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ANTIMICROBIAL PROPERTIES OF PLATELET-RICH PLASMA

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ABSTRACT

Autologous platelet concentrates have been extensively used in a variety of medical fields to promote hard and soft tissue regeneration. The significance behind their use lies in the abundance of growth factors in platelets α-granules that promotes wound healing. In addition, antibacterial properties of platelet concentrates have been recently pointed out.

In this study, the antimicrobial effect of pure platelet-rich plasma (P-PRP) was evaluated against microorganisms isolated from oral cavity, such as *Candida albicans, Enterococcus faecalis, Pseudomonas aeruginosa, Streptococcus agalactiae* and *Streptococcus oralis*. The effect of the platelet concentration, of the $CaCl_2$ activation step and of the presence of plasmatic components on the antimicrobial activity of P-PRP was also investigated.

Blood samples were obtained from healthy donors. The antibacterial activity of P-PRP, evaluated as the minimum inhibitory concentration, was determined through the microdilution two-fold serial method.

Results showed that P-PRP was able to inhibit the growth of *E. faecalis, C. albicans, S. agalactiae* and *S. oralis*, but not of *P. aeruginosa* strains. The antimicrobial activity of P-PRP seemed to be sustained by a co-operation of plasma components and platelet-derived factors. Moreover, the activation of coagulation with $CaCl_2$ appeared to represent a fundamental step for the antibacterial activity of P-PRP.

The antimicrobial properties of P-PRP might represent a valuable adjunct to the enhancement of tissue regeneration.

1. INTRODUCTION

1.1. Platelets

Besides red blood cells, platelets are the second anuclear blood compartment. They play a crucial role in primary hemostasis, as well as in a variety of other physiological processes. To fulfill these functions, they contain a variety of mediators, which are stored in intracellular vesicles or granules. In this chapter, the biology and function of platelets will be discussed in detail.

1.1.1. Platelet biology

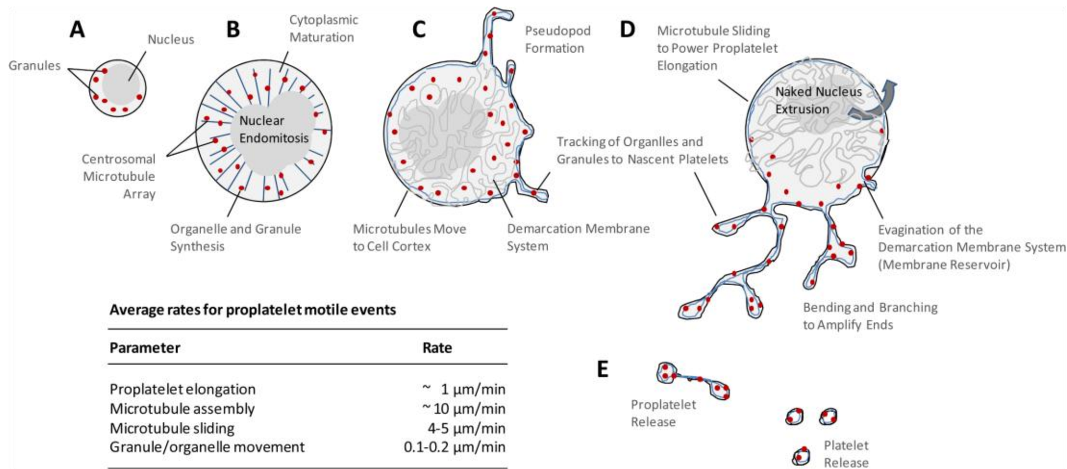
Platelets are small anucleate cell fragments that originate from megakaryocytes in the bone marrow. Megakaryocyte and platelet production is regulated by thrombopoietin, a hormone produced mainly by liver and kidneys. Each megakaryocyte gives rise to 5.000-10.000 platelets. An average healthy adult produces around 10^{11} platelets per day. The physiological platelet concentration in whole blood is 150.000-400.000/µl. If the number of platelets is too low, excessive bleeding can occur; if the number of platelets is too high, blood clots can form and obstruct vessels. Reserve platelets are stored in the spleen and released when needed by sympathetically-induced splenic contraction. The lifespan of circulating platelets is 5-9 days. Old platelets are destroyed by phagocytosis in the spleen and in the liver.

1.1.1.1. Platelet formation

Platelets originate from the cytoplasm of megakaryocytes, their precursor

cells, which reside in the bone marrow.(1) Megakaryocytes are the largest (50–100 μm) and one of the rarest cells in the bone marrow; they account for about 0,01% of nucleated bone marrow cells.(2) They are highly specialized precursor cells that function solely to produce and release platelets into the circulation. To assemble and release platelets, megakaryocytes become polyploid by endomitosis (DNA replication without cell division) and then undergo a maturation process in which the bulk of their cytoplasm is packaged into multiple long processes called proplatelets, and the nucleus is extruded. A megakaryocyte may extend 10–20 proplatelets, each starting as a blunt protrusion that over time elongates, thins, and branches repeatedly. Platelets form selectively at the tips of proplatelets.(3) As platelets develop, they receive their granule and organelle content as streams of individual particles transported from the megakaryocyte cell body.(4) Platelet formation can be arbitrarily divided into two phases. The first phase of megakaryocyte maturation and development takes days to complete and requires megakaryocyte-specific growth factors. During this time, massive nuclear proliferation and enlargement of the megakaryocyte cytoplasm occur as the cell is filled with cytoskeletal proteins, platelet-specific granules and sufficient membrane to complete the platelet assembly process. The second phase is relatively rapid and can be completed within hours. During this phase, megakaryocytes generate platelets by remodeling their cytoplasm first into proplatelets and then into preplatelets, which undergo subsequent fission events to generate discoid platelets (Figure 1). The time required for megakaryocytes to complete polyploidization, mature, and release platelets

is about 5 days in humans.(5-7)



Parameter	Rate
Proplatelet elongation	~ 1 μm/min
Microtubule assembly	~ 10 μm/min
Microtubule sliding	4-5 μm/min
Granule/organelle movement	0.1-0.2 μm/min

Figure 1. Megakaryocytes transition from immature cells (A) to released platelets (E). From *Thon and Italiano, Semin Hematol 2010; 47:220–226.*

1.1.1.2. Platelet structure

Platelets are small anucleate cell fragments that have a characteristic discoid shape and range from 1 to 3 μm in diameter (Figure 2). The platelet cell can be divided into a) a peripheral zone (glycocalyx, plasma membrane, submembrane area), b) a sol-gel zone (microtubules, microfilaments, glycogen, smooth and coated vesicles), c) an organelle zone (α-granules, dense bodies, electron-dense chains and clusters, lysosomes, glycosomes, tubular inclusions, mitochondria) and d) membrane systems (Golgi complexes, surface-connected open canalicular system, dense tubular system, membrane complexes).

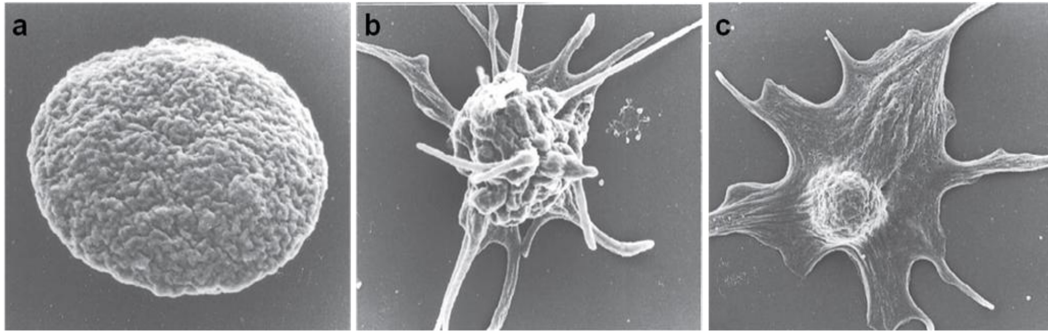


Figure 2. Discoid platelet (a), dendritic platelet (b) and spread platelet (c) photographed in the low-voltage, high-resolution scanning electron microscope. Magnification 30.000X, 13.000X and 11.000X. From Michelson, *Platelets (Second Edition) 2006; p 46.*

a) Peripheral zone

Platelet plasma membrane has a thick external coat called glycocalyx. This coating consists of the carbohydrate moieties of membrane glycolipids and glycoproteins. Glycoproteins play a role in platelet adhesion to damaged surfaces, platelet activation, aggregation and interaction with other cells.(8, 9) The main glycoproteins involved in hemostasis are the glycoprotein (GP) Ib-IX-V complex and the integrin αIIbβ3 (GP IIb-IIIa complex) (Figure 3).

The phospolipid bilayer of the plasma membrane is morphologically similar to that of the membranes covering other cells.

The area lying just below the plasma membrane is important to platelet function. Here the cytoplasmic domains of transmembrane receptors interact with proteins regulating the signaling processes of platelet activation. Many of these proteins are associated with calmodulin, myosin and short actin filaments making up the membrane contractile cytoskeleton.(10, 11) The

contractile system of this area is involved in platelet shape changes and in the translocation of receptor complexes to the platelet outer surface, both critical processes for cellular adhesion and spreading at sites of vascular injury.

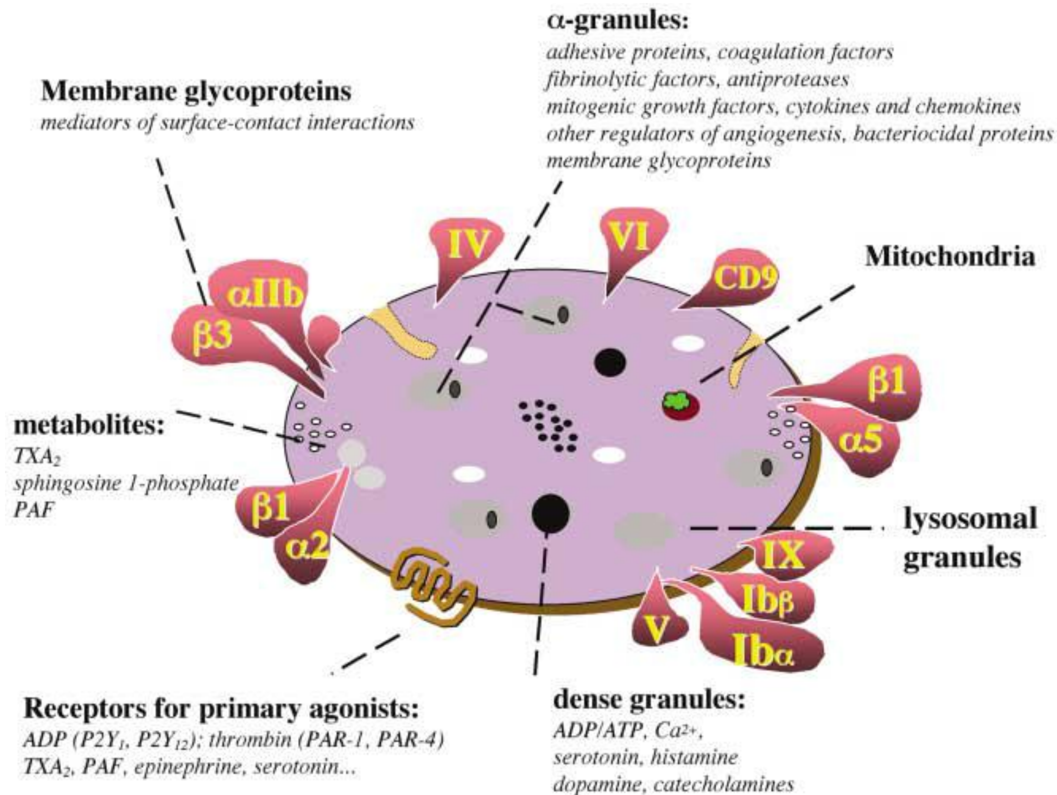


Figure 3. Schematic representation of platelet structure. From *Anitua et al., Thromb Haemost 2004;91:4–15.*

b) Sol-gel zone

In addition to the subsurface membrane contractile filament system discussed earlier, two other filament systems are present in platelet cytoplasm. One is the circumferential coil of microtubules, which is the major cytoskeletal support system responsible for maintaining the discoid shape of

platelets.(12, 13) The other is the actomyosin filament system, that serves as the matrix on which organelles and other structural components are suspended and maintained separate from each other and the cell wall in resting cells.(14) Following platelet activation, the cytoplasmic actomyosin cytoskeleton constricts the circumferential coil of microtubules and drives α-granules and dense bodies into close association in platelet centers.(15) If stimulation is strong enough, α-granules and dense body contents are secreted to the exterior of the cell via channels of the surface-connected open canalicular system (OCS).(14, 16) The cytoplasmic actomyosin cytoskeleton is also involved in shape changes, hemostatic plug contraction and clot retraction.

Glycogen particles and smooth and coated vesicles are randomly distributed in platelet cytoplasm.(17-19) Their origin and function are unknown.

c) Organelle zone

Platelets contain three major types of secretory organelles: α-granules, dense bodies and lysosomes. Occasional multivesicular bodies (MVBs) are also present. MVBs develop in megakaryocytes by the fusion of small vesicles budding from the trans-Golgi network (TGN) and serve as sorting stations in the development of α granules, dense bodies, and lysosomes.(20)

Among platelet granules, α-granules are the most abundant. There are approximately 50-80 α-granules per platelet, ranging in size from 200 to 500 nm.(21) The development of α-granules takes place in megakaryocytes.(22) α-granules cargo derives from budding of the TGN and from endocytosis of

the plasma membrane, both clathrin-mediated processes; pinocytosis of cargo can also occur. Vesicles are subsequently delivered to MBVs, where sorting occurs. MVBs then mature to produce α-granules (Figure 4a).(17, 23, 24) During platelet formation, α-granules are transported along microtubules from the megakaryocyte cell body through proplatelets (Figure 4b). Platelets form as bulges along the length of these extensions and α-granules are maintained in the nascent platelets by coiled microtubules.(25, 26) α-granule content is released when the vescicle membrane fuses with surface-connected membranes of the OCS or with the plasma membrane.(27) SNAREs represent the core of the fusion machinery. Coiled-coil domains within vesicle (v-SNAREs) and target SNAREs (t-SNAREs) interact, forming a twisted 4-helical bundle, bringing the opposing membranes of granule and cell into close apposition. Binding of vSNAREs and tSNAREs generates energy required for membrane fusion. Pore formation with release of granule contents subsequently ensues (Figure 4c).(28, 29) α-granules appear to be heterogeneous with regard to cargo.(30, 31) Hundreds of bioactive proteins, playing roles in various biological functions, are released from α-granules. Such molecules will be discussed in detail later.

Dense bodies are smaller and fewer than α-granules.(32) They are rich in ADP, ATP, Ca^{2+}, serotonin and histamine. ADP promotes platelet aggregation while ATP participates in platelet response to collagen. Ca^{2+} is a necessary cofactor for platelet aggregation and fibrin formation. Serotonin release leads to vasoconstriction and increased capillary permeability. Histamine can have pro- and anti-inflammatory effects.(33)

Platelets also contain few lysosomes, which can secrete acid hydrolases, cathepsins D and E, elastase and other degradative enzymes.(34)

Small numbers of relatively simple mitochondria are present in the platelet cytoplasm. They serve an important role in energy metabolism.(35) Other membrane-enclosed organelles or structures are also present in the cell, such as glycosomes, electron-dense chains and clusters(36), and tubular inclusions.(19, 36, 37) Their origin and function is unknown.

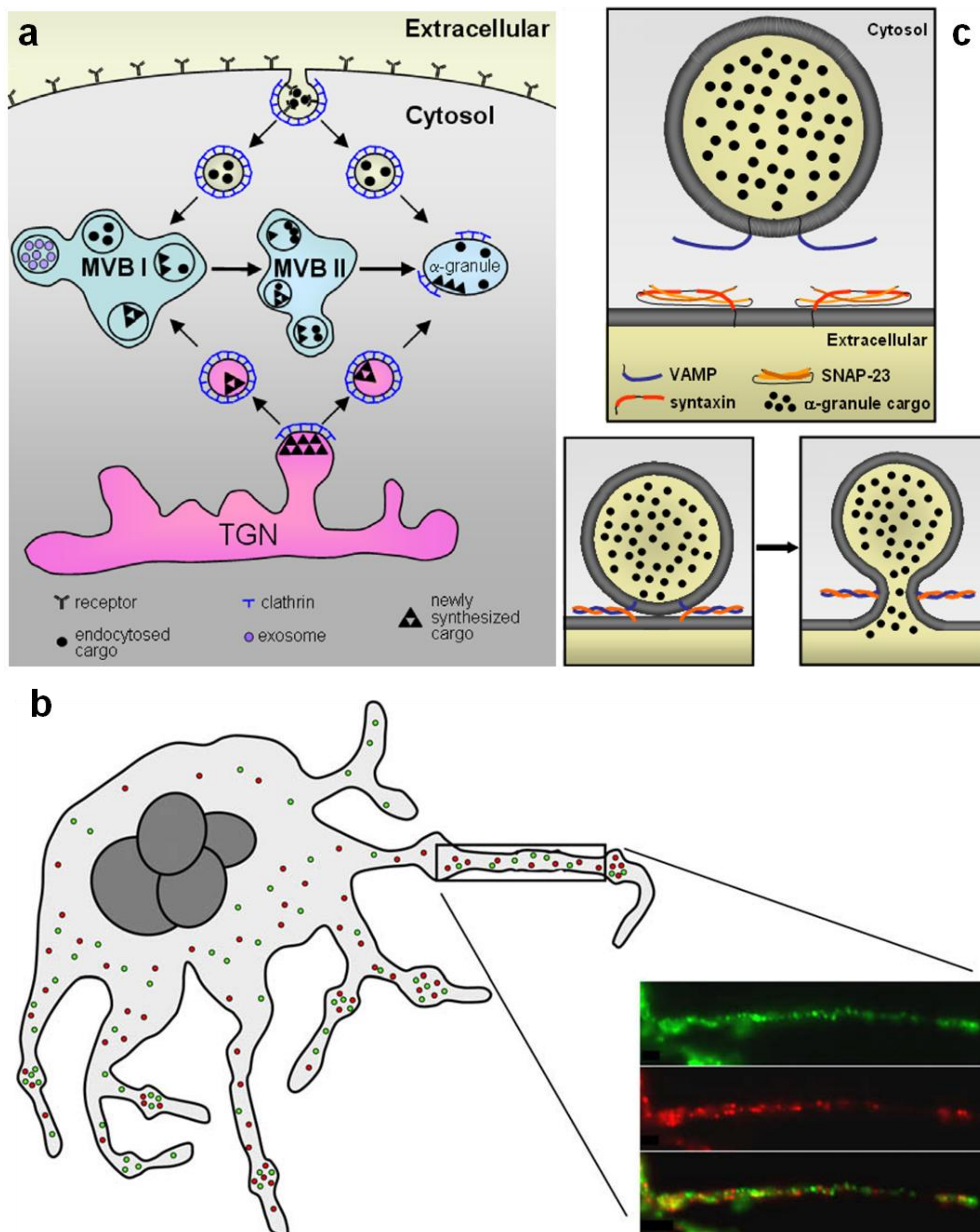


Figure 4. α-granules formation in megakaryocytes (a), transport during proplatelet formation (b) and degranulation in mature platelets (c). Adapted from *Blair & Flaumenhaft, Blood Reviews 2009;23:177–189.*

d) Platelet membrane system

Golgi complexes are membrane systems ordinarily confined to

megakaryocytes. However, residual membrane elements of the Golgi apparatus, consisting of a few parallel-associated, flattened sacules with no budding vesicles, are found in less than 1% of circulating platelets.(38)

The OCS is part of the platelet surface membrane.(39-41) OCS channels are tortuous invaginations of surface membrane tunneling through the cytoplasm in a serpentine manner. Channels of the OCS greatly expand the total surface area of the platelet exposed to circulating plasma and provide a means for chemical and particulate substances to reach the deepest recesses of the cell.(42, 43) As a result, the OCS may be the major route for the uptake and transfer of products from plasma granules and serve as conduit for the discharge of products stored in secretory organelles.(44-46) Channels of the OCS have also major roles in the hemostatic reaction. After adhesion to a damaged vascular surface, the platelet extends filopodia to bind firmly to the injured area. This is followed rapidly by the assembly of cytoplasmic actin filaments and by the spreading of the platelet to cover as much area as possible. The result is an increase of up to 420% of the exposed surface area during conversion from a discoid platelet to a fully spread cell. Evaginated channels of the OCS are the major source of membrane for the expanded surface area of the spread platelet.(47)

Channels of the dense tubular system (DTS) are randomly dispersed in platelet cytoplasm.(39) They represent residual smooth endoplasmic reticulum of the parent cell.

OCS and DTS are not completely isolated membrane systems. Canaliculi of the OCS and DTS form intimate physical relationships in nearly every

cell.(48)

The rarest of the membrane systems found in circulating platelets is rough endoplasmic reticulum (RER). The RER in the parent megakaryocyte has disappeared by the time granulopoiesis has been completed and before proplatelets are formed. Platelets RER channels are usually in parallel association. Ribosomal complexes are also present in cytoplasm adjacent to the RER.(49)

1.1.2. Functional roles

Platelets are known for their role in hemostasis, where they help prevent blood loss at sites of vascular injury. Yet, increasing evidence indicates that platelets fulfill a much wider role in health and disease. In fact, platelets are also involved in the modulation of innate immune response, inflammation, angiogenesis, wound healing and in the pathogenesis of various diseases (e.g. atherosclerosis, Alzheimer’s disease and cancer) (Figure 5). The role played by platelets in hemostasis, wound healing and antimicrobial host defense will be discussed in the following paragraphs.

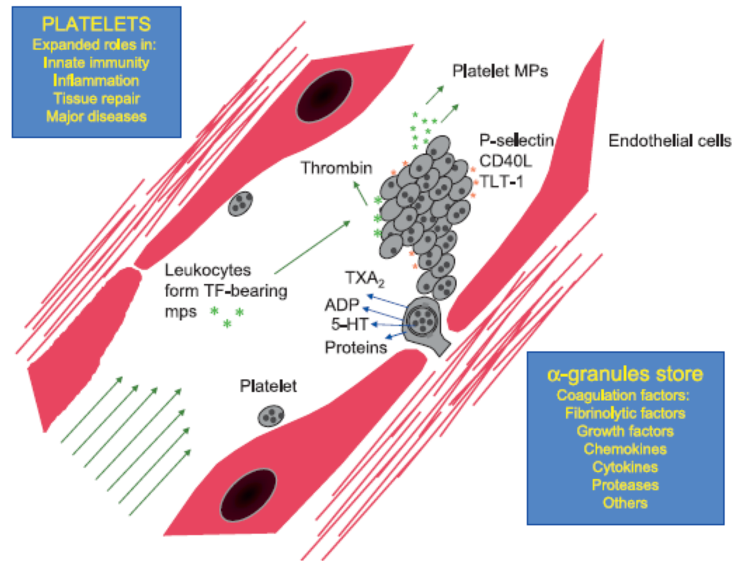


Figure 5. Role of platelets in hemostasis and other functions. From *Nurden, Thromb Haemost 2011;105 Suppl 1:S13-33.*

1.1.2.1. Hemostasis

Much is known about how platelets fulfill their function in hemostasis. After disruption of the integrity of the endothelial cell lining of a blood vessel, circulating platelets localize, amplify and sustain the coagulant response at the injured site. Platelets provide the first line of defense in plugging the leaky vessel by adhering to the site of injury. Platelet adhesion and activation are mediated primarily by their interactions with the subendothelial proteins von Willebrand factor (vWf) and collagen. Platelets bind to subendothelial vWf primarily via the platelet membrane glycoprotein GPIb-IX-V complex and to collagen via the integrin $\alpha_2\beta_1$ and GPVI.(50-52) Platelet adherence to collagen via GPVI triggers the intracellular signaling events that result in platelet activation.(53-55) One platelet activation event, platelet aggregation, serves to prevent additional blood loss from a damaged vessel in the earliest

stage of hemostasis. Platelet aggregation is a result of the cross-linking of activated integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) molecules expressed on the surface of two different platelets by fibrinogen to form a temporary platelet plug that prevents additional blood loss from the leaky vessel.(56) Platelet adherence and aggregation at a site of vascular injury serve not only to plug the damaged vessel temporarily, but also to localize subsequent procoagulant events to the injured site to prevent systemic activation of coagulation. Furthermore, activated platelets actively regulate the propagation of the coagulation reaction by releasing a number of bioactive peptides and small molecules from their α- and dense granules that recruit additional platelets to the growing thrombus, as well as participate directly in procoagulant events. Whereas platelet dense granules contain high concentrations of low molecular weight compounds that potentiate platelet activation (e.g., ADP, serotonin, calcium), α-granules concentrate large polypeptides that contribute to both primary and secondary hemostasis. α-granules secrete:

- adhesive proteins (e.g. fibrinogen, vWf) which mediate platelet–platelet and platelet–endothelial interactions;(57, 58)
- adhesive receptors (e.g. GPIbα-IX-V, GPVI, integrin αIIbβ3) that also participate in platelet adhesion;(59, 60)
- coagulation factors and co-factors (e.g. factor V, XI, XIII, prothrombin, high molecular weight kininogens) which participate in secondary hemostasis;(61-65)
- proteins that contribute to hemostatic balance by limiting the progression of coagulation (e.g. antithrombin, C1-inhibitor, tissue factor

pathway inhibitor, protein S, protease nexin-2, plasmin and plasminogen).(66-68)

This overall process provides for rapid fibrin polymerization at the wound site, transforming blood in the fluid phase to a solid phase plug.

1.1.2.2. Wound healing

Platelets are crucial for vascular remodeling and tissue repair.(69) Platelet proteomes are extremely complex.(70, 71) Activated platelets release, or expose at their surface, biologically active molecules that promote the recruitment, growth, and differentiation of cells. Platelet bioactive substances are entrapped within α-granules, dense granules and lysosomal granules, or are present in the cytoplasm. Growth factors that are mostly contained within α-granules are key factors orchestrating tissue regeneration. Growth factors act binding to specific receptors on the cell membrane; a second messenger transmits the signal into the cell, eliciting a physiological response (Figure 6). The role played by platelet growth factors in hard and soft tissue regeneration will be discussed in detail later.

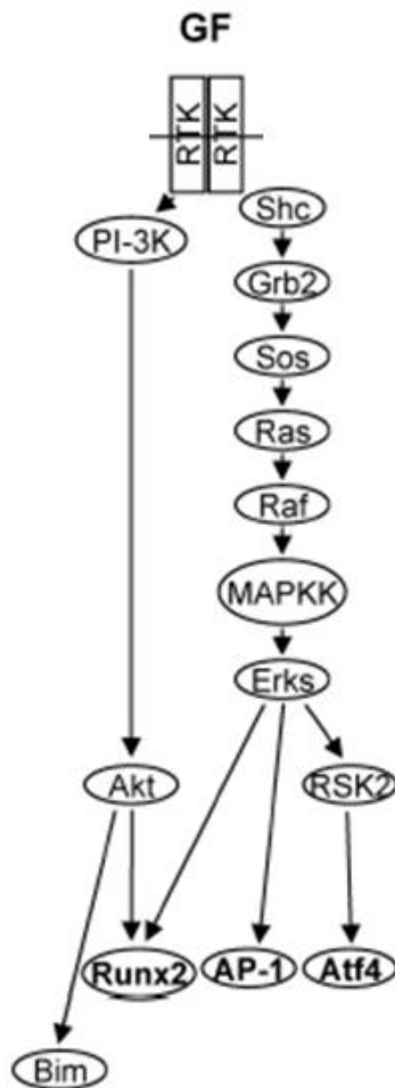


Figure 6. Growth factors' signalling pathway. Adapted from Chau et al., Histol Histopathol 2009;24:1593-1606.

1.1.2.3. Antimicrobial host defense

Platelets play a significant role in host defense against pathogenic microorganisms.(72, 73) Numerous studies have demonstrated that platelets are among the first blood cells to recognize endothelium damaged by microbial colonization and to accumulate at sites of infected endovascular

lesions.(72, 74) Platelets interact directly with viruses, bacteria, fungi, and protozoa.(75-77) Platelet α-granules contain proteins with direct microbicidal properties, a group collectively referred to as "platelet microbicidal proteins".(78) Many of the chemokines secreted by activated platelets - including CXCL4, thymosin-β4, the derivatives of CXCL7 (PBP, CTAP-III, NAP-2) and CCL5 (RANTES) – are microbicidal.(79, 80) Truncation of CTAP-III and NAP-2 at their C-terminus generates two additional peptides, thrombocidins-1 and -2, which are bactericidal *in vitro* against some strains of *Bacillus subtilis* and *Staphylococcus aureus*, and fungicidal for *Cryptococcus neoformans*.(81) Another microbicidal protein, thymosin-β4, localizes to platelet α-granules.(80) While it is debatable whether the local concentration of these secreted proteins are more important for antimicrobial activity or chemoattraction of leukocytes, multiple studies using combined *in vitro* and *in vivo* techniques have demonstrated that platelets are clinically relevant to host defense.(82, 83) α-granules also contain complement and complement binding proteins, which facilitate the clearance of microorganisms from the circulation. Platelet α-granules secrete C3 and C4 precursors, which participate in the complement activation cascade. P-selectin binds C3b, localizing the inflammatory response to sites of vascular injury.(84) Platelets α-granules also contain regulators of complement activation, such as C1 inhibitor.(85) Platelet factor H secreted from α-granules regulates C3 convertase in the alternative pathway.(86)

1.2. Platelet concentrates

As discussed earlier, platelets contain growth factors and other bioactive metabolites which are able to stimulate cell chemotaxis, proliferation and differentiation. For this reason, they have been adopted in clinical situations requiring rapid healing and tissue regeneration. Their administration in the form of platelet concentrates provides an adhesive support that can confine secretion of such bioactive substances to a chosen site. In this chapter, the history, classification, biological effects and therapeutic applications of platelet concentrates will be discussed, together with the evidence gathered from our previous work.

1.2.1. History of the medical use of platelet concentrates

The use of blood-derived products to seal wounds and stimulate healing started with the use of fibrin glues, which were first described nearly 100 years ago. Their use is very limited owing to the complexity and the cost of their production protocols. Consequently, the use of platelet concentrates to improve healing and to replace fibrin glues has been explored considerably during the last 20 years. Several types of platelet concentrates were developed. They can be basically distinct into two categories: platelet-rich plasma (PRP) and platelet-rich fibrin (PRF).

1.2.1.1. Fibrin glue

Fibrin glue has been studied for decades, originally for its use in surgery as a hemostatic agent and a sealant.(87-89) More recently, it has been used as carrier for cells, growth factors and drugs in tissue engineering

applications.(90-92) Fibrin glue consists of two components, a fibrinogen solution and a thrombin solution rich in Ca_2^+. When the two components are mixed, thrombin cleaves fibrinogen to form fibrin and factor XIII to form factor XIIIa, which then crosslinks fibrin to form a gel.(93) The fibrinogen component is obtained from pooled human plasma through cryoprecipitation and ethanol fractionation.(94) The cryoprecipitation uses a citrate buffer in a cold environment to precipitate fibrinogen. The ethanol fractionation method precipitates fibrinogen with 10% ethanol. The obtained fibrinogen is then lyophilized and sterilized before reconstitution. Initially, a commercial bovine enzyme was used for the thrombin component; For safety reasons, nowadays human and recombinant thrombin have replaced bovine one in commercial products.

1.2.1.2. Platelet-rich plasma

In transfusion medicine, platelet concentrates were originally used for the treatment and prevention of haemorrhage due to severe thrombopenia. The standard platelet concentrate for transfusion has been named “platelet-rich plasma” (PRP). The use of PRP to improve healing and to replace fibrin glues was first described by Whitman et al. in 1997.(95) Several techniques for the production of PRP are available. All available techniques have some points in common: blood is collected with anticoagulant just before surgery and is immediately processed by centrifugation. A first centrifugation step is designed to separate the blood into three layers: red blood cells at the bottom, platelet-poor plasma (PPP) at the top, and a “buffy coat” layer rich in

leukocytes and platelets in the middle. The next steps vary among the numerous protocols but are always an attempt to discard erythrocytes and to concentrate platelets, including or not leukocytes (Figure 7). Finally, the obtained platelet concentrate is applied to the surgical site, together with thrombin and/or CaCl2 (or similar factors) to trigger platelet activation and fibrin polymerization. Although bovine thrombin has been used by some authors, this is now avoided because of the risk of the development of antibodies to factors V, XI and thrombin, resulting in the risk of life-threatening coagulopathies.(96) Human and recombinant thrombin, or $CaCl_2$ (or similar factors) alone are used instead. Originally, PRP was prepared using a plasmapheresis system and required the collection of a large amount of blood (up to 500 ml). PRP obtained with this method classically contains 0,5 x 10^{11} platelets per unit. Recent centrifuges allow to collect smaller amounts of blood (usually about 60 ml) and obtain a lower platelet concentration in PRP. In fact, the best results *in vitro* have been obtained with physiological platelet amounts, that is, for a PRP with a platelet concentration that was equivalent to 2,5 times the blood concentration.(97) Higher platelet concentrations seemed to induce negative effects. However, the data on the dose-dependent effects is limited and should be investigated further.

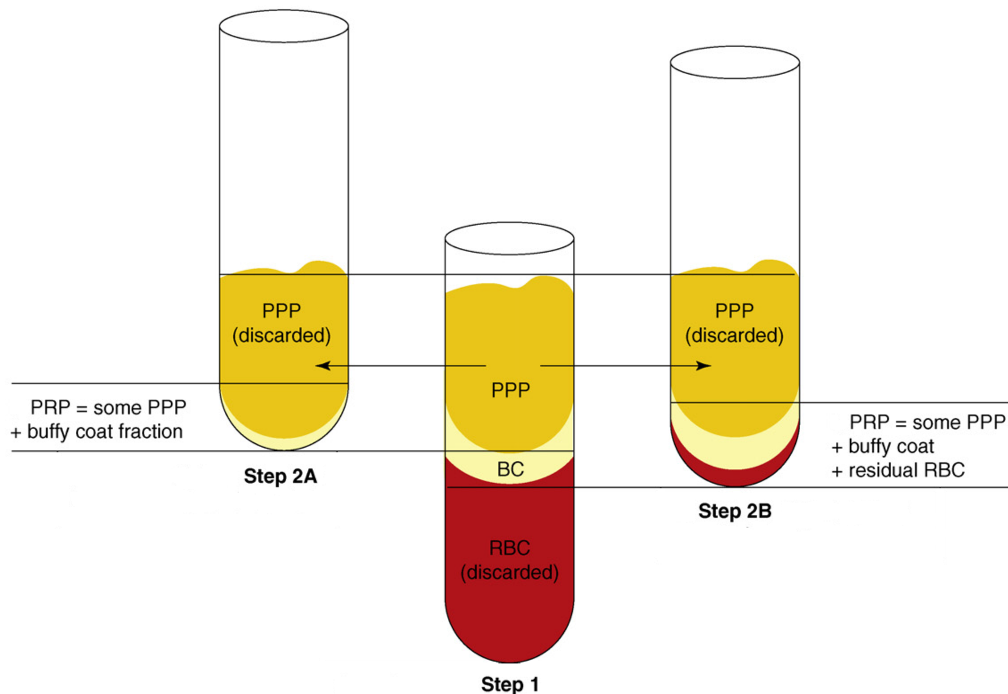


Figure 7. Production of PRP. BC = buffy coat, PPP = platelet-poor plasma, RBC = red blood cells. Adapted from *Dohan Ehrenfest et al., Trends Biotechnol 2009;27:158-167.*

1.2.1.3. Platelet-rich fibrin

PRF was developed by Choukroun et al. in 2001.(98) For the production of PRF, blood is collected without any anticoagulants and centrifuged. In the absence of anticoagulants, platelet activation and fibrin polymerization are triggered immediately. After centrifugation, three layers are formed: red blood cells at the bottom, acellular plasma at the top and a PRF clot in the middle (Figure 8).(99) The PRF clot is a strong fibrin matrix in which most of the platelets and leucocytes are concentrated.(100, 101) Unlike PRP, PRF does not dissolve quickly after application, but is slowly remodeled in a similar way to a natural blood clot. Platelets are activated during the centrifugation,

leading to a substantial embedding of platelet and leukocyte growth factors into the fibrin matrix.

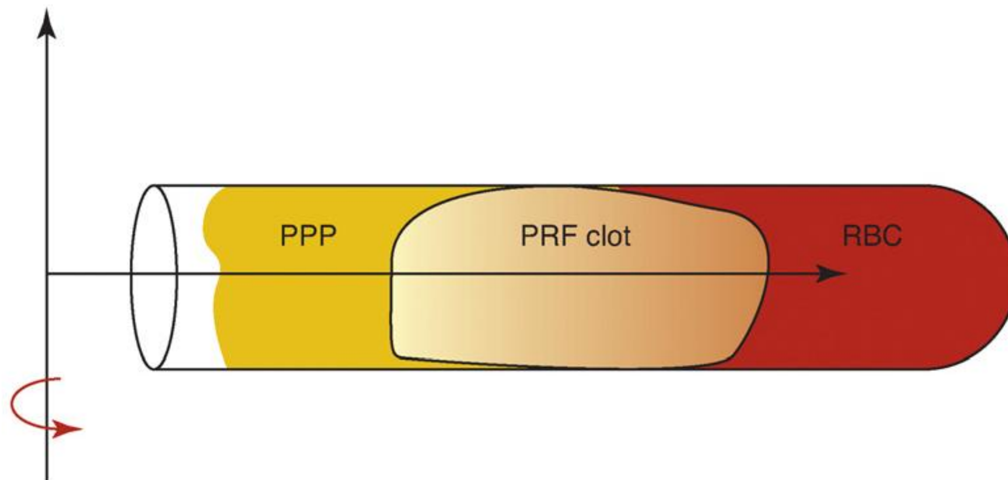


Figure 8. Production of PRF. Adapted from *Dohan Ehrenfest et al., Trends Biotechnol 2009;27:158-167.*

1.2.2. Classification

Nowadays many platelet separation systems that produce distinct types of platelet concentrates are available. It would be important to better characterize the content of platelet concentrates produced by the various commercial systems to make more informed decisions regarding their use in the clinical setting. A recent series of letters were published about platelet concentrates, where both terminology and content were hotly debated.(102-106) The definition and classification of platelet concentrates are indeed very important issues, as many misunderstandings are widely spread in the literature on this topic. Such techniques were initially gathered under the name "PRP" in reference to the generic term used in transfusion hematology,

but this name is too general for the qualification of the many products availabe today. A proposal for terminology and for a wide classification system for platelet concentrates has been improved and systematized in two recent publications.(107, 108)

According to Dohan Ehrenfest et al., four main categories of products can be defined, depending on their leukocyte content and fibrin architecture: pure platelet-rich plasma (P-PRP), leukocyte- and platelet-rich plasma (L-PRP), pure platelet-rich fibrin (P-PRF) and leukocyte- and platelet-rich fibrin (L-PRF).(107)

De Long et al. proposed the so called "PAW classification", based on three components: the absolute number of platelets ("P"), the manner in which platelet activation occurs ("A") and the presence or absence of white cells ("W").(108)

Achieving a terminology consensus on platelet concentrates technologies is important to avoid confusion in this wide and complex field of research. In many articles, the lack of characterization of the tested products made the literature in this field very difficult to sort and interpret. Without a consensus, this field will remain unclear and this situation will considerably restrain its development.

1.2.3. Biological effects

The advantages of using platelet concentrates in the clinical setting are many. The regenerative potential of platelet concentrates on hard and soft tissues has been explored considerably during the last two decades.

Recently, novel properties have been also pointed out, such as anti-inflammatory and antimicrobial effects.

1.2.3.1. Hard tissue healing

Platelet α-granules release many growth factors that may favor bone regeneration. Among them there are: platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), epidermal growth factor (EGF) and platelet-derived epidermal growth factor (PDECGF). These factors are variously involved in stimulating chemotaxis, cell proliferation and differentiation (Table).(33) A multitude of cytokines and chemokines are also stored and released by α-granules.(69, 109)

Growth factor	Biological activity useful for hard tissue regeneration
PDGF	Stimulates proliferation of mesoderm-derived cells such as fibroblasts, vascular smooth muscle cells, glial cells and chondrocytes. Attracts and activates neutrophils, monocytes and fibroblasts. Increases the synthesis of phospholipids, cholesterol esters, glycogen and prostaglandins. Regulates the production and degradation of extracellular matrix proteins. Stimulates the synthesis of additional growth factors. Interacts with TGF-β.
TGF-β	Stimulates proliferation of fibroblasts, marrow stem cells and preosteoblasts. Sustains and amplifies osteoblastic activity during bone healing, as target cells are able to synthesize and secrete their own TGF-β to

	act on themselves or on adjacent cells.
VEGF	Promotes angiogenesis.
bFGF	Stimulates proliferation and differentiation of preosteoblasts and osteoblasts.
IGF	Stimulates proliferation and differentiation of osteoblasts. Enhances the synthesis of bone matrix proteins such as alkaline phosphatase, osteopontin and osteocalcin.
EGF	May exert a positive activity through its mitogenic characteristics.
PDECGF	Promotes angiogenesis.

1.2.3.2. Soft tissue healing

Platelets release at the site of injury a variety of substances that are involved in all aspects of the wound healing process.(110) Among them there are: EGF, PDGF, TGF-a, TGF-b, keratinocyte growth factor (KGF), aFGF, bFGF, VEGF, connective tissue growth factor (CTGF) and IGF (Table). For this reason, platelet concentrates have been extensively used for topical therapy of various clinical conditions, including wounds and soft tissue injuries.(111-113)

Growth factor	Biological activity useful for soft tissue regeneration
EGF	Stimulates proliferation of fibroblasts, epidermal and epithelial cells. Chemoattractant for fibroblasts and epithelial cells. Enhances re-epithelialization and angiogenesis. Influences the synthesis and turnover of extracellular matrix.
PDGF	Stimulates proliferation of fibroblasts, smooth muscle cells, chondrocytes, epithelial and endothelial cells. Chemoattractant for fibroblasts, muscle cells, hematopoietic and mesenchymal cells. Stimulates neutrophils and macrophages.

	Enhances collagen synthesis, collagenase activity and angiogenesis.
TGFα	Stimulates proliferation of mesenchymal, epithelial and endothelial cells. Chemoattractant for endothelial cells. Controls epidermal development.
TGFβ	Enhances collagen synthesis. Decreases dermal scarring. Inhibits proliferation of fibroblasts, keratinocytes, hematopoietic, epithelial and endothelial cells. Antagonizes the biological activity of EGF, PDGF, aFGF and bFGF.
KGF	Most potent growth factor acting on skin keratinocytes. Plays a role in tissue repair following skin injuries.
aFGF	Stimulates proliferation of skin keratinocytes, dermal fibroblasts and vascular endothelial cells.
bFGF	Stimulates proliferation of fibroblasts, myoblasts, keratinocytes, chondrocytes and endothelial cells. Enhances angiogenesis, collagen synthesis, wound contraction, matrix synthesis, epithelialization. Stimulates KGF production.
VEGF	Stimulates proliferation of endothelial cells. Induces neovascularisation. Stimulates the synthesis of metalloproteinases.
CTGF	Stimulates proliferation and migration of endothelial cells.
IGF	Stimulates proliferation of mesodermal cell types. Promotes the synthesis of collagenase and prostaglandin E2 in fibroblasts. Stimulates collagen and matrix synthesis. Regulates the metabolism of joint cartilage.

1.2.3.3. Novel properties

Control of the inflammatory reactions that takes place in a wound can reduce pain and enhance the healing process. A reduction of post-surgical

inflammation has been reported when using platelet concentrates.(114) Several platelet-derived factors may contribute to the anti-inflammatory properties of platelet concentrates, such as RANTES and lipoxin A4.(114) TGF-β may also exert anti-inflammatory actions. In fact, in an animal model of asthma, platelet concentrate lysate rich in TGF-β exhibited immunosuppressive and anti-inflammatory properties and enhanced the development of $FOXP3^+$ regulatory T cells.(115) Platelet concentrate lysate have also shown to counteract the inflammatory effect mediated by IL-1β in chondrocytes by inhibiting the activation of nuclear factor-κB, a major pathway in the pathogenesis of osteoarthritis.(116, 117) A decrease in the expression of cyclooxygenase-2 and CXCR4 target genes was also observed. The role played by leukocytes is a matter of intense debate. It is possible that they might increase the inflammatory response at the wound site because of the metalloproteases and acid hydrolases secreted.(118) Further studies are needed to establish a better scientific understanding of the anti-inflammatory properties of platelet concentrates.

Platelet concentrates are also believed to exhibit antibacterial properties, thereby potentially limiting the risk of infection of surgical wounds. Recent studies have evaluated clinical and *in vitro* antimicrobial activity of platelet concentrates, showing inhibitory effect against various bacteria.(119-132) To date, the components responsible for the antimicrobial activity of platelet concentrates are poorly understood. Several antimicrobial factors have been proposed, including platelet antimicrobial proteins and peptides of the innate immune defense, or platelet α-granules components, such as complement

and complement-binding proteins.(80, 109, 126, 127, 133-135) Direct interaction of platelets with microorganisms, participation in antibody-dependent cell cytotocity, release of myeloperoxidase, activation of the antioxidant responsive element and antigen-specific immune response have also been suggested.(125, 136, 137) In fact, platelet concentrates are a complex mixture of platelets, leukocytes and plasma, and the respective impact of the plasma and cellular components has not been studied in detail yet.

1.2.4. Therapeutic applications

The use of autologous platelet concentrates has gained large popularity in a variety of medical fields, which will be briefly discussed in the following paragraphs.

1.2.4.1. Dentistry, oral and maxillofacial surgery

In the field of dentistry, oral and maxillofacial surgery, platelet concentrates have been used in different clinical procedures (e.g. sinus floor elevation, alveolar ridge augmentation, mandibular reconstruction, maxillary cleft repair, treatment of periodontal defects and extraction sockets), where it was applied alone or in addition to bone substitutes. Some papers reported positive effects of platelet concentrates on bone formation.(138-143) Others reported limited or no beneficial effect.(144-148) Currently, there is a lack of scientific evidence in the dental literature in favour of or against the clinical use of platelet concentrates for bone regeneration. Several reviews about the

application of platelet concentrates in dentistry have been published so far.(143, 148-155) Only a few of them followed a systematic approach.(155-157) Their general conclusion is that several methodological limitations are present in the available studies. Future research should focus on strong and well-designed randomized controlled clinical trials.

1.2.4.2. Orthopedics and sports medicine

Reconstruction of anterior cruciate ligament(158), tendon surgery, treatment of osteoarthritis, joint injuries and muscle tears are some examples of the applications of platelet concentrates in the field of orthopedics and sports medicine.(158-161) Ultrasound-guided injection of platelet concentrates may offer an alternative treatment over palliative or surgical treatments. Clinical benefits of platelet concentrates in this field remain controversial. Studies of various levels of evidence have tested their safety and efficacy, but standardization of the preparation methods and application procedures is necessary for further advancements. The growing emphasis on an evidence-based approach in the sports medicine and orthopedic settings demands additional research efforts before incorporating this technology in routine clinical care.

1.2.4.3. Treatment of skin lesions

Many reports about the application of platelet concentrates on chronic wounds are available. Results of clinical studies treating small-sized non-healing diabetic ulcers with platelet concentrates are encouraging.(162-164)

Combination with skin grafting may be required for large, deep ulcers. A recent systematic review assessing the effect of platelet concentrates on the treatment of cutaneous wounds showed that healing was improved compared to control wound care.(165)

Few reports are also available about the application of platelet concentrates on burns, but the value of their use in this field remains unclear. (166-168) A definitive assessment will require further studies.(169)

1.2.4.4. Other medical areas

Platelet concentrates in the form of eye-drops have been successfully used in ophthalmology for the treatment of many eye disorders, such as dry eye symptoms or corneal perforations.(170-172)

Platelet concentrates are also applied in plastic surgery and aesthetic medicine.(173-175)

Another interesting approach of this technology is the use of platelet concentrates for nerve regeneration purposes.(176, 177)

Moreover, the pool of growth factors released by platelets has been used as a powerful substitute of foetal calf serum in the culture of a wide range of cells, including fibroblasts and osteoblasts, mesenchymal stem cells and stromal cells.(178-180)

1.2.5. Evidence from our previous work

Our research group has used P-PRP in the field of dentistry for some years. Several clinical studies and systematic reviews of the literature have been

performed to evaluate the efficacy and safety of P-PRP in this clinical setting. Such studies will be briefly described in the following paragraphs.

1.2.5.1 Systematic reviews of the literature

In a systematic review, we evaluated the effect of autogenous platelet concentrates on the clinical and histomorphometric outcomes of maxillary sinus augmentation.(181) Only prospective comparative clinical studies were included. No difference in implant survival was found between test and control groups. Some studies reported a beneficial effect of platelet concentrates based on histomorphometric outcomes, while other studies found no significant effect. Favorable effects on soft tissue healing and postoperative discomfort reduction were often reported but not quantified.

In another systematic review, we evaluated the effects of autogenous platelet concentrates on clinical outcomes of the surgical treatment of periodontal diseases.(182) Only randomized clinical trials were included. A significant positive effect of the adjunct of platelet concentrates was found for intrabony defects. Such an effect was magnified in studies in which guided tissue regeneration (GTR) was not used, whereas in studies using GTR the use of platelet concentrates had no adjunctive effect. No significant effect of platelet concentrates was found for gingival recession nor for furcation treatment.

We also systematically assessed if the use of autologous platelet concentrates might be beneficial to the healing of extraction sockets.(183) Only prospective comparative studies were included. Based on the reports of the selected studies, we concluded that the use of platelet concentrates

could be beneficial for reducing postoperative pain and inflammation, thereby improving quality of life in the early period after extraction. No systematic acceleration of osseous healing at the post-extraction site could be demonstrated, suggesting that platelet concentrates *per se* probably exert a negligible effect on bone regeneration.

In conclusion, a clear advantage of platelet concentrates could not be evidenced from these reviews. A large heterogeneity was found regarding study design, sample size, surgical techniques, grafting materials, methods for preparing platelet concentrates and outcome variables. Standardization of experimental design is needed in order to detect the true effect of platelet concentrates in regenerative procedures in dentistry.

1.2.5.2. Clinical studies

Three studies showed that P-PRP produced significant beneficial effect to patients' quality of life, decreasing pain and surgery side effects as well as swelling, after sinus lift and endodontic surgery.(184-186)

A case report study showed that the addition of P-PRP to anorganic bovine bone improved the regenerative process after the surgical treatment of a large through-and-through periapical bone lesion.(187)

A prospective study showed the clinical success of a treatment protocol for the rehabilitation of edentulous posterior maxilla consisting of the positioning of short implants in combination with transcrestal sinus lifting with the adjunct of P-PRP. (188)

P-PRP was also used in immediate implant placement procedures,

demonstrating to be a safe, effective, and predictable treatment option for the rehabilitation of fresh post-extraction sockets.(189, 190)

1.3. Aim of this thesis

The aim of this thesis was to evaluate the antimicrobial activity of P-PRP. In particular, we assessed whether P-PRP displayed an inhibitory activity against microorganisms colonizing the oral cavity such as *Enterococcus faecalis, Candida albicans, Streptococcus agalactiae, Streptococcus oralis* and *Pseudomonas aeruginosa*. We also evaluated the effect of platelet concentration, of the activation step and of the presence of plasmatic components on the antimicrobial activity of P-PRP against *Enterococcus faecalis, Streptococcus agalactiae* and *Streptococcus oralis.*

2. MATERIALS AND METHODS

2.1. Donors

Blood samples were obtained from 47 healthy donors. All subjects were in general good health (ASA 1-2 according to the American Society of Anaesthesiologists classification) and older than 18 years. No patient took antibiotics during the month before surgery, nor was under anticoagulant or immunosuppressive therapy. Written informed consent for participation in the study was obtained from all patients. The present research was performed within the guidelines of the Helsinki Declaration for biomedical research involving human subjects. The study was approved by the Review Board of the Galeazzi Orthopedic Institute.

2.2. Blood collection

Peripheral blood from each donor (about 4,5 ml) was taken by venipuncture into 5 ml sterile tubes (Vacutainer, BD, Franklin Lakes, New Jersey, U.S.A) containing 3,8% (wt/vol) trisodium citrate as anticoagulant. Tubes were immediately gently inverted three times, as clotting may occur when the blood is not thoroughly mixed with the anticoagulant additive. Tubes were inverted gently to prevent hemolysis.

2.3. Production of pure platelet-rich plasma (P-PRP)

2.3.1. Production of single donor P-PRP

Fresh human whole blood from 17 donors was processed using a laboratory centrifuge (Megafuge 1.0 R, Heraeus, Hanau, Germany) to obtain P-PRP,

following Anitua's protocol.(191) Peripheral blood (4,5 ml) from each donor was centrifuged at 460 g for 8 min at room temperature. After centrifugation, 3 components were obtained: red blood cells at the bottom of the tube; a thin layer of leukocytes referred to as "buffy coat" in the middle; and plasma, which contains most of the platelets, at the top. The 1 ml plasma fraction located above the red cell fraction, but not including the buffy coat, was collected. Care was taken to avoid creating any turbulence and to prevent the aspiration of leukocytes and erythrocytes. The remaining plasma was discarded. P-PRP was stored at -20°C until use.

2.3.2. Production of pooled donor P-PRP

Fresh human whole blood from donors was processed using a laboratory centrifuge (Megafuge® 1.0 R, Heraeus, Hanau, Germany) to obtain P-PRP, following Anitua's protocol.(191) Four pools of blood were produced: two pools (one of 16 ml and the other of 72 ml) were created with blood collected from 22 patients with the baseline platelet count falling into the physiological range (150.000-400.000 platelets/µl), while two pools (each of 16 ml) were created with blood collected from 8 patients with the baseline platelet count above (4 patients) or below (4 patients) the physiological range, respectively. Pooled blood was transferred into 9 ml tubes and centrifuged at 580 g for 8 min at room temperature. After centrifugation, three components were obtained: red blood cells, a thin layer of leukocytes referred to as "buffy coat" and plasma. The 2 ml plasma fraction located above the red cell fraction, but not including the buffy coat, was collected. Care was taken to avoid creating

any turbulence and to prevent the aspiration of leukocytes and erythrocytes. P-PRP was stored at -20°C until use.

2.4. Production of platelet-poor plasma (PPP)

To prepare PPP, an aliquot of 2 ml was collected from the P-PRP obtained from the 16 ml blood pools and centrifuged at 3000 g for 10 minutes in Megafuge 1.0 R (Heraeus Holding, Hanau, Germany). The supernatant (almost 2 ml) was recovered. The platelet pellet (PLT) was resuspended in 2 ml of phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, Missouri, U.S.A.). In this way, a pure platelet solution without plasmatic components was obtained. PPP and PLT were stored at -20°C until use.

2.5. Production of 2-fold and 4-fold concentrated PRP (PRPx2, PRPx4)

To further increase platelet concentration, an aliquot of 16 ml was collected from the P-PRP produced from the 72 ml blood pool and centrifuged at 3000 g for 10 minutes in Megafuge 1.0 R (Heraeus Holding, Hanau, Germany). The supernatant (about 16 ml) was recovered, and the platelet pellet was resuspended in half of the starting plasma volume in order to obtain a platelet concentration two times higher than that of P-PRP (PRPx2). The procedure was repeated twice in order to produce a platelet concentration four times higher than that of P-PRP (PRPx4) (Figure 9). PRPx2 and PRPx4 were stored at -20°C until use.

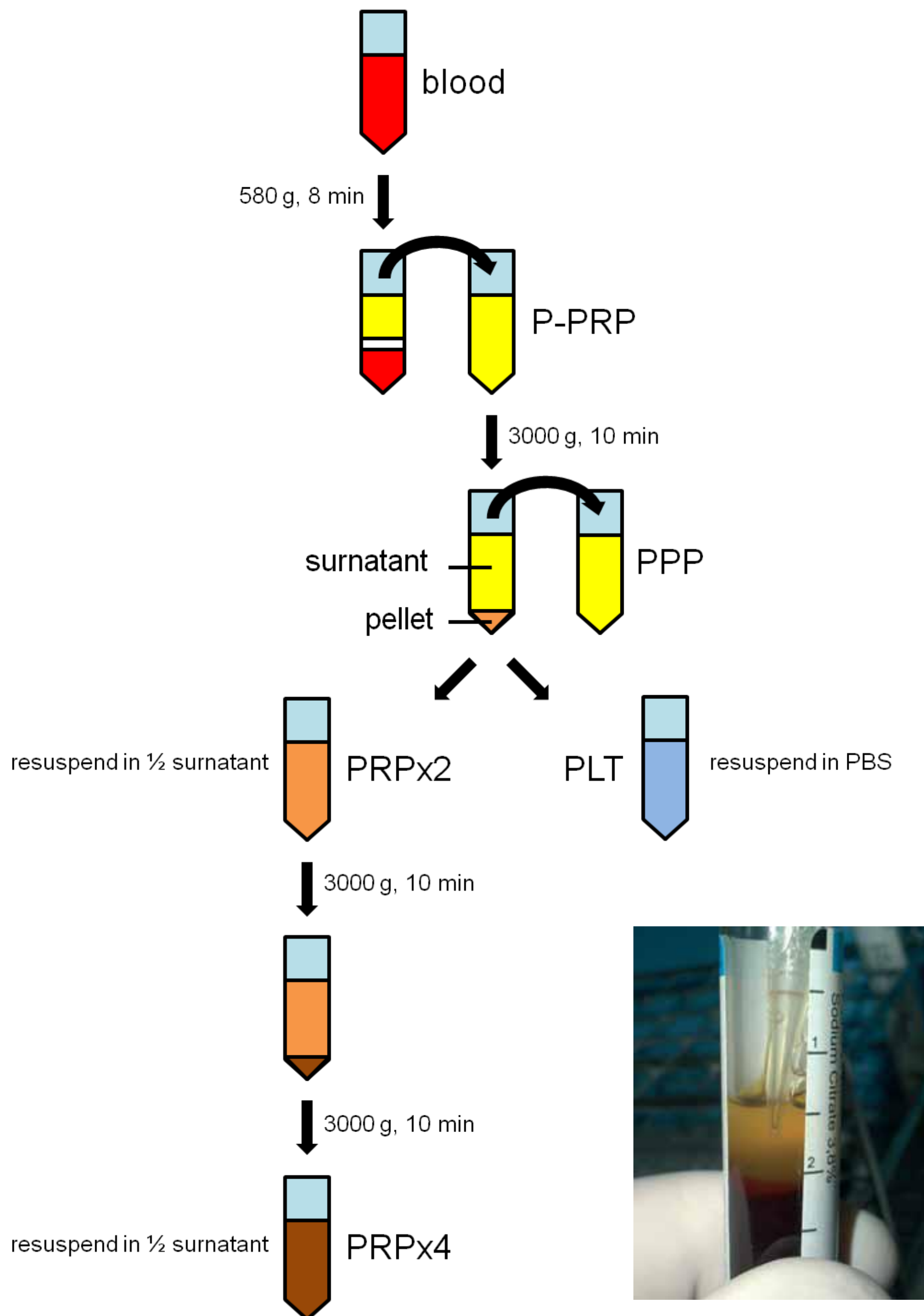


Figure 9. Experimental design of the study. The image illustrates the procedure used to obtain P-PRP, PPP, PLT, PRPx2 and PRPx4 from whole blood.

2.6. Determination of platelet and leukocyte count

Platelet concentration in whole blood, P-PRP, PPP, PRPx2 and PRPx4 was counted automatically using a hematology analyzer (XE-2100, Sysmex Europe, Norderstedt, Germany). To evaluate the purity of P-PRP, white blood cells count was also performed both in whole blood and P-PRP. According to Anitua et al., leukocyte levels in P-PRP are consistently lower than in whole blood (< 10^3 white blood cells/µl).(191)

2.7. Activation of coagulation

P-PRP, PPP, PLT, PRPx2 and PRPx4 were activated shortly before use. In order to initiate clotting and trigger the release of platelet content, the activator, consisting of 10% $CaCl_2$ (BTI, Vitoria, Alava, Spain) was added (50 µl per ml).

2.8. Microbial strains

Microbial strains previously isolated from clinical samples were used. In particular, five representative microorganisms chosen among those colonizing the oral cavity were used: three aerobic gram-positive bacteria such as *Enterococcus faecalis*, *Streptococcus agalactiae* and *Streptococcus oralis;* one aerobic gram-negative bacteria such as *Pseudomonas aeruginosa*; and one yeast such as *Candida albicans*. This strains were previously identified by biochemical identification (API system and Vitek 2 Compact (bioMériéux, Marcy l'Etoile, France)) and confirmed by DNA sequencing of 80 bp of the variable regions V1 and V3 of the 16S rRNA gene by pyrosequencing (PSQ96RA, Diatech, Jesi, Italy). For each species, five

strains were used. Each strain presented different characteristics (e.g. different antibiotic resistance). In addition, ATCC strains were used as controls: *E. faecalis* ATCC #29212, *C. albicans* ATCC #928, *S. agalactiae* ATCC #13813, *S. oralis* ATCC #35037 and *P. aeruginosa* ATCC #27853.

2.9. Microbial cultures

Microbial strains were kept stored in cryoprotectant medium (brain-heart infusion broth (BHI; bioMériéux, Marcy l'Etoile, France) with 10-15% glycerol (Sigma-Aldrich, St. Louis, Missouri, U.S.A.)) at -80°C to suspend metabolism. Before use, microbial cultures were recovered by thawing and seeding on appropriate solid medium. *E. faecalis* and *P. aeruginosa* were seeded on Trypticase Soy Agar (bioMériéux, Marcy l'Etoile, France) and incubated for 24 hours at 37°C. *C. albicans* was seeded on Sabouraud Glucose Agar (bioMériéux, Marcy l'Etoile, France) for 24 hours at 37°C. *S. agalactiae* and *S. oralis* were seeded on Columbia Blood Agar (bioMériéux, Marcy l'Etoile, France) and incubated in a 5% CO_2 atmosphere for 24 hours at 37°C.

2.10. Determination of antimicrobial activity

2.10.1. Determination of the minimum inhibitory concentration (MIC)

The MIC, defined as the lowest concentration of an antimicrobial substance that inhibits the visible growth of a microorganism, was determined by broth microdilution method (Figure 10). A suspension in BHI (or BHI + 5% blood for *S. agalactiae* and *S. oralis*) was prepared for each strain, with an optical

density equal to 0,5 McFarland ($1x10^8$CFU/ml). After obtaining a concentration of $1x10^4$ CFU/ml using appropriate dilutions, 20 µl of each suspension were inoculated in a 96-wells microplate containing 180 µl of BHI (or BHI + 5% blood for *S. agalactiae* and *S. oralis*) and a serial dilution of the material under evaluation. Positive controls were performed inoculating the microbial suspension in BHI (or BHI + 5% blood for *S. agalactiae* and *S. oralis*) alone. For pooled donor P-PRP, PPP, PLT, PRPx2 and PRPx4, non activated samples (that is, $CaCl_2$ was not added) were also tested. In order to assess that $CaCl_2$ did not possess antimicrobial activity per se, controls were performed inoculating microbial suspension in BHI (or BHI + 5% blood for *S. agalactiae* and *S. oralis*) + $CaCl_2$. MIC values were read after incubation for 24 hours at 37°C (in a 5% CO_2 atmosphere for *S. agalactiae* and *S. oralis*). Assays were performed in duplicate for each strain and, if the two MICs differed by more than two wells, the assay was repeated. MIC values were expressed as platelet concentration (n° platelets/µl) and dilution (%).

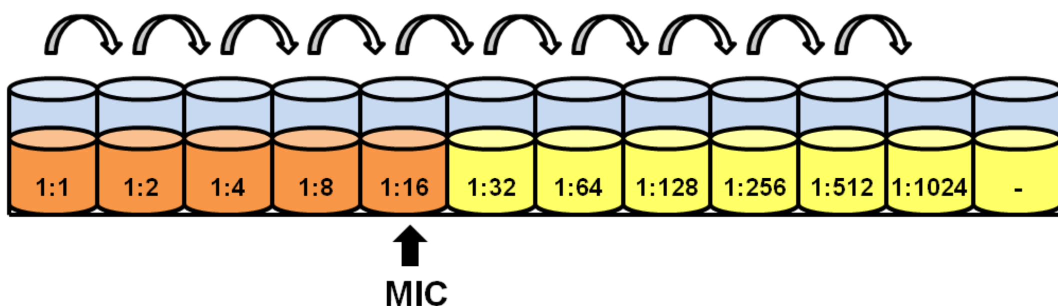


Figure 10. Schematic representation of a MIC test. The image illustrates the row of a 96-well microplate. Numbers in each well represents the ratio between antimicrobial substance and culture medium. In the first well, antimicrobial substance and culture medium are mixed in 1:1 ratio. A serial

dilution is performed until the last well, in which only culture medium is present (positive control of growth). Microbial suspension is then inoculated in each well. The MIC corresponds to the dilution present in the last well in which microbial growth is observable (colored in orange) after incubation for 24 hours in appropriate culture conditions.

2.10.2. Determination of the minimum microbicidal concentration (MMC)

The MMC, defined as the lowest concentration of an antimicrobial substance that kills a microorganism, was determined. MMC test involves a series of steps that that place once MIC test is performed. Samples (20 µl) of microbial suspension were collected from each well of the plate used for the MIC assay and subcultured on appropriate solid medium (Figure 11). Trypticase Soy Agar (bioMériéux, Marcy l'Etoile, France) was used for *E. faecalis* and *P. aeruginosa*; Sabouraud Glucose Agar (bioMériéux, Marcy l'Etoile, France) was used for *C. albicans*; and Columbia Blood Agar (bioMériéux, Marcy l'Etoile, France) was used for *S. agalactiae* and *S. oralis*. MBC value were read after incubation for 24 hours at 37°C (in a 5% CO_2 atmosphere for *S. agalactiae* and *S. oralis*).

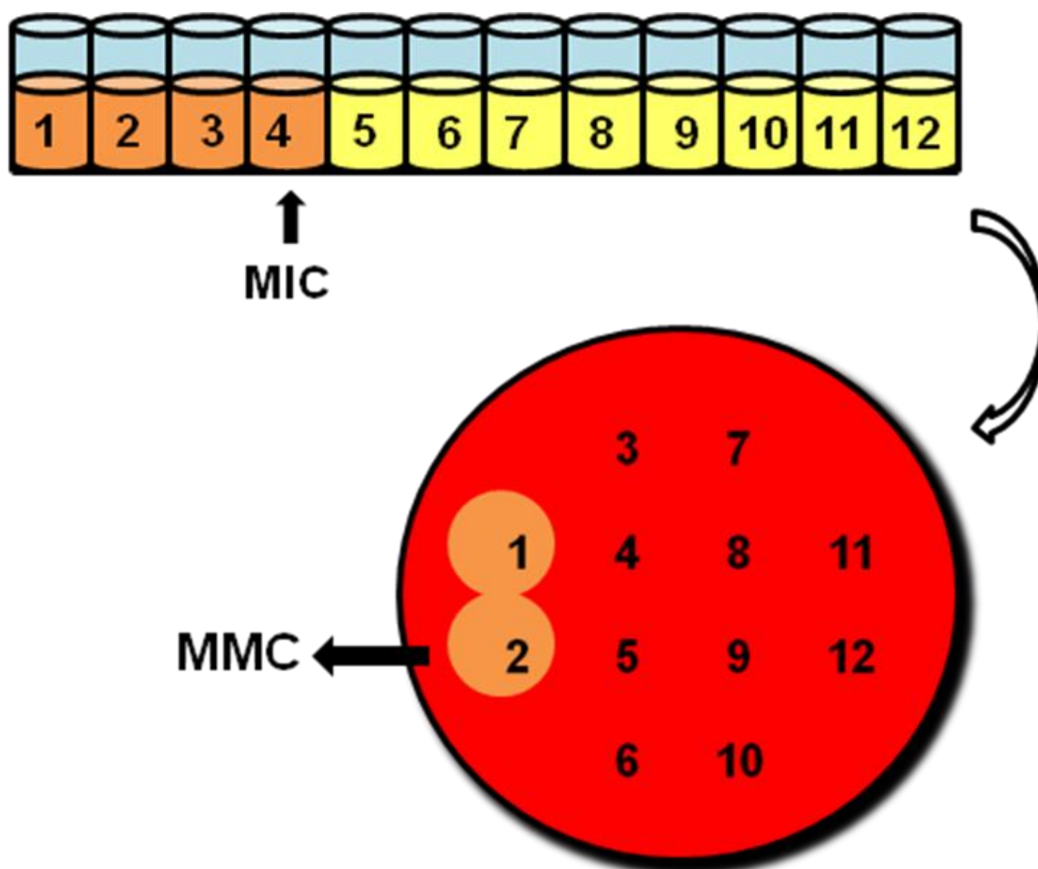


Figure 11. Schematic representation of an MMC test. The image illustrates the row of a 96-well microplate in which a MIC test has been performed. A sample of microbial suspension from each well (numbers from 1 to 12) is inoculated onto an agar plate. The MMC corresponds to the dilution present in the last well that permits the growth of microbial subcultures (orange circles) after incubation for 24 hours in appropriate culture conditions.

2.11. Statistical Analysis

Results were expressed as means ± standard deviations. When two groups were compared, an analysis was done using unpaired t test. For data in which there were more than two comparisons, an analysis was done using

one-way ANOVA followed by Bonferroni's post-test. A probability value of P = 0,05 was used as the significance level. Data analysis was performed using a statistical software package (GraphPad Prism, GraphPad Software Inc., La Jolla, California, U.S.A.).

3. RESULTS

3.1. Platelet and leukocyte count

3.1.1. Platelet and leukocyte count in single donor P-PRP

Mean platelet and leukocyte concentrations in whole blood were (239.000 ± 77.000)/µl and (6.400 ± 2.300)/µl, respectively. Mean platelet and leukocyte concentrations in P-PRP were (350.000 ± 120.000)/µl and (340 ± 270)/µl, respectively. Platelets were 1,5 times more concentrated in P-PRP than in whole blood, while leukocytes were 18,8 times less concentrated.

3.1.2. Platelet and leukocyte count in pooled donor P-PRP, PPP, PRPx2 and PRPx4

Platelet concentrations in whole blood, P-PRP, PPP, PRPx2 and PRPx4 are shown in Table 3. Platelet concentration in P-PRP was 1,5 to 2,2 times higher than in whole blood. PRPx2 and PRPx4 were 1,5 and 3,1 times more concentrated than P-PRP, respectively.

Mean leukocyte concentrations in whole blood and P-PRP were (7500 ± 3200)/µl and (802 ± 123)/µl, respectively. White blood cells in P-PRP were 9,4 times less concentrated than in whole blood. The percentages of the five types of leukocytes (neutrophils, lymphocytes, monocytes, eosynophils and basophils) in P-PRP differed from percentages in whole blood (Table 4). In particular, white blood cells in P-PRP consisted mainly of lymphocytes (72%), while neutrophils were the most abundant leukocyte type in whole blood (62,3%).

	Pool 1	Pool 2	Pool 3	Pool 4
Blood	184.000	252.000	535.000	149.000
P-PRP	328.000	565.000	787.000	227.000
PPP	15.000	/	14.000	11.000
PRPx2	/	825.000	/	/
PRPx4	/	1.768.000	/	/

Table 3. Platelet count in pooled donor P-PRP, PPP, PRPx2 and PRPx4. Concentrations are expressed as n°platelets/μl. Slashes indicate that the count was not performed.

	Blood		**P-PRP**	
	Mean	SD	Mean	SD
Neutrophils	62,3	3,8	18,7	13,1
Lymphocytes	25,9	2,7	72,0	13,0
Monocytes	9,3	1,1	8,4	1,4
Eosinophils	2,1	0,4	0,8	0,4
Basophils	0,4	0,1	0,2	0,1

Table 4. Leukocyte count in pooled donor P-PRP. Concentrations are expressed as percentages (%). SD = standard deviation.

3.2. Antimicrobial activity of single donor P-PRP

MIC are shown in Figure 12. For each microorganism, results were expressed as the mean of the MIC calculated for 5 clinical strains and one ATCC strain. As can be seen from the figure, MIC were fairly uniform among microorganisms, except for *C. albicans*, whose MIC was about 4 times higher than the others. *E. faecalis, S. agalactiae* and *S. oralis* were inhibited by a

platelet concentration of about 20.000 platelets/µl (corresponding to a dilution of about 10% from the initial concentration), while *C. albicans* was inhibited by a platelet concentration of about 80.000 platelets/µl (corresponding to a dilution of about 40% from the initial concentration). The difference among effective concentrations was statistically significant (P value < 0,001). *P. aeruginosa* is not depicted in figure because it was not inhibited by P-PRP at the platelet concentrations tested.

The MMC for *E. faecalis, S. agalactiae* and *S. oralis* was 3-4 times higher than the MIC. In contrast, the MMC of *C. albicans* couldn't be estimated at the platelet concentrations tested (Figure 13).

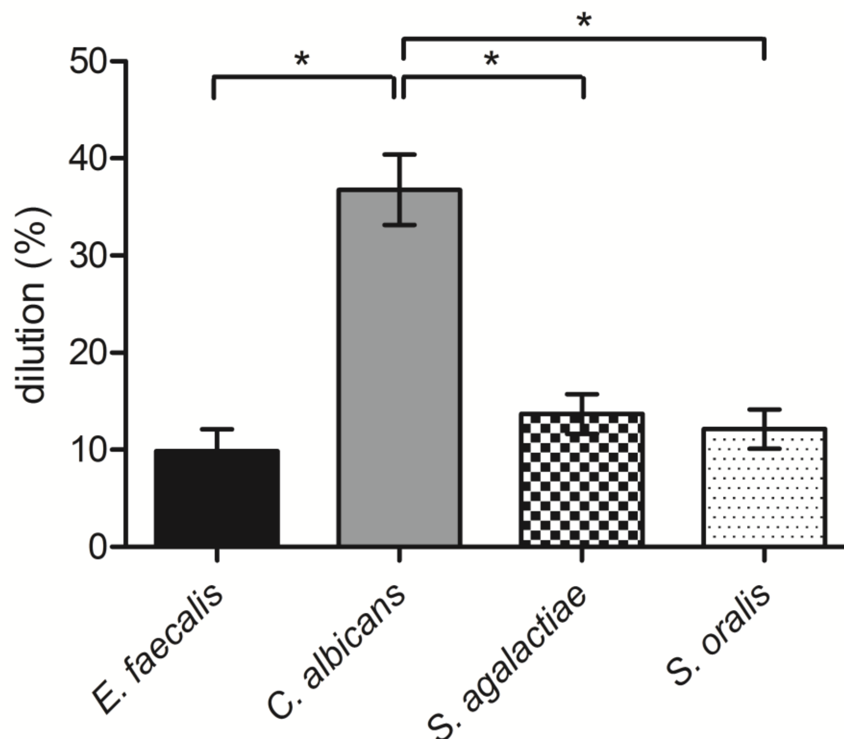


Figure 12. MIC of P-PRP. Error bars represent standard deviations. *P value < 0,001.

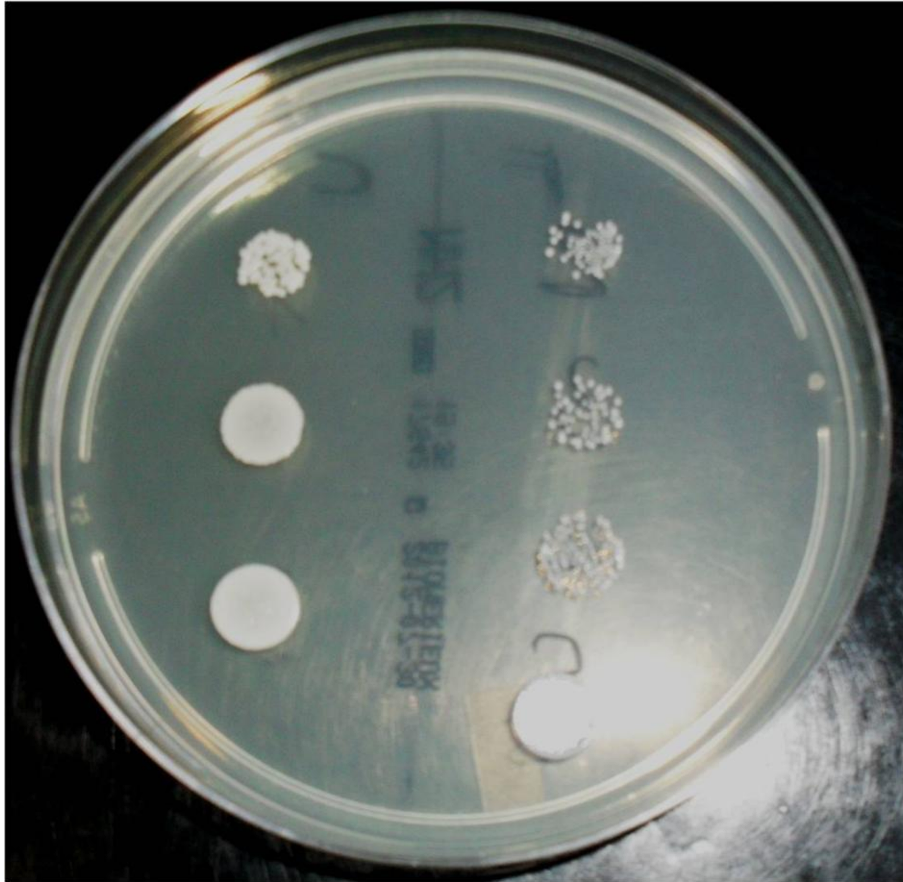


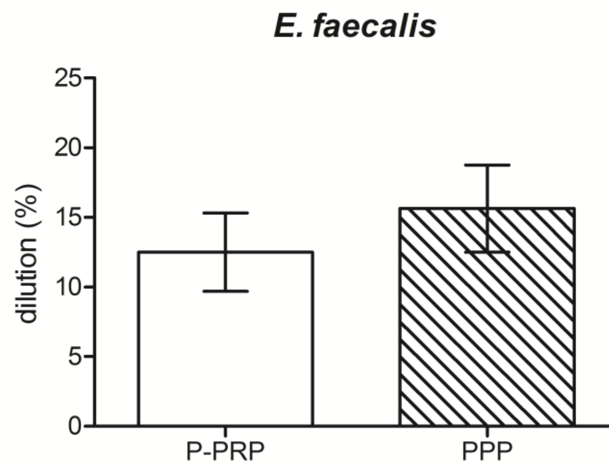
Figure 13. MMC of P-PRP. Exemplificative photograph of an agar plate on which was performed the MMC test for *C. albicans* (left) and *E. faecalis* (right). As can be seen from the figure, *E. faecalis* was more susceptible to P-PRP effect than *C. albicans*.

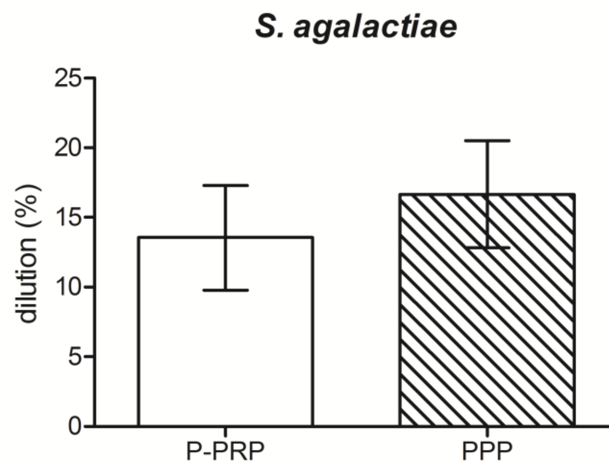
3.3. Antimicrobial activity of pooled donor P-PRP

3.3.1. Comparison among P-PRP, PPP and PLT

MIC of P-PRP and PPP are shown in Figure 14. For each microorganism, results were expressed as the mean of the MIC calculated for 5 clinical strains and one ATCC strain. As concentration of P-PRP and PPP expressed as n°platelets/µl is substantially different, MIC was expressed as dilution (%)

from the initial concentration. As can be seen from the figure, P-PRP and PPP were effective in inhibiting the growth of all bacterial strains tested at a dilution of about 15% (corresponding to a concentration of about 40.000 platelets/µl for P-PRP and 2.000 platelets/µl for PPP). Differences between effective concentrations were not statistically significant (P = 0,47 for *E. faecalis*, P = 0,57 for *S. agalactiae*, P = 0,84 for *S. oralis*). PLT alone did not show any antibacterial activity and then was not depicted in figure 14. Moreover, only $CaCl_2$-activated samples were able to inhibit bacterial growth. $CaCl_2$ per se alone did not show any antibacterial activity.





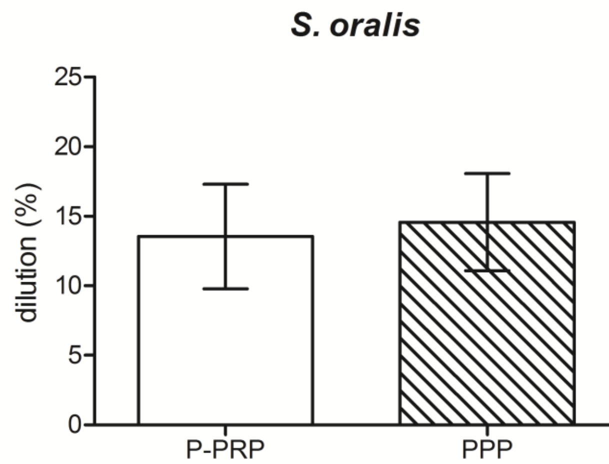
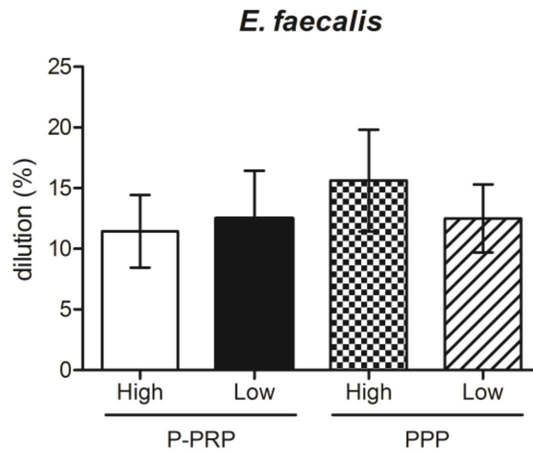
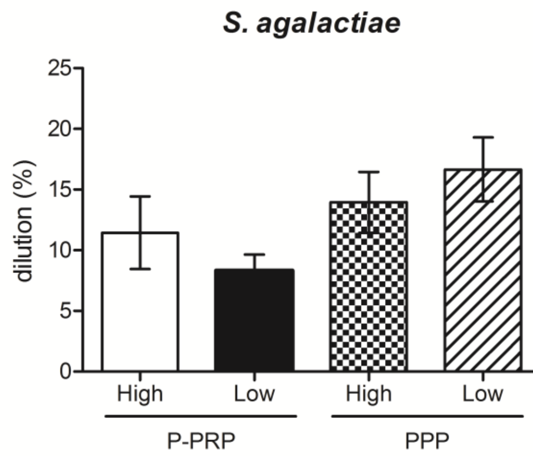


Figure 14. MIC of P-PRP vs. PPP. Error bars represent standard deviations.

3.3.2. Comparison between P-PRP and PPP from donors with different baseline platelet count

MIC of P-PRP and PPP obtained from patients with different baseline platelet count are shown in Figure 15. For each microorganism, results were expressed as the mean of the MIC calculated for 5 clinical strains and one ATCC strain. As concentration of P-PRP and PPP expressed as n°platelets/µl is not comparable due to a substantial difference, MIC was expressed as dilution (%) from the initial concentration. As can be seen from the figure, P-PRP and PPP were effective in inhibiting the growth of all bacterial strains tested, independent of baseline platelet concentration. The effective dilution of P-PRP and PPP was about 15% (corresponding to a concentration of about 90.000 and 2.000 platelets/µl for P-PRP and PPP from donors with baseline platelet count > 400.000 platelets/µl, and to 20.000 and 1.000 platelets/µl for P-PRP and PPP from donors with baseline platelet count < 150.000 platelets/µl, respectively) for all strains. Differences between effective concentrations were not statistically significant (P = 0,85 for *E. faecalis*, P = 0,13 for *S. agalactiae*, P = 0,99 for *S. oralis*). As seen before, only $CaCl_2$-activated samples were able to inhibit bacterial growth. $CaCl_2$ per se alone did not show any antibacterial activity.





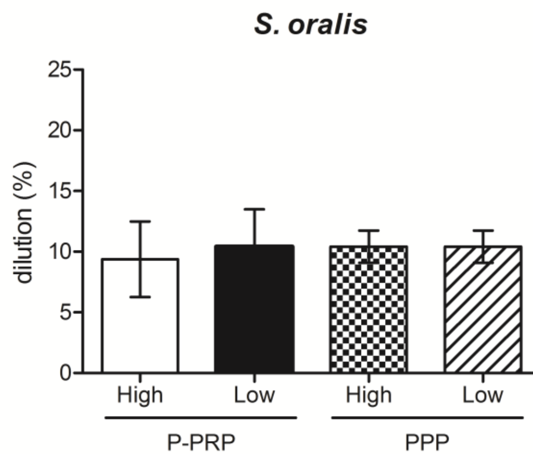
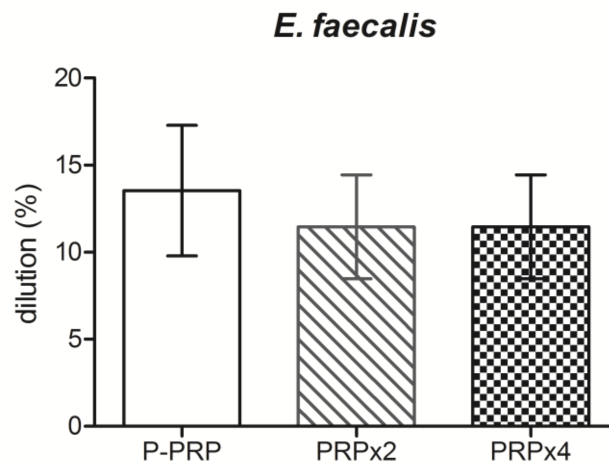
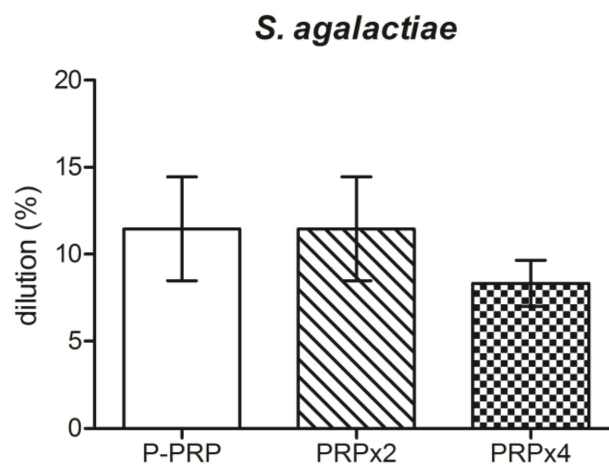


Figure 15. MIC of P-PRP vs. PPP from donors with different baseline platelet count. Error bars represent standard deviations. High = baseline platelet count > 400.000/µl. Low = baseline platelet count < 150.000/µl.

3.3.3. Comparison among P-PRP, PRPx2 and PRPx4

MIC of P-PRP, PRPx2 and PRPx4 are shown in Figure 16. For each microorganism, results were expressed as the mean of the MIC calculated for 5 clinical strains and one ATCC strain. As concentration of P-PRP, PRPx2 and PRPx4 expressed as n°platelets/µl were substantially different, MIC was expressed as dilution (%) from the initial concentration. As can be seen from the figure, P-PRP, PRPx2 and PRPx4 were effective in inhibiting the growth of all bacterial strains tested. The effective dilution of P-PRP, PRPx2 and PRPx4 was about 15% (corresponding to a concentration of about 50.000, 100.000 and 200.000 platelets/µl for P-PRP, PRPx2 and PRPx4, respectively) for all strains. Differences between effective concentrations were not statistically significant (P = 0,71 for *E. faecalis*, P = 0,87 for *S. agalactiae*, P = 0,62 for *S. oralis*). As seen before, only $CaCl_2$-activated samples were able to inhibit bacterial growth. $CaCl_2$ per se alone did not show any antibacterial activity.





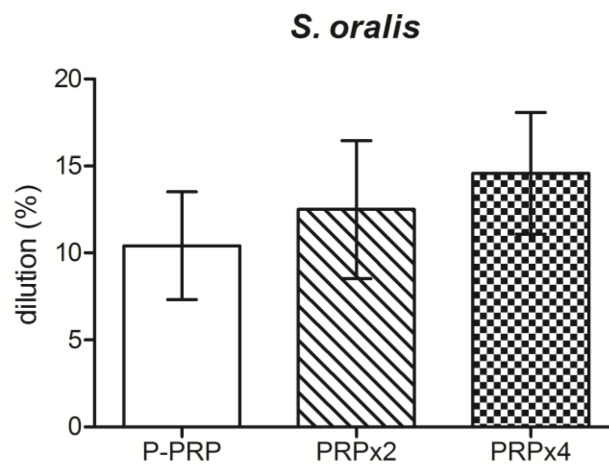


Figure 16. MIC of P-PRP vs. PRPx2 vs. PRPx4. Error bars represent standard deviations.

4.DISCUSSION

The regenerative potential of platelet concentrates has been explored considerably during the last two decades. On the contrary, in the available literature only few reports can be found about their antimicrobial effects. To date, the components responsible for the antimicrobial activity of platelet concentrates remain poorly understood, in particular because these materials are a complex mixture of platelets, white blood cells and plasma. The respective impact of the plasma and cellular components has not been studied in detail yet. Several antimicrobial factors have been proposed, including platelet antimicrobial proteins and peptides of the innate immune defense, or platelet α-granules components, such as complement and complement-binding proteins.(80, 109, 127, 133-135) Direct interaction of platelets with microorganisms and participation in antibody-dependent cell cytotocity and white blood cells in direct bacterial killing, release of myeloperoxidases, activation of the antioxidant responsive element and antigen-specific immune response have also been suggested.(125, 136, 137) The role of leucocytes within platelet concentrates is a matter of intense debate. Some authors have suggested that inclusion of white blood cells in platelet concentrates may help to improve the stability of the scaffold and increase the antimicrobial potential.(107) However, Anitua et al. results showed that the addition of leukocyte fraction did not significantly improve the antimicrobial properties of P-PRP.(191) It is also possible that the additional leukocyte content might increase the inflammatory response at the site because of the metalloproteases, pro-inflammatory proteases and acid hydrolases secreted by white blood cells.(192)

The first part of this study has shown that P-PRP was active against some of the microorganisms isolated from the oral cavity such as *E. faecalis, C. albicans, S. agalactiae* and *S. oralis*, but not against *P. aeruginosa.* Except for *E. faecalis* and *P. aeruginosa*, platelet concentrates have never been tested against such microorganisms.

E. faecalis is associated with different forms of periradicular disease, including primary extraradicular and post-treatment persistent infections.(193) Such microorganism possesses the ability to survive the effects of root canal treatment and persists as a pathogen in the root canals and dentinal tubules of teeth. Implementing methods to effectively eliminate *E. faecalis* from the dental apparatus is a challenge. We found that P-PRP was active at low platelet concentration ranges (1-2 orders of magnitude lower than the baseline blood values) against this microorganism, while Bielecki et al. observed no activity of platelet concentrate.(121) The reasons for this discrepancy may lie in the different protocol used for platelet concentrate production, which can lead to products with different biological characteristics, or in the different sensibility of the method (Kirby-Bauer disc-diffusion method) used to evaluate the susceptibility to platelet concentrate.

Oral candidosis is the most common fungal infection encountered in general dental practice. It manifests in a variety of clinical presentations and can occasionally be refractory to treatment. It is caused by commensal *Candida* species. While a large majority of healthy individuals harbor strains of *Candida* intraorally, only selected groups of individuals develop oral candidosis. The most commonly implicated species is *C. albicans*, which is

isolated in over 80% of oral candidal lesions.(194) In the present study, we observed that P-PRP was active against *C. albicans* at higher platelet concentration ranges (same order of magnitude of the baseline blood values) than those effective against the other bacteria tested. This result is consistent with the findings of Tang et al. who tested *in vitro* antimicrobial activity of seven antimicrobial peptides isolated from human platelets, and noticed that they were more potent against bacteria than fungi.(80)

Streptococcus strains are consistently present in the human oral cavity and are the major early colonizers of dental biofilm. Strains of many species of oral streptococci are generally commensal in humans. However, strains of other species, including those of *S. oralis* and *S. agalactiae*, may be more commonly associated with significant infections.(195, 196) In this study, we observed that P-PRP was active against *S. agalactiae* and *S. oralis* at platelet concentration ranges similar to the range which inhibited *E. faecalis*.

P. aeruginosa is an opportunistic bacterial pathogen that has been shown to integrate into oral biofilms.(197) Its presence has been associated with the onset of ventilator-associated pneumonia.(198) We found no activity of P-PRP against *P. aeruginosa* at the concentrations used in this experiment. This result is in line with the findings of Bielecki et al. and Burnouf et al., who even observed that platelet concentrate induced growth of this microorganism, suggesting that platelet concentrate may induce a flare-up of infection from *P. aeruginosa*.(121, 122) The value of platelet concentrates in the presence of a co-existing infection with this bacterium is therefore uncertain.

In our study we also used standard ATCC bacterial strains, which may behave in a way different from isolates, in order to assure reliability of results and reproducibility of experimentation. Results were similar to those obtained with clinical isolates of bacteria.

In addition, we performed a MMC test. We found such test difficult to perform, as P-PRP coagulates at high concentrations. MMC values couldn't be obtained for *C. albicans*, while MMC values for the other microorganisms were 3-4 times higher than MIC values. Further studies are necessary to investigate the true potential bactericidal effect of P-PRP.

The second part of this study was designed to elucidate which components of P-PRP can inhibit bacteria. We tested 1) P-PRP, which is the formulation commonly used in dentistry and oral surgery 2) PLTs resuspended in PBS, in order to determine the role of platelets isolated from the plasmatic components, and 3) PPP in order to define the antimicrobial role of plasma innate or humoral immune response.

Moreover, we tested the above-mentioned products before and after activation with $CaCl_2$, in order to evaluate their antimicrobial activity in the absence of activation of coagulation and release of platelets granules content.

Products were tested against *E. faecalis, S. agalactiae* and *S. oralis*, which have been shown to be susceptible to P-PRP activity in the first part of this study. All materials were prepared from the same pool of donors to limit the impact of individual donor variations. $CaCl_2$-activated P-PRP inhibited all strains, confirming the observations made in the first part of this study. Also

$CaCl_2$-activated PPP exerted a comparable antibacterial activity against all strains tested. By contrast, PLTs alone did not show any antibacterial activity. These data seems to suggest that the antimicrobial activity against *E. faecalis, S. agalactiae* and *S. oralis* is sustained by plasma components rather than platelet-derived factors. Another study tried to further understand which plasma components might bear the antimicrobial activity, testing samples after heat complement inactivation.(122) The absence of any bacterial inhibition by any of the heat-treated plasma and platelet materials supported the idea that the plasma complement (and/or other heat-sensitive compounds) played the major role in the bacteriostatic properties of concentrates.

Moreover, we observed that only $CaCl_2$-activated materials were able to inhibit bacterial growth, suggesting that the activation of coagulation is a fundamental step.

We also evaluated the effect of platelet concentration on the antimicrobial activity of platelet concentrate, comparing the activity of P-PRP and PPP obtained from a pool of patients having the baseline platelet count higher or lower than the physiological range. Sex and age were not significantly different between the two groups so as to reduce variability due to donor's individual features. As seen before, results showed that $CaCl_2$-activated P-PRP and PPP had a similar antibacterial activity against all bacteria tested, independent of the baseline platelet concentration.

Moreover, we compared $CaCl_2$-activated P-PRP, PRPx2 and PRPx4. All products were prepared from the same pool of donors to limit the impact of

individual donor variations. We observed that all of them had a similar antibacterial activity against the strains tested, independent of their platelet concentration. These results are consistent with the findings of previous studies showing no correlation between antimicrobial activity and the concentration of platelets in the blood and PRP.(121, 191)

Bacterial infection is one of the most serious complications impairing wound healing and tissue regeneration. Even when applying strict disinfection, bacteria can infiltrate and colonize the underlying tissues of the wound. The combination of proteolytic enzymes, toxin-rich bacterial exudates and chronic inflammation can alter growth factors and metalloproteinases, thereby affecting the cellular machinery needed for cell proliferation and wound healing.(199, 200) Developing approaches and strategies that may help to control or prevent the problem of wound infections would have considerable clinical, social and economic effects.

In addition to its established regenerative properties, in this study P-PRP demonstrated to possess also an antibacterial activity. Therefore P-PRP could be a potentially useful substance in the fight against postoperative infections and might represent the linking of osteoinductive and antimicrobial activity. The findings of the second part of this study may also have practical implications in the modality of application of P-PRP. In particular, it would be interesting to determine whether applications of the platelet-poor fraction on a wound, after the initial application of the platelet-rich fraction that enhance healing, may limit the risk of bacterial infection. Further research should investigate P-PRP antimicrobial capacity compared to antibiotics, its exact

antibacterial spectrum and prove its efficacy in the in vivo situation. The influence of patients' characteristics (sex, age, blood parameters like hematocrit, plasma proteins, platelet count, drug assumption, etc…) on antimicrobial activity should be also clarified.

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