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Joint pathology and platelet-rich plasma therapies

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Introduction: At the beginning of the new millennium, there was a breakthrough in platelet-rich plasma (PRP) therapy for tissue repair. The mechanisms governing the effects of this therapy in joint pathology remain largely unexplored.

Areas covered: This review is primarily based on PubMed and Web of Knowledge searches with the terms osteoarthritis in combination with PRP, treatment, cartilage, synovium, platelets, inflammation and/or angiogenesis. This search was completed by a manual search for relevant studies. We mainly include papers from the last 5 years. The concept of dynamic reciprocity is used to shape understanding of the spatial relationship between cells and their microenvironments as well as between tissues within the joint. We describe the processes of joint injury and pathology relevant to the mechanism of action of PRP, and elaborate insights into how PRP components may influence inflammation, angiogenesis, cell death and cartilage chondroprotection.

Expert opinion: PRP therapies are more complicated than previously acknowledged, and an understanding of the fundamental processes and pivotal molecules involved will hopefully be elucidated soon. This challenge is to provide a comprehensive description of the relationship between PRP components, healing mechanisms and clinical outcomes. Although PRP therapies in clinical trials await assessment, they have shed light on new avenues of management because of their effects on repair functions.

Keywords: joint injuries, osteoarthritis, platelet-rich plasma, tissue healing

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1. Introduction

The dramatic increase in the incidence and prevalence of joint pathology over the past two decades has focused attention on therapeutic interventions to reverse or ameliorate progressive joint damage and pathology. Degenerative osteoarthritis is the most common form of arthritis, and affects nearly 27 million adults in the USA [1]. In general, its diagnosis is established too late in the disease process to expect much from current therapies. Moreover, different conditions have been included in the same category, erecting a barrier against treatment development. The lack of effective treatments to slow disease progression worsens patient prognosis and results in a significant economic burden. For example, post-traumatic osteoarthritis in the USA costs more than $12 billion annually [2].

In the past decade, the development of platelet-rich plasma (PRP) therapies to improve tissue repair has rapidly expanded and has intrigued clinicians and scientists [3]. This is especially true in orthopedics and sports medicine. These new therapeutic approaches involve the delivery of an efficient combination of bioactive compounds to the site of injury to trigger diverse biological mechanisms involved in the healing process [4]. Although the clinical readiness of these therapies is questionable, some PRP therapies are currently used in anterior cruciate ligament (ACL) reconstruction [5], arthroscopic cartilage treatment [6] and in the management of

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chondropathies and osteoarthritis [7-12]. Herein, we discuss the current state of research in this field and highlight the properties of PRP, as an increased understanding of the biological terms in question will aid in the quest to tailor PRP therapies for the treatment of joint pathology.

2. Dynamic reciprocity

The concept of dynamic reciprocity is used to shape an understanding of the spatial relationships between joint components. Dynamic reciprocity describes the bidirectional interactions between cells and the extracellular matrix (ECM) or other components of the cell microenvironment (**Figure 1**). These numerous interactions regulate cellular functions and maintain tissue/organ architecture during periods of homeostasis. When injury modifies this microenvironment, cells receive signals to differentiate or dedifferentiate, to proliferate or remain quiescent and to initiate the processes of angiogenesis, inflammation, anabolism/catabolism and apoptosis/necrosis. Accordingly, two primary sources of environmental signals determine the biological nature of dynamic reciprocity within the joint capsule.

2.1 Cell–ECM interactions

The first component regulating dynamic reciprocity is the extracellular matrix (ECM), a fundamental component of all joint tissues, particularly in cartilage. The ECM is an evolving structure that rapidly responds to mechanical disruption and inflammation. Under normal conditions, chondrocytes not only synthesize ECM components (predominantly type 2 collagen and proteoglycans) but also degrade them via MMPs and the production of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). Thus, ECM degradation can affect cellular adhesion as well as the bioavailability of signaling and adhesive molecules [13]. Pericellular proteolysis of the ECM can lead to chondrocyte detachment and death, but conversely, cell-to-matrix engagement can increase the expression of anti-apoptotic proteins [14]. This combination of matrix destruction and cell death is a hallmark of degenerative joint diseases. Several proteinases, including MMPs, ADAMTS, neutrophil elastase and cathepsin G, are capable of degrading aggrecan. Type 2 collagen is resistant to most proteinases because of its triple helix structure, although it can be degraded by MMP-13. When the collagen molecules are cleaved, this molecule is denatured and becomes gelatin, which can be further digested by the gelatinases MMP-2 and -9.

2.2 Interactions between the joint tissues and synovial fluid

Another powerful source of extrinsic signaling factors is the synovial fluid, which bathes all joint tissues and produces a unique framework for communication between different cell phenotypes at the organ level. Essentially, synovial fluid is a dialyzed form of blood plasma, which contains hyaluronan, lubricin, systemic signaling factors and cytokines produced locally [15]. As synovial fluid is pumped through intra-capsular tissues, it transmits signals from one tissue to another to constitute a dynamic network of cytokines signals with multidirectional pathways of influence. Indeed, synovial fluid enables signals to reach cells in different tissues at the organ level. Signaling molecules enter the articular cartilage not only from the synovial fluid but also from the calcified cartilage of the subchondral bone, which makes each of these locations a functional unit [16]. Several compounds can diffuse through cartilage from the synovial fluid as well as from the subchondral bone. Thus, a detailed understanding of dynamic reciprocity requires the consideration of cartilage as well as all of the tissues within the joint, which include the menisci, subchondral bone, synovium, ligaments, infrapatellar fat pad, periarticular muscles, the joint capsule and the neural tissues. Furthermore, the mutual interactions between each of these components must be continually redefined [13].

A theoretical framework for the role of PRP in joint pathology has not yet been described. In this context, deciphering which cells interact and how they interact with PRP will require a nuanced understanding of joint injury and pathology. The nexus between the actions of PRP and those in joint injury and pathology occurs at many levels from the modulation of the immune response and angiogenesis to interfering with cell death and matrix destruction.

3. Joint injuries

The growing epidemic of joint injuries among today's athletes most commonly pertains to injuries of the knees and hips, although injuries to the acromioclavicular joints are also frequent in some sports. Joint injuries vary in severity from mild sprains (of a ligament, tendon or damage to muscle) to overt fractures and dislocations. Traumatic joint injuries initiate a dynamic sequence of early biological events that include bleeding, cell death and prominent inflammation (**Figure 2**).

3.1 Bleeding

Intra-articular bleeding occurs in many patients following joint trauma and surgical procedures, including osteochondral fractures and ligament or meniscus tears. Intra-articular blood can have a directly noxious effect on the tissue following the formation of iron-catalyzed oxygen metabolites that induce the pro-inflammatory activation of macrophages [17] as well as hemoglobin-stimulated MMP-2 and MMP-9 production by synovial cells. From an experimental study in dogs, a single joint injury that resulted in bleeding led to disruption of the cartilage matrix turnover for at least 2 weeks [18]. Moreover, a single joint hemorrhage (4 day exposure of cartilage to 50% v/v) resulted in chondrocyte death and an inability to restore proteoglycans PG during recovery [19-21]. These findings are also further supported by studies on hemophilia that have shown how repetitive intra-articular bleeding episodes caused the progressive destruction of the joint [22,23]. Thus, the aspiration of blood from the joint should be considered

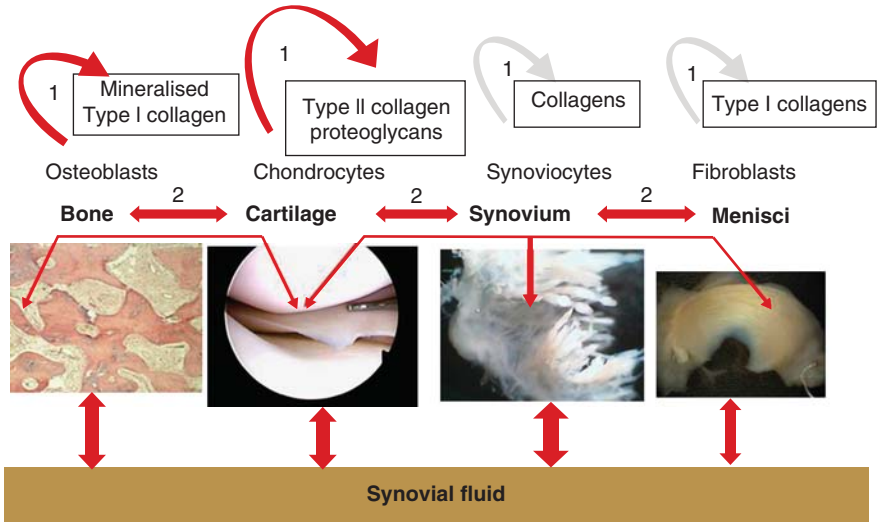

Figure 1. Dynamic reciprocity. Applying dynamic reciprocity in an intra-articular context may help us to understand the social interactions and relations of mutual dependence between different cell phenotypes. The concept of dynamic reciprocity is instrumental in understanding the bidirectional interactions between cells and the extracellular matrix and molecular cross-talk between different cell phenotypes through synovial fluid and the bone-cartilage unit (through calcified cartilage).

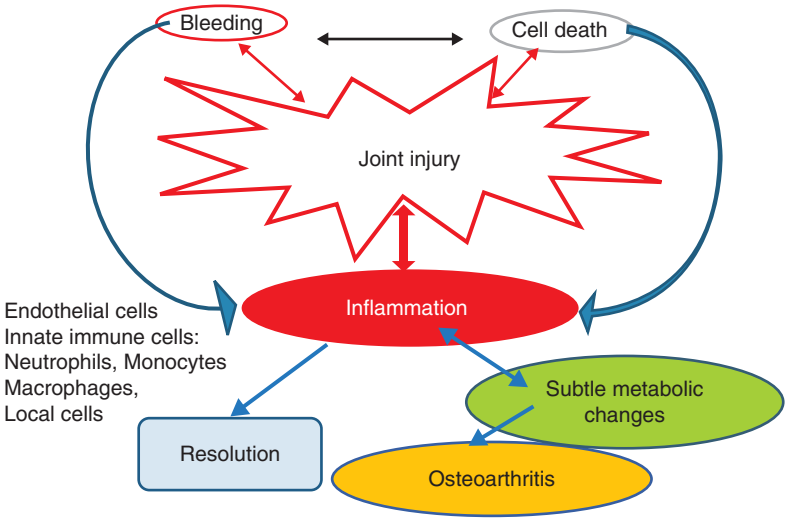

Figure 2. Previous joint injury is a major risk for the development of post-traumatic osteoarthritis, at 10 – 20 years post-injury. Subtle metabolic changes in the synovium, cartilage and/or bone slowly progress during a long sub-clinical period to a symptomatic phase characterized by pain and joint dysfunction.

to prevent long-term blood-induced joint damage. In addition, PRP injections following joint trauma may be beneficial to retard the degeneration of cartilage and the development of post-traumatic osteoarthritis [19,24].

3.2 Cell death

Apart from bleeding, joint injury is further augmented by cell death [25]. Cell death resulting from both controlled and uncontrolled mechanisms (apoptosis versus necrosis) has a marked effect on inflammation. The mechanism of cell death depends on the nature of the death signals present in the local milieu as well as the tissue/cell type [26]. Cellular death also results in the generation of various phagocytic signals, which determine the immunological consequences of dead cell removal. For example, apoptosis (controlled programmed cell death) evokes an anti-inflammatory response that suppresses immune responses [27]. Indeed, apoptosis is a common cellular process at all locations within the human body;

approximately 2 – 400 billion cells die each day without evoking inflammation. In the context of apoptosis, phagocytes take up apoptotic cells while releasing anti-inflammatory cytokines such as IL-10, TGF-β, platelet factor 4 (PF4) and prosraglandin E2 (PGE2) and inhibiting the production of pro-inflammatory cytokines such as TNF-α, GM-CSF, IL-12, IL-1β and IL-18 [28]. Conversely, cellular necrosis (a catastrophic form of cell death) is a passive, uncontrolled process toxic to neighboring cells, which evokes a potent pro-inflammatory response. In experimental acute mechanical trauma [29,30], chondrocytes primarily die by necrosis in the first 12 h after trauma and there is minimal cell death in the subsequent days. However, the picture has become more complicated, as biologists are now struggling not only to identify necrosis but also other intricate types of cell death including anoikis (cell death induced by loss of adhesion) and autophagy (the process of cellular self-digestion), which occur during prolonged periods of stress and can result in cell senescence and death. Furthermore, cross-talk between the various cell death pathways exists at multiple levels [31].

3.3 Inflammation

Joint bleeding and cellular death contribute to the onset of inflammation, a protective mechanism to remove damaged cells and initiate healing. Upon injury, endothelial cells in the vessel walls become activated and express cell adhesion molecules (selectins, intercellular cell adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM)). In doing so, these cells recruit specific populations of leukocyte subsets that respond to gradients of chemoattractants to transmigrate towards the inflammatory stimulus in the articular cavity. Neutrophils, which can be detrimental to the healing process [32,33], transmigrate across gradients of newly synthesized chemokines including regulated upon activation, normal T cell expressed and secreted (RANTES)/CCL5 and CCL2 [34]. There are several key ways in which neutrophils are detrimental to joint health. First, these cells release reactive oxygen species, cationic peptides, proteases and inflammatory cytokines such as IL-1β and TNF-α, which increase MMP and ADAMTS production by synovial cells and chondrocytes, and promote catabolism in the ECM of cartilage and menisci [35]. In addition, neutrophils produce elastases, which alter the lubricating ability of the synovial fluid through the degradation of lubricin and hyaluronan and aggravate cartilage damage [36]. Moreover, lysosomal enzymes and elastases can impair tissue repair because of the degradation of the growth factors that are essential for healing. Finally, there is a proven link between neutrophil migration and inflammatory pain [37]; nerve terminals can influence neutrophil recruitment through the molecular signals responsible for so-called neurogenic inflammation.

In addition to their role as macrophage precursors, monocytes influence the nature of an immune response in tissues as well as the blood. Macrophages are highly heterogeneous and their activation state is context-dependent. For example, upon stimulation with lipopolysaccharide or IFN-γ, macrophages become a major source of IL-6, IL-1β and TNF-α. Alternatively, the presence of IL-4/IL-23 in the tissue microenvironment is associated with macrophage synthesis of the growth factors TGF-β, TGF-α, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and VEGF [38,39].

The resolution of inflammation and the return to joint homeostasis requires a cessation of inflammatory cell recruitment and efficient dead cell clearance by macrophages, which also release factors that suppress inflammation, promote tissue recovery and reduce pain. Another essential aspect of this return to homeostasis involves the role of the stromal cell. Although these cells contributed to the inflammatory event, they revert to a non-inflammatory phenotype during recovery [40].

No single hypothesis can explain why previous trauma is a major risk factor for the development of post-traumatic osteoarthritis 10 to 20 years after the injury. One hypothesis is that trauma sequelae (i.e., subtle metabolic changes in the cartilage, synovium and/or bone) progress slowly during a lengthy sub-clinical period towards the symptomatic phase characteristic of joint pain and dysfunction. For example, central bone marrow lesions that abut the ACL are strongly related to osteoarthritis [41,42]. In addition, individuals with traumatic knee disorders and HA exhibited similar alterations in synovial tissue inflammation, which suggests the likely progression from trauma to disease [43]. Recently, Atkas *et al.* [44] demonstrated elevated levels of plasma TNF-α post-trauma, which is consistent with articular cartilage damage and the role of this cytokine as a serum marker for ongoing joint inflammation. Furthermore, aberrant loading, or mechanopathology (both macroscopic and microscopic), instigates pathology in these tissues [45].

4. Osteoarthritis

Osteoarthritis results from a failure of the damaged cartilage repair process due to biomechanical and biochemical changes in the joint (**Figure 3**). Studies of arthritic cartilage demonstrated chondrocyte hypertrophy, increased production of ECM molecules and increased bone turnover to be components of this disease. Furthermore, the development of osteophytes as part of this attempted repair process is a major feature of osteoarthritis. In parallel to the episodes of inflammation and vascular pathology, disease aspects such as cell death, menisci changes and the remodeling of subchondral bone create a vicious cycle of progressive joint degeneration. The clinical signs associated with these changes include pain, rigidity and decreased functionality.

4.1 Inflammation

Over the past two decades, the classification of osteoarthritis as an inflammatory disease in which inflammation can

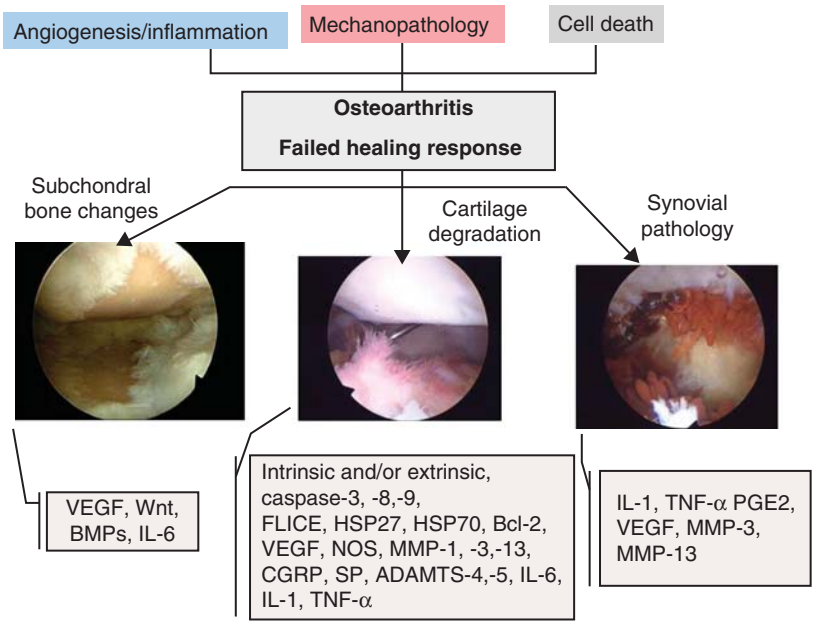

Figure 3. The prevailing paradigm suggests that osteoarthritis is a disease of the entire joint. Subchondral bone changes with increased metabolism and sclerosis; chondrocyte death and extracellular matrix catabolism; primary or secondary changes in the synovium including endothelial cell proliferation, macrophage infiltration and inflammation with subsequent alterations in the molecular composition of the synovial fluid.

ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs; Bcl-2: B-cell leukemia associated protein 2; BMP: Bone morphogenic protein; CGRP: Calcitonin gene-related peptide; FLICE: Fas-associating death domain- IL-1β-converting enzyme-like; HSP: Heat shock protein; PGE: Prostaglandin E; SP: Surfactant protein.

contribute to disease progression has gained popularity because of the results of imaging studies. Inflammation can affect the synovium and the subchondral bone separately. Whereas subchondral bone changes are identified by MRI, arthroscopy provides a direct view of the heterogeneous alterations of the synovium and has shown that knee effusion is associated with the presence of synovitis, which is directly linked to the clinical symptoms of joint swelling and inflammatory pain [46]. Alternatively, subchondral bone cysts and edema compromise the integrity of the articular cartilage, and endogenous products resulting from matrix degradation, including type 2 collagen fragments, cartilage oligomeric matrix protein (COMP), fibronectin (FN) and fibromodulin, can further activate immune cells to perpetuate inflammation [47].

Synovial macrophages play a role in triggering inflammatory and destructive states through the production of IL-1β and TNF-α, which stimulate chondrocytes to produce inflammatory mediators such as PGE2, NO, superoxides and proteolytic enzymes (MMPs and ADAMTs) [48,49]. The macrophage inflammasome also triggers the production of the alarmins S100A8 and A9, which are expressed in early osteoarthritis [50], and function as endogenous danger signals to elicit synovial activation. IL-1β and TNF-α (as well as mechanical stress and ECM degradation products) signals trigger NF-κB transcription factors to promote an inflammatory, activated state in synovial fibroblasts. Activated NF-κB regulates the expression of additional inflammatory regulators, such as IL-6, IL-10, GM-CSF, IL-8 and monocyte chemoattractant protein MCP-1, which, in turn, trigger matrix degrading enzymes such as MMP-1, -3 and -13 and aggrecanases such as ADAMTS-4 [51]. The subsequent fragmentation of the ECM (type 2 collagen, fibronectin and hyaluronan) reveals cryptic epitopes that stimulate the release of NO, chemokines and cytokines and the activation of the MAPKs [52].

As a result of their central roles in inflammation and joint destruction, TNF-α and IL-1β have become important therapeutic targets. In contrast to the TNF-α-driven IL-1β production during rheumatoid arthritis (RA), blocking either TNF-α or IL-1β in the treatment of osteoarthritis is futile, because both cytokines transmit inflammatory messages independently (redundancy). Thus, the use of autologous preparations containing soluble receptors that simultaneously block the activities of IL-1 and TNF (e.g., Ortokine or autologous protein solution (APS)) may further reduce joint catabolism [53,54].

The inflammatory milieu is further augmented by adipose tissue present in osteoarthritis synovium and the infrapatellar fat pad. This adipose tissue is the main source of adipokines, such as visfatin, adiponectin and leptin, which are capable of modifying the inflammatory and destructive responses in cartilage and synovium [55]. Inflammation present in the

synovium can also trigger changes in the peripheral nervous system. These can affect the afferent processing of nociceptive signals from the joint and surrounding tissues to cause pain, the main clinical symptom of osteoarthritis.

4.2 Angiogenesis

Angiogenesis is differentially regulated in cartilage versus subchondral bone by the very sensitive interplay of pro-angiogenic growth factorss and inhibitors, the imbalance of which can lead to the onset of osteoarthritis. In physiological conditions, chondrocytes are a source of endogenous inhibitors of angiogenesis, such as troponin 1, chondromodulin and tissue inhibitors of metalloproteases (TIMPs). However, in pathological conditions, pro-angiogenic factors are upregulated. Angiogenesis is often accompanied by inflammation, which facilitates plasma extravasation and the recruitment of inflammatory cells. The link between angiogenesis and inflammation is underscored by the upregulation of VEGF, also known as permeability factor (PF) because it facilitates plasma extravasation, endothelial cell proliferation and the outgrowth of capillaries. The expression of VEGF is augmented in osteoarthritis-affected joints by several mechanisms. For example, IL-1β and TNF-α can directly stimulate VEGF production by synovial cells, bone cells and chondrocytes. Moreover, low oxygen tension or hypoxia in the inflamed synovial joint promotes the activation of hypoxia-inducible factor 1α (HIF-1α), which is followed by VEGF expression [56]. VEGF and its receptors are expressed in an autocrine fashion by osteoarthritis chondrocytes, and these cells also produce increased levels of MMP-1, MMP-3 and MMP-13 and decreased levels of TIMP-1 and TIMP-2. Furthermore, neovascularization of the non-calcified articular cartilage is associated with its innervation by fine, unmyelinated sensory nerves. Nerve growth factor (NGF) expression and sensory nerve growth may link osteochondral angiogenesis to pain in osteoarthritis [57].

In contrast to the avascular physiology of non-calcified cartilage, subchondral bone vessels provide nutrients to calcified cartilage [16]. Thus, in addition to osteocyte death, ischaemia in subchondral bone causes bone resorption and articular damage. In particular, patients with hip osteoarthritis exhibit a high prevalence of vascular-related comorbidities, and a causal link between the progression of osteoarthritis and atheromatous vascular disease has recently been proposed [58]. Subchondral angiogenesis is also associated with growth factor expression by cells within subchondral spaces and vascular channels and with molecular changes in the environment [59].

4.3 Cell death and matrix catabolism

Osteoarthritis is characterized by, but not limited to, chondrocyte senescence and a loss of cartilage integrity. There is mounting evidence that excessive mechanical damage and/or stimulation by cytokines induces a senescent phenotype in chondrocytes. Extrinsic inducers of cell senescence and subsequent telomere shortening include oxidative DNA damage caused by reactive oxygen species (ROS). Recent findings suggest that, under metabolic or cytotoxic stress conditions, autophagy, the process of cellular self-digestion that protects cells during stress responses, can be upregulated to induce cell senescence. Autophagy is a protective mechanism in normal cartilage, but aging-related changes have also been shown to link autophagy with cell death and osteoarthritis [60]. Deranged autophagy leads to cell death and an impairment of the regenerative capacity of cartilage.

In addition to causing cell-cycle arrest, senescence is associated with the secretory phenotype with production of IL-6, IL-1, MMPs and EGF. Aging-induced senescence also reduces the ability of a cell to cope with stress. For example, IL-1β or fibronectin fragments stimulated chondrocytes from older adults to produce more MMP-13 (a major mediator with the highest activity for type 2 collagen) than those from young adults. Another feature of chondrocyte senescence is the decline in the proliferative and anabolic responses to growth factor stimulation. For example, there is a substantial age-related decline in the ability of IGF-I (a well-known anabolic factor) to stimulate proteoglycan and collagen synthesis. Furthermore, the accumulation with age of advanced glycation end products (AGE) in the cartilage ECM affects its mechanical properties and matrix integrity [50].

4.4 Pain

Although pain is the main clinical symptom of osteoarthritis, its relationship with joint damage is poorly understood and is confounded by the co-morbidities associated with age and stage of the disease. The source of the pain is not the cartilage, which has no pain fibers. Nociceptive fibers have, however, been described in the joint capsule, synovium, meniscus, bone marrow, periosteum and subchondral bone (and in the marrow cavities of osteophytes) [61]. For example, pain has been correlated with bone marrow lesions and synovitis. Inflammation in the synovium triggers changes in the peripheral nervous system, which affect the afferent processing of nociceptive signals from the joint and the surrounding tissues. Classical pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, are associated with pro-algesic effects [62]. Furthermore, neurotrophins, and especially NGF, mediate injury-induced and inflammatory pain by regulating the excitability of nociceptor fibers and altering the expression of key sodium channels, receptors and neuropeptides involved in the transmission of pain stimuli [63]. Anti-NGF (Tanezumab) is a potential analgesic therapy for central pain that has been recently tested in osteoarthritis patients [64]. Recent studies have indicated that endomorphins are expressed in T-cells, macrophages and synovial fibroblasts of patients with osteoarthritis [65]. However, there remain crucial gaps in our knowledge, as little is known about the role of the adaptive immune system in

chronic inflammatory conditions and the contribution of the immune system to chronic pain.

PRP therapies could use pro-anabolic or anti-catabolic mechanisms as well as pain relief for the treatment of these diseases, although the mechanisms of action are undoubtedly multifaceted and incompletely understood [66].

5. PRP-therapy

5.1 Modeling cross-talk at the organ level

Essentially, the methods of producing PRPs determine the composition and concentration of leukocytes, erythrocytes and platelets in a given plasma volume. There are three methods: i) the double spinning method using automated machines and commercial kits, ii) the single spinning method using conventional laboratory centrifuges followed by manual PRP separation, and iii) selective blood filtration using commercially available technology. Single spinning yields a one-fold to threefold change in platelet concentration over baseline levels, and double spinning yields a fourfold to eight-fold change in platelet concentration over baseline levels. Double spinning also concentrates leukocytes. Accordingly, platelet concentrates have been categorized as pure PRP (P-PRP), in which leukocytes are purposely eliminated from the PRP, and leukocyte and platelet-rich plasma (L-PRP), which contains a high concentration of leukocytes [67]. Growth factors and catabolic cytokine concentrations are influenced by the cellular composition of PRPs. Platelets increased anabolic signaling and, in contrast, leukocytes increased catabolic signaling molecules [66]. Moreover fibrin stability diminishes in the presence of leukocytes; the latter release elastases that destroy fibrin. Thus, we have used the terms 'L-PRP' and 'pure PRP therapies, to distinguish between the two formulations.

Prior to joint injection, PRP can be slowly activated by setting in motion the coagulation cascade with the addition of calcium ions, a necessary cofactor for endogenous prothrombin conversion to thrombin. Alternatively, coagulation and platelets can be instantly activated by adding a standard solution of bovine or human thrombin with 10% calcium chloride to the PRP. On the other hand unactivated PRP can be injected and activation will take place *in situ* induced due to the presence of tissue factors. Depending upon the activation mechanism, induced by Ca^{2+}, or thrombin or tissue factors we can achieve a gradual release or a. sudden burst of growth factors and cytokines. The way these proteins are released may influence signaling events and specific cellular functions. However this issue has not been investigated in joint injections.

One of the conceptual changes made in regards to PRP therapy has been the realization that growth factor signaling cannot predict the response of the entire molecular pool present in PRP (**Figure 4**). Recent studies on platelets have undermined the primacy of growth factors by demonstrating new classes of cytokines which are crucial to several biological processes involved in tissue healing [4,68-70]. Given the redundancy and pleiotropy of the PRP cytokine network, the specific actions of individual cytokines and the molecular mechanisms behind their functions have not yet been identified. Although we do not completely understand the biology of PRP, we aim to understand enough to be able to interfere with and lessen joint pathology [48,49]. To examine the theoretical links between PRP biology and joint pathology, we analyzed the following common relevant concepts: i) the regulation of inflammation, ii) the modulation of angiogenesis, and iii) anabolic actions and chondroprotection.

5.1.1 *Inflammation*

Platelets contain pre-stored and rapidly releasable proteins that play a major role as first-line inflammatory mediators. Platelets modulate the magnitude and duration of the inflammatory response by influencing at least two critical events. First, platelets control the *trans*-endothelial migration and activation of leukocytes. Second, these cells aid in the restoration of the tissue to its prior healthy state. PRPs influence not only neutrophil and monocyte transmigration, but also macrophage differentiation and activation. The major platelet chemokines include β-thromboglobulin (β-TG/CXCL7), PF4/CXCL4 and RANTES/CCL5 [71]. The release of high concentrations of CXCL7 [or neutrophil-activating peptide-2, (NAP-2)] is an example of how PRP marks the onset of an inflammatory reaction by affecting neutrophil migration. Platelets secrete two CXCL7 precursors, platelet basic protein (PBP) and connective tissue-activating peptide-III (CTAP-III). However, proteolytic processing by neutrophils is required to form chemotactically active CXCL7. These concepts have implications for the development of PRP formulations because they suggest that pure PRPs with a moderate platelet concentration may have less potential than leukocyte PRPs for the attraction of detrimental neutrophils. Unfortunately, there are few published studies on pre-defined PRP products and application protocols, and there is a lack of compelling evidence for the preferential use of either pure PRP or leukocyte PRP.

PF4/CXCL4 is another major cytokine that functions as a key regulator of cellular traffic; at low concentrations, PF4 inhibits the effect of CXCL7, and at high concentrations PF4 synergizes with CXCL7 [72]. Platelets also function to augment the local milieu by stimulating the production of other relevant chemokines and molecules involved in neutrophil recruitment (i.e., CXCL1/melanoma growth stimulating activity, alpha (GRO) and epithelial-derived neutrophil-activating protein 78 (ENA78)/CXCL5, CD-40L, MCP-1 and IL-8 receptor) [73].

The effect of platelet-released molecules on monocyte migration has been studied recently. In support of the complexity of the platelet secretome, some 315 proteins were identified; of these, 32 were present in the fraction that stimulated monocyte migration. Well characterized molecules such as MCP-1 collaborate with CCL5 to recruit monocytes. PRP stimulates monocyte chemotaxis

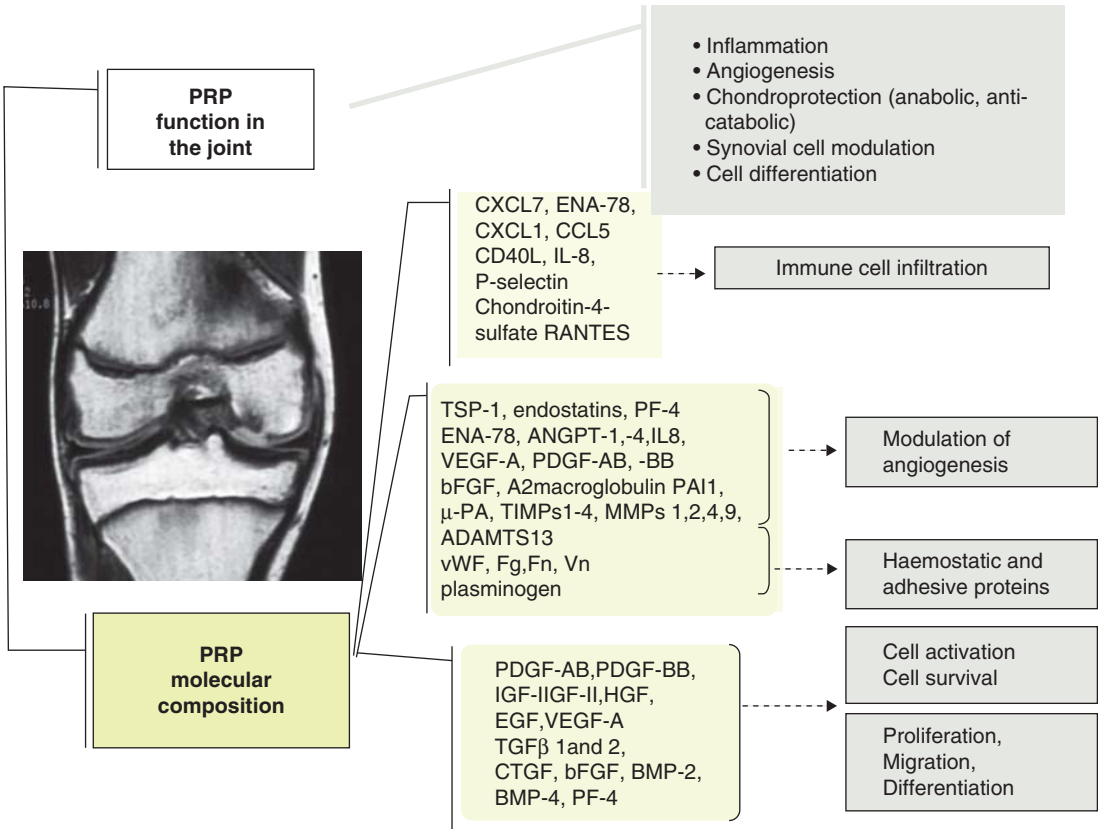

Figure 4. Changes in our conceptualization of PRP therapies. GFs cannot predict the behavior of the entire molecular pool, which, in addition to GFs, includes chemokines, enzymes, haemostatic and adhesive proteins that underlie the biological mechanisms of inflammation, angiogenesis, anabolic and anti-catabolic actions, cell differentiation and the modulation of synovial cell activities.

ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs; ANGPT-1: Angiopoietin; bFGF: Basic fibroblast growth factor; BMP: Bone morphogenic protein; CTGF: Connective tissue growth factor; ENA-78: Epithelial-derived neutrophil-activating protein 78; Fg: Fibrinogen; FN: Fibronectin; GF: Growth factor; HGF: Hepatocyte growth factor; PAI1: Plasminogen activator inhibitor 1; PDGF: Platelet-derived growth factor; PF: Platelet factor; PRP: Platelet-rich plasma; RANTES: Regulated upon activation, normal T cell expressed and secreted; TIMP: Tissue inhibitor of metalloprotease, TSP-1 thrombospondin-1; u-PA: Plasminogen activator, urokinase; Vn: Vitronectin; vWF: von Willebrand factor.

in a dose-dependent fashion, whereas RANTES is partially responsible for PRP-mediated monocyte migration [74]. PRP also promoted significant changes in monocyte-mediated pro-inflammatory cytokine/chemokine release. Furthermore, PF4, present in micromolar concentration in PRPs, prevents monocyte apoptosis and promotes macrophage differentiation [75]. Microarray technology has shown that PF4 induces a unique macrophage transcriptome distinct from the known macrophage activation patterns (classical and inflammatory) that shares diverse molecular similarities with both the pro- and anti-inflammatory activation patterns [76]. These findings were consistent with preliminary research on PRP that demonstrated an initial suppression of inflammation followed by an increase in IL-1β [77].

PRPs may terminate inflammation by restoring cells to a non-inflammatory phenotype. This effect could be mediated by various GFs, including hepatocyte growth factor (HGF), VEGF and TGF-β, which protect the function of the endothelial barrier [78]. For example, HGF treatment induces an anti-inflammatory cytokine profile in endothelial cells specifically by suppressing E-selectin [79]. Coudriet *et al.* [80], in a model of inflammatory activation (LPS-stimulation of macrophages), showed that, when HGF was present in the milieu, there was a decrease in the pro-inflammatory cytokine IL-6 and an increase in the anti-inflammatory cytokine IL-10. Furthermore, these changes were a result of signaling through the MET receptor. Interestingly, the molecular basis of the anti-inflammatory action of PRP on human chondrocytes relies on the action of HGF to inhibit NF-κB [81]. Specifically, HGF disrupted the expression of NF-κB-dependent pro-inflammatory mediators such as COX-2 and CXCR4. Recently, using a 3D *in-vitro* model, Wu *et al.* [82] showed that PRP associated with collagen scaffolds to restore type 2 collagen and aggrecan synthesis, which

was inhibited by IL-1β and TNF-α. PRPs also stimulate the synthesis of HGF by synoviocytes [83,84]. HGF is primarily found in plasma, and very little is found in platelets. Therefore, it is important to consider the balance between plasma and platelet proteins when formulating PRPs. These insights on the role of PRP in inflammation may lead to tailored and targeted formulations able to discriminate between the beneficial and harmful effects of this relationship between PRP and inflammation.

5.1.2 *Angiogenesis*

The influence of platelets on angiogenesis has been well established. However, because platelets contain both stimulators and inhibitors of angiogenesis, the mechanism by which platelets regulate angiogenesis remains unclear. Theoretically, PRP could change the angiogenic status of the joint by releasing small molecules from dense granules that could modulate the vascular tone or by secreting a pool of modulatory proteins (including both pro- and anti- angiogenic proteins). Substances released from these dense granules include serotonin, histamine, prostaglandin E2, prostaglandin D2, ADP/ATP and Ca^{2+}, and can alter the permeability of vessels to allow for plasma extravasation and the entry of blood-borne cells. Vascular cells have receptors for serotonin, and its release from platelets leads to vasoconstriction and an increase in capillary permeability. Histamine can have both anti- and pro-inflammatory effects, whereas the roles of catecholamines such as noradrenaline and dopamine require further investigation [85]. Angiogenic activators collectively promote vessel wall permeability and the recruitment, growth and proliferation of endothelial cells. Furthermore, the release of the biologically active lipid sphingosine-1-phosphate can regulate endothelial cell barrier function, vessel stability and angiogenesis, in part, by cross-talk with the PDGF and VEGF receptors [86].

Additionally, PRP can mediate the secretion of modulatory proteins packaged in alpha granule subpopulations, such as the pro-angiogenic proteins pPDGF A, B or C, TGF-β1, VEGF and HGF as well as the pro-angiogenic mediators angiopoietin, CXCL12 and MMP 1, 2 and 9). Paradoxically, other subpopulations of alpha granules contain established inhibitors of angiogenesis, such as PF-4, angiostatin, endostatin, fibronectin and the TIMPs 1 – 4 [87]. Importantly, the alpha granules of platelets constitute the major rapidly releasable reservoir of thrombospondin-1 (TSP-1) in humans (3.5 + 0.5 μg/10^8 platelets) [88]. TSP-1 is a potent inhibitor of endothelial cell proliferation and stimulates endothelial cell apoptosis. TSP-1 acts in an anti-angiogenic manner by preventing VEGF from binding to its cellular receptor and by interfering with the mitogenic effects of FGF [89]. Using an experimental model of osteoarthritis (the ACL transection model in rats), Hsieh *et al.* [68] examined the effects of thrombospondin-1-intraarticular gene transfer and reported reduced microvessel density, inflammation and suppression of osteoarthritis progression in treated animals compared with controls.

Intriguingly, pro- and anti-angiogenic proteins can be released from different sub-populations of alpha-granules, and the different secretion patterns are agonist-specific [90]. Secretion of either type of granule occurs following the selective engagement of the thrombin receptors proteinase activated receptor (PAR)-1 and PAR-4. For example, PAR1-activating peptide, ADP and the glycoprotein-VI-targeting collagen-related peptide induced massive release of SDF-1α and VEGF but modest release of PF4 or endostatin. In contrast, PAR4-activating peptide triggered marked PF4 and endostatin release [91,92]. This suggests that different activation mechanisms and environment stimuli evoke distinct secretion patterns of pro- and anti-angiogenic factors. Considering these data, further research is required to better understand the effect of PRPs on osteoarthritis. As it is uncertain whether the pathophysiology of osteoarthritis is the same in all patients, the joint response to PRP infiltration may be patient-dependent.

5.1.3 *Chondroprotective potential of PRP*

PRP therapies can delay joint deterioration by interfering with the early catabolic and inflammatory events and by subsequently promoting anabolic responses. PRP mediates chondro-protective actions by two mechanisms; it delivers anabolic factors and inhibits the actions of MMPs. PRP-released anabolic cytokines include connective tissue growth factor (CTGF/CCN2), TGF-β1 and -β2, IGF-I and IGF-II, FGF-2 and others [68]. The abundant levels of CTGF/CCN2 (20-fold higher than any other growth factor) mediate collagen deposition and promote cartilage regeneration [93]. Furthermore, TGF-β1, TGF-β2 and TGF-β3 stimulate the synthesis of type 2 collagen and proteoglycan *in vitro* as well as in experimental osteoarthritis [94,95]. Moreover, TGF-β signaling promotes the differentiation of mesenchymal stem cells into chondrocytes and enhances the repair of cartilage thickness defects by triggering chondrocyte differentiation. Although the pro-anabolic strategy of PRP therapy is attractive, molecular changes that occur with aging could lead to growth factor dysfunction. For example, TGF-β is a crucial anabolic factor that acts through the activin A receptor type 2-like kinase 5 (ALK5) receptor and the downstream small and mothers against decapentaplegic (SMAD)2/3 signaling pathway, counteracts IL-1 by the induction of TIMP and prevents hypertrophy. However, aging induces a shift towards a pathogenic role of TGF-β that consists of an ALK1 interaction and signaling through SMAD1, 5 and 8, and aging also enhances osteophyte formation [96]. IGF-I functionality is also declined as a result of the aging-related changes discussed above. Assuming an impaired anabolic GF stimulation with increased age, PRP actions would differ when administered to older patients. Although growth factors are generally associated with protective effects, a dual positive and negative role for TGF-β in osteoarthritis has been suggested. For example, in experimental osteoarthritis, osteophyte formation is driven by the presence of TGF-β, and is further enhanced by HGF under non-inflammatory conditions [97].

Anabolic factors cannot counteract enzymatic catabolic actions. MMPs destroy cartilage and are transcriptionally regulated by pro-inflammatory cytokines such as IL-1β and TNF-α. However, MMPs are secreted in an inactive form, and their activity is regulated by the molecular microenvironment. Plasma is a major source of the endogenous MMP inhibitor, α2-macroglobulin, which binds MMPs and leads to their clearance by endocytosis. Moreover, tissue inhibitors of MMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) that are released by platelets bind to and inactivate most MMPs. Additionally, TIMP-3 and α2-macroglobulin have been identified as potential endogenous inhibitors of the aggrecanases ADAMTS-4 and -5 [70].

5.1.4 *PRP and cartilage engineering ex vivo*

Evidence of the effects of PRP on cellular proliferation and differentiation comes mainly from studies of tissue engineering. Indeed, chondral lesions represent a clinical challenge, given the limited capacity of chondrocytes to proliferate *in vivo*. Thus, autologous cells can be harvested from a small tissue biopsy and sufficiently expanded *ex vivo* for re-implantation. When articular chondrocytes are the cellular source, PRP improves *ex vivo* proliferation but also causes de-differentiation [98]. Importantly, PRP-expanded cells retain their capacity to re-differentiate and synthesize cartilage-specific proteins when transferred to a 3D environment [98,99].

For the surgical treatment of symptomatic chondral defects, microfractures are used as a first-line treatment to promote the invasion of mesenchymal progenitor cells from the underlying subchondral bone. PRP therapy combined with the microfracture technique enhances the regeneration of neo-cartilage in a sheep model [100]. The cultivation of stem cells is another alternative under clinical investigation for the treatment of osteoarthritis, given their capacity to differentiate into chondrocytes and secrete a wide array of biologically active factors that support cell proliferation and tissue formation. The sources of these stem cells include the bone marrow and the synovial fluid [101]. In addition, the Hoffa fat pad contains stem cells with chondrogenic potential [102]. Stem cells derived from the meniscus, synovium, Hoffa fat, synovial fat and ACL share similar gene expression profiles [103]. Culturing these cells under hypoxic conditions has been shown to enhance their differentiation into cartilage-like tissue [104].

To avoid contact of the cells with bovine products and to implement good manufacturing practice (GMP)-compatible protocols, PRP releasates or lysates provide a feasible alternative to fetal calf or bovine serum in the expansion of these cells for cartilage engineering purposes [105]. The addition of PRP (compared with fetal calf serum) improves cellular expansion and imparts a differentiation capacity towards the osteogenic chondrogenic and adipogenic lineage [106]. In addition, PRPs can be used as carriers for chondrocyte delivery during re-implantation [107].

Alternatives to cellular therapy involve the controlled delivery of the PRP molecular pool. Indeed, the mode of delivery has been shown to influence the kinetics of signaling and cellular function. Improved osteochondral healing is evident when PRP was delivered in a poly-lactic-glycolic acid carrier [108]. Moreover, the combined delivery of PRP with collagen matrices may repair large cartilage defects that currently require autologous chondrocyte implantation (ACI) or osteochondral grafting [109]. Natural fibrin (formed during PRP coagulation with $CaCl_2$) also provides a physiological slow release of the compound and allows cells to experience a gradual increase in ligand concentration.

5.2 Summary of clinical data

Distinctions should be made between chronic and acute pathologies and between surgical and non-surgical conditions. Clinical studies are shown in **Table 1**. Conservative management of osteoarthritis and chondropathies is becoming increasingly popular, but clinical evidence is preliminary and modest, and is limited mostly to observational case studies that have used patient-reported outcomes as end-points (Western Ontario and McMaster Universities Index of Osteoarthritis (WOMAC), Knee injury and Osteoarthritis Outcome Score (KOOS), International Knee Documentation Committee (IKDC) and Visual Analogue Scale (VAS)). In a retrospective cohort study of knee osteoarthritis, decreased pain and enhanced function was reported 6 months after the intra-articular injection of PRP (compared with hyaluronan) [8]. In a case series study involving 115 young patients with low degrees of articular degeneration in the knee, Kon *et al.* [9] reported reduced pain and improved function that was maintained at 12 but not 24 months after treatment [110]. More recently, Kon *et al.* [111] reported that PRP showed more and longer efficacy than hyaluronan injections in reducing pain and recovering function. Better results were achieved in younger and more active patients with a low degree of cartilage degeneration. Sampson *et al.* [10], also in a small case series (n = 13), reported significant pain and symptom relief but did not find any significant change in the daily activities or quality of life of patients treated. PRP injections for hip osteoarthritis produced clinically significant reductions in pain and improvements in function, although this was only seen in 40% of patients [12]. When discussing PRP therapies, differences between the preparations and the re-administration procedures used should be acknowledged. Although pure PRP [6-8,11,12] and leukocyte PRP [9,10] formulations are not comparable to each other in terms of leukocyte content, platelet count and plasma volume, the resulting improvements in pain and function were not exclusive to any one formulation. For each study, the volume and number of injections was empirically determined, and all studies involved three injections, although the period between the injections ranged from 1 to 4 weeks. PRP is generally injected in the femorotibial compartment, although Sampson injected PRP into the suprapatellar bursa. Whether the differences in the clinical results are secondary to the differences in the formulation

Table 1. Studies on platelet-rich plasma and joint pathology.

Ref.	Articular pathology /intervention	Type of PRP/ volume	Study design, N/outcome index	Results	Follow up	Level of evidence
[6]	Knee cartilage avulsion/arthroscopic surgery	Pure-PRP (1.5 – 3×) $CaCl_2$ activated	Case Report/MRI, return to competition	Cartilage healing and functional recovery	12 months	IV
[8]	Knee osteoarthritis/three injections weekly	Pure-PRP (1.5 – 3×) $CaCl_2$ activated/8 ml	Case-control, n = 30/WOMAC	Significant differences in overall WOMAC and in function and pain subscales at 5 weeks	6 months	III
[9,110]	Knee osteoarthritis/3 injections every 2 weeks	L-PRP (4 – 6×) $CaCl_2$-activated/5 ml	Case series, n = 91/IKDC and VAS	Significant improvement at 6 – 12 months	2 – 6 months 12 – 24 months	IV
[111]	Knee osteoarthritis/3 injections Every 2 weeks	L-PRP (4 – 6×) $CaCl_2$ activated/5 ml	Prospective comparative study, PRP n = 50 High- and low-molecular weight hyaluronan, n = 50 each group	Similar at 2 months PRP better than hyaluronan at 6 months in pain and function	2 and 6 months	II
[10]	Knee osteoarthritis/3 injections every 4 weeks	Biomet Thrombin activated/6 ml in supra-patellar bursa	Case series, n = 13/KOOS, VAS, cartilage thickness US	Longitudinal improvement in KOOS and VAS 6 out of 13 increased femoral cartilage thickness	2-, 5-, 11-, 18- and 52-weeks	IV
[7]	Chondral/patellar degenerative lesions/open surgery AMIC *plus** grafting	PRPgel (biomet) collagenI/III membrane	Case series, n = 5/KOOS, VAS, Tegner MOCART	Improvement in KOOS and VAS, no changes in Tegner Complete integration border zone, incomplete filling in three cases	12 – 24 months	IV
[11]	Knee osteoarthritis/three injections every 2 weeks	Pure-PRP (1.5– 3×) $CaCl_2$ activated/ml	Case series, n = 261/WOMAC,VAS, SF-36, Lequesne	Significant improvement in all scores	6 months	IV
[12]	Hip osteoarthritis/three injections weekly	Pure-PRP (1.5 – 3×) $CaCl_2$ activated/8 ml	Case series, n = 40/WOMAC, VAS, HARRIS	Significant improvement in all scores (except WOMAC rigidity) in 40% of patients	6 months	IV

*AMIC *plus* :autologous matrix-induced chondrogenesis combined with platelet-rich plasma gel.

IKDC: International Knee Documentation Committee; KOOS: Knee injury and Osteoarthritis Outcome Score; MOCART: MRI score assessing morphology and signal intensity of the repaired tissue; PRP: Platelet-rich plasma; SF-36: Quality of life and health survey; US: Ultrasound; VAS: Visual Analogue Scale; WOMAC: Western Ontario and McMaster Universities Index of Osteoarthritis.

Level II: controlled trial without randomization; Level III: case control studies; Level IV: descriptive studies, case series, case reports.

requires clarification. Furthermore, a refinement of the imaging or biochemical endpoints for the clinical trials would help to assess the efficacy of PRP and avoid the placebo effects and artifacts from intermittent symptomatology. The pursuit to identify a unifying therapy for osteoarthritis would be enhanced by refining the end points in future clinical studies.

The proper identification of responders is another challenge, as PRP therapy is effective in only approximately 50% of patients. Theoretically, PRP application may well be much more efficacious in patients with early post-traumatic osteoarthritis before the radiographic signs are severe, but this needs further confirmation. In patients with significant irreversible bone and cartilage damage, the effect of PRPs would most probably be less impressive. However, PRP therapy would still probably improve the patients' quality of life [11]. Trials evaluating PRP therapies are ongoing, and publicly available details can be found at http://www.clinicaltrials.gov. There is urgent need to evaluate the therapeutic claims made about PRP therapies. However, to better define the conditions of clinical trials, we need to know more about differences, not only between PRP formulations but between injection protocols, including applied volumes, treatment schedules and patient selection criteria.

Given the biocompatibility of using the patient's own proteins, safety is guaranteed. Nonetheless, care must be taken to avoid injections close to the main blood vessels. Although risk with any injection include potential for infection and bleeding, no local or superficial infections, allergic reactions or any other complication have been reported in clinical studies. However, when combining PRP with biomaterials research efforts should be dedicated to characterizing these novel compounds before introducing them into clinical use. Indeed, the composite PRP-biomaterial can have different properties, and, in some instances, the novel composite can hinder repair mechanisms as recently shown in animal experiments [112].

6. Expert opinion

No simple rule can be generalized to describe how PRP works for the treatment of joint pathologies. PRP therapies are much more complex than previously thought, and their discovery has opened the door to a vast labyrinth of new questions. Complexity arises when the molecular content and function of platelets are dissected. The effectors mediating the beneficial effects of PRPs have not been identified, and platelets contain more than 300 proteins. Thus, the primacy of growth factors may be undermined by the identification of new classes of molecules. Thus, identifying critical factors as a basis for improving formulations may reduce the number of components so that the mechanisms of action could be better elucidated. The combined use of laboratory models and current techniques in molecular biology will reveal the central signaling patterns of PRPs and will greatly assist in the elucidation of many fundamental mechanisms defining PRP function in joint pathology. To improve clinical and therapeutic benefits, the principle of dynamic reciprocity should be exploited and the cellular contexts and microenvironment alterations resulting from PRP therapy should be taken into account.

One concept central to PRP therapy is context dependency. In fact, PRP therapy is a degenerate system, which can produce different results under different conditions. For example, whether PRP has pro- or anti-angiogenic effects would depend on its interactions with the host tissue. One potentially interesting area of research would be focused on how intercellular and intracellular signaling events are modified by PRPs in the different stages of osteoarthritis. This research would be followed by the integration of these data into a framework that explains which processes (inflammation, angiogenesis, cell death) are primarily affected. Moreover, establishing the cascade of events and the molecular and cellular hierarchy that produce the therapeutic effects of PRPs may identify novel applications for PRP therapies.

A major goal for the development of PRP therapy lies in increasing the variation of formulation options tailored to trigger each of these biological processes. In fact, PRP technologies may involve the use of different formulations for different tissues, for different stages of healing or for patients with different histopathological or clinical features. The barriers to treatment development and research include the insufficient understanding of the pathology and the fact that the physiopathology of osteoarthritis may not be identical for all patients. Hence, one major goal should be to improve diagnostic specificity and to stratify patients according to any of the previously described biomarkers indicative of various biological processes, which could include the ECM-derived molecules released into fluids during matrix catabolism of articular cartilage or subchondral bone remodeling, the synovial inflammatory biomarkers or any others utilizing advanced imaging techniques. Identifying PRP responders based on objective biological or structural biomarkers will be crucial for clarifying how PRP therapy works and the clinical conditions for which it is indicated.

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