Platelets

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# Platelets in wound healing and regenerative medicine

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**Abstract**

Although platelets are widely recognized as having a critical role in primary hemostasis and thrombosis, increasing experimental and clinical evidence identifies these enucleated cells as relevant modulators of other physiopathological processes including inflammation and tissue regeneration. These phenomena are mediated through the release of growth factors, cytokines, and extracellular matrix modulators that sequentially promote (i) revascularization of damaged tissue through the induction of migration, proliferation, differentiation, and stabilization of endothelial cells in new blood vessels; (ii) restoration of damaged connective tissue through migration, proliferation, and activation of fibroblasts; and (iii) proliferation and differentiation of mesenchymal stem cells into tissue-specific cell types. For these reasons, platelet-rich plasma (PRP) derivatives are used in regenerative medicine for the treatment of several clinical conditions including ulcers, burns, muscle repair, bone diseases, and tissue recovery following surgery. The benefits of PRP administration are associated with an economical advantage, taking into consideration that PRP administration does not require complex equipment or training for its execution. Moreover, due to their primary autologous origin, concerns of disease transmission or immunogenic reactions can be disregarded. Thus, platelet-enriched materials have become highly relevant in the last decade and constitute a growing focus of experimental and clinical study in the context of wound healing and tissue regeneration. However, despite the diverse applications, the efficacy of regenerative treatments using PRP is being called into question due to the lack of large controlled clinical trials and the lack of consensus regarding the PRP preparation techniques. This review describes the biological mechanisms underlying PRP's regenerative effects, the different methods of preparation and application of these biomaterials, and the controversies and future prospects related to the use of PRP in regenerative medicine.

**Keywords**

Growth factors, inflammation resolution, platelet-rich plasma, platelets, regenerative medicine, wound healing

**History**

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## Wound healing and tissue regeneration

Wound healing is a dynamic and physiological process for restoring the normal architecture and functionality of damaged tissue. This involves the sequential phases of (i) acute inflammation (minutes–hours–days after injury); (ii) proliferation and new tissue formation (days–weeks); and (iii) remodeling (weeks–months–years) (reviewed in (1–3)). The first stage of wound repair occurs immediately after tissue damage and begins with the formation of a platelet plug, followed by consolidation of a fibrin matrix that becomes the scaffold for infiltrating cells. Neutrophil and monocyte recruitments, followed by monocyte differentiation to the M1 macrophage phenotype, are triggered during the first 2–3 days of injury. These inflammatory and immune pathways are crucial to remove cellular debris and devitalized tissues and to prevent infection. After 3 days following injury, a switch occurs from an inflammatory profile to a resolutive one, which is characterized by a reduction in neutrophil recruitment, initiation of the switch from the M1 to M2 macrophage phenotype, and a reduction in proinflammatory cytokines release (reviewed in (1–3)). Resolution of inflammation overlaps with the initiation of the second stage of wound repair, which includes new tissue formation. This phase occurs between 1 and 3 weeks after injury, and it mainly involves the migration and proliferation of different cell types. One of the important processes of this stage is angiogenesis, defined as the growth of new blood vessels from preexisting ones, involving the action of endothelial cells (reviewed in (1-3)). Simultaneously, fibroblasts replace the fibrin matrix with granulation tissue composed of type-III collagen, elastin, proteoglycans, and hyaluronic acid (HA), which form a provisional scaffold for ingrowing blood vessels that provide nutrition and oxygen to the tissue (2). The third stage of wound repair is remodeling, which begins within 2–3 weeks of injury and could last for years depending on the wound characteristics. During this stage, blood vessels formed in the granulation tissue are no longer required and are removed by apoptosis. Due to the action of matrix metalloproteinases secreted by fibroblasts, macrophages, and endothelial cells, type-III collagen is replaced with the highly structured type-I collagen (2). The triad, which involves the immune system, extracellular matrix remodeling, and mesenchymal stem cells, orchestrates the tissue repair/regeneration processes depending on the intrinsic

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regenerative capacity of each tissue. Considering this, if the injury is caused in a highly regenerative tissue/organ (e.g., epithelium, liver, gut), a complete regeneration of damaged cellular components will be achieved through the differentiation and proliferation of resident stem cells (4). Depending on the lesion features, a complete regeneration would also occur in tissues with intermediate regenerative capacity (e.g., skin, bones, cartilage, tendons, and muscle). In contrast, if the injury was caused in tissues with a low regenerative capacity, such as the heart or central nervous system, tissues will be repaired but not regenerated, thus leaving a scar in the wound area (4,5).

## Wound healing challenges

A rapid resolution of the proinflammatory phase and transition into the regeneration phase is crucial to the outcome of tissue damage, and its dysregulation may aggravate complex diseases and prevent repair (3). Several aspects can affect wound healing, including local factors (presence of foreign bodies in the wound, tissue maceration, ischemia, or infection) as well as systemic factors (age, chronic inflammatory diseases including diabetes, medication, and malnutrition). These factors directly affect the physiological mechanisms of tissue regeneration causing several clinical complications including abnormal scarring (hypertrophic, keloid, and atrophic), pain, pruritus, tissue malignancy (Marjolin syndrome), hemorrhage, ulcer, infection, and amputation (5). Today, there are several methods to promote wound repair including synthetic matrices, biological tissue replacement, recombinant growth factors, and stem cell therapy (5–7). There are also local methods to promote circulation in patients with chronic wounds associated with neuropathies and vasculopathies. These include mechanical/physical methods (negative wound pressure therapy and intermittent pneumatic compression) and ionic methods (hyperbaric treatment with ozone) (8).

The use of growth factors derived from platelet-rich plasma (PRP) is another alternative to promote tissue regeneration (9–11). Like other methods, the molecular bases underlying the use of PRP, as well as the possible adverse effects and efficacy of these treatments, have not yet been fully elucidated. However, unlike other regenerative therapies, the use of PRP is an economical method since it does not require complex equipment or training for its execution. Moreover, due to their primary autologous origin, concerns of disease transmission or immunogenic reactions are neglected when modern procedures for donor screening and donation viral testing are in place and implemented following best practices (10). Thus, platelet-enriched materials have become highly relevant in the last decade and constitute a growing object of experimental and clinical study in the context of wound healing.

## Wound healing mediated by platelet-derived growth factors

Although platelets are widely recognized as having a critical role in primary hemostasis and thrombosis, increasing experimental and clinical evidence identifies these enucleated cells as relevant modulators of other physiopathologic processes including inflammation and tissue regeneration (12). These phenomena are mediated through the release of platelet's alpha-granule storage including growth factors, cytokines, and extracellular matrix modulators. The joint action of these factors sequentially promotes (1) revascularization of damaged tissue through the induction of migration, proliferation, differentiation, and stabilization of endothelial cells in new blood vessels; (2) restoration of damaged connective tissue through migration, proliferation, and activation of fibroblasts; and (3) proliferation and differentiation of mesenchymal stem cells into tissue-specific cell types (Table I) (11–15). Growth factors are also released by other cells, including endothelial cells, macrophages, fibroblasts, granulocytes, and mesenchymal cells. These cells synthesize growth factors *de novo* after hours or days of receiving a stimulus, and although these factors can be isolated *ex vivo*, this process is complex and expensive as it involves isolation, expansion, differentiation, and activation of each cellular type in culture (16). In contrast, growth factors are already preformed and stored in circulating platelets, and their release occurs within a few minutes. In this way, and unlike the other cells, obtaining growth factors derived from platelets is simple, which minimizes the time, manipulation steps, and risks associated with a transfusion component (17,18).

Although platelets also secrete antiangiogenic factors, levels of these molecules are negligible compared to the high levels of antiangiogenic molecules found in plasma, which contains both matrix-derived inhibitors (e.g., endostatin, thrombospondin, and tumstatin) and nonmatrix-derived inhibitors (e.g., angiostatin, soluble version of VEGFR-1 (sFlt-1), pigment epithelium-derived factor (PEDF), and prolactin), which can competitively and reversely interfere with several growth factors receptors (19–22). Moreover, our previous findings regarding the release of pro- and antiangiogenic molecules after the activation of thrombin receptors (PAR-1 or PAR-4) demonstrated that plasma-free platelets are more efficient than PRP as inducers of angiogenesis, suggesting that plasma interferes with angiogenesis induced by platelets (19). Further investigations are needed to evaluate the possible contribution of the antiangiogenic molecules present in plasma in the context of regenerative treatments with PRP.

In addition to its regenerative action, platelets also release molecules that promote defense against microbes. These include chemokines and cytokines that induce the recruitment and activation of immune cells, as well as microbicide proteins including kinocidins (e.g., PF4 (CXCL4), CXCL7 (also known as PBP), and CCL5); defensins (e.g., human β-defensin 2 (BD2)); thymosin β4 (Tβ4); and antimicrobial peptides (e.g., fibrinopeptide A or fibrinopeptide B and thrombocidins (which are proteolytic derivatives of CXCL7)) (23,24).

## PRP in regenerative medicine

Since the 1990s, platelet-derived products have been used in regenerative medicine. Early registers indicate that it was initially used in humans to promote macular hole healing followed by the application in odontology as grafting for dental implants (25,26). Application of PRP derivatives in regenerative medicine has rapidly diversified to the treatment of several clinical conditions, including ulcers, burns, muscle repair, bone diseases, and tissue recovery after surgery (9–11). PRP influences the migration, proliferation, and differentiation of several cell types, although the temporal features and molecular basis of this effect remain unclear. Cellular mechanistic insight into how PRP may be operating depends of the clinical scenario. An overview of PRP application and action on different medical fields is schematized in Figure 1. Specifically, the osteoinductive and collagen synthetic properties have led to the use of platelet derivatives in combination with standard medical and orthopedic treatments of tendon and ligament ruptures (10,27). In addition, PRP promotes bone, tendon, and cartilage regeneration by modulation several mechanisms including promoting MSC proliferation and chondrogenic differentiation (28); bone precursor cell proliferation (29,30); bone marrow mesenchymal stem cell proliferation and differentiation into osteoblast (30); mobilization of circulation-derived cells for tendon healing (31); bone cell proliferation and differentiation (32); chondrocyte proliferation and matrix biosynthesis (33); and angiogenesis as a crucial process in acute

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Table I. Effect of platelet-derived growth factors and cytokines (11–15).

| | Angiogenesis | Connective tissue healing and regeneration | Immune system | Others |
|---|---|---|---|---|
| PDGF (platelet-derived growth factor) | Growing vessel stabilization by supporting recruitment of pericytes (76,77) | Chemotaxis and mitogenesis of mesenchymal stem cells, fibroblast, osteoblastic cells, and oligooligodendrocytes (77,78)<br>Modulation of collagen synthesis and secretion by fibroblast (77). | Chemotaxis of monocytes and neutrophils (77)<br>Promotes M2 differentiation (77). | |
| bFGF (basic fibroblast growth factor) | Migration and proliferation of endothelial cells (79) | Chemotaxis and mitogenesis of mesenchymal stem cells, osteoblastic cells, chondrocytes, and fibroblasts (79) | | |
| TGF-β (transforming growth factor β) | Modulates angiogenesis (80) | Regulates osteoblastic cell mitogenesis as well as collagen synthesis and collagenase secretion (81)<br>Inhibits osteoclast formation and bone resorption (81) | Regulates adaptive immunity (82) | Maintains skin homeostasis by suppressing keratinocyte proliferation (83) |
| IGF-I (insulin-like growth factor-1) | | Chemotaxis of fibroblasts and stimulates protein synthesis (84)<br>Enhances bone formation by proliferation and differentiation of osteoblasts (85) | | |
| VEGF (vascular endothelial growth factor) | Migration and proliferation of endothelial cells (86,87) | Modulates activity of MMP and collagen secretion (87) | Chemotaxis of macrophages and granulocytes (86,87) | Vascular permeability (87)<br>Promotes lympho- and vasculogenesis (86,87) |
| EGF (epidermal growth factor) | Proliferation of endothelial and epithelial cells (88) | Promotes proliferation of fibroblast and collagen synthesis (88)<br>Differentiation of keratinocytes (90) | Promotes M2 differentiation (89) | |
| Angiopoietin-1 | Migration and proliferation of endothelial cells (91)<br>Growing vessel stabilization by supporting recruitment of pericytes (91) | | | Vascular permeability (91)<br>Vasculogenesis (91) |
| MMP (metalloproteinases) | Basal membrane dissolution promoting mobilization of endothelial cells (92) | Connective tissue remodeling (92) | | |
| SDF-1α (stromal cell-derived factor 1-α) (CXCL12) | Promotes chemotaxis, homing, proliferation, and differentiation of CD34+ cells into endothelial progenitor cells (93) | Chemotaxis of mesenchymal stem cells (94) | Chemotaxis and activation of leukocytes (95) | |
| CD40L (CD40 ligand) | Promotes survival, migration, and synthesis of growth factors in endothelial cells (96,97) | | Chemotaxis and activation of leukocytes (97) | |
| PF-4 (platelet factor 4) (CXCL4) | Interferes with the binding of VEGF and other growth factors on endothelial cells (98) | | Chemotaxis and activation of leukocytes (98) | Microbicide (99) |

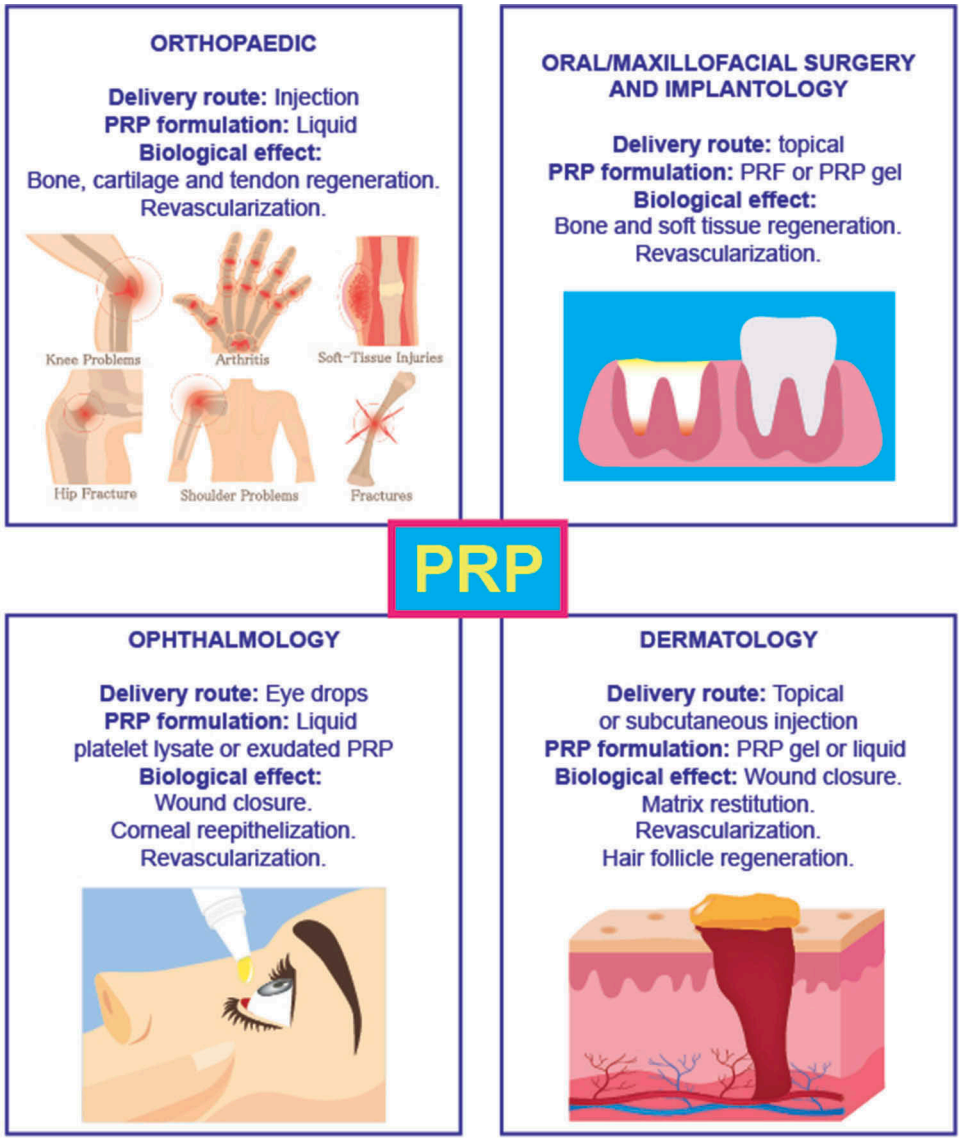


Figure 1. PRP on regenerative medicine. An overview about the application method and biological action of PRP on each medical field.

tissue injury repair (34). In the context of oral/maxillofacial surgery and implantology, PRP is able to regenerate bone as well as to induce proliferation of periodontal ligament cells (35), mesenchymal stem cells (36), and skeletal muscle satellite cells (37).

PRP is also used to regenerate dermal and epidermal damage tissues. PRP is widely applied in dermatology for the treatment of acute and chronic ulcers. Most works indicate that this phenomenon is mainly mediated by the effect of platelets on fibroblast. Indeed, PRP induces the migration, proliferation, and biosynthetic activity of dermal fibroblast promoting the extracellular matrix restauration as well as the differentiation of human dermal fibroblasts into myofibroblasts (38–40). Platelets also promote dermal revascularization (41) and restitution of dermal annex structures including hair follicle (42). Regarding restauration of epidermal damage tissue, several evidences indicate the use of platelet-derived biomaterial in ophthalmology. Platelet-derived products have shown to play a key role in the treatment of dry eye syndrome, dormant ulcers (epithelial defects of the cornea that fail to heal), and ocular surface syndrome after laser *in situ* keratomileusis and for surface reconstruction after corneal perforation associated with amniotic membrane transplantation (10). Several groups have focused their attention on understanding the different processes related to wound healing on the ocular surface. PRP promotes proliferation and migration of keratinocytes, conjunctival fibroblast cells, and endothelial cells (43,44). Human serum has a lubricating and mechanical-refractive action, but above all, it has a tropic effect for the epithelial cells. It contains a variety of growth factors, vitamins, and immunoglobulins, even in higher concentrations compared with natural tears (10,45).

Despite these diverse clinical applications, there are currently several controversies about the effectiveness of regenerative therapies with PRP. This is due to the absence of multicentre randomized trials with large sample sizes to validate these therapies. In this regard, De Pascale, Sommese and collaborators have performed a systematic review regarding platelets in regeneration by analyzing clinical trials that were indexed from 2010 to 2014 on PubMed, controlled-trials.com, clinicaltrialsregister.eu, eudract.ema.europa.eu, and clinicaltrials.gov (10). The conclusions from this analysis postulated that several individual studies found favorable treatment effects including three dentistry studies that showed an improvement in bone density; seven studies that showed positive results in joint regeneration; five studies that demonstrated an improvement in the wound healing; and two reports that found an

improvement of eye epithelial healing (10). Despite these individual studies, randomized controlled trials as well as meta-analyses have found no constant clinical benefit from the application of platelet-derived products for the prevention of tissue lesions (10). Furthermore, 14 clinical trials in phase 3 or phase 4 were ongoing at the time of this publication (10). Some of these clinical trials have been suspended due to slow enrolment of patients or study redesign; others have been concluded and the results not yet published, and the rest are still ongoing. Promisingly, the register of new clinical trials in phase 3 or phase 4 since 2015 has duplicated the total and historical registers, indicating that a certain insight about these therapies will be achieved when these large randomized trials will be completed. An update of the published clinical studies showing an improvement of outcome after platelet-derivative treatments is listed in Table II.

Although regulation regarding the use of PRP-based products varies in each country, a consensus regarding contraindications has been reached around the world. The use of PRP-based products is contraindicated in patients with syndromes of platelet dysfunction, thrombocytopenia, hemodynamic instability, sepsis, and local wound infection. Relative contraindications include avoiding use of PRP within 48 h of nonsteroidal anti-inflammatory drugs (NSAID) treatment or 2 weeks of treatment with systemic corticosteroids. Tobacco is also discouraged, or treatment is initiated in patients with fever, hemoglobin <10 g/dl, and platelet count $<10^5$/µl, and in patients who have had cancer (especially hematopoietic or bone cancer). To date, there is no compelling evidence of any systemic complications of local PRP injection including risk of carcinogenesis or thrombotic events (11).

Allogenic PRP therapy should be indicated by the physician after assessing the risks involved in each patient (46). The advantages of autologous over allogeneic platelet concentrates include avoiding any possible risks of contamination by donor-derived plasma viruses or prions and immune reactions associated with the internalization of allogeneic proteins. In practice, however, the volume of autologous platelets may be insufficient for clinical doses (9). Further limitations of autologous platelets include a lack of standardization due to variations in individual platelet count and growth factor composition that can be influenced by patients' age and overall biological condition. In contrast, allogeneic platelet concentrates, which can be processed as a pooled product by large-scale manufacturing, are produced according to national and international regulatory requirements for donor qualification, testing, and processing of blood components (9,47). These manufacturing protocols guarantee a product enriched with platelets and with minimal contamination of red blood cells (RBCs) and leukocytes (9,48). Thus, this allows large-scale, cost-effective, and standardized manufacturing of an allogeneic "off-the-shelf" product of platelets for regenerative treatments.

## Preparation of PRP-based biomaterials

Beyond the absence of large randomized trials, another challenge associated with these therapies is the lack of consensus on PRP preparation techniques. These procedures derive from classical protocols for obtaining platelet concentrates for transfusion, coagulation assays, or platelet functionality. Currently, there are several manual, automatic, and semiautomatic methods for this purpose (49). With some exceptions, the general methods for preparing PRP-based products for regeneration include three sequential steps: (i) blood collection, (ii) PRP separation, and (iii) PRP activation.

### Blood collection

Blood is collected with an anticoagulant using both open and closed systems. Acid-citrate-dextrose (ACD) is recommended for these protocols since it is approved for transfusion and available in most commercial kits designed for this purpose. Trisodium citrate or citrate-phosphate-dextrose (CFD) is also accepted (46,49). The effect of these citrated anticoagulants is antagonized by the addition of exogenous calcium. Most authors agree on not using ethylenediamine tetraacetic acid (EDTA) because it could damage the platelet membrane and transfused tissues (46,49).

### PRP separation

Platelets are concentrated after blood collection, and there is a high variability in protocols for this purpose. Most of these protocols include centrifugation steps with different times (4–20 min), velocities (100–3000 *x g*), temperatures (12–26°C), and cycles of centrifugation (one or two cycles) (9,10,49). Consequently, the concentration of platelets in the recovered PRP ranges from 300 to 1900 $x10^3$/µl (49). Several of these protocols aim to concentrate platelets between 5 and 9 times, to then obtain a final product that is more concentrated in platelet-derived growth factors. However, some controversies have been postulated about this issue because high platelet concentrations in PRP are reached by a combination of high centrifugation speeds, low temperatures, and various cycles of centrifugation (10,15,49). These conditions could induce a premature activation of platelets during centrifugation, altering the regenerative capacities of the final PRP-based product. Therefore, the increased number of platelets does not always ensure a high concentration of growth factors in the PRP-based final product.

### Classification of PRP

Upon centrifugation, blood is separated into three layers: the bottom layer with RBCs; the middle layer with platelets and white blood cells (buffy coat); and the top layer composed of plasma as a gradient of platelet concentration (platelet poor on the top, platelet intermediate on the middle, and PRP close to the buffy coat). Depending on which of these phases is collected, the volume of recovered PRP will be 2–40% of the total blood volume (49). The composition of PRP will also vary depending on whether the leukocyte-containing phase is collected or not. The contribution of leucocytes in PRP is currently seen as a double-edged sword, since although leucocyte-rich PRP (L-PRP) contains leukocyte's microbicide substances and enzymes that could contribute to prevent infections, these substances might also be capable of inducing inflammation, altering the extracellular matrix and damaging the cells involved in wound healing (50). Accordingly, an *in vitro* study has shown that the presence of leukocytes in PRP induces a proinflammatory phenotype in human fibroblasts and osteoblasts and interferes with cellular proliferation (51). Along the same line of evidence, a recent work performed *in vivo* using a model of wound healing in rabbits demonstrated that the implantation of mesenchymal stem cells combined with pure platelet-rich plasma (P-PRP) yielded better cartilage repair than that combined with L-PRP. This effect was due to the increased presence of proinflammatory cytokines derived from leucocytes (52). In addition to this possible deleterious effect of leukocytes, a recent systematic literature review performed by D'asta *et al.* indicated that there is not enough evidence to attribute the microbicide effect to the presence of leucocytes in PRP (53), arguing against the addition of leukocytes to PRP. Future studies are required to understand the beneficial or detrimental contribution of leucocytes in PRP. In particular, these investigations should be focused on the treatment of chronic

Table II. Published interventional clinical studies on platelet derivatives (years 2010–2017).

| Medical field | Platelet derivative | Delivery route | Outcome | Reference | Clinical trial register |
|---|---|---|---|---|---|
| Oral/maxillofacial surgery and implantology | PRF | Topical | Improves bone regeneration and soft-tissue healing after oral and maxillofacial surgery | (100) | - |
| | L- and P-PRP gel | Topical | Improves the quality and quantity of newly formed bone tissue on dental surgery | (101) | - |
| | L-PRP gel | Topical | Improves bone regeneration after cyst enucleation | (102) | |
| | L-PRP gel | Topical | Improves bone density after third molar surgery | (103) | - |
| | P-PRP gel | Topical | Improves regeneration of mandibular osteoradionecrosis defect | (104) | - |
| | L-PRF | Topical | Improves implant stability | (105) | - |
| | P-PRP gel | Topical | Reduces gingival recession | (106) | - |
| | P-PRP retracted clot (fibrin elastic matrix) | Topical | Reduces pain and inflammation and promote soft-tissue healing score after extraction socket | (107) | - |
| | P-PRP gel | Topical | Improves bone fill and bone density after dental implant | (108) | - |
| | P-PRP liquid activated immediately during application | Topical | Enhances the rate of bone formation in grafted maxillae | (109) | - |
| | L-PRF | Topical | Reduces pain and inflammation and promotes soft-tissue healing of simple postextraction socket | (110) | - |
| | P-PRP gel | Topical | Enhances bone formation on idiopathic bone cavity | (111) | - |
| | P-PRF and P-PRP gel | Topical | Improve healing of mandibular degree II furcation defects | (112) | - |
| | PRP gel | Topical | Improves bone regeneration in mandibular fractures | (113) | - |
| | PRP gel | Topical | Promotes revascularization of nonvital, immature, anterior teeth | (114) | - |
| Dermatology | ? | Topical | Accelerates complete wound healing after skin graft | (115) | NCT00856934 |
| | L-PRP gel | Topical | Improves heling of large-size recalcitrant ulcers | (116) | - |
| | L-PRP gel | Topical | Reduces ulcer wound size and improves the quality of patient's life | (117) | - |
| | L-PRP gel and liquid activated immediately during application | Topical and subcutaneous injection | Reduce wound size and healing time of diabetic foot ulcer | (118) | NCT03026855 |
| | L-PRF | Topical | Improves healing of postoperative wounds | (119) | NCT00931567 |
| | P-PRP liquid activated immediately during application | Intradermal injection | Stimulates histological changes in human skin | (120) | NCT00956020 |
| | P-PRP gel | Topical | Promotes complete healing and reduces the rate of wound infection in the treatment of diabetic foot ulcers | (121) | - |
| | P-PRP gel | Topical | Enhances wound closure rates of chronic skin ulcer therapy | (122) | - |
| | P-PRP gel | Topical | Improves score of diabetic chronic refractory cutaneous ulcers | (123) | - |

(*Continued*)

Table II. (Continued).

| Medical field | Platelet derivative | Delivery route | Outcome | Reference | Clinical trial register |
|---|---|---|---|---|---|
| Orthopedics | ? | Injected into the tendon | Reduces pain in tendinopathy and rheumatic diseases | (124) | NCT01600326 |
| | L-PRP liquid/inactivated | Injected into the paratenon sheath | Improved ankle range of motion after acute Achilles tendon rupture | (125) | - |
| | P-PRP liquid activated immediately during application | Injected into the posterior recess, gutters, and capsule | Prevents blood loss and postoperative pain and reduces narcotics indication after total knee arthroplasty | (126) | NCT01563380 |
| | P-PRP gel | Topical | Improves healing after transmetatarsal amputations in diabetic dysvascular patients | (127) | - |
| | PRP liquid activated immediately during application | Injection | Decreases pain after degenerative disorders of knee | (128) | - |
| | L-PRP gel | Topical applied to both the patellar and tendon bone plug | Reduces pain after patellar tendon graft harvesting | (129) | - |
| | P-PRP liquid/inactivated | Injection | Anti-inflammatory effect of PRP in the treatment of knee osteoarthritis | (130) | NCT02588872 |
| | P-PRP liquid activated immediately during application | Intraoperative injection during arthroscopy | Reduces pain and improves function after arthroscopy for knee degeneration | (131) | NCT02189408 |
| | P-PRP liquid/inactivated | Infiltrated into the lesion of the supraspinatus tendon | Reduces pain in rotator cuff disease | (132) | - |
| | P-PRP liquid activated immediately during application | Intra-articular injection | Reduces pain and improves clinical score on the treatment of hip osteoarthritis | (133) | - |
| | P-PRP liquid activated immediately during application | Intra-articular injection | Reduces pain on the treatment of knee osteoarthritis | (134–136) | - |
| | P-PRP liquid activated immediately during application | Injected into the capsule | Reduces blood loss after total knee arthroplasty | (137) | - |
| | P-PRP liquid/inactivated | Injection | Improves clinical and structural outcome after arthroscopic repair of medium-sized to large rotator cuff tear | (138) | - |
| | PRP gel | Topical | Reduces pain on large rotator cuff tear | (139) | NCT01266226 |
| | P-PRP liquid activated immediately during application | Injection | Reduces pain after anterior cruciate ligament injury | (140) | - |
| | P-PRP liquid activated immediately during application | Intra-articular injection | Improves self-reported pain, symptoms and lower extremity function in patients with knee osteoarthritis | (73) | ACTRN12611000651987 |
| | PRP liquid/inactivated | Injection | Promotes clinical improvement in pain, disability, and tendon pathology in degenerative tendinopathies | (141) | ISRCTN68341698 |
| | PRP liquid/inactivated | Injected into each of the affected discs | Reduces pain and improves function and patient satisfaction after lumbar intradiscal treatment | (142) | - |
| Ophthalmology | Autologous serum | Topical | Improves graft re-epithelialization after penetrating keratoplasty | (143) | NCT01075347 |
| | Allogenic serum | Topical (eyedrops) | Improves dry eye in patients with chronic graft-versus-host disease | (144) | - |
| | Autologous platelet lysate | Topical (eyedrops) | Improves treatment of macular holes | (145) | - |

inflammatory wounds (such us foot-diabetic ulcers) where physiological control mechanisms of leucocyte proteases are locally and systemically altered (50,54).

Moreover, L-PRP derived from buffy coat contains a considerable number of RBCs (55). The role of RBCs in PRP-regenerative treatments is largely unknown, with little data on the specific effects of this component of PRP. A recent study performed by Braun *et al*. showed that using PRP rich in RBC promotes synoviocytes cell death resulting in the release of catabolic mediators that may increase cartilage damage and contribute to joint degeneration (56). These data suggest that RBCs can be deleterious, particularly with intra-articular PRP injections for cartilage degeneration and osteoarthritis (55,57,58). Further studies will be required to elucidate the precise individual contributions of platelets, RBCs, and leukocytes to the beneficial or deleterious effects of PRP in different clinical scenarios.

Besides the cellular composition, PRP is also classified according to the amount of fibrin. In this regard, PRP was first classified into four groups: P-PRP; leukocyte- and platelet-rich plasma (L-PRP); pure platelet-rich fibrin (P-PRF); and leukocyte- and platelet-rich fibrin (L-PRF) (59). In 2012, P-PRP and L-PRP were also subclassified as activated or non-activated (60,61). More recently, Alsousou and Harrison have postulated a more detailed and complete classification system. This included a combination of numeric and alphabetical characters to identify the class of PRP based on the presence or absence of leukocytes (L or P), the fibrin content (high: PRF; low: PRP), activated or not activated (I or II), platelet concentration (A: $<900x10^3$/μl; B: 900-$1700x10^3$/μl; C: $>1700x10^3$/μl), and the preparation category (gravitational platelet sequestration technique; standard cell separators; and autologous selective filtration) (11). This simple, accurate, and pragmatic system of classification results in a unique term (e.g., L-PRP IB1) that reflects the product properties. This classification system could contribute to clarifying the confusion in the scientific literature and misleading conclusions currently observed regarding these treatments.

## Handling of PRP

Once PRP is collected, it can be applied immediately or stored. Some authors suggest using PRP within no longer than 6 h of blood collection (62), others indicate that an additive solution could allow it to be used for up 7 days after collection (63), and others suggest that it could be used even after long-term storage for years if PRP is frozen (64). Novel protocols include using freeze-drying PRP alone or in combination with more sophisticated methods such as three-dimensional (3D)-printed scaffolds (65). Although it is currently unknown whether not-fresh PRP is therapeutically effective, an approach has been recently published by Shiga et al. describing the measurement of growth factor levels in PRP samples that were either fresh or maintained at room temperature (RT), frozen or freeze-dried up to 8 weeks. They found that while growth factor levels markedly decrease after 2 weeks of storage at RT, they were maintained for 4 weeks followed by a significant decrease at 8 weeks in the frozen group. In contrast, the freeze-dried group maintained baseline levels of growth factors for the entire 8 weeks, and it is conceivable that they could remain intact for longer periods of time (66). These data indicate that freeze-drying enables PRP storage while maintaining bioactivity and efficacy for extended periods and could be considered for therapies that *a priori* necessitate multiple applications of PRP in the same patient.

## Activation and formulation of PRP

PRP is activated to induce the release of platelet alpha-granule storage. This process involves the generation of thrombin for inducing fibrin formation as well as platelet activation. The most used activation methods include the addition of calcium chloride ($CaCl_2$) or calcium gluconate ($C_{12}H_{22}CaO_{14}$) solutions to restore the levels of calcium chelated by anticoagulants. The equation describing the chemical interaction between calcium, trisodium citrate ($Na_3C_6H5O_7$), and citric acid ($C_6H_8O_7$) indicates that 22–25 mM of calcium is required to antagonize the effect of anticoagulants. Specifically, while trisodium citrate anticoagulant required 22 mM of calcium, CFD and ACD required 24–25 mM of calcium since they contain both trisodium citrate and citric acid. Controversially, the published protocols do not provide accurate information on this topic. For example, some protocols indicate that 0.2–0.5 mL of $CaCl_2$ should be added per 1 mL of anticoagulated PRP with ACD (10) or 1 mL of $CaCl_2$/thrombin mix for every 6 mL of anticoagulated PRP with CFD (67). In these examples, the final concentration of calcium is not specified; this is a critical experimental condition since the excess of calcium could affect coagulation and platelet activation by dissociation of FXIII and alteration of platelet membrane integrity (68). On the other hand, a recent publication has shown that the concentration of anticoagulants currently used for blood extraction protocols could be reduced by half to optimize platelet concentrates for regenerative medicine (69). Accordingly, a reduction in $CaCl_2$ concentrations is also considered for these settings (69).

Autologous thrombin is another activation method that is used alone (70) or in combined with $CaCl_2$ (67). Activation with bovine thrombin is not recommended as this was associated with coagulopathy due to the cross-reactivity of antibodies against human coagulation proteins (71). Depending on the protocol, the activation of PRP is induced for 20 min to 1 h either at 37°C (14) or at RT (72). A novel activation method described as "photo-activated PRP" involves exposing platelets to ultraviolet light (UV) irradiation. The underlying mechanisms of activation by UV are not completely elucidated, but the intra-articular injection of UV-activated PRP is growing for orthopedic treatments (73). Injecting inactivated PRP into soft tissues may also induce platelet degranulation by action of extracellular matrix collagen (74). Of note, calcium levels chelated by anticoagulants are not restored either by UV-activated or by inactivated PRP. Whether anticoagulants may locally damage the nonvascularized tissues that characterized wounds is not yet known.

Unlike the activation methods described above, special considerations apply for the activation of the platelet-rich fibrin (PRF) products, in which coagulation is induced during centrifugation. For obtaining P-PRF, blood is collected with anticoagulant and centrifuged, and then, plasma is transferred to a second tube. Clotting is triggered by the addition of $CaCl_2$, and the tube is immediately centrifuged, allowing the formation of a stable-rich fibrin clot during centrifugation (60). On the other hand, L-PRF is obtained through Choukroun's protocol, where blood is collected without anticoagulant and centrifuged. Thus, platelet activation and fibrin polymerization are triggered immediately during centrifugation. In this case, three layers are formed: the RBC layer, the acellular plasma top layer, and the PRF clot in the middle (75). The PRF clot forms a strong fibrin matrix with a complex 3D architecture that works as a scaffold for regeneration, and it is mainly used in oral, maxillofacial, ENT (ear, nose, throat), and plastic surgery (59).

Unlike the PRFs, the fibrin matrix is not strong in PRP, and it is contracted after activation, resulting in four different formulations previously described by Anitua *et al*. (14): (1) the liquid

PRP; (2) the "gel" of PRP or 3D scaffold; (3) the liquid-exudate PRP; and (4) the elastic and dense autologous fibrin membrane. The liquid PRP is activated at the time of use and applied by injection or embedded biological substitutes. In contrast, the 3D matrix or PRP "gel" is obtained after 15–20 min of activation and is used for the treatment of ulcers, wound closure, and tissue engineering. This formulation can be combined with other materials, such as autologous bone, demineralized freeze-dried bovine bone, or collagen, adjusting the resulting characteristics of the scaffold. After 40 min of activation, clot retraction becomes evident, allowing the formation of the liquid exudate (3) and the retracted clot (4). The exudate contains plasma proteins and platelet releasate, and it can be used as eyedrop treatment for dry eye disease and other corneal defects. The elastic fibrin membrane obtained after clot retraction is used as a socket sealant after tooth extraction and to promote the epithelialization of soft tissues (14).

Although there is no specific information about how the differences in PRP preparation could lead to mechanistically different actions, there is an association between the type of PRP-derived material with some clinical treatments as is specified in Table II. In summary, for oral/maxillofacial surgery and implantology, most protocols use the solid (PRF) or gel formulations. On the other hand, both gel and liquid formulations are used in desmatology (for wound healing and ulcer treatment) and orthopedics (for the treatment of knee osteoarthritis, chondropathies, and tendinopathies). In contrast, in ophthalmology the PRP releasates or platelet lysates are the first choise ftreatment for penetrating keratoplasty, macular holes, and dry eye. Regarding the cellular composition of these biomaterials, the presence of leucocytes is avoided in intra-articular treatments protocols probably for avoiding local tissue inflammation (56–58).

## Conclusions and future perspectives

PRP is used in regenerative medicine for treating several clinical conditions, including ulcers, burns, muscle repair, bone diseases, and tissue recovery after surgery. Despite diversity in applications, the efficacy of the regenerative treatments using PRP is being called into question due to the lack of large controlled clinical trials and the lack of consensus regarding PRP preparation techniques. These procedures derive from classical protocols for obtaining platelet concentrates for transfusion, coagulation assays, or platelet functionality, which are focused on preserving platelet hemostatic but not regenerative responses. Despite these controversies, recent clinical trial results demonstrate promising clinical benefits in dermatology, dentistry, ophthalmology, orthopedics, and densitometry. A certain insight into these therapies will be achieved when the large randomized trials currently ongoing in phase 3 or phase 4 will be completed.

Further investigations are needed to understand the questions surrounding the mechanisms of PRP in tissue regeneration, including the following: What is the effect of antiaggregating drugs on these treatments? What are the biological mechanisms underlying the use of these therapies? What is the regenerative capacity of PRP derived from patients with chronic inflammatory diseases? What are the possible long-term adverse effects associated with the use of PRP? Which is the optimum composition required to induce the maximal regenerative response? The answer to these questions will contribute to achieving the best efficacy of regenerative therapies mediated by PRP.

## Image source

In Figure 1, drawing of odontology images were adapted from <a href = "https://www.freepik.com/free-photos-vectors/icon">Icon vector created by Photoroyalty - Freepik.com</a> under the free for commercial use with attribution license. The drawings of orthopedic, ophthalmology, and dermatology were purchased and adapted from www.shutterstock.com under standard license (orthopedic from Rvector/shutterstock.com; ophthalmology from solar22/shutterstock.com; and dermatology from gritsalak karalak/shutterstock.com).

## Declaration of interest

The author declares no competing financial interests.

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