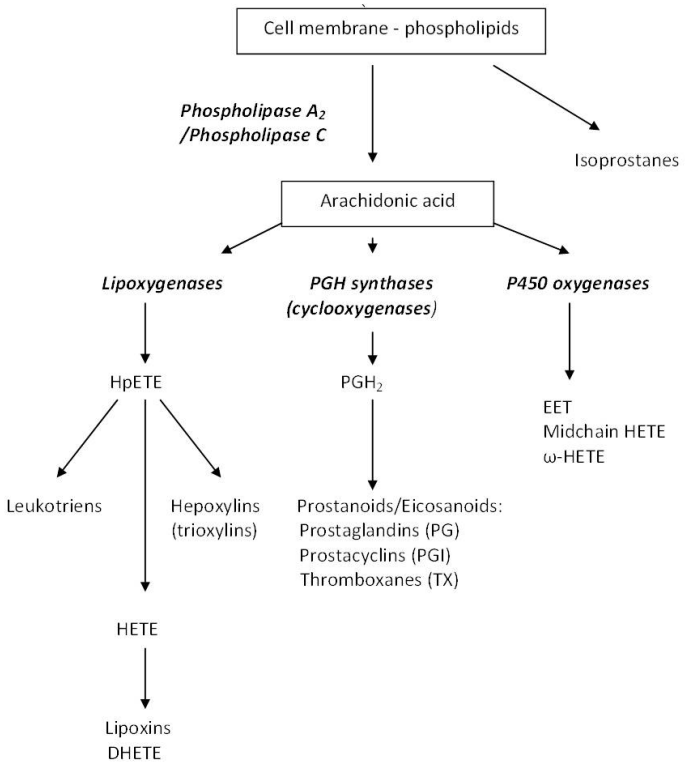

Fig. 1. *General pathways of arachidonic acid`s metabolism*

transports the aminophospholipids, phosphatidylserine and phosphatidyethanolamine, from the outer to the inner leaflet of the plasma membrane [2]. Because uncatalyzed transbilayer movement of lipids is slow, lipid asymmetry is stable in quiescent cells. The collapse of lipid asymmetry is exposure of phosphatidylserine in the external leaflet of the plasma bilayer, where it is known to serve at least two major functions: providing a platform for development of the blood coagulation cascade and presenting the signal that induces phagocytosis of apoptotic cells. Lipid asymmetry is collapsed by activation of phospholipid scramblase(s) that catalyse bidirectional transbilayer movement of the major classes of phospholipid [3]. The transbilyer movement of phosphatidylserine is responsible for the platelet procoagulant activity [4]. Exposure of phospahtidylserine is a flag for macrophage recognition and clearance from the circulation [5,6]. Platelets, stored at room temperature for transfusion for more than 5 days, undergo changes collectively known as platelet storage lesions [7]. These changes result not only in reduced responsiveness toward agonists but also in an accelerated clearance from the circulation following transfusion [8].

Prostanoids

Polyunsaturated fatty acids (PUFA) may affect cell functions after their release from cell lipid storage sites, especially phospholipids, and specific oxygenation. Most of lipid mediators are oxidation products, formed either through specific oxygenases or by non-enzymatic peroxidation. Both types of oxidation require the presence of double bonds within the acyl chain of the fatty acid (FA) precursor. Monounsaturated FA might be substrates of the monooxygenase cytochrome P450, polyunsaturated fatty acids (PUFA) are the major precursors of oxygenated derivatives so far described, notably as products of dioxygenases (Fig. 1). The products from FA nowadays justify the name oxylipins as a generic term [9].

Monohydroxy derivatives of most PUFA, which can be converted by the platelet 12-lipoxygenase, appear to be able to inhibit thromboxane-induced aggregation in the μM range. This results from the antagonism of the thromboxane receptor site. The effects of the hydroperoxy intermediates are also to be considered in the frame of their contribution to the peroxide tone, which affects the PUFA oxygenation [10]. Indeed, 12-HpETE was found to activate platelet cyclooxygenase, lipoxygenase [11], and phospholipase A_2, making it an intermediate able to potentiate the whole AA cascade [9].

Numerous di- and tri-hydroxy derivatives of docosahexaenoic acid (DHA) have been described [12]

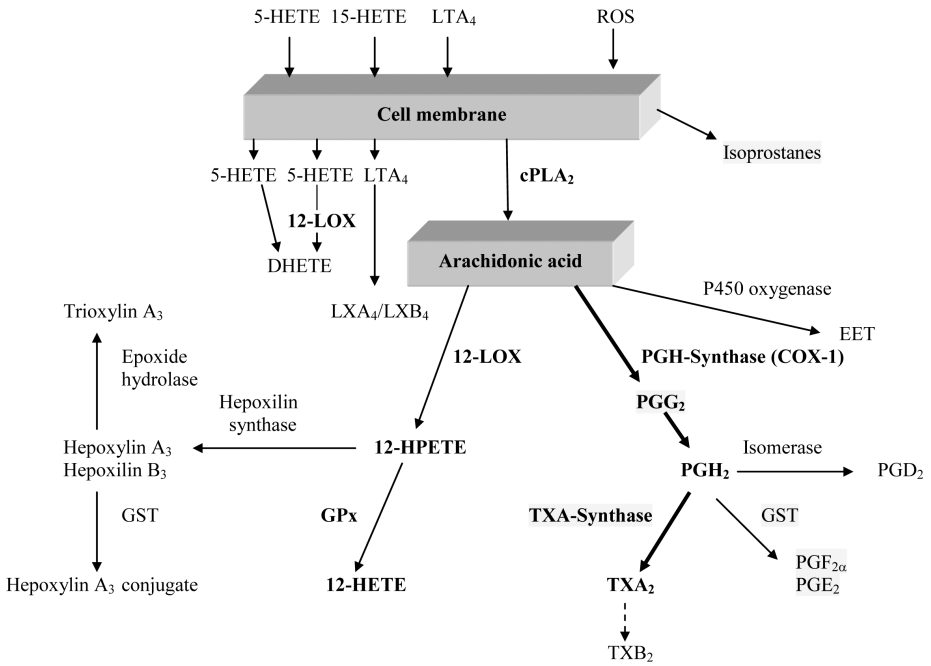

Fig. 2. *Platelets' pathways of arachidonic acid`s metabolism.*

with some of them being able to accelerate the resolution of inflammation, and they have been named resolvins [13]. Among the dihydroxydocosatrienes, focus has been put on 10(R),17(S)-diOH-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid, called protectin D1 (PD1) or neuroprotectin D1 (NPD1) [14,15]. This compounds is described as a product of animal 15-lipoxygenases. However, plant lipoxygenases may convert DHA to 10(S),17(S)-diOH-docosa-4Z,7Z,11E,13Z,15E,19Z-hexaenoic acid (PDX). It appears as an isomer of PD1/NPD1 for the stereochemistry of carbon 10 (S versus R) and the geometry of the conjugated triene (11E, 13Z, 15E versus 11E, 13E, 15Z) [9]. Lagarde et al. described that PDX inhibits aggregation with submicromolar concentration. This indicates that PDX inhibits downstream to the phospholipase A_2 stage. Further investigation revealed that PDX inhibits cyclooxygenase 1 (COX-1) in platelets, also at submicromolar concentrations, whereas it does not affect 12-lipoxygenase. Therefore PDX behaves as a COX-1 inhibitor [9].

Eicosanoids

Eicosanoids are lipid signaling mediators generated by the action of lipoxygenase (LOX), cyclooxygenase, or cytochrome P450 on arachidonic acid (AA) hydrolysed from membrane phospholipids (PLs) by phospholipase A_2 (PLA_2) or phospholipase C (PLC) [16].

Thromboxane A_2

Arachidonic acid is metabolized by COX-1 into the prostaglandin endoperoxides, PGG_2 and PGH_2 (Fig. 2). Next, thromboxane synthase further metabolizes PGH_2 into TXA_2, which is a potent activator of platelet aggregation, with a half-life 20-30 seconds [17]. Thromboxane A_2 is rapidly degraded into an inactive form of TXB_2 [16]. Thromboxane A_2 (TXA_2) released by activated platelets acts to recruit additional platelets to the thrombus, and it contributes to stabilizing the thrombus. TXA_2 biosynthesis is known to be activated by signaling from both of the platelet thrombin receptors, protease-activated receptor 1 (PAR1) and PAR4 [18-20]. The rate-limiting step in TXA_2 biosynthesis by platelets is activation of cytosolic phospholipase $A_{2\alpha}$ ($cPLA_{2\alpha}$) with production of free arachidonic acid (AA) as substrate for cyclooxygenase-1 (COX-1). The product of COX-1, prostaglandin H_2, is then metabolized to TXA_2 by thromboxane synthase. Generated TXA_2 binds to its G-protein coupled receptor (GPCR) known as TXA_2 receptor (abbreviated as TPR). There two splice variants for TPR with distinct tissue expression, α-isoform and β-isoform [17]. Platelets were

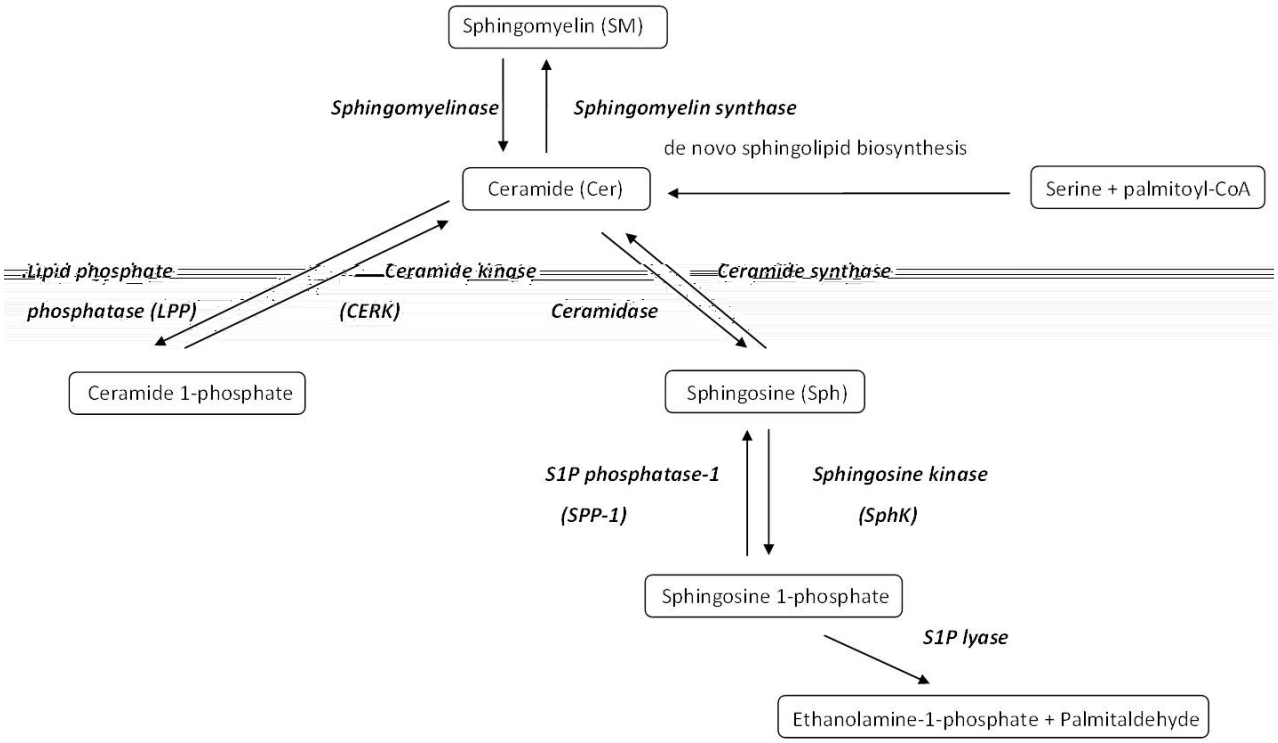

Fig. 3. *General pathways of sphingolipids' metabolism*

found to express high levels of mRNA for the α-isoform and low levels of β-isoform [16].

Platelet-derived TXA_2 can act in a paracrine manner to up-regulate endothelial COX-2 expression and prostacyclin (PGI_2) synthesis. TXA_2 via selective activation of the p44/42 mitogen-activated protein kinase (MAPK) pathway, is the soluble platelet product that regulates endothelial COX levels and PGI_2 synthesis [17,21].

Prostaglandins

The prostaglandins PGE_1 and PGE_2 are known to modify platelet function. PGE_1 has been shown consistently to inhibit platelet function in a concentration-dependent manner [22-31]. In contrast, the effects of PGE_2 are less consistent and have been reported to be either stimulatory or inhibitory of platelet function depending on the concentration of PGE_2 and the conditions used [32,33]. Activated human platelets synthesize and release prostaglandin (PG) E_2 during whole-blood clotting, although at lower rate than thromboxane A_2. PGE_2 can bind at least four structurally different receptor subtypes (EP1-4), resulting in diverse and often oposite final biological responses [34]. The presence of several EPs on the same cell, exerting different effects, is common for PGE_2 [35,36]. The variable PGE_2 concentration within the microenvironment possibly elicits an activatory or inhibitory modulation, finely tuning platelet responsiveness. The net effect of PGE_2 at low concentrations consists of enhancing stabilization and amplification of platelet response. This seems to result from a complex balance between EP3 variants- and EP2-mediated responses that positively and negatively modulate platelet aggregation, respectively [37].

12-Hydroxyeicosatetraenoic acid

Another major eicosanoid released by platelet activation is 12-hydroperoxyeicosatetraenoic acid (12-HpETE), a product of the oxygenation of AA by 12-lipoxygenase (12-LOX). 12-HpETE has been shown to stimulate 12-LOX but inhibit COX-1 in lysed platelets. Platelet glutathione peroxidase rapidly reduces 12 HpETE into 12-hydroxyeicosatetraenoic acid (12-HETE). The biological relevance of this pathway is not as clear as that of prostanoids, although it may represent around one third of the whole AA oxygenation after its release from platelet membrane phospholipids in response to aggregating agents [9]. 12-HETE is released by collagen and also by

higher concentration of thrombin [38]. Holinstat et al. found that the protease-activated receptor (PAR)-induced signaling pathway that activates the $cPLA_{2\alpha}$, that provides AA to COX-1 is different from that activating the $cPLA_{2\alpha}$ that liberates substrate for 12-LOX. Both COX-1 and 12-LOX are functionally coupled to a discrete subset of platelet $cPLA_{2\alpha}$ that provides AA separately to each of these oxygenases [39].

Isoprostanes

The family of eicosanoids called isoprostanes possess a chemical structure that is isomeric to the classical prostanoids [40]. While the prostaglandins are produced as a result of cyclooxygenase enzyme activity, isoprostanes are generally thought to form nonenzymatically by free radical-mediated peroxidation of arachidonic acid (AA), and bound up with phospholipids of cells membranes and lipoproteins (for example LDL) [41-44]. Those incorporated in membrane phospholipids and lipoproteins are released to the circulation by phospholipase-A_2 and/or PAF-AH (platelet-activating factor acetylhydrolases) [45,46]. Separate evidence has suggested that cyclooxygenase activity may also contribute to isoprostane production in selected tissues [47].

Four classes of isoprostanes are produced as a result of the free-radical oxidation of AA, with each class containing 16 subtypes of isoprostanes resulting in 64 individual isoprostane molecules [16]. Of the isoprostane members, 8-iso-$PGF_{2\alpha}$ appears to be the most abundantly produced in vivo [40]. Isoprostanes are of clinical interest for two main reasons: they are ligands for prostaglandin receptors, and thus may exhibit biological activity like TXA_2 and other AA metabolites [44]; and they have been found to associate with the oxidative status of an organism [47].

Minuz et al. found isoprostanes compound a modulator of platelet function. Isoprostanes can circulate in vivo at concentrations orders of magnitude higher than other AA metabolites such as TXA_2 and remain much more chemically stable [48,49]. Human platelets possess two 8-iso-$PGF_{2\alpha}$ binding sites, which is consistent with the finding that 8-iso-$PGF_{2\alpha}$ signals through two separate pathways (one stimulatory and one inhibitory). While the stimulatory signaling pathway is thromboxane A_2 receptor (TPR)-dependent, the inhibitory pathway is TPR-independent and associated with elevation of platelet cAMP levels [50].

Endocannabinoids

Endocannanbinoids are derivatives of arachidonic acid and belong to the eicosanoid family. The two main endocannabinoids, 2-arachidonoyl glycerol (2-AG) and anandamide, are synthesized in response to increasing intracellular calcium concentrations by diacylglycerol lipase [51]. Hydrolysis of 2-AG produces AA, which is the main substrate for cyclooxygenase (COX) [52].

Platelets express two cannabinoid receptors (CB_1 and CB_2) and possess the metabolic pathways to both synthesize and degrade endocannabinoids [53]. 2-AG can stimulate platelet activity, but that effects are mediated through an aspirin-sensitive pathway (COX-mediated mechanism) rather than through cannabinoid receptors [54].

Epoxyeicosatrienoic acids

Epoxyeicosatrienoic acids (EETs) are synthesized from arachidonic acid by cytochrome P450 epoxygenases, and converted to dihydroeicosatrienoic acid (DHETs) by soluble epoxide hydrolase (sEH). The molecular "family" of EETs includes 5,6-, 8,9-, 11,12- and 14,15-EET. Human platelet phospholipids can release EET that might play a role in stimulus-response coupling in platelets. EETs have a potent inhibitory effect on platelet aggregation. Krotz et al. reported that EETs hyperpolarized platelets and inactivated them by inhibiting adhesion molecule expression and platelet adhesion to cultured endothelial cells [56].

Oxidized phospholipids

Maskrey et al. observed, that human platelets generated four analogous phosphatidyletanolamine lipids that contained 12-HETE (12-HETE-PE) and increased significantly in response to ionophore, collagen, or convulxin [55]. Their generation by platelet 12-LOX was stimulated by triggering protease-activated receptors-1 and -4 and signaling via Ca^{+2} mobilization secretory phospholipase A_2, platelet-activating factor – acetylhydrolase, *src* tyrosine kinases, and protein kinase C [57]. Unlike free 12-HETE that is secreted, esterified HETEs remain cell-associated, with HETE-PEs migrating to the outside of the plasma membrane [58,59]. Because the majority of the PC is already on the outside in resting cells, it is likely that 12-HETE-PC is also facing the external side.

Specific molecular families of oxidized phospholipids form on acute receptor/agonist-dependent activation of human platelets, indicating that they are physiological products and of likely importance in cell signaling during both health and disease [57].

Phospholipid growth factors

Sphingolipids

Sphingolipids are major components of plasma membrane. Phospholipids, including sphingomyeline (SM), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are converted from plasma

lipoproteins supplied by selective transfer, not by endocytosis. Platelets are rich in SM, which represents 21% of their total phospholipids and 13% their total lipids [60]. The majority of the SM to be located at the outer leaflet of the plasma membrane of the platelets [61].

Sphingolipid metabolites have been implicated as modulators of membrane signal transduction systems and shown to be involved in diverse cellular processes [62,63].

Ceramide, the central molecule in sphingolipid structure and metabolism, is composed of sphingosine and a saturated or nonsaturated long-chain fatty acid. It is produced via de novo biosynthetic pathway beginning with the condensation of serine and palmitoyl-CoA by , sphingomyelin by sphingomyelinases (Fig. 3). It can be phosphorylated by ceramide kinase (CERK) to ceramide 1-phosphate (C1P), or utilized for the synthesis of sphingomyelin or glycosphingolipids. Ceramide can also be broken down by ceramidases to sphingosine, which in turn is phosphorylated by sphingosine kinases (SphKs) to generate sphingosine 1-phosphate (S1P). S1P is degraded by specific phosphatases that regenerate sphingosine or by a lyase that cleaves it irreversibly into ethanolamine 1-phosphate and palmitaldehyde [61-64].

Ceramide is an important second messenger in various stress responses and growth mechanisms, and formation of ceramide occurs in response to many inducers of stress, such as TNFα, γ-interferon, 1α,25-dihydroxyvitamin D_3, interleukin 1, UV light, heat, chemotherapeutic agents, FAS antigen, and growth factor (NGF) [65,66]. Ceramide has been shown to mediate many cellular events including growth arrest, differentiation and apoptosis. Sphingosine may also be an important physiological regulator. It can inhibit protein kinase C as well as induce cell-cycle arrest and apoptosis [64]. Sphingosine in platelets is supplied from at least two sources: generation in the plasma followed by incorporation, and generation at the outer leaftlet of the plasma membrane, initiated by cell surface sphingomyeline degradation [61].

The ceramide-derived metabolites S1P and C1P, represent an important class of bioactive lipid mediators. Activation of CERK and formation of C1P triggers the eicosanoid cascade. This mechanism would ensure coordinated activation or/and translocation of $cPLA_2$ and induction of COX-2, the enzymes that generate and metabolize arachidonic acid, respectively, in the pathway for formation of prostaglandins as well as other eicosanoids [64,67].

S1P functioning as an intracellular second messenger and as a ligand for five G protein-coupled receptors, named $S1P_1$ to $S1P_5$. S1P also has intracellular actions independent of these receptors [64,68]. In contrast to ceramide and sphingosine, S1P was found to stimulate cell proliferation and antagonize ceramide-induced apoptosis. It also plays critical roles in regulation of trafficking of various immune cells. S1P has been shown to have significant effects on lymphocytes. Both B- and T-cells require S1P activity to allow their movement out of lymphoid organs, and thymocytes require S1P receptor activation for release from the thymus [69,70]. It affects other biological processes including vascular development, carcinogenesis, and atherosclerosis. Recent studies have also revealed the involvement of S1P signaling in coagulation and in tumor necrosis factor α-mediated signaling [71].

Platelets lack S1P lyase activity and have highly active sphingosine kinase, so they accumulate high concentration of S1P [71-73]. The concentration of S1P in serum are usually higher than that in plasma [71,74], indicating that S1P is released from platelets during the coagulation processes. In fact, several reports showed that platelets secreted S1P upon stimulation, such as thrombin, collagen, and phorbol ester. Sphingosine 1-phosphate can be released extracellulary after stimulation by protein kinase C activators [72,75].

Very high concentration of S1P (>10μM) was shown to induce platelet activation such as intracellular calcium increase, shape change, and aggregation, but physiological range of S1P does not activate platelets. Although S1P is not a major activator of platelet, S1P signaling seems to play a role in the platelet production [76]. $S1P_4$ is specifically up-regulated during the development of human megakaryocytes from hematopoietic progenitor cells, and plays a role in shaping the terminal differentiation megakaryocytes and proplatelet development [71].

Lysophosphatidic acid

Lysophosphatidic acid (LPA), a member of the phospholipid growth factors family. LPA targets cells through at least six well-characterized extracellular receptors and one nuclear receptor (the PPARγ receptor) [77-79]. A minor portion of serum LPA originates within platelets, whereas the majority of LPA is generated from lysophospholipids secreted from activated platelets in part of the action of lysophospholipase D (lysoPLD), also known as autotoxin [80]. LPA has been found to stimulate cell division, migration, and to inhibit apoptosis [81].

Platelet-activating factor

Platelet-activating factor (PAF) is a proinflamatory phospholipid mediator and a potent platelet agonist [82]. PAF is degraded and inactivated by PAF-acetylhydrolase (PAF-AH), an enzyme that exhibits a Ca^{+2}-independent phospholipase A_2 activity and in plasma, it is associated with lipoproteins [83]. PAF-induced platelet aggregation participates in activation of the 12-LOX pathway and is accompanied by the release of vasodepressor nerve-

stimulating substances from platelets [84].

Lp(a) inhibits PAF-induced platelet activation in a nonspecific manner. The Lp(a)-associated PAF-AH does not play any important role in this effect, whereas the apo(a) moiety of Lp(a) significantly reduces its inhibitory effect in an apo(a) isoform size-independent manner [85].

Atherogenic role of platelets

Beside their classical role in thrombosis, platelets contribute to endothelial dysfunction, initiate atheromatous plaque formation, and modulate various inflammatory responses [86,87]. Continously elevated blood levels of LDL exert "proatherogenic" activating effects on platelet function resulting in hyperaggregability. In contrast, HDL desensitizes platelets and has "anti-atherogenic" effects. When platelets adhere to the inflamed or functionally disturbed, but otherwise uninjured endothelium, they are able to initiate and accelerate atherosclerosis. Adhering platelets recruit monocytes that transmigrate into the subendothelial space and develop into macrophages and foam cells. Platelets also recruit endothelial progenitor cells when they adhere to the endothelium. These progenitor cells differentiate into endothelial cells for vascular regeneration but also into foam cells depending on the microenvironmental conditions. The way of LDL transport and deposition into the arterial vessel wall and subsequent foam cell formation by the following mechanisms: adhering platelets bind and take up modified LDL via scavenger receptors, such as CD36 or LOX-1. They contribute to the further oxidation of lipoproteins by a substantial release of ROS. Platelets transfer bound modified LDL into macrophages by which they are phagocytosed and further processed [88].

Mildly oxidized LDL (mox-LDL) and minimally modified LDL (mm-LDL) which escape the uptake of macrophage scavenger receptors accumulate in the atherosclerosis intima. Mox-LDL and mm-LDL, but not native LDL be able to induce platelet shape change and aggregation. LDL oxidation generates lipids with platelet stimulatory properties such as lysophosphatidylcholine, certain oxidized phosphatidylcholine molecules, F_2-isoprostanes and lysophosphatydic acid (LPA). LPA has been identified as the lipid responsible for platelet stimulation by mox-LDL, mm-LDL and also mox-HDL. These lipoproteins activate platelets by stimulating G-protein coupled LPA receptors and a Rho/Rho kinase signalling pathway leading to platelet shape change and subsequent aggregation. LPA-mediated platelet activation might contribute to arterial thrombus formation after rupture of atherosclerosis plaques and to the increased blood thrombogenicity of patients with cardiovascular diseases [89]. Lp(a) is considered an atherogenic lipoprotein, which is also implicated in thrombosis, although the underlying mechanisms remain incompletely understood. Lp(a) does not influence platelet activation induced by collagen or thrombin. Intact Lp(a) inhibits collagen induced platelet aggregation or ADP-induced platelet aggregation. Furthemore, it has been shown that intact Lp(a) or recombinant apo(a) promotes the aggregation response of platelets to subaggregant doses of arachidonic acid, whereas recombinant apo(a) does not affect the platelet response to low doses of collagen or thrombin [90].

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PLATELET RICH PLASMA THERAPY: INFLAMMATORY MOLECULES INVOLVED IN TISSUE HEALING

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Inflammation represents a fundamental aspect of the healing process. Besides their primary role in hemostasis, platelets play an active role in the immunological and inflammatory aspect of tissue healing. Indeed , they can be directly involved in the inflammatory response by the production and release of several inflammatory mediators, including a variety of cytokines, such as TGF-β, IL-1 β, CD40L, and chemokines, such as CXCL7, CXCL4, CXCL4L1, CCl5, CXCL1, CXCL8, CXCL5, CXCL12, CCL2, CCL3. Platelet are not only a source of several chemokine involved in the inflammatory response and tissue healing, but they also express chemokine receptors, in particular CCR1 CCR3 CCR4 and CXCR4, thus being able to being able to regulate the inflammatory response associated to the healing process. However, this local inflammation must be taken under control, and platelets can prevent the excess of leukocytes recruitment by anti-inflammatory cytokines, such as TGF-β. For this biological properties of platelets, platelet rich plasma therapy (PRP) is considered an innovative and promising approach that has been extended to many field of medicine, ranging from non-union defects, bone fractures, spinal fusion, bone implant and osteointegration, joint arthroplasty, to the treatment of several traumatic or degenerative pathologies of tendons, cartilage and ligaments.

Platelet and tissue healing

The repair process after tissue injury occurs through an ordered sequence of events , aimed to restore tissue homeostasis and function. Tissue repair involves various cell types, ranging from immune to vascular cells as well as resident or migratory leukocytes and fibroblasts, which cooperate through a complex of extracellular and intracellular signaling molecules. This complex series of events can be divided into three main phases: an inflammatory phase, a proliferative or reparative phase and a final remodeling phase [1]. The initial exposure of tissue cells to physical, mechanical or chemical damage leads to the start of acute inflammatory response. This process is modulated by local regulatory mechanisms, in order to restrict the site of inflammation to the area of tissue injury and the magnitude of the response to the amount of tissue damage. Endothelial cells limit to the site of injury the formation of the clot containing activated platelet and leukocytes, which release growth factors and cytokines, leading to the onset of inflammation. In the first two days the main infiltrate is constituted by leukocytes, then replaced by monocytes which turn into macrophages [2]. Then, according to the severity of tissue damage, macrophages differently activate through the inflammatory pathway, characterized by the production of IL-6, IL-1β and TNF-α [3] or the classical activation through IL-4 /IL-23 and characterized by the synthesis of healing factors such as transforming growth factor (TGFβ),basic fibroblastic growth factor (bFGF), platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF)[4]. The high concentration of these

Key words: platelets, cytokines, inflammatory response

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molecules initially released by leukocytes and platelets and then by macrophages lead to the proliferative phase, characterized by an increase of specific cell populations, including fibroblasts, endothelial cells and resident specific tissue cells. In addition , the production of VEGF, bFGF and PDGF stimulates angiogenesis , while TGFβ, in association with bFGF and PDGF, induces collagen synthesis and extracellular matrix (ECM) deposition, which, in the last remodeling phase of the healing process, is reorganized by ECM proteases, such as matrix metalloproteinases (MMPs) [2]. Among blood components involved in tissue healing, platelets have a crucial role in tissue healing. Platelets are anucleated , cellular fragments derived from megakariocytes in the bone marrow and in arteries that have a primary function on hemostasis and host defense [5, 6] .Immediately after tissue damage , platelets are essential for the clot formation, the first event after trauma or surgery starting the healing cascade. Platelet activation after tissue damage leads to the formation of platelet plug and blood clot to provide hemostasis. Moreover, activated platelets contribute to the healing process by the secretion of several biologically active molecules stored in platelet granules. Platelet dense granules contains ATP, ADP, GDP, GTP, serotonin and histamine, released in the first phase of healing response, to increase vascular permeability and leukocytes extravasation from the blood vessel and infiltration into the tissue damage site. Platelet α-granules contain the majority of the biologically active molecules, ranging from adhesion molecules, coagulation factors, protease inhibitors, growth factors and inflammatory mediators [5].

Platelet and inflammatory mediators in tissue healing

Inflammation represents a fundamental aspect of the healing process. Besides their primary role in hemostasis, platelets play an active role in the immunological and inflammatory aspect of tissue healing [7]. A growing body of evidences indicates that platelets play a main role in the inflammatory response [8, 9]. Platelets usually circulate in a quiescent state, but upon activation they can secrete and present a variety of molecules, change their shape from discoidal to globular one and their expression pattern of adhesion molecules, leading to the adhesion to vessel wall and leukocytes. Activated platelets can interact with neutrophils, through P- selectin and integrins, with endothelial cells and monocytes [10]. Moreover , platelets can be directly involved in the inflammatory response by the production and release of several inflammatory mediators, including a variety of cytokines and chemokines[11] [6], summarized in Table 1.

Platelet expressed cytokines

TGF-β

Transforming growth factor β (TGF-β) is produced by nearly every cell type of the body , but the main source of this cytokine are bone and activated platelets [1]. Nearly every cell type exhibits a cellular response to TGF-β, but this cytokine exerts a different role in each cell type [11]. In bone tissue, TGF-β stimulates the proliferation of osteoblasts precursor cells and it has direct stimulatory effect on bone collagen synthesis. In addition to osteoblasts, TGF-β activates fibroblasts to induce collagen formation, endothelial cells for angiogenesis, chondrocytes for cartilage production [11]. As inflammatory mediator, TGF-β stimulates leukocytes chemiotaxis and regulates the transcription of integrin receptors, as well as a wide spectrum of matrix protein including matrix metalloproteinases (MMPs) and their inhibitors. Despite its prohealing effect, TGF-β is also a key cytokine in the pathogenesis of fibrosis and scar formation [12, 13], characterized by excessive collagen deposition.

IL-1β

Platelets contain unspliced heteronuclear IL1β RNA, which undergoes splicing and translation upon activation [14] . The agonist for this platelet response is LPS, which binds TLR4 expressed on platelets membrane and induces a rapid splicing translation and secretion of mature IL1β after caspase-1 processing. In particular, LPS stimulates microparticles shedding and IL1β secretion which, in turn, induces endothelial cells to produce adhesion molecules such as VCAM-1, ICAM-1 –E-selectin, α2β3 integrin. Beside the stimulation of these adhesion molecules, IL1β produced by activated platelets induces the secretion of others inflammatory mediators such as IL-6, IL-8 and CCL2 from endothelial cells [15, 16], thus amplifying the inflammatory response.

CD40L

The platelet cytokine CD40 ligand (CD40L) is stored into α-granules and rapidly translocated to the cell surface upon activation, where it can either remain as a transmembrane protein or it can be secreted in a truncated form, after cleavage by MMP-2 [17]. In both cases, platelet CD40L binds to the cognate receptor CD40, expressed on endothelial cells, inducing chemokine secretion and up-regulation of adhesion molecules by these cells and finally , leukocytes recruitment [18].

Platelet expressed chemokines

Among various platelet secreted soluble factors, chemokines represent a significant portion of α-granule contents, which are released within seconds after platelet activation [19] . Chemokines represent a family of chemotactic cytokines which can be divided into four families (CXC, CC, CX3C, C) according to the

position of the first two cysteine residues. This structural categorization corresponds to different actions on leukocyte subpopulations. Thus, CXC chemokines mainly act on neutrophils, in the early phase of inflammatory response, while CC chemokines mainly act on monocytes, in the later phase. Chemokine action is mediated by the binding with their cognate receptors, which are 7 transmembrane G-protein coupled receptors (7TM GPCR). Platelet interplay with chemokines system is multifaced : they may be activated by chemokines, they can release directly chemokine after activation or can induce chemokine expression by other cell types [20] .

CXCL7

Among the chemokines secreted by platelets, CXCL7 is the most abundant and representative. After platelet activation, CXCL7 is released into the serum reaching micromolar concentration (1.6 to 4.8 μM) and it is considered a measure of platelet activation. There are several molecular variants of CXCL7, resulting from N-terminal truncation: β thromboglobulin (β-TG), platelet basic protein (PBP), connective tissue active peptide (CTAP III) and neutrophil activating peptide 2 (NAP-2) [6]. Additional CXCL7 derived molecule can origin from a CXCL7 precursor molecule, called pre PBP. Following platelet activation most of the CXCL7 is released in the CTAP III form, while PBP represent only about 10% [21-23] . Otherwise, NAP-2 and β-TG are virtually absent from supernatants of thrombin stimulated platelets. In particular, NAP-2 is formed only in the presence of leukocytes and represents the only CXCL7 protein that may be addressed as a functional chemokine, being able to support leukocytes chemiotaxis [24] . Consequently, PBP and CTAPIII may be addressed as inactive precursors of NAP-2, although they exhibit a variety of other biological functions, such as supporting fibroblast metabolism and synthesis of matrix components [25]. NAP-2 exerts its chemotactic function by binding to CXCR1 and CXCR2 expressed on neutrophils membrane. The same receptors are expressed by megakaryocytes [26] , suggesting a role of NAP-2 also in the maturation of these cells into platelets. NAP-2 activity can be regulated at different levels. CXCL7 processing into NAP-2 occurs by neutrophils themselves [27] and NAP-2 itself may downregulate CXCR2 in neutrophils, by a negative feedback mechanism. Desensitization of CXCR2 limits and prevents an excessive neutrophils recruitment and degranulation into the injury site. In addition, a heterologous desensitization of neutrophils degranulation occurs on CXCR2 with platelet activating factor (PAF) and IL-8, both CXCR2 ligands. Following CXCR2 desensitization, since the affinity of NAP-2 to CXCR2 is much higher than to CXCR1, NAP-2 can attract neutrophils over a wide range of concentrations [28].

CXCL4, CXCL4L1

CXCL4, also known to as platelet factor 4 (PF4), is stored in α granules and it is released into the plasma quickly reaching concentrations ranging from 0.4 to 1.9 μmol/L after platelet activation. This chemokine binds to a 200-kDa chondroitin sulfate proteoglycan on the surface of human neutrophils, but it also can bind the splice variant of CXCR3, CXCR3B, expressed on endothelial and T cells [29]. Intriguingly, CXCL4 signaling through CXCR3B is not Pertuxis toxin (PTX) sensitive, suggesting a signaling mechanism different from that characteristic of the other chemokine receptors. PF4 exerts different biological function and, according to the cell type, it can promote activating or deactivating cellular functions involved in acute host defense as well as long term regulatory process. CXCL4 inhibits cell proliferation of fibroblasts, endothelial cells as well as angiogenesis, while it can promote monocyte differentiation to macrophages or antigen presenting cells [30] . At T cell level, CXCL4 inhibits cell proliferation and Th1 cytokine production, shifting the T cell phenotype toward a Th2 response. In this way CXCL4 plays an essential role in maintaining an efficient immune response against microbial infection. One of the peculiar features of this platelet derived chemokine is the ability to cooperate with other stimuli. PF4 enhances adhesion of neutrophils to endothelial cells induced by NAP-2. Furthermore, PF4 enhances the CCL5-induced monocyte arrest on endothelial cells and induces phagocytosis and oxygen radical formation by macrophages [31]. This cooperation of PF4 with regulatory chemokines of monocytes leads to the differentiation of a physiologically different population of macrophages with high capacity of microbial uptake and killing, accompanied by a release of TNF and proinflammatory chemokines like CXCL8, CCL4 or CCL3, thus enabling a high degree of flexibility of the inflammatory response. Besides CXCL4, human platelets contain the product of a gene homologous to CXCL4, called CXCL4L1, which differs from CXCL4 by a substitution of three aminoacids in the C-terminal portion. The functional features of CXCL4L1 differs from those of CXCL4; in particular, it is a stronger inhibitor of endothelial cells [32]

CCL5

CCL5, also known as RANTES, is mainly secreted by lymphocytes, but platelet represents an important source of this chemokine which binds to the receptors CCR1 CCR3 and CCR5, inducing the adhesion and transmigration of monocytes and T lymphocytes across the endothelium. This transendothelial migration is regulated by the expression of integrin and adhesion molecules like ICAM and VCAM [33]. One of the peculiar features of CCL5 is the ability to form aggregates and to interact with

Table 1. *Plateler cytokines , chemokines and chemokine receptors*

Cytokines		
Name	**Main target cell**	**Biological function**
TGF-β	endothelial cells, osteoblasts , fibroblasts,leukocytes, chondrocytes	collagen syntesis, angiogenesis, chemiotaxis
IL-1β	endothelial cells	production of adhesion molecules and inflammatory cytokines
CD40L	endothelial cells	Chemokine secretion and up-regulation of adhesion molecules
Chemokines		
Name	**Main target cell**	**Biological function**
CXCL7	megakaryocytes ,neutrophils	megakaryocytes differentiation ,neutrophils chemotaxis
CXCL4	neutrophils, endothelial cells	inhibition of fibroblast and endothelial cells proliferation, inhibition of angiogenesis and Th1 response. Induction of monocytes and neutrophils arrest, macrophage activation
CXCL4L1	endothelial cells	inhibition of endothelila cells proliferation
CXCL8	neutrophils	neutrophils recruitment
CXCL5	monocytes	monocytes recruitment and activation
CXCL12	platelet, endothelial cells	angiogenesis, platelet activation
CXCL1	monocytes	monocyte arrest to endothelium
CCL3	smooth muscle cells	Monocyte adhesion
CCL5	T cells , eosinophils, basophils, NK cells	migration of monocytes anf T lymphocytes
Chemokine receptors		
Name	**Ligands**	**Biological function**
CXCR4	CXCL12	megakaryocytes differentiation, platelet aggregation and activation
CCR1	CCL3,5,7,8,13,14,14,23,	platelet activation, leukocytes recruitment
CCR3	CCL5,7,8,11,13,15,,24,26	platelet activation, leukocytes recruitment
CCR4	CCL7,CCL22	megakaryocytes differentiation, platelet aggregation and activation

glicosaminoglycans present on the surface of endothelial cells [34, 35]. Besides promoting cell migration, CCL5 acts as a high concentration as a T-cell mitogen factor and induces the release of proinflammatory mediators, thus amplifying the inflammatory response.

CXCL1

CXCL1, also known as GRO-α, is expressed by neutrophils, monocytes, macrophages and endothelial cells, but it is also secreted in small quantities by platelets [19]. This chemokine binds to CXCR2 and, with lower affinity, to CXCR1. Upon binding to CXCR2, CXCL1 induces the arrest of monocytes and their adhesion to the vessel wall, but it remains unclear the role and contribution of the platelet derived amount of CXCL1 to the whole process [36, 37].

CXCL8

CXCL8 or IL-8 is one of the main inflammatory chemokines and it is produced by almost all the main cell types involved in the inflammatory response, ranging from monocytes, neutrophils, T lymphocytes, to fibroblasts and endothelial cells [38]. It is also stored into α-granules of platelets and it is released in micromolar concentrations after platelet activation. It is the main chemoattractant for neutrophils through the receptors CXCR1 and CXCR2. CXCL8 also attracts T lymphocytes and basophils and stimulates histamine release [39, 40].

CXCL5

Together with CXCL1 and CXCL8, CXCL5 , firstly described as ENA-78, is a chemoattractant acting specifically on neutrophils, by binding the chemokine receptors CXCR1 and, with lower affinity, CXCR2 [36]. It has been demonstrated to have a high sequence affinity with other chemokines like NAP-2 or CXCL1 [41]. Differently from the other chemokines, CXCL5 has been demonstrated to have no effect on monocyte adhesion, suggesting a specific role in the recruitment of neutrophils into the site of inflammation [42].

CXCL12

CXCL12, also called stromal derived factor -1 (SDF-1), is expressed in smooth muscle cells, macrophages, endothelial cells and platelets [43]. This chemokine plays a dual role in platelet biology. On the one hand, it is directly involved in platelet activation, on the other hand is produced by activated platelet and it promotes neointima formation after arterial injury [44, 45]. In particular CXCL12 has been demonstrated to induce adherence

of CD34+ progenitors cells and their differentiation into endothelial cells in vitro and in vivo [43, 45] .

Other chemokines

CCL2 is mostly produced by endothelial cells and smooth muscle cells, but is also secreted by platelets. This chemokine has been demonstrated to support monocyte adhesion in vitro and neointima formation in vivo [46]. Also CCL3, also known as MIP-1 α, has been shown to be secreted by platelets [20]. It binds to CCR1 and CCR5, expressed in smooth muscle cells and is present at high levels in the blood after acute myocardial infarction, suggesting a role for this chemokine in the inflammatory event after this kind of tissue injury [47].

Platelet expressed chemokine receptors

Platelet are not only a source of several chemokines involved in the inflammatory response and tissue healing, but they also express chemokines receptors, being themselves target of some of the chemokines they produce [36]. In particular, the chemokine receptors CCR1 CCR3 CCR4 and CXCR4 have been detected on platelet surface, and they have been demonstrated to be functional receptors, being able to respond to their cognate chemokines. Indeed, the different agonist of chemokine receptors present on platelet are able to induce several phases of platelet activation, including Ca+ signal, aggregation, ADP release from dense granules [48]. In particular, the ability of some CXCR4 and CCR4 agonist (CXCL12, CCL17 and CCL22, respectively) to induce platelet aggregation is promoted by low levels of ADP and thrombin. In addition, CCR4 and CXCR4 ligands may also be expressed in megakariocytes and regulate the differentiation of this precursor into mature platelet [49, 50].

PRP therapy and its role in the inflammatory response during tissue healing

Platelet rich plasma (PRP) is defined as a portion of the plasma fraction of autologous blood having a platelet concentration higher than physiological one [51]. As such, PRP contains not only high levels of platelets but also clotting factors and platelet secretory proteins. As autologous platelets concentrate , it has been recognized as a powerful hemostatic agent since 1970 and a topical adjuvant therapy to enhance tissue repair process since 90s [52]. Platelet rich plasma provides an intriguing alternative to surgery by promoting physiological tissue healing and its use has been extended to many fields of medicine [11]. In particular, in the orthopedic field , PRP applications are numerous and heterogeneous: it has been used as adjuvant to promote bone healing in non-union defects, bone fractures, spinal fusion, bone implant and osteointegration , joint arthroplasty, and in the treatment of several traumatic or degenerative pathologies of tendons, cartilage and ligaments [53-55]. The rationale of PRP use in so many fields is that platelets, as mentioned above, are a reservoir of growth factors and bioactive molecule crucial for the healing process [56]. Moreover, the advantage of PRP is to be an autologous blood product, avoiding host immune response. For this reason PRP therapy is considerated a safe therapy, since deleterious effects have not been reported in the clinical practice. However, caution must be taken about different preparations of PRP, the appropriate dose, timing and length of application of PRP therapy in order to ensure a reliable and reproducible use of PRP [53]. In particular, different methods of PRP preparation may include not only platelets but also leukocytes (leukoplatelets concentrates), in particular neutrophils. These cells are key factors of the inflammatory response and may exacerbate tissue damage via several mechanisms: they are able to secrete cytokines such as TNF-α, IFN-γ, IL-6 or IL-1β that cause matrix destruction through the production of matrix metalloproteinases MMP-1, MMP-3 and MMP-13 [57, 58]. In addition, the interaction of leukocyte with platelet among PRP may induce an increase of further leukocytes recruitment into the injury site, through the secretion of platelet-stored chemokines, and an amplification of the inflammatory response, through the production of platelet cytokines. The inflammatory response is part of the tissue healing process, but it must be of appropriate magnitude and timing to ensure correct tissue repair and homeostasis. Therefore in the PRP therapy application it is crucial to control the inflammatory response to avoid adverse effects for patient safety. So far, no surgical inflammation has been reported in association with PRP therapy. Moreover, it has been shown that, in PRP activated by thrombin, platelet-derived TGF-β counteracts TNF-α effect on chemokine transactivation, preventing an excess of monocytes recruitment into the site of tissue damage [59] . The final effect of thrombin activated PRP is a balance of pro-inflammatory and anti-inflammatory cytokines and chemokines. The control of inflammatory response is also mediated by other PRP elements, such as HGF acting on the principal transcription regulator of inflammatory process, NFkB [59].

CONCLUSION

Platelet rich plasma therapy is an innovative and promising approach that has been extended to many field of medicine. Besides the hemostatic properties of platelets, PRP plays an active role in the inflammatory response associated with the tissue repair process. Platelets are not only a reservoir of several inflammatory molecules, but they also express chemokine receptors, thus being able

to be actively involved in the inflammatory response. However, this local inflammation must be taken under control, and PRP itself has the property to prevent the excess of leukocytes recruitment by anti-inflammatory cytokines, such as TGF-β. So far PRP therapy has been described in a little number of controlled clinical trials, and further studies will increase the knowledge about the molecular mechanism and the molecules involved in PRP-mediated tissue repair process.

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MICROBICIDAL PROPERTIES OF LEUKOCYTE- AND PLATELET-RICH PLASMA/FIBRIN (L-PRP/L-PRF): NEW PERSPECTIVES.

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Platelets, as main actors of the first stage of the healing process, play an important role in tissue repair. Their granules contain many active substances, particularly over 30 growth factors with significant effects on the resident cells at the site of injury, such as mesenchymal stem cells, chondrocytes, fibroblasts, osteoblasts. This potential may be increased by the concentration of the platelets, using platelet-rich plasma/fibrin products. In the four families of platelet concentrates, 2 families contain also significant concentrations of leukocytes: L-PRP (Leukocyte- and Platelet-Rich Plasma) and L-PRF (Leukocyte- and Platelet-Rich Fibrin). Inductive properties of platelet concentrates were widely described. However, they present also antimicrobial effects. The antibacterial effects of L-PRP were highlighted in only a few in vitro studies. Strong activity comparable to gentamicin and oxacillin for L-PRP against methicillin susceptible Staphylococcus aureus (MSSA) was already demonstrated. L-PRP also inhibited the growth of methicillin resistant Staphylococcus aureus (MRSA) and Escherichia coli. Some authors also reported clinical observations about the reduction of infections and the induction of healing processes after the use of platelet concentrates in cardiac, orthopaedic, oral and maxillofacial surgery. However, very little is yet known about the antibacterial effects of these concentrates. In this manuscript, the current data about the antimicrobial agents and cells present in the platelet-rich plasma/fibrin are highlighted and discussed, in order to introduce this new key chapter of the platelet concentrate technology history.

Healing, platelets and leukocytes.

The healing process of bones and soft tissues is one of the most complex mechanism among the vital functions of the living tissues. Despite intensive scientific investigation, this process remains still little-known. Nevertheless, it is known that the healing process of the osseous tissue is organized in 3 separate, self-overlapping phases: early inflammatory reaction, new callus production and remodeling, which is the scar tissue rebuilding (1, 2).

Platelets (thrombocytes) have strong properties of adhesion, aggregation and forming a precoagulation surface leading to the creation of a blood clot. They play a specific key role not only in the hemostasis, but also in the process of tissue healing (2). Thrombocytes are cytoplasmic fragments of megakaryocytes with a diameter of approximately 2 μm, developing in the bone marrow. In the physiological conditions there are 140-400 X 10^9/l of thrombocytes in the blood. Platelets do not have a nucleus, however, their structure includes granules of a diameter of 200-500 nm. The number of these granules varies between 50 and 800. Alpha granules are the crucial ones for the process of healing as they constitute a peculiar intracellular storehouse for the biologically active proteins, particularly more than 30 growth factors.

Key words: Leukocyte, platelet concentrate, PRP (Platelet-Rich Plasma), PRF (Platelet-Rich Fibrin).

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Moreover, inside the granules, peptides with antibacterial activity are also stored (1).

Apart from the platelets, leukocytes are also very active in the process of healing. One of their main role is to produce inflammatory cytokines and a battery of growth factors at injury site. Macrophages also produce collagenase, which stimulates the process of cleaning the wound, release transforming growth factors (TGF) to stimulate the keratinocytes, as well as platelet-derived growth factors (PDGF), which formerly was considered to be specific of thrombocytes. They also release interleukin-1 (IL-1), fibroblast growth factor (FGF) and tumor necrosis factor (TNF), which are substances that stimulate fibroblasts to produce collagen, and improve angiogenesis. Granulocytes and macrophages engage the production of inflammatory mediators such as leukotriene B4 and platelet activating factor (PAF), stimulating the expansion and the increased permeability of blood vessels, and stimulating the production of inflammatory cytokines and proteolytic enzymes. These factors act on endothelial cells of blood vessels, stimulating the adhesion of neutrophils and lymphocytes and their migration outside the vessel (1, 3). Lymphocytes also produce growth factors, and they may contribute to tissue remodeling during the late phase of wound healing (1). Toulon et al. reported that human epidermal T cells are able to produce insulin-like growth factor 1 (IGF-1) upon activation and promote wound healing in a skin organ culture model (4).

Platelet concentrates for regenerative medicine.

Due to their numerous key properties, platelets started to be used in the contemporary medicine in the strategies of stimulation of tissue healing, as an autologous cell and plasma fraction extracted from the peripheral blood (5). The autologous whole blood undergoes centrifugation in order to obtain a concentrate of platelets - called platelet-rich plasma (PRP) like in transfusion medicine - which could be transformed into a fibrin gel after activation by the addition of thrombin and calcium ions (or equivalent technologies). Other methods called Platelet-Rich Fibrin (PRF) were also developed with a natural activation procedure, in order to obtain stronger fibrin architecture (5-8). Most authors believe that the gel activation and the release of the growth factors lead to the acceleration of the wound healing process (5). The impact of platelet growth factors was particularly intensively investigated and debated in oral and maxillofacial applications (9-14), and PRP/PRF technology for regenerative medicine treatments is one of the most active field of research in the dental/maxillofacial and orthopedic literature, with implant surfaces/designs (15-20) and bone bioengineering (21-23).

Since the early development of these technologies, researchers were mostly focused on the platelet count in the concentrates. However, PRPs can contain a significant number of leukocytes. A lack of pertinent terminology and classification led to severe confusions between the many available products and to inadequate methodologies of analysis (5-7).

Recently, a full classification of platelet concentrate technologies was designed (5, 6), and allowed to classify the main available techniques in 4 families, depending on their leukocyte content and fibrin architecture:

- Pure Platelet-Rich Plasma (P-PRP) and Leukocyte- and Platelet-Rich Plasma (L-PRP) are liquid platelet suspensions, respectively without and with leukocytes. They can be used as injectable suspension, particularly in sports medicine. After activation (with thrombin, calcium chloride, batroxobin or others agents), these preparations become respectively P-PRP and L-PRP fibrin gels, with a brutal and incomplete fibrinogen polymerization and a light final fibrin architecture.
- Pure Platelet-Rich Fibrin (P-PRF) and Leukocyte- and Platelet-Rich Fibrin (L-PRF) are solid fibrin biomaterials, respectively without and with leukocytes. In these techniques, the platelet activation is part of the production process: it can be natural (L-PRF) or artificial (P-PRF) but always occurs during the centrifugation, and leads to a strong final fibrin architecture.

The concept of this classification is to define and regroup the products through their main features and associated biological mechanisms. This is only a first step, since platelet concentrations, leukocyte concentration and formula can also vary within a family of products. This classification is very attractive and logical from a strict theoretical standpoint, but it is now necessary to highlight and clarify the different biological properties and mechanisms associated to each family.

As we have written, 2 families contain significant amounts of leukocytes: L-PRP (Leukocyte-and Platelet-Rich Plasma)(1, 2) and L-PRF (Leukocyte- and Platelet-Rich Fibrin)(7). These 2 families of products are the most often used technologies in this field, but little is known about the impact of leukocytes in these preparations. Therefore, it is now very important to highlight the huge number of properties, factors and pathways that these cells can use to influence their environment, to regulate the tissue reactions in a wounded site and finally to promote a better healing.

Antimicrobial agents of the platelet concentrates.

As the use of platelet concentrates in medicine became more common, many attempts to explain their properties of stimulating and accelerating the tissue healing have been made. For years it was considered that the properties of the concentrates were mainly related to the high

concentrations of growth factors in their composition. However, several authors started to insist on the key role of the fibrin architecture and leukocyte content of these preparations, and to develop the concept that the growth factors alone cannot be considered as the main actors of these products. In the recent years the discussion was enriched by few studies focusing on platelet and leukocyte concentrates and their antimicrobial properties related to the presence of the natural antibacterial peptides (1, 2).

The antibacterial peptides (HDP – Host Defense Peptides) are produced by the macrophages, epithelial cells, as well as neutrophils and thrombocytes, and are one of the most important elements shaping the so-called human natural immunity. These proteins are synthesized with the participation of lipopolysaccharides from the cellular membrane of gram-negative bacteria and possess a wide spectrum of activity against gram-positive and gram-negative bacteria as well as viruses and fungi (24-26). They act through the PRR (pathogen recognition receptors), while their production is regulated by lymphocytes Th17 as well as IL-17 and IL-22. The antibacterial proteins initiate the lysis of the bacterial cell, stimulating the release of lipopolysaccharides and the lipoteichoic acid, which are the bacterial mediators of the inflammation. During the following stages they lead to the mast cell degranulation and, as a result, to histamine release, as well as vasodilation and the chemotaxis of neutrophils. The antibacterial peptides inhibit the processes of fibrinolysis using the tissue-type plasminogen activator, and – what is even more important – they stimulate the processes of wound healing by increasing the chemotaxis of fibroblasts and their growth; they also inhibit tissue destruction by blocking the activity of the bacterial proteases. Unlike antibiotics, which are specifically related to the particular elements of the bacterial cells, the antibacterial peptides act destructively against the cellular membranes of bacteria, where they directly enter, which reduce the risk of the development of bacterial resistance (25).

According to the contemporary knowledge, platelets through their properties of adhesion, aggregation and clot forming play a key role not only in the hemostasis, but also in the process of tissue healing. From the healing standpoint, the alpha granules - located in the cytoplasm of platelets - are the main intracellular storage for the biologically active proteins. We know at least 30 growth factors active in each stage of the tissue healing process and 7 human platelet antimicrobial proteins (HPAPs): fibrinopeptide A and B, thymosin beta 4, platelet basic protein, protein activating the connective tissue 3, RANTES (Regulated on Activation Normal T Cell Expressed and Secreted) and platelet factor 4, which have antibacterial properties acting against methicillin susceptible Staphylococcus aureus (MSSA) and methicillin resistant Staphylococcus aureus (MRSA), Escherichia coli, Candida albicans, Cryptococcus neoformans (27). In the available literature there is only little information on the structure and function of platelet antibacterial proteins.

On the basis of the conducted studies, RANTES protein, together with the platelet factor 4, was classified in the group of chemokines, which play a key role in the bacterial infections caused by the intracellular pathogens. It is considered that the level of RANTES increases in the acute phase of infection in tuberculosis and in Pneumocystis carinii infection, as well as in the gastritis and the concomitant Helicobacter pylori infection (28-30). The presence of chemokine RANTES was observed in the cerebrospinal fluid in children with viral and bacterial inflammations (28). The platelet factor 4 (PF4) is a protein produced in megakaryocytes, and stored like many other HPAPs in the platelet alpha granules. Its release during the activation of thrombocytes intensifies their aggregation under the influence of ADP in vitro. The most important task of the platelet factor 4 is to neutralize the heparin-like compounds in the blood plasma and on the surface of endothelium and to decrease the activity of antithrombin III (AT III). It also shows a strong antibacterial activity (31).

Tang et al. - on the basis of the studies conducted on the separated platelet peptides - have stated that the antibacterial platelet proteins are activated with the cooperation of thrombin. What is even more important, they have proven that the platelet factor 4 and the protein activating the connective tissue 3 show synergic antibacterial activity against Escherichia coli. They have also observed that each of the enumerated peptides reveals antibacterial activity against at least 2 bacteria, and that the proteins show an increased activity against the bacteria in comparison with their antifungal activity. Moreover, their activity is said to increase in the acid environment, with the exception of tymosin β-4 which remains active in the alkaline environment (32). On the basis of studies conducted on the antibacterial properties of gelling leukocyte- and platelet-rich plasma (L-PRP), Bielecki et al. have demonstrated its antibacterial activity against MSSA and MRSA and 2 strains of Escherichia coli (ATCC35218 and ATCC25922), while on the other hand it causes the increase in growth of Pseudomonas aeruginosa (33).

The influence of the leukocytes on the mechanism of L-PRP action has not been entirely evaluated; its supporters claim that their influence on the inflammatory state is beneficial, while opponents – on the other hand – notice their negative effect in the form of solid enzymes release (5, 6, 34). At the time being it is a known fact that leukocytes, and especially neutrophils, are a rich source of not only the growth factors (35-37), but especially of

the natural antibacterial proteins (1). Neutrophils are the most common form of leukocytes with strong phagocytic properties. They constitute the first line of antibacterial defense. The cytoplasm of the neutrophil granulocytes contains numerous granules. The most important ones are primary (azurophil) granules connected to the process of intracellular bacteria destruction and containing numerous bactericidal factors, including defensins, cathelicidins, serprocidins, Bactericidal/permeability-increasing protein (BPI) of gram-negative bacteria, myeloperoxidase and cytoplasmic calprotectin. The secondary (specific) granules are rich in antibacterial proteins such as lysozyme, collagenase, gelatinase, lactoferrin, phospholipase A2, transcobalamin-1 and membrane proteins (38). Neutrophils release enzymes which destroy some specific components of the damaged tissues (to allow cell migration, tissue cleaning and finally tissue reconstruction), and first of all damage microbes, especially bacteria. Neutrophils circulating in the blood become activated by chemotactic factors and this constitutes a signal for the merging of the secretory granules with the superficial membrane. The process of phagocytosis starts by surrounding the organism by pseudopodia, and then closing it inside a phagosome, which undergoes a merge with granules, mainly the primary. The granules release their content exposing the microbe to the activity of a strong mixture of antibacterial proteins (38). The destruction of the phagocytosed bacteria takes place with the participation of oxygen species, or without oxygen with the use of lactoferrin or lysozyme.

The main feature of antimicrobial proteins is their ability of destroy the cell body of microbes with the use of positive charges, which allow the proteins to bond with the negatively charged surface of the target cell (39). The mechanism is based on the disintegration of the cellular membrane by forming channels inside the membrane, depolarization and fragmentation, which can lead to the death of the cell. The peptides interact with the membrane of the target cell by "carpet", "barrel-stave" or "toroidal pores" mechanism and using a detergent-like model (39-41).

In case of interaction with the cell body of gram-negative bacteria, the antibacterial proteins react with lipopolysaccharides, and in case of the gram-positive bacteria with the teichoic acid and peptidoglycan (PG), supporting the divalent ions (39-41).

Defensins, also called lysosomal cationic proteins, are crucial element of the innate immunity and are found in neutrophils, thrombocytes and tissues of liver, skin, eye, as well as the gastrointestinal system, respiratory system and urogenital system (42, 43). Defensins constitute 15 % of the proteins from a human neutrophil and 50 % of the proteins of the primary granules of neutrophil granulocytes (42). In the mammals, it was described:

- 12 α-defensins: HNP1 to 4 ((human neutrophil peptides 1 to 4), HD5 and 6 (human defensins 5 and 6), NP1 and 5 (neutrophil peptides 1 and 5), criptidin 3 and 4, RMAD3 and 4 (rhesus macaque myeloid a-defensins 3 and 4),
- 25 β-defensins: HBD1 to 4 (human β-defensins 1 to 4), SBD1 and 2 (sheep β-defensins 1 and 2), TAP (tracheal antimicrobial peptide), LAP (lingual antimicrobial peptide), EBD (enteric β-defensin), BNBD1 to 13 (bovine neutrophil β-defensins 1 to 13), PBD1 and 2 (porcine β-defensins 1 and 2), mBD-1 (murine β-defensin 1),
- and 5 θ-defensins: retrocyklin 1 and 2, RTD1 to 3 (rhesus θ-defensins 1 to 3).

These molecules display biological roles which are not only antibacterial, but also antiviral, antiparasitic, and antifungal (43). The mechanism of antimicrobial activity of defensins is related to their merging with the cellular membrane of a microbe, and then initiating several metabolic processes leading to apoptosis (43, 44). For example α-defensins HNP1 to 4 (human neutrophil peptide) are located, with others, in the granules of neutrophils and act by connecting to the teichoic acids located in the cell wall of gram-positive bacteria (43).

On the basis of the current knowledge, the following has been stated: β-defensin HBD-1 does not show any bactericidal effect against Listeria monocytogenes, Mycobacterium tuberculosis, paratuberculosis, Pasteurella haemolytica, Pseudomonas aeruginosa or Enterococcus faecalis. Nonetheless, it reveals a strong activity against Escherichia coli (43, 45, 46). On the other hand, β-defensin HBD-2 in human has a strong inhibiting activity against gram-positive and gram-negative bacteria, Candida albicans, and it influences also the production of TNF-α (43, 47). It has also been stated that the β-defensin HBD-3 shows bactericidal activity against gram-positive and gram-negative bacteria, but its effect on some aggressive species like Burkholderia cepacia has not been proven (48). In the literature, it has been proven that defensins show not only antibacterial but also antiviral, antifungal, and even antiparasitic effect (49). Their antineoplastic activity has also been stated (50).

Until now one cathelicidin (hCAP-18) has been discovered in human. It is stored as an inactive precursor protein and undergoes synthesis in the specific granules of neutrophil granulocytes, natural killer cells and mast cells (42, 51). After proteolysis, hCAP-18 undergoes a transformation into an active peptide LL-37. A high concentration of this peptide (together with a decreased ionic strength of the environment and the presence of divalent ions) causes an increase in the antibacterial activity (52). hCAP-18 shows a wide spectrum of antibacterial activity both against gram-positive and

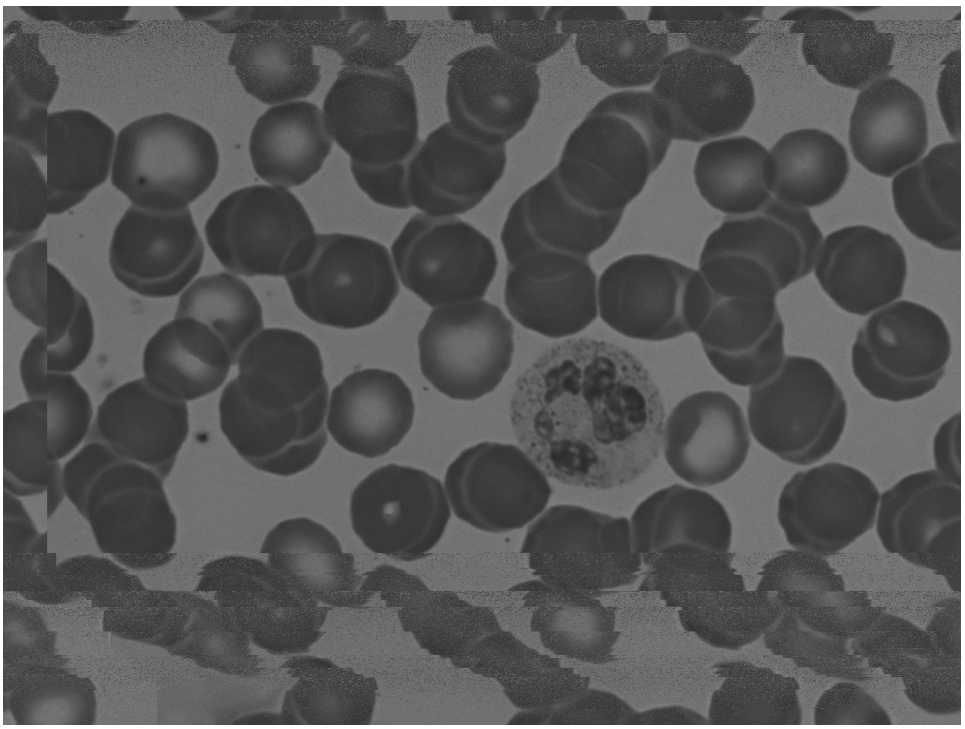

Fig. 1. *Neutrophil with granules in its cytoplasm*

gram-negative microbes.

The mechanism of cathelicidin activity against microbes is identical to the mechanism of defensins and consists of the disintegration of the membrane of the bacteria on which these proteins have influence. Cathelicidins show antibacterial effect against gram-negative bacteria such as Escherichia coli, Salmonella enterica serovar typhimurium, Pseudomonas aeruginosa and gram-positive bacteria including Staphylococcus aureus. Cathelicidins show also an antiviral effect against the influenza viruses and HIV (40, 42, 53, 54).

According to the current knowledge, bacteria developed several defense mechanisms against defensins and cathelicidins, preventing the antibacterial peptide bonding and its incorporation into the cell wall, and by the change of the permeability of the bacterial membrane. Scientists conduct many studies trying to use human defensins and cathelicidins in the treatment of some diseases, and a wide range of synthesized proteins are still in the phases of clinical trials (42, 43).

Serprocidins are considered to be part of a group of digestive enzymes having influence on the bacterial structures. They are located in osteocytes, myocytes, macrophages, blood vascular cells, neoplastic cells, and especially in the granules of neutrophil granulocytes. The antibacterial function of these cells is dependent on the high proteolytic and catalytic activity of serprocidins (55). The group of serprocidins includes: elastase, which has antibacterial effects against gram-negative bacteria and also an antifungal activity; proteinase 3, which influences activation and disintegration of hCAP-18; and cathepsin G, which stimulates lymphocytes and monocytes and increases the susceptibility of macrophages to HIV-1 virus infection (55, 56).

Bactericidal/permeability-increasing protein (BPI) is mainly located in the granules of neutrophils, as well as - in smaller amounts - in fibroblasts, monocytes, eosinophil granulocytes and epithelial cells. Its gram-negative antibacterial function is related to the strong affinity to lipid A located in the wall of the bacterial cell, due to which BPI leads to disintegration of the bacterial membrane, and as a result to the bacterial death (57). It has been proven that this protein may show antibacterial activity against gram-positive microbes in specific conditions (58).

Myeloperoxidase is a hemoprotein stored in the granules of neutrophils and lysosomes of monocytes. In the 1960s, some studies demonstrated that its mechanism of activity is based on the stimulation of the catalysis of chloride ion and hydrogen peroxide into hypochlorite, which is 50 times stronger (in terms of bactericidal activity) than H_2O_2 (59). Myeloperoxidase shows also destructive effects on fungi, viruses, as well as natural

Fig. 2. *Agranular lymphocyte*

killer and neoplastic cells.

Calprotectin is a protein stored and produced by neutrophils and constitutes approximatively 50% of their cytoplasmic proteins. Calprotectin exerts strong antibacterial effects in the mechanism of inhibition of zinc-dependent metalloproteinases, which stimulates the migration of neutrophils and initiation of the process of phagocytosis (60). Within the inflammatory processes, it plays a regulatory role as an antibacterial and antiproliferative protein (60).

The basis for the antibacterial properties of the blood serum is the cooperation between the complement system and lysozyme, which constitute an important barrier protecting the organism against the entry of microbes. Lysozyme belongs to non-specific humoral immune system reaction and occurs mainly in the azurophil granules, specific granules and tertiary neutrophil granules, and also in the granules of monocytes and macrophages. Lysozyme exerts antibacterial effect mainly against gram-positive bacteria; on the other hand its cytolytic function against gram-negative bacteria has not been entirely evaluated yet (61, 62). Lysozyme destroys the outer membrane of gram-negative bacteria leading, afterwards, to its death.

Lactoferrin, together with defensins and cathelicidins, belongs to the so-called cationic peptides; it is produced by neutrophils, and its antibacterial properties are related to the possibilities of bonding the ferrous ions (25).

Platelet-leukocyte aggregates (PLAs) form frequently in the human organism, and this aggregation is controlled by the expression of selectins on the cell surfaces (63). Platelet-leukocyte aggregates (PLAs) are activated far more often than the circulating single cells (64). In pathological conditions, the number of platelet-leukocyte aggregates significantly increases in comparison with the physiological levels (63-65). For example, neutrophil-platelet complexes are activated more easily than single neutrophil granulocytes, and therefore they stimulate the development of adhesion and phagocytosis (66). The role of the activated leukocytes in the process of PLAs formation has not been entirely explained so far.

Antimicrobial activities of the PRP/PRF: perspectives.

In contemporary medicine, the innate immunity is a central parameter to consider in the therapeutic strategies, and this immunity is closely related to the constantly increasing resistance of bacteria to various medications, particularly antibiotics. Many recent investigations aim at overcoming the resistance of microbes to antibiotics by the use of natural proteins from the human organism. Their undeniable advantage is minor cytotoxicity and absence of resistance to their activity. The use of platelet concentrates technologies for the collection and use of the

natural antimicrobial agents of the organism is therefore a very interesting approach, but the topic is almost not investigated in the literature. Most authors are still debating about growth factors contents and neglect the many other components of these autologous complex materials.

Several studies about the composition and application of platelet-leukocyte gels have shown already that these gels present high concentration of leukocytes and platelets rich in natural antimicrobial peptides. Classical 2-stages L-PRP preparation protocol was used in Cieslik-Bielecka's study in rats [67]. She identified these cells through flow cytometry after labeling T lymphocytes for CD3, CD4, CD8, CD27, CD28, TCRab, TCRgd and their active form for CD25, RT1B ; B lymphocytes for CD45R, CD45RA, CD90 ; NK for CD161a ; monocytes and granulocytes for CD18, CD11bc. In gravitational platelet separation systems developed by companies, where automatic partition of the buffy coat from the red blood cells and the acellular plasma layer is reached, a huge number of leukocytes can also be obtained. Their phenotype was identified through flow cytometry after labeling leukocytes for CD45: monocytes (CD14+/CD33+), granulocytes (CD15+), lymphocytes B (CD19+), lymphocytes T (CD3+), T helper cells (CD3+/CD4+), suppressor and cytotoxic T cells (CD3+/CD8+), cytotoxic T cells (CD3+/CD16+/CD56+), NK (CD45+/CD3-/CD16+/CD56+) and their progenitor form (CD34+), with fluorochrome conjugated antibodies [1]. Therefore, the proper term to use as a nomenclature for these products is clearly "leukocyte and platelet-rich plasma (L-PRP)".

Moreover, in unpublished investigation on 20 healthy volunteers with another L-PRP protocol, we showed a slightly weaker antimicrobial activity of L-PRP gel against MRSA and MSSA in comparison with the previous study [33]; however, this second L-PRP gel presented a strong activity against Enterococcus faecalis and Pseudomonas aeruginosa. The statistical analysis demonstrated no relationship between the platelet counts and microbial inhibition. However, significant correlation between leukocytes and growth inhibition zone in 3 strains; MRSA, MSSA and Pseudomonas aeruginosa was noted. The leukocyte concentration had no influence on inhibition of Enterococcus faecalis growth, but a strong correlation of this effect with the amount of active T-lymphocytes (CD3+/CD16+/CD56+ with CD25+) was found.

However, this is only a first step; the analysis of the biological activity of these natural antibacterial proteins in the PRP/PRF is necessary to develop new preventive and therapeutic strategies with this family of products. This is a new aspect and chapter of the PRP/PRF history.

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APPLICATIONS AND LIMITS OF PLATELET-RICH PLASMA IN SPORTS RELATED INJURIES

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Platelet-rich plasma (PRP) is a promising alternative approach based on the efficacy of autologous growth factors to accelerate tissue healing, allowing a fast recovery after muscles, ligaments, tendon or cartilage lesions. This literature review begin focusing on the role of platelets growth factors' in these tissue healing and on the available preparation methods for PRP. Moreover we consider the in vitro and in vivo study on PRP, some of the most important therapeutic applications and limitations. Although several preclinical studies show promising results, clinical studies still show controversial results. Further studies are required to define the efficacy and to specify the way of using PRP in the orthopaedic practice.

Platelet-rich plasma (PRP) represents a relatively new biotechnology procedure used for enhancing the healing response in musculoskeletal injuries which is often a slow and sometimes incomplete process (1,2,3). The term PRP is preferred to autologous platelet gel, plasma-rich growth factors (PRGFs), or a mere autologous platelet concentrate (4). PRP is a portion of the plasma fraction of autologous blood having a platelet concentration above baseline, that contains a number of proteins, cytokines and growth factors that initiate and regulate basic aspects of wound healing (2,3).

The use of platelet rich plasma as a potent source of growth factors has been developed by Marx at the end 1990's for maxillofacial surgery as ancillary therapy in oral surgery for bone grafts (4, 5). The rising interest in PRP now appears in many fields including orthopedics, dentistry and wound healing as well as cosmetic and cardiothoracic surgery. In fact, the autologous origin, easy preparation and the reduced cost of platelet-based preparations is higher attractive for sports medicine where a fast recovery and return to competitions are often critical outcomes in patient care. The use of PRP in elite athletes have been discussed for a long time due to its content of growth factors that may be considered a doping violation. The World Anti-Doping Agency (WADA) released in 2011 a statement regarding the acceptable uses of PRP in athletes (6). This is an important issue to render the athletes treated with PRP therapy eligible for athletic participation.

Several basic science studies in the last decade have focused on the clinical use of PRP, but there are only a few controlled clinical studies and with a small number of subjects. In addition, a variety of reports regarding the platelet concentration and the presence of different growth factors in the PRP concentrate have been published, as well as many preparation protocols, kits, centrifuges and methods with a lack of standardization in PRP dosing are currently reported.

To better understand how PRP may assist in healing we purpose to analyze the existing published studies which support the efficacy of PRP and its use in orthopaedic sports medicine for the treatment of tendinous, ligamentous and cartilaginous injuries.

Background

Tissue healing is an orchestrated biological phenomenon consisting of three sequential phases, inflammation, proliferation, and remodeling. After tissue injury platelets are stimulated to aggregate and generate thrombin, which triggers the production of a fibrin matrix from fibrinogen in the injured site. When platelets are activated they release growth factors, cytokines and hemostatic factors that reside within their alpha and dense granules and that are critical in the early stage of the clotting cascade pathways (Figure 1).

In particular, alpha granules contain platelet-derived growth factors (PDGF-AA, BB, and AB isomers), transforming growth factor-β (TGF-β), vascular

Key words: Platelet Rich Plasma, Sports injury, Growth factors

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Table I *Published Studies on PRP Clinical Applications and their outcomes*

Authors	Site	Design	System	Results
Sanchez et al[46]	Achille's Tendon	Case-control study (n=6)	Platelet-rich fibrin matrix; 460g[§] for 8 min using PRGF System II, BTI, Vitoria-Gasteiz, Spain	Significant improvement in earlier range of motion, earlier return to gentle running and earlier return to sports, smaller cross-sectional tendon area
Gaweda et al[44]	Achille's Tendon	Case series (n=15)	Two centrifugations at 2400 RPM[§] for 10 min and 3600 RPM[§] for 15 min, respectively	Significant improvement in functional measures (VISA-A and AOFAS)
de Vos et al[36]	Achille's Tendon	Prospective randomized controlled trial (n=27 PRP group; n=27 control group)	GPS® System Biomet Biologics Indiana, USA 3200 RPM for 15 min	No difference in pain scores or functional measures (VISA-A)
de Jonge et al[49]	Achille's Tendon	Randomized controlled trial (n=27 PRP group; n=27 placebo group)	Recover™ Platelet Separation Kit Biomet Biologics, Indiana, USA 3200 RPM for 15 min	No clinical and sonographic benefit of platelet-rich plasma injection compared with placebo injection at 1 year follow-up
Peerbooms et al[50]	Elbow Tendon	Randomized controlled trial (n=51 PRP group; n=49 control group)	Recover™ Platelet Separation Kit, Biomet Biologics Indiana, USA 3200 RPM for 15 min	Significant decrease of pain (VAS) and function (DASH) scores after 26 weeks and 1 year from platelet application
Mishra and Pavelko[51]	Elbow Tendon	Cohort study (n=15 PRP group; n=5 control group)	GPS® System Biomet Biologics Indiana, USA 3200 RPM for 15 min	Significant decrease of pain (VAS) and function (modified Mayo elbow score)
Kon et al[52]	Patellar Tendon	Case series (n=20)	Two centrifugations at 1800 RPM for 15 min and 3500 RPM for 10 min, respectively	Significant improvement in quality of life scores (EQ-VAS and SF-36) and function (Tegner score)
Filardo et al[53]	Patellar Tendon	Prospective randomized controlled trial (n=15 PRP group; n=16 control group)	Two centrifugations at 1800 RPM for 15 min and 3500 RPM for 10 min (pellet spin), respectively	No significant improvement in PRP group with pain (VAS) and function (Tegner score)
Randelli et al[56]	Rotator cuff Tendon	Case series (n=14)	GPS® II Platelet Concentration System, Biomet Biologics Indiana, USA 3200 RPM for 15 min	Significant improvement in pain (VAS) and function (UCLA and Constant scores)
Castricini et al[57]	Rotator cuff Tendon	Randomized controlled trial (n=45 PRP group; n=45 control group)	Two centrifugations at 1100 RPM for 6 min and 3500g for 10 min, respectively	No significant improvement in shoulder function (Constant score) or structural outcome (MRI)
Jo et al[59]	Rotator cuff Tendon	Cohort study (n=19 PRP group; n=23 control group)	Plateletpheresis system with leukoreduction set COBE spectra LRS Turbo Caridian BCT Colorado, USA	No improvement in pain (VAS), in the recovery of range of motion, strength, or function (ASES, Costant system, UCLA, DASH, SST, SPADI)
Nin et al[65]	Anterior Cruciate Ligament	Prospective randomized controlled trial (n=50 PRP group; n=50 control group)	Two centrifugations at 2.217g for 8 min and 380g for 6 min, respectively	No significant differences between the groups for inflammatory parameters (perimeters of the knee and C-reactive protein level), magnetic resonance imaging appearance of the graft, and clinical evaluation scores (VAS, IKDC)
Cervellin et al[66]	Anterior Cruciate Ligament	Prospective randomized controlled trial (n=20 PRP group; n=20 control group)	GPS® II Platelet Concentration System, Biomet Biologics Indiana, USA 3200 RPM for 15 min	Significant decrease subjective knee pain and donor-site level (VISA score)
Kon et al[70]	Cartilage	Case series (n=90, 114 knees)	Two centrifugations at 1800 RPM for 15 min and 3500 RPM for 10 min, respectively	Reduce of pain and improve knee function and quality of life with short-term efficacy (IKDC, EQ-VAS)

§= RPM is indicated where g is not available (RPM = revolutions per minute; g = gravitational force)

endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived epidermal growth factor (PDEGF), insulin-like growth factor-1 (IGF-1), platelet factor 4 (PF4), interleukin-1, 2 (IL-1, IL-2) (7-12). Many of these factors have been shown to enhance bone and soft tissue healing. Insuline-like growth factor is thought to stimulate osteoblast proliferation and differentiation. PDGF, EGF and bFGF have been shown to stimulate proliferation of osteoblastic progenitors, mesenchymal stem cells and epidermal cells. Transforming growth factor beta stimulate collagen synthesis, a component of extracellular matrix, and VEGF and bFGF enhance angiogenesis and revascularization.

After release, the cytokines bind to transmembrane receptors on the surface of local or circulating cells, and they initiate an intracellular signaling. The tissue regeneration through angiogenesis, extracellular matrix production, and collagen synthesis is orchestrated by the autocrine and paracrine effects of the growth factors. Alpha granules are also a source of fibrinogen (Fg), fibronectin (Fn), vitronectin (Vn), thrombospondin-1 (TSP-1), osteocalcin (Oc), osteonectin (On) and of many other proteins variously involved in stimulating chemotaxis, modulating inflammatory molecules and

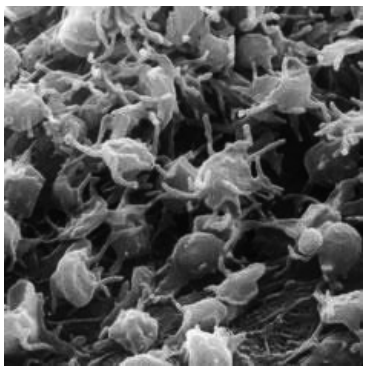

Fig. 1. *Microphotograph of activates platelets at microscopy electron scansion; magnification 10x when printed at 10cm wide; scale bar is 5µm*

attracting leucocytes (13, 14).

Besides alpha granules also dense granules play a role in tissue modulation and regeneration. The dense granules contain histamine, serotonin and adenosine that allow inflammatory cells wider access to the wound site and activates macrophages and modulates inflammation during wound healing (15, 16). Calcium is also a component of the dense granules and its involvement in wound healing is mainly in keratinocyte proliferation and differentiation. The calcium content within the dense granules of platelets may play a vital role in its delivery to the site of injury (17).

Moreover, platelets store antibacterial and fungicidal proteins, proteases such as metalloprotease-4 and coagulation factor (16), and lysosomal granules which can secrete acid hydrolases, cathepsins D and E, elastase and lisozima (18, 19).

PRP (Platelet Rich Plasma)

The combination and concentration of bioactive molecules that exist within PRP and the important role of these factors in wound healing, have strong clinical interest in tissue regeneration. Consequently, researchers worldwide are evaluating how PRP produces these effects. PRP contains about one million platelets per microliter (3) and various cytokines, that have been not all characterized. The optimal concentration of platelets for bone regeneration has been shown to be between $5.0x10^5$ and $1.73x10^6$ platelets/µl of PRP, after wise the concentration above $1.8x10^6$ platelet/µl may have a paradoxically inhibitory effect. Moreover, leading in vitro studies have also found that PRP significantly inhibits the growth of Staphylococcus aureus and Escherichia coli (20, 21).

PRP is easily produced and in literature different methods of collection, preparation and administration were reported. The available PRP techniques include two processes. Whole blood is collected with anticoagulant just before or during surgery and is immediately processed by centrifugation for separating the blood into three layers; red blood cells (RBCs) are found at the bottom, acellular plasma (PPP, platelet-poor plasma) in the supernatant and a "buffy coat" layer appears in between, in which platelets are concentrated. Then an additional centrifugation separates PRP from platelet-poor plasma (PPP). The platelet concentrate is then activated with the addition of bovine thrombin or $CaCl_2$, resulting in a gelatinous platelet gel which finally is applied on the surgical site by a syringe.

The several techniques for platelet concentrates lead to a different product with different biology and potential uses. David M et al. suggest the classification of the different platelets concentrate into four categories, depending on their leucocyte and fibrin content: pure-PRP (P-PRP), leucocyte-rich PRP (L-PRP), pure plaletet-rich fibrin (P-PRF) and leucocyte-rich PRF (L-PRF)(22).

Pure platelet concentrates were first developed for maxillofacial surgery (5). The first automated method of producing platelet concentrates was plasmapheresis, and recently, more compact system have been developed, such as Vivostat PRF (Vivolution, Denmark) which allows the production of a leucocyte-poor platelet concentrate for surgical use but is very expensive for daily practice. Anitua's PRGF method (plasma rich in growth factors) is money saving manual protocol commercialized by BTI (BioTechnology Institute, Vitoria, Spain) for producing P-PRP but this technique is characterized by low reproducibility (22, 23). PRP rich in leucocytes is composed of a high quantity of platelets, leucocytes and circulating fibrinogen and it can be generated both by manual protocols such as Curasan® (Fa. Curasan, Kleinostheim, Germany), Regen® (Regen Laboratory, Mollens, Switzerland) or Plateltex® (Bratislava, Slovakia) and automated methods such as SmartPReP® 2 APC+™ (Harvest Technologies, Plymouth, USA), Magellan® (Arteriocyte, Cleveland, USA) or Biomet GPS® III (Biomet Manufacturing Corp, Warsaw, IN). The presence of leukocytes within PRP for surgical use is a controversial issue widely debated in literature. In fact, leukocytes may play an important role in enhancing the tissue repair process since are able to produce VEGF (24,25) and it is known that neutrophils and monocytes contain granules filled with myeloperoxidase, a substance which contributes to antimicrobial activity of platelet concentrate at the site of application (20). A recent research showed that L-PRP was able to stimulate anabolism and remodeling potential of tendons and could be used for the treatment of tendinosis (26-29). However, other studies reported that leucocytes may lead to increased

local inflammation in injured tissue, especially in muscle strains or joint infiltrations by proinflammatory proteases and acid hydrolases they release (30, 31). While the use of L-PRP has never been systematically studied, a positive or negative effect of white blood cells cannot be generalized to during clinical application; however, there is no proof now of the undesiderable side effects. Plaletet rich fibrin (PRF) or platelet gel (PLG), is a product with an high-density fibrin network some with leukocytes (L-PRF) and some without leukocytes (P-PRF) (22). L-PRF is produced with Fibrinet PRFM kit by Cascade Medical (New Jersey, USA) while P-PRF by Choukroun's PRF protocol developed in France by Choukroun et al (32). The fibrin matrix is generated by addition of calcium chloride during the second centrifugation step resulting in formation of a very dense gel (33) that may contribute to healing by providing a conductive scaffold for cell migration and new matrix formation.

Other parameters should be considered for an effective classification of platelets and for the use of these techniques in daily surgical practice: the speed and number of centrifugations, the use of anticoagulant in the sample, the use of an activator together with calcium chloride and the density of the fibrin matrix obtained. These differences highlight the suggestion that the various platelet preparations may result in different clinical effects and the confounding factors when comparing the results obtained in different studies (TABLE I).

CURRENT CLINICAL APPLICATIONS

Tendon

Tendons are dense parallel-fibered collagenous connective tissue containing an organized fibrillar matrix which consists primarily of type I collagen, proteoglycans and glycoproteins. Tenocytes are fibroblast-like cells within the tendon and are responsible for tissue maintenance, matrix remodeling and consequently for pathological response to changes in the biological and mechanical environment. The low metabolic rate and the poor vascularization predispose the tendon to slow healing. (34-39).

One of typical problem in orthopeadic sports medicine is tendinopathy that is considered as a syndrome characterized by tendon pain, localized tenderness and swelling which impair performance. The causes are long-term overuse and deterioration of the tendon rather than an acute injury which causes inflammation of the tendon. The correct diagnosis is important because the inflammation of tendinitis is differently treated from tendinopathy. Inflammation in tendinitis quickly responds to medication or anti-inflammatory treatment. However, in chronic tendon injuries due to degeneration with little or no inflammation present, treatment may be quite lengthy and focuses on improvement of the strength of the tendon and of tissue rebuilding. The most common tendon injuries in sports include Achilles tendon, patellar tendon, tractus iliotibialis, hamstring tendon, rotator cuff tendon and tennis elbow. Current nonoperative treatments are rest, stretching, non-steroidal anti-inflammatory drugs, gradual return to exercise, and shock wave therapy but often they offer only a partial recovery. Surgery is also an option, but return to sports after surgery is reported in only 60-85% of cases (35).

In the last years, sports physicians have increasingly been using PRP in tendon disorders to restore the normal tissue composition and to avoid further degeneration. Recent basic science research has shown that the growth factors delivered by platelets stimulate cell proliferation of human tenocytes and chondrocytes (34). Other studies showed that autologous growth factors are able to change collagen production and degradation by influencing matrix regulating enzymes (35-39). Moreover TGF-β induces an increase on the procollagen types I and III and mechanical properties on tendon cell cultured, whilst PDGF-BB, IGF-1, VEGF and B-FGF promote tendon cell proliferation and tendon healing. Anitua et al studied the effects of platelet-rich and platelet-poor clots on human tendon cells in culture. Their results suggest that the pool of growth factor released from an autologous PRP (PRGF) increased significantly the proliferation of human tendon cells and stimulated them to produce VEGF and HGF at the site of injury also promoting therapeutic angiogenesis (33, 34). In rat models PRP injected into the hematoma resulted in increases tendon callus strength and stiffness (40). In another study, Kajikawa et al, showed that locally injected PRP in patellar tendon of chimeric rat expressing green fluorescent protein in circulating cells and bone marrow cells, enhanced the recruitment of circulation derived cells to the injury site, with concomitant increased levels of collagen I and III, which is consistent with repair and remodeling tendon (41). Lyras et al. recently described an overexpression of IGF-1 following PRP treatment of rabbit patellar tendon in the first 3 weeks of healing in both epitenon and endotenon also confirmed by histological evaluation that revealed a superior healing process in PRP group compared with the controls (42). Further research in a bigger animal model showed that repetitive injection of PRGF within Achilles tendon fascicles in sheep triggered a healing response assessed by increased cell number and angiogenesis, and did not provoke fibrosis (43, 44). Bosh et al reported that a single intratendinous PRP treatment in horses determined a significant effect on biochemical, biomechanical, and histological properties of tissue repair. Moreover, PRP-treated tendons was stronger and had a higher collagen, GAG, and DNA content than control-

treated tendons (45).

Overuse injury of the Achilles tendon is a frequent problem that often affects sportsmen but also sedentary middle-aged individuals (46-48). Patients with Achilles tendinopathy who have failed physical therapy and multiple modalities of conservative treatment are candidates for PRP injection. Reports in human Achilles tendon repair are controversial. Sanchez et al showed a shorter time in the recovery of motion and return to sporting activities in athletes treated with PRP. Moreover, they observed no wound complication and a faster return to jogging and training activities (46). This study used a fibrin scaffold in addition to PRP, in a small patient group numbers (N=6); controlled clinical trials with wide patient cohorts are necessary to assess the efficacy of this treatment. Additionally, Gaweda et al reported that a single injection of PRP, under ultrasound guidance into the hypoechoic area of the tendon, determined a significant improvement of American Orthopedic Foot and Ankle Society (AOFAS) and of Victorian Institute of Sport Assessment-Achilles (VISA-A) scores in 14 patients (15 Achilles tendon) affected of a non-insertional Achilles tendinopathy (47). The authors also reported an improvement in qualitative gray-scale ultrasound characteristic. On the other hand, de Vos and colleagues reported that a PRP injection in patients did not have any improvement in pain and activity when compared with saline treatment (48) and also more recently, de Jonge et al showed, in a double-blind randomized placebo-controlled clinical trial, that PRP did not improved clinical symptoms after 1 year in chronic Achilles tendinopathy, in comparison to placebo injection (49).

Epicondylar tendinoses are frequent injuries in athletes who perform repetitive wrist motions and strong gripping. PRP has advantages in the treatment of tennis elbow as demonstrated by a double-bind randomized controlled trial of Peerbooms et al (50). They observed that a single injection of PRP in a population of 100 patients with lateral epicondylitis, improve pain and function at 6 months and 1 year follow-up better than corticosteroid treatment and without any complications. In particular, one year after the procedure, PRP-treated patients reported a mean improvement of 63.9% in their visual analog score (VAS) compared with the initial values, whereas the corticosteroid-treated patients reported a 24.0% improvement ($p<.001$). Moreover, after 1 year, Disabilities of the Arm, Shoulder, and Hand scores (DASH) improved 66% in PRP patients versus a 17.4% improvement in corticosteroid-treated patients ($p=.001$). Previously, also in a cohort study, reported a clinical decrease of pain after PRP treatment in patients having chronic elbow tendinosis (51).

Jumper's knee (patellar tendinopathy) is common in athletes of sport disciplines where jumping is frequent e.g. basketball, soccer and volleyball. Kon et al presented the results of a case series (pilot study) for the treatment of patellar tendinopathy with multiple PRP injections (N=20) and reported statistically significant improvement in pain and physical function at 6 months follow-up (52). The same group, treated 15 patients who, after a failure of nonsurgical or even surgical treatments, were able, through the combination of multiple PRP injections and physiotherapy, to achieve a good recovery and to return to their previous sporting activity (53).

Rotator cuff repair

Rotator cuff tears are more and more frequent due to the population age increase; it is common injury in athletes who play tennis, baseball, softball and swimming. Failure to heal and retearing after surgery is not uncommon and perhaps depends on the size of the tear and the level of tendon degeneration (54, 55). PRP augmentation of rotator cuff may improve healing and clinical outcome even if early studies results appeared contentions. A prospective study by Randelli et al evaluated the safety and the outcome of local administration of autologous PRP (N=14) activated by autologous thrombin, in arthroscopic rotator cuff repair. They reported pain decrease, UCLA, and Constant scores at 6, 12, and 24 months improvements after surgery when compared with preoperative values, without adverse events (56). In contrast, Castricini et al recently performed a randomized controlled trial on patients with small and medium rotator cuff tears, using a platelet rich fibrin (PRFM). They reported no differences in shoulder function (Constant score) or structural outcome (magnetic resonance imaging, MRI) (57). However, Arnoczky using the Castricini data, and comparing only those individuals considered to have normal values in each MRI outcome measurement, finds that the use of a PRFM actually results in a statistically significant return to normal footprint area ($P<.05$) and MRI signal intensity ($P<.001$) when compared with non-PRFM–treated individuals (58). Moreover, Jo et al in a prospective cohort study, reported that the addition of PRP gel to arthroscopic rotator cuff repair did not accelerate clinical recovery when compared with conventional treatment. Nevertheless, the authors found several weaknesses on their study and considered their results not sufficient to reach a conclusion about the effect of PRP on rotator cuff repair, suggesting the need for further research (59).

Anterior Cruciate Ligament

Platelet concentrate may be employed also to stimulate and sustain anterior cruciate ligament (ACL) healing. ACL reconstruction involves the use of a tendon/ligament autograft which is transplanted into bone tunnels

at its femoral and tibial insertions. Successful ACL reconstruction requires a good healing process of the tendon graft in the bone tunnel as soon as possible after surgery. The rate of failure of ACL healing is included in a range from 40% to 100% in contrast with medial collateral ligament healing (60). Consequently, enhancement of tendon graft healing to the bone is crucial to induce an early rehabilitation and a rapid return to activity. The graft-tunnel healing is a complex and poorly understood healing process, that slowly occurs and it is influenced by several factors, including the type of used graft (auto versus allograft, and soft tissue versus bone-plug grafts), method of graft fixation and tensioning, graft motion, and tunnel placement. In this context PRP application may improve biological allograft integration reducing the rate of surgical failure; it may reduce the donor-site morbidity and the delay of return to sports activities in athletes. The application of a collagen hydrogel scaffold containing PRP has been found to improve the final result in several animal models. Murray and colleagues, in a canine and porcine animal model, reported an improvement in primary healing of the ACL using a scaffold of collagen-PRP hydrogel in partial ACL ruptures in comparison with untreated controls (61, 62). The same authors, in a different study, used PRP alone added to suture repair without the collagen scaffold and did not report any improvement on maximum load and stiffness in Yorkshire pig. They concluded that PRP should be combined with a scaffold to stabilize it within the wound site to be effective (63). Orrego et al reported a significant mature graft signal (100%) in magnetic resonance imaging from the PRP group respect to the control group, but did not find statistical differences in the osteoligamentous interface (64). Moreover, Nin et al, in a prospective, randomized, double blind study did not show statistically significant differences in clinical and inflammatory parameters in patients treated with the addition of platelet-derived growth factor (PDGF) in primary anterior cruciate ligament (ACL) reconstruction with BPTB allograft (bone–patellar tendon–bone) (65). On the other hand, in a recent prospective, randomized, controlled study by Cervellin et al PRP application was able to significantly decrease subjective knee pain and donor-site level (VISA score) in patients treated for ACL reconstruction with BPTB (66).

Cartilage

Articular cartilage injuries remain a challenging problem for orthopaedic surgeons and sports physicians due to limited self repair potential of this tissue. Cartilage contains only one cell type and is not vascularized, and its regeneration, according to tissue engineering principles, requires the joint expertise of biologists, chemists and engineers to provide highly potent cells, specific growth factors and optimized physical environments (67).

In cartilage tissue, chondrocytes are responsible for the synthesis and renew the matrix that surrounds them with a constant turnover mechanism (68). Current knowledge on the effect of growth factors on the behavior of chondrocytes is rapidly increasing. TGFβ, PDGF, IGF, FGF and HGF, could be considered suitable tools to enhance cartilage repair via different pathways including recruitment of chondrogenic cells, stimulation of chondrogenic cell proliferation and enhancement of cartilage matrix synthesis (69).

Regarding the use of PRP as source of growth factors for the treatment of cartilage injuries and for intra-articular application to address knee pain, Kon et al treated with three intra-articular PRP injections 91 patients (115 knees) who presented a chronic knee degenerative condition. The health status was evaluated by IKDC- and EQ-VAS scores which showed that PRP treatment is safe, reduces pain, improves knee function with low degree of articular degeneration and quality of live in younger patients. Nevertheless, this study is limited by the lack of control groups and short follow-up periods up to 12 months (70). Recently, the 24-month follow up results of this study were published but the outcome (IKDC score) worsened from 67% to 59% (71).

CONCLUSIONS

PRP represents a regenerative approach based on the use of autologous growth factors for the treatment of joint and cartilage disease to accelerate tissue healing allowing a fast recovery and return to competition of athletes. Several preclinical studies show promising results and recently, some randomized controlled trials have being published, but results are still controversial and further studies are required to support the use of PRP in clinical practice. The major limitations in human studies are low sample number, the absence of control group and levels of evidence. Moreover, is mandatory to clarify and standardize the different phases of PRP preparation, the optimal concentration of platelets, the use of PRP rich in leucocytes and the site and number of injections; however, this is not easy to be performed for ethical and practical considerations. In conclusion, many factors, such as the quality of PRP preparations, may influenced the efficacy of the treatment in clinical studies.

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THE USE OF AUTOLOGOUS PLATELET AND PLASMA PRODUCTS IN SALVAGE NECK DISSECTIONS: A PROSPECTIVE CLINICAL STUDY EVALUATING EARLY AND LATE WOUND HEALING

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Objectives: To evaluate the effect of autologous platelet and plasma adhesives (APA) on postoperative drainage and soft-tissue fibrosis following neck dissections. Design: This was a blinded comparative prospective cohort study done as two parts: part one evaluated early post-surgical outcomes and part two evaluated late tissue fibrosis. Method: Salvage neck dissections were stratified into two groups based on severity of prior treatment. High risk patients were defined as those who had previously undergone chemoradiation therapy and autologous platelet adhesives were administered to the surgical wound intraoperatively. The low risk group consisted of patients undergoing salvage neck dissections following radiation only and acted as controls. Part one evaluated postsurgical wound drainage as the primary outcome as well as length of hospital stay and complications. Part two evaluated late postoperative tissue fibrosis by comparing neck skin using the Cutometer. R2 and F0 were the specific Cutometer parameters for quantifying the viscoelastic properties of the skin. Results: Postoperative wound drainage was significantly less (253.7 vs. 345.8) in the autologous platelet adhesive group as compared to the control group ($p < 0.03$). Length of stay in the APA group versus the control group was 3.13 and 3.86 days respectively ($p<0.004$). Both R2 and F0 measurements showed improved viscoelastic properties of the skin in the APA group (R2 $p<0.05$, F0 $p<0.05$). Conclusions: APA application following salvage neck dissections may reduce early postoperative wound drainage and improve long-term skin quality

Tissue adhesives have been used with increasing frequency in surgery to promote adhesion, improve hemostasis and wound healing. With the availability of commercial systems that allow for isolation of autologous platelet and plasma adhesives (APA) from the patients' own blood, a recent trend has seen the increasing use of autologous products [1]. High concentration of platelets or fibrin can be obtained readily at the time of surgery by incorporating centrifugation technology that allows for immediate application. These processes can separate the blood into platelet-rich plasma (PRP) and platelet-poor plasma (PPP), with packed red blood cells as a byproduct (Figure 1). Both PRP and PPP contain the full complement of coagulation proteins; while PRP may contain up to 10 times the concentration of platelets per volume over whole blood [2]. When PRP and PPP are activated with thrombin these products can be applied as an adhesive and hemostatic agent (Figure 2). Perhaps most compelling is that since platelets play such a vital role in the initial phase of wound healing, platelet adhesives such as PRP may have potential benefits on both short and long-term healing characteristics.

Various biocompatible adhesives such as Tiseel™ (Baxter Health Corp., Glendale, CA) have been used previously to promote tissue healing and reduce wound drainage [3-12]. Most of these products have the

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disadvantage of using pooled human plasma, but have a well-established safety profile and have been used widely as internal tissue sealants and for wound adhesion. However such products lack the potential benefits of high platelet concentrations.

APA containing high concentrations of platelets per volume has been used in a wide variety of soft and hard tissue procedures to promote wound healing [1,13-20]. Platelets represent the body's initial response to injury and become activated in large numbers at sites of tissue trauma by actively synthesizing and releasing growth factors for 7 to 10 days, thereby setting the stage for the body's healing response. While platelets do not survive past this interval, they create a local environment that attracts other cell types that are integral to the latter stages of the healing cascade. Proposed benefits to wound healing include enhanced tissue adhesion and hemostasis through platelet activation and fibrin clot formation. Furthermore growth factors released from platelets such as platelet-derived growth factor, transforming growth factor-beta, insulin-like growth factor–1, and vascular endothelial growth factor, attract fibroblasts, stimulate angiogenesis and signal the differentiation and proliferation of fibroblasts to rebuild tissues [21-24]

Although studies support the theoretical benefits of APA, clinical evaluations have been limited with few prospective studies evaluating the efficacy of these products [14,19,20]. Furthermore, there is little evidence that demonstrates any long-term quantifiable benefits in wound healing with the use of APA.

In order to evaluate the effects of APA in a high risk compromised wound, only neck dissections following radiation therapy (RT) or chemoradiotherapy (CRT) were studied. Neck dissection was selected as the surgical model based on the standardized nature of the procedure. Patients with residual or recurrent cervical metastases after primary CRT require salvage surgical dissection. Although complication rates are acceptable in the salvage setting, the incidence of postoperative complications such as increased wound drainage, infection, delayed healing, wound dehiscence, fistula formation, and late tissue fibrosis have been reported to be significantly higher [25-30].

The ability to evaluate, monitor, and perhaps improve tissue fibrosis following surgery hold substantial promise from a clinical perspective. Quantifying the viscoelastic characteristics of the neck secondary to medical intervention may serve as the initial step in understanding tissue changes over time. Being able to quantify tissue fibrosis may enable assessment of therapies that can objectively improve outcomes.

The purpose of the present study was to evaluate the early and long-term effects of APA application on the complex soft-tissue surgical wound. The objectives of the present work were two-fold. Our first objective was to compare the effect of intraoperative application of PRP/PPP on wound drainage, length of hospital stay, and perioperative complications with the standard protocol. The second objective was to compare the effect of intraoperative application of PRP/PPP with respect to the standard protocol on long-term neck fibrosis by measuring the viscoelastic properties of skin.

To our knowledge this is the first investigation of autologous adhesives on wound healing and neck fibrosis following neck dissection.

MATERIALS AND METHODS

The study was approved by the institutional Ethics Review Board. The study was designed as a two-part blinded prospective cohort study. All consecutive patients over 18 years of age undergoing salvage neck dissection following either RT or CRT for malignant tumours of the head and neck were eligible for enrollment. Patients undergoing concurrent resection of a mucosal primary cancer or patients with known platelet disorders were excluded from the study. Demographic and cancer related information were collected. 14 patients undergoing neck dissections following CRT were considered the highest risk group and received intraoperative APA (PRP and PPP) application (Group 1, PRP/PPP group). 16 patients who were treated with RT alone were considered lower risk and did not receive APA application (Group 2, control group). Patient demographics for both groups are provided in Table 1A and 1B.

In the first part of the study, the early postoperative outcomes in the two cohorts were compared by assessing the volume of wound drainage, the length of hospital stay, and the postoperative complications. PRP and PPP were prepared intraoperatively from 60cc of the patient's whole blood using the Gravitational Platelet Separation System™ (Biomet Inc. Warsaw, IN). The preparation method has been previously described [19]. PRP and PPP were each separated after centrifugation. In a separate centrifugation, the patient's own thrombin was isolated from 10cc's of whole blood. Immediately following the neck dissection but prior to skin closure, PRP and PPP were applied using thrombin for activation. First PRP and thrombin were applied concurrently to the entire surgical wound immediately prior to skin closure. A closed suction drain was then inserted but was not immediately connected to suction. Once the skin was closed, PPP and thrombin were delivered together under the skin flaps. Five minutes of pressure was gently applied to the neck to aid in wound adhesion, after which suction was connected to the drain at -100cc H2O pressure. In the control group, the same procedure was followed without the application of PRP and PPP. The postoperative care was standardized for all patients. Attending nurses measured drain output at 12 hour intervals and were blinded to the treatment group. Drains were removed when less than 30 cc was collected in a 12 hour period. Patients were discharged upon removal of the drain. Major complications such as perioperative infection requiring intravenous antibiotics causing discharge delay, wound

dehiscence and hematoma requiring reoperation were collected. It was felt that minor wound infections were difficult to diagnose and confirm; hence these rates were not measured.

In the second part of the study, long-term neck skin fibrosis in the two cohorts was compared. In order to objectively measure the viscoelastic changes to the skin, the Cutometer® MPA 580 (Courage + Khazaka Electronic GmbH, Köhn, Germany) was used. The Cutometer is a validated, non-invasive instrument that measures viscoelastic properties of skin using a controlled suction probe on a defined region of the skin flap [31,32] and has been used to quantify radiation skin fibrosis [33]. The Cutometer measures skin elasticity of compliance by using a suction device for determining deformation of the skin in response to creation of a negative pressure that is applied directly to skin and then measuring the restitution to resting state. The Cutometer parameters that correspond to skin elasticity and restitution are represented by "R2" and "F0", respectively. The R2 parameter can range in value from 0 to 1, with values closer to 1 indicating increased viscoelasticity. The F0 parameter has no upper limit but in contrast to R2, a lower value for F0 indicates better viscoelasticity. R2 and F0 have been validated to represent the viscoelastic properties of skin and were used as the primary outcomes to measure late effects of tissue fibrosis. All Cutometer measures were obtained by the same technician who was blinded to the population under investigation.

RESULTS

Thirty patients undergoing thirty-one neck dissections (one patient had bilateral neck dissections) were enrolled into the study. There were 14 patients in PRP/PPP group (15 neck dissections) with age range from 46 to 79 years (mean age = 57.5). There were 16 patients in the control group with age range from 28 to 77 years (mean age = 54 years). All patients in the PRP/PPP group underwent curative-intent CRT to the neck and/or primary tumour site whereas patients in the control group underwent preoperative RT only. The average time of follow-up for the PRP/PPP and control groups was 13 and 21 months, respectively.

Part One: Early Postoperative Outcomes

The average amount of postsurgical drainage in the PRP/PPP group was significantly less than the group who did not receive PRP/PPP (253.7 cc versus 345.8cc, p=0.03). The average length-of-stay was significantly lower in the PRP/PPP group (3.13 days versus 3.86 days, p =0.004). There were no major complications in either group.

Part Two: Tissue Fibrosis and the Viscoelastic Properties of the Skin

A significant reduction in neck skin fibrosis following salvage neck dissection was found in CRT patients treated with PRP/PPP as compared to the control group of neck dissection patients treated with RT alone. An assessment of the R2 and F0 parameters generated by the Cutometer revealed the following: for the R2 measure, a mean value of 0.856 was found for the PRP/PPP group and a value of 0.772 was found for the control group ($p < 0.05$); F0 revealed a mean value of 12.9 for the PRP/PPP group versus 16.0 for the control group ($p<0.05$).

DISCUSSION

This is the first known investigation that has sought to evaluate both the early and late effects of autologous platelet adhesives on wound healing for salvage neck dissections. The study found significant differences in early wound drainage and late tissue fibrosis in patients who received APA at the time of surgery. In order to specifically evaluate the effects on a high risk complex wound, the salvage neck dissection was used as an ideal model based on the standardization of the operation and the known risk profile. The positive findings of this study are particularly compelling due to the methodology whereby only the higher risk (CRT) patients received APA while the lower risk (RT) patients acted as controls.

Although the study was not randomized, the study was biased against the group that received PRP/PPP. Only patients who underwent CRT received intraoperative PRP/PPP while patients treated with RT alone underwent standard neck dissection. This methodology was used with the anticipation that the highest risk group receives the potentially beneficial intervention. We acknowledge that this fact may be a significant confounder to our findings. The primary outcome parameters of wound drainage and Cutometer readings were assessed by blinded evaluators. Although surgeons were not blinded, the rigorous study protocol on drain removal and patient discharge was implemented in order to mitigate this potential bias.

The early postoperative outcome of wound drainage was significantly reduced in the PRP/PPP cohort by approximately 90cc. Clear instructions were given to the nurses to ensure that the drainage tubes did not become plugged. Although the volume of drainage reduction was statistically significant, it is debatable whether this represents an important clinical difference. Nevertheless, the present study showed that using PRP/PPP did reduce wound drainage. Similar finding have been seen in uncomplicated wounds using PRP/PPP [19,20] but this is the first study that demonstrated drainage reduction in a complex wound, such as the chemoradiated field. Although length-of-stay data favoured the treatment group, this was not an independent variable. The study was designed so that amount of drainage was used as the criterion for drain removal and hospital discharge. Therefore these two outcome measures were directly associated. Many centres have practices that allow earlier patient discharge with the drainage tube in place

Table 1A. *Group 1: Chemoradiated Salvage Neck Dissections (PRP/PPP Applied)*

Age	Gender	TNM Staging	Surgical Procedure
79	F	T1N0 Left tongue	Left modified radical
67	F	T2N0 Right Tongue	Right modified radical
67	F	T2N0 Right Tongue	Left modified radical
63	M	T1N0M0 Lip	Right modified radical
46	F	T2N0 Right Tongue	Right modified
46	M	T2N0M0 Left Tongue	Bilateral modified radical
47	M	T2N2aM0 BOT	Left modified radical
54	M	T2N2bM0 Tonsil	Right modified radical
48	M	T1N2C BOT	Right modified radical
51	M	T3N2BM0 BOT	Right modified radical
50	M	T3N2a Left Tonsil	Left modified radical
64	M	T3N2a Left Tonsil	Left modified radical
56	M	T2N0M0 FOM	Right modified radical
67	M	T1N0 Lip	Left modified radical

Note: TNM (Tumor, Node, Metastases) Staging was based on the American Joint Committee on Cancer (AJCC) Criteria

Table 1B. *Group 2: Radiation-Only Salvage Neck Dissections (No PRP/PPP)*

Age	Gender	TNM	Surgical Procedure
58	M	T1N0M0 Lip	Left modified radical
56	M	T3N3M0 Right Piriform	Right modified radical
46	M	T2N2aM0 Tonsil	Left radical
46	M	T2N2aM0 Tonsil	Right modified radical
57	M	T3N2aM0 Right Soft Palate	Right modified radical
61	M	T2N0 Left Tongue	Left radical
59	M	T2N3 L Tonsil	Right radical
46	F	T2N0 Right Tongue	Left radical
60	M	T3N2b Supraglottic	Left modified radical
62	M	TXN2C	Right modified radical
40	M	T3N2 Left Tonsil	Left modified radical
56	M	T2N0M0 FOM	Left modified radical
77	M	T1N1M0 Lip	Right modified radical
56	M	T1N0M0 Tongue	Left modified radical
58	M	T1N0M0 Lip	Right modified radical
28	M	T4N0 L Alveolus/Mandible	Left modified radical

Note: TNM (Tumor, Node, Metastases) Staging was based on the American Joint Committee on Cancer (AJCC) Criteria

Table 2: *Results*

	PRP/PPP Group	Control Group	p-value
Wound Drainage (ml)	253.7	345.8	0.03
Length of Stay (days)	3.13	3.86	0.004
R2 Cutometer	0.856	0.772	< 0.05
F0 Cutometer	12.9	16.0	< 0.05
Follow up (months)	13	21	< 0.05

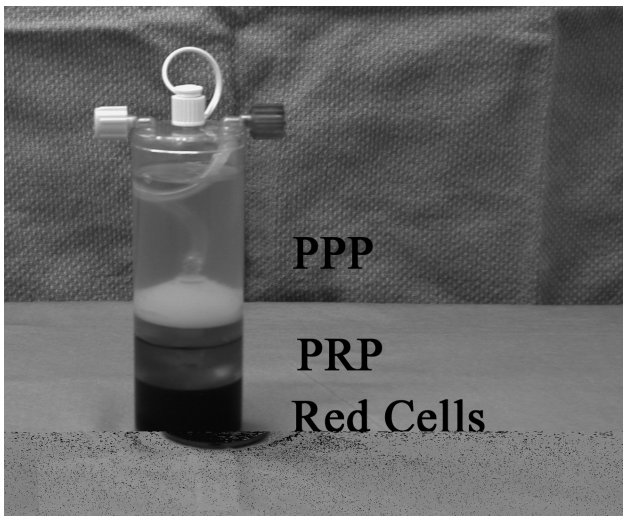

Fig. 1. *Autologous PRP and PPP after centrifugation*

Fig. 2. *PRP and PPP isolated and ready for application*

and therefore the generalizability of reducing length of hospital stay may not be realized by the application of PRP/PPP. No major complications were seen in any of the 30 patients studied and the application of PRP/PPP does not appear to pose any additional risks to the patient relative to the rate or severity of complications. While it is well known that use of autologous products virtually eliminates any risk of infectious disease transmission that may exist a risk with pooled blood products, it would not appear to predispose the patient to other infections at any greater level than that noted in those who did not receive PRP/PPP.

There is now considerable evidence to support that tissue adhesives reduce postoperative drainage in head and neck surgery [3-5]. Application of Tisseel™ into the surgical bed following thyroidectomy and parathyroidectomy was associated with significant reduction in early post-operative drainage. Other benefits included earlier drain removal and reduced length of hospital stay. Similar advantages were seen following parotidectomies and rhytidectomies [6-12]. Yoo et al. conducted the first randomized controlled trial evaluating the effects of APA on post-operative drainage following hemithyroidectomy [19]. Twenty-four hour cumulative drainage was significantly lower in the treatment group compared to controls. Furthermore, there was a trend towards decreased length of hospital stay in the treatment group. This present study however, is the first to demonstrate reduced drainage in the CRT surgical wound.

Quantitative evaluation of soft-tissue fibrosis following surgery is challenging and several instruments have been used in an attempt to quantify skin viscoelastic properties [34,35]. However, the Cutometer remains the most widely accepted measure and has been a validated instrument used to assess the viscoelastic properties of skin in various clinical situations [31,32]. Furthermore, only the Cutometer has been used to quantify changes to skin following RT [33].

The present study is the first to evaluate tissue fibrosis following CRT and surgery. Skin properties were used as the surrogate marker for skin fibrosis and only long-term (>6 months follow up) results were measured. Regarding the changes in the viscoelastic properties of skin as measured by the Cutometer, several interesting findings emerged. Both R2 and F0 measurements in the treatment group suggested better tissue compliance. It should be highlighted that the PRP/PPP group was the CRT group as compared to the RT-only control group. Although the follow up periods were different between groups, the finding of better skin quality in the higher risk PRP/PPP group is compelling. The measured difference in R2 and F0 was approximately 20 percent. Whether these measurable differences translate into significant clinical advantages was not evaluated in this study and thus, remains to be confirmed through additional empiric study. Nevertheless, this present study is the first report in the literature that demonstrated quantitative difference in a long-term tissue tissue fibrosis with the application of PRP/PPP.

This study was not able to determine the respective effects of PRP and PPP on early and late wound outcomes. Furthermore, this study did not evaluate nor define the mechanism of action that accounts for the results seen in this study. It is well understood that APA acts as an

adhesive and tissue sealant, which intuitively may explain the reduction in wound drainage. One can speculate that since both PRP and PPP possess the coagulation factors including fibrin, each would be expected to improve tissue adhesion thereby reducing early wound drainage. However, given our understanding of late effects such as fibrosis, changes seen in the viscoelastic characteristics of the skin may not be explained by early differences of wound drainage amounts or PPP alone. It has also been demonstrated that activated factors contained within platelets are essential for wound healing. Supraphysiologic concentrations of platelets applied to the surgical wound may therefore improve tissue healing. Perhaps late benefits in wound healing may be attributed to the action of platelet. However, a clear delineation of the mechanism has been difficult to prove and was beyond the scope of the present study. Although additional research is required to fully document and validate the present findings, our data provide initial levels of support to the clinical utility of APA in association with soft-tissue surgery of the head and neck.

There are inherent limitations to surgical trials including the difficulty in blinding the surgeon with respect to treatment group. While careful patient selection was employed, this was not a randomized trial and may suffer from selection bias. Although the study was designed to potentially bias against the treatment group, a true randomization methodology may have served to more accurately measure outcomes in the present study. Another limitation to the study is the relatively small sample size. Particularly in ascertaining important differences such as wound infections and hematomas, the lack of events precluded finding any effect.

CONCLUSION

This is the first study that evaluated the early and late effects of APA on tissue healing in the CRT surgical wound. The present data provide initial support that the application of autologous plasma products was associated with measurably better outcomes of wound drainage and skin fibrosis. Whether these differences translate into important clinical benefits remains to be seen through both quantitative and qualitative patient outcomes.

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TREATMENT OF KNEE CHONDROPATHY WITH PLATELET RICH PLASMA. PRELIMINARY RESULTS AT 6 MONTHS OF FOLLOW-UP WITH ONLY ONE INJECTION

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Application of new biological treatments in orthopaedics is controversial nowadays. Surgeons and practitioners know how difficult can be to choose a solution for chondral injuries. Joint damages are from little contusions, osteochondral fractures, avascular necrosis, osteochondritis and degenerative processes like osteoarthritis and rheumatisms. All mentioned have a common problem: the lack of regenerating hyaline cartilage by themselves. Recently, PRP have been used to treat early moderate chondropathies. Here we show the preliminary results of 30 patients affected by chondropathy of the knee after 6 months treated with a single intrarticular injection of PRP. Thirty patients, 18-65 years old, with a diagnosis of I to III Outerbridge chondropathy in the knee, pain for more than 3 months following conservative treatment and no bone axial defect, were treated with one intraarticular injection of PRP (GPS mini set, BIOMET®), after written consent and Ethic and Legal Committee approval. VAS and KOOS scores were evaluated before PRP injection and at 1, 3 and 6 months after the treatment. ANOVA with repeated measures using the SPSS® showed significantly better results in term of KOOS and VAS scores at 1, 3 and 6 months respect to the pre-injection value (p<0,05) We think that PRP treatment is a promising alternative for the treatment of knee chondropathy; however its efficacy has to be demonstrated with more clinical works, with longer follow up and with greater number of patients, even with controlled and randomized trials. In our study only one injection of PRP has been able to allow a clinical improvement, suggesting the possibility to avoid multiple injections protocols, and consequently reducing the health expenses. Until the efficacy of PRP will not be definitely demonstrated, surgeons should be very prudent in indications.

Hyaline cartilage is a specific form of connective tissue, formed by cells (chondrocytes), and an extracellular matrix very organized, which include collagen type II, proteoglycans (chondroitin 4-6 sulphate, keratan sulphate), glycoproteins and water (fig.1). Up to 80% of the weight of normal cartilage is water (1, 2). Mechanical properties of articular cartilage are explained by the interaction between solid and liquid structures: when an axial force is applied against the articular surface, water flows out the matrix in order to absorb the stress; if the load is continuous, then solid phase must bear the stress, and subsequently begins to change its structure, producing damage (3). (Figs. 2, 3).

Outerbridge, in 1961, described a classification to understand the consequences of articular cartilage injuries, based on operative findings for patellar chondromalacia (4). The lack of this classification is that there is not included the depth and location of the injury, but it is still the most widely used. Noyes and Stabler (5), in 1989, established their classification including those factors, based on arthroscopic findings. Standard tissue repair after an injury begins immediately with cell necrosis, followed by inflammation, proliferation and cell regeneration. Vascular supplementation is mandatory to occur in this process (6).

Articular damage can derive from a direct impact or repetitive trauma, producing in most cases, a cartilage flap which microscopically corresponds to a chondrocyte necrosis and a disruption of the matrix. Because cartilage is avascular, normal process of repairing is not possible, except if there is a connection of the flap with subchondral bone, which undergoes a bleeding that promotes the

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presence of growth factors (PGDF, *platelet derived growth factor,* and TGF-β, *transforming growth factor beta*) (2), necessary for tissue repair, although the scar is not sufficient in time and space. Moreover, primary response of chondrocytes is ineffective and short lived. Small superficial lesions remain stable over time, and do not progress to a degenerative arthritis. Larger ones, cause joint effusion with mechanical symptoms and have the potential to finish in a degenerative arthritis (7).

Cartilage injuries are a challenge when diagnosis is performed. Joint effusion is not always present, pain is usually related with movement, maybe a point of tenderness is present, and is frequent to associate it to a chronic anterior cruciate instability (8). It is important to add that when treating a patient arthroscopically for another reason, usually a meniscus tear, surgeon diagnoses at the same time of surgery a cartilage damage which initially was not seen in magnetic resonance films. New specialized MRI using fast spin echo sequences has been shown to have enhanced accuracy for articular injuries and may allow preoperative diagnosis (9).

Little flaps and Outerbridge grade I should be left without performing maneouvers. Laser and thermal chondroplasty improve the surface appearance, but they have not shown to stimulate healing. For full thickness injuries, is mandatory to distinguish osteochondritis dissecans (OCD) and focal chondral defects. In OCD lesions, it is very difficult to decide the ideal treatment. Size and location of the injury should be helpful to determine which treatment is the best. In some cases performing treatments indicated for focal chondral defects could be the best option (5). For symptomatic focal full thickness chondral defects, marrow stimulation technique is still the most common approach. These arthroscopic procedures are generally easy and inexpensive. In the recent years, Pridie drilling has been increasingly replaced by the microfracture technique according to Steadman (10). Both these approaches rely on the same biological principles of promoting resurfacing with the formation of fibro-cartilaginous repair tissue. For the treatment of smaller cartilage defects (<2.5 cm), microfracture still remains the first choice treatment. The clinical results after microfracture of the knee are age dependent and seem to be not much durable: younger and active patients (<40 years) with smaller isolated traumatic lesions on the femoral condyles have the best long-term results; the decline of the clinical results begins after 18 months and is significantly more pronounced in older patients with defects on the patella-femoral joint and tibia (5). Lower quality of the repair tissue, partially incomplete defect filling and calcified cartilage formation in the defect area seem to be the major limitations of this method. The AMIC (Autologous Matrix Induced Chondrogenesis) technique was developed to enable treatment of larger defects by the application of a collagen type I/III matrix, in particular when cell-engaged procedures such as autologous chondrocyte implantation (ACI) cannot be used for economical reasons or because not indicated. AMIC seems to be particularly suitable for treating retro patellar cartilage, where microfractures alone are not sufficient. The results of the ongoing studies are awaited to establish whether better results with this technology are achievable in the long term (11, 12, 13).

Tissue grafting techniques include mosaicplasty, periosteal and perichondrial grafting, autologous chondrocytes implantation (ACI) and allograft tissue grafting. Mosaicplasty is well indicated in injuries less than 2,5 cm in size, with the actual arthroscopic sets available for performing it. For larger defects, one should suggest a periosteal and perichondrial grafting, due to the presence of mesenchymal cells to differentiate to chondrocytes, but this technique is very meticulous for harvest and fixation, and good outcome is only evident in young patients (5, 14). ACI technique is meticulous, expensive and surgical demanding procedure; it needs a careful patient selection and surgeon experience. Nevertheless good outcomes have been reported, better if ACI was performed as a primary treatment, not as a secondary or rescue technique (15). Use of synthetic scaffolds is a potentially attractive alternative to traditional cartilage procedures as they are readily available and, unlike allogenic tissue transplants, are associated with no risk of disease transmission. Their efficacy, however, has not been definitely proven clinically (16).

Surgeons and practitioners know how difficult can be to choose a solution for chondral injuries. When considering treatment, one must have in mind factors such as size, location, age, activity level and etiology. One of the main problems is how to treat all of these patients who suffer a mild or moderate chondropathy, mostly when surgery has been performed with the techniques described above and symptoms remain present. Reduction of weight bearing, anti-inflammatory drugs, physiotherapy and viscosupplementation are among the common conservative the treatments which can be recommended, but none of them have shown to be completely effective. Moreover, actual social pressure obliges us to offer other possibilities, but lack of protocols, well designed clinical studies and medical evidence make us to investigate the successful of these new treatments. Platelet rich plasma (PRP) intraarticular injections are suggested as a supplement of the main treatment in chondral defects after surgery when painful situation is still present. Only in patients with low degrees of chondropathy, PRP could be injected as a primary treatment if surgeon is sure that the outcome would be successful, but this situation

is a challenge. Lack of literature about this method in cartilage, lack of protocols, health social system pressure to shorten the notes of sick, and sensibility for the treatment expenses made us to investigate the use of PRP to improve pain and function in chondropathies of the knee, in which other treatments failed. Knowing that PRP is defined as the concentration of 1,1 million platelets/ µl or greater, and there is a release of growth factors derived from alpha granules, it is interesting to notice the importance to enhance the homeostasis and the potential reparative effect in cartilage injuries, but understanding their effects not only in hyaline cartilage, but also in meniscus, tendons, ligaments, synovial lining and any exposed subchondral bone as well as on mesenchymal stem cells that gain access to the joint. In fact, Fortier et al (17), in their study about the effect of growth factors in cartilage repair, shows the TGF superfamily (including TGF β1, bone morphogenetic protein BMP-2 and BMP-7), insulin growth factor IGF I, fibroblast growth factor FGF-2 /FGF-18 and PDGF, as the main proteins which have the major potential in cartilage repair, being BMP-7 (also called osteogenic protein 1) the gold standard growth factor with the ability to both decrease catabolic cytokines (interleukins and matrix metalloproteinases) and stimulate cartilage matrix synthesis. Chondrocytes and mesenchymal stem cells exposed to PRP are able to increase cell proliferation and cartilage extracellular matrix synthesis of proteoglycans and type II collagen compared with controls (18). Fukumoto et al found that IGF-I has a synergistic effect with TGF- β in promoting mesenchymal cell chondrogenesis (19).

Here we show the preliminary results in a sample of 30 patients affected by chondropathy of the knee after 6 months from a single intrarticular injection of PRP.

MATERIAL AND METHODS

Patients' inclusion criteria were: grade I to III chondropathy according to Outerbridge (by image or arthroscopy), age between 18-65 years old, pain for more than 3 months, patients not responsive to other treatments. Exclusion criteria were: bone axial defects and/or limb length differences, use of non-steroidal anti-inflammatory drugs (NSAID) in the last 15 days, viscosupplementation, cortisone injections and/or surgery in the last three months, local infection , concomitant oncology disease, known platelets disease , history of anemia, pregnancy, breastfeeding, patient under treatment of mental health, patients with difficulty for follow up. Every patient was clinically examined and body mass index (BMI) calculated; then the KOOS (*Knee Injury and Osteoarthritis Outcome Score*) and VAS (*Visual Analogical Score*) were self administrated, and a specific written consent for acceptance of PRP treatment was signed by the patient and surgeon; one intraarticular injection of PRP was planned. We use the GPS Mini system (BIOMET®, Warsaw, IN) always prepared by the same nurse, without activation of platelets. In every patient 27 ml of venous blood was harvested and mixed with 3 ml citrate, then centrifuged with the disposable kit (3,200 rpm/15 min); the PRP was obtained by aspiration of the exclusive layer; immediately the PRP was injected in the surgical room under aseptic and antiseptic conditions into the joint, with the knee in extension.. No anaesthetic drugs were administered during the injection neither NSAID were prescribed after it. No specific physiotherapy was followed by the patients, who were suggested to avoid exercises with direct impact against the knee or with maximal bending. Clinical examinations were performed at 1, 3, and 6 months after the PRP injection; every time KOOS and VAS scores were calculated. Secondary effects were registered too.

RESULTS

Descriptive analysis is showed in tables I and II. 22 men and 8 women were included in the study, the mean age 44 years (range 19-62) and the mean BMI 27 (range 22-36). 3 patients were affected by grade I Outerbridge, 15 by grade II and 12 by grade III. No adverse events were reported after the injection of PRP. ANOVA with repeated measures using the SPSS® (Statistical Package for the Social Sciences) showed a statistically significant effect of time on KOOS and VAS scores from the pre-injection situation to 1,3 and 6 months follow-up ($p<0,05$) .(Tables III-VI).

DISCUSSION

Application of new biological treatments in orthopaedics is controversial nowadays, although in animal models positive results in the treatment of cartilage have been demonstrated. Sustained release PRP injections have also been found to improve cartilage matrix metabolism, which suggests its use in osteoarthritis management (20). Intraarticular injections of PRP have been studied in osteoarthritis model; it has been found an enhancing of hyaluronic secretion, being useful in joint homeostasis (21). Other authors (22) have described the use of plasma rich in growth factors to treat chondral defects in athletes and reported good outcomes, demonstrating that this plasma is effective for the functional recovery of articular cartilage, and concluding that local treatment is safe. Recently, clinical review about studies in cartilage (23) has noted no randomized controlled trials, but only an observational retrospective study which used hyaluronic as a control; investigators used serial PRP injections with modest outcomes. It has been reported a better effect of PRP intraarticular injections when compared to viscosupplementation (24). In their work Kon et al have reported 115 cases diagnosed of knee osteoarthritis treated with four articular injections of PRP every three weeks, with promising results, especially for young patients with

Table I. *Descriptive statistical data. BMI: body mass index.*

	Age	BMI	OUTERBRIDGE
Mean	44,63	27,40	2,30
Std. deviation	10,759	3,37	,651
Variance	115,757	11,352	,424
Asymmetry	-,243	,613	-,385
Kurtosis	-,385	,110	-,609
Minimum	19	22	1
Maximum	62	36	3

Table II. *Descriptive statistical data. 1, 3 and 6 = 1, 3 and 6 months. PRE: score pre-injection of PRP.*

	KOOS PRE	KOOS 1	KOOS 3	KOOS 6	VAS PRE	VAS 1	VAS 3	VAS 6
Mean	70,05	80,23	85,56	85,16	6,53	4,63	3,37	2,77
Std. deviation	11,23	10,42	10,25	13,88	1,43	1,65	1,90	2,19
Variance	126,80	108,60	105,11	192,89	2,05	2,72	3,62	4,80
Asymmetry	-,529	-,586	-,835	-1,564	,79	,439	,333	,803
Kurtosis	-,886	-,167	,134	2,415	-,809	-,997	,486	-,235
Minimum	48,50	54	62	41	4	2	0	0
Maximum	85	95,50	100	100	9	8	8	8

low BMI, males and milder degrees of chondropathy (25). Another clinical study has suggested PRP as a possibility of treatment in primary and secondary osteoarthritis but with a small sample of patients receiving three doses of PRP (26).

After the literature analysis, several open questions still persist: 1) the lack of blinded, controlled, randomized studies about effectiveness of PRP in knee chondropathy. In order to reach a good level of evidence, it would be necessary to perform a well-designed study, with a large number of enrolled patients. 2) Efficacy of serial injections. We are agree with Nguyen et al (23), who has suggested in their clinical review, that they have not found to be necessary serial injections of PRP at intervals of 2-4 weeks. Tissue healing is in an active state for at least 6 weeks, and remodelling occurs over months. Because there are not protocols about this question, we have established to treat our patients with a single injection; patient is informed about the possibility of a second injection, but only if is justified by a worsening or a non expected relieving of symptoms over three to six months. Every decision has to be always individualized. In fact, Peerbooms et al (28) have already published satisfactory results with only one PRP injection for chronic lateral epicondylitis over 6-12 months. As our preliminary results suggests, treatment of chronic chondropathy with only one intraarticular injection at 6 months follow-up allow obtaining a good outcome, with pain relief and a progressive functional improvement of the joint. Definitive results over one year follow-up are necessary to understand if a second injection is indicated or if there is a worsening which should justify it. 3) There is not

Table III. *ANOVA with repeated measures for KOOS scores with a 95% confidence interval.*

Times	Times	p
PRE	1 month	,000
	3 months	,000
	6 months	,000
1 month	PRE	,000
	3 months	,001
	6 months	,099
3 months	PRE	,000
	1 months	,001
	6 months	1,000
6 months	PRE	,000
	1 month	,099
	3 months	1,000

sufficient evidence with the actual literature to decide if insurance companies (public or private) should cover this therapy. Different costs between various disposable sets of PRP are dependent on the distributing company, size of the kit and diverse number of elements inside the sterile boxes which sometimes are not necessary; therefore medical fees can increase the final cost substantially, even if at the moment lack of medical protocols and unified criteria are not present for the use of PRP. However, PRP injections may be a good alternative when compared to surgical intervention if cost effectiveness is studied. 4) Rehabilitation program after PRP injection. There is not any shared physiotherapy protocol described after PRP injections, so we inform the patient about the importance to avoid direct impacts (i.e. when running, kneeling or jumping) and maximal bending positioning. We encourage to practice exercises actively such as cycling, elliptic, walking inside a swimming pool without resistance. In case the patients are enrolled in a study, we of course ask them avoid ultrasound or magneto therapy, in order to not affect the final evaluation. 5) Acute or chronic situation. We usually treat patients with a clinical situation over three months which has not results with standard therapies such as NSAID or physiotherapy programs. We believe that not all acute injuries need to be managed with PRP. In acute condition (i.e. after an accident with a bone contusion in MRI) we strongly suggest standard therapy. The situation is different when we have to deal with professional or semi-professional sportsmen/women. Social and team pressure encourages us to treat acute conditions with PRP, and although there is not scientific evidence to do it routinely, we have recently treated several osteochondral contusions without fracture with a PRP injection with good results. However each case needs to be study singularly before deciding the treatment. 6) Level of pain. Everybody knows how much difficult is

Table IV. *Graphic showing the estimated means evolution of KOOS scores. Numbers 1 to 4 are the references to the times of follow up (1: pre- injection; 2: 1 month; 3: 3 months;4 : 6 months).*

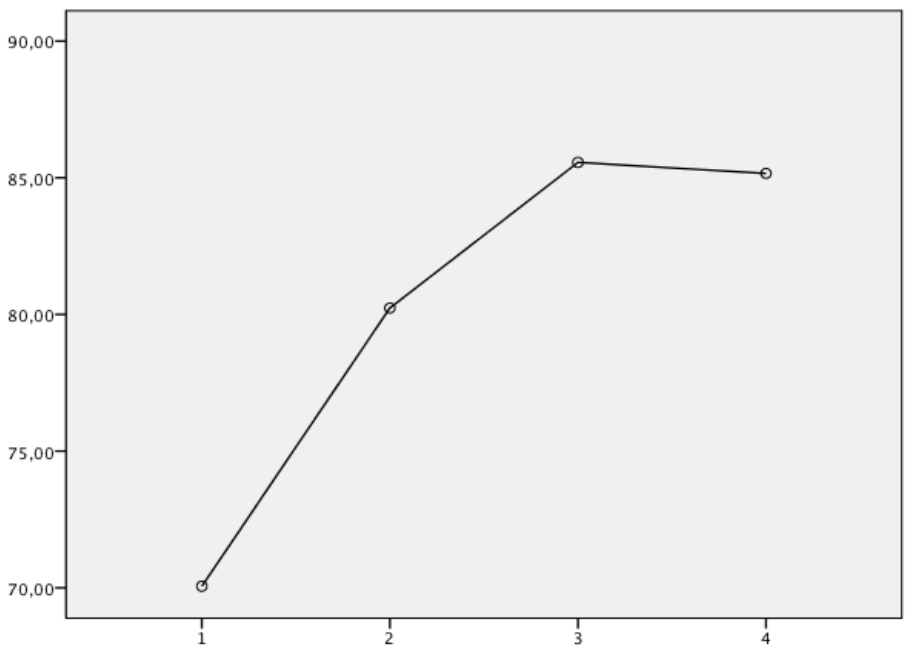

Table V. *ANOVA with repeated measures for VAS scores with a 95% confidence interval.*

Times	Times	p
PRE	1 month	,000
	3 months	,000
	6 months	,000
1 month	PRE	,000
	3 months	,000
	6 months	,000
3 months	PRE	,000
	1 months	,000
	6 months	,252
6 months	PRE	,000
	1 month	,000
	3 months	,252

to determine the level of pain in each patient. This is the reason for using more complex scales and scores to validate objectively when would be necessary to treat a patient with PRP. Although VAS score is more subjective, we usually reserve PRP treatment to patients with a VAS scores higher than 4. KOOS and WOMAC scores are more objective, and in our study a KOOS score of 85 was the limit to indicate it to as a case deserving this kind of solution. 7) Use of NSAIDs and modulation of pain. It seems that use of NSAIDs can alter the effect of growth factors due to the related inhibition of prostaglandin necessary in the inflammation phase. Therefore, if pain is high, we advise patients to be free to take NSAIDs until 10 days before the injection of PRP and at least after 3 weeks from the treatment. Paracetamol is an alternative as pain killer which can be always used. Moreover, release of serotonin has been proposed as a modulator of pain, which is present in the dense granules of platelets. However, given the short duration of release and the short half life of serotonin, this effect is unlikely sustained (23). 8) Activation of platelets. We do not activate platelets when PRP is injected inside a joint, because it is well known that the main injection promotes a bleeding which releases thrombin, necessary for the process of activation, and therefore when platelets meet collagen, this process is naturally activated. 9) Quantity of PRP injected. With the kit we used in our study, 4 ml of PRP are obtained. The knee is sufficiently wide to receive this volume. However, if a little joint is treated (i.e. articular vertebral joints), one should be aware of a possible intraarticular overpressure.

CONCLUSIONS

Our opinion about the application of PRP to treat cartilage injuries is based on the actual experience and clinical evidence, although more clinical scientific studies should be performed to determine the indications and protocols; at the moment, our preliminary results show a progressive improvement of pain and functionality with only one articular injection of PRP when treating chondral injuries in the knee, compared to actual literature which is based in serial injections of PRP. The main limitation of our

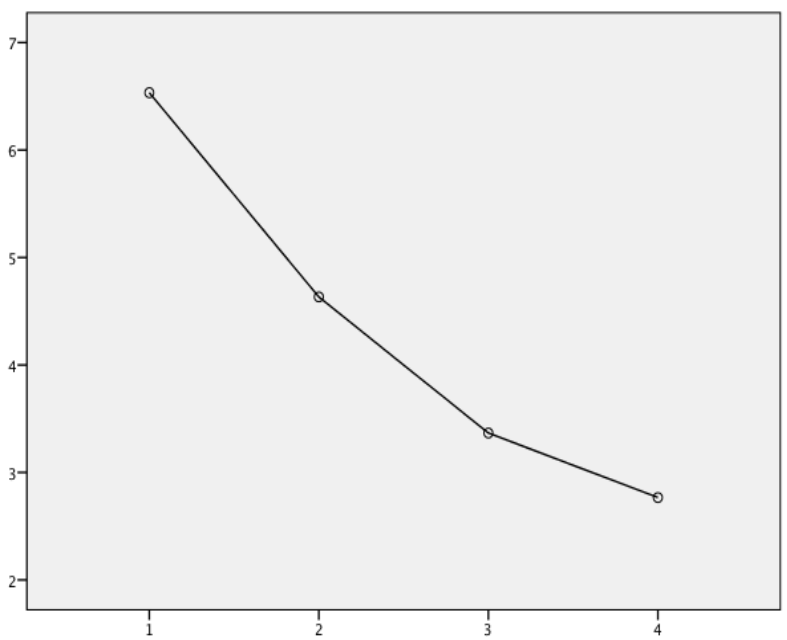

Table VI. *Graphic showing the estimated means evolution of VAS scores. Numbers 1 to 4 are the references to the times of follow up (1: pre- injection; 2: 1 month; 3: 3 months;4 : 6 months).*

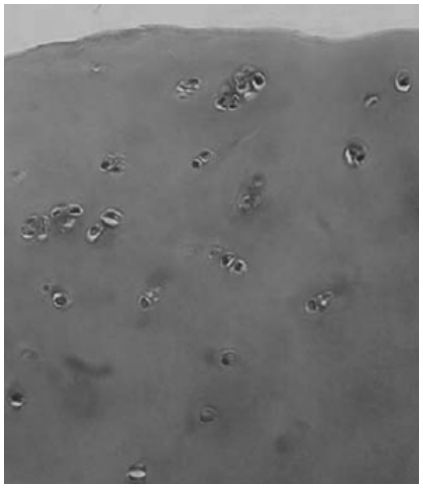

Fig.1. *Area of hyaline cartilage showing chondrocytes inside their typical lagoons. Haematoxylin eosin 100X.*

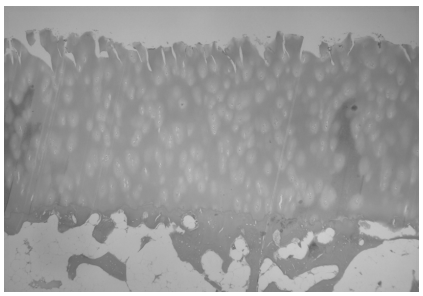

Fig. 2. *Degenerative surface of hyaline cartilage with fragmentation. Haematoxylin eosin 40X.*

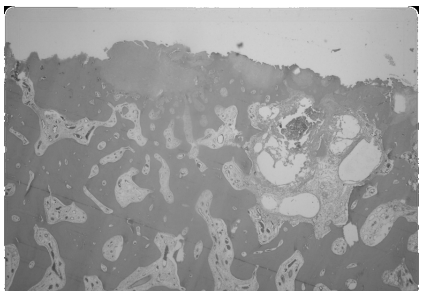

Fig. 3. *Osteoarthritis. Note the lack of cartilage surface and bone cysts. Haematoxylin eosin 10X.*

study lays on the lack of a control, and the consequent lack of randomization and blinded evaluation. However, there is already literature which has compared viscosupplementation with PRP and better results have been found for the last one. Therefore, we encourage to our colleagues to propose PRP as an alternative when standard treatments have failed, but it is important to follow with researching and publishing as soon as possible and to consider multicenter studies in order to set up effectiveness of PRP. It is important to determine the grade of chondropathy and to differentiate from patients affected by advanced osteoarthritis with potential indication for joint replacement since we believe that PRP should not be indicated for all patients. Regarding the economical aspect, we have not found literature about codes for PRP injection in Europe; in our environment, actual public health care insurance covers 75% of the procedures, and we have proposed to perform the technique with one PRP injection for treating cartilage, tendons, muscle and ligaments injuries (including harvesting, preparation, surgeon fees and image guidance when indicated) in a specific area to avoid unnecessary costs (like a minor surgical process), and after previous study, good results have been obtained. Furthermore, if protocol with only one injection is added, final billing could be more interesting, even when patient should pay it without reimbursements. Maybe our definitive results should explain us some of the questions described above.

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