EXPERT OPINION

Knee osteoarthritis: hyaluronic acid, platelet-rich plasma or both in association?

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Introduction: Bidirectional interactions between cells and fluidic surroundings regulate cellular functions and maintain tissue or organ architecture. Accordingly, the synovial fluid is the primary source of environmental signals and determines to a great extent the molecular interactions within the joint capsule, both in homeostasis and pathology.
Areas covered: We provided an update on hyaluronic acid (HA) and platelet-rich plasma (PRP) concepts necessary to build the rationale for creating a combined treatment. The information is based on a PubMed search using the terms 'platelet-rich plasma', 'hyaluronic acid', 'knee pathology', 'knee osteoarthritis' (OA).
Expert opinion: In OA, a deleterious fluidic microenvironment is established, with presence of HA fragments, catabolic enzymes and inflammatory molecules. The central concept underlying intra-articular injection is to modify deleterious fluidic microenvironments. PRP administration has shown pain remission and function improvement, but less than half of the patients showed clinically significant improvement. PRP exceeds HA, the comparator used in PRP clinical trials, albeit both HA and PRP alleviate symptoms in mild-to-moderate OA patients. Combining PRP and HA may benefit from their dissimilar biological mechanisms and help in controlling delivery and presentation of signaling molecules. Three armed randomized studies, using both HA and PRP as comparators, will provide information about the impact of this approach.

Keywords: hyaluronic acid, joint pathology, knee, osteoarthritis, platelet-rich plasma, synovial fluid

Expert Opin. Biol. Ther. (2014) 14(5):635-649

1. Introduction

Hyaline cartilage is essential for joint function, enabling an almost frictionless movement, dissipating the mechanical stress and protecting the bone from excessive load and trauma. Unfortunately, it has limited healing potential due to the partial isolation from systemic control, attributable to the lack of nerves and vessels and to its complex microscopic configuration. Moreover, articular cartilage is considered a postmitotic tissue with almost no cell turnover, and the loss of type II collagen, the main structural protein in cartilage extracellular matrix (ECM), is considered to be irreparable [1]. So, small and confined injuries can progress into degenerative changes, leading to osteoarthritis (OA).

With population ageing, OA becomes a rising public health problem expected to affect 20% of adults in Europe and North America by 2020 [2]. Knee OA is the most cumbersome in terms of prevalence and disability. Its burden is not limited to pain; it also compromises overall health, and quality of life of the affected

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patients [3]. Financial burden is enormous, both from a societal and patient perspective.

OA treatment is a well-known clinical challenge, and currently, no medical therapy has shown to halt or reverse OA progression [4]. Several conservative treatments, including oral and topical NSAIDs, glucosamine, chondroitin-sulfate and intra-articular corticosteroids, have been proposed as noninvasive alternatives for pain reduction and enhancement of function, with varying success rates. However, they are not able to modify the normal evolution of the disease, and some of them, while efficacious in the short-term pain management, may have deleterious local and systemic consequences [5].

There is a critical need for improved therapies to aid not only in managing clinical symptoms but also in halting the disease process. With the increasing understanding of cell signaling networks, current research is investigating new conservative methods seeking to provide an instructional environment for stimulating joint repair. In this framework, platelet-rich plasma (PRP) is used to manipulate the complex spatiotemporal signaling within and between the joint tissues [6]. The increasing understanding of the roles of cytokines and their interactions with cells and components of the ECM has shown how closely PRP may mimic the natural healing microenvironment. In the last decades, not only PRP but also hyaluronic acid (HA) injections have been extensively used to improve lubrication, modulate inflammation and modify the catabolic microenvironment. In doing so, these conservative therapies not only aim to reduce clinical symptoms but also interfere with OA progression.

In this article, we seek to summarize the current preclinical and clinical knowledge on this topic, reporting comparative studies between HA and PRP injections, and suggesting the possibility of the combined use of these therapeutic agents. The intention of this manuscript is not to fully review HA and PRP in joint pathology but to provide an update on HA and PRP concepts necessary to build the rationale for creating a combined treatment. A second goal of this review is to reconsider some hypotheses that attempt to explain the relevance of PRP and HA injections as it relates to the modification of the joint microenvironment.

2. Hyaluronic acid supplementation

2.1 Basic concepts and experimental research

Hyaluronan is a polyanionic, unbranched glycosiaminoglycan polymer composed of disaccharide subunits of *N*-acetyl-D-glucosamine and D-glucuronic acid. Although, the correct designation for the native polymer is hyaluronan, in this review, for convenience, we use the term HA all through the text.

Sound basic research supports the therapeutic potential of injecting exogenous high molecular weight (HMW) HA. This substance, endogenously produced by synoviocytes, fibroblasts, chondrocytes and mesenchymal stem cells (MSCs) [7], is the major compound of synovial fluid. Its high viscosity grants viscoelastic properties to the fluid, and along with lubricin, contributes to the boundary lubrication [8], which is necessary for low friction levels on the articular surface. Thus, it has a shielding effect on cartilage surfaces and other joint components. Furthermore, due to its abundant negative charges, HA absorbs a large amount of water, and this vast water domain helps to create the spaces through which the cells move and signaling molecules diffuse to reach their targets.

Native HA polymers are undetermined in weight. Actually, HA can be synthesized by three different synthases termed HAS (HAS-1, -2 and -3), that create polymers of different sizes. Genes codifying the three HAS isoforms are located in different chromosomes and differentially regulated at transcriptional, translational and posttranslational levels [9,10]. For example, HAS2 is present in the inner membrane of actively secreting synoviocytes and assemble up to 20,000 disaccharide units producing a large polymer of $2 \times 10^6 - 7 \times 10^6$ Da, as measured by size exclusion chromatography [11]. The expression of HAS can be stimulated by IL-1β, TGF-β and PDGF which activate phospholipase-6-diacylglycerol-proteinkinaseC (PKC)-ERK1/2 signal pathway [12,13]. HMW HA is immunosuppressive, anti-inflammatory and anti-angiogenic [14,15].

However, in OA, the concentration and the molecular weight of endogenous HA are decreased within the joint, due to the impaired hyaluronan synthesis, free radical degradation [16] and dilution in intra-articular effusions [17]. As a consequence, the SF viscoelasticity declines, and the transfer of mechanical stress to cartilage increases its vulnerability to mechanical damage. Furthermore, HA fragments are pro-angiogenic and have pro-inflammatory effects signaling through Toll-like receptors TLR2 and TLR4 expressed in macrophages and chondrocytes [18-20].

Besides HA role in viscosupplementation, basic research, performed in OA models in animal (rats, rabbits, dogs and sheep), has shown that HA has several pleiotropic signaling properties, such as anti-inflammatory, anti-apoptotic, anti-angiogenic and anti-fibrotic effects, with normalization of endogenous HA synthesis and chondroprotection [21]. Actually, HA binds to a number of cell-membrane receptors termed hyaladherins. The predominant and more widely expressed is CD44, a membrane glycoprotein made of 10 stable exons and 10 variable exons inserted in different combinations at a particular extra-membrane site. The biology of CD44 is complex, apart from the constant isoform named CD44S, there are an enormous number of splicing alternatives designated as CD44v, some of which have well-defined activities in embryogenesis, matrix assembly, malignant transformation and metastatic dissemination [22].

CD44 is essential to its antigenic properties and protective effects on remodeling and degeneration in OA evolution [23]. Indeed, CD44 decrease in articular cartilage is related to progression of knee OA [24]. Besides, both CD44 and RHAMM (CD168) (the HA receptor for HA-mediated motility), are

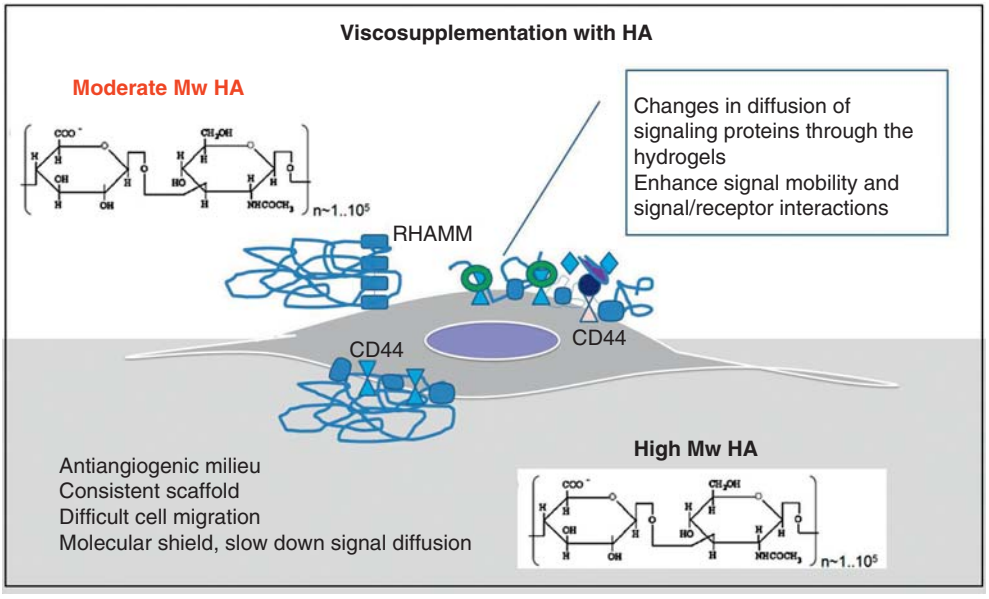

Figure 1. In addition to viscosupplementation, hyaluronic acid has pleiotropic actions by binding to CD44 and hyaluronan-mediated motility receptor receptors. Moreover, hyaluronic acid (HA) can modify the accesibility of signaling proteins to their target receptors in the cell membrane. Concerning HA molecular weight and concentration, high molecular weight HA may slow down the diffusion of signaling molecules. These properties may be relevant in the HA+PRP combination.

involved in the regulation of growth factor signaling (**Figure 1**). Moreover, CD44 interacts in parallel with several different ligands including collagens, osteopontin and MMPs [25]. A recent study has been performed in an experimental model of murine OA (TGF-β1 injection and treadmill running), which displays many OA-like changes, including synovial activation. HA injection, 24 h after TGF-β1 injection, hinders neovascularization and fibrosis of the synovium, and kept in good condition articular cartilage in wild-type, but not in CD44 knockout mice. This finding suggests that the injected HA enhances the synthesis of chondrogenic proteins, and blocks that of fibrogenic or degradative proteins in both the cartilage and subchondral bone [26].

The analgesic properties of HA, besides to the activities previously described, could be also attributed to a specific activity on opioid receptors [27]. Pain in OA is likely to have multiple sources, including subchondral bone marrow lesions, synovium and the periostium as well as soft tissues surrounding the joint including extra-articular bursae and infra-patellar fat pad. Pain in OA is classified as nociceptive, based on the presence of opioid receptors in the synovial lining and sublining cells. Various molecules present in extracellular space modulate nociceptor sensitivity by targeting different receptor types. The biological mechanisms responsible for HA analgesic activity have been partially elucidated by Zavan *et al.* [27] in experiments performed with CHO (Chinese Hamster Ovary) cells that express a panel of opioid receptors. The results demonstrated that HA stimulates the k receptor (KOP), also expressed on fibroblast-like synoviocytes, in a concentration-dependent manner, but not on the DOP, MOP and NOP receptors. This selective activity could be due to the singular conformational structures of HA compared to morphine, more closely related to dynorphin organization.

The pain threshold also increases, due to the direct analgesia through inhibition of pain receptors and by a direct action on synovial nerve endings and stimulation of synovial lining cells [28,29].

At present, HA compounds with diverse molecular weight are commercially offered. The enhanced diffusion of low MW (LMW) preparations (0.5 – 1.5 millions Da) through the ECM of the synovium makes possible the interaction with synovial cells, thereby modulating the synovial inflammation [30]. However, because of the modest elastoviscosity of these compounds, compared to native hyaluronic in the synovial fluid, HA preparations with HMW (6 – 7 millions Da), have been developed [31]. These formulations retain higher amounts of fluid in the articular space using their hydrophilic properties, and also have a greater anti-inflammatory activity, as shown by reduced prostaglandin E 2 and bradykinin concentration attributed to a reduced migration of inflammatory cells [32].

Currently, other long-acting compounds are being examined. NASHA (non-animal stabilized HA) is manufactured by a two-stage procedure: culturing bacteria that synthesize HA, in conjunction with a subsequent mild stabilization process [33]. Stabilization involves creating ionic or covalent bonds between HA chains, by chemical processing; HA cross-linking does not alter the biochemical properties of

HA, instead it provides improved viscoelastic properties and a longer dwelling time in joint, compared with nonstabilized HA preparations. Actually, while unmodified HA has a residence time of 12 – 24 h following delivery, cross-linked HA can dwell up to 48 h [34].

Alternatively, a natural, highly purified solution of HA has been obtained by fermentation, which also contains 0.5% of mannitol, a free-radical scavenger which helps to stabilize sodium hyaluronate chains, thus increasing their residence time within the joint without increasing their MW [35].

Then again, many types of particulate carriers have been investigated aiming to increase the retention time of therapeutic agents within the knee capsule: among them, cationic polymeric nanoparticles that form scattered ionically connected filamentous arrangements ('ionically cross-linked hydrogels') linked with local hyaluronic [36]. These hydrogels, formed by mixing nanoparticles with synovial fluid, do not appreciably alter its viscosity *in vitro*, with a release of the conjugated peptide approximately 20% per week. Certainly, after intra-articular injection in rat knees, about 70% of the nanoparticles are retained in the joint for 1 week. Thus, cationic polymeric nanoparticles increase the retention of HA in joints and are suitable for therapeutic use.

Another medical device [37] combined chondroitin sulphate and HA. The role of chondroitin sulfate is twofold: i) it creates specific interactions designed to optimize the rheological behavior of HA; and ii) it regulates cartilage metabolism by performing as a substrate for polysulphated glycosaminoglycans synthesis as well as an inhibiting the synthesis of catabolic cytokines and metalloproteinases.

A new product combines HA of synthetic origin with a high concentration of sorbitol, which is an oxygen-free radical scavenger. When combined, these substances form an injectable gel after developing a dense network of hydrogen bonds. Sorbitol delays the degradation of this gel and decreases the migration of macrophages in the synovium by means of its capacity as a scavenger and neutralizer of oxygen-free radicals [38].

To ameliorate OA treatment while avoiding adverse effects, a mixture of celecoxib-loaded liposomes embedded in HA gel has been formulated [39]. Celecoxib is a COX-2 selective inhibitor with analgesic and anti-inflammatory properties. Liposomes are good candidates for local delivery of therapeutic agents because they are derived from naturally occurring, biodegradable and nontoxic lipids. The combination of two drugs, both efficient in the treatment of OA, but with different mechanisms, injected into the joints, is expected to have synergistic effect. Indeed, in a rabbit knee OA model, the liposomal combination was more effective than a single drug in pain control and cartilage protection, as shown by the histopathological study [40,41].

These pharmaceutical studies taken together show how intense is nowadays the research aiming to ameliorate the therapeutic efficacy of HA. Open-label, noncontrolled studies have proven the clinical efficacy of some of these products [42]. However, high-quality clinical studies proving their superiority toward the available preparations of HA are still lacking.

2.2 Clinical trials

Viscosupplementation with HA in knee OA has been approved by the FDA [43] and is recommended by OARSI for nonsevere OA [44]. Guidelines are based on a meta-analysis of randomized saline-controlled trials, including a total of 29 studies representing 4,866 unique subjects (intra-articular HA: 2673, saline: 2193) [45].

Prospective single or double-blind trials have been done using different types of HA (LMW and HMW). The number of injections varied from 3 to 5 weekly, with a maximum of 11 in 23 weeks, the doses ranged from 15 to 60 mg, and follow-up periods ranged from 4 weeks to 18 months. Pain outcome was followed using the visual analogue scale and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC). A minor number of studies evaluated the functional outcomes (WOMAC [physical function], Lequesne Index, the range of motion), the subjective global assessment and the quality of life of the patients.

HA injection resulted in very large treatment effects between 4 and 26 weeks for knee pain and function compared to preinjection values. Compared to saline controls, standardized mean difference values with intra-articular HA ranged from 0.38 to 0.43 for knee pain and 0.32 – 0.34 for knee function. There were no statistically significant differences between intra-articular HA and saline controls in any safety outcome, including serious adverse events and study withdrawal. The authors concluded that intra-articular injection of US-approved HA products are safe and effective in patients with symptomatic knee OA [45].

Clinical changes were similar in the trials where LMW or HMW HA was used [46-51]. However, the number of injection considered necessary was lower for HMW preparation, and this is an important advantage for the patients [50].

Another meta-analysis [52], comparing the time course of symptoms (the 'therapeutic trajectory') in HA and corticosteroid-treated patients, highlights that from the baseline to week 4 intra-articular corticosteroid were more effective than HA. By week 4, the two approaches have equal efficacy, but beyond week 8 HA was superior.

It must be noted that, in all trials, some of the patients were nonresponders to therapy. Currently, the characteristics of responders have not been clearly identified, but a greater benefit was observed in patients with low-grade OA [53]. This is indirectly confirmed by changes of the serum levels of specific OA biomarkers (Coll2-1 and Coll2-1 NO2) after viscosupplementation. The serum concentrations of the biomarkers were significantly higher in Kellgren Lawrence (KL) III/IV patients compared to KL I/II patients, and were significantly lower at baseline in responders than in nonresponders [54]. Therefore, a rapid decrease of type II collagen degradation and joint inflammation after HA injection, supports the

utility of serum biomarkers as predictive factors for response to treatment.

The role of age in predicting the therapeutic response is debated: some authors report no significant difference among subjects of different ages, whereas others claim that intra-articular joint HA injections are effective in both young and old patients with regard to pain and functional status over a short-term period, but that, in the long term (12 months), the benefit declines rapidly in the elderly subjects [51]. Essentially, advanced OA may be a non-return back irreversible condition.

3. Platelet-rich plasma

More recently, a new intriguing prospective is gaining ground, marking the transition from the traditional modalities focusing on the concept of using a single molecule approach to the innovative idea of co-delivering multiple bioactive factors mimicking the complexity of microenvironments that regulate repair mechanisms. Assuming that tissue repair involves the sequential signaling of multiple factors, the delivery of a single type of molecule is insufficient. Co-delivery of various proteins is most likely required to building an efficient and proper regenerative environment and can break the vicious circle based on failure of the repair process that progressively leads to OA (**Figure 2**).

In this scenario, intra-articular PRP injections have been attracting attention as an innovative and promising treatment to modulate inflammation and stimulate repair. The basic principle underlying the PRP therapeutic activity is to release a collection of signaling proteins, including growth factors (GFs) and chemokines among other proteins, to the joint environment, thereby inducing tissue regeneration mechanisms [55]. Briefly, regulatory proteins released from PRP must be capable of interfering with the catabolic microenvironment in OA joints while modulating the inflammatory response, inducing cell migration and proliferation and regulating angiogenesis and cell differentiation.

Since PRPs can have a broad range of functions, it is difficult to decide which function or aspect is the most relevant for knee pathology outcome. A full description of signaling proteins released from PRP and their role in modulating inflammation and vascular pathology have been recently appraised in two excellent reviews [6,56]. Here we focus on describing the properties of PRP on mechanisms that help in building the rationale for creating a combined HA/PRP treatment.

3.1 Precursor cell migration, proliferation and differentiation

Precursor cell migration, proliferation and differentiation are intended biological effects theoretically related to the PRP clinical response. Cartilage is composed of postmitotic cells incapable of proliferation. Therefore, regeneration may be based on migration of mitotic stem cells or their progeny (precursor cells) to the cartilage surface, followed by differentiation and the synthesis of ECM components. However, the avascular nature of cartilage hinders migration of circulating stem cells, thus precursor cells identified in other joint tissues, such as the synovium [57] or the Hoffa fat [58], although with distinct differentiation potency, are candidates to repair cartilage defects; alternatively cells can home cartilage lesions by migrating from the subchondral bone [59].

3.1.1 *Cell migration*

PRP exploits the ability of cells to migrate. Actually, by inducing changes in the cell microenvironment, PRP facilitates the motility of BMSCs, ADSCs and chondrocytes [60-62]. In fact, cells direct their migration toward or against a gradient of a molecular stimulus such as GFs and cytokines, including SDF-1a (CXCL12), PDGF-BB and HGF (**Figure 3**).

The ability of PRP to create gradients of GFs and chemokines is based on three central features: first, on the kinetics of release of chemokines from alpha-granules in platelets, second on the structural and chemical properties of the fibrin scaffold, and finally, on the plasmin degradation of the fibrin.

Platelets release upon activation a huge repertoire of chemokines, GFs and other cytokines prestored in their alpha granules. In parallel upon PRP activation, a specialized provisional fibrin network is formed. Fibrin binds several plasma proteins including vitronectin, fibronectin, vWF and thrombospondin. Recent research has identified fibronectin as a major factor in human serum to recruit subchondral progenitor cells [63].

Additionally, these proteins within the fibrin bind GFs and form molecular complexes that can dramatically enhance the potency of GFs [64]. Moreover, some of these structural proteins have RGD sequences, which can engage integrin receptors and improve tissue morphogenesis [65]. Structural properties govern the mechanical degradation and cell infiltration properties of fibrin. The plasmin-induced degradation of fibrin, that is, the rate of fibrinolysis, is important for full delivery of immobilized molecules, such as PDGF-BB, TSP, fibulin, albumin, SPARC, vWF, VEGF, FGF-2, IL-1, that are released upon proteolytic cleavage [66]. Importantly, cell migration within fibrin matrices depends upon the cell-associated proteolytic activity by plasmin and MMPs due to the high density of covalent cross-links within fibrin.

Besides these effects PRP stimulates HA synthesis, as shown by '*in vitro*' experiments performed on synovial fibroblasts isolated from the synovium of patients with OA undergoing prosthetic surgery [67], and the newly synthesized HA may help to improve cell motility.

3.1.2 *Cell proliferation*

PRP also supports other mechanisms necessary for cartilage repair, such as proliferation [68]. In fact, PRP has been deeply tested and shown to be a safe and suitable supplement to achieve large-scale expansion of MSC for cell therapy purposes. Moreover, PRP not only supports MSC proliferation

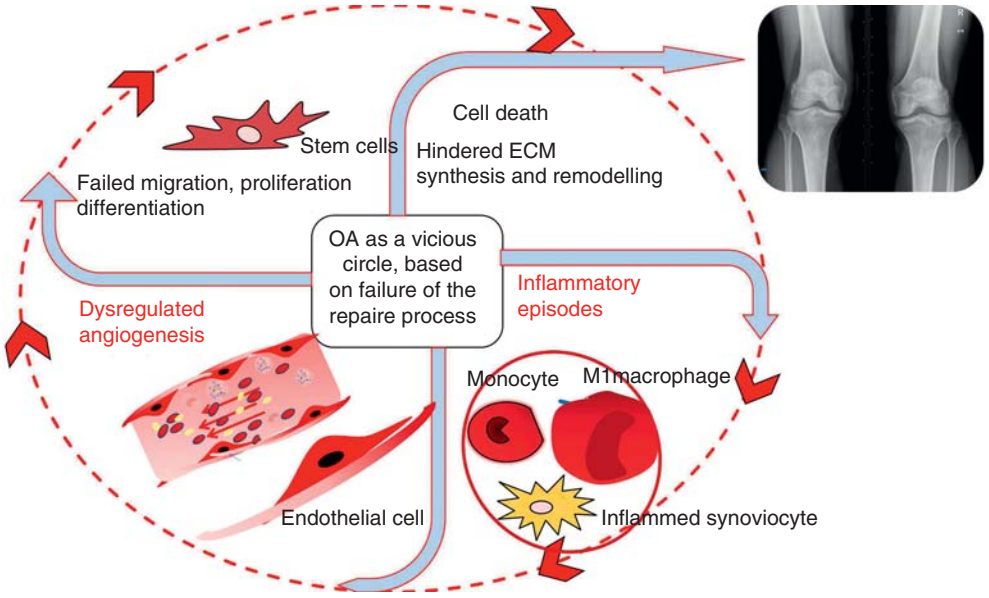

Figure 2. Failed healing of the knee. An effective biological treatment would involve modifying the fluidic microenvironment using a multimolecular approach. In doing so, we target the various failed healing processes that drive osteoarthritis progression, including inflammation, angiogenesis, cell migration and differentiation and the synthesis of extracellular matrix proteins.

ECM: Extracellular matrix; OA: Osteoarthritis; PRP: Platelet rich plasma.

but it is also safer and more effective than FBS. However, there is no consensus on which PRP formulation is more proliferative: the PRP releasate or the platelet lysate. The former is the supernatant extruded after PRP coagulation, and it may be considered as a PRP serum; the activation method to achieve granule secretion may introduce variability between products [69-71]. Alternatively, the platelet lysate is obtained after several freeze and thaw cycles of either PRP or L-PRP [72,73].

As occurs with PRP commercial systems, centrifugation forces and time, as well as the number of spins (double vs single), alter the PRP product in terms of platelet count and leukocyte concentration [74,75]. Moreover, in cell cultures, not only the platelet count, but also the percentage of PRP used to supplement the media influences proliferation rates. It was shown that supplementing cell media with 5 – 10% of PRP yield the highest expansion rates [76], illustrating that 'the more is better' assumption does not concern PRP as both pro- and anti-proliferative molecules are present in the mixture. Importantly, studies demonstrate that MSCs expanded in PRP-derived formulations maintain pluripotency along the passages [75,77].

3.1.3 *Chondrogenic differentiation*

Differentiation is of paramount importance to cartilage regeneration. Differentiated cells synthesize specific molecules unique to hyaline cartilage ECM, such as collagen type 2 and aggrecans, in adequate proportion conferring exceptional biomechanical properties to the ECM. Noteworthy, an inflammatory synovial fluid hinders the differentiation of human subchondral progenitor cells, decreasing the expression of aggrecan, collagen II and COMP [76]. However, PRP stimulate migration and differentiation of human subchondral cells into chondrocytes [62] highlighting the consequences of manipulating the biological milieu.

Whether PRP drives the cell to chondrogenic differentiation *in vitro* depends on the precursor cell characteristics along with the culture system (i.e., monolayer culture, micromass, three-dimensional scaffolds). Proteomic studies demonstrated that chondrocytes cultured with PRP in either mono- or three-dimensional conditions maintained the chondrocyte phenotype or at least de-differentiation was inhibited after several days in culture [78]. Buffered PRP enhanced MSCs chondrogenic differentiation as assessed by expression of chondrogenic markers [79]. Not only the system of culture but also the percentage of PRP used to supplement the media influences differentiation. Optimal concentrations of PRP for chondrogenic differentiation of ADSCs were 5 – 25% [80,81], and cells maintain their differentiated phenotype through cell passages [80].

3.2 Donor variability

Occasionally, results cannot be validated due to two main reasons; first because GF and cytokine profiles are very different in pure PRP and leukocyte-PRP, and secondly, because of inter-donor variability [82]. The later can be overcome *in vitro* by using pooled PRP of at least 10 donors [70-76,78-85]. Most researchers do not pool PRPs, instead they use blood

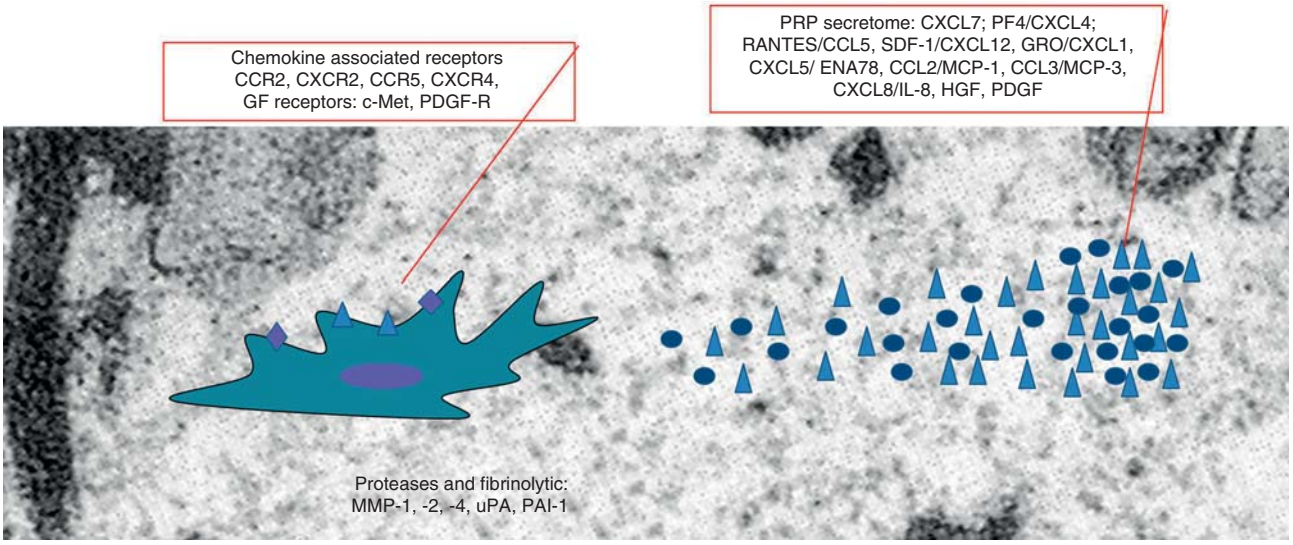

Figure 3. Cell migration. Cartilage repair may be based on migration of mitotic precursor cells from the synovium, hoffa fat or subchondral bone in response to chemotactic gradients. Platelet-rich plasma induces cell migration by releasing a pool of chemotactic proteins and migration is ehnanced in the presence of hyaluronic acid.
HGF: Hepatocyte growth factor; PDGF: Platelet derived growth factor; PRP: Platelet rich plasma.

from single donations [60,79,80,86,87]. Many authors do not consider the age of donors as a relevant parameter. Cho *et al.* [86] and Lohmann *et al.* [87] found that MSCs' proliferation is higher with PRP from young donors. Moreover, they confirmed that MSCs cultured with PRP from elder donors presented a senescence phenotype [87]. This fact proves that age of donors should be taken seriously as a parameter that may affect PRP composition and *in vitro* effects. At the time of writing, our laboratory is researching for the chief characteristics of pedigree PRP donors for osteoarticular and tendon pathologies.

3.3 Nonclinical proof of concept

Cartilage lesions may be classified according to severity in partial and full thickness or osteochondral defects. The therapeutic approaches to treat these lesions vary, depending on the severity, from PRP injections associated to microfractures (a strategy designed to recruit stem and progenitor cells to the lesion), scaffolds associated to PRP, PRP with cells, or PRP with cells and scaffolds [88]. The utilization of PRP injections or PRP gel associated with micro-fractures showed promising results in a sheep model, probably by enhancing subchondral cell migration [89]. Moreover, repeated intra-articular injections (up to five) produced better results, even if they did not generate hyaline cartilage [90].

Intra-articular injections of collagenase are used as a reproducible model to induce the wanted OA severity in the knee. Using this model, moderate OA was induced in rabbits, treated with intra-articular PRP injections which resulted in higher scores in gross morphology and histology [91]. Intra-articular injections of PRP with gelatin microspheres prevented OA progression in a rabbit model [92]. Instead, three weekly PRP injections provoked slow modifications that could not be appraised 2 weeks post-treatment [93].

3.4 Clinical trials

Compared to HA, the use of PRP in the treatment of knee OA is more recent. Unexpectedly, there are more clinical than animal studies [94]. Up to now, five RCT studies [95-99], three controlled nonrandomized studies [100-102], and several case series have been published (**Table 1**) [103-109].

In an open study [107], 50 patients with knee OA were treated with two intra-articular injections of autologous PRP; of them 25 had undergone a previous operative intervention for cartilage lesions (shaving or micro-fracture), whereas 25 had not. After 12 months follow-up, the PRP treatment showed positive effects in all subjects. Both operated and nonoperated patients showed significant improvement in terms of pain reduction and improvement of symptoms and quality of life. In another open study, the efficacy of PRP administration and the duration of the positive effects were tested in 65 patients suffering from knee OA. The symptoms relapsed up to 6 months as mean, but deteriorate again 1 year after the PRP injection. Increasing age and progressive patella-femoral joint degeneration resulted in a decreased potential for PRP injection therapy [109]. Patel *et al.* [97] compared the effects of the active treatment (two groups: single injection of PRP and two injections 3 weeks apart) versus placebo (saline). A significant improvement in WOMAC scores (pain, stiffness, physical function, and total score) was observed without any difference between the two groups in active treatment. The efficacy lasted until

Table 1. Clinical studies comparing platelet-rich plasma versus hyaluronic acid injections.

Autor (year) [Ref.]	Type of HA	Type of PRP (platelet enrichment)/volume	Outcome index	N, follow up	Results	Level of evidence
Vaquerizo *et al.* 2013 [95]	Cross-linked High-MW HA Hylan GF-20 20 mg/ml, 3 ml	Pure-PRP (1.5 – 2×) $CaCl_2$ activated/8 ml PRP/3 injections 1-week interval	WOMAC Lequesne	n = 48 per group 24 and 48 weeks	Higher rate of response (30% reduction in WOMAC) in PRP patients at 24 and 48 weeks	I
Sanchez *et al.* 2012 [96]	Low-MW HA 1% sodium hyaluronate/2 ml	Pure-PRP (1.5 – 2×) $CaCl_2$ activated/8 ml PRP/3 injections 1-week interval	WOMAC	n = 89 PRP, n = 87 HA 1,2,6 months	Higher rate of response (50% decrease in pain) in PRP patients at 24 weeks	I
Cerza *et al.* 2012 [98]	Low-MW HA 20 mg/2 ml 4 weekly injections	Pure-PRP/5.5 ml/ 4-weekly injections	WOMAC	n = 60 per group 1,3,12 months	Sustained improvement up to 24 weeks in the PRP group, better than HA	I
Spakova *et al.* 2012 [100]	Low MW HA/ 3 weekly injections	PRP/3 ml/3 weekly injections	WOMAC 11 point pain intensity	n = 30 per group 3 and 6 months	Significantly better results in PRP group at 3 and 6 months	II
Kon *et al.* 2011 [101]	High- and Low-MW HA	L-PRP (4 – 6×) $CaCl_2$ activated/5 ml/3 injections 2-week interval	IKDC, VAS	n = 50 each group (3 groups) 2 and 6 months	Similar at 2 months, at 6 months PRP better than HA in pain and function	II
Sánchez *et al.* 2008 [102]	Low-MW 2% HA /2 ml	Pure-PRP (1.5 – 2×) $CaCl_2$ activated/8 ml PRP/3 injections 1-week interval	WOMAC	n = 30 each group matched by age and sex 6 months	Significant differences in function and pain at 5 weeks	III

HA: Hyaluronic acid; IKDC: International Knee Documentation Committee; KOOS: Knee injury and Osteoarthritis Outcome Score; L-PRP: Leukocyte platelet-rich plasma; MW: molecular weight; P-PRP: Pure platelet-rich plasma; VAS: Visual analogue scale; WOMAC: Western Ontario and McMaster Universities Index of Osteoarthritis.

the final follow-up at 6 months, with slight worsening thereafter. In the control group WOMAC scores deteriorated from baseline to the final follow-up. There was no influence of age, sex, weight, or BMI on the outcome.

Of particular interest are the trials where PRP was compared to HA. Filardo *et al.* [99], in an open trial including 109 patients (55 treated with HA and 54 with PRP), at the follow-up evaluations (up to 12 months), observed in both groups a clinical improvement, but not statistically significant difference inter-groups. Merely those patients with low-grade articular degeneration (KL score up to II) treated with PRP showed an encouraging positive tendency. Similarly, Spakova *et al.* [100] in a prospective study, including a total of 120 patients with KL grade I, II, or III knee OA, found significant better results in the WOMAC and Numeric Rating Scale scores in PRP-treated patients, after a 3 and 6 months follow-up, compared to those who were treated with HA. Finally, Cerza *et al.* [98] performed a randomized controlled trial, examining the effect of four intra-articular injections of PRP versus four intra-articular injections of HA. Treatment with PRP had a significant effect shortly after the last injection and a continuously sustained improvement up to 24 weeks, as shown by WOMAC score. The clinical outcomes were better when compared with HA-treated patients. In the HA group, patients with severe OA (grade III) had the worst results, whereas stratifying patients according to OA severity in the PRP group did not show any statistically significant improvement in the outcome.

A recent systematic review with quantitative synthesis supports the conclusion that at 6 months PRP is superior to HA treatment in adult patients with mild to moderate knee OA [110]. A more recent multicenter randomized trial involving 90 patients (not included in the above quantitative synthesis), showed enhanced outcomes 6 months and 1 year after 3 weekly injections of pure PRP compared to a single injection of cross-linked HMW HA [95]. A latter meta-analysis [111] indicated that PRP-treated patients show a continual improvement until 12 months, while HA showed a highest point of efficacy at 2 months post-treatment. There were no differences in adverse reaction between both treatments. Reviews on PRPs do not mention other blood derivatives, such as Autologous Conditioned Serum (ACS, marketed as Orthokine), based on IL-1Ra anti-inflammatory properties, a receptor antagonist for IL-1β [112].

Whether inter-patient inconsistencies in response to treatment can be attributed to procedural differences, such as the number and volume of injections, along with the type of PRP and the interval between injections is not clear. Other important sources of unpredictability include the conditions of the host tissue, that is to say the severity of OA (e.g., full-thickness vs superficial OC defects, or different OA severity as assessed by radiographic scales). Another potential disadvantage of using autologous PRP is its intrinsic biological variability. Kruger *et al.* [113] tested the main basis for PRP injections, that is the local application of molecules active in chondrogenic differentiation, by quantifying Bone Morphogenetic protein 2 (BMP-2), CTGF, FGF-2, TGF-β3 in a pooled PRP from six donors. However, BMP-2 and CTGF were present in five out of six donors in very variable concentrations when the PRPs were analyzed one by one by ELISA. Indeed, this variability hinders reaching valid conclusions about the potency of PRP injections for knee OA.

4. HA plus PRP in association?

Recent basic research supports the idea that HA and PRP treatments can be advantageously associated without altering the original relevant characteristics of both products. Recently, in a co-culture model, involving synovial cells and chondrocytes, Sundman *et al.* [114] compared the effects of PRP or HA on inflammation, as measured by TNF-α, IL-6 and IL-8 proteins; they found that, although both treatments decreased TNF-α production, IL-6 was decreased only in HA cultures, but not in PRP-treated cells suggesting that both treatments influence inflammation through different mechanisms. The expression of catabolic enzymes such as MMP-13 was reduced in synoviocytes and chondrocytes treated with PRP but not in cells treated with HA. Moreover, the synoviocytes treated with PRP but not those treated with HA expressed HAS 2. Thus, separately HA and PRP are beneficial for joint cells although they function through different mechanisms. Therefore, we may infer that their advantages might be additive when both products are injected in the knee OA. Anitua *et al.* [61] evaluated the potential of pure PRP to induce tendon cells and synovial fibroblasts migration and examined whether the combination of PRP with HA improves their motility *in vitro*. Human fibroblast cells were isolated from synovium and tendon biopsies and cultured by standard procedures. Therefore, PRP was obtained from a young healthy donor and added to the culture medium at different doses. Finally, the migratory capacity induced by PRP, HA and both in association were tested. PRP stimulated the migration of fibroblasts, as well as HA, but this effect was more prominent when HA was combined with PRP. Indeed, an increase of 335% in motility was observed in the case of HA+PRP treatment compared with HA. Therefore, this '*in vitro*' study definitely proves that PRP improves the biological properties of HA. CD44 has been implicated in the migratory signal transduction, as well as receptor for HA-mediated motility in several cell lineages. Plasma-derived GFs increase the CD44 expression, and this favors cell migration through the interaction of this receptor with extracellular HA.

In another study [115], the outgrowth of rabbit chondrocytes from cartilage fragments, loaded onto a composite scaffold made of a HMW HA and autologous PRP, was evaluated. Defects were created by means of a medial arthrotomy, in rabbit knees and cartilage fragments were collected. Therefore, membrane scaffolds with HA were prepared and cartilage fragments were loaded into the membrane. Finally, the

Table 2. Similarities and differences between platelet-rich plasma and hyaluronic acid treatments.

	Intra-articular injections	
	HA	**PRP**
Clinical effects	Pain reduction and function improvement in the short term (3 – 6 months)	Pain reduction and function improvement in the short term (3 – 6 months) exceeds HA effects
Indications	Mild-moderate OA	Mild-moderate OA (but used in the full spectrum of severity)
Treatment schedule	Weekly three to five injections*	Weekly, biweekly, monthly one to five injections
Formulations	Various MWs available, native or cross-linked	Variations based on platelet and leukocyte counts, the latter are depleted, L-PRP or P-PRP
Presentation	Ready to use	Blood withdrawal and centrifugation (minimal manipulation)
Biological mechanisms	Lubrication Cell signaling through hyaladherins	Multiplex cell signaling, various cell targets Cell migration, proliferation, differentiation, anabolism

*Except cross-linked HMW, 1 injection.
HA: Hyaluronic acid; OA: Osteoarthritis; PRP: Platelet-rich plasma.

in vivo defects were filled with cartilage fragment load HA scaffolds alone, or adding PRP. A histological evaluation at 6 months showed that this latter group had better results, being filled by a repair tissue with some features of hyaline cartilage. This repair tissue was better quality than that of the lesions treated with scaffold only and untreated lesions.

The idea of positive interactions between HA and PRP is supported by an elegant experiment where HA was added to a GFs present in platelets. BMP2 plays a critical role in the embryologic development of normal cartilage, thus it may enhance reparative processes by setting in motion morphogenetic processes, including the formation of ECM. Unfortunately, the high chondrogenic potency of BMP2 is hampered by its short half-life and rapid degradation *in vivo*. Perlecan/HSPG2, a heparan sulfate proteoglycan, represents an essential component of cartilage ECM. Due to specific receptors Perlecan can act as a depot for BMP2 storage and controlled kinetics, protecting BMP2 from proteolytic cleavage. To avoid its own diffusion and susceptibility to degradation, PlnD1 was immobilized through conjugation to a larger biocompatible carrier forming HA-based microgels (PlnD1-HA) in order to preserve BMP2 activities [116]. The efficiency of this system was tested using an experimental OA model in mice. It was observed that knees treated with PlnD1-HA/BMP2 had lesser damage compared to control knees. Moreover, they had, in comparison to controls treated with Perlecan+HA, higher mRNA levels of type II collagen, proteoglycans and xylosyltransferase 1, a rate-limiting anabolic enzyme involved in the biosynthesis of glycosaminoglycans. In conclusion, this study shows that HA can favor the stabilization of some GFs, enabling their therapeutic potential. Currently, no clinical studies support this basic research.

There are reports, which claim excellent results of the HA+PRP association in the healing of pressure ulcers and surgical wounds [117-119], and in Morton neuroma surgery [120], but these anecdotal findings need confirmation by controlled trials, comparing the composite PRP/HA with HA or PRP, used alone. Currently alternate cycles of three injections of PRP and three injections of HA are performed in clinical practice, although results have not been reported yet. **Table 2** shows, differences and similarities between HA and PRP therapies.

5. Expert opinion

Since the first intra-articular use of PRP in arthroscopic surgery one decade ago [121], PRP intra-articular injections have become an intriguing treatment for managing knee OA. Human trials, thus far, have shown pain remission and improvement of function in the mid-term (3 – 6 months), but less than half of the patients reach at least a symptom reduction by 40% [96]. Significantly, PRP exceeds HA, the comparator used in every PRP clinical trials. Albeit, both HA and PRP alleviate symptoms in patients with mild–moderate OA. Both treatment schedules most often involve repetitive injections, although the injected volume of PRP is three- to fourfold, the volume of HA. Indeed, both approaches are comparable in terms of the route of administration and safety.

The concept behind HA application is to mimic the properties of synovial fluid, that is to lubricate the hinge joint. Nevertheless, more recently, the idea that in homeostasis the synovial fluid not only lubricates the joint but also provides a positive biological microenvironment has prompted research on HA signaling through hyaladherins located in the cell membrane. Unfortunately, in OA, a deleterious fluidic microenvironment is already established, with the presence of HA fragments, catabolic enzymes, and inflammatory molecules. In this context, the central concept underlying intra-articular injection is to modify this deleterious fluidic microenvironment.

Alternatively, the joint microenvironment can be modified with PRP injections that deliver multiple factors that modulate angiogenesis and inflammation as well as cell anabolism. In fact, the relationships between joint tissues (meniscus, synovium, ligaments, articular surface) and the synovial fluid

are bidirectional meaning that they both (the tissues and the synovial fluid) modify and are modified. So, by injecting PRP and HA we aim to replace the ill-fluid with an engineered fluid providing lubrication and able to control the delivery and presentation of signaling molecules. HA and PRP may improve OA symptoms through dissimilar biological mechanisms.

Since HA and PRP are not mechanical but biological approaches, the ability of PRP+HA to change the biological status of the joint and promote tissue healing will be particularly critical during the initial stages of OA, before the onset of structural changes. Although mixing HA and PRP involves minimal manipulation, studies to verify critical aspects of the character and performance of the composite are mandatory. Several key aspects such as the molecular weight of HA and the concentration to be mixed with PRP should be analyzed. Ideally, HA tertiary structure should let spaces through which molecules can diffuse and approach the cell membrane to interact closely with their specific receptors. Whether or not HA may help to retain PRP in the joint cavity by exerting osmotic pressure on the joint surface calls for exploration. Considering that the HA chains are constantly moving in solution, and that effective pores in this meshwork will depend on HA concentration and MW, molecules may reach the articular surfaces with different kinetics, depending on their size and hydrodynamic volumes.

The future impact of HA+PRP would depend on the capacity of delivering molecules that meet the requirements of the injured joint. An efficient intra-articular therapy would be achieved if modulatory proteins released from PRP+HA are capable of interfering with the catabolic microenvironment, while modulating the inflammatory response, enhancing cell migration and proliferation, and controlling the angiogenic status as well as cell differentiation. Prospective randomized double blind studies, preferably using both HA and PRP as comparators (three armed), and a selected stage of OA severity, preferably early OA, will provide information about the impact of this novel approach.

The development of 'PRP+HA' therapy may be a value-based approach that does not incur high costs, but may provide high benefits for patients suffering OA, and potential savings for health systems. In the first instance this therapy is directed to nonsevere OA, positioning for market entry, at a sector where the probabilities of success are higher. The actual economic context makes imperative to measure patient-relevant end points, and translate health outcomes into pecuniary value. It would be essential to calculate at what price this technology will be likely to meet cost-effectiveness requirements by developing frameworks that captures as many value-relevant features as possible. Recent advances in personalized medicine suggest that to be effective therapies should be directed in an individualized manner to treat specific subgroups of patients. Thus, the expansion of molecular and imaging biomarkers to stratify patients and identify the responders to this therapy should accompany the development of 'PRP+HA' therapy.

Over the next few years we hope to see the value of combined therapies, that is, PRP+HA, to meet critical health needs such as knee OA. If successful, although initially focused on knee OA, 'PRP+HA' therapy could be extended to other joints, mainly the hip and the hands.

Acknowledgement

I Andia and M Abate both authors contributed equally to this work.

Declaration of interest

The authors state no conflict of interest. I Andia is partially supported by Basque Government Grant Saio12-PE12BF007.

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