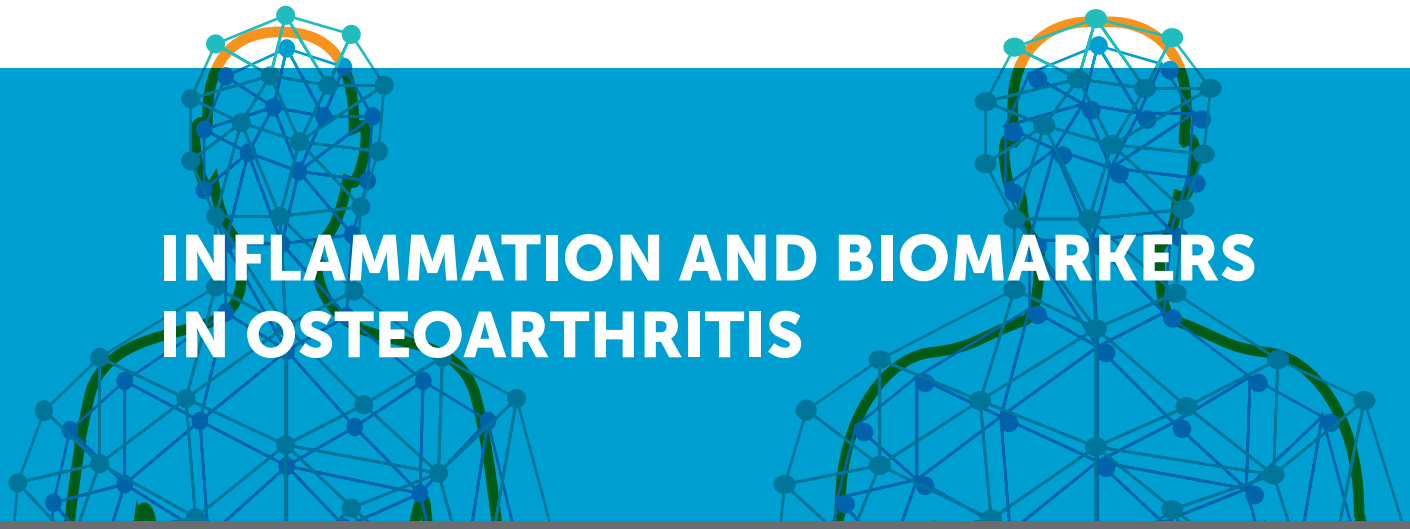

INFLAMMATION AND BIOMARKERS IN OSTEOARTHRITIS

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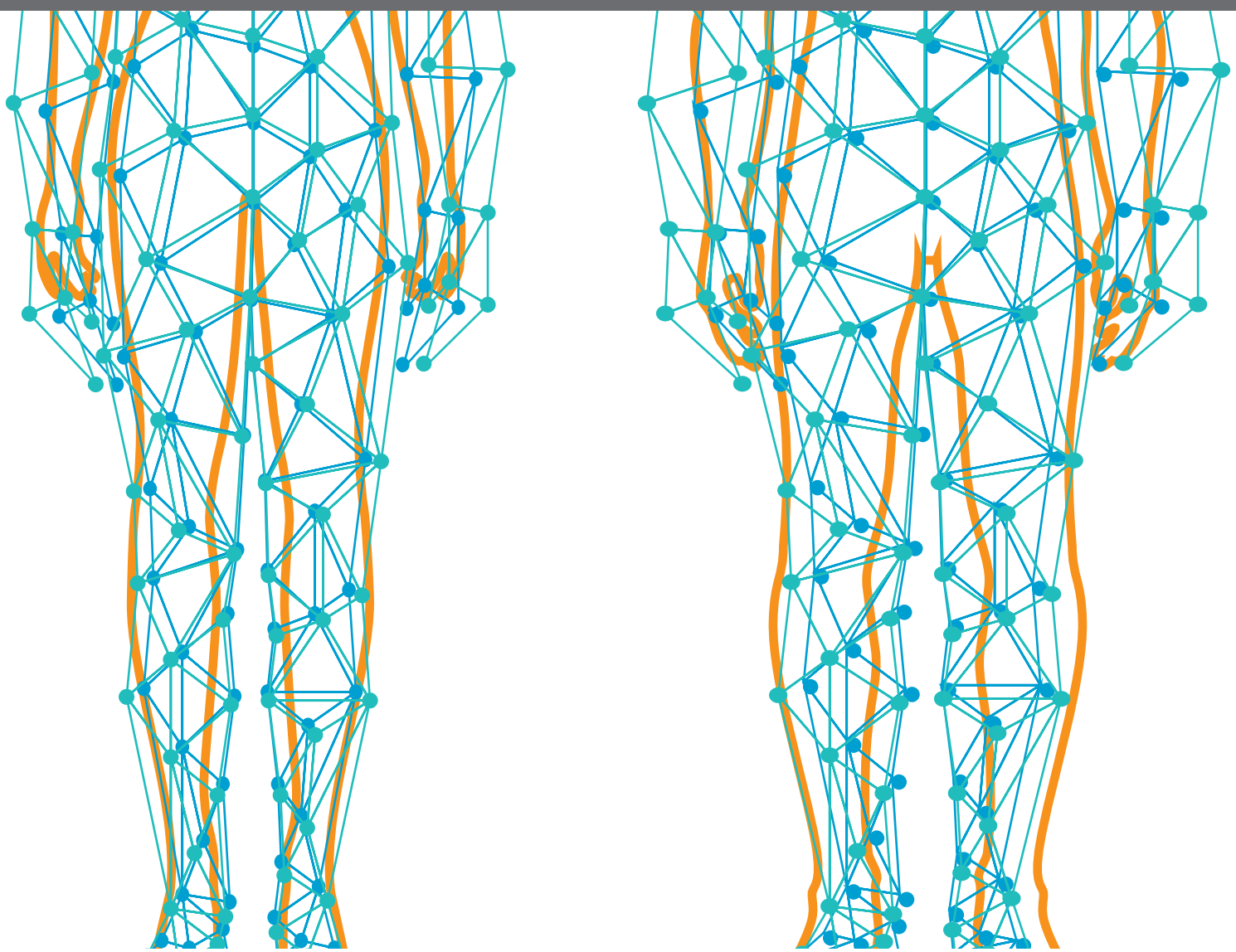
PUBLISHED IN: Frontiers in Medicine



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frontiers

ISSN 1664-8714
ISBN 978-2-88971-338-7
DOI 10.3389/978-2-88971-338-7

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INFLAMMATION AND BIOMARKERS IN OSTEOARTHRITIS

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Citation: Rocha, F. A. C., Fonseca, J. E., Mobasheri, A., Gualillo, O., Henrotin, H. E., Largo, R., Herrero-Beaumont, G., eds. (2021). Inflammation and Biomarkers in Osteoarthritis. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-338-7

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frontiers in Medicine

EDITORIAL
published: 27 July 2021
doi: 10.3389/fmed.2021.727700

Editorial: Inflammation and Biomarkers in Osteoarthritis

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Keywords: osteoarthritis, biomarkers, cartilage, synovium, inflammation

OPEN ACCESS

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Specialty section:
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

Received: *19 June 2021*
Accepted: *30 June 2021*
Published: *27 July 2021*

Citation:
Mobasheri A, Fonseca JE, Gualillo O, Henrotin Y, Largo R, Herrero-Beaumont G and Rocha FAC (2021) Editorial: Inflammation and Biomarkers in Osteoarthritis. Front. Med. 8:727700. doi: 10.3389/fmed.2021.727700

Editorial on the Research Topic

Inflammation and Biomarkers in Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis affecting more than 500 million people globally (1). It accounts for more pain and functional disability than any other musculoskeletal disease and is an important source of high societal and economic costs (2). Although the pathophysiology of OA is poorly understood (3), the risk factors associated with disease development are well-established. They include age (4), obesity (5), sex (6), previous incidence of joint injuries (7, 8), meniscal damage (9), joint instability (10), malalignment (11), genetics (12), bone shape (including anatomical deformities) (13), muscle weakness and sarcopenia (14), and metabolic disease (15–17). Although OA can affect any synovial joint, including joints in the hand, according to studies on the global burden of disease in 2010 (18) and 2017 (19), knee OA represents the greatest societal burden.

Beside mechanical derangement, inflammation plays a key role in the pathogenesis and progression of OA (20, 21). However, the inflammation associated with OA is not the same type and grade that is associated with rheumatoid arthritis (RA) and other inflammatory diseases of joints (22). It is becoming increasingly accepted that "low-grade" inflammation and the mechanisms that regulate it are relevant not only to joint pain and disability in OA (23), but also to joint trauma and the biomechanical damage sustained to joint tissues (24–26). Persistent synovitis as well as damage to the subchondral bone have been considered to play major roles in joint destruction, particularly in knee OA (27, 28). The association of meniscal damage with OA progression has highlighted the role of the meniscus and its biomechanical role in the joint (29–31). Therefore, the menisci may also participate in the inflammatory scenario of joints affected by OA (32).

Another important contributor to the process of "low-grade" inflammation in OA is the synovium (23, 33). There is evidence of cross-talk between articular cartilage, subchondral bone and synovium. Mechanistic evidence comes from *in vitro* and animal studies and clinical evidence

from studies on patients with OA (34, 35). Synovial cells, particularly type A macrophage-like synoviocytes, are likely to be the major source of pro-inflammatory mediators within the joint (36). Moreover, there are differences in the profile of pro-inflammatory cytokine production in classically activated (M1) and alternatively activated (M2) macrophages (37, 38). Macrophage polarisation is an issue that may be relevant not only to emerging targeted therapies but also to ongoing efforts aimed at discriminating the different molecular endotypes and clinical phenotypes of OA (39, 40).

Biochemical markers (also called molecular markers, signature molecules or biomarkers) are biological molecules found in body fluids, or tissues that may be used as indicators of physiological and pathophysiological processes. They can be defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." (41). Biomarkers may be used to see how well patients respond to new treatments and interventions for a disease or condition. In OA biomarkers may be used to understand disease pathogenesis, study progression and define the molecular endotypes (42, 43). Biomarkers have been used very effectively to identify molecular endotypes and clinical phenotypes in other disease areas. For example, in asthma, biomarkers have been used to identify phenotypes and endotypes that characterise severe asthma (44, 45). However, in the field of OA we are lagging behind and need to catch up in order to enhance clinical trials and facilitate drug development. Biomarkers of early OA represent a major unmet need and more research needs to be done to identify biomarkers that characterise early events in the pathogenesis of OA.

The aim of this Research Topic was to assemble a comprehensive collection of authoritative articles focusing on fundamentals of the inflammatory scenario in OA joints and their relevance to existing and emerging biomarkers in this disease. One of the key priorities is the identification, characterisation and validation of biomarkers that define molecular endotypes of OA, serving as tools to discriminate different OA phenotypes.

MicroRNAs (miRNAs) are post-transcriptional regulators that are dysregulated in osteoarthritic tissues including the synovium. miRNAs are important contributors to OA synovial changes and to act as novel therapeutic targets. Tavallaee et al., reviewed the recently published literature investigating the roles that miRNAs play in OA-related synovial pathologies including inflammation, matrix deposition and cell proliferation. Their analysis of the literature has revealed that miRNAs contribute to synovial homeostasis, inflammation, fibrosis, angiogenesis, cell survival and cell apoptosis, contributing to OA synovial pathology.

The inflammation fuelled by metabolic imbalance, also known as "meta-inflammation," is a type of chronic (long-lasting), persistent but "low-grade" systemic inflammation caused by multiple components involved in metabolic syndrome (MetS), including central obesity, adipokine dysregulation, and impaired glucose tolerance. Gratal et al., reviewed the literature focusing on purinergic regulation in OA cartilage and how different components of MetS modulate the purinergic system in OA. They described the critical role of receptors, such as adenosine A2A receptor (A2AR) and ATP P2X7 receptor in OA and assess how nucleotides regulate the inflammasome in OA.

Villalvilla et al. conducted an animal study using rabbits to investigate the effect of hypercholesterolemia induced by high-fat diet (HFD) in cartilage from OA rabbits, and how oxLDL affect human chondrocyte inflammatory and catabolic responses. They found that HFD intake does not modify cartilage structure or pro-inflammatory and catabolic gene expression and protein presence, both in healthy and OA animals. Their study concluded that dietary cholesterol intake may not be deleterious for articular cartilage but altered cholesterol metabolism may be involved in the associations observed in human disease.

Although biomarkers are important in OA research, clinical trials, and drug development, they have not yet had any significant impact on the clinical management of the OA and follow-up. Bernotiene et al., argued that emerging nano-technologies and immunoassay platforms that are already impacting on routine diagnostics and monitoring in other diseases could potentially serve as technological and strategic examples for enhanced clinical management of OA. Their review article explored the implementation of such technologies in OA research and therapy and discussed the challenges that hinder the development, testing, and implementation of new OA biochemical marker assays utilising emerging multiplexing technologies and biosensors.

Rajandran et al., evaluated the association between biomarkers of innate immunity and magnetic resonance imaging (MRI) features of early and late stages of knee OA. They investigated biomarkers of innate immunity associated with meniscal extrusion and synovial inflammation in earlier stage and bone marrow lesions (BMLs) in later stages of knee OA. They also observed associations between pro-inflammatory biomarkers and various MRI features in the early stages of knee OA. Their exploratory study supported the association between biomarkers of activated macrophages and synovial inflammation in the early stages of knee OA.

Lambert et al., reviewed the literature focusing on damage-associated molecular patterns (DAMPs) as biomarkers and potential therapeutic targets for OA. Their paper highlighted the central role of DAMPs in the interplay between immune responses and inflammation in OA.

Sun et al., used a rat model of OA to determine whether switching from an obesogenic diet to a normal chow diet can mitigate the detrimental effects of inflammatory pathways that contribute to OA pathology. Their results indicated that dietary switching from an obesogenic diet to a normal diet reduces body weight and restores metabolic parameters and suppresses synovial inflammation. They concluded that obesogenic diets induce systemic and synovial inflammation and dietary switching may be used as an intervention to slow down the progression of OA.

Work by de Melo Nunes et al. examined the chemical composition of glycosaminoglycans (GAGs) from normal and osteoarthritic cartilage and a reported reduced sulphur content

in GAGs from OA patients, which is associated with a reduced zeta potential.

Finally, Zhang et al., reviewed the literature on synovial fibrosis in OA, establishing the concept that fibrosis is an eventual outcome of inflammation in OA. Therefore, new interventions are needed to slow the progression of fibrosis in OA and associated co-morbidities. They proposed the combined use of anti-fibrotic drugs with potential for therapy in OA.

We hope that you enjoyed reading these papers as much as we enjoyed editing them for this Research Topic in the rheumatology section of Frontiers in Medicine.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing, editing, and revision of this editorial.

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frontiers in Medicine

MINI REVIEW
published: 11 August 2020
doi: 10.3389/fmed.2020.00376

MicroRNAs in Synovial Pathology Associated With Osteoarthritis

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Osteoarthritis (OA) is the most common type of arthritis, a disease that affects the entire joint. The relative involvement of each tissue, and their interactions, add to the complexity of OA, hampering our understanding of the underlying molecular mechanisms, and the generation of a disease modifying therapy. The synovium is essential in maintaining joint homeostasis, and pathologies associated with the synovium contribute to joint destruction, pain and stiffness in OA. MicroRNAs (miRNAs) are post-transcriptional regulators dysregulated in OA tissues including the synovium. MiRNAs are important contributors to OA synovial changes that have the potential to improve our understanding of OA and to act as novel therapeutic targets. The purpose of this review is to summarize and integrate current published literature investigating the roles that miRNAs play in OA-related synovial pathologies including inflammation, matrix deposition and cell proliferation.

Keywords: osteoarthritis, synovium, microRNA, inflammation, fibrosis

OPEN ACCESS

Edited by:
Francisco Airton Castro Rocha, Federal University of Ceará, Brazil

Reviewed by:
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Specialty section:
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

Received: *17 April 2020*
Accepted: *18 June 2020*
Published: *11 August 2020*

Citation:
Tavallaee G, Rockel JS, Lively S and Kapoor M (2020) MicroRNAs in Synovial Pathology Associated With Osteoarthritis. Front. Med. 7:376. doi: 10.3389/fmed.2020.00376

INTRODUCTION

Osteoarthritis (OA) is the most common chronic debilitating disease imposing a significant socioeconomic burden and affecting the quality of life of millions of people worldwide (1). OA affects the whole joint and involves progressive articular cartilage degradation, subchondral bone remodeling, ectopic bone formation, ligament degeneration, menisci degradation, and synovial inflammation and hypertrophy (2). Many OA studies largely focus on cartilage health as it facilitates joint movement and is highly susceptible to OA; however other tissues, notably the synovium, are now recognized to be involved in OA pathology (3, 4). OA alters the homeostatic functions of cells residing in the synovium, but we are only starting to elucidate the underlying gene expression and regulatory mechanisms responsible, and how these changes contribute to disease progression. Gene expression profiles of the synovium are also altered during OA, which is accomplished by multiple regulatory mechanisms. At the post-transcriptional level, gene transcripts are regulated by a class of small non-coding RNAs called microRNAs (miRNAs). A single miRNA can target a large number of transcripts contributing to tissue specific gene expression (5). The complex network of miRNAs that regulate the pathophysiology of cartilage degeneration during OA has been previously reviewed (6); however, very little is known regarding the role of miRNAs in regulating synovial gene expression during OA. In this review, we summarize the contributions of the synovium to OA pathology and how focusing on the role of miRNAs in regulating the activity of fibroblast-like synoviocytes (FLS) warrants further study to further elucidate mechanisms contributing to OA pathologies.

CELLULAR INTERACTIONS IN THE OA SYNOVIUM

The synovium is a loose connective tissue that encapsulates the joint and aids in maintaining joint homeostasis through the functions of its resident cells: synovial macrophages and the more abundant FLS [reviewed in (3, 4)]. FLS are mesenchyme-derived cells that share characteristics with other fibroblasts, such as the expression of collagens IV and V, vimentin and CD90, but also show unique expression that differentiates them from other resident fibroblasts, notably cadherin-11 expression by FLS in the synovial lining (7). In healthy synovium, FLS are the major source of extracellular matrix (ECM) and synovial fluid, while resident macrophages remove metabolites and products of matrix degradation (4). As OA progresses, the synovium undergoes hyperplasia, sublining fibrosis, increased vascularization, and increased cell proliferation, migration and invasion (3).

In the context of OA, FLS are the major contributors to the observed excessive synovial ECM deposition and fibrosis (8). While they are involved in the production of proinflammatory and profibrotic mediators, resident synovial macrophages also respond and contribute to OA progression and inflammatory responses (9). Accumulation of macrophages in the synovium is a defining characteristic of synovitis, notably adjacent to areas of cartilage degradation (10, 11). Macrophages are highly plastic cells; and although a broad spectrum of activated states exists, macrophages are generally classified as pro-inflammatory (M1) and inflammatory resolving (M2) (12). In healthy conditions, macrophages are thought to be in an M2-like phenotype that maintains tissue homeostasis and repair (13). Inflammatory mediators, such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α), as well as catabolic enzymes, such as matrix metalloproteases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs), are produced by synoviocytes and secreted into synovial fluid in quantifiable levels (9). These changes contribute to the excessive ECM deposition and increased synovial thickness detected in OA patients and animal models, and impact joint integrity.

In the synovial fluid of patients with knee OA, the balance of M1 and M2 macrophage markers is skewed toward a pro-inflammatory state, and the degree of the shift is positively associated with the OA severity (14). In the OA synovium, the majority of macrophages possess pro-inflammatory profiles, driving responses that promote synovitis and osteophyte formation (10, 11, 15). In addition to modulating local inflammatory responses, activated macrophages secrete various MMPs and ADAMTSs, which remodel the synovial matrix, and enhance fibrosis-promoting activities of FLS (16). The master driver of fibrosis is transforming growth factor-beta 1 (TGF-β1) as it stimulates FLS expression of other profibrotic mediators, including α-smooth muscle actin (α-SMA), vascular endothelial growth factor (VEGF), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), tissue inhibitors of metalloproteinases-1 (TIMP-1), and collagen type I, as well as OA FLS proliferation and migration (17). FLS in turn influence macrophage activity (18). Thus, interactions of FLS with macrophages can also contribute to the pathological changes in the synovium during OA and is an important consideration for future studies.

miRNA BIOGENESIS AND FUNCTION

MicroRNAs (miRNAs) are single stranded endogenous small non-coding RNA molecules of 21–24-nucleotide (nt) length that are transcribed by RNA polymerase II. MiRNAs are expressed in polyadelynated and capped nascent transcripts ∼ 200 nt (pri-miRNA) with hairpin structures. Pri-miRNAs are recognized by DiGeorge syndrome critical region gene 8 (Dgcr8, an RNA binding protein) and cleaved into ∼70 nt stem loop precursors (pre-miRNA) in the nucleus by Drosha, a nuclease of the RNase III family, and transported to cytoplasm by Exportin 5. Pre-miRNAs are processed into miRNA duplexes in the cytoplasm by the enzyme Dicer. One strand (mature miRNA) asymmetrically assembles into the Argonaute (AGO) protein of the RNA-induced silencing complex (RISC) and the other one is destroyed. Mature miRNAs then bind mRNAs of target genes in a sequence-specific manner via "seed" sequences, 2–8 nucleotides from the 5′ end of miRNAs, usually resulting in cleavage of target mRNAs or translational repression [reviewed in detail in (19, 20)].

miRNAs IN OA SYNOVIAL PATHOLOGY

OA studies to date mostly focus on the role of miRNAs in regulating cartilage maintenance and degradation. However, miRNAs also regulate other aspects of OA, including synovial pathology. This is an understudied area and consequently, much less is known. For the purpose of this review, we searched PubMed using "Osteoarthritis + synovium + miRNA" and "Osteoarthritis + synovitis + miRNA" for studies published until March 2020. A total of 83 articles were identified. Thirty-five articles focused exclusively on articular cartilage or tissues other than synovium or on OA symptoms, rather than synovial pathologies, leaving 48 articles relevant to this review.

Considering FLS as essential participants in joint homeostasis and contributors to OA synovial pathology, it is not surprising that OA FLS show differential miRNA profiles. Recently, deep sequencing identified 245 differentially expressed genes in OA FLS and bioinformatics analyses highlighted "ECM organization and altered cellular movement" as one of the most enriched OA FLS functions connected to the differentially expressed genes and miRNA network (21). OA FLS also exhibit an independent miRNA signature from rheumatoid arthritis (RA) FLS, negatively correlating to the expression levels of their putative target genes (22). Elevated levels of miR-625 and miR-124 in OA FLS are associated with decreased expression of their target genes, while miR-155b and miR-203 are expressed at lower levels concomitant with higher expression of their target genes (22). In addition to *in vitro* studies, animal models aid in the understanding of differentially expressed miRNAs in OA synovium. Kung et al. found 394 miRNAs transiently expressed at 1 vs. 6 weeks in the synovium of the destabilization of the medial meniscus

TABLE 1 | Role of some miRNAs in the synovial pathology during OA.

MiRNA	Species/model system	Role in OA	References
miR-181c	Human OA FLS	Suppresses expression of MMP13, IL-6, and IL-8 and targets OPN to reduce FLS proliferation.	(24)
miR-770	Human OA FLS	Suppresses proliferation of OA FLS.	(25)
miR-26a-5p	Human OA FLS	Targets COX2 to reduce Bcl-2, IL-6, TNF-α, and IL-8 expression.	(26)
	Rat instability model of OA	Alleviates synovial inflammation.	
miR-146a	Human OA FLS	Dampens IL-1β signaling.	(27–29)
	Mouse Knockout	Exhibits synovial hyperplasia.	(30)
miR-122	Human OA FLS	Reduces IL-1α levels.	(31)
miR-381a-3p	Human OA FLS	Targets IκBα to enhance NF-κB activity.	(32)
	Rat MIA	Upregulates in the synovium of MIA rats.	
miR-34a miR-146a miR-181a	Human OA FLS	Promote inflammatory mechanisms and oxidative stress.	(33)
miR-29a	Human OA FLS	Targets VEGF and suppresses ECM production.	(34)
	Mouse CIOA	Protects the synovium from hyperplasia and macrophage infiltration.	
miR-338-3p	Human OA FLS	Targets TRAP-1 to regulate TGF-β responsive genes.	(35)
miR-125	Human HUVEC	Enhances glycolysis and angiogenesis.	(36)
miR-128	Mouse ACLT	Promotes synovial membrane thickness and fibroblast activation.	(37)
miR-101	Rats MIA	MiR-101 inhibition reduces cytokine expression in the MIA rats synovium.	(38)

ACLT, anterior cruciate ligament transection; CIOA, collagenase-induced osteoarthritis; FLS, fibroblast-like synoviocytes; HUVEC, human umbilical vein endothelial cells; OA, osteoarthritis; MIA, monosodium iodoacetate; ECM, extracellular matrix; MMP13, matrix metalloprotease 13; IL-6, interleukin-6; IL-8, interleukin-8; OPN, osteopontin; COX2, cyclooxygenase-2; Bcl-2, B-cell lymphoma-2; TNF-α, tumor necrosis factor alpha; IL-1β, interleukin-1 beta; IL-1α, interleukin-1 alpha; IκBα, inhibitor of nuclear factor kappa B alpha; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; VEGF, vascular endothelial growth factor; TRAP-1, TNF receptor-associated protein-1; TGF-β, transforming growth factor-beta.

(DMM) mouse model of knee OA (23). Thus, several miRNAs modulated in the synovium potentially contribute to joint destruction, synovial inflammation, and fibrosis (summarized in **Table 1** and **Figure 1**). However, the individual and combined contributions of these miRNAs to synovial pathology warrant further investigation to comprehensively understand their role and signaling mechanisms in OA.

miRNAs AND SYNOVIAL INFLAMMATION IN OA

MiRNAs play key roles in OA-related synovial inflammation. The expression levels of inflammatory-related miRNAs measured in the synovium from OA patients and animal models show unique signatures when compared to normal controls. When comparing inflamed areas with normal areas of synovium from OA patients, 31 miRNAs are identified in an OA-specific regulatory network comprised of 97 interactions of 38 transcription factors and 35 genes (39). Many miRNAs are upregulated during OA that exacerbate inflammatory responses in the synovium. MiR-381a-3p is upregulated in the synovium of both OA patients and in the monosodium iodoacetate (MIA)-injected rat model of OA pain; and miR-381a-3p enhances nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity in cultured human OA FLS by targeting inhibitor of nuclear factor kappa B alpha (IκBα) (32). Inhibition of miR-101 in MIA-injected rats reduces cytokine expression in the synovium (38). Furthermore, blocking miR-128 reduces both synovial membrane thickness and fibroblast activation protein (FAP)-positive FLS accumulation in the mouse anterior cruciate ligament transection (ACLT) model of OA (37). Thus, fine-tuning synovial inflammation through miRNA modulation is a promising avenue of research for future OA therapeutic targets.

Some miRNAs have been shown to exhibit anti-inflammatory effects in the synovium during OA. For instance, administration of human bone mesenchymal stem cell-derived exosomes overexpressing miR-26a-5p to cultured OA FLS targets cyclooxygenase-2 (COX2), reducing B-cell lymphoma 2 (Bcl-2), IL-6, TNF-α and IL-8 expression, and increasing Bcl-2-associated X protein (Bax) expression and caspase cleavage, alleviating synovial inflammation in a rat joint instability model of OA (26). MiR-146a is highly expressed in the synovium during OA and when knocked-down in mouse models, NOTCH1 and IL-6 expression are increased in the synovium concomitant with synovial hyperplasia (30). When overexpressed in OA FLS, miR-146a decreases the expression of inflammatory mediators, including IL-1-induced TNF receptor associated factor 6 (TRAF 6), IL-1 receptor-associated kinase 1 (IRAK 1), COX2, IL-8, MMP13, and ADAMTS5 expression (27). Denbinobin, a naturally occurring 1,4-phenanthrenequinone, promotes histone acetyltransferase activity, resulting in increased miR-146a expression and inhibition of nuclear factor (NF)-κB activity, dampening IL-1β-elicited expression of cell adhesion molecules and monocyte adhesion to OA FLS (28). Intriguingly, histone deacetylase inhibitors also promote miR-146a expression in IL-1β-treated OA FLS by facilitating NF-κB binding to miR-146a promoter, which reduces downstream responses including IL-6 secretion (29). Thus, the acetylation pattern of miR-146a is an important aspect to its expression and function in OA FLS. MiR-122 is another miRNA with anti-inflammatory potential as its overexpression in OA FLS reduces IL-1α levels (31). Taken together, miRNAs have the potential to regulate inflammation positively or negatively in the OA synovium; but timing, source, and context of their expression in relation to OA-related inflammatory responses needs to be better understood.

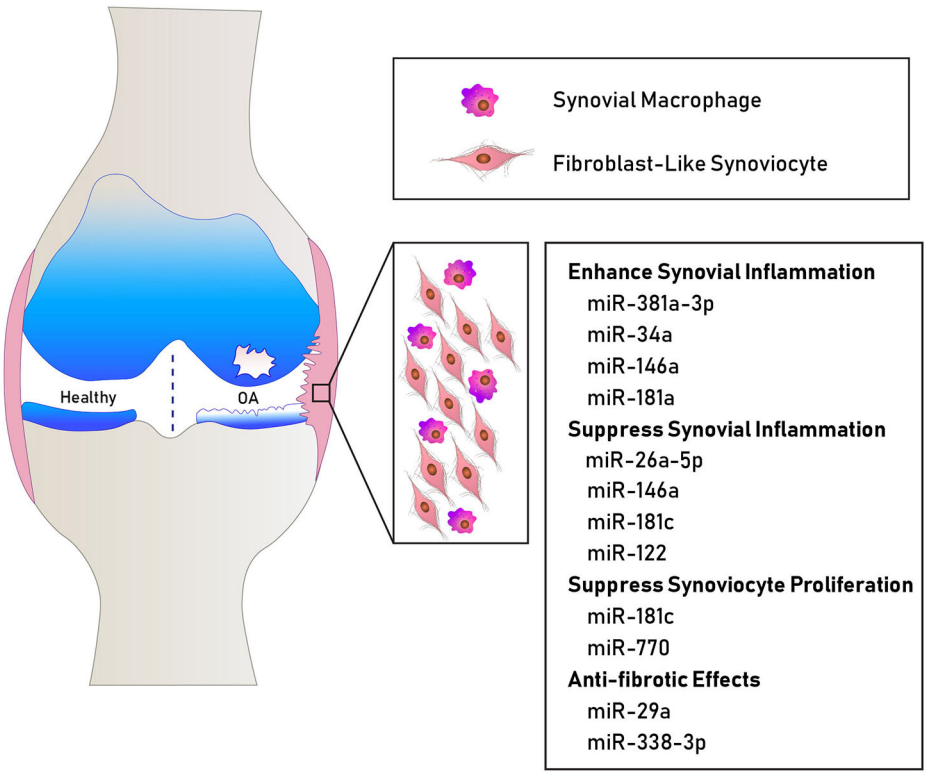

FIGURE 1 | Schematic showing some miRNAs involved in human OA synovial pathology. MiR-381a-3p, miR-34a, miR-146a, and miR-181a promote inflammatory mechanisms (32, 33). MiR-26a-5p, miR-146a, miR-122, and miR-181c suppress the expression of inflammatory cytokines (24, 26, 27, 31). MiR-181c and miR-770 suppress fibroblast-like synoviocyte proliferation (24, 25). MiR-29a and miR-338-3p exhibit anti-fibrotic effects (34, 35).

RA FLS have been shown to mount greater inflammatory responses compared to OA FLS, expressing higher levels of certain inflammation-inducing miRNAs. For instance, miR-146a, miR-155, and miR-223 are expressed at higher levels in synovial tissues and RNA extracted from paraffin embedded RA synovial sections relative to OA samples (40, 41). OA tissue is routinely used as control comparisons in these instances. As a result, much more is known about the role of miRNA in RA FLS and synovial tissue. In RA FLS, miR-155 suppresses MMP1 and MMP3 expression (42). Inhibition of miR-155 in synovial fluid-derived macrophages reduces TNF-α production *in vitro* (43). Mir-221-3p is also expressed at higher levels in RA synovial tissue and fluid, and inhibits the anti-inflammatory arm of macrophages by suppressing the JAK3/STAT3 axis and increasing the expression of inflammatory mediators such as IL-6 and IL-8 (44). Similarly, miR-145-5p and miR-143-3p are expressed at higher levels in RA synovium and FLS compared to OA (45). MiR-145-5p targets osteoprotegerin, aggravating bone erosion in collagen-induced arthritis, and also regulates semaphorin 3A (SEMA3A) to modulate the phenotype of RA FLS (45, 46). MiR-143-3p targets insulin-like growth factor1 receptor (IGF1R) and insulin-like growth factor binding protein 5 (IGFBP5) expression, regulating the Ras/p38 MAPK signaling pathway, contributing to FLS proliferation and apoptosis (45, 47). Additionally, miR-203 promotes NF-κB activation and secretion of MMP1 and IL-6, thereby accelerating RA FLS activation (48). Overall, miRNAs clearly modulate the inflammatory profile of synovial macrophages and FLS in RA.

However, it is now appreciated that OA FLS exhibit an independent miRNA signature from RA FLS (22). Intriguingly, several miRNAs that negatively regulate inflammation or FLS proliferation are expressed at higher levels in OA synovium and FLS compared to RA, including miR-34a-3p, miR-124a, miR-30a, miR-10a, miR-140-3p, and miR-140-5p (49–53). MiR-34a-3p expression is decreased in RA FLS, leading to increased inflammation and proliferation (49). Downregulation of miR-34a passenger strand (miR-34a*) in RA FLS, due to methylation of its promoter, promotes apoptosis resistance (54). MiR-124a also suppresses proliferation and inflammation by directly targeting cyclin-dependent kinase 2 (CDK-2) and monocyte chemoattractant protein-1 (MCP-1) in RA FLS (50). Furthermore, decreased levels of miR-30a in RA synovium correlate with reduced apoptosis and enhanced autophagy (51), while lower expression of miR-10a is thought to promote excessive secretion of inflammatory cytokines via NF-κB regulation (52). OA is considered a low-grade inflammatory disease compared to RA or other types of inflammatory arthritis (55); thus, it is not surprising that many miRNAs are differentially expressed in RA compared to OA synovial cells. However, it does not preclude the

possibility that these miRNAs also contribute to synovial inflammation and OA progression. Detailed comparisons of the differential miRNA profiles detected in RA and OA synovial cells coupled with mechanistic studies could offer a jumping point for future investigations into their contributions to OA pathogenesis.

miRNAs AND SYNOVIAL FIBROSIS

In general, a limited number of studies have investigated the role of miRNAs in processes associated with OA synovial fibrosis. For instance, miR-29a targets VEGF and its inhibition in OA FLS promotes the expression of ECM genes (collagen III, TGF-β1, PLOD2, TIMP1, ADAM12, MMP9, MMP13, and ADAMTS5) (34). Conversely, miR-29a overexpression decreases VEGF and ECM gene expression levels. In a mouse model of collagenase-induced OA (CIOA), intra-articular administration of a miR-29a precursor protects the synovium from hyperplasia and macrophage infiltration (34). Thus, miR-29a, which is decreased in OA synovium, appears to reduce profibrotic activities in the healthy synovium by tightly regulating angiogenesis and ECM production. MiR-338-3p is another ECM-regulating miRNA decreased in OA synovium and synovial effusions compared to synovial tissues from patients with joint trauma. MiR-338-3p counteracts TGF-β1-induced expression of vimentin, type I collagen and TIMP1 in FLS by directly targeting TNF receptor-associated protein 1 (TRAP-1) and regulating Smad 2/3 signaling pathways (35). Overall, these miRNAs exhibit anti-fibrotic regulatory effects; however, there are likely more miRNAs with similar activities that remain to be identified in addition to miRNAs with profibrotic effects that exacerbate synovitis associated with OA.

Profibrotic mediators have also been shown to regulate miRNA expression, contributing to OA synovial pathology. For instance, TGF-β1 enhances the expression of anti-inflammatory factor hemeoxygenase 1 (HO-1) by reducing the expression of miRNA-519b in human OA FLS (56). TGF-β1 also inhibits miR-92a to promote the expression of forkhead box class O 3 (FOXO3) in OA FLS, lowering mRNA and protein levels of TNF-α, IL-1β, VEGF, and C-C Motif Chemokine Ligand 2 (CCL2) (57). Another profibrotic growth factor, connective tissue growth factor (CTGF), increases miR-210 expression in OA FLS by activation of PI3K, AKT, ERK, and NF-κB/ELK1 pathways, contributing to VEGF-dependent angiogenesis (58). It is noteworthy that profibrotic mediators such as TGF-β1 and CTGF modulate select miRNAs to regulate certain aspects of synovitis including inflammation and angiogenesis. This effect can be counteracted by other miRNAs. MiR-125a is expressed at higher levels in OA synovium compared to psoriatic arthritis and modulates glycolysis in human umbilical vein endothelial cells (HUVEC) to inhibit angiogenesis (36). MiRNAs are dysregulated in the synovium during OA, but the way in which they regulate inflammation, angiogenesis or ECM modulation, and how they interact to maintain the joint homeostasis, remains poorly understood and requires extensive investigation in near future.

MECHANISMS REGULATING miRNAs IN OA SYNOVIUM

Adipocyte-derived molecules (adipokines) are elevated in the joint during OA and play an important role in cartilage and bone turnover (59). In addition, adipokines alter miRNA expression levels, modulating synovial inflammatory responses. Visfatin and resistin upregulate miR-34a, miR-146a and miR-181a in OA FLS, which when inhibited, decreases proinflammatory responses and oxidative stress (33). Adipokines can also inhibit miRNAs to enhance inflammatory responses. For instance, visfatin inhibits miR-199a-5p expression in OA FLS through ERK, p38, and JNK signaling pathways, which promotes IL-6 and TNF-α production (60). Similarly, resistin suppresses miR-33a and miR-33b in OA FLS resulting in increased MCP-1 transcription, facilitating the migration of monocytes (61). Thus, select miRNAs are regulated by adipokines influencing OA-related inflammatory responses.

Just as miRNAs regulate mRNAs, miRNAs are also regulated through interaction with RNA partners, specifically long non-coding (lnc) RNAs and circular (circ) RNAs (24, 25, 62). Both act as sponges, binding directly to miRNAs and regulating their free concentration. Evidence suggests that these regulatory RNAs have the capacity to fine-tune miRNA activity in OA FLS. For example, lncRNA nuclear enriched abundant transcript 1 (NEAT1) binds miR-181c, inhibiting osteopontin (OPN) expression and regulating OA FLS proliferation (24). Similarly, the lncRNA prostate cancer gene expression marker 1 (PCGEM1) binds miR-770, promoting OA FLS proliferation and survival (25). In fact, 122 circRNAs are differentially expressed in the OA synovium, with over 1,000 miRNAs and 28,000 circRNA-miRNA interaction pairs. Intriguingly, 641 miRNAs are predicted to interact with six circRNAs (62). These findings indicate that many miRNAs can be modulated by a handful of circRNAs, adding complexity to the network that regulates synoviocyte expression profiles. CircRNAs and lncRNAs represent an opportunity to modulate several miRNAs simultaneously, and thus hold great therapeutic potential to modulate OA synovial pathology.

FUTURE DIRECTIONS

An important aspect overlooked in many OA studies using animal models is that OA is an age-related disease and experiments are routinely conducted in young animals. As with other organ systems, cellular activity in joint tissues is altered with age, including abnormal ECM, cytokine and reactive oxygen species (ROS) production, which likely contribute to OA pathology differently than post-traumatic or metabolic-induced OA (63, 64). Little is known regarding how aging alters synovial homeostasis and function over time, and how that might contribute to OA progression. Expression of many miRNAs change with age in various tissues, altering processes like cell senescence (65). MiR-126-3p, which is important for cell attachment to the ECM, is downregulated in aged OA chondrocytes relative to their younger counterparts (66). Improving our understanding of how miRNAs are

differentially expressed with age, and how this alters joint homeostasis and OA progression will be essential for future translational success.

Integrated analyses examining miRNA and transcript profiles in parallel will help elucidate dysregulated miRNA and RNA interactions occurring in OA. In OA, Chen et al. performed RNA sequencing alongside small RNA sequencing in OA FLS compared to those derived from healthy tissue (21). Putative targets of dysregulated miRNAs were predicted by bioinformatic approaches, including 14 genes (11 upregulated and 3 downregulated) that require further biological validations (21). Another study attempted to identify differential mRNA and miRNA expression in the DMM mouse OA model using microarray and RT-qPCR, but found no evidence of differential expression of miRNAs and RNAs levels between sham and DMM-induced OA mice at 1 or 6 weeks after surgery (23). However, the time after surgery examined, the small sample size used and variability observed within the groups, might be masking some relevant changes, and further investigation is warranted.

In addition to holding therapeutic potential, miRNAs in the synovial fluid or synovium-derived extracellular vesicles (EVs) might also act as biomarkers (67, 68). Increased levels of miR-23a-3p, miR-24-3p, miR-186-5p, miR-29c-3p, miR-34a-5p, and miR-27b-3p are found in the synovial fluid of OA patients with late-stage compared to early-stage radiographic knee OA (69). Some of these miRNAs are highly expressed in the OA synovium. MiR-210 is increased in the synovial fluid of both early- and late-stage radiographic knee OA patients compared to healthy donors and positively correlates with VEGF levels (70). Other synovial fluid miRNAs suggested as OA biomarkers include miR-29b-3p and miR-140, which show positive and negative correlations with radiographic knee OA severity, respectively (71, 72). As we continue to unearth the biomarker potential of some of these miRNAs, understanding the release mechanism as well as the exact cellular source of secreted miRNAs in the joint will advance our understanding of miRNA contributions to OA pathology. Profiling miRNA content of cells and tissues using next generation sequencing not only helps to identify the source of miRNAs, but also has the added advantage of identifying novel miRNAs, expanding the rapidly-growing human miRNA repository and promoting investigations into new regulatory mechanisms and therapeutic targets. Sequencing datasets are routinely deposited on-line, and this open format is not only idea-generating but can also be used to substantiate novel findings. MiRNAs are currently being explored as potential therapeutic targets to counteract cartilage degeneration and synovitis in OA. For example, inhibition of miR-101 and miR-128 has been shown to rescue cartilage degeneration and synovitis in MIA and ACLT animal models of OA, respectively (37, 38). Extensive research is underway to identify the best mode of delivery of miRNA-based therapies (mimics or inhibitors) in preclinical models of OA.

CONCLUSIONS

Taken together, miRNAs contribute to synovial homeostasis, inflammation, fibrosis, angiogenesis, cell survival and cell apoptosis, contributing to OA synovial pathology. MiRNAs have been a focus of OA research since their discovery and they are attracting more attention due to their biomarker and therapeutic potential. However, research on the role of miRNAs in OA-related synovial pathology is only in its infancy. Most research on synovitis is performed in samples from RA patients or animal models where OA tissues are often used as a control reference. This has hampered our understanding of the mechanisms modulated by miRNAs in OA synovitis. Additional studies are needed to comprehensively understand the role miRNAs play in OA-related synovial pathology and to identify novel disease modifying targets for therapeutic development.

AUTHOR CONTRIBUTIONS

GT and SL performed the relevant literature searches. GT, JR, and SL wrote the manuscript. MK critically reviewed the manuscript and provided important intellectual and funding contributions. All authors have critically read and approved the manuscript.

FUNDING

This work is supported by grants to MK by The Natural Sciences and Engineering Research Council of Canada (NSERC; Grant #RGPIN-2017-06360). MK is the recipient of a Tier 1 CRC from the Canada Research Chairs Program. GT is the recipient of PhD scholarships from the Arthritis Society (Grant #: TGP-15-116) and Queen Elizabeth II/Canadian Arthritis Network Graduate Scholarships in Science and Technology.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

REVIEW
published: 28 August 2020
doi: 10.3389/fmed.2020.00506

Purinergic System Signaling in Metainflammation-Associated Osteoarthritis

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Inflammation triggered by metabolic imbalance, also called metainflammation, is low-grade inflammation caused by the components involved in metabolic syndrome (MetS), including central obesity and impaired glucose tolerance. This phenomenon is mainly due to excess nutrients and energy, and it contributes to the pathogenesis of osteoarthritis (OA). OA is characterized by the progressive degeneration of articular cartilage, which suffers erosion and progressively becomes thinner. Purinergic signaling is involved in several physiological and pathological processes, such as cell proliferation in development and tissue regeneration, neurotransmission and inflammation. Adenosine and ATP receptors, and other members of the signaling pathway, such as AMP-activated protein kinase (AMPK), are involved in obesity, type 2 diabetes (T2D) and OA progression. In this review, we focus on purinergic regulation in osteoarthritic cartilage and how different components of MetS, such as obesity and T2D, modulate the purinergic system in OA. In that regard, we describe the critical role in this disease of receptors, such as adenosine A2A receptor (A2AR) and ATP P2X7 receptor. Finally, we also assess how nucleotides regulate the inflammasome in OA.

Keywords: purinergic system, A2AR, P2X7 receptor, osteoarthritis, metainflammation, mitochondrial metabolism, Inflammasome, rheumatic diseases

OPEN ACCESS

Edited by:
Ying Ying Leung, Duke-NUS Medical School, Singapore

Reviewed by:
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Specialty section:
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

Received: *15 May 2020*
Accepted: *22 July 2020*
Published: *28 August 2020*

Citation:
Gratal P, Lamuedra A, Medina JP, Bermejo-Álvarez I, Largo R, Herrero-Beaumont G and Mediero A (2020) Purinergic System Signaling in Metainflammation-Associated Osteoarthritis. Front. Med. 7:506. doi: 10.3389/fmed.2020.00506

INTRODUCTION

The biological actions of purine nucleotide and nucleoside signaling have been recognized since 1929. ATP was proposed as responsible for non-adrenergic, non-cholinergic intestinal and bladder transmission. However, it was not until 1972 that *G. Burnstock,* the founding father of purinergic scientific research, introduced the term "purinergic signaling" (1) and in 1976 specific receptors for extracellular nucleotides were defined (2).

ATP is known for being the universal energy currency. An equilibrium between the intracellular and extracellular amount of ATP is maintained in basal conditions, but in certain physiological and pathological situations, such as apoptosis, infections, mechanical stress, and inflammation (3), cells release ATP from intracellular deposits to the extracellular space. This process is mediated by pannexin (e.g., Pannexin-1) (4) and connexin hemichannels (e.g., Connexin-43) (5–7), but also other ion channels, such as calcium homeostasis modulator 1 (CALHM1) (8), volume-regulated anion channel (VRAC) and maxi-anion channel (MAC) (9), vesicular exocytosis (10) and autophagy-dependent lysosomal exocytosis (11, 12), and through uncontrolled release in apoptotic processes (3). In the extracellular compartment, ATP has an entirely different function and

activates purinergic signaling via ion channel and transmembrane purinergic receptors in the cell membrane (3) (**Figure 1**).

ATP is rapidly degraded to adenosine, which also exerts an important role in purinergic signaling. Outside the cell, several enzymes hydrolyze ATP and limit nucleotide availability for purinergic signaling. Ecto-nucleoside triphosphate diphosphohydrolase (CD39) converts ATP into ADP, and ADP into AMP. Ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) also converts ATP directly into AMP and hydrolyzes ADP directly to adenosine. Separately, ecto-5'-nucleotidase (CD73) transforms AMP into adenosine (13–16). Additionally, one of the main regulators of the purinergic system in the extracellular space is adenosine deaminase (ADA), which anchores to the plasma membrane through the dipeptidyl peptidase IV (CD26) (17) and metabolizes adenosine to inosine (7, 18). Adenosine kinase (AK) converts adenosine back to AMP (19). Adenosine can also be directly released outside the cell through nucleoside transporters (ENT) (18) (**Figure 1**).

Pharmacology recognizes two families of purinergic receptors: P1 receptors, selective for adenosine (20), and P2 receptors, selective for nucleotides and dinucleotides of purines and pyrimidines (21).

P1 or adenosine receptors are G-protein coupled receptors (GPCR) currently divided into four subtypes, named A1R, A2AR, A2BR, and A3R, whose activation is dependent on the presence of extracellular adenosine (20). A1R and A3R differ from A2AR and A2BR in the particular G protein they interact with, with A1R and A3R being inhibitors (coupled to Gi), and A2AR and A2BR promoters (coupled to Gs) of cAMP synthesis via adenylate cyclase (AC). The modulation of cAMP levels controls multiple signaling pathways, including mitogen-activated protein kinase (MAPK) and serine-threonine specific kinases (22). Adenosine receptors are desensitized via through their internalization by β-arrestins (18, 23) (**Figure 1**).

Within the P2 or nucleotide receptors, P2X are ionotropic and P2Y are metabotropic receptors. P2X receptors are activated by ATP, and seven subtypes (1–7) have been identified. P2X receptors are homo- or heterotrimers, which combine differently (e.g., P2X2/3, P2X1/2, P2X1/5, P2X2/6, P2X4/6, and P2X1/4) and form an ion channel for Na^+, K^+ and Ca^{2+} (18, 23). P2Y receptors (P2Y1/2/4/6/11/12/13/14) are selective for ATP, ADP, UTP, and UDP, and couple with G proteins (21). Like adenosine receptors, P2Y receptors are internalized by β-arrestin, resulting in desensitization of the purinergic signal (18, 23, 24) (**Figure 1**).

Purinergic signaling is evolutionarily conserved (25), and is involved in several physiological and pathological processes, such as neurotransmission, cell proliferation, platelet aggregation, vasodilatation, and inflammation (23, 24).

Purinergic signaling contributes to the pathophysiology of several bone and cartilage diseases, such as OA, rheumatoid arthritis (RA), and osteoporosis (18).

Osteoarthritis (OA) is the most frequent and most disabling rheumatic disease in developed countries (26, 27). Around 250 million people suffer from knee OA worldwide, with a 33% prevalence in the population aged over 65 years, and with women being more affected than men (28, 29). OA is characterized by the progressive degeneration of diarthrodial joints (30), involving all the joint tissues, especially articular cartilage (AC), which suffers erosion and progressively becomes thinner. OA has traditionally been considered a "wear and tear" disease, caused by mechanical cartilage breakdown. However, it is now well-accepted that inflammation plays a critical role in the disease's progression both in cartilage and the synovium (31). Among the multifactorial etiology of OA, metabolic syndrome (MetS) and aging are critical risk factors for the onset of the disease, due in part to a state of low-grade chronic inflammation (32). The interaction between tissue damage and destruction by mechanical and biological mechanisms, together with the activation of innate immunity by multiple local and systemic inflammatory factors, are responsible for the chronicity of pathological processes in OA.

In this review, we present the important contribution of MetS and purine metabolism to the chronic pro-inflammatory status during OA progression. Moreover, we provide an integrated view of the mechanisms triggered by low-grade chronic inflammation as a pivotal axis in the pathogenesis of OA.

METAINFLAMMATION IN OA

MetS is an increasingly prevalent condition, generally diagnosed when more than two of the following risk factors are present: central obesity, lowered HDL cholesterol, high triglycerides, hypertension, and/or impaired glucose tolerance (33–35).

Metainflammation, the inflammation triggered by a metabolic imbalance (36), is a low-grade inflammation generated by components implicated in MetS. This phenomenon is mainly caused by excess nutrients and therefore, by an energy surplus, and it contributes to the pathogenesis of OA (37).

Recent studies have focused on identifying the relationship between MetS components and OA in order to understand its influence on the inflammatory process involved in the pathogenesis of the disease. An epidemiologic association between MetS and OA has been observed in clinical studies, especially with knee OA (38). Animal studies have also demonstrated that OA pathogenesis can be led by metabolic dysregulation (38). Despite increasing evidence of an association between OA and MetS, the mechanisms linking these diseases are not fully understood (39).

Obesity and Metainflammation in OA

Central obesity may lead to OA pathogenesis by promoting systemic and local inflammation and by increasing load and consequent mechanical wear of the joints (40). There is an association between obesity and OA both in weight bearing and non-weight bearing joints, which highlights the key role of chronic inflammation in the disease's development (41).

Increased adipose tissue on the body exerts metainflammation through the production of adipokines and cytokines (42, 43). Some secreted adipokines are leptin, resistin and adiponectin (44). They are able to modulate the immune system and induce synthesis of pro-inflammatory and catabolic mediators, which leads to chondrocyte dysfunction and aggravates OA progression (45). These adipokines are distributed systemically and can be found in high amounts in osteoarthritic synovial fluid (45).

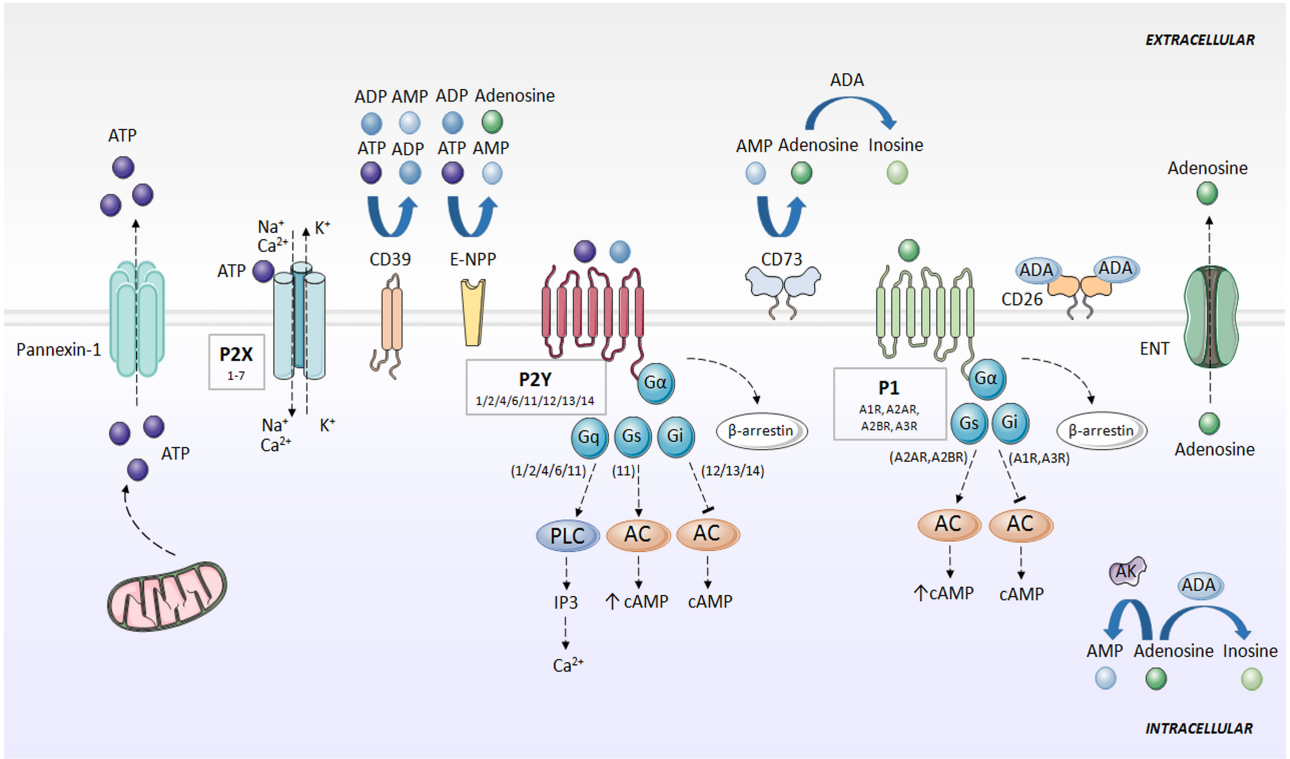

FIGURE 1 | Purinergic signaling and specific receptors for extracellular nucleotides. ATP is released to the extracellular space mainly through pannexins (e.g., Pannexin-1). Then, ATP is rapidly degraded to ADP, AMP, and adenosine by the action of CD39, E-NPP and CD73. Adenosine can be converted to AMP through AK action, or degraded to inosine by ADA. Adenosine is also directly released outside the cell through ENT. P2X receptors activated by ATP, forming an ion channel for Na^+, K^+, and Ca^{2+}. P2Y receptors are activated by ATP and ADP, and associate with G proteins, promoting intracellular second messengers' cascades, including Ca^{2+} and cAMP. Adenosine activates P1 receptors, coupled to G-proteins. A1R and A3R interaction with Gi, and A2AR and A2BR with Gs. P1 and P2Y receptors desensitized through internalization by β-arrestins. AC, adenylate cyclase; ADA, adenosine deaminase; ADP, adenosine diphosphate; AK, adenosine kinase; AMP, adenosine monophosphate; ATP, adenosine 5′-triphosphate; cAMP, cyclic adenosine monophosphate; CD26, dipeptidyl peptidase IV; CD39, ecto-nucleoside triphosphate diphosphohydrolase; CD73, ecto-5′-nucleotidase; E-NPP, ecto-nucleotide pyrophosphatase/phosphodiesterase; ENT, nucleoside transporter; G, G protein; IP3, inositol trisphosphate; P1, adenosine receptors; P2X, ionotropic nucleotide receptors; P2Y, metabotropic nucleotide receptors; PLC, phospholipase C.

They are synthesized by synoviocytes, articular chondrocytes and adipocytes of intra-articular fat tissue (37) (**Figure 2**).

Leptin plays an essential role in energy metabolism, leading to energy consumption. It affects growth factor synthesis and anabolism, and it is present in human chondrocytes, synovium, osteophytes and infrapatellar fat pads (44). In OA, this protein shows a marked expression in cartilage and osteophytes in comparison with healthy tissue (46). Furthermore, leptin contributes to the pathogenesis of OA, since it has a pro-inflammatory and catabolic role in cartilage metabolism, its expression being directly associated with the degree of cartilage destruction, and through the stimulation of growth factor synthesis (46, 47). Leptin induces the synthesis of matrix metalloproteinases (MMPs) in primary cultures of human chondrocytes. Accordingly, in patients with OA its concentration correlates with MMP-1 and MMP-3 levels in the synovium (48, 49). Furthermore, leptin promotes the activation of monocytes, and a subsequent increase in macrophages in the synovium (32).

Resistin is mainly expressed in white adipose tissue, but also by macrophages, osteoblasts, osteoclasts, and chondrocytes (40). Resistin is capable of inducing cytokine and chemokine expression in chondrocytes (50), being a potential regulator of the pro-inflammatory cytokines that activate transcription nuclear factor kappa B (NF-κB) (51). Moreover, it is upregulated during monocyte-macrophage differentiation (52) and tumor necrosis factor (TNF)-α, interleukins (IL)-1β, IL-6, and lipopolysaccharide (LPS) stimulation increases its levels (53). Resistin is also involved in angiogenesis via the induction of endothelial cell growth and migration (54), and can endorse osteoclastogenesis via modulation of bone turnover mediators (51). Thus, bone remodeling and resistin-stimulated pro-inflammatory cytokines in cartilage may promote OA pathogenesis. Nevertheless, further studies are necessary to determine the role of resistin in the onset and progression *in vivo* of OA (40).

Adiponectin, another adipokine produced by adipose tissue, shows higher concentrations within the joint, which suggests an important role in the onset and progression of OA. The synovial fluid of patients with OA shows a 100-fold increase in adiponectin expression as compared with their plasma (55).

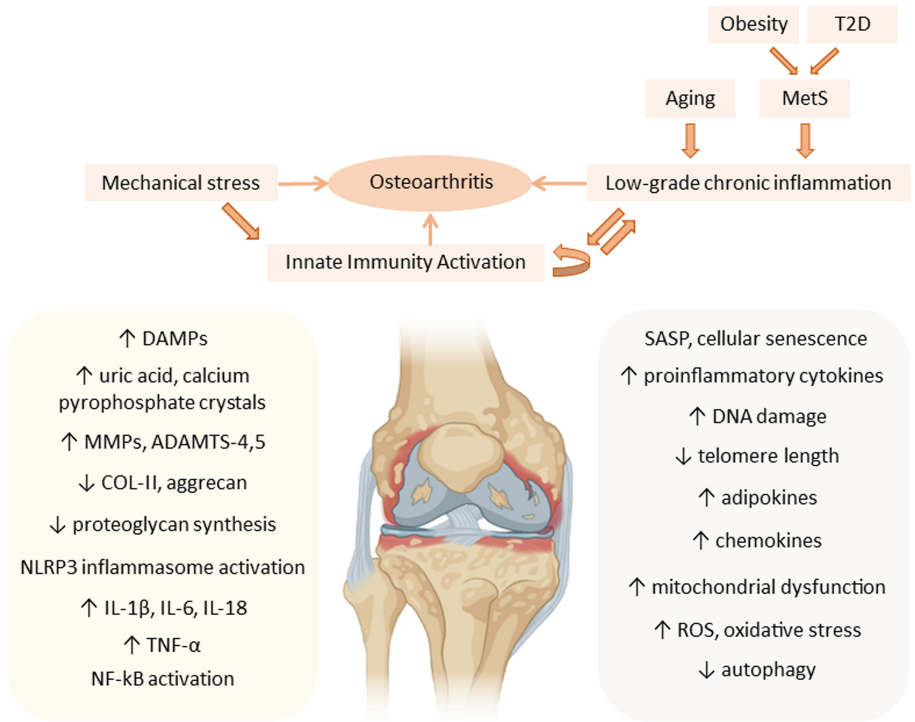

FIGURE 2 | Metainflammation in the pathogenesis of osteoarthritis (OA). Mechanical stress contributes, together with the low-grade chronic inflammation associated with aging and the components of the metabolic syndrome (MetS), to the chronic activation of innate immunity in the joint, mainly affecting articular cartilage, during OA progression. Aging-associated factors and senescence together with metabolic factors, such as an increase in adipokines, maintain a systemic low-grade chronic inflammatory status that contributes to the pathogenesis of OA, increasing the expression of cartilage catabolic enzymes (MMPs, ADAMTS), and pro-inflammatory cytokines (IL-1β, IL-6, IL-18, and TNF-α), thus perpetuating inflammation in the joint. ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; COL-II, type II collagen; DAMPs, danger-associated molecular patterns; DNA, deoxyribonucleic acid; IL, interleukin; MMP, matrix metalloproteinases; MetS, metabolic syndrome; NF-kB, nuclear factor kappa B; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; ROS, reactive oxygen species; SASP, senescence-associated secretory phenotype; T2D, type 2 diabetes; TNF, tumor necrosis factor.

In addition, adiponectin receptor 1 (AdipoR1) is expressed in cartilage, bone, and synovial tissues (44). Adiponectin may protect against progression of OA since it induces the downregulation of MMP-13 and upregulation of an associated MMP-13 inhibitor (44). Another study suggests that adiponectin may exert an anti-inflammatory effect on cartilage, upregulating the tissue inhibitor of metalloproteinases 2 (TIMP-2) (40, 55). However, serum adiponectin levels are not associated with OA (56).

Experimental models, such as rats, which developed OA after been fed with a high-fat diet (HFD), show that there is a close relationship between obesity and the local immune response in synovial tissue (57). Synovial resident macrophages play a key role in HFD-induced inflammation, which produces an increase in M1 macrophages compared with M2 polarized cells. This highlights the importance of M1 macrophage subsets in the development of obesity-associated OA (57). Furthermore, HFD aggravates synovial inflammation during OA, by increasing macrophage infiltration and metabolic-mediated remodeling of adipose tissue, together with a significant presence of pro-inflammatory factors (58). Additionally, free fatty acids induce activation of Toll-like receptor 2 (TLR2) or TLR4, trigger macrophage recruitment, and activate the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome (59).

In addition to the secretion of metabolic molecules, obesity contributes to a major mechanical load on the joint. Mechanical loading studies in human and animal models show that abnormal loads can lead to changes in the composition, structure, and mechanical properties of articular cartilage (44), together with inflammatory responses throughout the joint. Mechanoreceptors on the surface of chondrocytes detect increased loading on the knee, triggering intracellular signaling cascades of cytokines, growth factors and MMPs (44).

Osteoarthritic chondrocytes respond differently to mechanical stimulation when compared with cells from healthy joint cartilage (60). The production of catabolic cytokines IL-1β and IL-6, the lack of an increase in mRNA aggrecan levels, and persistent mechanical stimulation are the main differences (61, 62). The mechanotransduction pathway involves recognition of the mechanical stimuli by integrins and activation of integrin-mediated signaling pathways leading to the production of cytokines, in both healthy and osteoarthritic chondrocytes (63).

When we focus on the effect of mechanical loading in OA through the stimulation of pro-inflammatory mediators,

in vitro mechanical loading experiments show that injurious compression leads to proteoglycan depletion, destruction of the collagen network and cartilage degradation. In response, pro-inflammatory products are released and are postulated to cause synovitis (64). Interestingly, joint movement induces the expression of the anti-inflammatory cytokine IL-10. Therefore, not only the induction of inflammation, but also the lack of resolution of inflammation could play a role in OA (65).

Type 2 Diabetes and Metainflammation in OA

Type 2 diabetes (T2D) is a metabolic disorder characterized by high blood glucose levels that cause an inadequate β-cell response to the progressive insulin resistance (66). The incidence and prevalence of the most common T2D has nearly doubled in the last two decades, and its presence is reported in a high proportion of knee OA cases (67). Both diseases have many risk factors in common, which may explain the increased prevalence of musculoskeletal diseases in diabetic patients (67).

A considerable association between T2D and OA has been observed in several meta-analyses (68, 69), which is why T2D is the most commonly studied component of MetS as a risk factor for OA (70). Accordingly, epidemiological and experimental evidence suggests that T2D could be an independent risk factor for the onset and progression of OA, that is, the diabetes-induced osteoarthritic phenotype (71, 72). However, other studies show that diabetes mellitus is associated with accelerated degeneration of the cartilage matrix (67) and that T2D is a strong predictor for the development of severe OA, independent of age and body mass index (BMI). This suggests that longstanding diabetes *per se* is detrimental for knee and hip joints (73). According to this, increased levels of the cartilage oligomeric matrix protein (COMP) can be found in the synovial fluid of T2D subjects. Therefore, treating T2D may minimize glycemic control parameters and inflammation, along with synovial fluid COMP levels and OA progression (74).

Furthermore, inflammation plays a key role in the pathogenesis of T2D (59). T2D is associated with low-grade inflammation of adipose tissue in obesity and auto-inflammation in pancreatic islets (75). Indeed, in T2D, pancreas and adipose tissue, as well as other tissues, are infiltrated by macrophages and other immune cells, switching their profiles from an anti-inflammatory to a pro-inflammatory phenotype (76). Pro-inflammatory cytokines inhibit insulin signaling in peripheral tissues and induce β-cell dysfunction, contributing critically to the pathogenesis of T2D (76).

Inflammation in T2D can be triggered by excessive levels of nutrients, like glucose, which leads to the local production and release of cytokines and chemokines: IL-1β, TNF-α, CC-chemokine ligand 2 (CCL2), CCL3 and CXC-chemokine ligand 8 (CXCL8) in the pancreatic islets and insulin-sensitive tissues. In T2D patients, IL-1 receptor antagonist (IL-1RA) production by β-cells is decreased and IL-1β induces inflammation in pancreatic islets through NF-κB activation, which plays a critical role in the development of OA (59). Consequently, immune cells (macrophages and mast cells) are recruited and contribute to tissue inflammation (59, 77) (**Figure 2**).

Other authors postulate that high levels of glucose stimulate intercellular adhesion molecule 1 (ICAM-1) overexpression in the endothelium. ICAM-1 is a hallmark of diabetes-related inflammation and a crucial driver for cartilage degradation in T2D models. ICAM-1 enables and facilitates the adhesion and entrance of macrophages from serum or the synovium and facilitates the release of cytokines like IL-1β by the chondrocytes or synoviocytes This event triggers the production of MMP-13 and the recruitment of more macrophages, creating a vicious circle (78).

Senescence and Metainflammation in OA

Aging is a critical risk factor for the onset and progression of many chronic diseases, from metabolic disorders to neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (32, 79). However, the aging process itself does not seem to be the direct cause of these disease states. Aging results from an imbalance between a variety of environmental and cellular stressors, and an insufficient capacity of cellular mechanisms to resolve stress processes. This results in the accumulation of unrepaired damage, which makes individuals more susceptible to developing chronic diseases (80). Also, there is an energy imbalance as a result of an age-associated decrease in the resting metabolic rate, in part due to a loss of lean mass, which does not synthesize enough ATP in older individuals. Finally, there is a dysregulation of signaling cascades that guarantee tissue homeostasis, including hormones, inflammatory mediators and antioxidants, thus triggering an inflammatory state characteristic of the aging process (80) (**Figure 1**).

This imbalanced situation ultimately leads to decreased adaptation to stress, loss of proteostasis, cellular senescence, inflammatory responses, metabolic disarrangement, DNA damage, detrimental epigenetic modifications, and impaired cell - cell communication. These defective responses trigger a state of low-grade chronic inflammation associated with aging that has been termed "inflammaging," which plays a major role in the pathogenesis of age-related diseases (32, 79–83).

Aging and age-related diseases share diverse mechanisms that promote low-grade chronic inflammation (32). These mechanisms include inflammatory processes, mainly the chronic activation of innate immunity, but also changes in the adaptive immune system, such as defective T cell functioning, leading to immunosenescence (79). Another prominent mechanism actively contributing to inflammaging is cellular senescence. Senescent cells are unable to proliferate but remain metabolically active and acquire a senescent-associated secretory phenotype (SASP). This SASP is characterized by altered secretion of pro-catabolic enzymes, such as MMP-1, MMP-3, MMP-9, and MMP-13; pro-inflammatory cytokines, such as IL-6, IL-1α and β; chemokines (IL-8, CCL2, CCL5, and CCL19); and the following growth factors: transforming growth factor-β (TGF-β), fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF) (45, 83). Furthermore, senescent cells exhibit DNA damage, shortening of telomeres, defective autophagy, mitochondrial dysfunction, and increased oxidative stress (32, 41, 83) (**Figure 1**).

In osteoarthritic cartilage, chondrocytes suffer cellular senescence, known as "chondrosenescence," as a result of inflammatory signals that increase with aging and mechanical stress (32, 84). An accumulation of senescent cells is also found in the osteoarthritic synovium (41, 83). These factors, together with chondrocyte inability to repair the damaged tissue, are responsible for cartilage degeneration in OA (82). Aging in cartilage is also associated with lower cell density and loss of proteoglycans, joint stiffness, increased chondrocyte size and protein glycosylation (32, 85, 86). Advanced glycation end products (AGEs) increase with age in the cartilage extracellular matrix (ECM) and modify the mechanical properties of cartilage. Their receptor can also interact with TLRs on the surface membrane, thus enhancing innate immune activation (83). Chondrosenescence also occurs through the loss of chondrocyte autophagy. Chondrocytes closely depend on autophagy for the maintenance of cartilage homeostasis, since it is the protective mechanism responsible for the removal of cellular debris and macromolecules through lysosome-mediated degradation (32). In the OA pathogenic context, autophagy reduces inflammation in the joint tissues (87–89). However, the loss of chondrocyte autophagic capacity associated with aging results in further destruction of the cartilage ECM and increased oxidative stress (32, 90). The senescent phenotype in chondrocytes and synoviocytes contributes to perpetuating a local pro-inflammatory state in the joint, due to the release of cytokines and increased cartilage degradation. At the same time, the SASP favors a state of low-grade systemic inflammation through the release of chemokines that also contributes to the pathogenesis of OA (32) (**Figure 2**).

IMMUNITY AND THE PURINERGIC SYSTEM IN OA

As mentioned above, OA displays low-grade chronic inflammation, primarily mediated by innate immunity (45). Articular cartilage damage and degeneration occurs mainly because of an imbalance between the mechanical load it receives and its ability to absorb and distribute it. Altered mechanical joint loading is often associated with being overweight, anatomical misalignment or post-traumatic joint instability, and these are all critical risk factors for joint destruction (91, 92). The progressive destruction of the cartilage matrix leads to the appearance of tissue fragments in the articular cavity, known as "damage-associated molecular patterns" (DAMPs). These signals are detected by "pattern recognition receptors" (PRRs), present in chondrocytes and synoviocytes. Toll-like receptors (TLRs) are the PRRs that mainly mediate this signaling, particularly TLR2 and TLR4 in OA cartilage (41, 93). TLRs trigger a signaling cascade that results in innate immunity activation, by leading the activation of interferon-regulatory factors, NF-κB, and activator protein 1 (AP-1), and inducing pro-inflammatory mediators, such as cyclooxygenase (COX)-2; cytokines, such as IL-6 and TNF-α; components of the inflammasome, such as caspase-1, NLRP3, pro-IL-1 and pro-IL-18; MMPs and aggrecanases, such as ADAMTS-4 and−5 (41, 45). The activity of MMPs and ADAMTS, enzymes that degrade type II collagen and aggrecan in the cartilage matrix, promote the appearance of more DAMPs, thereby perpetuating tissue damage and innate immunity activation.

Varying extracellular concentrations of different purinergic metabolites, such as ATP, ADP and adenosine, are reliable indicators of the tissue status during inflammation, entailing a robust regulatory system of the immune response (94–97).

Adenosine is a potent modulator of inflammation and immune responses (98). Adenosine regulates function, proliferation and activation of immune cells, and promotes the resolution of inflammation dampening the immune response in physiological and pathological situations, acting as a self-limiting signal (96). Platelets, endothelial cells, neutrophils and macrophages are considered an important source that contributes to increasing extracellular adenosine during inflammation (99) (**Table 1**). It is well-known that adenosine biases TLR4. Adenosine, by A2AR activation, can inhibit LPS-induced TLR4-mediated responses, by inhibiting Th1-polarizing responses (TNF-α, IL-12p70) and activating anti-inflammatory cytokine production and Th2-polarizing responses (IL-6 and IL-10) (111, 112) and inflammation-resolving properties (113). Adenosine produces increased levels of cAMP, and this second messenger inhibits LPS-induced IL-12 production in murine peritoneal macrophages (114), decreases LPS-induced TNF but enhances IL-10 in human monocytes (115). Nevertheless, the effects of cAMP on TLR-mediated cytokine production can be dependent on cell type. Recent evidence suggests that cAMP levels in chondrocytes are an indicator of the metabolic function, with low levels indicating cartilage degradation, and increased levels suggesting an increased cartilage synthesis (116).

As mentioned, TLRs are activated during OA progression, particularly TLR4 (93). Different drugs have been shown to decrease the inflammatory and catabolic response in OA chondrocytes stimulated with different DAMPs by inhibiting the TLR4/MyD88/NF-κB signaling pathway (93). In murine articular chondrocytes, hyaluronan (HA) fragments have been found to induce inflammation via CD44 and TLR4 and NF-kB activation; adenosine can attenuate this inflammation process via A2AR activation (117). Although the exact mechanism has not yet been fully elucidated, it is known that adenosine produces high levels of cAMP that activate protein kinase A (PKA) and inhibit NF-κB (118). No data has been found in the literature, but this mechanism must be altered in OA progression.

In contrast to the anti-inflammatory role of adenosine-mediated purinergic signaling, nucleotide receptors promote inflammatory mechanisms (3). Elevated concentrations of ATP are usually a warning sign of cell death detected by immune cells at sites of active inflammation. Extracellular ATP plays an important role in the innate immune response, upregulating the inflammatory pathways (119). Extracellular ATP (in the millimolar range) predominantly induces proinflammatory effects through activation of the low affinity receptor P2X7 (120), but low (micromolar) extracellular ATP concentrations exert immunosuppressive action through the activation of the high affinity P2Y11 receptor (121, 122).

TABLE 1 | Effects of adenosine and ATP through their specific purinergic receptors in inflammation.

	Adenosine		ATP	
Cell type	**Receptor**	**Effect**	**Receptor**	**Effect**
Endothelial cells	A2AR, A2BR	Blockade of IL-6, IL-8, E-selectin, VCAM-1 (99) Inhibition of the recruitment of leukocytes and neutrophils adhesion to the endothelium (95)	P2	↑ intracellular Ca^{2+}, ↑ mitochondria ATP production (100) ↑ NO production (100)
Neutrophils	A2AR	↓ oxidative stress response (99) ↓ leukotriene B4 secretion (99) ↓ IL-8 production (99) ↓ apoptosis (99) ↓ neutrophil-induced NETosis (101)	P2X7	↑ adhesion to endothelial cells (100)
	A2BR	Suppression of neutrophil transmigration across the endothelial vessel wall (102) Inhibition of VEGF secretion (102)	P2Y2, P2Y6, P2Y14	↑ chemotaxis (103) ↑ oxidative stress (104)
	A1R	↑ neutrophil chemotaxis and phagocytosis (105)		
	A3R	Neutrophil migration to injury site (106) ↓ ROS production and chemotaxis (106)		
Macrophages and monocytes	A2AR	Suppression of TNF-α, IL-6, IL-12, IL-8, NO and MIP-1α production (106) ↑ IL-10 production (107) ↑ VEGF, angiogenesis (95, 108)	P2X7	↑ IL-1β production (109) NLRP3 inflammasome activation (109)
	A2BR	↑ IL-10 secretion (95) Inhibition of iNOS and MHC-II expression (99)	P2X4	↑ CXCL5 secretion (110) ↑ ROS production (104) ↑ MIP-2 secretion (104)
	A3R	Regulates macrophage migration (105)	P2Y1, P2Y6, P2X	↑ phagocytosis of apoptotic cells (104)
Dendritic cells	A2AR	Suppression of the capacity to activate naïve T cells (106) Differentiation of T cells into Th1 helper leukocytes (106)	P2X7	Differentiation (100) Secretion of inflammatory cytokines (IL-1β, IL-18, TNF-α, IL-23) (100) Activation of TH-17 lymphocytes (100)
	A2BR	↑ pro-angiogenic activity under hipoxic conditions (105)	P2Y11	↑ cAMP, inhibition of T lymphocyte activation (104) ↓ pro-inflammatory cytokine and chemokine production (104)
	A1R	Loss of the phagocytic capacity (106)		
NK cells	A2AR, A2BR	↓ TNF-α expression (106) ↓ pro-inflammatory cytokine (105)	P2Y11	↓ cytotoxic activity of endothelial CX3CL1 (104)
	A3R	↑ cytotoxic activity (106)		

↑ means increase; ↓ means decrease.

ATP is considered like a DAMP in OA as it activates TLR4 and leads to NLRP3 inflammasome activation and caspase-1-mediated IL-1β secretion (123) and activates NF-κβ signaling (124), leading to cartilage degradation and synovial inflammation.

PURINERGIC SYSTEM IN OA AND MODULATION BY METAINFLAMMATION

The direct effect on the joint of purinergic system activation in different cell subtypes has been described extensively (18, 125). Therefore, we shall only concentrate on the modulation of the purinergic system in chondrocytes, since they play a key role in OA.

Adenosine and Its Receptors in OA

The presence of adenosine receptors in human articular chondrocytes was first discovered in 1999 (126), and they were further characterized in bovine chondrocytes in the presence or absence of low-frequency low-energy pulsed electromagnetic fields (PEMFs) (127). Since then, much importance has been conferred to the role of adenosine in the regulation of inflammatory processes in cartilage and the maintenance of joint homeostasis, modulating the release of pro-inflammatory mediators and cytokines (128). All four adenosine receptors are expressed in chondrocytes, but A2AR and A3R are particularly relevant (18, 22, 128, 129).

A2AR has a broad range of physiological activities, and is known to play an essential role in the maintenance of articular cartilage homeostasis (130). Biophysical interventions, such as PEMFs, for stimulation of bone and cartilage via A2AR are currently being studied (131, 132). There are different modalities of *in vivo* adenosine delivery: polydeoxyribonucleotides, liposomes (129), and functionalized nanoparticles (133). All of them are for intra-articular administration and activate adenosine receptors. When cartilage explants are treated

with A2AR antagonists (ZM241385 as a specific antagonist; CGS15943 and theophylline as broad receptor antagonists), cartilage matrix degradation occurs, which is evidenced by increased glycosaminoglycans (GAG), MMP-3, MMP-13, Prostaglandin E2 (PGE2) and NO release (134). It has been observed that in mouse articular chondrocytes stimulated with IL-1β, an A2AR agonist (CGS21680) can counteract the upregulation of the inflammatory markers NF-κB, TNF-α, IL-6, MMP-13, and NO (135). It has also been reported that in the presence of IL-1β, osteoarthritic chondrocytes release less adenosine and ATP, suggesting that inflammation reduces purinergic signaling via A2AR. This may be contradictory to another study which reported that in equine articular chondrocytes, LPS stimulates accumulation of extracellular adenosine working as an inflammatory blocker on chondrocytes (136). The divergence between studies could be due to the nature of the stimuli or species-dependent differences. Mice lacking A2AR develop OA, which can be seen in increase in MMP-13 and Col10a1 expression, fibrillation and thinning of cartilage, disordered chondrocytes, less GAG and loss of sulfated proteoglycans and collagen in cartilage (130). These events are not present in the preventive group or in the treatment group, after intra-articular injection of liposomal adenosine in a rat model of post-traumatic OA (130). Adenosine released by osteoarthritic chondrocytes can also signal via A2AR to limit the production of intracellular NO, which is associated with ECM degradation and chondrocyte apoptosis (129, 137) (**Table 2**).

A3R exerts anti-inflammatory effects on different experimental OA models (185). A3R knockout (KO) mice develop progressive loss of articular cartilage. Agonists for A3R downregulate key genes implicated in OA pathology, such as RUNX2 (151). In a recent study CF101, a highly selective A3R agonist, was orally administered twice daily to monosodium iodoacetate OA-induced rats. It was found that CF101 downregulated the signaling pathway of NF-κB, which led to decreased levels of TNF-α. This effect prevented cartilage damage and chondrocyte apoptosis, osteophyte formation, and bone destruction. It also reduced pannus formation and lymphocyte infiltration. On the other hand, all these effects were counteracted by the A3R antagonist MRS1220 (155) (**Table 2**).

During inflammatory processes, ADA is secreted to the extracellular space altering adenosine levels. Synovial fluid ADA measurement, in association with C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) levels, makes it possible to distinguish OA from other rheumatic diseases, like RA (186). In cartilage, when extracellular adenosine levels decrease, cartilage damage markers appear, e.g., increase in GAG release, and higher production of MMPs, PGE2 or NO (187). In some rheumatic diseases like RA, ADA1 levels are increased, compromising extracellular adenosine levels and possibly contributing to the severity of the disease (188).

Pain intensity correlates with OA progression. At low concentrations, adenosine binds to A1R inducing analgesia by decreasing nociceptor nerve conduction, and to A2AR, which triggers an anti-inflammatory response by secreting anti-inflammatory mediators. However, excessive adenosine concentration can be detrimental (138). In the case of excessive joint motion, which leads to excessive activation of CD73 via hypoxia inducible factor (HIF)-1α, extracellular adenosine accumulates. The consequent activation of the low-affinity A2BR seems to play a harmful role in cartilage homeostasis, possibly due to its capacity of stimulating inflammatory pathways involving MAPK (128, 151). In OA, as a low-grade chronic inflammatory disease, increased extracellular adenosine levels may switch selectivity of receptor binding toward A2BR, generating hyperalgesia (138) (**Table 2**).

As mentioned above, inflammation reduces signaling via A2AR in chondrocytes. However, in response to metabolic stress and inflammation, adenosine accumulates extracellularly, like in obesity (189). The activation of the adenosine A1R increases lipogenesis (139, 140), adipogenesis (141) and leptin production (142, 143). Accordingly, overexpression of A1R in adipose tissue in mice protects from obesity-induced insulin resistance (144), whereas A1R KO mice show anti-lipolytic effects (139). A1R KO mice exhibit increased fat mass and body weight, and impaired glucose tolerance and insulin sensitivity (145, 146). Contrary to A1R, the activation of A2BR, which is highly expressed in human primary pre-adipocytes (148), can inhibit both adipogenesis and lipogenesis *in vitro* (141, 190). Moreover, in rodent models, the overexpression of A2BR seems to correlate with parameters of obesity, being upregulated in visceral adipose tissue of mice fed HFD (152). Finally, studies in obese patients show that A2BR expression in subcutaneous fat is positively associated with BMI and other parameters of obesity (149). On the other hand, activation of A2AR in human and murine adipose tissue not only activates lipolysis, but also induces increased energy expenditure and protects mice from diet-induced obesity (148). Finally, the receptor A3R is also expressed in adipocytes, and KO animals for this receptor present less abdominal and total body fat (148) (**Table 2**).

Furthermore, obesity is closely related to a state of insulin resistance, which is considered to be a key step in the development of diabetes and MetS (190). The stimulation of A1R induces insulin sensitivity and reduces insulin secretion (147). On the other hand, the activation of A2BR contributes to increased insulin resistance by affecting the production of IL-6 and other cytokines (153). Animal studies confirm that A2BR activation increases serum IL-6 levels (154), which may be involved in the development of insulin resistance and improve insulin sensitivity (191). A2BR affects inflammatory processes in adipose tissue through the activation of macrophages, and indirectly inducing the development of insulin resistance (153) (**Table 2**).

In view of the above, these changes in the purinergic system mediated by MetS could be involved in the development of OA, which would partially explain the relationship between both pathologies. It has been stated that in adipose tissue there is a low stimulation of A1R and A2AR in obesity conditions (148). Interestingly, this situation is similar in chondrocytes, which could induce a loss in cartilage homeostasis, since they play an essential role in the maintenance of tissue, and consequently promote the development of OA (130). Moreover, the activation of A2BR in obesity contributes to inflammatory processes and secretion of pro-inflammatory molecules (148),

TABLE 2 | Description of purinergic receptor roles in osteoarthritis (OA), obesity and type 2 diabetes (T2D).

Purinergic receptors	Role in OA	Role in obesity	Role in T2D
A1R	It is able to induce analgesia by decreasing nociceptor nerve conduction (138).	A1R activation increases lipogenesis (139, 140), adipogenesis (141) and leptin production (142, 143). Over-expression of A1R in adipose tissue in mice protect from obesity-induced insulin resistance (144). A1R KO mice show increased fat mass and body weight, and impaired glucose tolerance and insulin sensitivity (145, 146).	A1R stimulation induces increased insulin sensitivity and reduces insulin secretion (147).
A2AR	Maintenance of AC homeostasis. Mice lacking A2AR develop spontaneous OA and chondrocytes lacking A2AR develop an OA phenotype (130). Adenosine replacement by intra-articular injection of liposomal suspensions containing adenosine prevents development of OA in rats (130).	A2AR activates lipolysis, induces increased energy expenditure and protects mice from diet-induced obesity (148).	Signaling through the A2AR increases proliferation and survival of β-cells and promotes β-cell regeneration (149, 150).
A2BR	A2BR plays a harmful role in cartilage homeostasis due to its capacity of stimulating inflammatory pathways (128, 151).	A2BR inhibits adipogenesis and lipogenesis and correlates with parameters of obesity. Mice fed with HFD show A2BR upregulated in visceral adipose tissue (152). Obese patients show a positive association between A2BR expression in subcutaneous fat and BMI (149).	A2BR activation increases insulin resistance by affecting the production of IL-6 and other cytokines (153, 154). A2BR affects inflammatory processes in adipose tissue through the activation of macrophages, and indirectly inducing the development of insulin resistance (153).
A3R	It shows an anti-inflammatory effect in OA. KO mice develop progressive loss of AC. OA rat model orally treated with A3R agonist prevented cartilage damage and apoptosis of chondrocytes, osteophyte formation, bone destruction, reduced pannus formation and lymphocyte infiltration (155).	KO animals for this receptor present less abdominal and total body fat (148).	A3R activation induces β-cell necrosis, but the role of this receptor in the regulation of glucose and lipid homeostasis in T2D is unknown (149).
P2X1	At high concentrations of ATP, P2X1 facilitates the release of NO and PGE2, which are involved in inflammatory processes and cartilage resorption (156).	P2X1 is expressed on immune cells and its activation contributes with an inflammatory response. However, its expression during overweight or obesity has not been described (157).	P2X1 is expressed in rodent β-cells, but it cannot be detected in human β-cells (158). T2D does not affect the distribution or the gene expression of P2X1 (159).
P2X2	As P2X1, at high concentrations of ATP, P2X2 facilitates the release of NO and PGE2, which are involved in inflammatory processes and cartilage resorption (156).	Obesity promoted a decrease in the expression of P2X2 receptors on enteric neurons of obese male mice (160).	P2X2 is expressed in rodent β-cells, but it cannot be detected in human β-cells (158). P2X2 expression is decreased in the retina of diabetic rats (161).
P2X3	P2X3 activation in chondrocytes induces NO and PGE2 release, suggesting a role in modulating the inflammatory process and playing an important role in the development of articular hyperalgesia in arthritic joints (162).	-	P2X3 receptors are present in isolated single mouse β-cells, in rat pancreas (158) and in human β-cells (158). P2X3 activation leads to enhanced insulin secretion (158).
P2X4	P2X4 is involved in excessive ATP efflux when ANK gene is overexpressed in OA (163), causing accumulation of crystals (164).	As P2X1, P2X4 is expressed on immune cells and its activation contributes with an inflammatory response. However, its expression during overweight or obesity has not been described (157).	P2X4 is expressed in rodent β-cells, but it cannot be detected in human β-cells (158). At higher extracellular ATP concentrations (more than 1 μM), cell viability decreased and P2X4 is implicated (165).
P2X5	Although it has not been described in OA, it is known that P2X5 signaling contributes to bone loss in experimental periodontitis via promotion of inflammation and direct regulation of osteoclast maturation (166).	P2X5 is present in the surface of brown/beige human adipocytes, with very low expression in white fat. It is also expressed in brown preadipocytes, and its expression is further increased upon differentiation (167).	P2X5 is present in human β-cells, but further investigation is needed to highlight its role (158).
P2X6	–	–	P2X6 is expressed in rodent β-cells, but it cannot be detected in human β-cells (158). Further investigation is needed to highlight its role (158).

(Continued)

TABLE 2 | Continued

Purinergic receptors	Role in OA	Role in obesity	Role in T2D
P2X7	P2X7 activation in OA chondrocytes aggravates inflammatory process, pain and cell death (168). Overactivation of P2X7 by high concentrations of ATP in OA cartilage areas leads to cell death (74). Blocking P2X7 in rats exert pain-relieving and anti-inflammatory effects (169). P2X7 causes accumulation of crystals (164).	P2X7 shows anti-adipogenic effects (170). P2X7 KO mice present increased body weight and adipocyte hyperplasia in fat pads (171). P2X7 is expressed on immune cells and their activation contributes with anti-inflammatory response. However, its expression during overweight or obesity has not been described (157).	P2X7 is present in β-cells, and it is down-regulated in T2D. In human islets, P2X7 seems to be involved in secretion of insulin (165). The P2X7 KO mice have lower β-cell mass, impaired glucose tolerance and defective insulin and interleukin secretion (172).
P2Y1	Mice P2Y1 KO show reduced trabecular bone in the long bones. Cells derived from mice P2Y1 KO has increased osteoclast formation and resorption (173).	P2Y1 induces adipogenic differentiation of stem cells (170, 174). P2Y1 is able to induce leptin production in murine adipose tissue (175).	P2Y1 is present on intra-islet capillaries and in small pancreatic blood vessels. At higher extracellular ATP concentrations (more than 1 μM), cell viability decreased and P2Y1 is implicated (165).
P2Y2	It is involved in the signaling of mechanical forces coming from the extracellular matrix articular via integrins. OA chondrocytes do not increase the amount of extracellular ATP via P2Y2 after mechanical stimulation, and do not stimulate anabolic responses (63, 176).	P2Y2 induces the adipogenic differentiation of stem cells (170, 174). Mice with depletion of P2Y2 show dependant resistance to develop HFD-induced obesity accompanied with an improvement of the metabolic status (177).	P2Y2 is expressed in small pancreatic blood vessels, but T2D does not affect the distribution or the gene expression of P2Y2 (159).
P2Y4	In the periodontal ligament, P2Y4 induces phosphorylation of ERK and consequently type I collagen and OPG release, essential for remodeling of the alveolar bone. However, its role in OA has not been describe yet (18).	P2Y4 is able to induce the adipogenic differentiation of stem cells (170, 174). Activation of P2Y4 inhibits adiponectin expression, and P2Y4 KO mice show increased adiponectin secretion (178).	P2Y4 has been identified in pancreatic duct cells of the young rat (α and β-cells), but its role in T2D must be clarified (179).
P2Y6	As P2Y4, in the periodontal ligament P2Y6 induces phosphorylation of ERK and consequently type I collagen and OPG release, essential for remodeling of the alveolar bone. But its role in OA has not been describe (18).	Selective P2Y6 deficiency in mice AgRP neurons prevents diet-induced hyperphagia, adiposity, and insulin resistance in the long term (180). P2Y6 deficiency blunts macrophage-inflammatory responses and limits atherosclerosis development (180).	Mouse islets cells possess P2Y6 receptor, whose activation lead to the modulation of insulin secretion. It may play a role as autocrine regulator of insulin release (181).
P2Y11	–	P2Y11 induces the adipogenic differentiation of stem cells (170, 174), but further research is necessary to understand its role in obesity.	In human and murine tissue there is evidence that P2Y11 is a key upstream component in the signaling cascade regulating vascular reactivity during diabetic hyperglycemia (182).
P2Y13	–	P2Y13 shows anti-adipogenic effects (170). Mice P2Y13 KO improve outcome in metabolic syndrome with an increased protection against developing an insulin resistance as shown through an improved glucose tolerance and basal glucose levels, a decelerated weight gain and a better metabolic turnover (183).	P2Y13 is involved in the induction of β-cell apoptosis in presence of high glucose and free fatty acids levels (184).
P2Y14	–	P2Y14 shows anti-adipogenic effects (170).	P2Y14 deficiency mice significantly changed expression of components involved in insulin secretion.

AC, Articular cartilage.

which possibly contribute to the cartilage alteration seen in OA. According to this, new studies are being developed and show that intra-articular stimulation of A2AR can reverse not only OA induced by anterior cruciate ligament injury, but also obesity-related OA in experimental models of the disease (192).

As described above, T2D is the most commonly studied component of MetS as a risk factor for OA. Metformin, a first-line drug for T2D treatment, is known to affect the energy state of the cell. It has been recently proposed that metformin may be beneficial in obese patients with knee OA (193) and can also inhibit respiratory chain complex 1, activating AMPK and inhibiting AMP deaminase, which results in an increase in extracellular adenosine (194). The increased adenosine levels might activate A2AR and could explain the beneficial effect of metformin in obese patients with knee osteoarthritis, but this needs to be explored further.

P2 Receptor Modulation in OA

Currently, it is known that extracellular nucleotides are fundamental in the regulation of biological processes in many

tissues, including the musculoskeletal system, and could work as potential therapeutic targets (195).

We have known that nucleotide receptors are expressed by chondrocytes and are associated with PGE2 release since 1991 (196). However, their role in chondrocytes may be controversial, possibly depending on the amount of extracellular ATP available and the physiological/pathological conditions of the joint (18). Regarding the physiological conditions, prechondrogenic condensation occurs, a phenomenon necessary for chondrogenic differentiation and skeletal patterning. This process of prechondrogenic condensation is mediated by extracellular ATP signaling via the P2X4 receptor, which leads to Ca^{2+}-driven ATP oscillations, ensuring the correct constitution of the joint (197). P2X1 and P2X2 receptors were identified in primary bovine chondrocytes, showing that they can facilitate the release of NO and PGE2, which are involved in inflammatory processes and cartilage resorption, in the presence of high concentrations of ATP (156). Upregulation of proteoglycan levels and reduction in NO release after dynamic compression occurs in bovine articular chondrocytes via P2 and has an anabolic effect on the cartilage (198). P2X2 and P2X3 receptors are also expressed in chondrocytes. ATP levels in OA have been linked to pain intensity (199). Activation of P2X3 receptors in chondrocytes induces NO and PGE2 release, suggesting their function in modulating the inflammatory process and playing an important role in the development of articular hyperalgesia in osteoarthritic joints (162) (**Table 2**).

The P2X7 receptor is highly expressed in cells of the immune system, and participates in regulating inflammation and pain, although the exact mechanisms are not yet understood (200). Interestingly, the P2X7 receptor needs a high concentration of ATP for full activation, suggesting a specific role under pathological conditions (201). Accumulation of ATP released by osteoarthritic chondrocytes could act as a warning signal via P2X7, aggravating the inflammatory process and pain and causing cell death (168). Low concentrations of extracellular ATP produce a positive response of the P2X7 receptor activation toward cell proliferation. However, when the concentration of ATP is high, as it is supposed to be in cartilage areas affected by OA, there is an overactivation of the P2X7 receptor, leading to cell death (47). The strategy of blocking the P2X7 receptor could benefit the survival of chondrocytes in OA. It has been found that signaling via P2X7 in rabbit articular chondrocytes leads to chondrocyte apoptosis through the activation of the phospholipase A2 (PLA2)/cyclooxygenase-2 (COX-2) pathway (45). Numerous studies have focused on pain mitigation, one of the main consequences of OA, and their results show that targeting purinergic receptors may be considered as a therapeutic alternative to stop or slow down articular cartilage degeneration. Blocking the P2X7 receptor would have an impact on cartilage itself since it has been observed that its inhibition by the selective antagonist AZD9056 exerts pain-relieving and anti-inflammatory effects. This antagonist counteracts the induction of MMP-13 or NF-κB, both upregulated in osteoarthritic chondrocytes, in an OA rat model. In this study, AZD9056 also reversed the upregulation of IL-1β, IL-6, TNF-α, substance P and PGE2 (169) (**Table 2**).

Extracellular inorganic pyrophosphate, whose levels regulate physiological and pathological mineralization, are also influenced by P2 receptors. Suramin, a putative antagonist of P2 receptors, can block the stimulation generated by ADP on the production of inorganic pyrophosphate in chondrocytes, with the consequent blockade of excessive deposition of calcium pyrophosphate dihydrate (CPPD) in the articular cartilage, which would hinder the correct mineralization of the bone (202). Similarly, and consolidating the idea that high concentrations of extracellular ATP promote cartilage damage, another study on primary articular chondrocytes has shown that P2X7 and P2X4 may be implicated in excessive ATP efflux when the ANK gene is overexpressed, as in OA (163), causing accumulation of CPPD (164). Modulation of these receptors would facilitate the reduction of joint damage and cartilage regeneration (**Table 2**).

However, activating or blocking P2 receptors in OA does not only act on the inflammatory onset of the disease. As mentioned above, abnormal mechanical loading of the joint exerts changes in cell structure and mechanical properties that lead to OA. Physiological joint loading and articular cartilage compression seem to play a key role in ATP release and P2 signaling. Mechanical forces coming from the ECM are transmitted to rat articular chondrocytes via integrins, activating different molecular mechanisms, such as stimulation of the P2Y pathway, mainly by P2Y1, P2Y2, and P2Y4 receptors (203). A candidate to transfer these forces is the primary cilium, which modulates ATP-induced Ca^{2+} signaling via P2X (P2X4, P2X7) and P2Y (P2Y1, P2Y2) receptors (204). As an overview, the forces applied to chondrocytes lead to an increase in ATP release, which downregulates the expression of MMPs, such as MMP-13 (205). On the contrary, when sustained pressure is maintained on chondrocytes, ATP release is suppressed, causing cartilage degeneration (206). This mechanical stimulation is essential for maintaining cartilage integrity, and different responses to this input have been observed between healthy and osteoarthritic chondrocytes. Healthy chondrocytes increase the amount of extracellular ATP after mechanical stimulation via the P2Y2 receptor exerting anabolic responses, while osteoarthritic chondrocytes do not increase these ATP levels (63, 176) (**Table 2**).

From another perspective, the activation of some receptors, such as P2Y1, P2Y2, P2Y4, and P2Y11, induces the adipogenic differentiation of stem cells (170, 174), whereas, other receptors, like P2Y13, P2Y14, and P2X7 show anti-adipogenic effects (170). In addition, the P2Y1 receptor can induce leptin production in murine adipose tissue (175). On the other hand, activation of the P2Y4 receptor inhibits adiponectin expression, and P2Y4 KO mice show increased adiponectin secretion (178). P2X7 KO mice have increased body weight and adipocyte hyperplasia in fat pads (171) (**Table 2**).

Rat models have revealed that the P2Y1 receptor is present on intra-islet capillaries, and P2X4 receptors appear in β-cells. Moreover, P2X1, P2X3, P2Y1, and P2Y2 receptors are expressed in small pancreatic blood vessels, and β-cells present P2X7 receptors, which are downregulated in T2D. In human islets, the receptor seems to be involved in the secretion of insulin and IL-1RA (165). The P2X7 KO mice show lower β-cell mass, impaired glucose tolerance, and defective insulin and interleukin

secretion (172). Furthermore, the P2Y13 receptor is involved in the induction of β-cell apoptosis in the presence of high glucose and free fatty acid levels (184). Extracellular ATP (1 μM) increases insulin secretion in mouse β-cell lines, but at higher ATP concentrations, cell viability decreases, with involvement of the P2Y1 and P2X4 receptors (165) (**Table 2**).

There is some controversy in the modulation of these receptors both in OA and MetS. Extracellular concentrations of ATP modulate the activation of these receptors, which act differently according to the tissue where they are expressed. The P2X7 receptor is related to the inflammatory processes during OA development, with high levels of extracellular ATP (168). However, in adipose tissue, this receptor shows an anti-adipogenic effect (171). In T2D, the P2X7 receptor has been reported to be decreased (172). Furthermore, activation of the P2Y1 receptor increases in obesity in the adipose tissue (175) and in T2D in β-cells (165). In chondrocytes, the P2Y1 receptor plays a key role in the response to mechanical forces, and when the pressure is maintained, increased ATP release may cause cartilage degeneration (203). A deeper knowledge of these receptors in these three conditions is necessary to better describe the close relationship among them (**Table 2**).

Mitochondrial Metabolism, Purinergic Signaling, and OA

During chondrosenescence, the glycolysis pathway is overstimulated, trying to generate ATP promptly, which is necessary for repairing damaged cartilage (207). Chondrocytes are mainly glycolysis-dependent cells, but they keep the ability to use mitochondrial respiration in certain cases to enhance cell survival and ECM biosynthesis in periods of nutrient stress to sustain ATP synthesis (208, 209). However, under OA conditions, mitochondrial biogenesis and activity are disrupted, leading to a greater amount of ROS production. This can be linked to AMPK deficiency in OA, a molecule responsible for regulating cellular metabolism and energy balance (210). AMPK is also a chondroprotective molecule that is capable of inhibiting procatabolic responses to inflammation and biomechanical injury via its downstream targets, peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α and forkhead box O (FOXO)3A (211). AMPK/sirtuin (SIRT)-3 signaling protects mitochondria from oxidative stress by deacetylating superoxide dismutase (SOD2) and decreasing ROS. This pathway also prevents mitochondrial DNA damage by activating mitophagy (212). Some approaches have focused on enhancing the AMPK/SIRT1 pathway, treating an OA rat model with quercetin, and finding overall improvement in mitochondrial function, higher ATP levels in mitochondria, increased mitochondrial copy number and attenuation of ROS levels (213). Other studies have observed that pharmacological stimulation of AMPK increases PGC-1α via SIRT1, reversing impairments in mitochondrial biogenesis, oxidative phosphorylation (OXPHOS) and intracellular ATP in human knee OA chondrocytes (214). It is established that extracellular adenosine can contribute to AMPK activation and regulation (215). Adiposomal injections and CGS21680 A2AR agonist have demonstrated FOXO1/3 activation and retention in the nucleus, which is implicated in increased autophagy and cartilage homeostasis. This recent study provides a mechanism in which A2AR can activate SIRT1 and AMPK through PKA (216) (**Figure 3**).

Age-associated mitochondrial dysfunction and mitochondrial dysfunction in OA contributes to perpetuating the low-grade systemic pro-inflammatory status by further promoting cellular senescence and SASP, and with it, the pathological processes associated with inflammaging (32, 210, 217). In this context, mitochondria increase the production of superoxide anion and hydrogen peroxide, and the amount of AMP increases with respect to ATP, and mitochondrial danger-associated molecular patterns (DAMPs) are released. The increase in ROS, DNA damage, oxidized fatty acids, proteins, and cofactors, ultimately alters cellular function (32). This chondrocyte ROS increase is partly due to mitochondrial dysfunction and reduced SOD2 activity, which is an antioxidant enzyme and a by-product of the mitochondrial electron transport chain. The altered redox environment promote disruptions in signaling, increasing pro-catabolic MAP kinases p38 and ERK and inhibition of anabolic insulin growth factor 1 (IGF-1) or bone morphogenic protein 7 (BMP-7) signaling, leading to an aging phenotype and chondrocyte death (218). In OA, this increase in oxidative stress also disrupts proteoglycan and collagen network, favoring the appearance of residues that further activate innate immunity. The accumulation of ROS accelerates cellular senescence processes and apoptosis by decreasing ATP levels (32). Sustained mechanical loading is essential in OA for reducing ATP levels and respiratory activity, and for increasing ROS production on bovine osteochondral explants (206) (**Figure 3**).

Inflammation and mitochondrial disruption are closely related and affect OA progression. Inhibition of the mitochondrial respiratory chain induces inflammatory responses. In primary human chondrocytes the inhibition of complexes III and V induces COX-2 expression and PGE2 production, the generation of ROS and the activation of NF-κB (219). Therefore, COX-2 levels are upregulated in inflamed joint tissues and are located in the superficial layers of human cartilage with OA, where damage firstly appears (219). COX-2 overexpression is related to higher PGE2 production in osteoarthritic cartilage, which is partly responsible for inflammation and pain (220). Nimesulide, a COX-2 inhibitor, mediates its anti-inflammatory effects *in vivo* and *in vitro* by increasing CD73 activity and AMP hydrolysis so that higher levels of adenosine are available for A2AR activation (221). This mechanism of action positions adenosine signaling as a major player in inflammatory-derived pain mitigation by non-steroidal anti-inflammatory drugs (NSAIDs) and points out an correlation between mitochondrial alterations, inflammation and pain.

Osteoarthritic chondrocytes exhibit a depletion in the mitochondrial production of ATP with a consequent reduction of extracellular adenosine and A2AR stimulation, disrupting chondrocyte homeostasis (222). This ATP level decrease in OA contributes to an increase in glycolysis with pyruvate conversion to lactate and defects in OXPHOS, leading to mitochondrial dysfunction (209). A2AR-null mice develop spontaneous OA with increased mitochondrial dysfunction, increased ROS burden and reduced ATP production via OXPHOS (22, 130).

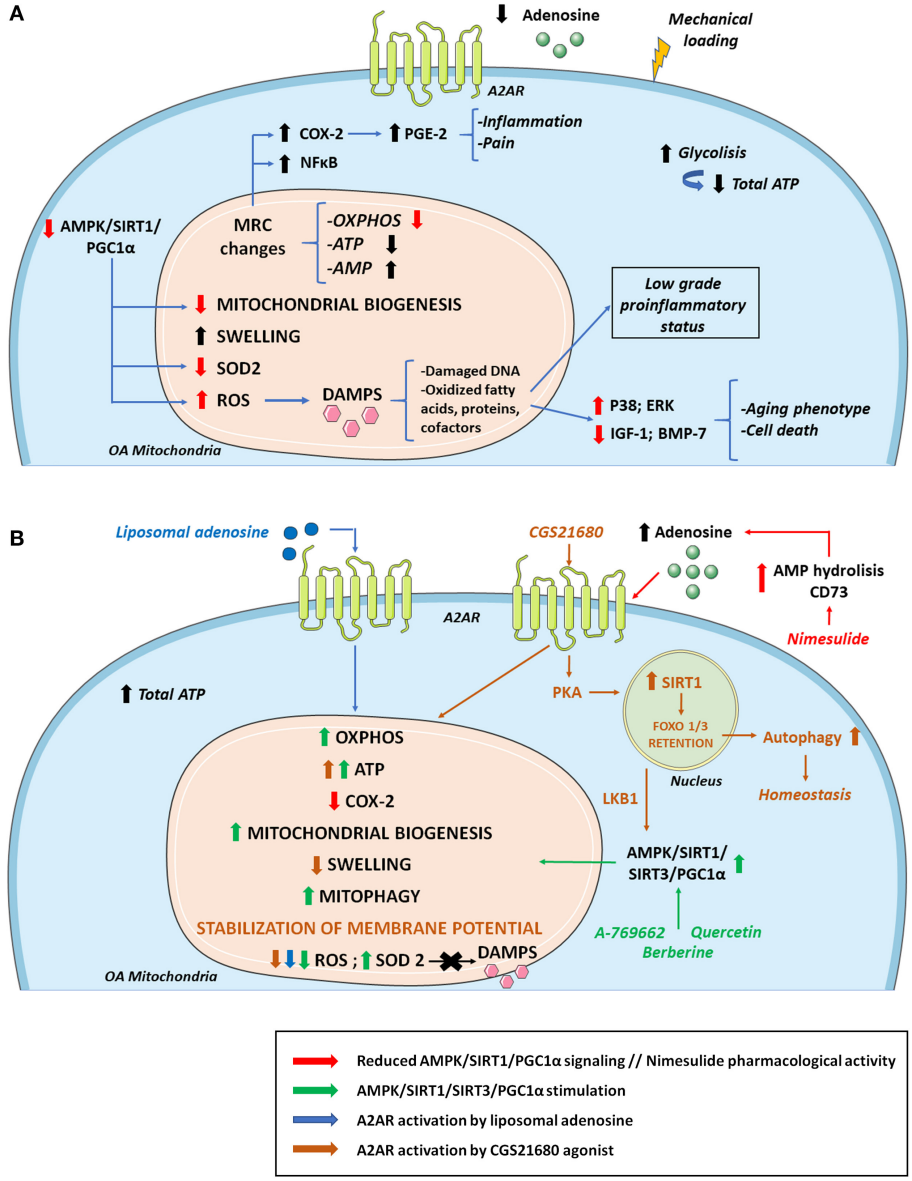

FIGURE 3 | Osteoarthritis (OA) mitochondrial metabolism and its modulation by A2AR and AMPK stimulation. **(A)** During the development of OA, cellular metabolic alterations occur. There is an increase in glycolysis activity to compensate the energy requirement necessary to repair damaged cartilage. However, total ATP levels decrease due to mitochondrial defects together with sustained mechanical loading. Changes in the MRC and its complexes impair ATP and AMP production. As a result, there are lower extracellular levels of ATP and adenosine which diminish A2AR signaling. These alterations in the MRC cause an increase in proinflammatory molecules and transcription factors such as COX-2 and NF-κB, respectively, aggravating inflammation and pain. Reduced AMPK/SIRT1/PGC-1α signaling compromises mitochondrial biogenesis and OXPHOS. There is also a decrease in antioxidant enzymes such as SOD2 that cause increases in ROS production, contributing to the release of DAMPS that aggravate the inflammatory status, stimulating catabolic pathways (p38; ERK) and reducing anabolic pathways (IGF-1; BMP-7), which supposes cell death and an aging phenotype. **(B)** Mitochondrial OA impairment can be partly reversed by AMPK and A2AR stimulation. Quercetin, berberine and pharmacological AMPK activator A-769662 can activate AMPK/SIRT1/PGC-1α and AMPK/SIRT3 pathways increasing OXPHOS, ATP, mitochondrial biogenesis, mitophagy, and SOD2 activity. AMPK stimulation also promotes a decrease in ROS production with reduced DAMPs secretion diminishing inflammation and aging phenotype. Regarding ROS level reduction, similar effects are found when A2AR is activated by agonists like liposomal adenosine. Moreover, A2AR agonist CGS21680 stimulates SIRT1 via PKA allowing FOXO1/3 retention and increased autophagy. CGS2168 indirectly activates AMPK through LKB1. Nimesulide increases extracellular adenosine levels favoring a reduction in the expression of COX-2 and the associated inflammation and pain. AMP, adenosine monophosphate; AMPK, AMP activated protein kinase; BMP-7, bone morphogenetic protein 7; DAMPS, damage associated molecular patterns; IGF-1, insulin growth factor 1; LKB1, liver kinase B1; MRC, mitochondrial respiratory chain; OXPHOS, oxidative phosphorylation; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PKA, protein kinase A; SIRT1, sirtuin 1; SIRT3, sirtuin 3; SOD2, superoxide dismutase.

This model also shows mitochondrial depolarization, swelling, fragmentation and mitophagy (222). ROS burden is mitigated in a mouse model of obesity-induced OA by intra-articular injections of liposomal adenosine or CGS21680. This A2AR agonist also increases mitochondrial basal rates of respiration and ATP production *in vitro*, with increased ATP release in IL-1β-treated human chondrocytic T/C-28a2 cells. A2AR activation also stabilizes mitochondrial membrane potential and reduces mitochondrial swelling after IL-1β exposure (222) (**Figure 3**).

Purinergic System, Inflammasome and OA

The NLRP3 inflammasome is considered an important component involved in OA pathogenesis (223). Inflammasomes are multi-protein complexes mainly present in the cytosol of myeloid cells, such as neutrophils, monocytes and DCs, but they are also found in non-hematopoietic lineage cells such as endothelial fibroblasts or chondrocytes (224).

Assembly and activation of the inflammasome components by different stimuli (e.g., DAMPs) triggers a regulated pro-inflammatory reaction and induces cellular pyroptosis as a protection mechanism orchestrated by innate immunity to restore tissue homeostasis (225). In the joint, inflammasome dysregulation can lead to synovial inflammation and cartilage destruction, characteristic of OA pathogenesis (226).

It has been established that at least two signals are necessary to induce NLRP3 activation: a first priming signal, where TLRs recognize DAMPs or PAMPs promoting synthesis of inflammasome components mediated through NF-kB; and a second activation step, which leads to the assembly of the scaffold inflammasome proteins and recruitment of pro-caspase-1. This second signal is stimulated by cellular stress or pathogen-derived molecules (227) (**Figure 4**).

Although the exact role of NLRP3 is still controversial, several studies have pointed out that overactivation of the NLRP3 inflammasome is involved in low-grade chronic inflammation during OA development (226, 228). Inflammasome secretion of pro-inflammatory IL-1β leads to synovial inflammation and cartilage degradation (229). IL-18, another cytokine processed by the NLRP3 inflammasome, is elevated in synovial fluid and serum of patients with OA, and has been proven to decrease type II collagen and aggrecan expression, disrupt autophagy and induce apoptosis in chondrocytes (230, 231). A recent work shows that inhibition of the mTOR pathway after IL-18 treatment has protective effects in chondrocytes both *in vitro* and *in vivo* (232). Data suggest that IL-1β and IL-18 are mainly produced in the damaged synovium rather than cartilage (233). Analyses of knee joint explants from patients with primary OA have demonstrated that the synovium expresses high levels of NLRP3 inflammasome components (234), and NLRP3 and NLRP1 inflammasomes are overexpressed in synovium derived from knee OA patients (235). In addition, oxidative stress-mediated NLRP3 inflammasome activation was enhanced after inhibiting HO-1 expression (236). Pyroptosis mediated by NLRP3 inflammasome have also been found in chondrocytes *in vitro* and *in vivo* (234).

All these data support the idea that the NLRP3 inflammasome is involved in the onset and progression of OA and can even be considered a potential new biomarker for diagnosis and tracking in patients with OA (228).

Usually, CPPD and basic calcium phosphate (BCP) crystal deposits are found in the synovial fluid and cartilage of patients with OA. Both crystals are believed to be involved in joint inflammation and may correlate with disease harshness (237, 238). BCP crystals can promote NLRP3 inflammasome-dependent or -independent inflammation (239). Although some reports challenge this assumption, like one performed in a mouse meniscectomy OA model, overwhelming data support the notion that BCP and CPPD crystals induce synovial inflammation (240). *In vivo* and *in vitro* models have shown that these crystals are recognized by innate immune system cells, activating the NLRP3 inflammasome (237, 241). TLRs can recognize CPPD and BCP crystals, and their phagocytosis may induce ATP release, K^+ efflux and ROS production (242), suggesting that the purinergic system plays an important role in crystal-mediated inflammasome activation processes in OA.

ATP, its derived metabolites and their recognition by P2X7, P2Y or adenosine receptors are a fine-tuned regulatory mechanism of inflammasome activity, sustaining a chronic inflammatory condition in different diseases. However, their exact role during OA development remains uncertain. Below we address different mechanisms of inflammasome activation mediated by purinergic signaling. This may open a new insight for understanding OA pathogenesis.

Adenosine, Its Receptors and the Inflammasome

Extracellular adenosine activates inflammasome-mediated IL-1β production through A2AR, A2BR, and A3R (243). Adenosine influx through nucleoside transporter ENT2 was converted to ATP by AK, increasing intracellular ATP concentration, which led to ATP secretion from macrophages and consequent upregulation of NLRP3 and IL-1β secretion. On the other hand, ADA reduced IL-1β secretion mediated by nanoparticle-induced NLRP3 inflammasome activation in macrophages (243). Another paper demonstrated that after LPS or ATP stimulation of mouse peritoneal macrophages, adenosine prolonged inflammasome activation, upregulating NLRP3 and IL-1β and enhancing caspase-1 activity. Adenosine stimulated A2AR, activating the downstream cAMP/PKA and CREB/HIF-1α pathway (**Figure 4**). In this study, adenosine was able to overcome a tolerant unresponsive state acquired by macrophages after previous LPS stimulation, re-inducing high levels of IL-1β secretion (244).

ATP, Its Receptors and the Inflammasome

In normal conditions, excessive extracellular ATP is degraded by ecto-ATPases to adenosine. However, when homeostasis is disrupted, high levels of extracellular ATP released to the milieu from stimulated or necrotic cells acts as a warning signal, being detected by the P2X7 receptor. This results in a massive efflux of K^+ across the porous cytoplasmic membrane (245). The P2X7 receptor mediates NLRP3 inflammasome activation in different inflammatory diseases and conditions, and is highly expressed in leukocytes and in other cell types (246–248). Pannexin-1 forms hemichannels across the plasma membrane permeable to ATP, releasing it to the extracellular milieu (249). Pannexin-1 directly

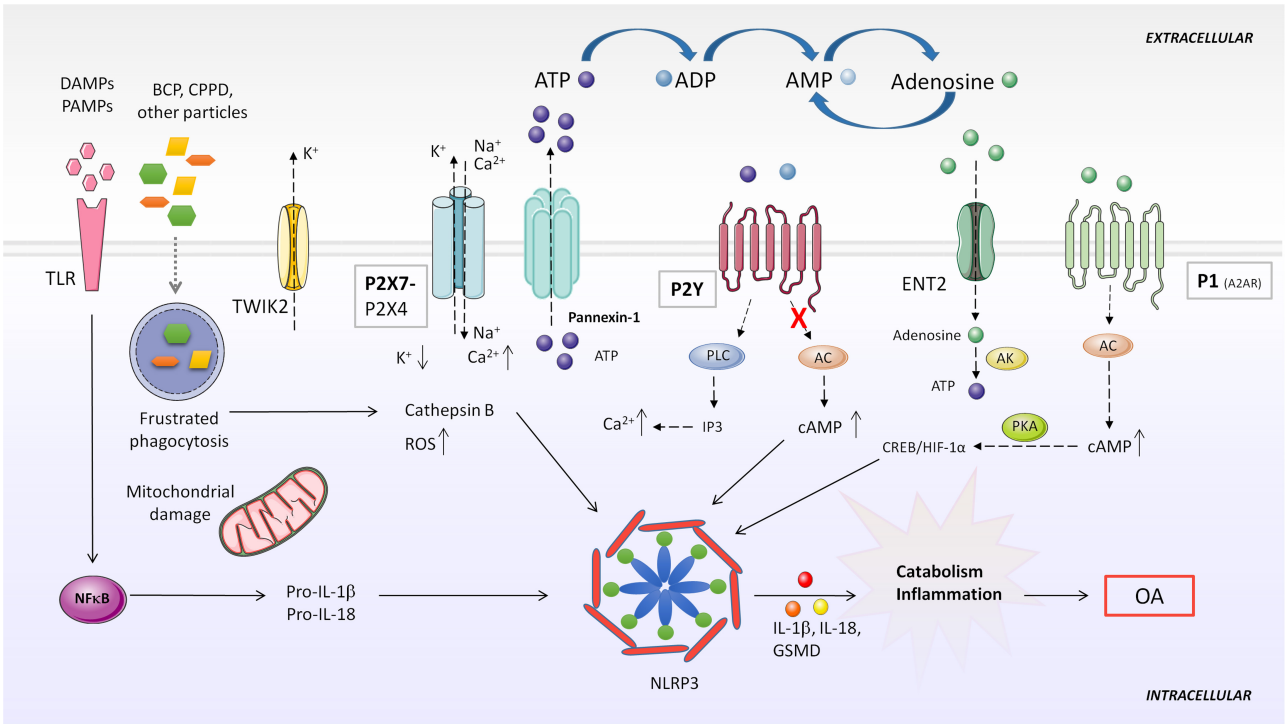

FIGURE 4 | Purinergic system implication in NLRP3 inflammasome activation. DAMPS or PAMPs present in the microenvironment are recognized by TLR receptors, which in turn, upregulate pro-IL-1β and pro-IL-18 expression through NF-κB activity. BCP and CPPD crystals can be both recognized by TLRs and phagocytized by the cell. Impaired particle or crystal phagocytosis by phagosomes leads to cathepsin B release and mitochondrial dysfunction, which induce elevated ROS production and consequent NLRP3 activation. Additionally, frustrated phagocytosis can lead to massive ATP release through Pannexin-1, activatingP2X7 and causing ionic cytoplasmic imbalance. Excessive K^+ efflux, especially through TWIK2, and Na^+ and Ca^{2+} influx through P2X7 activates NLRP3 inflammasome. Activation of different P2Y by ATP or ADP increases intracellular Ca^{2+} concentration. Particles can also impede induction of purinergic AC-cAMP pathway allowing NLRP3 activity. A2AR activation by extracellular adenosine induces HIF-1α upregulation, which activates NLRP3. AC, adenylate cyclase; AK, adenosine kinase; BCP, basic calcium phosphate; CPPD, calcium pyrophosphate dehydrate; DAMP, danger-associated molecular patterns; ENT2, equilibrative nucleoside transporter 2; GSMD, gasdermin D; IP3, inositol 1,4,5-trisphosphate; NLRP3, NOD-like receptor pyrin domain containing 3; PAMP, pathogen-associated molecular patterns; PKA, protein kinase A; PLC, phospholipase C; ROS, reactive oxygen species; TLR, toll like receptor; TWIK2, two-pore domain weak inwardly rectifying K^+ channel 2.

interacts with the P2X7 receptor in different cell lines, and this has been associated with NLRP3 inflammasome activation (249–251) (**Figure 4**).

Riteau et al. demonstrated that several sterile particles induced endogenous ATP release from human macrophages in a P2X7 receptor-dependent way. They proposed that ATP release through Pannexin-/Connexin hemichannel activation could be a consequence of cathepsin B leakage from lysosome disruption. Extracellular ATP could then act in an autocrine/paracrine way, activating the P2X7 receptor and amplifying ATP release and inflammasome activation response (252) (**Figure 4**).

An alternative mechanism was described by Di et al. through which the P2X7 receptor could mediate K^+ efflux in macrophages. The two-pore domain weak inwardly rectifying K^+ channel 2 (TWIK2) is involved in NLRP3 inflammasome activation in an LPS-induced lung inflammation mouse model. They proposed that ATP activated P2X7 receptor-favored influx of Na^+ and Ca^{2+} cations, modulating membrane potential and promoting efflux of K^+ through the TWIK2 channel (253).

ROS are considered another well-known stimulus through which the NLRP3 inflammasome can be activated. It has been reported that Ca^{2+} influx mediated by the activated P2X7 receptor can phosphorylate NADPH oxidase complexes in different cell types, including macrophages, stimulating the production of ROS (246) (**Figure 4**).

Although the P2X7 receptor has been described as the main purinergic receptor involved in NLRP3 inflammasome activation, a recent study in rat urothelial cells showed that treating cells with both P2X7 and P2X4 inhibitors eliminated intracellular caspase-1 activity, suggesting the involvement of the P2X4 receptor in inflammasome activity (254).

Riteau et al. also observed that at low ATP doses, the addition of the metabolites ADP and UTP, which act specifically on P2Y receptors, enhanced particle-mediated IL-1β production (252). Oxidized ATP, an inhibitor of P2X and P2Y receptors, was able to decrease IL-1β production in a dose-dependent manner, but P2X7 receptor selective inhibition did not impair IL-1β production. Interestingly, P2Y1 and P2Y2 receptors, together with A2AR and A2BR expression are upregulated and IL-1β synthesis increased by ATP metabolites and their stable forms. As these purinergic receptors couple to G proteins, in this study the authors suggested that the signaling pathway involved in this

process was mediated by phospholipase C (PLC)-IP3 activation or inhibition of AC-cAMP. Therefore, an increase in cytoplasmic Ca^{2+} or a decrease in cAMP levels were sensed by the NLRP3 inflammasome, increasing its activity (243).

CONCLUSIONS AND FUTURE DIRECTIONS

In this review, we have described the relevance of the purinergic system in osteoarthritic cartilage and how MetS components associated with OA influence this system (18, 160). It is well-established that A2AR activity mediated by adenosine is involved in articular cartilage homeostasis maintenance, and is a key modulator during OA (134). Obesity-triggered stimulation of this receptor could contribute to cartilage loss and OA development in obese patients (141).

On the other hand, a more in-depth study of the contribution of nucleotide receptors in OA and MetS is needed to understand better the underlying mechanisms that activate purinergic signaling in the pathogenesis of OA and elucidate confounding data. For example, the P2X7 receptor mediates ATP related inflammation during OA (169, 251), but this receptor exerts an anti-adipogenic effect (148). Therefore, it remains unclear whether the link between obesity and OA might be lie in ATP receptors.

Apart from the activation or inhibition of adenosine/ATP receptors, other components of the purinergic system are involved in OA progression and may be potential modulators of metabolic inflammation. AMPK is an energy sensor involved in inflammation, metabolism and T2D, which is able to adapt cellular metabolism in response to the cellular nutritional and environmental stage (255). AMPK activity is constitutively present in healthy articular chondrocytes and decreases with age and during OA progression (213, 256). AMPK activity has been described to prevent articular cartilage degeneration during mouse aging (257), and is also required to maintain mitochondrial function and prevent OA (213). As we have described earlier in this review, metformin can activate AMPK, resulting in an increase in extracellular adenosine (194). It is known that metformin is beneficial in obese patients with knee OA, but the contribution of AMPK to this effect requires further study.

Another component that needs further analysis is the contribution of ADA to this disease. ADA is an indicator of cellular immunity, and its levels are increased in the following diseases: RA, psoriasis, sarcoidosis, cancer and tuberculosis (258). Synovial ADA fluid measurement, in association with CRP and ESR levels, can distinguish OA from other rheumatic diseases, like RA (186). There is a study in the Indian population that links increased ADA activity with being overweight and obesity (150). This link should be studied in obese patients with OA to understand better the role of ADA as an adenosine level regulator.

As mentioned above, several purinergic receptors are involved in inflammasome activation in different diseases. The study of these mechanisms in the inflammation of OA could be an interesting field of research.

In conclusion, the purinergic system is a key modulator of metainflammation, and its contribution to the pathogenesis of OA opens future therapeutic approaches for the treatment of the disease.

AUTHOR CONTRIBUTIONS

AM, GH-B, and RL conceptualized, wrote, and revised the manuscript. PG, AL, JM, and IB-Á were primarily responsible for writing, editing, and revising the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from Instituto de Salud Carlos III through the Miguel Servet program (CP15/00053), co-funded by Fondo Europeo de Desarrollo Regional (FEDER) and research grants from the Spanish Instituto de Salud Carlos III (PI16/00991 and PI19/00744). PG and AL are recipients of a research fellowship from the Fundación Conchita Rábago. JM and IB-Á are funded by the Instituto de Salud Carlos III through PIE15/00048 and FI19/00060, respectively.

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Conflict of Interest: AM has filed a patent for the use of adenosine A2AR agonists to prevent prosthesis loosening (pending) and a separate patent concerning the use of A2AR agonists and agents that increase adenosine levels to promote bone formation/regeneration. RL and GH-B have filed a patent on the use of 6-shogaol in osteoarthritis.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 25 September 2020
doi: 10.3389/fmed.2020.566250

Modulation of the Inflammatory Process by Hypercholesterolemia in Osteoarthritis

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Objective: Several studies have linked metabolic syndrome to the development of osteoarthritis (OA) through hypercholesterolemia, one of its components. However, epidemiological studies showed contradictory results, and it is not clear how hypercholesterolemia itself, or oxidized LDL (oxLDL)—a pathological molecule potentially involved in this relationship—could be affecting OA. The objectives of this study were to investigate the effect of hypercholesterolemia induced by high-fat diet (HFD) in cartilage from OA rabbits, and how oxLDL affect human chondrocyte inflammatory and catabolic responses.

Design: New Zealand rabbits were fed with HFD for 18 weeks. On week 6, OA was surgically induced. At the end of the study, cartilage damage and IL-1β, IL-6, MCP-1, MMP-13, and COX-2 expression in articular cartilage were evaluated. In addition, cultured human OA articular chondrocytes were treated with oxLDL at concentrations equivalent to those expected in synovial fluid from HFD rabbits, in the presence of IL-1β and TNFα. The effect of oxLDL on cell viability, nitric oxide production and catabolic and pro-inflammatory gene expression was evaluated.

Results: HFD intake did not modify cartilage structure or pro-inflammatory and catabolic gene expression and protein presence, both in healthy and OA animals. OxLDL did not affect human chondrocyte viability, ADAMTS5 and liver X receptor (LXR) α gene expression, but decreased the induction of IL-1β, IL-6, MCP-1, MMP-13, iNOS, and COX-2 gene expression and MMP-13 and COX-2 protein presence, evoked by cytokines.

Conclusions: Our data suggest that cholesterol intake *per se* may not be deleterious for articular cartilage. Instead, cholesterol *de novo* synthesis and altered cholesterol metabolism could be involved in the associations observed in human disease.

Keywords: osteoarthritis, cartilage, hypercholesterolemia, chondrocyte, LDL, diet

OPEN ACCESS

Edited by:
Kayo Masuko,
Sanno Medical Center, Japan

Reviewed by:
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Specialty section:
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

Received: *27 May 2020*
Accepted: *28 August 2020*
Published: *25 September 2020*

Citation:
Villalvilla A, Larrañaga-Vera A, Lamuedra A, Pérez-Baos S, López-Reyes AG, Herrero-Beaumont G and Largo R (2020) Modulation of the Inflammatory Process by Hypercholesterolemia in Osteoarthritis. Front. Med. 7:566250. doi: 10.3389/fmed.2020.566250

INTRODUCTION

Osteoarthritis (OA) is one of the main causes of pain and disability, with high impact on life quality but also on national economies (1). It is a multi-factorial disease, and several risk factors like trauma, aging, gender, obesity and genetic predisposition have been associated with the development and progression of OA. In fact, new studies deepening in OA pathophysiology have

revealed that this disease could be divided in different phenotypes (2). Among them, metabolic OA is of high importance. This phenotype arises from the wide association of OA with metabolic syndrome, with higher incidence of this condition in OA patients than in the population without the disease, and a more severe progression of OA in patients with metabolic syndrome (3).

Metabolic syndrome is defined by the presence of insulin resistance, obesity, hypertension and dyslipidaemia. Several studies have analyzed the role of each component in the development of OA, concluding that hypercholesterolemia and OA are significantly associated (4, 5). In addition, dysregulated lipid metabolism and cholesterol accumulation have been found in chondrocytes during OA (6, 7), and knockout mice with altered HDL metabolism have higher predisposition to the development of OA (8).

Despite the evidence showing a relationship between hypercholesterolemia and OA, epidemiological studies have found contradictory results about the use of statins to control circulating cholesterol levels and OA development. While a reduced progression of knee OA or lower progression of generalized OA in statin users have been reported (9, 10), no improvement of knee pain, joint function, or structural progression has been observed (11, 12). Therefore, there is insufficient data to prove an effect of circulating cholesterol levels on the onset and progression of OA.

Experimental models of OA allow to analyse the specific effect of dietary cholesterol in the development of the disease, disregarding other factors that are linked to hypercholesterolemia in humans. However, just a few studies have used this approach and obtained conflicting results (13–16). Since rabbits have been claimed as the best animal model for lipid-related research (17, 18), we employed rabbits with normal cholesterol metabolism in our study, in order to analyse the effect of high fat diet (HFD) on OA progression. Furthermore, we carried out an experimental model of high lipid intake that is not associated to an increase in weight gain, that allows to adequately address the contribution of added mechanical load and hyperlipidaemia in cartilage alterations (19–21).

Another suggested hypothesis is that oxidized low density lipoproteins (oxLDL) could promote inflammation during OA development, being the linking molecule between atherosclerosis and OA and explaining the association between this clinical complication of metabolic syndrome and OA (4). Different studies suggest that oxLDL could be deleterious for chondrocytes, inducing decreased cell viability and increased senescence (22–24), as well as hypertrophic-like changes (25). However, these studies did not asses these changes driven by oxLDL on an already inflamed environment such as that found in OA joints. Additionally, other studies suggest an anti-inflammatory and protective role of oxLDL in macrophages activated with LPS (26), as well as a proliferation-triggering effect of human quiescent fibroblasts (27).

Therefore, we aimed to investigate the effect of hypercholesterolemia induced by HFD in cartilage damage associated to OA in rabbits, and how oxLDL affect human chondrocyte inflammatory and catabolic responses.

MATERIALS AND METHODS

Experimental Model in Rabbits

Four months old male New Zealand rabbits ($n = 36$) weighing 3–3.5 kg (Granja San Bernardo, Navarra, Spain) were used in accordance to local and European regulations on care and use of research animals, after approval of the protocol by the Institutional Ethics Committee and following ARRIVE guidelines.

The environmental and temperature conditions were kept constant throughout the study, with 12-h light and 12-h darkness cycles. Rabbits were kept in individual transparent $50 \times 40 \times 40$ cm cages that allow social interactions. After 2 weeks of acclimatization with *ad-libitum* access to water and standard commercial chow, rabbits were randomly separated into four groups (20).

Sixteen randomly selected rabbits were fed HFD containing 0.5% cholesterol and 4% peanut oil (Sniff Spezialdiäten GmbH, Soest, Germany), and 20 rabbits received regular chow, both administered *ad-libitum*. Six weeks later, hypercholesterolemia was established and bilateral OA knee was induced in 10 randomly selected regular chow fed (OA group) and 10 randomly selected HFD animals (OA-HFD group) by anterior cruciate ligament transection (ACLT) and partial medial meniscectomy following an established protocol (20, 28). The surgery was performed on overnight fasted animals, in the morning, under general anesthesia by intramuscular injection of a combination of 2 mg/kg xylazine (Rompun, Bayer) and ketamine hydrochloride (Ketolar, Pfizer) in a 3:1 ratio. Procedures were carried out under aseptic conditions in an operating room. Antibiotic prophylaxis was performed with intramuscular cefonicid injections (100 mg/kg) (Smith K. Beecham) before surgery and the following 3 days. Six HFD animals (HFD group) and 10 regular chow animals (control group) did not undergo experimental intervention. Two OA-HFD animals died due to surgery complications. Blinding was performed by using a non-consecutive numerical code for animals in each group.

Our group has previously verified in several studies that the inflammation generated by the ACLT surgery does not affect the outcome of the joint injury (28). Therefore, we decided not to include a simulated surgery (Sham) group in this study to reduce the total number of animals used.

Body-weight was monitored at baseline and every other week throughout the study. Twelve weeks after OA induction, blood samples were collected from the marginal ear vein after overnight fasting and rabbits were then euthanized by intra-cardiac injection of Tiobarbital (B. Braun, Barcelona, Spain) 1 g/20 ml, following general anesthesia as previously described (28). Articular cavity was reached and left and right tibia cartilage samples were collected independently and immediately frozen to be used for molecular biology studies. Both femurs from each rabbit were also dissected and immersed in 4% paraformaldehyde for histological analysis.

As previously reported, feeding with this HFD did not induced weight gain, glucose metabolism alteration, or systolic blood pressure elevation (19, 20). However, these animals showed a significant increase both in circulating total cholesterol (control:

32 ± 10; HFD: 1,876 ± 287*; OA: 28 ± 4; OA-HFD: 2,050 ± 196* mg/dl; *$p < 0.05$ vs. control) and triglyceride concentration (control: 48 ± 7; HFD: 253 ± 97*; OA: 67 ± 12; OA-HFD: 290 ± 81* mg/dl; *$p < 0.05$ vs. control) (20).

Histopathological Study

After fixation in 4% paraformaldehyde, femurs were decalcified for 4 weeks in a solution containing 10% formic acid and 5% paraformaldehyde. The decalcified femoral condyles were cut in a sagittal plane along the central portion of the articular surface of each medial femoral condyle corresponding to the weight-bearing area, and then embedded in a paraffin block. Sections of 4 μm were stained with Alcian-Blue PAS to assess pathological changes in cartilage. These samples were evaluated using a modified version of Mankin's grading score system, which analyses four different parameters with a total score up to 21: cartilage structure (0–8), proteoglycan staining (0–6), loss of chondrocytes (0–4), and clone formation (0–3) (29). Two blinded observers evaluated the samples (AL-V and RL), and each data presented in **Figure 1** was the mean for each sample between these observers.

Immunohistochemical Studies

Immunohistochemical analyses were done in 4 μm femur cartilage samples. Antigen retrieval was performed by incubation with 0.1% trypsin from bovine pancreas (Sigma-Aldrich, USA) in 0.1% $CaCl_2$. Inflammatory markers were visualized using anti-metalloproteinase (MMP)-13 (R&D systems, USA, MAB511, 30 μg/ml) and anti-ciclooxigenase (COX)-2 (sc-1745; Santa Cruz Biotechnology, USA, 1/100 dilution) antibodies as previously described (30). A secondary biotinylated anti-mouse and anti-goat IgG was used respectively for detection of positive signal through a horseradish peroxidase linked to an avidin/biotin complex (ABC) (Vector Laboratories, USA) using 3,3 diaminobenzidine tetra-hydrochloride as chromogen (Dako, Denmark). Sections were counterstained with Haematoxylin, dehydrated and mounted in DPX (Merck Millipore, USA). Positive immunoreactivity was evaluated in x20 magnification photographs obtained using a Leica DM3000 LED digital micro-imaging instrument (Leica Microsystems, USA). Each image was analyzed using the Image J software (National Institutes of Health, USA) and the percentage of positive area was calculated as previously described (30, 31).

Human Chondrocyte Culture

Human chondrocytes were obtained from the FJD-Biobank (IIS-Fundación Jiménez Díaz, Madrid, Spain) and came from OA patients who underwent total knee replacement surgery. Cells were grown in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 U/ml of penicillin and streptomycin (Lonza, Belgium). At least four independent experiments were performed for each determination. Each replicate corresponds to an independent experiment carried out with chondrocytes extracted from a different donor. Cells were used within the first or second passage (32, 33).

LDL Isolation and Cell Treatment

Human LDL were isolated from fresh plasma by sequential ultracentrifugation (34). After removing VLDL and IDL at 1.019 g/ml, LDL fraction between 1.019 and 1.063 g/ml was collected. LDL were then oxidized using 5 μM $CuSO_4$ at 37°C for 2 h and the reaction was stopped by adding 0.3 mM EDTA. OxLDL were dialysed, sterile filtered and quantified using Bio-Rad protein assay (Bio-Rad Laboratories, USA). Then, they were diluted to 1 mg/ml with sterile PBS and stored at 4°C for no longer than 90 days. LDL from three different healthy donors were pooled after isolation, and then oxidized and quantified. Three different oxLDL pools were employed for all the experiments.

After 20 h in serum-free conditions, chondrocytes were treated with 10 or 40 μg/ml of oxLDL, in the presence or absence of 1 ng/ml of IL-1β and 10 ng/ml of TNFα (Peprotech, NJ, USA). Control cells were treated with LDL vehicle (PBS).

Cell Viability Assay

Chondrocyte viability was tested using the methyl-thiazolyl-tetrazolium assay (MTT reagent, Sigma-Aldrich, MO, USA). Briefly, 7×10^3 cells/well were plated in 96-well plates and treated as described above for 20 h. Then, the cells were incubated for 4 h with 10 μl/well of MTT (5 mg/ml). After formazan salt was dissolved with 10 mM HCl in 10% SDS, absorbance was measured at 570 nm.

Nitrite Production

Nitrite accumulation was determined in the culture medium using the Griess reaction as previously described (33). Therefore, 4×10^4 cells/well were seeded in 24-well plates and grown until confluence. Then, cells were treated as described above for 72 h. Nitrite content was evaluated in 50 μl of culture supernatant by incubating for 5 min with Griess reagent (equal volumes of 1% sulphanilamide in 5% phosphoric acid and 0.1% Naphtylethylenediamine–HCl) and absorbance was measured at 540 nm. All measurements were performed in duplicate.

Gene Expression Analysis

Human primary chondrocytes were seeded in 6-well plates at 2×10^5 cells/well and cultured until confluence. Then, cells were treated as described above for 24 h and lysed with Tripure Reagent (Roche, IN, USA). Rabbit cartilage samples were ground into a powder and mixed with Tripure Reagent (32).

RNA was isolated according to manufacturer's protocol and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). TaqMan Gene expression assays were used to quantify mRNA expression in StepOnePlus™ detection system using StepOne™ software v2.2 (Applied Biosystems) (19, 34). HPRT was used as endogenous control and data was presented as relative expression to IL-1β and TNFα-stimulated chondrocytes or to healthy rabbits.

Western Blot Studies

Briefly, 20 μg of total cell lysates were separated by 12% SDS–PAGE and transferred to a nitrocellulose membrane as described elsewhere (35). The following primary antibodies were applied: anti-COX-2 antibody (Santa Cruz Biotechnology;

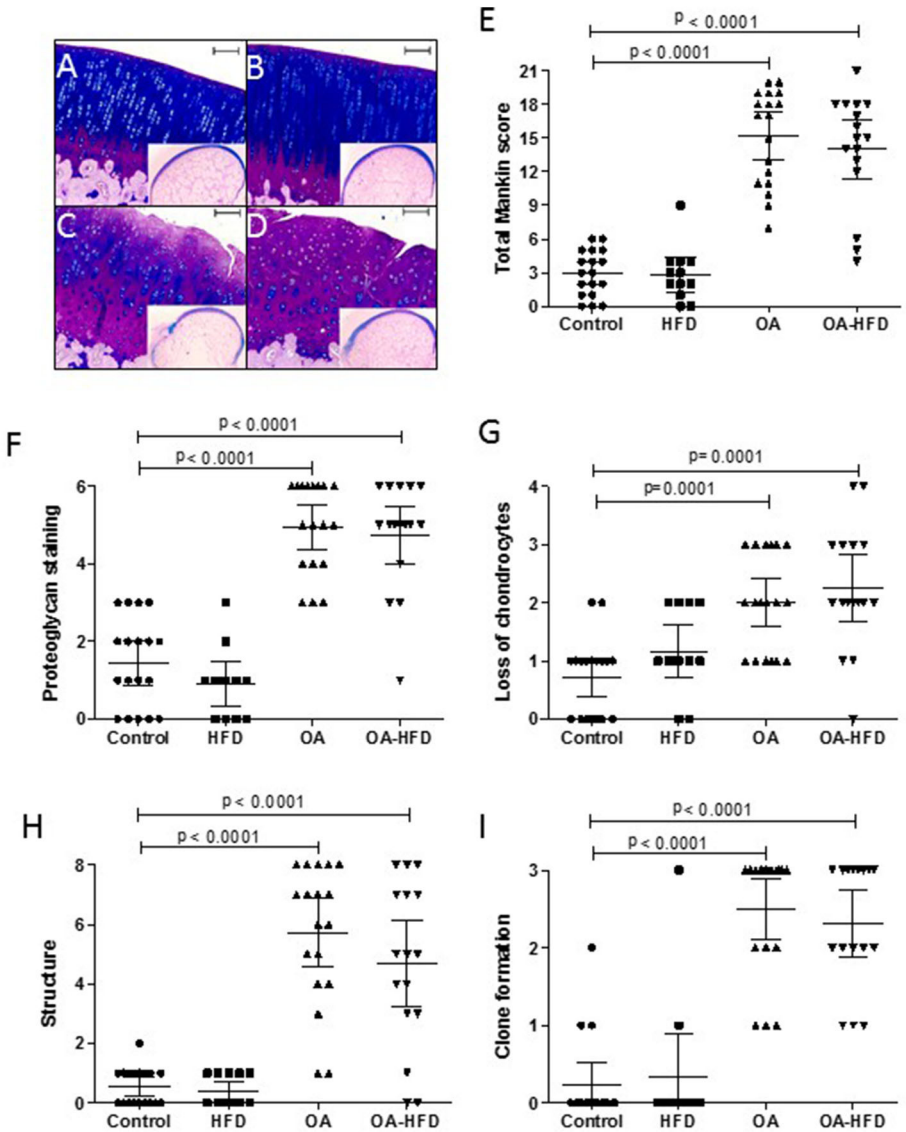

FIGURE 1 | Histological characterization of cartilage damage. Representative sections of femur cartilage stained with Alcian-Blue PAS from control **(A)**, high fat diet (HFD) **(B)**, osteoarthritic (OA) **(C)**, and OA fed HFD **(D)** rabbits. An image of the whole femur section is presented in miniature. Scale bar = 100 μm. Histopathological changes in cartilage were measured using a modified Mankin score as described in Methods **(E)**, evaluating proteoglycan staining **(F)**, chondrocyte loss **(G)**, alteration of cartilage structure **(H)**, and clone formation **(I)**. Data are expressed as mean ± confidence interval; n = 12–18 samples per group.

sc-1745; 1/100 dilution) and anti-MMP-13 antibody (Abcam; ab39012; 1/6000 dilution) in 3% BSA, overnight at 4°C. Antibody binding signal was detected by chemoluminescence through horseradish peroxidase-linked secondary antibodies. α-Tubulin (Sigma-Aldrich; T5168; 1/5000 dilution) was used for protein loading control. Densitometric measurements were normalized relative to protein presence in IL-1β and TNFα-stimulated chondrocytes using Quantity One software (Bio-Rad) (20).

Statistical Analysis

Data obtained from each animal, articular tissue or *in vitro* experiments were analyzed using Kruskal-Wallis multiple comparison test followed by *post-hoc* analysis or Mann-Whitney tests, where appropriate, with Prism software (v5.01, GraphPad software, Inc). Results were expressed as mean ± confidence interval. $P < 0.05$ was considered statistically significant.

RESULTS

Cartilage Damage in OA Rabbits Fed HFD

Histological parameters were assessed as previously described (29). At the end of the study, 18 Control, 12 HFD, 18 OA and 16 OA-HFD femurs were evaluated. Two control and two OA

femurs could not be evaluated due to technical difficulties during sample processing.

The surgical induction of OA was successful in all knees of OA animals, with all the studied histological parameters significantly increased compared to non-OA animals (**Figure 1**). The administration of HFD did not modify the cartilage histology, with the HFD animals showing a score similar to control animals. In addition, OA-HFD animals showed the same damage than the standard chow fed OA rabbits (**Figure 1**).

Molecular Changes in Cartilage From OA Rabbits Fed HFD

Considering the absence of worsening in cartilage histopathology in the presence of HFD, we decided to analyse molecular mediators of the OA process in cartilage from one tibia of each animal from the different study groups, to find out if hypercholesterolemia induced by HFD could be modifying molecular mediator expression in this tissue at a level not appreciable by histology.

HFD did not modify IL-1β, IL-6, MMP-13, MCP1 nor COX-2 gene expression in comparison to Controls. OA animals showed a significant increased expression of these mediators (**Figure 2**). In OA-HFD animals, the expression of these pro-inflammatory and catabolic mediators was not significantly modified (**Figure 2**) in comparison to OA rabbits.

We also tested the presence of MMP-13 and COX-2 proteins in the articular cartilage of the rabbits. As can be observed in **Figure 3**, the tissue staining of both proteins was significantly enhanced in the cartilage of OA and OA-HFD when compared to controls (**Figures 3E,J**), although the tissue localization for these proteins was similar between these two groups. Furthermore, OA-HFD rabbits showed no differences in the intensity of the staining for MMP-13 and COX-2 when compared to OA animals (**Figures 3C–E,H–J**).

Effect of oxLDL on Human Chondrocyte Viability

Given that oxLDL have been suggested as a potential link between metabolic syndrome complications and OA (4), we decided to study the effect of oxLDL on chondrocytes during an OA-like inflammatory environment. The selected doses were in the range of oxLDL levels expected in the synovial fluid of our hypercholesterolemic rabbits in accordance with published data (36–38).

Human primary chondrocytes were stimulated with the selected concentrations of oxLDL in the presence or absence of IL-1β and TNFα pro-inflammatory stimuli. The concentrations used in this study did not affect chondrocyte viability as measured by MTT assay (**Figure 4A**).

Effect of oxLDL on Pro-inflammatory and Catabolic Mediator Expression in Chondrocytes

As they did not affect chondrocyte viability, we studied the effect of the oxLDL on different pro-inflammatory and catabolic mediators produced by human OA primary chondrocytes.

Firstly, we measured nitric oxide (NO) production as nitrite accumulation in culture media. Both 10 and 40 μg/ml of oxLDL reduced the production of NO induced by pro-inflammatory stimuli in human primary chondrocytes, and they did not affect NO production in non-stimulated cells (**Figure 4B**).

Then, we quantified gene expression of different mediators in human chondrocytes. The presence of oxLDL did not significantly modify the gene expression of the different mediators studied. However, the induction of IL-1β, IL-6, and MCP-1 gene expression by a pro-inflammatory cocktail of IL-1β and TNFα was significantly reduced in the presence of both 10 and 40 μg/ml of oxLDL (**Figures 5A–C**). We also analyzed the expression of inducible NO synthase (iNOS) and COX-2, as they are important enzymes for the production of pro-inflammatory mediators, and oxLDL also decreased the expression of these enzymes (**Figures 5D,E**). In addition, we analyzed the expression of catabolic mediators. MMP-13 gene expression was significantly decreased by oxLDL, while ADAMTS5 was not modified by the presence of the lipid (**Figures 5F,G**).

We also measure the protein presence of MMP-13 and COX-2 in the cell extracts from human OA chondrocytes incubated with the pro-inflammatory cocktail in the presence or absence of oxLDL. As can be observed in **Figure 6**, 40 μg/ml of oxLDL was able to significantly decrease the synthesis of these proteins induced by cytokines (**Figures 6A–C**).

Effect of oxLDL on Liver X Receptor (LXR) α in Chondrocytes

Different anti-inflammatory effects of oxLDL have been attributed to LXRα activation. Therefore, we measured the gene expression of this nuclear receptor, that is able to auto-regulate its own expression (39). As can be observed in **Figure 5H**, the incubation with the cocktail of cytokines significantly diminished the gene expression of LXRα, although no significant effect was observed with the addition of oxLDL.

DISCUSSION

The present study shows that hypercholesterolemia induced by HFD does not alter knee cartilage structure in rabbits. In fact, HFD has no effect on cartilage histopathological score and OA-associated gene expression and protein presence after surgically-induced OA. Accordingly, oxLDL does not modify human articular chondrocyte viability, and decreases the expression of pro-inflammatory and catabolic factors under inflammatory conditions *in vitro*.

Experimental models of OA allow to analyse the specific effect of dietary cholesterol in the development of the disease, disregarding other factors that are linked to hypercholesterolemia in humans. However, just a few studies have used this approach. Two studies analyzed the effect of HFD in mice with altered cholesterol metabolism (15, 40), and found that exposure to high cholesterol levels increased spontaneous cartilage damage in healthy animals. A recent work showed that even wild-type Wistar rats presented OA-like changes when fed HFD (16).

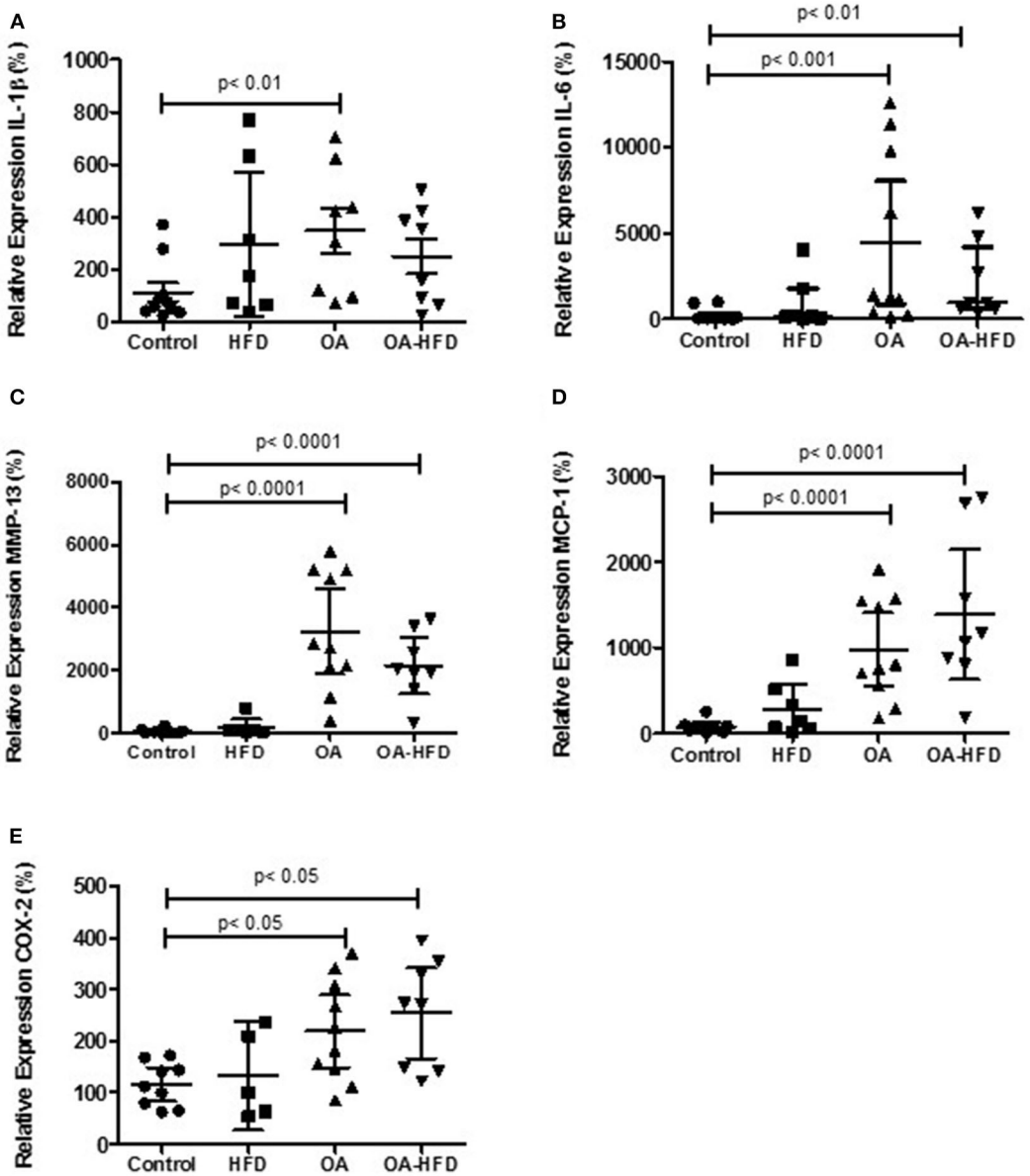

FIGURE 2 | Differential gene expression examined by real time PCR in cartilage. IL-1β **(A)**, IL-6 **(B)**, MMP-13 **(C)**, MCP-1 **(D)**, and COX-2 **(E)** were measure in control, high fat diet (HFD), osteoarthritic (OA), and OA fed HFD (OA-HFD) rabbits. HPRT was used as endogenous control and results were expressed as fold change comparing to healthy rabbit. Data are expressed as mean ± confidence interval; $n = 6–10$ animals per group.

However, de Munter et al. observed that HFD did not worsen cartilage structure in wild-type mice and in transgenic animals with altered cholesterol metabolism after OA induction (13, 14). In our study, rabbits with normal cholesterol metabolism were used to analyse the effect of HFD on OA progression.

As mentioned before, rabbits have been claimed as the best animal model for lipid-related research (18). In contrast to mice and rats, rabbits have a lipoprotein metabolism similar to humans, where the main cholesterol pool is from a hepatic origin and LDL is the predominant plasma lipoprotein (18). The apoenzyme (Apo) profile and the hepatic LDL receptors are also similar in rabbits and humans (17, 41). In contrast to rodents, rabbits present cholesteryl ester transfer protein (CETP) activity, which is also active in humans and essential for HDL metabolism (18, 42). Higher activity of this enzyme has been associated with anti-inflammatory properties, reduced adipogenesis and lower body mass index, and may also be involved in reducing circulating oxLDL levels. In fact, pharmacological inhibition of CETP has been linked to increased sepsis and mortality (43). These similarities between rabbits and humans suggest that rabbits are a better model to analyse cholesterol effects on OA than mice and rats used in previous studies.

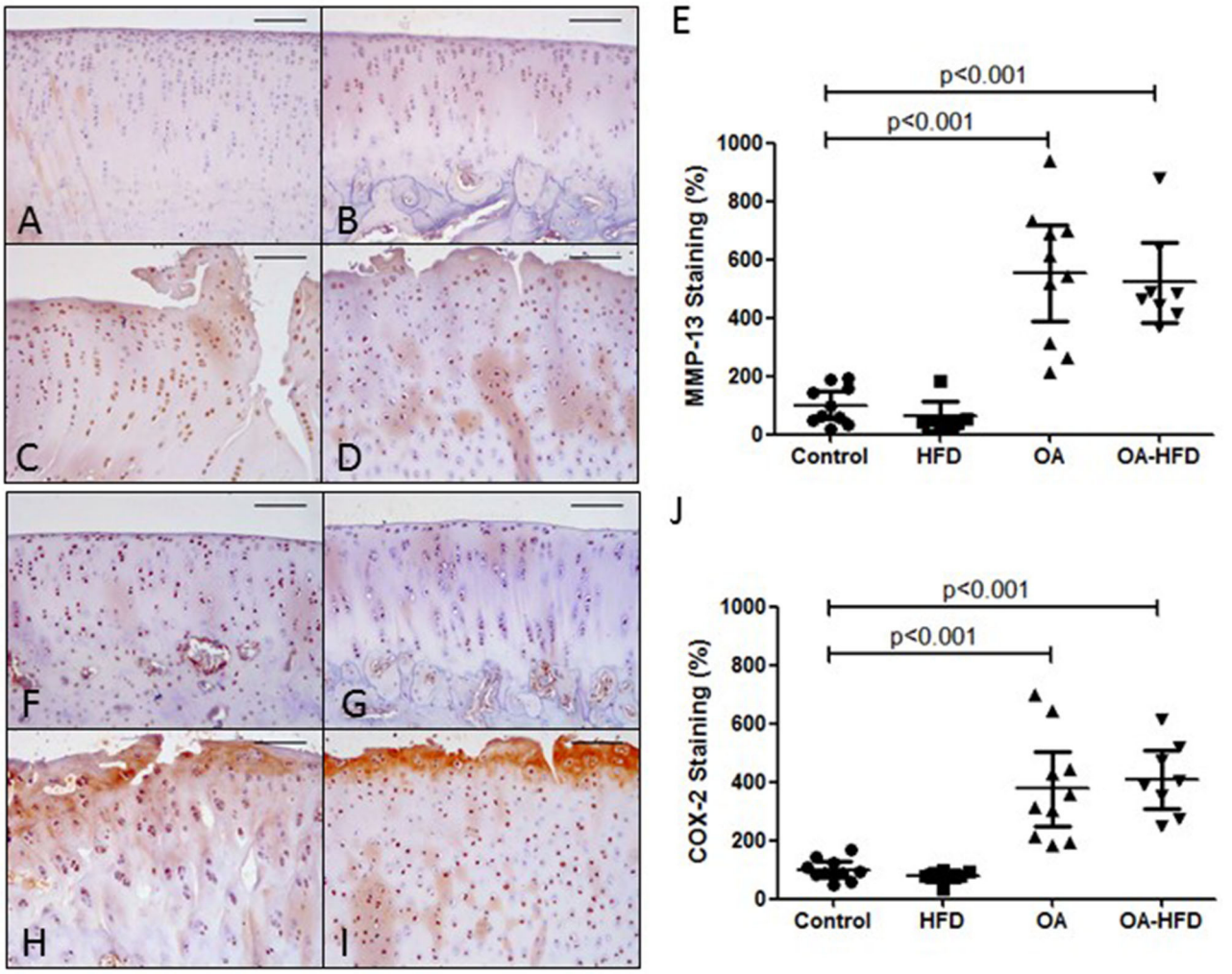

FIGURE 3 | Inflammatory markers in rabbit cartilage. Representative sections of MMP-13 immunohistochemical staining in control **(A)**, high fat diet (HFD) **(B)**, osteoarthritic (OA) **(C)**, and OA fed HFD (OA-HFD) **(D)** femur cartilage, and MMP-13 staining cuantification **(E)**. Representative sections of COX-2 immunohistochemcal staining in control **(F)**, HFD **(G)**, OA **(H)**, and OA-HFD **(I)** femur cartilage, and COX-2 staining cuantification **(J)**. Scale bar = 100 μm. Results were expressed as fold change comparing to healthy rabbit. Data are expressed as mean ± confidence interval; $n = 7–10$ animals per group.

Results found in the present study using a rabbit model for diet-induced hypercholesterolemia suggest that under normal cholesterol metabolism, external cholesterol intake does not affect cartilage structure. These data may reflect human hypercholesterolemia associated with bad eating habits in the absence of altered cholesterol metabolism more strictly than rodent models. Inconsistent results from epidemiological studies trying to link statin use and cholesterol levels with OA might be associated with the results showed in this study (9–12). In fact, several articles have described an anti-inflammatory and anti-catabolic effect of statins in cartilage *in vivo* and *in vitro*, which is independent of their cholesterol lowering properties (44–46). This protective effect has been associated with inhibition by statins of protein prenylation during cholesterol biosynthesis, which is able to regulate collagenase expression and cartilage breakdown in chondrocytes and cartilage explants (47, 48). This way, statins have been shown to reduce spontaneous cartilage damage in aging mice (49) and OA development in several animal models of the disease (44, 50–52). These data suggest that cholesterol biosynthesis, instead of cholesterol intake, may be relevant to cartilage metabolism, supporting the absence of a direct effect of HFD on cartilage damage progression in our study.

OxLDL may be produced in an inflammatory environment such as the arthritic joint (53), and they have been suggested to be a key player in the relationship between atherosclerosis, a clinical complication of metabolic syndrome, and OA (4). It has been previously demonstrated that human OA cartilage express LOX-1, indicating that this tissue is able to respond to oxLDL (22, 54). It has been reported that chondrocytes

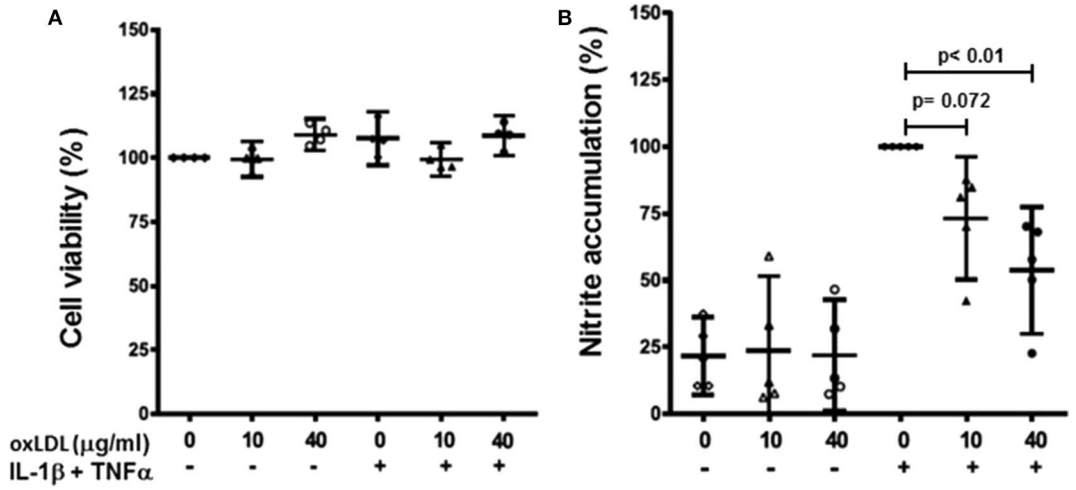

FIGURE 4 | Effect of oxLDL on human chondrocyte viability and nitric oxide production. Human chondrocytes were cultured in the presence or absence of IL-1β and TNFα with 10 and 40 μg/ml of oxLDL. Cell viability was measured using MTT assay **(A)** and nitric oxide production was evaluated as nitrite accumulation in culture medium after 72 h using Griess reaction **(B)**. Data are expressed as mean ± confidence interval. $n = 4$ experiments for cell viability, and $n = 5$ for nitrite accumulation (replicates employing different tissue donors).

treated with oxLDL showed decreased cell viability and increased senescence (22–24, 54), as well as hypertrophic-like changes (25). However, we observed that oxLDL stimulation did not affect human primary chondrocyte viability, and even decreased pro-inflammatory and catabolic mediator expression induced by IL-1β and TNFα. LOX-1 expression in chondrocytes has been shown to be disease-specific (22, 54) and LOX-1 levels increase in human OA cartilage (55). The expression of this receptor and, therefore, the ability of chondrocytes to respond to oxLDL could also be dependent on the inflammatory state. In the same sense, other studies have demonstrated similar protective roles of oxLDL by decreasing the pro-inflammatory state, in a model of macrophages activated with LPS (26). This fact could determine how chondrocytes behave after oxLDL treatment in the different studies where it has been assessed. In addition, it has been shown that the degree of oxidation may have different effects on cells. In osteoblasts, mild oxLDL did not decrease cell viability and even increased proliferation, while standard oxLDL at high concentrations decreased cell viability and proliferation (56). Regarding the degree of oxidation, in an independent study Zettler and collaborators showed that a stimulation of oxLDL increased cell proliferation in quiescent human and rabbit fibroblasts, through the up-regulation of regulatory proteins of the cell cycle (27). In contrast to other published works, our study uses LDL that have been mildly oxidized by reducing the time of Cu^{2+} treatment. This means that these oxLDL better reflect the joint environment, as it has been reported that, even in a high inflammatory environment like that observed in rheumatoid arthritis joints, LDL are mildly oxidized in synovial fluid (53). Therefore, different species and degree of LDL oxidation could account for the differences observed between experiments.

To our knowledge, this is the first study using mild oxLDL to treat human primary chondrocytes that are stimulated by pro-inflammatory cytokines associated to the OA environment. Our results suggest that the oxLDL that could be present in OA synovial fluid may not be detrimental for cartilage, and even have anti-inflammatory properties, which might be a compensatory response to counteract the inflammatory environment. In fact, activation of LXR in human OA chondrocytes has been reported to have anti-catabolic effects under inflammatory stimulation (57). In murine chondrocytes, LXR induction with agonists decreases ADAMTS4, MMP-2, and MMP-13 gene expression (58). OxLDL have been shown to have a biphasic effect on NF-κB activation, depending on cell type and incubation time. Oxysterols, which are thought to be at least partially responsible for the biological effect of oxLDL, are able to inhibit NF-κB activation by pro-inflammatory agents, probably through LXRα (59). Oxysterols trigger both LXRα and LXRβ activation, inducing their target genes, which control lipid synthesis and metabolism, such as ApoE, CETP, ABCA1 (ATP Binding Cassette A1) transporter, ABCG1 transporter, and LXRα (39). We tested whether mildly oxLDL could be triggering an anti-inflammatory response through LXRα, as the activation of this nuclear receptor is able to induce its own gene expression (39). According to our data, oxLDL presence was not able to modify the decrease in LXRα expression induced by cytokines. These data suggest that the anti-inflammatory effect of oxLDL could not be mediated by LXR activation. However, these effects, which have been attributed to the inhibition of NF-κB activation, could be exerted by other bioactive oxidized lipids that are present in oxLDL, such as oxidized phospholipids, oxidized poly unsaturated fatty acids or different aldehydic products (59). Therefore, further studies are needed in order to identify the oxLDL component that could drive a protective effect on OA progression.

In contrast to the *in vitro* results, pro-inflammatory gene expression in cartilage from rabbits was not reduced

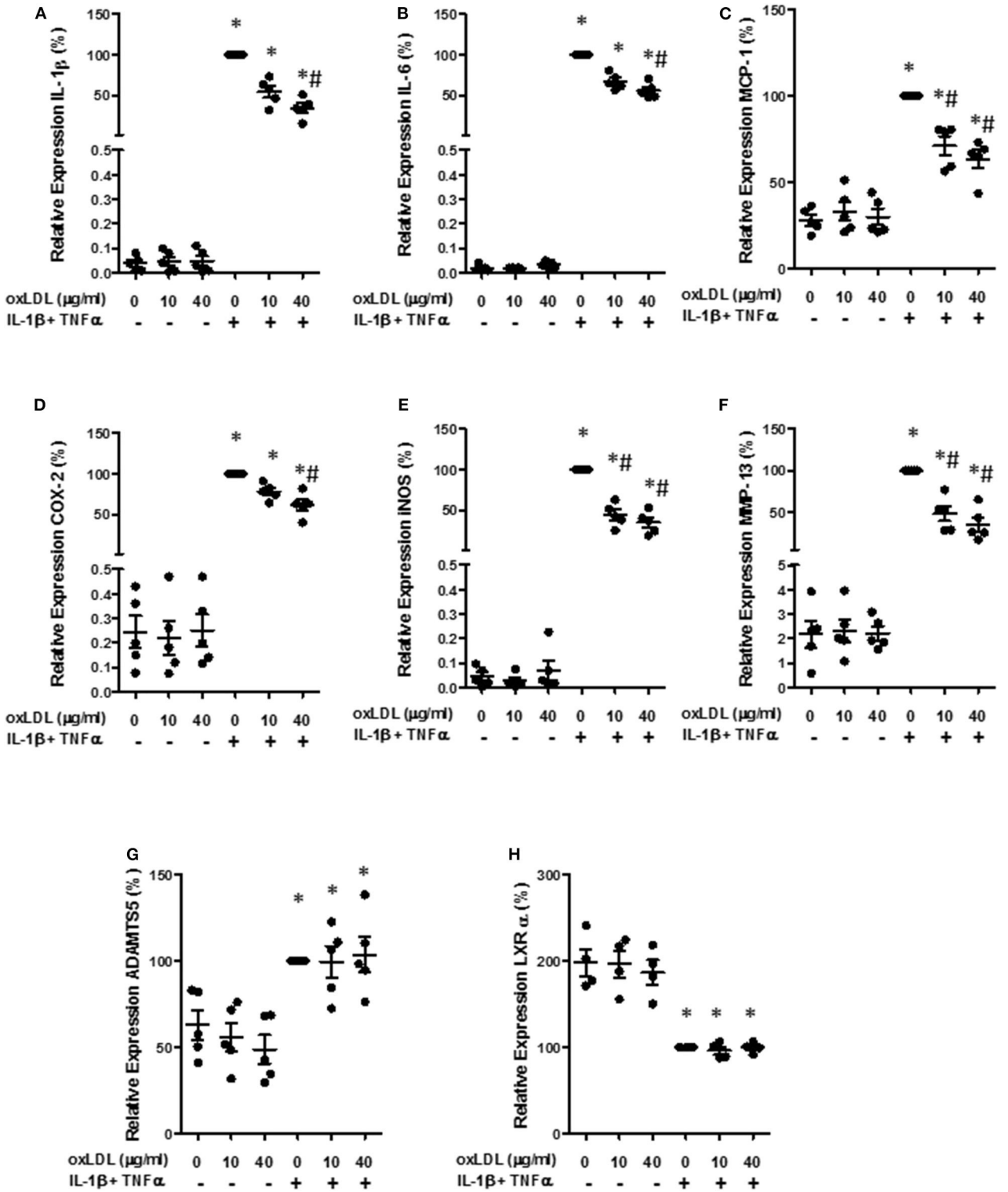

FIGURE 5 | Effect of oxLDL on inflammatory and catabolic mediator expression in human chondrocytes. Human chondrocytes were cultured in the presence of IL-1β and TNFα and 10 or 40 μg/ml of oxLDL, and IL-1β **(A)**, IL-6 **(B)**, MCP-1 **(C)**, COX-2 **(D)**, iNOS **(E)**, MMP-13 **(F)**, ADAMTS5 **(G)**, and LXRα **(H)** gene expression was analyzed using real time PCR. HPRT was used as endogenous control and results were calculated as a percentage over IL-1β and TNFα-stimulated control. Data are expressed as mean ± confidence interval. $^{*}p < 0.05$ vs. basal medium; $^{\#}p < 0.05$ vs. IL-1β+TNFα. $n = 4$–5 replicates, employing different donors.

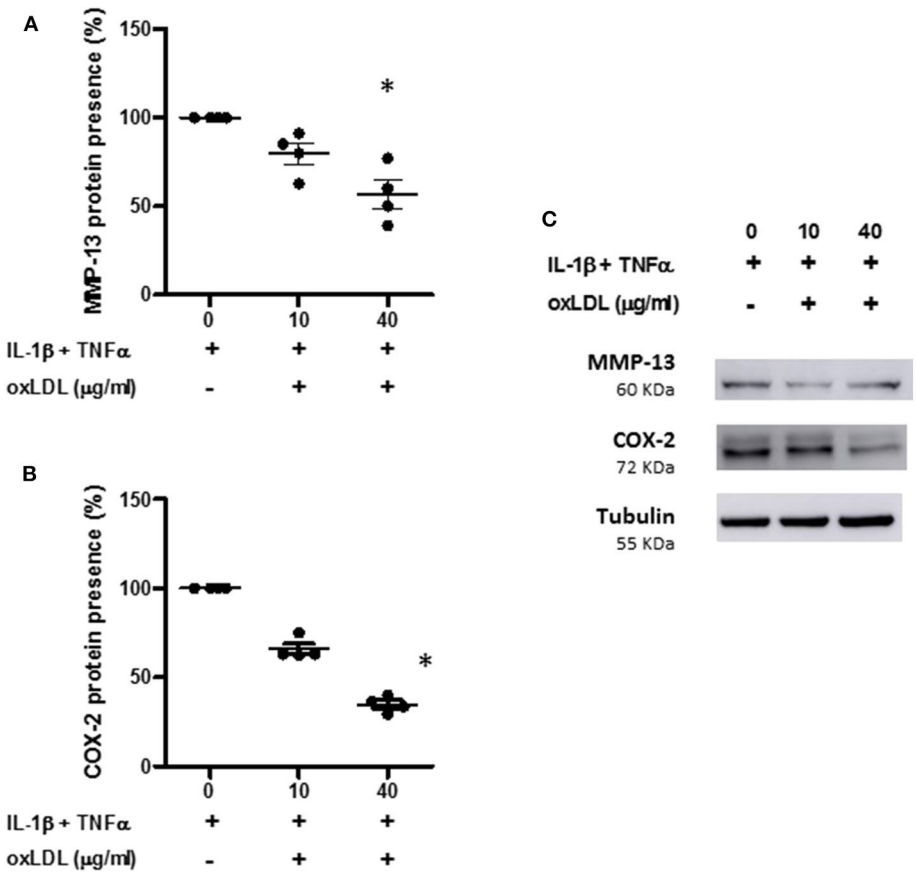

FIGURE 6 | Effect of oxLDL on MMP-13 and COX-2 protein presence in human chondrocytes. Human chondrocytes were cultured in the presence of IL-1β and TNFα and 10 or 40 μg/ml of oxLDL, and MMP-13 **(A)** and COX-2 **(B)** protein expression was analyzed by Western-blot experiments in total cell proteins. Tubulin was used as protein loading control and results were calculated as a percentage over IL-1β and TNFα-stimulated control. Data are expressed as mean ± confidence interval. $^*p < 0.05$ vs. IL-1β+TNFα. $n = 4$ replicates, employing different donors. A representative Western blot is shown in **(C)**.

by the increased cholesterol levels induced by HFD. Very recently, de Munter et al. observed that oxLDL uptake by synovial macrophages prevented other cell types from being activated by this lipid (60). These results suggest that, in our model, synovial macrophages could hoard the available oxLDL and impede their anti-inflammatory actions on cartilage.

In conclusion, the present study shows that, on an *in vivo* model of hypercholesterolemia in rabbits, cholesterol intake does not worsen cartilage degeneration and neither promotes structural and histopathological changes in rabbits with OA. These results are consistent with our *in vitro* model, where mildly-oxidized LDL do not show lipotoxic effects on human articular chondrocytes; conversely, they decrease the expression of pro-inflammatory and catabolic factors under inflammatory conditions. Therefore, these data suggest that cholesterol *per se* may not be deleterious for knee cartilage; instead, the effect of this molecule on OA progression might derive from secondary processes occurring during *de novo* biosynthesis, as suggested by previous studies using statins to inhibit this pathway.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Welfare Committee IIS-Fundacion Jimenez Diaz.

AUTHOR CONTRIBUTIONS

AV: design of the study, acquisition, analysis, interpretation of data, and drafting the article. AL-V: acquisition, analysis, interpretation of data, and drafting the article. AL: acquisition, analysis of data, literature revision, and data discussion. SP-B: acquisition of data. AL-R: literature revision and data discussion. GH-B and RL: conception, design of the study, interpretation of data, and drafting the article. All authors critically revised the manuscript for important intellectual property and approved the final version to be submitted.

FUNDING

This work was supported by research grants from the Instituto de Salud Carlos III (PI15/00340; PI18/00261; and PI16/00065), co-funded by Fondo Europeo de Desarrollo Regional (FEDER). Fundación Jiménez Díaz Biobank was funded by grants from the Instituto de Salud Carlos III- RETICS Biobanks Network, with FEDER founds. AV, AL-V, and AL were recipients of a research fellowship from the Fundación Conchita Rábago. SP-B and RL's work were funded by the Instituto de Salud Carlos III through a PFIS or a research staff stabilization program, respectively. The funders had no influence on the design of the study, collection, analysis, interpretation of the data, and writing the manuscript, or the decision to submit the manuscript for publication.

ACKNOWLEDGMENTS

The authors thank Dr. Juan Antonio Moreno (Renal and Vascular Research Laboratory, Fundación Jiménez Díaz, Madrid) for his help during oxLDL isolation.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 21 October 2020
doi: 10.3389/fmed.2020.572977

Emerging Technologies and Platforms for the Immunodetection of Multiple Biochemical Markers in Osteoarthritis Research and Therapy

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Biomarkers, especially biochemical markers, are important in osteoarthritis (OA) research, clinical trials, and drug development and have potential for more extensive use in therapeutic monitoring. However, they have not yet had any significant impact on disease diagnosis and follow-up in a clinical context. Nevertheless, the development of immunoassays for the detection and measurement of biochemical markers in OA research and therapy is an active area of research and development. The evaluation of biochemical markers representing low-grade inflammation or extracellular matrix turnover may permit OA prognosis and expedite the development of personalized treatment tailored to fit particular disease severities. However, currently detection methods have failed to overcome specific hurdles such as low biochemical marker concentrations, patient-specific variation, and limited utility of single biochemical markers for definitive characterization of disease status. These challenges require new and innovative approaches for development of detection and quantification systems that incorporate clinically relevant biochemical marker panels. Emerging platforms and technologies that are already on the way to implementation in routine diagnostics and monitoring of other diseases could potentially serve as good technological and strategic examples for better assessment of OA. State-of-the-art technologies such as advanced multiplex assays, enhanced immunoassays, and biosensors ensure simultaneous screening of a range of biochemical marker targets, the expansion of detection limits, low costs, and rapid analysis. This paper explores the implementation of such technologies in OA research and therapy. Application of novel immunoassay-based technologies may shed light on poorly understood mechanisms in disease pathogenesis and lead to the development of clinically relevant biochemical marker panels. More sensitive and specific biochemical

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Specialty section:
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

Received: *15 June 2020*
Accepted: *31 August 2020*
Published: *21 October 2020*

Citation:
Bernotiene E, Bagdonas E, Kirdaite G, Bernotas P, Kalvaityte U, Uzieliene I, Thudium CS, Hannula H, Lorite GS, Dvir-Ginzberg M, Guermazi A and Mobasheri A (2020) Emerging Technologies and Platforms for the Immunodetection of Multiple Biochemical Markers in Osteoarthritis Research and Therapy. Front. Med. 7:572977. doi: 10.3389/fmed.2020.572977

marker immunodetection will complement imaging biomarkers and ensure evidence-based comparisons of intervention efficacy. We discuss the challenges hindering the development, testing, and implementation of new OA biochemical marker assays utilizing emerging multiplexing technologies and biosensors.

Keywords: osteoarthritis (OA), biochemical marker, multiplexing technologies, biosensors, nanotechnology, immunodetection, magnetic resonance imaging (MRI)

INTRODUCTION

Osteoarthritis (OA) is the most common form of joint disease and a major cause of pain and chronic disability in older individuals (1). Although OA is primarily associated with aging in the human population (2), there are other key contributing risk factors, including obesity, gender, a history of joint trauma or repetitive use, genetics, heritable metabolic disorders, muscle weakness, underlying anatomical and orthopedic disorders (i.e., congenital hip dislocation), previous joint infection, crystal deposition (i.e., gout), previous rheumatoid arthritis (RA), and various disorders of bone turnover and blood clotting (3).

Biomarkers have been defined in the literature with considerable overlap (4). In general, biomarkers are any quantifiable measurement that can be objectively assessed as an indicator of a biological process, including anatomic, physiologic, biochemical, or molecular parameters. These markers can be associated not only with the presence and severity of specific diseases but also the effects of medical treatments and interventions. The use of laboratory-based biochemical markers in clinical settings is relatively new, and the best strategies to this application are still being developed at medical research level and as well at technological level (i.e., development of reliable detection methods).

Currently, there are no reliable, quantifiable, and easily measured biochemical markers capable of providing an earlier diagnosis of OA, inform on the prognosis of OA disease, and monitor responses to emerging therapeutic modalities (5). The evaluation of structural changes in articular damage via imaging biomarkers [as determined by radiograph or magnetic resonance imaging (MRI)] is the most frequently used in clinical trials to evaluate subject eligibility, and/or efficacy of intervention, supporting decision making in OA drug development by ascertaining treatment effects on joint structure (6). The European League Against Rheumatism (EULAR) has formulated a set of guidelines for imaging applications for the clinical management of OA in peripheral joints (7). However, radiography seems inadequate in the case of OA, as the utility for objective clinical picture evaluation is limited, and in most of the cases, there is no correlation between radiographical and clinical features, especially during early OA stages. This is an example of how radiographic imaging has hampered OA research endeavors (8, 9). MRI thanks to its high sensitivity in showing all involved joint tissues that are clinically relevant at a much earlier disease stage is gaining greater recognition. The importance of MRI in OA diagnosis and prognosis is increasingly emphasized (10–12). However, early detection of structural changes in the joint by MRI does not necessarily serve as an indicator of the existence of clinically defined OA, especially in the absence of symptoms. The potential outcome domains to assess for early OA include patient reported outcomes, features of clinical examination, objective measures of physical function and pain, levels of physical activity, imaging, and biochemical markers in body fluids.

In addition to imaging biomarkers, biochemical markers of joint tissue turnover have the capacity to reflect disease-relevant biological activity and provide useful diagnostic and therapeutic information, enabling a more rational and personalized approach to healthcare management (13). Expert consensus groups have proposed a generally accepted classification of OA biochemical markers according to the disease pathogenesis (14, 15). They include markers of cartilage, bone, and synovial metabolism, which comprise several collagenous proteins, their epitopes and cleavage peptides, various enzymes and non-collagenous proteins, as well as markers of low-grade inflammation: cytokines, chemokines, lipid mediators, and other biochemicals (15). The ratio between the synthesis and breakdown of both articular cartilage and bone can provide an insight into the underlying pathological processes involved in OA, albeit this process may be detected only in late stages of disease pathogenesis. The importance of inflammatory response in OA has also been increasingly highlighted during the past decade, shifting the focus of research investigations on the correlation between biochemical markers of "low-grade inflammation" and disease progression, especially in the context of emerging inflammatory endotypes and phenotypes of OA (16).

The complex evaluation of biochemical markers of extracellular matrix (ECM) turnover, low-grade inflammation, and other biological processes may lead to more specific evaluation of the catabolic and inflammatory aspects of OA. However, most of the studies carried out with biochemical markers to date have focused on late stages of the disease in humans or animal models. Studies in early stages of OA are rare due to the lack of biochemical markers that permit detection of early OA stages.

Early OA refers to the earliest disease stage characterized by emerging clinical symptoms. Early OA does not have a mutually agreed-upon definition, but a group of specialists have proposed a draft classification based on the existence of patient-reported symptoms, clinical examination findings, and minimal to no radiographic signs (17). The definition of early OA is still evolving, and there are ongoing efforts to move toward a consensus definition. Although there is no consensus on an internationally accepted classification, it is

generally agreed that identification of the early stages would enable the development of new therapeutics, allowing more targeted and efficacious clinical intervention. However, this requires establishing a clear link between molecular changes in the preradiographic stages, before the onset of clinical and radiographic manifestation of OA (18). Biomarkers indicative of early disease processes and the criteria by which OA is stratified into "early clinical" and "early OA" stages require validation by extensive longitudinal studies that take into account different risk factors, patient subgroups, and types of affected joints (18).

At the present time, none of the currently available biochemical markers are sufficiently capable of discriminating OA diagnosis or prognosis in individuals or provide a consistent outcome measure in OA clinical trials (19). The need for stratification of distinct OA subtypes (the so-called clinical phenotypes) has been recently highlighted (20). This relates to the need for gaining a better understanding of the pathogenesis of OA, the identification of molecular endotypes, and the prospect of developing personalized treatments. Hence, stratifying OA biochemical markers for the detection of molecular endotypes and the enhanced definition of the clinical phenotypes will inevitably expedite the development of personalized OA treatment approaches, as proposed recently (11, 21, 22).

The presence of inflammation has been recognized as a leading component of different OA subtypes (23). Contrast-enhanced MRI of pre-radiographic OA joints revealed that presence of moderate synovitis is very common in knees with clinical OA and is also commonly found in joints without exhibiting concomitant joint effusion (24). Systemic inflammatory mediators, adipokines, released by the adipose tissue, are also involved in the pathogenesis of OA (25), which suggests the convergence, overlap, and interaction of these phenotypes, especially in older individuals with multiple comorbidities.

The inability to assess changes in the joint in the early stages of OA is a major obstacle to making further progress in OA diagnosis and prognosis. Moreover, this stagnancy in biochemical marker development is hampering drug development and thwarting efforts to identify the disease early and mitigate its huge socioeconomic impacts. Therefore, there is an acute need to establish biochemical marker panels or methods for efficient detection to facilitate earlier diagnosis of OA, inform OA prognosis, and monitor therapeutic efficacy of the disease (16, 26). The development of biochemical marker immunoassays has been an ongoing area of activity in OA research and clinical development, but it is still far from being incorporated into a unified framework for disease characterization.

The present article reviews the relevant literature and outlines the main challenges faced by the scientific, medical, and engineering communities in the establishment of the relationship between biochemical markers and OA, as well as the development and design of emerging immunodetection methods such as multiplexing technologies, biosensors, and nanotechnology platforms.

BIOCHEMICAL MARKERS FOR OA DIAGNOSIS AND PROGNOSIS

The currently accepted classification criteria for OA biochemical markers developed by the OA Biomarkers Network has assigned biomarkers into five categories, including burden of disease, investigative, prognostic, efficacy of intervention, diagnostic, and safety (BIPEDs) (27, 28). This categorization provides a framework to describe the potential application of biochemical markers as tools for the early identification of the disease, differentiation of patients based on the extent of disease severity, pre-symptomatic identification of individuals with the disease, or a clinical endpoint used to determine the efficacy of treatment. Biochemical marker detection in serum or urine samples is the least invasive procedure, therefore standing in the first line of clinical interest, while the synovial fluid (SF) biochemical markers are expected to be more reflecting local processes in the joint but have gained more traction in recent years with the increased developmental focus on intra-articular therapies. Although a series of different biochemical markers were assessed for their potential utility, only minimal success was achieved in their clinical validation, highlighting the need for new biochemical markers, representative of joint tissue damage or even linked to particular joints (22, 29). Nevertheless, the major challenge for OA drug development remains the lack of biochemical markers indicating the efficacy of treatment and OA progression (30).

The establishment of normal range intervals of well-phenotyped age-matched controls for 18 separate biochemical markers that were used by the Foundation for the National Institutes of Health/Osteoarthritis Research Society International (FNIH/OARSI) consortium project was an important step in the development of biochemical marker for the diagnosis of OA (31). However, even though some of these biochemical markers showed different distributions between OA subjects and non-OA controls, the overlap of concentration values remains a problem for diagnostic and prognostic applications, as well as the differences between reference intervals based on race, gender, and age, which might necessitate further research into factors that might affect biochemical marker concentrations (31). The variables in baseline levels between individuals pose a significant challenge for biochemical marker development, especially since OA pathogenesis does not embody a singular etiological trajectory, as previously indicated (i.e., inflammatory, bone, metabolic, or age).

BIOCHEMICAL MARKERS OF CARTILAGE TURNOVER IN OA

Most OA biochemical markers are dedicated to characterizing cartilage turnover. The most commonly investigated biochemical markers includes the following: ECM degradation—CTX-II, Coll2-1, C2C, C2M, Coll2-1NO2, cartilage oligomeric matrix protein (COMP), aggrecan epitopes (ARGS, TEGE, FFGV), fibulin-3 epitopes (Fib3-1, Fib3-2, Fib3-3), etc.; ECM synthesis—PIIANP, PIIBNP, CPII, CS846, and many others (32) (**Figure 1**).

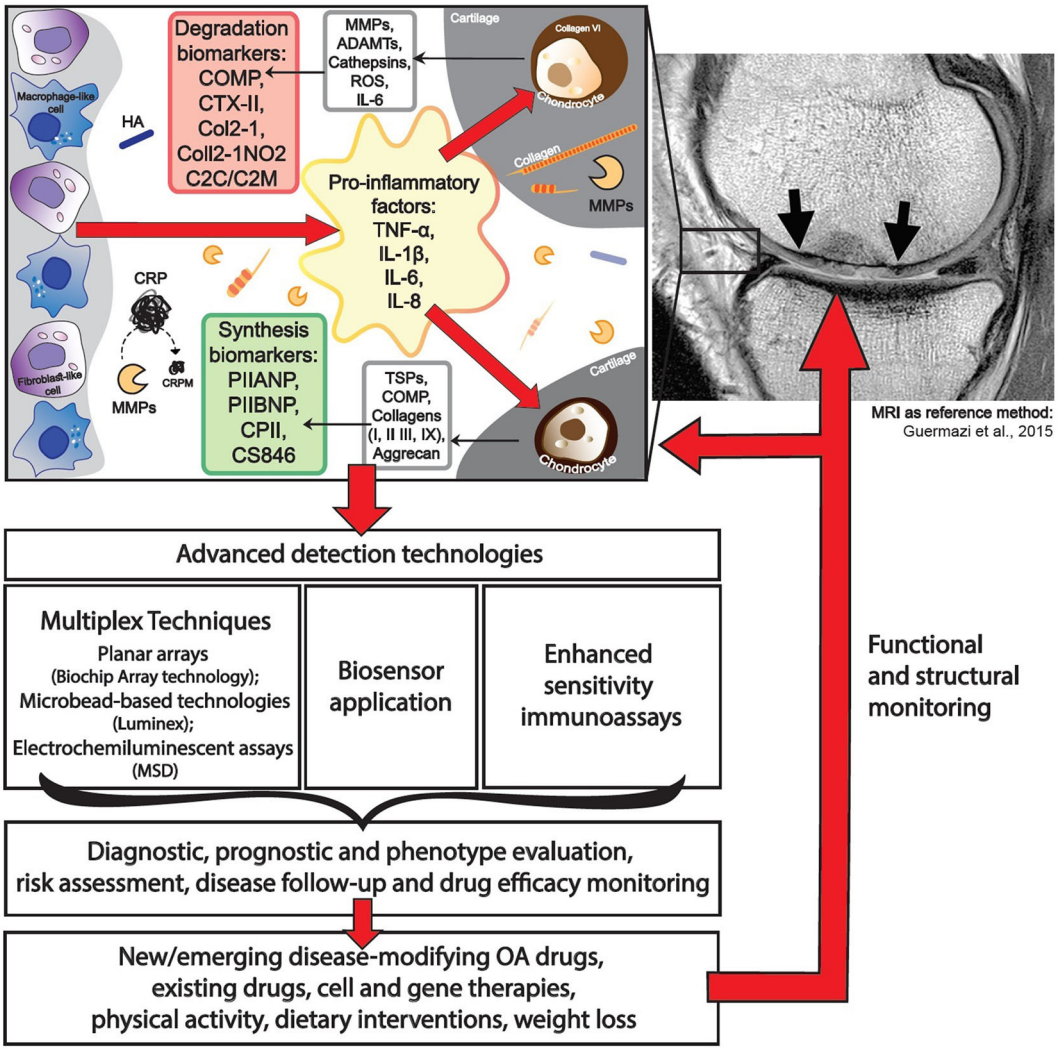

FIGURE 1 | Application of advanced technologies for immunodetection of osteoarthritis biomarkers.

Some of those cartilage metabolism biochemical markers have gained recognition in the field. For instance, urinary CTX-II (C-telopeptide fragments of type II collagen) is one of the better-known OA biochemical markers that has achieved a superior predictive profile when compared to others (33). Both urine and SF CTX-II were found to be associated with radiographic severity (34), while urine CTX-II was associated with pain in patients with early OA (35).

Another potential biochemical marker widely reported in the OA literature is COMP. Serum COMP is a biochemical marker that has been used in numerous studies because of its ability to distinguish between healthy subjects and OA patients. Furthermore, in the case of COMP, possible prognostic capabilities have been observed (36). Although some studies reported conflicting results, a meta-analysis showed that these biochemical markers (i.e., COMP and CTX-II) could be effective for OA diagnosis and prognosis of progression and differentiation between healthy groups and individuals affected with OA (37). Variation in COMP levels seems more pronounced in SF samples, as compared to serum levels (36). In addition, levels of COMP in SF showed a strong correlation with OA severity, while only a weak inverse correlation to serum COMP levels was found, reinforcing that SF samples are strongly reflective of COMP as an OA biochemical marker (36). Levels of COMP in SF, specifically intact forms of the molecule, were found to be higher in OA patients than in those suffering from other articular disorders, like RA, reactive arthritis, or acute trauma (38). As such, intact COMP appears to better associate with slow progressing and chronic joint pathogenesis, rather than acute proteolytic processes that are associated with severe inflammation in other arthritides, which cause COMP degradation and cleavage. This also highlights the fact that neo-epitopes from the same protein could be generated in diverse manners and thus serve as indicators of various pathological

mechanisms related to distinct diseases or disease subtypes (39). Such phenomena should be considered when designing OA analysis platforms, as appropriate antibody selection (with respect to targeted regions) could potentially be linked to better pathogenetic profile validation.

BIOCHEMICAL MARKERS OF LOW-GRADE INFLAMMATION IN OA

The most recent definition of OA describes it as disease characterized by low-grade inflammation, distinguishing it from other inflammatory joint diseases. Identification of biochemical markers of low-grade inflammation may identify subtypes of OA, thus contributing to the choice of treatment strategies, particularly with non-steroidal anti-inflammatory drugs, which currently remains among the primary options from the scarce choice of clinically relevant therapies for OA. Notable inflammation-related biochemical markers of OA include CRP, tumor necrosis factor-α (TNF-α), IL-6, and IL-1β, recurring in multiple study reports and systematic reviews (15, 22, 29). The concentrations of these biochemical markers are low, especially compared with other inflammatory diseases of the joint, with TNF-α, IL-6, and IL-1β levels detected at picogram-scale quantities (40), making it challenging to detect their fluctuations in the circulation. For example, CRP levels are consistently elevated in OA patients, with modest variations in its levels among various studies (41). CRP levels were shown to differ between OA patients and non-OA controls and correlated with symptoms of OA, such as pain and function loss; however, these were not found to reflect radiographic findings such as joint space narrowing and KL scores (41). Measurement of hsCRP is a routine laboratory test and the gold standard for evaluation of inflammation (15), making it a relatively translational and accessible transition to OA diagnosis. It is important to note that more attention should be given to known confounding factors of CRP such as body mass index (BMI), race, and gender when interpreting data (42–44). Distinct from full-length CRP are fragments derived from CRP, such as CRPM, which are generated by endopeptidases, such as matrix metalloproteinases (MMPs), which are activated during OA. Indeed, CRPM has been evaluated in RA (45), axial spondyloarthritis (46), and OA (47–49) as a marker of inflammation. The Rotterdam cohort study revealed for the first time that CRPM predicts the risk of OA progression independently of the established biochemical markers uCTX-II and COMP (50).

Elevated levels of IL-1β, IL-5, IL-6, IL-10, IL-13, and TNF-α have been observed in the plasma of patients with knee OA, as compared to SF samples showing unchanged levels (27). Authors of the study attribute either the fluctuation in cytokines in the circulation to the systemic nature of OA or the enhanced permeability of the synovial membrane (40). Similarly, serum TNF-α concentrations were shown to be predictive for radiographic knee OA progression (41) and together with IL-6, were indicative of cartilage loss and joint space narrowing (51). On the other hand, increased SF IL-6, IL-8, and TNF-α levels were associated with pain during movement, while only TNF-α correlated with pain at rest (34).

The selection of inflammatory biochemical markers for OA assessment remains challenging and inconsistent and depends on the purpose, hypothesis, and concept of each study. As there is no consensus on the parameters for the evaluation of low-grade inflammatory activity in OA, a combination of inflammatory and cartilage turnover biochemical markers, in addition to imaging biomarkers, may be the best choice to characterize OA (see section Choice of the Reference Method for Biochemical Marker Detection).

EMERGING EXPLORATORY AND INVESTIGATIVE BIOCHEMICAL MARKERS OF OA: FROM MICE TO LARGE ANIMAL MODELS OF OA

As previously stated, there is a lack of novel biochemical markers to predict OA clinical status. Moreover, fewer biochemical marker candidates enable clinical decision making about the efficacy of various therapies. The current situation requires pairing between investigative knowledge regarding the disease pathogenesis and the utility of this knowledge to develop novel biochemical markers and therapies.

Searching the current literature for the following phrases including "investigative biomarkers" "osteoarthritis," and "mice" showed 48 results. Among some of the reports, higher serum levels of FGF21 were displayed in an ACL model of mice, which appeared to be markedly higher in adipsin-deficient mice (52). Another interesting therapeutic target is transglutaminase (TG2), which was shown in various preclinical models to mediate chondrocyte hypertrophy and interleukin-1-induced calcification (53–56). As such, TG2 protein levels in synovial fluid protein have been reported to correlate with the histological grade of OA (57). As the activity of TG2 may be altered in non-OA-related synovitis, better characterization of its levels and bioavailability are needed to assess its potential utility as an OA biochemical marker for human subjects (58). MMP3, an enzyme that degrades proteoglycans and collagens, was associated with early structural OA changes in STR/ort mice (59), which are consistent with human studies showing that its levels correlate with joint width narrowing (60). Another catabolic matrix modifier, cathepsin B, was shown to display increased activity in synovial fluid in various studies (61, 62) and could be paired with imaging of the joint using activity-based probes. Mechanistically, cathepsin B is linked with Sirt1 cleavage and inactivation (63), as well as collagen degradation in cartilage ECM (64). Recently, fragments of Sirt1 were shown to be predictive of chondrosenescence and OA severity using an enzyme-linked immunosorbent assay (ELISA)-based method (65), linking the loss of SIRT1 activity in OA cartilage to its emergence in serum. Lipid profiles in plasma are also reported as biochemical markers of pain and cartilage destruction using the DMM mice models (66). In particular, Pousinis and colleagues, reported six lipid species that increased

during posttraumatic OA, identified as cholesterol esters-CE(18:2), CE(20:4), CE(22:6), phosphocholine-PC(18:0/18:2), PC(38:7), and sphingomyelins-SM(d34:1) (66). Phospholipid species were also shown to be increased in human OA plasma (67, 68), indicating that these lipid species may undergo aberrant biosynthesis during OA pathogenesis. The study of lipid mediators as potential marker of OA is an important and promising area of research that highlights the potential for using lipidomics in future studies. However, at the present time, there are no standardized low-cost platforms and easy-to-perform assays for identifying a wide-range of lipid biomarkers.

The use of mouse models for biomarker screening is often advantageous over large animals due to its capacity to undergo skeletal maturation or develop OA more rapidly than larger animals. Moreover, the abundance of genetic mouse models for the validation of pathways and biomarkers related to OA pathophysiology make them good cost-efficient models for initial screening. Indeed, there are many novel findings related to biomarker discovery in mouse models as compared to those available in large animals. Conversely, the use of large animals is justified because they are translational models due to their physiological and anatomical biomechanical similarity to load-bearing human joints and is documented mostly in dogs, sheep, and horses, which exhibit naturally occurring OA (69). When assessing dogs undergoing surgical induction of OA, a significant decrease in TIMP-2 in SF and serum is reported, while MMP-2 was elevated in SF during the progression of OA (70). Coll2-1 and Coll2-1 NO_2 were correlated with OA changes in the canine ACLT model (71), consistent with the decrease in these serum fragments after HA intra-articular administration to OA patients in a separate study (72).

Assessment of spatial changes in serum proinflammatory cytokines and ECM-related biomarkers was reported for a 4-year training period of posttraumatic OA racehorses. While COMP and CTX-II showed an early increase in serum already at year 2 of training, IL-1β, TNF-α, and IL-6 were mostly elevated at the fourth year of training (73). These data are in line with the approximate 4-fold increase reported for lame training horses vs. non-lame training horses in a separate study using ELISA for the detection of COMP neoepitope (74). NMR analysis of synovial fluid from sheep subjected to anterior cruciate ligament (ACL) reconstruction injury vs sham, revealed significant changes in the following metabolites: isobutyrate, glucose, hydroxyproline, asparagine, serine, and uridine (75). Another study examining the synovial fluid of sheep with OA of the temporomandibular joint detected elevated levels of the active MMP2 in pathogenic SF samples (76). Overall, large animals screened for OA biomarkers exhibit increased levels of matrix-degrading enzymes and ECM fragments (MMP2, COMP, and CTXII), associated with early stages of the disease, while cytokines emerge in the circulation at latter stages, which resembles some of the findings reported for human cohorts (77). In summary, changes in biomarkers are more readily apparent in the synovial fluid of large animals compared to that in serum, as is the case in humans.

Altogether, these biochemical markers, and possibly more, discovered primarily in animal models could contribute to our understanding of OA pathogenesis, become potent drug targets, and serve as potential biochemical markers of OA. The capacity to use genetic mice models as affirmation of a biochemical marker is particularly an important approach for validating candidate investigative and exploratory biochemical markers to associate them with OA and its accompanied joint tissue damage.

CONVENTIONAL IMMUNOASSAYS AND EMERGING MULTIPLEXING IMMUNODETECTION-BASED TECHNOLOGIES

Disease prediction by biochemical marker analysis is one of the most promising research areas nowadays. By far, the most widely applied method for biochemical marker quantification is ELISA, which are frequently used to quantify antibodies, peptides, proteins, and hormones in the plasma, serum, urea, or supernatants. ELISA is a highly sensitive, specific, and accurate method. However, ELISAs can only measure a single molecule of interest at a time, which is a major challenge for simultaneous quantification of multiple antigens in huge cohorts of patient samples, in terms of both workload and sample amount. The detection of a single biochemical marker in the serum, intracranial fluid, synovial fluid, urine, or other bodily fluids is not a reliable prognostic or diagnostic indicator in the vast majority of diseases. Single analyte assays are widely applied in protein measurements, while running at least a couple of experiments in parallel increases the time consumption and risk for an error to occur, and a larger volume of analytical samples are needed (78, 79).

Analysis of multiple biochemical marker molecules in every patient could potentially shed better light on disease characteristics and status. However, the measurement of multiple parameters in a single precious sample from a patient in a single run has challenged the scientific and medical community and led to the development and design of assays utilizing multiplexing technologies. Multiplex immunoassays have gained traction in recent years and have already been successfully used in diagnostic tests and cohort screening. Although the majority of developed platforms is still under investigation in the preclinical phase, these techniques can lead to a strong technological revolution in the future as reliable and cost-efficient systems for diagnosis, disease prediction, and monitoring. Two basic assay formats have been developed to facilitate simultaneous detection of multiple analytes: planar arrays (i.e., biochip array technology) and bead-based arrays (e.g., Luminex Technology), and the applications of these assays has been extensively reviewed (78, 80).

The basic principles of biochip planar arrays based on ligand-binding assays were first described in the 1980s (81). Since then, the technology has advanced and attracted the attention from biochemical marker research to clinical diagnoses and prognoses. In antibody microarray systems, large amounts of different antibodies are printed on the planar microarray surface where multiplexed affinity reagents are used to detect and quantify proteins in complex with biological samples (82). In addition to the advantage of detecting multiple biochemical markers, antibody microarrays assays are high throughput

and highly sensitive by using small sample volumes and delivering fast results (<24 h from sample preparation to data analysis). Despite the remaining challenges associated with multiplex immunoassay platforms, such as cross-reactivity to off-target biochemical markers and their clinical applicability, there are several systems commercially available (83). For instance, the company MesoScale Discovery® (MSD) offers a multiplex immunoassay platform built upon planar array technology utilizing electrochemiluminescence detection (83, 84). The platform is made in a 96-well format with integrated electrodes to deliver an electric impulse to each well and specific antibodies spotted at the bottom. The detection reagent contains electrochemiluminescent labels that bind to the detection antibody and are only activated by an electric charge, eliminating any background interference by non-specific label detection. This kind of detection system was validated and the sensitivity compared favorably to well-validated single-plex ELISAs (84).

Among planar-array-based technologies, bead-based or cytometric bead array (CBA) platform has also been developed by combining ELISA-based technology with flow cytometry. Beads of different sizes or colors are used for those multiplexed immunoassays (85). Initially, the assay was developed for conventional flow cytometers, but the design of a CBA analyzer by Luminex Company (Luminex Corporation, Austin, TX, USA) enabled streamlining of the workflow and data analysis, making it more accessible. Luminex technology has been adopted by many leading bioscience companies, and various biochemical marker panels have been developed. The Luminex xMAP (multianalyte profiling) technology is a bead-based flow cytometric platform for multiplex analysis. It uses magnetic or polystyrene particles, incorporating two fluorophores in 100 different ratios, giving the possibility to detect 100 analytes (86). The technology offers a greater reproducibility, as compared to the planar arrays, and sensitivity comparable to that of ELISA (87, 88).

The development of a multiplex assay requires overcoming many difficulties, including insufficient detection limits and standardization of the biochemical marker panels for diagnosis and treatment monitoring (89). At the research level, non-traditional methods in clinical settings have been considered as novel strategies to enable high sensitivity and simultaneous detection of a multitude of biochemical markers. Proximity ligation assay (PLA) and, more recently, the proximity extension assay (PEA) have been reported as a sensitive and selective immunoassay method for protein quantification using a pair of DNA oligonucleotides linked to antibodies against the target molecule (90). A multiplexed platform containing 96-plex PEA-based immunoassay was developed to achieve simultaneous measurement of 92 biochemical markers related to cancer, and its performance was evaluated in comparison with benchmark bead-based immunoassays (91, 92). Very recently, a digital PLA (dPLA) has been proposed to simultaneously detect Gram-negative and Gram-positive bacterial DNA as well as the inflammatory biochemical markers IL-6 and TNF-α from patient samples (93). A major advantage of this innovative platform is the use of a digital amplification method, which enables the quantification of very small changes in concentration of the biochemical markers (i.e., subfemtomolar resolution for protein targets). As an outcome, those analyses showed that temporal changes in several biochemical markers, rather than the absolute concentrations, are reliable predictors of patient outcomes.

Surface-enhanced Raman scattering (SERS) has also been considered as a potential detection method of multiple biochemical makers. SERS combines nanostructures made of noble metals (e.g., silver and gold) with Raman spectroscopy, providing a dramatic increase in the characteristic molecular fingerprint offered by Raman spectrum (94). Particular attention has been given to SERS immunoassays, which ensure high sensitivity by SERS detection and high specificity from the antigen–antibody binding. Furthermore, the combination of nanotags and characteristic spectrum of the target molecule makes SERS detection a very attractive strategy to achieve multiplexing as recently demonstrated (94, 95). Photon-upconversion nanoparticles have been used to develop a microtiter plate immunoassay capable of detecting PSA at 1.2 pg/ml, which is 10 times more sensitive than commercial ELISA and covers a dynamic range of three orders of magnitude (96). Such technology has the potential to develop into a new generation of digital immunoassays.

A multiplexed protein detection technology resembling an immunodetection method was developed by SomaLogic Company. The SOMAScan assay utilizes nucleic acid ligands with protein-like side chains—aptamers (SOMAmers) (97). The principle resembles antibody–antigen interaction detection, with affinities comparable and often superior to those of antibodies. SOMAmers have distinct advantages for such applications, including selection conditions not tied to *in vivo* immunization, thermal and chemical stability, smaller size, ease of manufacturing, reliable supply, and full control of lot-to-lot variability (98).

Development of the multiplexed detection assays should inevitably focus on the selection of the detection system/technology and reagents that are the key factors in obtaining sensitivity and specificity. For example, four high-sensitivity cytokine multiplex assays on a Luminex or electrochemiluminescence (MSD) platform were evaluated for their ability to detect circulating concentrations of 13 cytokines as well as for laboratory and lot variability (99). The study showed that no single multiplex panel detected all cytokines, and there were highly significant differences between laboratories and/or lots with all kits. The detection of single IL-6 cytokine was assessed by means of four different immunoassays/platforms (100). IL-6 was measurable in all plasma samples by MSD, while 35, 1, and 4.3% of samples were out of range when measured by Luminex assay, Ultrasensitive Luminex assay (Invitrogen), and High-Sensitivity ELISA (R&D), respectively. Again, it emphasizes the importance of the reagents used for the detection, not only the detection platform.

APPLICATION OF MULTIPLEXING TECHNOLOGIES IN DIAGNOSTICS OF VARIOUS DISEASES

This section describes the lessons from research experiences and challenges observed in various diseases applying multiplexing

and diverse innovative technologies for biochemical marker identification and analysis. One of the main challenges is the choice of the most relevant biochemical markers for the prediction and monitoring of a disease. For instance, automated and multiplex biochemical marker assay has been developed to reliably distinguish between RA patients and healthy individuals (101). This study included serum samples from 120 patients. The multiplexed assay was considered to be a relevant and specific method to diagnose RA by using a biochemical marker panel with three biochemical markers yielding a sensitivity of 84.2% and a specificity of 93.8% and using four biochemical markers a sensitivity of 59.2% and a specificity of 96.3%. In another study, quantification of 12 biochemical markers was performed, utilizing a multiplexed sandwich immunoassay in three panels for RA diagnosis (102). This study has demonstrated that the 12 individually selected biochemical markers exhibit a high level of precision with minimal cross-reactivity and interference by substances commonly seen in RA patients. Interestingly, among these two multiplex panels, only two biochemical markers, IL-6 and TNF-α, were included in both studies, while analysis of other biochemical markers generated inconsistent results. Despite that, both studies conclude that their methods provide highly reproducible results, are effective, and can even stratify RA patients into clinically relevant subtypes (102, 103).

Additional analytical capacity has been introduced to the RA biochemical marker field when the recently developed multibiomarker disease activity (MBDA) test was validated in a clinical study on RA patients. The MBDA scores (from 1 to 100) were capable of monitoring changes in disease activity over time and effectively discriminating clinical responders from non-responders in diverse RA cohorts (104). This scoring system is most commonly used to assess the response of biological therapy in RA, however with varying success and utility for the process of tapering and the ceasing of disease-modifying anti-rheumatic drugs (DMARDs). In the AMPLE study of the biological agents Abatacept and Adalimumab, the MBDA score did not reflect clinical disease activity (105), while in the *post hoc* analysis of three cohort studies on Rituximab, the same score was confirmed to represent the clinical response to treatment in RA patients (106). Currently, a prospective, a randomized study of the Vectra DA MBDA blood test is under investigation for Food and Drug Administration (FDA) authorization (107) in diverse RA cohorts.

Disease phenotyping in multiple sclerosis (MS) served as another example of biochemical marker selection. Cases of different subtypes of MS were compared by simultaneous analysis of serum IL-1β, IL-6, IL-8, and TNF-α levels via comparison of two commercially available multiplex platforms (i.e., Luminex-xMAP and Meso Scale Discovery) (108). Although the presence of these biochemical markers was detected in all the subtypes of MS, the levels varied. The significant increase in IL-6 and IL-8 in all the MS subtypes was determined, but a significant increase in TNF-α was observed only in one of the subtypes, as compared to the controls. In addition to the biochemical markers that can be used to diagnose MS, this parameter could be included into the biochemical marker panel for specification of the MS subtype.

Protein array chip immunotechnology has been applied in osteoporosis diagnosis as an alternative method for single biomarker concentration evaluations (109). Individual biomarker assays in osteoporosis, similarly to other multimodal diseases, fail to describe such complex diseases. Single biomarker concentration measurement is currently used to evaluate the progression of osteoporosis (OP) as well as the measurement of bone mineral density (BMD) by dual-energy X-ray absorptiometry (110). The same Immunological Multiparameter Chip Technology (IMPACT) platform that has been applied in RA diagnosis (101) is used to simultaneously measure OP biomarkers CTX-I, procollagen type I N-terminal propeptide (PINP), osteocalcin, and intact parathyroid hormone (PTH). The choice to measure these specific biomarkers was made due to their high sensitivity and suitability to evaluate bone resorption and formation changes. Although the results demonstrated similar analytical performance characteristics to single biomarker assays with an increased sensitivity, the necessity for larger numbers of OP patients as well as inclusion of more biomarkers associated with bone metabolism change indicators in the panel is further needed (109, 111).

Biochemical marker analysis is also becoming a crucial step in cancer diagnostic and predictive/prognostic characterization of the disease stages. The 96-plex PEA immunoassay has been developed and shown to be both sensitive and specific, as well as more scalable, in comparison to traditional immunoassays (91). Such PEA immunoassay has been applied for multiplex analysis of patients with colorectal cancer, which determined the significant correlation of the expression of CEA, IL-8, and prolactin with specific colorectal cancer stage (92). The identical set of 1 μl of plasma samples from patients with colorectal cancer or unaffected controls was run for both assays. Similar quantitative expression patterns were determined for 13 plasma antigens common to both platforms, while the potential efficacy of proximity extension assay was endorsed, as it only demonstrated that the expression of CEA, IL-8, and prolactin are significantly correlated with colorectal cancer stage. Later, another PEA platform for an expanded panel of 275 biochemical markers has been developed and produced a 12 biochemical marker signature algorithm that was comparable to a clinically approved blood-based screening test (112).

Antibody array systems have also been applied in cancer biomarker screening, where simultaneous detection of multiple breast cancer and ovarian cancer biochemical markers, relevant to clinical diagnosis was achieved (113, 114).

It is evident that biochemical marker research in cancer is highly advanced compared to other disease areas, and these studies may serve as good examples of simplified ways for sensitive and specific detection of different cancer types, for instance breast, colorectal, etc. Such state-of-the art technologies as multiplexing a combination of biochemical markers and implementation of biosensors save time and resources for the prediction of treatment response. Other kits mostly cover single-antigen protocols that have been implemented in most clinical laboratories. Cancer biochemical markers profile a panel of different cancer subtypes, where a single biochemical marker might indicate a particular subtype of a disease. However, only 15–20% of patients develop a response to biochemical markers of different cancer subtypes (115). Therefore, larger numbers of

biochemical markers can be included, more sensitive and specific diagnostic tool can be developed, and there will be a higher likelihood of positive responses for at least one of the biochemical markers (116). Taken together, the application of multiplex biochemical marker technologies in other disease areas may offer insights that could be implemented as a framework for clinically important OA biochemical marker combination research.

MULTIPLEXING ADVANCES IN OA RESEARCH

Due to the capability of measuring up to 100 analytes in one relatively small sample, many different companies have utilized the Luminex xMAP technology platform and created different panels of multiplex assays. Although only a few studies applied this or other multiplex technologies in screening samples from OA patients so far, they generated important data on disease pathogenesis and progression. These data are summarized in **Table 1**.

A cytokine/chemokine panel was measured in serum from patients with hip and knee OA and compared with that in healthy controls using Luminex platform (117). Endothelial growth factor (EGF), FGF2, MCP-3, MIP-1α, and IL-8 were differentially expressed between hip and knee OA cohorts. In the knee OA samples, EGF was undetectable while MCP-1 and MIP-1β were highly expressed compared with that in hip OA and control samples, suggesting specific differences that may be related to differential disease processes within a given joint. Thus, different inflammatory biochemical marker combinations may represent OA lesions of different joints. These data support findings from an earlier pilot study in human knee synovial fluid (118), showing that among 21 cytokines screened, elevated MCP-1 and MIP-1 in SF were also increased in subjects with advanced arthritis, based on the International Cartilage Repair Society (ICRS) criteria.

Recently, five of the biochemical markers examined in synovial fluid, using cytokine multiplex assay (Luminex), significantly correlated with both knee pain and function (119) (**Table 1**). Furthermore, significant associations between OA radiographic severity (KL scoring) and some molecules in the synovial fluid were observed. Another 10-plex Luminex assay was applied not on SF but on synovial protein extracts of OA patients undergoing knee replacement surgery (30). Noteworthy, among the proteins analyzed, vascular endothelial growth factor (VEGF) was decreased in the synovium of symptomatic compared with asymptomatic OA samples, which is in contrast to the results of previously mentioned study on synovial fluid (117). Additionally, MMP-1 protein expression was increased in OA compared to postmortem synovial tissue controls.

The importance of evaluating SF biochemical markers as indicators of a symptomatic inflammatory OA endotype has been highlighted in a recent study on 25 patients with radiographic knee OA (47). Levels of 47 different cytokines, chemokines, and growth factors related to inflammation were screened using multiplex immunoassay (Luminex technology). A subset of six SF biochemical markers (**Table 1**) was associated with synovial inflammation, as well as radiographic and clinical severity, in OA. These six OA-related SF biochemical markers were specifically linked to indicators of activated macrophages and neutrophils. These results attest to an inflammatory OA endotype that may serve as the basis for therapeutic targeting of a subset of individuals at high risk for knee OA progression.

A first detailed comparison between Luminex and MSD multiplex platforms for the analysis of real clinical SF samples from end-stage knee OA was performed on inflammatory cytokines IL-1β, TNF-α, IL-6, and IL-8 (123). Both systems were capable of detecting the selected cytokines, while the MSD platform had a significantly lower limit of detection (LOD) for all four analytes. The authors concluded that MSD platform was better able to detect and quantify low-level analytes (IL-1β and TNF-α) in OA SF samples compared to Luminex, but due to the differences in the antibody pairs and their affinities, such comparisons of technologies are not very conclusive. Noteworthy, the cytokine measurements in OA samples were at best semi-quantitative and depended on the applied platform, assay, and its manufacturer, thus making the comparisons between the technologies complicated.

Flow cytometry and multiplex flow cytometry-based ELISA were employed for the analysis of cell composition and soluble protein production in synovium collected from OA patients undergoing knee replacement surgery (120). Here, IL-6 expression was highest in mesenchymal cells, although in a handful of patients, hematopoietic immune cell (mainly macrophage) expression was more dominant. Using a novel approach, patient-specific inflammatory clusters were identified: they broadly separated into T cell/lymphocyte (1) and myeloid (2 and 3) clusters, with cluster 3, in particular, associated with high tissue and mesenchymal cell IL-6 and IL-8 release. There are preliminary suggestions that these clusters reflect different patient phenotypes, with cluster 2 trending with female sex and cluster 3 with a history of prior joint surgery (arthroscopy/arthroplasty). It remains to be determined if these clusters can be better defined and how they are related to disease progression and clinical phenotypes.

A highly sensitive multiplex assay based on surface plasmon resonance imaging (SPRi) was first developed for the analysis of four cytokines in synovial fluid (IL-1β, IL-6, IFN-α, and TNF-α) (124). Later, by adding several early OA biochemical markers, including complement C3 peptide fragment (C3F), the assay was applied to detect early OA (121). Technologically, specific capture antibodies were spotted on a gold sensor and loaded into the SPRi machine (IBIS MX96). A sample with biochemical markers reached the sensor through a flow cell, and the interactions with the antibodies were measured in real time. The signal was enhanced by adding biotinylated detection antibodies, followed by neutravidin and biotinylated gold nanoparticles, resulting in a signal improvement of over 200 times and an increased sensitivity of more than 10,000 times. The assay is currently undergoing validation in a small patient cohort.

There is a growing evidence supporting the importance of biochemical markers reflecting metabolic changes in cartilage and bone during OA. The panel of metabolic products of cartilage and bone ECM molecules, representing the processes of breakdown (catabolism) or synthesis (anabolism), has been

TABLE 1 | Multiplex assays-based studies in OA.

Assay	Platform	Samples tested	Detected valuable markers in OA (correlations, associations etc.)	References
Milliplex MAP human cytokine/chemokine panel (42 analytes)	Luminex	Serum and synovial fluid, hip and knee	IL-6, MDC and IP-10 correlated with hip OA. IL-6, MDC, and IP10 were associated with pain in the hip cohort. MCP-1 and MIP-1β were highly expressed in the knee OA	(117)
LINCOplex™ Immunoassay (21 analytes)	Luminex	Knee synovial fluid	MCP-1, MIP-1, IL-2, IL-5 elevated in advanced OA (ICRS scale)	(118)
Pro-human cytokine multiplex assay (33 analytes)	Luminex	Knee synovial fluid	IL-10, IL-12, IL-13, SCGF-β, VEGF correlated with knee pain and function. IL-6, IL-8, IFN-γ, SCGF-β, VEGF, CXCL1 were associated with OA severity (KL scoring)	(119)
Human Luminex Screening Assay (10 analytes)	Luminex	Knee synovial protein extracts	VEGF was decreased in symptomatic OA vs. asymptomatic OA patients' samples. MMP-1 protein increased in OA vs. postmortem controls	(30)
Myriad Human InflammationMAP® 1.0 multiplex immunoassay (47 analytes)	Luminex	Synovial fluid	VEGF, MMP-3, TIMP-1, sICAM-1, sVCAM-1, MCP-1 related to synovial inflammation in OA, radiographic and symptom severity	(47)
BioLegend LEGENDplex human adipokine flow cytometry-based ELISA (13 analytes)	Flow cytometer	Knee synovium cells (24 h cultures *in vitro*/supernatants)	IL-6 expression was highest in mesenchymal cells vs. hemopoietic. One of the patient-specific inflammatory clusters identified had high tissue and mesenchymal cell IL-6 and IL-8 release	(120)
SPRi multiplex assay (9 analytes)	IBIS MX96	Serum and synovial fluid	Early OA markers—IL-1β, IL-6, TNF-α, IFN-γ, IL-10, CCL2, IL-8, IL-4, and C3F high sensitivity (low pg/ml) detection system. Undergoing validation in patient cohort	(121)
Microfluidic FMGC (2 analytes)	Microfluidics	Serum and urine	Simultaneous detection of sCTX-II and uCTX-II. 24-fold and 3.5-fold shorter completion time than the ELISA for urinary and serum CTX-II	(122)

IL-1β, 2, 4, 5, 6, 8, 10, 12, 13, interleukin 1β, 2, 4, 5, 6, 8, 10, 12, 13; MDC, macrophage-derived chemokine; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemoattractant protein-1; MIP-1, 1β, macrophage inflammatory protein 1, 1β; ICRS, International Cartilage Repair Society; SCGF-β, stem cell growth factor β; TIMP-1, metallopeptidase inhibitor 1; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; IFN-γ, interferon gamma; CXCL1, C–X–C motif chemokine ligand 1; KL score, Kellgren and Lawrence score; MMP-1, 3, matrix metallopeptidase 1, 3; TNF-α, tumor necrosis factor-α; CCL2, C–C motif chemokine ligand 2; C3F, complement C3 peptide fragment; FMGC, microfluidic fluoro-microbeads guiding chip; sCTX-II and uCTX-II, serum/urinary C-telopeptide fragments of type II collagen.

extensively reviewed (28, 32, 33). The prognostic value of the peptides arising from molecular breakdown or synthesis of cartilage ECM is still under investigation. An attempt to multiplex such markers via sandwich and competition immunoassays has been made (122). The new strategy aimed to simultaneously detect the C-telopeptide fragments of type II collagen (CTX-II), which has heterogeneous epitope structure in serum (sCTX-II; homodimers) and urine (uCTX-II; monomers or variant monomers). For the detection of both serum and urinary CTX-II peptides, a microfluidic fluoro-microbeads guiding chip (FMGC) was developed. It has one inlet for sample insertion and four separate chambers, two of which are dedicated for the sandwich-based detection, while the other two were for the competitive immunoassay. The proposed FMGC-based multiple sensing system accurately detected CTX-II, and the results obtained using this assay correlated well with those obtained using commercial ELISA kits. A combination of inflammatory cytokines/chemokines/MMPs together with cartilage/bone synthesis/degradation markers in a single multiplex assay would provide a powerful tool for the classification of OA subtypes and individualized OA treatment strategies.

Taken together, although several novel multiplexing technologies have been tested for biochemical markers associated with OA, the number of such studies remain very limited. The ability to measure the inflammatory cytokines in a multiplexed manner is not yet translated to the combined multiplex assays for cartilage/bone metabolic markers and not well-controlled with a sensitive reference method as MRI. These data could lead to understanding the role and importance of inflammation in the

TABLE 2 | Enhanced immunoassays for detection of biochemical markers relevant to OA.

Method	Samples tested	Biomarker	Advantages	Characteristics	References
Quantitative lateral flow immunoassay using antibody-conjugated gold nanoparticles	OA patient SF	COMP	Cost effectiveness	Dynamic detection range: 0.6–20 μg/mL	(127)
Quantum dot-linked immunosorbent assay (with immobilized orientation-directed half-part antibodies)	Antigen solution	IL-6	High sensitivity	Lower LOD: 50 pg/Ml	(128)
SENSIA	Pooled human serum	15-Plex (including IL-1β, IL-2, IL-4, IL-6, IL-10, MMP-9, TNF-α)	Cost effectiveness	IL-1β, IL-2, IL-6, and 2 other markers were in good agreement with FLISA ($>0.9R^2$)	(129)
Surface-enhanced Raman scattering based immunoassay	Healthy volunteer blood samples	IL-6, IL-8, and IL-18	High sensitivity	LOD: IL-6, 3.8 pg/ml; IL-8, 7.5 pg/ml; and IL-18, 5.2 pg/ml	(130)
Electrochemiluminescence-based system	Serum samples	CRP	High sensitivity, good selectivity, good reproducibility, and low cost	Range: 0.05–6.25 ng	(131)
Microfluidic immunoassay with streptavidin–biotin–peroxidase nanocomplex	Unspecified patient serum samples	IL-6 (multiplexed with procalcitonin)	High sensitivity	Detection range, 5–1,280 pg/ml; LOD, 1.0 pg/ml	(132)
Combined electrochemiluminescent and electrochemical immunoassay	Serum samples	IL-6	Broad dynamic range, high sensitivity, and selectivity	Detection range, 10 ag/ml−90 ng/ml	(133)

COMP, cartilage oligomeric matrix protein; IL-1β, 2, 4, 6, 8, 10, 18, interleukin 1β, 2, 4, 6, 8, 10, 18; LOD, limit of detection; SENSIA, silver-enhanced sandwich immunoassay; MMP-9, matrix metalloproteinase 9; TNF-α, tumor necrosis factor-α; FLISA, fluorescence-linked immunosorbent assay; CRP, C-reactive protein.

processes of cartilage breakdown and regeneration and specify the need for intervention. Moreover, most of the multiplexed OA biochemical marker studies to date were performed on SF samples, while only few studies involved the analyses of serum or urine, which would arguably better serve the major clinical need for OA monitoring.

NANOTECHNOLOGY STRATEGIES TO ENHANCE SELECTIVITY AND SENSITIVITY OF BIOCHEMICAL MARKER DETECTION

The major drawback, particularly related to serum or urinary biochemical markers, is that some of them are found at lower than picomolar concentrations, which are too low to be detected by conventional methods such as ELISA. Therefore, the analytical techniques, offering high degrees of sensitivity and specificity, such as those employing nanomaterials, proximity ligation, or digital platforms (e.g., digital ELISA), might appear useful for the analysis of ultralow concentrations of biochemical markers in a clinical setting (125). The recent advances in material science, nanotechnology, and bioconjugation techniques have enabled the application of a large diversity of nanomaterials to enhance the sensitivity of advanced immunoassays (126). Only a few studies have been reported about enhanced immunoassays specifically designed for OA biochemical markers (**Table 2**). For instance, a quantitative lateral-flow immunoassay technique with antibodies conjugated to gold nanoparticles was used for the detection of COMP in the synovial fluid. The proposed method showed similar results to corresponding ELISA, with an average difference of <7% without the need of expensive equipment or complex procedures (127). However, most of the reported studies have investigated the use of advanced nanomaterial-enhanced immunoassays toward inflammatory biochemical markers.

Markers of inflammatory activity have been at the forefront of detection limit improvement, as the concentrations of immune analytes are notoriously low, particularly in the serum or urine (134). For instance, an ELISA-like method based on the nanometer-sized fluorescent semiconductor particles called quantum dots has detected concentrations of IL-6 as low as 50 pg/ml (128). Application of silver-enhanced sandwich immunoassay (SENSIA) showed comparable results to the fluorophore-linked immunosorbent assay for the detection of IL-6, IL-2, and IL-1β in serum samples while being more cost effective (129). IL-6 and IL-8 showed improved detection limits (2.3 and 6.5 pg/ml, respectively) with sensitive surface-enhanced Raman scattering-based immunoassays in comparison to ELISA counterparts (130).

TABLE 3 | Biosensors for detection of biochemical markers relevant to OA.

Method	Samples tested	Biochemical marker	Advantages	Characteristics	References
Quartz crystal microbalance biosensor	Urine of OA patients and healthy controls*	COMP	Reaction time advantage, high sensitivity	Detection range: 1–200 ng/ml	(136)
Nanoparticle amplified SPRi aptasensor	Human serum	CRP	High sensitivity	LOD: 5 fg/ml	(137)
Quartz crystal microbalance biosensor	MMP-1 controls	MMP-1	Reaction time advantage	Detection range: 2–2,000 nM	(138)
Fiber optic-particle plasmon resonance biosensor integrated with microfluidic chip	OA patient SF*	MMP-3	Cost-effectiveness, portability, high sensitivity	–	(139)
Fiber-optic particle plasmon resonance biosensor	OA patient SF*	TNF-α and MMP-3	Reaction time advantage, simple usage, high sensitivity, high selectivity	LOD: TNF-α, 8.2 pg/ml; MMP-3, 8.2 pg/ml	(140)
Fluoromicrobeads guiding chip-based system	Human SF and serum*	COMP	Reaction time advantage	Detection range: 4 and 128 ng/ml	(141)
Fluoromicrobeads guiding chip-based system	Human urine-based controls and artificial serum	uCTX-II and sCTX-II	Simultaneous detection, reaction time advantage	Linear detection range: sCTX-II, 0.1–2.0 ng/mL; uCTX-II, 200–2,800 ng/mmol	(122)
Ultraviolet–visible spectroscopy	uCTX-II controls	CTX-II (multiplexed with glucose)	Cost effectiveness and simple manufacturing	Detection range: 1.3–10 ng/ml	(142)
Ambient light-based optical biosensor	uCTX-II epitope controls	uCTX-II	Cost effectiveness, simple usage	LOD: 0.2 ng/ml	(143)

**Analyzed in OA patient samples.*

MMP-1, 3, matrix metallopeptidase 1, 3; TNF-α, tumor necrosis factor-α; sCTX-II and uCTX-II, serum/urinary C-telopeptide fragments of type II collagen; LOD, limit of detection; SENSIA, silver-enhanced sandwich immunoassay; COMP, cartilage oligomeric matrix protein; CRP, C-reactive protein.

Electrochemiluminescence-based systems have also been applied for the analysis of inflammatory markers. Application of such label-free electrochemiluminescent immunosensor that utilizes the poor conductivity of CRP molecules bound to antibodies has enabled its detection at the limit of 0.011 ng/ml (131). An immunoassay based on mesocrystal nanoarchitectures combined with electrochemiluminescent and electrochemical detection has been presented to quantify IL-6 (133). The results showed high sensitivity by achieving a broad linear dynamic range of 10 ag/ml−90 ng/ml.

A quantitative microfluidic immunoassay combined with the streptavidin–biotin–peroxidase (SA-B-HRP) nanocomplex-signal amplification system (MIS) has also been presented to detect IL-6 simultaneously to a second inflammatory biochemical markers (i.e., procalcitonin). In this case, the linear range for IL-6 detections was 5–1,280 pg/ml, and the limit of detection was 1.0 pg/mL, which was significantly improved compared with microfluidic immunoassays without amplification systems. Despite these promising outcomes toward inflammatory biochemical markers, to our knowledge, the enhanced-detection immunoassays mentioned above have not yet been applied toward OA inflammatory response.

NOVEL STRATEGIES TO ENABLE DIAGNOSTIC AND PROGNOSTIC MONITORING VIA BIOSENSORS

Biochemical markers have the potential to be used as indicators of changes in the course of the disease, which might signal the need for additional imaging tests or changes in the course of OA, which could require treatment option re-evaluation (11). However, as a monitoring tool, they will only be considered if there are methods available to easily detect the subtle changes in concentration values while maintaining consistency in data collection. A potential solution to this is the application of biosensors, which are analytical devices that analyze biological responses and convert them into measurable physicochemical signals, typically exhibiting high specificity and reusability (135). Biosensors are an expanding field toward rapid, easy, and reliable detection of relevant biochemical marker that have been implemented in the diagnosis of various diseases and have also started to be considered as potential tools for OA research and clinical application (**Table 3**).

COMP has been measured in both OA patient's synovial fluid and serum with an immunosensing device using FMGC

technology that has a reaction time advantage over conventional ELISA (141). As it shows good correlation to results obtained by conventional ELISA (coefficient of variation was only within 7%), it could potentially be used in clinical settings, more so if the technology advances to a multiplex format. FMGC-based system has also been used for uCTX-II and sCTX-II quantification, which not only analyzed both factors simultaneously but was also faster than the conventional ELISA by 25- and 3.5-fold, respectively (122). Both systems exhibited high sensitivity and very similar LODs to their respective ELISAs.

Several optics-based biosensing platforms have also been investigated. Low-cost optical biosensing platforms, based on ultraviolet–visible spectroscopy, have been developed for uCTX-II using just common office supplies for data reading (142). As the detection range falls into the clinically relevant intervals, this method of analysis could be introduced into clinical immunoanalysis. The same biochemical marker, uCTX-II, was analyzed using a smartphone-embedded illumination biosensor that had a high accuracy under various lighting conditions (143). Due to its low-cost fabrication requirements and satisfactory detection capabilities in both indoor lighting and in sunlight, it has the potential of being used as a point-of-care diagnostic tool. Real-time multiplexed analysis of MMP-3 and TNF-α was achieved using a single fiber-optic particle plasmon resonance biosensor, which could be used for monitoring both inflammatory and cartilage breakdown activity simultaneously (140). The same type of biosensor has been used for the detection of inflammatory biochemical marker IL-1β with results comparable to a corresponding ELISA method (limit of detection, 21 pg/ml), which, together with the relatively low cost, shorter analysis time, and small sample requirements, seems very attractive for the use in a clinical setting (144).

Other potential candidates for innovative biosensing approaches are technologies that use the piezoelectric effect and transform mechanical stress into quantifiable electrical current. For instance, a quartz crystal microbalance (QCM)-based biosensor for COMP was developed and, compared to ELISA data, showed high accuracy in a shorter analysis time frame (136). Similar biosensor has been demonstrated to detect MMP-1 levels at concentrations between 2 and 2,000 nM in <10 min with a lower detection limit of 2 nM (138). While the clinical relevance of COMP was demonstrated due to its association to the OARSI grades of OA progression, MMP-related biochemical markers might be useful in determining the activity of the processes of cartilage degradation. QCM-based strategies are user friendly and quicker than ELISA, making it a suitable application as a homecare device, comparable to those used for blood glucose monitoring.

Seeking to overcome the problems faced by conventional ELISA method, such as high cost and long process duration, electrochemical-impedance-based immunoassays have been applied to determine levels of the bone-related degradation biochemical marker CTX-I (145).

Advanced biosensors technology has also exploited nanotechnology to enable high sensitivity detection of biochemical markers, potentially leading to applications in clinical settings. Nanoparticle-enhanced plasmonic biosensor have been demonstrated to detect inflammatory marker CRP in only 2 h at concentrations four orders of magnitude lower than the clinically relevant concentrations (146). This ultrasensitive biosensor is fabricated using scalable and low-cost manufacturing, providing a powerful platform for multiplexed biochemical marker detection in several settings. Nanoplasmonic biosensor microarrays have been demonstrated for parallel multiplex immunoassays of six cytokines (i.e., IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ) in a complex serum matrix on a single device chip (147). The device was fabricated using easy-to-implement, one-step microfluidic patterning and antibody conjugation of gold nanorods. The proposed biosensor showed the capability to measure cytokine at concentrations down to 5–20 pg/ml from a 1-μl serum sample within 40 min. Electrochemical immunobased biosensors in combination with carbon nanomaterials have also been reported to detect inflammatory biochemical markers (148, 149). Simultaneous detection of IL-1β and TNF-α using human serum and saliva was achieved using dual-screen printed electrodes modified with carbon nanotubes (148). The proposed biosensor showed improved analytical performance with respect to previous approaches and ELISA methods by achieving limits of detection at 0.38 pg/ml (IL-1β) and 0.85 pg/ml (TNF-α), within 2 h and 30 min and significantly less reagents consumptions.

Diverse cost-effective biosensors for OA that minimize the duration of analysis or in other ways outperforming conventional methods are under development, with a subset of them proposed as clinically suitable biochemical marker detection tools or even potential point-of-care monitoring devices. Although there are multiple technological approaches that are currently under investigation to determine the applicability of various OA biochemical markers, studies that sensitively and consistently follow the changes in levels of OA biochemical markers throughout the course of the disease and elucidate diurnal variation, responses to physical activity, anti-inflammatory medication, or nutrition are still a lacking. For instance, despite that large diversity of biosensors have been developed to detect the inflammatory biochemical marker CRP as reviewed in Vashist et al. (150), CRP biosensors were not tested on OA patient serum samples so far. CRP biosensors created have already been designed reaching detection levels in the zeptomolar concentrations (137). Of note, it is likely that monomeric and multimeric forms of CRP may possess different catabolic and inflammatory profiles. Therefore, new assays need to be developed to distinguish between monomeric and multimeric forms of CRP and other proteins. The application of biosensors, ideally for several multiplexed biochemical markers, might lead to new insights into the role of inflammation in the pathological processes of cartilage turnover in OA.

CHOICE OF THE REFERENCE METHOD FOR BIOCHEMICAL MARKER DETECTION

One of the major drawbacks in biochemical marker development for OA is a lack of relevant reference methods to validate their efficacy. Most studies so far have employed radiographic

TABLE 4 | Correlation between biochemical marker levels and MRI data.

Study design, number of patients	MRI scoring system	Detection method/biochemical marker panel	Biochemical markers association with MRI scoring data	References
Case–control ($n = 600$) Follow-up points: baseline, 12 and 24 months	MOAKS: Hoffa synovitis and effusion synovitis	ELISA: HA, MMP-3, Coll2–1NO2	HA and MMP-3 were modestly associated with effusion-synovitis at baseline	(153)
Cross-sectional ($n = 89$)	WORMS	ELISA: COMP, MMP-3, Coll2-1, Turbidimetric analysis: CRP	COMP correlated positively with WORMS and MMP-3. WORMS scoring data are not provided	(154)
Case control ($n = 141$) Follow-up: 2 years	WORMS	ELISA: HA, MMP-3, COMP, Coll2-NO2, uCTX-II, PIIANP, CTXI, CS846, C2C, CPII, NTXI/uNTXI, C12C/uC12C	MRI data associated between HA, COMP, and MMP-3 biochemical markers of OA. The biochemical cartilage ECM (Coll2-NO2) degeneration reflects MRI T2 measures	(155)
Cross-sectional ($n = 160$) Follow-up: 2 years	WORMS	ELISA: IL-8, COMP, CTXI, NTXI, PIIINP, MMP-3, MMP-10, MMP-13	The positive association was between IL-8 and infrapatellar fat pad signal intensity	(156)
Cross-sectional ($n = 141$) Follow-up: 2 years	WORMS	ELISA: 100A8/A9, MMP-3, MMP-10, MMP-13	The levels of alarmins 100A8/A9 had positive associations with MRI score for total and local cartilage defects (lateral femoral, lateral tibial, and medial femoral sites)	(157)
Osteoarthritis Initiative Progression subcohort ($n = 583$) Follow-up: 2 years	MRI quantitative cartilage volume measurement	ELISA: adiponectin LUMINEX: adipsin chemerin, leptin, visfatin, IL-8, MCP-1, CRP	The ratio of adipsin/MCP-1 was associated with the MRI knee structural changes, and CRP/MCP-1 with symptoms in obese OA subjects	(158)
Cross-sectional ($n = 16$) Follow-up: 5 years	MRI semiautomatic segmentation method	ELISA: COMP, C1,2C, CS846	Long-term mechanical stimuli increase the cartilage degradation markers as C1,2C and CS846. Those biochemical markers correlate with cartilage damage (MRI)	(159)
Multicenter, double-blind, phase III clinical trial ($n = 163$) Follow-up: 1 year	WORMS	ELISA: CTX-I, COMP, PIIANP, MMP-3, C1M, C3M, C2M, CS846, CTX-II, uCTX-II/creatinine ratio	Clinical study of cell and gene therapy: no significant differences in MRI between the groups of treatment vs. placebo	(160)
Randomized, double-blinded, sham-controlled trial ($n = 55$) Follow-up: 3 months	MOAKS: Hoffa synovitis and effusion synovitis	ESR ELISA: hsCRP	Low-dose radiation therapy does not induce significant effects on inflammatory signs assessed by MRI, ultrasound and serum inflammatory markers	(161)

s100A8/A9, myeloid related protein 8/14, calprotectin; ESR, erythrocyte sedimentation rate; COMP, cartilage oligomeric matrix protein; Coll2-1, type II collagen degradation biomarker 1; Coll2–1NO2, nitrated epitope of the α-helical region of type II collagen; C12C/uC12C, collagen type I and II cleavage product and its urine form; C2C, type II collagen degradation biomarker, generated from C-terminus fragment; C1M, C2M, C3M, matrix metalloproteinase (MMP) mediated type I, II, III collagen degradation biomarker; CPII, C-propeptide of type II procollagen; CRP, C-reactive protein; CTX-I, serum C-terminal telopeptide of type I collagen; CS846, chondroitin sulfate synthesis marker; sCTX-II and uCTX-II, serum/urinary C-telopeptide fragments of type II collagen; HA, hyaluronic acid; IL-8, interleukin 8; MMP-3, 10, 13, matrix metalloproteinase 3, 10, 13; MCP-1, monocyte chemoattractant protein-1; MOAKS, MRI OA Knee Score; NTXI/uNTXI, crosslinked N-telopeptide of type I collagen and its urine form; PIIANP, serum N-propeptide of collagen IIA; PIIINP, N-terminal procollagen III propeptide; WORMS, whole organ magnetic resonance imaging score.

assessment of KL grade for scoring of OA stages, which is neither sensitive, specific, nor easily reproducible in longitudinal clinical trial (10). The lack of a sensitive reference biochemical marker (either imaging or biochemical marker) have likely led to difficulties in proving the utility of biochemical markers in OA. The close correlation between "wet" (biochemical analyte, genomic, etc.) and "dry" (radiography, MRI, or clinical evaluation finding, etc.) as a reference method in each case

is of major importance for the successful implementation of biochemical markers (**Figure 1**). The prospective CHECK study investigates five clusters of biochemical markers, related to specific pathogenic processes: "bone," "inflammation," "synovium," "adipokines," and "cartilage synthesis," which will be validated via early radiographic KL scoring of OA status as a reference method (151). Duration of this study is planned for 10 years, and it will finish in 2022.

MRI technology is increasingly implemented as a reference method for evaluation of OA status (152). Several studies involving the analysis of multiple biochemical markers in serum and synovial fluid of knee OA patients (22) use MRI as a reference method for knee damage evaluation. The relation of biochemical serum markers to MRI data in studies performed in 2018 and 2019 are summarized in **Table 4**.

The listed studies revealed associations of different biochemical markers with MRI scoring of synovial inflammation and cartilage degradation. It is important to note that these MRI techniques can detect early bone marrow lesions that may be associated with the onset of cartilage degradation and correlate with inflammation in joint tissue (162). As these data are ambiguous and associated with various aspects of synovial inflammation and/or cartilage damages, no perfect scoring system exists to date. In terms of complexity, heterogeneity, and size of knee OA-related data, it is considered a "big data" issue; therefore, machine learning and application of computer algorithms has attracted significant interest for the evaluation of biochemical and imaging markers (163). A recent publication by Emery et al. described outcome measures for early OA that could be useful in clinical practice and/or the research setting (12). A consensus-based OA phenotype framework was created with intent to facilitate research on OA phenotypes and increase combined efforts to attain effective OA phenotype classification, by providing a number of coherent definitions and statements and a set of reporting recommendations that were supported by a panel of experts in OA research field (164). Many other research groups are currently working with regulatory agencies across the world seeking to clinically qualify confirmed new biochemical markers and imaging biomarkers (165, 166).

DEVELOPMENT OF REFERENCE METHODS

Looking into the future of radiological reference method development, more attention will be focused on morphological MRI, observing "premorphological" biochemical compositional changes of articular tissues (12, 18). Compositional MRI techniques evaluate cartilage composition [glycosaminoglycans (GAGs), proteoglycans, or collagens] and hydration. Compositional MRI of cartilage matrix changes can be performed using advanced MRI techniques such as delayed gadolinium-enhanced MRI of cartilage (dGEMRIC), T1 rho, and T2 mapping (152). The chemical exchange saturation transfer of GAG (GagCEST) can detect cartilage endogenous GAG content without the need of intravenous contrast injections or special hardware (167). Negatively charged GAGs attract sodium cations and their distribution in sodium MRI correlates with GAG content in cartilage (152). It is consequently important to harness morphological MRI, compositional changes of the cartilage (GAGs, proteoglycans, or collagens), and hydration from images and to establish a correlation between them and quantitative biochemical markers.

In differential diagnosis for OA, it is important to evaluate the intensity of inflammatory component expression, which usually is not as high as in other inflammatory arthritides. The hybrid imaging techniques as the positron emission tomography (PET) method with 18F-fluorodeoxyglucose (FDG) uptake reflected the inflammatory activity, associated with elevated inflammatory cytokine levels, suggesting that FDG-PET may be effective for quantification of the inflammatory activity in different rheumatic diseases (168, 169). The inflammation may contribute to the increased FDG uptake in OA, reflecting the rate of disease progression and inflammatory phenotype. Single-photon emission computed tomography combined with the high-resolution computed tomography (SPECT/CT) technique can visualize folate receptor positive cells, representing activated macrophages and neutrophils, which is associated with the inflammation in the joints of OA patients (47).

These new technological approaches are may be systematically applied for the identification and characterization of OA phenotypes and, together with the relevant biochemical and imaging markers, might contribute to a better understanding of the role of low-grade inflammation in the development of OA, as well as facilitate the identification of the need for anti-inflammatory medication (11). The local inflammatory activity associated with elevated inflammatory cytokine levels (169) and folate receptor positive cells, representing activated macrophages and neutrophils, can already be visualized (47). The rapid progress of such sophisticated imaging methods should lead to the precision in imaging biomarker choice and implementation.

CONCLUSIONS

In this article, we have provided an overview of several technologies, platforms, and strategies that facilitate and improve biochemical marker detection. Application of such novel techniques in imaging and biochemical marker identification may lead to better definition of OA phenotypes and categories and add complementary value to radiologically validated outcome measures in clinical practice and/or the evidence-based comparisons into the effectiveness of therapeutic interventions.

OA biochemical marker immunoassays are potentially viable tools for OA evaluation, monitoring, and drug development. However, specific problems, such as low biochemical marker concentrations, patient-specific variation, limited utility of single biochemical markers to get definitive characterization of OA status, and application of relevant reference methods, require innovative approaches to produce clinically relevant biochemical marker biosensors. Such technologies are already on the way to establishment in routine diagnostics and monitoring of other diseases and could potentially serve as good technological

platforms for early OA characterization. Inflammatory markers are monitored in many inflammatory diseases, and novel technologies like biosensors dedicated to reducing costs or time of their detection may also be successfully implemented in OA. Multiplexing inflammatory biochemical markers in combination with biochemical markers of cartilage matrix turnover may shed light on poorly understood mechanisms involved in OA pathogenesis and lead to a better understanding of the role of low-grade inflammation in OA pathogenesis and better classify different clinical phenotypes and molecular endotypes of OA, leading to personalized therapeutic approaches for OA.

AUTHOR CONTRIBUTIONS

EBe: conceptualization and review methodology. EBa, GK, PB, IU, and UK: acquisition of resources. EBe, EBa, GK, PB, IU, and UK: writing—original draft. EBe, EBa, GK, PB, IU, UK, MD-G, and AM: writing—revision. EBe, EBa, GK, CT, HH, GL, AG, MD-G, and AM: review and further editing. EBe and AM: supervision. AM: final submission and funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

We wish to acknowledge financial support from the European Structural and Social Funds through the Research Council of Lithuania (Lietuvos Mokslo Taryba) according to the Programme Attracting Foreign Researchers for Research Implementation, Grant No. 01.2.2-LMT-K-718-02-0022. The research underpinning some of the work reviewed has received funding from a number of sources including The European Commission Framework 7 program (EU FP7; HEALTH.2012.2.4.5-2, project number 305815; Novel Diagnostics and Biomarkers for Early Identification of Chronic Inflammatory Joint Diseases); the Innovative Medicines Initiative Joint Undertaking under grant agreement no. 115770, resources of which are composed of financial contribution from the European Union's Seventh Framework program (FP7/2007-2013), EFPIA companies' in-kind contribution and the H2020 projects RESTORE (project number 814558; topic NMBP-22-2018 RIA), and MIRACLE (project number 780598; topic ICT-30-2017). Details of the D-BOARD FP7 Consortium are available at: http://www.d-board.eu. Details of the H2020-RESTORE Consortium are available at restoreproject.eu. Details on the H2020-MIRACLE Consortium are available at miracleproject.eu. AM was the coordinator of the D-BOARD Consortium funded by European Commission Framework 7 program (EU FP7; HEALTH.2012.2.4.5–2, project number 305815, Novel Diagnostics and Biomarkers for Early Identification of Chronic Inflammatory Joint Diseases). AM is a member of the Applied Public-Private Research enabling OsteoArthritis Clinical Headway (APPROACH) consortium, a 5-year project funded by the European Commission's Innovative Medicines Initiative (IMI). Details of the APPROACH IMI Consortium are available at: https://www.approachproject.eu. AM has also received financial support from the European Commission through a Marie Skłodowska-Curie Intra-European Fellowship for career development awarded to Dr. Csaba Matta (project number: 625746; acronym: CHONDRION; FP7-PEOPLE-2013-IEF). MD-G was supported by Rosetrees Trust (PGS19-2/10029) and the Israeli Science foundation (370/17).

ACKNOWLEDGMENTS

We would like to thank our collaborators and members of our research teams for useful discussions about the emerging applications of biochemical marker platform and nanotechnologies in OA research and therapy.

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Conflict of Interest: AG is a shareholder of BICL, LLC and consultant to Pfizer, Galapagos, TissueGene, MerckSerono, AstraZeneca, and Roche. AM has consulted for Abbvie, AlphaSights, Artialis SA, Flexion Therapeutics, Galapagos, Guidepoint Global, IAG, Kolon TissueGene, Pacira Biosciences Inc., Pfizer Consumer Healthcare (PCH), Servier, and Science Branding Communications and received research funding from the European Commission (FP7, IMI, Marie Skłodowska-Curie, ES Strukturines Paramos), Versus Arthritis (Arthritis Research UK), Pfizer Inc., Kolon TissueGene, and Merck KGaA. CT is an employee of Nordic Bioscience.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GLOSSARY

ACLT, anterior cruciate ligament transection; ARGS, TEGE, FFGV, Aggrecan epitopes; BIPEDs, burden of disease, investigative, prognostic, efficacy of intervention, diagnostic, and safety; C12C/uC12C, collagen type I and II cleavage product and its urine form; C1M, C2M, C3M, matrix metalloproteinase (MMP) mediated types I, II, III collagen degradation biomarker; C2C, type II collagen degradation biomarker, generated from C-terminus fragment; C3F, complement C3 peptide fragment; CA153, CA125, cancer antigen 153, 125; CBA, cytometric bead array; CCL2, C–C motif chemokine ligand 2; CEA, carcinoembryonic antigen; CHECK, Cohort Hip and Cohort Knee; Coll2-1, type II collagen degradation biomarker 1; Coll2-NO2, nitrated form of type II collagen degradation; COMP, cartilage oligomeric matrix protein; CPII, C-propeptide of type II procollagen; CRP, C-reactive protein; CRPM, neo-epitope of MMP-1 and MMP-8 mediated degradation of C-reactive protein; CS846, chondroitin sulfate epitope 846; CXCL1, C–X–C motif chemokine ligand 1; dGEMRIC, delayed gadolinium-enhanced MRI of cartilage; ECM, extracellular matrix; EGF, endothelial growth factor; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; FDA, Food and Drug Administration; FDG, 18F-fluorodeoxyglucose; FGF2, fibroblast growth factor 2; Fib3-1,2,3, fibulin-3 epitopes 1,2,3; FLISA, fluorescence-linked immunosorbent assay; FMGC, fluoro-microbeads guiding chip; FNIH, Foundation for the National Institutes of Health; GagCEST, chemical exchange saturation transfer of GAG; GAGs, glycosaminoglycans; HA, hyaluronic acid; hsCRP, high-sensitivity CRP; ICRS, International Cartilage Repair Society; IFN-γ, interferon gamma; IL-1β, 2, 4, 5, 6, 8, 10, 12, 13, interleukin-1β, 2, 4, 5, 6, 8, 10, 12, 13; IP-10, interferon gamma-induced protein-10; KL, Kellgren and Lawrence; LOD, limit of detection; MAP, multianalyte profiling; MCP-1,3, monocyte chemoattractant protein-1,3; MBDA, multibiomarker disease activity; MDC, macrophage-derived chemokine; MIP-1, 1α, 1β, macrophage inflammatory protein-1, 1α, 1β; MMPs, matrix metalloproteinases; MOAKS, MRI OA Knee Score; MRI, magnetic resonance imaging; MS, multiple sclerosis; MSD, Meso Scale Discovery; MSP, methylation-specific PCR; NTXI/uNTXI, crosslinked N-telopeptide of type I collagen and its urine form; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International; OPG, osteoprotegerin; PDGFR, platelet-derived growth factor receptors; PET, positron emission tomography; PIIANP, serum N-propeptide of collagen IIA; PIIBNP, serum N-propeptide of collagen IIB; RA, rheumatoid arthritis; SCGF-β, stem cell growth factor-β; sCTX-II and uCTX-II, serum/urinary C-telopeptide fragments of type II collagen; SENSIA, silver-enhanced sandwich immunoassay; SERS, surface enhanced Raman scattering; SF, synovial fluid; sICAM-1, soluble intercellular adhesion molecule-1; SIRT1, deacetylase sirtuin-1; SPECT/CT, single-photon emission computed tomography combined with high-resolution computed tomography; SPRi, surface plasmon resonance imaging; sVCAM-1, soluble vascular cell adhesion molecule-1; TIMP-1, metallopeptidase inhibitor-1; TNF-α, tumor necrosis factor-α;VEGF, vascular endothelial growth factor; WORMS, whole-organ magnetic resonance imaging score.

frontiers in Medicine

ORIGINAL RESEARCH
published: 12 November 2020
doi: 10.3389/fmed.2020.554669

Exploring the Association of Innate Immunity Biomarkers With MRI Features in Both Early and Late Stages Osteoarthritis

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Objective: To evaluate the association between biomarkers of innate immunity and the magnetic resonance imaging (MRI) features of earlier and later stages of knee osteoarthritis (KOA).

Methods: From 139 and 20 participants with earlier and later stages of KOA, respectively, we analyzed knee MRIs scored using the Boston Leeds Osteoarthritis Knee Score (BLOKS) at recruitment with biomarkers. In paired serum (s) and synovial fluid (sf), we quantified three biomarkers related to innate immunity: lipopolysaccharide binding protein (LBP), CD14 and Toll-like receptor 4 (TLR4), and three proinflammatory biomarkers [interleukin-6 (IL6), IL8, and tumor necrosis factor alpha (TNFα)].

Results: In participants with earlier KOA, (s) LBP was statistically significantly associated with meniscal extrusion, and (sf) CD14 was associated with effusion after adjustment with age, sex, and body mass index. In participants with later stage of KOA, (sf) LBP was associated with effusion. (sf) CD14 was associated with cartilage loss and BML. In earlier stage of KOA, the proinflammatory biomarkers IL6, IL8, and TNFα were associated with most MRI features.

Conclusion: Innate immunity biomarkers (s) LBP was associated with MRI meniscal extrusion; (sf) CD14 was associated with MRI synovial inflammation in earlier stage and BMLs in later stage of KOA. Associations between proinflammatory biomarkers and various MRI features in earlier stage of KOA were observed.

Keywords: osteoarthritis, biomarkers, innate immunity, pro-inflammatory, magnetic resonance imaging

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

Received: *22 April 2020*
Accepted: *28 September 2020*
Published: *12 November 2020*

Citation:
Rajandran SN, Ma CA, Tan JR, Liu J, Wong SBS and Leung Y-Y (2020) Exploring the Association of Innate Immunity Biomarkers With MRI Features in Both Early and Late Stages Osteoarthritis. Front. Med. 7:554669. doi: 10.3389/fmed.2020.554669

INTRODUCTION

Knee osteoarthritis (KOA) is the most common form of arthritis affecting about 250 million people worldwide and a leading cause of mobility and disability in the elderly (1, 2). Inflammation of the synovium may play a role in the pathophysiology of KOA, as evidenced by histological synovitis from the tissue harvested from patients undergoing arthroplasties (3–5). However, there is less information on earlier stages of KOA, and the pathophysiological characteristics of earlier

KOA could be different. Greater knowledge of the initial stages of the disease may help advance understanding of KOA pathogenesis and identification of therapeutic targets for early KOA, which may be more responsive to treatment. Magnetic resonance imagining (MRI) is an imaging modality that reveals the pathology of all joint tissues including the cartilage, synovium, meniscus, and bone marrow. MRI can detect synovial membrane inflammation in early KOA even when signs of joint inflammation were not obvious in physical examination (6, 7); it therefore provides a mean for studying earlier stages of KOA.

It has been hypothesized that macrophage-associated innate inflammation may play a role in the pathogenesis of KOA (8, 9). Systemic lipopolysaccharide (LPS) levels were shown to be higher in obese animals, possibly related to increased intestinal permeability (10, 11). LPS, being a pathogen-associated molecular pattern (PAMP), binds to lipopolysaccharide binding protein (LBP) (12). The cluster of differentiation 14 (CD14) biomarker is predominantly found on activated macrophages and serves as a receptor for the LPS–LBP complex (9, 13). The binding of the LPS–LBP complex to CD14 would then trigger the Toll-like receptor 4 (TLR4) of macrophages (14, 15), leading to the downstream production of inflammatory mediators and catabolism of chondrocytes (16). Recently, both serum (s) and synovial fluid (sf) LPS and LBP were shown to be associated with the abundance of activated macrophages in the and synovium as well as associated with radiographic severity and symptoms among patients with KOA (17), suggesting the possible role of LPS in triggering of the innate immunity in KOA pathogenesis. TNFα and IL1β have been implicated as the key drivers of inflammatory cascade in KOA (16). IL6 was shown to be elevated in (s) and (sf) in KOA patients, stimulating metalloproteinase expression and mediating cartilage extracellular matrix protein degradation (18, 19). IL8 is an inflammatory chemokine mediating neutrophil accumulation and activating leukocyte homing to the synovium (16, 20).

In this study, we aim to evaluate the association of inflammatory biomarkers related to innate immunity: LBP, CD14, TLR4 with different MRI features of the knee from two studies of participants with KOA. These biomarkers represent the possible trigger, receptor, and effector cells (activated macrophage) of the innate immunity. We also evaluated the association of downstream inflammatory biomarkers: IL6, IL8, and TNFα with MRI features. Participants from one of studies represent earlier stage of KOA and the other with later stage of KOA.

MATERIALS AND METHODS

Study Cohorts

We analyzed baseline clinical data and biological samples from two existing studies established in a tertiary hospital in Singapore.

The Study With Earlier Stage of KOA

Participants were recruited from a cross-sectional study of 145 participants with knee pain. The study protocol was read and approved by the SingHealth Centralized Institutional Review Board (Ref: 2012/837/E), and informed consent were obtained from all participants prior to the start of the study. The participants (40–79 years old) from this study were recruited from the community via social media, advertisements, and referrals from the primary health care clinics (Singhealth Polyclinics) and two departments at the Singapore General Hospital (Rheumatology and Immunology; and Orthopedics) (21). Interested participants were invited to call through a recruitment telephone hotline and were then screened by trained staff. Participants who experienced pain in at least one knee on most days during the past month were invited to a screening clinic visit. All participants were examined by a rheumatologist (YYL) at the clinic. The study inclusion criteria included adult above 40 years old and a positive response to the question "Do you have pain, aching or stiffness of the knee on most days of the past month." We excluded those who had prior knee replacement surgery or were planning for knee replacement surgery in the next 6 months. Radiography of both knees were taken in the clinic, and participants with Kellgren and Lawrence (KL) (22) grade 4 in either knee or isolated patellofemoral joint involvement on radiography as read by the attending rheumatologist were excluded. We excluded participants with significant joint injuries in the past 1 year and other joint diseases (rheumatoid arthritis, spondyloarthritis, Paget's disease, joint fractures, hyperparathyroidism, hyperthyroidism, hypothyroidism). We also excluded participants with contraindications to magnetic resonance imaging (MRI), such as significant renal impairment, pregnancy, metallic implants *in situ*, and claustrophobia. Participants using warfarin were excluded due to an increase risk of bleeding after joint aspiration. One hundred thirty-nine participants completed MRI of the knee. Sixty (43%) participants from this study fulfilled the American College of Rheumatology (ACR) Clinical and Radiographic Criteria for KOA (23). Among the 79 participants who did not fulfilled the ACR Clinical and Radiographic Criteria, 33 participants fulfilled classification criteria of early KOA suggested by Luyten et al. (24).

The Study With Later Stage of KOA

We used the baseline data and biological samples from the Colchicine effectiveness in symptom and inflammation modification in knee osteoarthritis (COLKOA) (NCT02176460) (25, 26). The COLKOA is a double-blind, placebo-controlled, randomized trial that compared a 16-week treatment with a standard daily dose of oral colchicine to placebo for symptomatic KOA. The study protocol was read and approved by the SingHealth Centralized Institutional Review Board (CIRB2012/659/E), and informed consent was obtained from all participants before beginning the study. A total of 109 participants with symptomatic KOA based on the ACR Clinical and Radiographic Criteria (23) with KL grading of $\geq$2 in at least one knee during the screening were recruited in this study. We included in this study 20 participants who had MRI of the knee performed at baseline. Ten participants each from the colchicine and placebo arms were randomly selected for knee MRI according to a pre-specified list (25).

Clinical Data Collection

In both studies, we collected age, sex, ethnicity, history of knee joint injury, comorbidities (hypertension, hyperlipidemia, diabetes mellitus, and coronary artery disease), and current medications. The body weight and height were measured using an electronic weight scale and an ultrasonic height sensor (Avamech B1000 Series) without shoes. The index knee was designated as the more symptomatic knee or the dominant knee if symptoms of both knees were similar. The clinical severity of KOA was evaluated using the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) of the index knee (27). The WOMAC consists of 24 items that are divided into three subscales, which are pain, stiffness, and physical function. Each domain was standardized to obtain a score ranging from 0 to 100; higher scores reflected greater clinical severity.

Radiographic Assessment

For both studies, all the participants underwent at recruitment posteroanterior fixed-flexion weight-bearing radiography on both knees with a SynaFlexer lower limb positioning frame (Synarc). The radiography was taken by starting with a 10° caudal beam angle until achieving alignment of the anterior and posterior margins of the tibial plateau to within 1.2 mm target (28). Each of the radiograph was scored according to the KL grading (0–4) of the tibiofemoral (TF) compartment (22) by an experienced musculoskeletal radiologist (SBW) who was blinded to the condition of the participants. A total of 70 radiographs (140 knees) from this study were rescored for KL grades by the original assessors 8–12 weeks apart and blinded to the original scores. The weighted kappa of KL grading of TF compartment was 0.60 [95% confidence interval (CI), 0.42–0.73].

Magnetic Resonance Imaging Assessment

A total of 139 participants from the earlier KOA study and 20 participants from the later KOA study had MRI on their index knees (25). The MRI imaging were performed in a 45-min session by a 3-T Philips Ingenia machine with the index knee immobilized in a dedicated dStream T/R 16ch knee coil. The sequences assessed were coronal T1w, coronal proton density fat saturated, sagittal proton density fat saturated, axial T2w fat saturated, and axial T1w fat saturated (pre- and post-gadolinium contrast). The slice thickness was 2 mm, and the in-plane resolution matched the T1-weighted water excite scan with interpolation. The repetition time was 20 ms, and fat suppression (by water excitation) was utilized to minimize chemical shift artifacts.

The MRIs were assessed for osteophytes (OST) size, cartilage integrity, bone marrow lesions (BMLs), effusion, Hoff's synovitis, and meniscal extrusion according to the Boston Leeds Osteoarthritis Knee Score (BLOKS) scoring system (29) by a musculoskeletal radiologist (JRT) who was blinded to the participant's clinical details. OST size, cartilage integrity, BMLs, and meniscal extrusion scores were calculated according to the number of subregions affected (30). All 20 MRIs from this study of later stage of KOA were read twice by the same assessor blinded to the sequence of MRI and participants' condition. The weighted kappas of MRI scoring for BMLs (median, 0.68; range, 0.60–0.72), cartilage integrity (median, 0.73; range, 0.44–0.91), OST (median, 0.88; range, 0.87–0.90), and meniscal extrusion (median, 0.84; range, 0.82–0.85) were satisfactory, and the weighted kappa for effusion and Hoff's synovitis were 0.61 (95% CI, 0.36–0.85) and 0.76 (95% CI, 0.63–0.88), respectively.

Biological Sample Collection

The protocol for biological sample collection and processing were standardized across both studies. Blood samples were collected at least 2 h postprandial from all the participants at recruitment. Knee synovial fluid was collected from all participants in the later KOA study and the earlier KOA study starting from the 41st participant onwards. The synovial fluid was aspirated directly from the index knee with a 20-gauge needle. In cases where no fluid was obtained, 10 ml of saline was injected into the joint and the fluid aspirated after gentle compression of the knee. Only participants with joint fluid obtained by direct aspiration was included in the current analyses (see below). The collected samples were centrifuged at 3,000 rpm for 15 min, aliquoted into separate vials, and stored in a −80°C freezer until analysis.

Biomarker Assessment

The following (s) and (sf) biomarkers were measured with commercially available enzyme-linked immunosorbent assay (ELISA) kits for innate immunity, LBP, CD14, and TLR4, and downstream proinflammatory biomarkers, IL6, IL8, and TNFα. The measurements were performed according to the manufacturer's guidelines. (s) biomarkers were measured in duplicates to obtain the intra-assay coefficient of variations (CVs), except that (s) TLR4 assays were run in singlicate due to exhaustion of sample volume. (sf) biomarkers were measured in singlicate. To ensure reliability for samples measured in singlicate, we measured pooled (s) or (sf) samples in each plate. The pooled (s) or (sf) samples and kit standards were run in duplicate to derive the CVs. All intra- and interassay coefficients of variation (CVs) of the kit standards and sample controls were within 15% limits (**Supplementary Table 1**).

As majority of the biomarkers of the lavage samples from knee aspirate fell below the lower limit of detection (LLOD), we decided to limit the analysis of (sf) biomarkers to those samples obtained via direct aspiration. Therefore, the analyses of (sf) biomarkers were limited to 47 participants in the earlier KOA study and 14 participants in the later KOA study. Less than 70% of (s) IL6 and IL8 concentrations were above the lower limit of detection (LLOD) in the earlier KOA study; these biomarkers in the earlier KOA study were analyzed as a categorical variable (0 = concentrations below LLOD and 1 = concentrations above LLOD).

Statistical Analysis

Majority of the biomarkers except (sf) and (s) IL6 and (s) IL8 were not normally distributed. Hence, we log transformed all biomarker data to achieve normality. We evaluated the associations between the (s) and (sf) biomarkers with MRI features, KL gradings, and WOMAC scoring using generalized linear models adjusted for age, sex, and body mass index (BMI). As MRI features and KL gradings were ordinal scales, the

ordinal logistic model type was used within the generalized linear models for these outcomes. Since we tested for two items within the domains of cartilage loss and BMLs, we adjusted the *p* values using *Bonferroni's* correction and considered $p < 0.025$ as statistically significant to reduce the likelihood of type I errors. For all other domains, $p < 0.05$ were considered as statistically significant. The analyses were conducted using SPSS version 25 (IBM Corp.).

RESULTS

Participants in Early KOA and Late KOA Cohort

The baseline characteristics of the 139 participants in earlier KOA study and 20 participants in later KOA study who had MRI data are summarized in **Table 1**. Characteristics of participants from both studies were similar to other KOA cohorts in terms of age, sex distribution, and BMI (31, 32).

Compared participants in the study with earlier stage of KOA, participants in the study of later stage of KOA were older with a higher BMI and higher KL grade of the index knee. Participants in the late KOA cohort also had higher percentages of diabetes mellitus and hypertension. The duration of KOA was longer for participants in the later KOA study. The concentration of biomarkers for innate immunity and inflammation were generally higher in the later KOA study.

Associations Between Synovial Fluid and Serum Biomarkers

Spearman's correlations between biomarkers are summarized in **Supplementary Table 2**. In both studies, (s) LBP correlated with the respective (sf) biomarkers. In the earlier KOA study, (s) and (sf) LBP correlated with proinflammatory markers IL6, IL8, and TNFα. (s) and (sf) TLR4 were correlated with each other in the earlier KOA study but not in the later KOA study. (s) and (sf) CD14 were not correlated with each other in both studies. In the later KOA study, (sf) CD14 was significantly correlated with (sf) IL6, IL8, and TNFα. The downstream proinflammatory biomarkers were generally correlated with each other.

Associations Between Inflammatory Biomarkers With MRI Features and Other Outcomes

In the earlier KOA study, (s) LBP was associated with meniscal extrusion, and (sf) CD14 was associated with effusion (**Table 2**). These associations persisted after adjustment for age, sex, and BMI. The presence of downstream proinflammatory biomarkers (s) IL6, was associated with osteophytes, synovitis, effusion, and meniscus extrusion, while that of (sf) IL6 was associated with effusion. Similarly, (s) TNFα was statistically significantly associated with osteophytes, cartilage loss, synovitis, and effusion after adjustment.

None of the biomarkers related to innate immunity was associated with symptoms or radiographic gradings. The presence of (s) IL6 was negatively associated with WOMAC function that marginally reached statistical significance.

In the later KOA study, (sf) LBP was associated with effusion and WOMAC function. (sf) CD14 was associated with effusion in univariate analysis but lost statistical significance after adjustments (**Supplementary Table 4**). (sf) CD14 was associated with BMLs after adjustment of age, gender, and BMI (**Table 3**). None of the biomarkers related to innate immunity was associated with radiographic gradings. (s) IL6 was associated with WOMAC pain and function, while (sf) IL8 was associated with BMLs.

DISCUSSION

Our study has demonstrated that biomarkers of innate immunity were associated with MRI features in both earlier and later stages of KOA. (sf) CD14 was positively associated with effusion on MRIs before and after adjustment for age, sex, and BMI in the earlier stage of KOA. In addition, (sf) CD14 was associated with BMLs in the later stage of KOA. Association between (s) LBP and meniscal extrusion was noted only in earlier stage KOA. In the earlier stage of KOA, the associations of a wider spectrum of downstream proinflammatory biomarkers with various MRI features were more prominently observed. In general, higher concentrations of all downstream proinflammatory biomarkers were observed in the participants with later stage of KOA than those with earlier stage of KOA.

Inflammation of the synovium has been observed in early KOA via synovial biopsies and MRI even before radiographical changes occurred (4, 33). There is growing evidence that synovitis is implicated in the symptoms and disease progression of KOA (34–37). Immunohistochemical studies have demonstrated that synovial tissue has a mononuclear cell infiltrate, particularly in early KOA (35, 38). This is consistent with our data that showed an association with (sf) CD14 and effusion in earlier stages of KOA. Soluble (sf) CD14 is a cellular marker that is predominantly found on activated macrophages and monocytes (39). Daghestani et al. have previously shown a positive association between baseline macrophage markers (sf) CD14 and (sf) and (s) CD163 with the abundance of activated macrophages in the synovium (9). These biomarkers were also associated with the radiographic progression of KOA after 3 years. This suggested that macrophage-related synovitis could be a driver of the structural damage in KOA and its progression. This is in line with murine models that have shown that synovial macrophages were involved in osteophyte formation and growth (40). Our current findings concur with the hypothesis that macrophages were associated with signs of inflammation (effusion) for the earlier stage of KOA, while (sf) CD14 was associated with BMLs in the later stage of KOA. The postulated upstream trigger, (sf) LBP, was associated with effusion in the later stage of KOA. In both studies, we did not observe an association of synovitis with innate immunity biomarkers. One possible explanation is that the synovitis scoring used in this study measures inflammation in the infra-patella fat pad, which may not be as in close relation to effusion or special imaging for activated macrophages used in other study (17, 41). In the later stage of KOA, (sf) CD14 continued to associate with a

TABLE 1 | Population characteristics of participants from early and late KOA studies.

	Earlier stage KOA study (*n* = 139)	**Later stage KOA study with MRI data (*n* = 20)**
Age, years[¶]	55.5 ± 7.8	56.1 ± 6.5
Female (%)	82.5	65.0
BMI, kg/m^2[¶]	26.0 ± 5.9	28.1 ± 4.8
Ethnicity, n, (%)		
Chinese	116 (81.1)	15 (75.0)
Malay	15 (10.5)	2 (10.0)
Indian	10 (7.0)	3 (15.0)
Others	2 (1.4)	0 (0)
Comorbidities, n, (%)		
Hypertension	43 (30.3)	7 (35.0)
Hyperlipidemia	55 (38.7)	7 (35.0)
Diabetes mellitus	12 (8.5)	1 (5.0)
Coronary artery disease	4 (2.8)	0 (0)
Duration of KOA, years[¶]	2.5 (4.9)	7.4 (7.1)
Fulfilled ACR Clinical and Radiographical Criteria for KOA (%)	43	100
KL grade of index knee, n, (%)		
0	40 (28.8)	1 (5.0)
1	44 (31.7)	5 (25.0)
2	35 (25.2)	6 (30.0)
3	18 (12.9)	5 (25.0)
4	2 (1.4)	3 (15.0)
WOMAC pain (0–100) [¶]	31.1 ± 18.4	42.1 ± 21.1
WOMAC function (0–100) [¶]	32.0 ± 19.9	44.9 ± 20.9
Biomarkers[¥]		
(s) LBP (ng/ml)	9079.0 (6745.0, 11794.0)	9466.5 (8072.3, 10514.3)
(sf) LBP (ng/ml) [£]	1796.9 (1448.5, 2416.4)	2012.9 (1579.6, 2315.6)
(s) TLR4 (pg/ml)	1049.0 (661.0, 1294.0)	699.5 (628.5, 1047.0)
(sf) TLR4 (pg/ml)[£]	875.2 (12.5, 1150.5)	3845.4 (3088.5, 4092.9)
(s) CD14 (ng/ml)	1254.8 (1145.0, 1374.7)	1327.5 (1223.8, 1555.5)
(sf) CD14 (ng/ml) [£]	255.3 (167.8, 400.8)	849.7 (681.5, 972.6)
(s) IL6 (pg/ml)	0.48 (0.48, 2.09)	3.35 (1.95, 5.65)
(sf) IL6 (pg/ml) [£]	87.45 (24.93, 248.06)	25.62 (12.43, 63.76)
(s) IL8 (pg/ml)	0.87 (0.32, 2.21)	4.95 (3.30, 9.15)
(sf) IL8 (pg/ml) [£]	5.09 (3.67, 9.22)	29.35 (24.02, 39.73)
(s) TNFα (pg/ml)	3.07 (1.10, 4.78)	4.75 (2.72, 5.66)
(sf) TNFα (pg/ml) [£]	1.62 (1.30, 1.90)	0.99 (0.74, 1.23)

Knee osteoarthritis (KOA) was classified according to the American College of Rheumatology Clinical and Radiographic Criteria for KOA.
[¶]Mean ± SD.
[¥] Median (interquartile range).
[£]Limit to participants with direct aspiration of synovial fluid.
BMI, body mass index; KL grade, Kellgren and Lawrence grading; WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index; (s), serum; (sf), synovial fluid; IL, interleukin; CD, cluster of differentiation; LBP, lipopolysaccharide binding protein; TNFα, tumor necrosis factor alpha; TLR4, Toll-like receptor 4.

wider spectrum of MRI features in different structures, including BMLs and marginally significant for cartilage loss. This suggested that innate immunity plays an important role in pathogenesis in different stages of KOA and may be particularly important in driving inflammation in the synovium and the downstream proinflammatory response in earlier KOA.

In the current study, we found that (s) LBP, which is a protein that binds to the LPS, was positively associated with meniscal extrusion on MRI in the earlier stage of KOA, while (sf) LBP was associated with effusion and WOMAC function in the later stage of KOA. It has been hypothesized that circulating lipopolysaccharide (LPS) may activate the synovial macrophages and contribute to the pathogenesis of OA (14). Studies have shown that systemic levels of LPS are elevated in obese patients even in the absence of an infection. Both animal and human studies have shown that obesity is linked to intestinal

TABLE 2 | Association of inflammatory biomarkers with MRI and other features in the earlier stage of KOA, adjusted with age, gender, and BMI.

Dependent variables	β (95% confidence intervals)											
	(s) LBP	**(sf) LBP**	**(s) TLR4**	**(sf) TLR4**	**(s) CD14**	**(sf) CD14**	**(s) IL6[§]**	**(sf) IL6**	**(s) IL8[§]**	**(sf) IL8**	**(s) TNFα**	**(sf) TNFα**
OST size	−0.43 (−2.33, 1.47)	0.15 (−1.59, 1.90)	0.13 (−0.43, 0.69)	−0.15 (−0.80, 0.50)	−0.63 (−4.93, 3.67)	−0.75 (−1.77, 0.28)	**0.87 (0.21, 1.54)**†**	−0.10 (−0.75, 0.55)	0.49 (−0.15, 1.13)	0.58 (−1.19, 2.35)	**0.91 (0.25, 7.21)**†**	−2.30 (−4.61, 0.01)
% Cart loss	0.47 (−1.42, 2.37)	1.07 (−0.86, 3.00)	0.11 (−0.41, 0.64)	−0.30 (−0.97, 0.38)	1.64 (−2.63, 5.90)	−1.34 (−2.66, −0.01)*	0.64 (−0.01, 1.29)	−0.26 (−0.87, 0.36)	0.31 (−0.31, 0.93)	0.23 (−1.51, 1.97)	**0.83 (0.18, 1.49)*†**	−2.78 (−5.23, −0.33)*
% Cart full thickness	−0.02 (−1.94, 1.91)	−0.19 (−2.16, 1.79)	0.52 (−0.04, 1.07)	0.40 (−0.28, 1.09)	0.97 (−3.30, 5.23)	−0.42 (−1.22, 0.37)	0.59 (−0.07, 1.25)	0.24 (−0.37, 0.85)	0.58 (−0.06, 1.23)	0.85 (−0.99, 2.69)	**0.91 (0.24, 1.58)**†**	−1.86 (−4.36, 0.64)
BML size	0.61 (−1.35, 2.56)	−0.13 (−1.81, 1.56)	0.38 (−0.15, 0.90)	0.05 (−0.61, 0.70)	0.73 (−3.51, 4.97)	−0.05 (−0.80, 0.69)	0.72 (0.06, 1.37)*	−0.08 (−0.74, 0.58)	0.55 (−0.09, 1.19)	1.01 (−0.88, 2.89)	0.73 (0.07, 1.38)*	0.36 (−1.82, 2.53)
% BML size	0.48 (−1.47, 2.44)	−0.14 (−1.83, 1.56)	0.33 (−0.20, 0.86)	0.11 (−0.54, 0.76)	1.08 (−3.17, 5.33)	−0.06 (−0.80, 0.69)	0.640 (−0.01, 1.29)	−0.07 (−0.73, 0.60)	0.59 (−0.05, 1.23)	1.06 (−0.84, 2.96)	0.71 (0.06, 1.37)*	0.44 (−1.73, 2.62)
Synovitis	1.38 (−0.75, 3.51)	1.03 (−0.79, 2.86)	0.49 (−0.18, 1.16)	0.06 (−0.59, 0.70)	2.93 (−1.90, 7.76)	0.71 (−0.18, 1.59)	**0.85 (0.17, 1.54)*†**	0.66 (−0.10, 1.41)	0.69 (−0.00, 1.38)	1.21 (−0.80, 3.22)	**1.21 (0.44, 1.99)**†**	0.07 (−2.56, 2.69)
Effusion	1.02 (−1.05, 3.08)	2.37 (−0.31, 5.05)	−0.19 (−0.74, 0.35)	0.20 (−0.51, 0.91)	2.45 (−2.22, 7.12)	**1.18 (0.123, 2.24)*†**	0.50 (−0.18, 1.17)	**1.20 (0.42, 1.98) **†**	0.68 (0.01, 1.35)*	1.16 (−0.85, 3.18)	**0.93 (0.22, 1.64)**†**	1.63 (−1.45, 4.72)
Meniscus extrusion	**3.10 (0.47, 5.74)*†**	−0.10 (−2.18, 1.98)	−0.48 (−1.20, 0.25)	0.32 (−0.46, 1.09)	3.02 (−2.57, 8.61)	0.02 (−0.80, 0.83)	**0.87 (0.07, 1.67)*†**	0.74 (−0.06, 1.54)	0.01 (−0.83, 0.85)	2.54 (−0.20, 5.28)	0.49 (−0.43, 1.42)	−0.61 (−3.25, 2.04)
KL grade	0.24 (−1.72, 2.19)	−0.30 (−2.25, 1.65)	0.47 (−0.13, 1.06)	0.50 (−0.18, 1.18)	2.32 (−2.11, 6.75)	0.10 (−0.67, 0.87)	0.53 (−0.14, 1.20)	0.10 (−0.59, 0.79)	0.19 (−0.45, 0.83)	−0.11 (−2.07, 1.85)	0.55 (−0.10, 1.20)	−2.03 (−4.67, 0.61)
WOMAC pain	3.51 (−16.32, 23.33)	−7.93 (−28.18, 12.32)	1.40 (−3.87, 6.66)	5.03 (−1.50, 11.55)	19.85 (−24.37, 64.06)	−0.70 (−9.89, 8.48)	−2.18 (−8.64, 4.27)	−3.57 (−9.67, 0.25)	2.06 (−4.21, 8.34)	−5.27 (−21.19, 10.67)	4.30 (−1.98, 10.58)	−13.71 (−35.84, 8.43)
WOMAC function	−11.85 (−32.72, 9.02)	−7.82 (−27.20, 11.57)	−0.50 (−6.06, 5.05)	5.23 (−0.90, 11.36)	13.24 (−33.38, 59.86)	−1.66 (−10.32, 7.00)	**−7.00 (−13.72, −0.25)*†**	−3.17 (−8.94, 2.61)	0.89 (−5.74, 7.52)	−3.77 (−18.86, 11.31)	−2.28 (−8.92, 4.36)	−6.16 (−27.31, 14.99)

**p < 0.05, **p < 0.01, † Bonferroni–adjusted p < 0.025 for cartilage loss and BMLs, p < 0.05 for all other domains (Bold).*

§ (s) IL6, IL8 and (sf) TLR4 were analyzed as categorical variable as more than 75% less than Lower limit of detection (0 = Below lower Limit of Detection and 1 = Above lower Limit of Detection).

MRI, Magnetic Resonance Imaging; %, percentage; BML, Bone Marrow Lesion; Cart, Cartilage; OST, Osteophyte; KL grade, KL, Kellgren and Lawrence grading; (sf), synovial fluid; (s), serum; IL, interleukin; CD, cluster of differentiation; TNFα, Tumor Necrosis Factor alpha; LBP, Lipopolysaccharide Binding Protein; TLR, Toll–like receptor; vs., versus.

TABLE 3 | Association of inflammatory biomarkers with MRI and other features in the later stage of KOA, adjusted with age, gender and BMI.

Dependent variables	β (95% confidence intervals)											
	(s) LBP	**(sf) LBP**	**(s) TLR4**	**(sf) TLR4**	**(s) CD14**	**(sf) CD14**	**(s) IL6**	**(sf) IL6**	**(s) IL8**	**(sf) IL8**	**(s) TNFα**	**(sf) TNFα**
OST size	3.30 (−8.98, 15.57)	11.33 (−3.60, 26.25)	−0.67 (−2.42, 1.09)	11.43 (−5.71, 28.57)	−4.67 (−20.86, 10.93)	4.41 (−5.30, 3.35)	0.42 (−1.44, 2.27)	−0.30 (−2.82, 2.22)	−2.37 (−5.43, 0.69)	6.72 (−5.37, 18.81)	−3.91 (−8.21, 0.38)	−2.69 (−14.48, 9.10)
% Cart loss	4.49 (−7.37, 16.36)	−24.84 (−1.75, 0.71)	−1.39 (−3.44, 0.65)	5.91 (−12.29, 24.10)	−12.87 (−30.91, 5.91)	2.08 (−0.26, 4.42)	0.59 (−1.85, 3.02)	−0.86 (−0.36, 1.89)	−3.50 (−7.48, 0.49)	6.64 (−5.94, 19.22)	−0.13 (−4.45, 4.18)	10.14 (−5.31, 25.58)
% Cart full thickness	4.69 (−7.34, 16.72)	17.01 (2.01, 32.05)*	−1.81 (−4.03, 0.40)	6.60 (−9.11, 22.31)	−15.84 (−33.03, 1.35)	2.79 (0.32, 5.25)*	0.51 (−1.91, 2.93)	−1.02 (−3.50, 1.46)	−1.84 (−5.05, 1.37)	6.21 (−4.65, 17.06)	−1.13 (−5.47, 3.21)	3.85 (−7.85, 15.55)
BML size	−0.15 (−11.06, 10.76)	0.38 (−8.92, 9.67)	−0.37 (−1.78, 1.04)	1.40 (−14.61, 17.41)	−5.88 (−23.49, 11.74)	**9.06 (1.62, 16.49)*†**	0.99 (−1.05, 3.03)	0.58 (−1.52, 2.67)	−1.92 (−5.32, 1.48)	15.90 (1.06, 30.75)*	0.44 (−3.56, 4.44)	7.18 (−7.63, 21.99)
% BML size	1.16 (−10.39, 12.71)	1.84 (−7.24, 10.92)	−0.42 (−1.86, 1.02)	−2.6 (−18.28, 13.04)	−9.76 (−27.80, 8.27)	**9.06 (1.65, 16.48)*†**	0.79 (−1.21, 2.80)	0.65 (−1.49, 2.79)	−2.04 (−5.51, 1.42)	**18.54 (3.68, 33.40)*†**	−0.07 (−4.12, 3.98)	11.57 (−3.27, 26.30)
Synovitis	1.81 (−9.33, 12.94)	5.83 (−4.39, 16.04)	−0.32 (−1.92, 1.28)	8.92 (−9.73, 27.57)	3.14 (−14.58, 20.86)	1.74 (−0.55, 4.03)	−0.60 (−2.86, 1.66)	−0.43 (−2.74, 1.88)	−3.04 (−6.67, 0.58)	8.13 (−3.93, 20.19)	1.54 (−2.74, 5.82)	7.02 (−6.34, 20.37)
Effusion	13.87 (−0.08, 27.81)	**14.07 (0.49, 27.66)***	−0.47 (−1.97, 1.05)	1.17 (−16.16, 18.50)	−10.57 (−28.29, 7.15)	8.25 (−12.66, 29.16)	0.46 (−1.60, 2.52)	0.07 (−2.49, 2.63)	−0.75 (−3.74, 2.25)	2.53 (−8.47, 13.53)	−0.30 (−4.47, 3.88)	15.91 (−1.67, 33.48)
Meniscus extrusion	3.02 (−9.65, 15.68)	7.32 (−2.91, 17.54)	−0.71 (−2.28, 0.86)	−5.70 (−20.36, 8.97)	−12.80 (−30.09, 4.48)	1.55 (−0.90, 4.00)	−0.60 (−2.68, 1.49)	−0.20 (−1.74, 2.40)	−2.82 (−6.16, 0.51)	2.56 (−7.95, 13.08)	−2.30 (−6.43, 1.83)	1.53 (−9.71, 12.77)
KL grade	2.84 (−8.17, 13.85)	−4.21 (−14.43, 6.01)	−0.62 (−2.19, 0.96)	−12.38 (−31.86, 7.09)	−9.04 (−26.06, 7.97)	2.19 (−0.77, 5.14)	0.52 (−1.53, 2.57)	343.7 (−38,869, 39,556)	−1.47 (−4.77, 1.83)	7.31 (−4.19, 18.81)	−0.83 (−4.92, 3.27)	8.96 (−5.32, 23.23)
WOMAC pain	88.40 (−9.30, 186.1)	−1.47 (−3.94, 1.00)	−3.58 (−18.60, 11.43)	27.26 (−102.1, 156.7)	**−148.8 (−290.4, −7.13)*†**	9.72 (−5.70, 26.15)	**26.91 (10.63, 43.20)**†**	−2.43 (−20.22, 15.37)	−16.45 (−44.92, 12.02)	−10.98 (−62.86, 40.90)	−8.05 (−46.38, 30.28)	26.54 (−44.63, 97.71)
WOMAC function	71.45 (−34.24, 177.15)	**97.60 (29.88, 165.3)**†**	−0.58 (−16.41, 15.25)	77.64 (−67.94, 223.2)	−66.05 (−227.0, 94.93)	−4.05 (−22.16, 14.05)	**25.50 (7.62, 43.39)**†**	−8.54 (−28.05, 10.98)	−10.12 (−40.60, 20.37)	−35.14 (−90.75, 20.48)	−16.17 (−55.92, 23.58)	−2.92 (-84.29, 78.45)

*$p < 0.05$, **$p < 0.01$.

†*Bonferroni-adjusted $p < 0.025$ for cartilage loss and BMLs, $p<0.05$ for all other domains bold).*

MRI, magnetic resonance imaging; %, percentage; BML, bone marrow lesion; Cart, cartilage; OST, osteophyte; KL grade, Kellgren and Lawrence grading; (sf) synovial fluid; (s), serum; IL, interleukin; CD, cluster of differentiation; TNFα, tumor necrosis factor alpha; LBP, lipopolysaccharide binding protein; TLR, Toll-like receptor; vs., versus.

permeability (10–12). In a healthy environment, the intestinal mucosa provides a selectively permeable membrane between the systemic circulation and the intestinal lumen (13). A high-fat diet, however, disrupts the expression and localization of the tight junction protein in the small intestine that results in an increased permeability of the intestine (10, 42) and causes the LPS in the gut to be released in the systemic circulation. The LPS in the systemic circulation binds to LBP and may subsequently activate the macrophage by binding to CD14 and TLR4 (13). The activation of the macrophage can result in the synthesis of proinflammatory cytokines such as IL6, IL8, and TNFα, leading to degeneration of articular cartilage (16, 43). Huang et al. have previously shown that both (s) and (sf) LPS and LBP in KOA patients were associated with the abundance of activated macrophages in the synovium, radiographic severity, and KOA symptoms (17). In another study, the change in (s) LPS/LBP over 12–18 months were associated with radiographic progression of KOA, while the change in (s) TLR4 over 18 months was associated with cartilage degradation marker (CTXII) (44). The finding of our study therefore concurs with the role of LPS/LBP pathways in association with the innate immunity in the pathogenesis of KOA.

Our study found that the associations between the downstream proinflammatory biomarkers with MRI-detected BMLs and synovial inflammation were more prominently seen in the earlier stage of KOA as compared to the later stage of KOA. A greater intensity of inflammation in OA during the early phase has been described in other studies (35, 38, 45). Although inflammatory biomarkers were found in all stage of KOA, Smith at al. have shown a decrease in the ratio of IL1 receptor antagonist to IL1α and β with increasing radiographic grades of KOA (38). Benito et al. have demonstrated a significantly greater CD4+ and CD68+ cell infiltration, number of cells producing TNFα and IL1β, together with greater blood vessel formation, vascular endothelial growth factor, and intercellular adhesion molecule-1 expression in the synovial tissue taken from participants undergoing arthroscopy for knee pain compared to those undergoing knee replacement surgery for late-stage KOA (35). Similarly, Ene et al. have observed a more extensive synovitis with mononuclear and macrophage infiltrates, diffused fibrosis, thickening of the lining layer, and neovascular formation in participants with earlier KOA who underwent arthroscopy compared to those with end-stage KOA who underwent knee replacement surgery (45). One possible reason that explains the relative less associations we observed between proinflammatory biomarkers and MRI features in the later stage of KOA in the current study lies in the semiquantitative MRI scoring system. The number of subregions affected introduces substantial ceiling effect in the later stage of KOA, which limited the capacity of showing association with increasing levels of proinflammatory biomarkers. Taken together, our study supports a prominent role of inflammation particularly in earlier stage of KOA.

The strength of our study is having two studies at different stages of KOA. Paired (s) and (sf) samples were collected together with MRI imaging that provided detailed assessment of various tissue structure of the knee as compared to radiography. The concentration of the measured biomarkers was consistent with other studies (17, 44). There are a few limitations in the interpretation for our study. The cross-sectional study design showed associations rather than causal relationship between the biomarkers and KOA. The associations may give insight into the pathogenesis of KOA, but the study design does not allow evaluation of pathogenic mechanisms. The associations between various biomarkers were not readily interpretable clinically. Further research is required for the validation of current findings. Nonetheless, it provides evidence to support the role of innate immunity in all stages of KOA. The sample size of the study in the later stage of KOA was small and may result in limited statistical power to demonstrate small associations. These results need to be validated with a larger cohort. The (sf) analyses were only limited to participants whose joint fluid was obtained via direct aspiration, as majority of biomarker concentrations from lavage samples fall in undetectable ranges. Hence, the results for (sf) may only apply to participants with more joint effusion. We recognize that the classification of earlier and later stages of KOA was arbitrary. In the continuum of progression of chronic illness like KOA, a cutoff between early and late stage would be difficult, and there is currently no standardized definition for early KOA. With the recruitment criteria in these studies, one with knee pain in the past 1 month while another seeking active treatment, we believe that participants from the two studies generally represented different stages KOA, and this is also reflected by the lower KL scoring in the index knees and shorter duration of illness in the earlier KOA studies. Associations of biomarkers with MRI features in either study were performed separately with adjustment for only age, sex, and BMI. We did not perform any multivariable analysis, and no formal statistical comparisons between studies have been made. Therefore, the study can only be considered exploratory, which gives preliminary insight into different stages of KOA. In addition, the recruitment was based on clinical justifications with assistive radiographies of both knees read by the attending rheumatologist, which resulted in 1.4% of participants in the study of earlier KOA who had KL = 4 and 30% of participants in study of the later KOA who had KL <2 in the index knee as read by the radiologist. Lastly, participants in both studies had KOA and persistent pain; no comparison of the biomarker concentrations with healthy controls has been conducted in the current study.

In conclusion, the present exploratory study supports the association between biomarkers of activated macrophages and synovial inflammation in the earlier stage of KOA. This may be related to activation of the innate immunity via LPS and LBP. In addition to synovial inflammation, innate immunity activities were associated with BMLs and cartilage loss in the later stage of KOA. This suggests the important role of innate immunity in different KOA stages.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The SingHealth Centralized Institutional Review Board E. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Y-YL and CAM conceptualized and designed the study. SR, CAM, JRT, SW, and Y-YL acquired the data. SR, Y-YL, and JL performed the data analysis. SR and Y-YL drafted the manuscript. All authors interpreted the data, critically revised the manuscript, and approved the final version of manuscript.

FUNDING

This study was supported by the National Medical Research Council, Singapore (NMRC/CSA-INV/0022/2017), and the Lee Foundation Grant. The funding sources had no role in the study design, data collection, analysis, interpretation of data, manuscript writing, or decision to submit.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.554669/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

REVIEW
published: 18 January 2021
doi: 10.3389/fmed.2020.607186

The Damage-Associated Molecular Patterns (DAMPs) as Potential Targets to Treat Osteoarthritis: Perspectives From a Review of the Literature

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During the osteoarthritis (OA) process, activation of immune systems, whether innate or adaptive, is strongly associated with low-grade systemic inflammation. This process is initiated and driven in the synovial membrane, especially by synovium cells, themselves previously activated by damage-associated molecular patterns (DAMPs) released during cartilage degradation. These fragments exert their biological activities through pattern recognition receptors (PRRs) that, as a consequence, induce the activation of signaling pathways and beyond the release of inflammatory mediators, the latter contributing to the vicious cycle between cartilage and synovial membrane. The primary endpoint of this review is to provide the reader with an overview of these many molecules categorized as DAMPs and the contribution of the latter to the pathophysiology of OA. We will also discuss the different strategies to control their effects. We are convinced that a better understanding of DAMPs, their receptors, and associated pathological mechanisms represents a decisive issue for degenerative joint diseases such as OA.

Keywords: osteoarthritis, cartilage, immunity, inflammation, synovitis

OPEN ACCESS

Edited by:
George Bertsias,
University of Crete, Greece

Reviewed by:
Mohit Kapoor,
University of Toronto, Canada
Changhai Ding,
Southern Medical University, China

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Specialty section:
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

Received: *16 September 2020*
Accepted: *11 November 2020*
Published: *18 January 2021*

Citation:
Lambert C, Zappia J, Sanchez C, Florin A, Dubuc J-E and Henrotin Y (2021) The Damage-Associated Molecular Patterns (DAMPs) as Potential Targets to Treat Osteoarthritis: Perspectives From a Review of the Literature. Front. Med. 7:607186. doi: 10.3389/fmed.2020.607186

INTRODUCTION

Osteoarthritis (OA) is the most common joint disease affecting more than 70 million people across the United States (*CDC: Arthritis: At a Glance*) and Europe (1). As underlined by many authors, it has long been considered as "a wear and tear disease" of cartilage associated with age, it is in reality a complex disorder affecting the "whole joint" (2) and the pro-inflammatory pathways of immunity that can culminate in illness (3–5).

During the osteoarthritis (OA) process, activation of immune systems, whether innate or adaptive, is strongly associated with low-grade systemic inflammation (4, 6–10) (**Figure 1**). This process was initiated and driven in the synovial membrane, especially by damage-associated molecular patterns (DAMPs) released from the extracellular matrix (ECM) to the joint cavity during cartilage degradation (4, 11–13). Briefly, these fragments released into the synovial cavity stimulate the production and release of inflammatory mediators (cytokines, chemokines, lipid mediators, and DAMPs themselves) by the synovial cells (macrophages and fibroblasts) into the

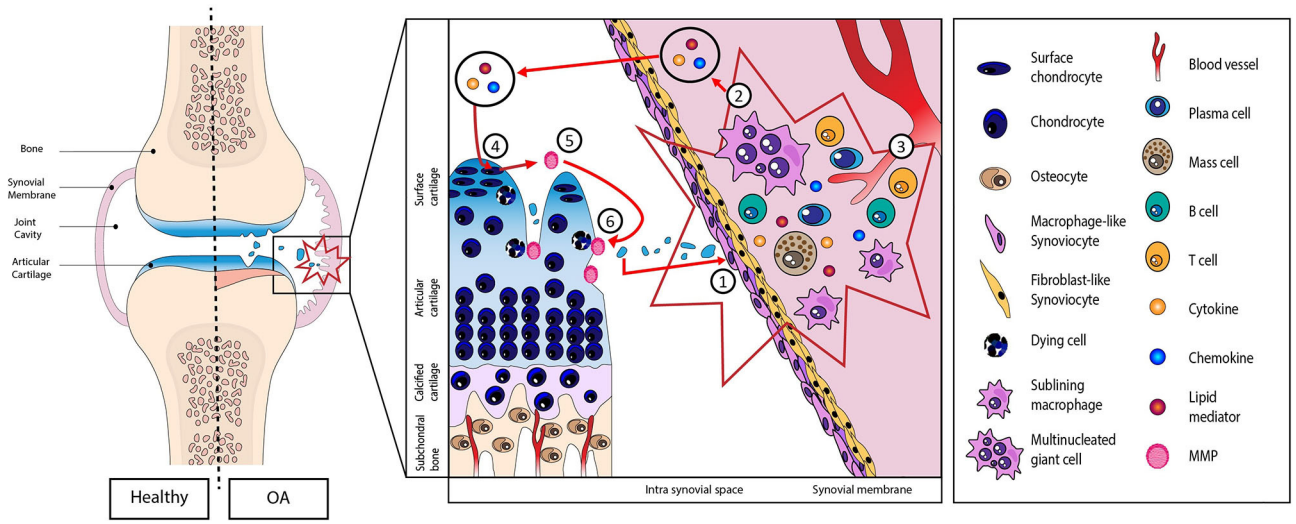

FIGURE 1 | Schematic representation of the role of damage-associated molecular patterns (DAMPs) in the initiation and perpetuation of the low-grade systemic inflammation. (1) DAMPs released from extracellular matrix to the joint cavity during cartilage degradation. (2) Proliferation and hyperplasia of the lining cells along with inflammatory cell infiltration and (3) neoangiogenesis. (4) Production of inflammatory mediators (cytokines, chemokines, lipid mediators, and DAMPs themselves) into the synovial fluid. (5) These mediators then activate chondrocytes that in turn produce metalloproteinase resulting in a vicious cycle (6) between cartilage and synovial membrane.

synovial fluid. These mediators, in turn, activate chondrocytes that produce metalloproteinase, resulting in a vicious cycle between cartilage and synovial membrane (12).

DAMPs are defined as endogenous stimuli that are released either from ECM or from dying cells (14). "*Intracellular*" DAMPs consist of a set of immunogenic molecules released from the breakdown of necrotic and apoptotic cells such as calcium-binding protein S-100, high-mobility group box protein 1 (HMGB1), or uric acid, while "*extracellular*" DAMPs correspond to the ECM components (glycoproteins, proteoglycans, or glycosaminoglycans). The biological activity of these DAMPs goes through pattern recognitions receptors (PRRs) including Toll-like receptors (TLRs), NOD-like receptors (NLRs), and Receptor for Advanced Glycosylation End products (RAGEs) (15). These PRRs have been identified, notably, on the surface of immune cells, chondrocytes, osteoblasts, and synoviocytes. The binding of DAMPs to these receptors initiates downstream signaling cascades leading to the activation of several transcription factors, such as notably, the nuclear factor-κB (NF-κB), an inflammatory response key regulator (16). This activation leads to the release of various factors like catabolic factors [matrix metalloproteinase (MMP)-1,−3,−9, and−13], cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6], chemokines [C-C motif chemokine ligand (CCL)-2,−5,−7,−8], cathepsins (B, K, and L), and complement cascade (17), factors described as essential in OA pathogenesis.

The aim of this review is to focus on the roles of DAMPs in the pathogenesis of OA. We have also researched the ways to block DAMP activity and summarized the current therapeutic approaches targeting DAMPs activity.

In this context, the literature search was performed using the Pubmed/Medline database between January 2010 and April 2020. All original papers, systematic and narrative reviews, were included. Searches were performed using the search terms "osteoarthritis," "cartilage," "synovium," "DAMP," and "immunity." Papers published in English and reporting on the search criteria were included in this manuscript, while duplicates were removed from the selection. As a consequence, 98 articles

Abbreviations: OA, osteoarthritis; DAMPs, damage-associated molecular patterns; ECM, extracellular matrix; HMGB1, high-mobility group box protein 1; PRRs, pattern recognition receptors; TLRs, Toll-like receptors; NLRs, NOD-like receptors; RAGEs, Receptor for Advanced Glycosylation End products; NF-κB, nuclear factor-κB; MMP, matrix metalloproteinase; TNF, tumor necrosis factor; CCL, C-C motif chemokine ligand; ADAMTS, A Disintegrin And Metalloproteinase with Thrombospondin Motifs; Fn, fibronectin; IL, interleukin; HA, hyaluronan; NO, nitric oxide; MyD88, myeloid differentiation primary response 88; NLRP3, NOD-like receptor family, pyrin domain containing 3; TN-C, tenascin-C; EGF-L, epidermal growth factor-like; FBG, fibrinogen-like globe; PRG4, lubricin/proteoglycan 4; PRELP, proline-arginine-rich-end-leucine-rich repeat protein; SLRP, small leucine-rich repeat protein; MAC, membrane attack complex; ERK, extracellular signal-regulated kinase; RA, rheumatoid arthritis; FnEDA, fibronectin extra domain A isoform; Col2A1, collagen type II alpha 1 chain; NC4, non-collagenous domain 4; COMP, cartilage oligomeric matrix protein; BSP-1, bone sialoprotein 1; SIBLINGs, small integrin-binding ligand N-linked glycoproteins; PGE_2, prostaglandin E2; VEGF, vascular endothelial growth factor; MCP-1, monocyte chemoattractant protein-1; S100A8, S100 calcium-binding protein A8; S100A9, S100 calcium-binding protein A9; S100A12, S100 calcium-binding protein A12; CPPD, calcium pyrophosphate deposition; BCP, basic calcium phosphate; TRIF, TIR domain-containing adaptor-inducing interferon; TRAM, TRIF-related adaptor molecule; Mal, MyD88-adaptor like; PI3K, phosphoinositide 3-kinase; ICAM-1, intercellular adhesion molecule 1; CMC-I, carpometacarpal-I; NODs, nucleotide-binding oligomerization domains; NALP, Nacht domain-containing, leucine-rich repeat-containing and pyrin domain-containing protein; ASC, C-terminal caspase recruitment domain; AGEs, advanced glycation end-products; PPARγ, peroxisome proliferator-activated receptor γ; PGD_2, prostaglandin D2; CXCL-1, chemokine (C-X-C motif) ligand 1; Cox-2, cyclooxygenase 2; mAb, monoclonal antibody; sRAGE, soluble RAGE.

were analyzed, and their relevant data were included in this narrative review.

EXTRACELLULAR DAMAGE-ASSOCIATED MOLECULAR PATTERNS FROM CARTILAGE EXTRACELLULAR MATRIX

Current evidences indicate that endogenous molecular products derived from ECM disruption can function as DAMPs to activate PRRs (14, 18). MMPs and/or aggrecanases [a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and−5] are able to cleave a large number of ECM molecules (**Table 1**), leading to the exposure of cryptic epitopes and recognition with ligand receptors (18). Inflammatory mediators produced may in turn stimulate the production of cartilage-degrading enzymes and recruitment of inflammatory cells, thus establishing a vicious cycle between cartilage and synovial membrane that contributes to OA progression.

Homandberg and Hui (19) suggested that ECM breakdown fragments may promote inflammation and cartilage loss. So, during cartilage degradation, proteolytic cleavage of fibronectin (Fn) generates fibronectin fragments with cartilage chondrolytic activities. These are exercised through the increase of MMP expression, the suppression of proteoglycan synthesis, or the increase of cytokines. They highlighted that an amino-terminal 29-KDa fibronectin fragment (Fn-f) was able to induce, in human articular cartilage explant cultures, the production of not only pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-1α, but also MMPs, MMP-1 and−3. In human chondrocytes, Hwang et al. (20) also demonstrated that Fn-f was able to regulate cartilage catabolism through TLR-2. Furthermore, Fn-f is also able to upregulate TLR-2 expression through IL-1ra, suggesting an autocrine/paracrine regulation of IL-1 activity (21).

Hyaluronan (HA) can be described as a non-sulfated component of the ECM, commonly and abundantly found in the synovial fluid. Exogenous HA is injected in knee joints with the aim to treat joint inflammation through a mechanical effect leading to the inhibition of inflammatory pathways, stimulation of cartilage anabolism, and reduction of free radical production (22). However, HA action seems related to its molecular mass, HA of high molecular weight being anti-inflammatory and inversely for low-molecular-weight HA (23). In this context, low-molecular-weight HA, resulting from the HA degradation at sites of inflammation and tissue injury, induced nitric oxide (NO) and MMP production by mechanisms dependent on CD44 and myeloid differentiation factor 88 (MyD88) through TLR-2,−4 (22). The fragmentation products of hyaluronic acid containing sugar units of 4–16 oligosaccharide size have also been demonstrated to act as potent activators of dendritic cells and macrophages *via* TLR-4 (24). Yamasaki et al. (25) also demonstrated that small HA oligosaccharides activate inflammasome through NOD-like receptor family, pyrin domain containing 3 (NLRP3) and release of IL-1β.

Tenascin-C (TN-C) belongs to the ECM glycoprotein family. It is involved in tissue injury and repair. In OA, its expression is upregulated in cartilage and synovium. TN-C is also elevated in OA synovial fluid when compared to healthy one. Sofat et al. (26) demonstrated that TN-C fragments [the epidermal growth factor-like (EGF-L) and Fn type III domains 3–8 of TN-C] contributes to cartilage matrix degradation by inducing aggrecanase activity. Recently, Midwood et al. (27) also highlighted that TN-C induces cytokine production (TNF-α, IL-6, and IL-8) through the activation of TLR-4 in human macrophages and synovial fibroblasts. Zuliani-Alvarez et al. (28) have identified three distinct sites within the C-terminal fibrinogen-like globe (FBG) domain of TN-C contributing to TLR-4 activation.

Lubricin/proteoglycan 4 (PRG4) is a mucin-like glycoprotein. It is present at the surface of articular cartilage and contributes to the maintenance and integrity of the joint. Decreased expression of PRG4 is associated with OA progression (29). However, recently, Iqbal et al. (30) demonstrated in synovial cells that the full-length recombinant human PRG4 can regulate the immune response *via* TLRs (TLR-2,−4, and−5) and, therefore, modifies cytokine and chemokine secretion. Thus, the PRG4/TLR binding activating the NF-κB pathway is involved in maintaining the homeostatic state of the cell. However, when TLR-2,−4, or−5 is bound to another agonist, in turn, PRG4 activates inflammatory responses *via* an alternative pathway that does not appear to be nuclear factor NF-κB dependent (30).

Decorin, biglycan, fibromodulin, lumican, PRELP (proline-arginine-rich-end-leucine-rich repeat protein), chondroadherin, and osteoadherin are members of the small leucine-rich repeat protein (SLRP) family, as reviewed by Zappia et al. (31). Fibromodulin is a keratan sulfate proteoglycan found in cartilage and tendon. Sjöberg et al. (32) showed that fibromodulin triggers complement activation. Sjöberg et al. (33) revealed that fibromodulin upregulated the membrane attack complex (MAC) from human OA sera. In addition, these authors also demonstrated that osteoadherin and chondroadherin, like fibromodulin binds C1q and activates classical pathway (33). In macrophages, biglycan, a small leucine-rich proteoglycan, has been demonstrated to act as an endogenous ligand of TLR-4 and TLR-2. This binding results in a rapid activation of p38, extracellular signal-regulated kinase (ERK), and NF-κB and, subsequently, the stimulation of TNF-α and macrophage inflammatory protein-2 (MIP-2) expression (34). Barreto et al. (35) also demonstrated that soluble biglycan is commonly detected in knee synovial fluid of patients with advanced knee OA or rheumatoid arthritis (RA). Soluble biglycan upregulates TLR-4 expression in human OA chondrocytes, increases both expression and concentrations of catabolic factors (ADAMTS-4, ADAMTS-5, MMPs, NO, cathepsin K, IL-6, and IL-8), and decreases the expression of matrix components (collagen type II, aggrecan), globally resulting in net loss of cartilage (35). Recently, Avenoso et al. (36) also reported that human chondrocytes treated with biglycan produces several inflammatory mediators (IL-1β, IL-6, MMP-13, and IL-17) and activates NF-κB and TLR-4 (36). Conversely, biglycan and decorin can also bind to C1q and then inhibit the classical pathway (37).

Fn, whose fragments were found increased in OA cartilage and synovial fluid, was also identified as an activator of TLR (38). Two Fn domains have been identified as TLR

TABLE 1 | Overview of DAMPs and their implications in the OA pathogenesis.

DAMPs	Receptors	Activated signaling pathway	Biological effects	Species	References
Extracellular DAMPs					
Fibronectin fragments	TLR-2	MyD88	- ↑ catabolic cytokines - ↑ MMPs Suppression proteoglycan synthesis	Human	(19)
	/	IL-1ra	- ↑ TLR-2 expression	Human	(20, 21)
Hyaluronan (Low molecular weight)	TLR-2;−4	CD44 and MyD88	- NO and MMP production - Dendritic cell and macrophage activation	Human, Mouse	(22, 24)
	NLRP3		- IL-1 release	Mouse	(25)
Tenascin-C	TLR-4		- Cytokine synthesis (TNF-α, IL-6,−8)	Human	(27, 28)
Lubricin	TLR-2,-4,-5		- Anti-inflammatory effect (↓ cytokine expression)	Human, Rat	(30)
Fibromodulin, Osteoadherin Chondroadherin	C1q	Classical pathway	- MAC upregulation	Human	(33)
Biglycan	TLR-2,−4	P38, ERK and NF-κB	- ↑ TNF-α and MIP-2 expression - ↑TLR-4 expression - ↑ Catabolic factor expression - ↓ Matrix component expression	Human, Mouse	(34–36)
	C1q	Classical pathway	- Inhibitory function on the classical pathway	Human	(37)
Fibronectin	TLR-4	P38 and NF-κB	- Cytokine release from mast cells and T cells	Mouse, Human	(39–41)
	TLR-2	MyD88	- Catabolic responses (MMP-3 upregulation, cleavage of fibronectin, or type II collagen)	Human	(20)
Native Type II collagen	DDR2	P38 and NF-κB	- Cytokine and MMP induction	Human	(42)
N-terminal telopeptide of collagen type II (29-mer)	/	Protein kinase C and p38	- ↑Cathepsins B, L, and K - ↑MMP-2,−3,−9, and -13	Human, Bovine	(43, 44)
24-mer synthetic peptide of type II collagen (CB12-II)	/	PI3K/Akt and NF-κB	- MMP-13 induction	Bovine, Human	(45, 46)
Coll2-1	TLR-4	NF-κB	- ↑ IL-8 - ↑ MMP-3	Human	(47)
Aggrecan 32-mer fragment	TLR-2	NF-κB	- ↑ Protease expression (MMP-13 and ADAMTS-5) - ↓ Col2A1 and aggrecan expression	Mouse, Human	(48)
Collagen IX (NC4)	C4, C3, and C9		- Inhibition of complement activation	Human	(49)
COMP	C3b	Alternative pathway	- Activation complement system	Human	(50)
	C1Q	Classical and lectin pathways	- Inhibition complement system	Human	(50)
Intracellular DAMPs					
Gc-globulin, α1-microglobulin, α2-macroglobulin	TLR-4		- ↑Inflammatory cytokine and growth factor production	Human	(56)
Fibrinogen	TLR-4	NF-κB	- ↑ Chemokine production Attraction of T cells, neutrophils, and additional macrophages	Mouse, Human	(58–60)
S100A8/S100A9	TLR-4	NF-κB	- ↓ Anabolic factor production - ↑Catabolic factor production Osteophyte formation Synovitis - ↑ Knee symptoms, cartilage defects, and MMP-3 serum levels	Mouse, Human	(61–64)
AIIt	TLR-4	MAPK and NF-κB	- Macrophage activation - Inflammatory mediator secretion	Human	(65, 66)
S100A12	RAGE	p38 and NF-κB	- ↑ MMP-13 and VEGF expression and release	Human	(67)

(Continued)

TABLE 1 | Continued

DAMPs	Receptors	Activated signaling pathway	Biological effects	Species	References
HMGB1	RAGE, TLR-2,-4	ERK and NF-κB	- Promotes chemotaxis - ↑ Cytokines, chemokines, and MMP expression	Human	(71–74)
CPPD, BCP	TLR-2, NRLP3	MAPK, NF-κB	- ↑MMPs, prostaglandin and inflammatory cytokine production - NO production - Neutrophil apoptosis inhibition	Mouse, Human, Bovine	(77–80)

OA, osteoarthritis; DAMPs, damage-associated molecular patterns; ADAMTS, A Disintegrin and Metalloproteinase with Thrombospondin Motifs; HMGB1, high-mobility group box protein 1; TLRs, Toll-like receptors; RAGEs, Receptor for Advanced Glycosylation End products; NF-κB, nuclear factor-κB; MMP, matrix metalloproteinase; TNF, tumor necrosis factor; IL, interleukin; HA, hyaluronan; NO, nitric oxide; MyD88, myeloid differentiation primary response 88; NLRP3, NOD-like receptor family, pyrin domain containing 3; MAC, membrane attack complex; ERK, extracellular signal-regulated kinase; Col2A1, collagen type II alpha 1 chain; NC4, noncollagenous domain 4; COMP, cartilage oligomeric matrix protein; BSP-1, bone sialoprotein 1; SIBLINGs, small integrin-binding ligand N-linked glycoproteins; VEGF, vascular endothelial growth factor; S100A8, S100 calcium-binding protein A8; S100A9, S100 calcium-binding protein A9; S100A12, S100 calcium-binding protein A12; CPPD, calcium pyrophosphate deposition; BCP, basic calcium phosphate; PI3K, phosphoinositide 3-kinase.

activators: the extra Type III domain and FnEDA. These domains stimulate TLR-4-dependent cytokine release from mast cells and T cells (39, 40). Kelsh et al. (41) also identified NF-κB and p38 signaling pathways as transducers of Fn-f/TLR signals. Hwang et al. (20) demonstrated in human chondrocytes the probable involvement of MyD88-dependent TLR-2 signaling pathway in Fn fragment release and mediated cartilage catabolic responses.

Type II collagen-derived peptides also seem to act as potent activators of innate immunity. In human chondrocytes, Klatt et al. (42) have observed the collagen II-dependent induction of both cytokines (IL-1β,−6, and−8) and MMPs (MMP-1,−3,−13, and−14) involved in p38 and NF-κB signaling. In human articular chondrocytes, an N-terminal fragment of type II collagen (29-mer fragment) stimulated the production of cathepsins B, L, and K through the activation of protein kinase C and p38 mitogen-activated protein kinase (MAPK) (43). Fichter et al. (44) demonstrated that mRNA and protein levels of MMP-2,−3,−9, and−13 were also upregulated by this 29-mer peptide. In a cartilage explant culture model, Tchetina et al. (45) reported that a 24-mer synthetic peptide of type II collagen (named CB12-II) was able to stimulate type II collagen cleavage through MMP-13 induction. Subsequently, in a study conducted in human OA chondrocytes, Yasuda (46) demonstrated that CB12-II stimulated phosphoinositide 3-kinase (PI3K)/Akt, leading to NF-κB activation. Recently, our team demonstrated that Coll2-1, a synthetic peptide located in the triple helical part of the type II collagen molecule and currently used as a biomarker of cartilage degradation, activates synoviocytes to produce IL-8 and chondrocytes to produce MMP-3. We also demonstrated that these Coll2-1 effects were mediated through TLR-4 and NF-κB signaling pathway activation (47).

Lees et al. (48) also examined the bio-activity of an aggrecan 32-mer fragment. They reported that it increased MMP-13 and ADAMTS-5 mRNA expression and decreased Col2A1 and aggrecan mRNA through TLR-2- and NF-κB-dependent signaling.

Type IX Collagen is located at the surface of fibrils formed by collagen II, playing roles in tissue stability and integrity. Collagen IX cleavage and loss of the N-terminal non-collagenous domain 4 (NC4) precede major damage of collagen II fibrils and can therefore be considered as key early steps in cartilage degradation. Kalchishkova et al. (49) showed that NC4 is able to bind C4, C3, and C9 and to directly inhibit C9 polymerization and MAC formation and can therefore be considered as a complement system inhibitor. NC4 interactions with fibromodulin and osteoadherin also inhibited complement activation by these proteins (49).

The cartilage oligomeric matrix protein (COMP), detected with abnormally high levels in OA synovial fluid, can also fix the complement system *via* C3b and C9 through an alternative complement pathway. COMP is also able to inhibit classical and lectin pathways through its interaction with C1q and mannose-binding lectin (50). The same observation is reported with cartilage fragments decorin and biglycan (51).

The bone sialoprotein I (BSP-1) is described as a non-collagenous ECM protein, member of the small integrin-binding ligand N-linked glycoproteins (SIBLINGs) family, expressed by many cell types among which are osteoblasts, osteoclasts, chondrocytes, synoviocytes, macrophages, and activated T cells (52). BSP-1 levels are increased in OA joint (synovial fluid and articular cartilage) compared to healthy controls, and these levels are correlated with the severity of joint lesion and the inflammatory status of patients (53). Furthermore, elevated levels of BSP-1 activate both an increase of MMP-13 expression and NF-κB activation and, consequently, the increased production of cytokines and chemokines, leading to NO, prostaglandin E2 (PGE_2), IL-6, and IL-8 production and imbalance the cartilage homeostasis (54). Moreover, BSP regulates T cell development, increases Th1 differentiation, suppresses Th2, and supports Th17 differentiation. Tardelli et al. (55) also demonstrated that BSP-1 has a key role not only in monocyte chemotaxis and macrophage differentiation but also in 4 macrophage proliferation.

INTRACELLULAR DAMAGE-ASSOCIATED MOLECULAR PATTERNS

Plasma Proteins

Sohn et al. (56) have recently identified by mass spectrometry in synovial fluid three plasma proteins of interest: Gc-globulin (vitamin D-binding protein), α1-microglobulin, and α2-macroglobulin. They showed that these plasma proteins induced TLR-4-dependent production of a large number of inflammatory cytokines and growth factors like IL-1β, IL-6, TNF-α, and vascular endothelial growth factor (VEGF). Fibrinogen, also found with increased levels in OA synovial fluid (57) and whose amount of fibrin deposited in the synovial membrane positively correlates with the severity of OA, is able to stimulate the production of chemokines [IL-8, monocyte chemoattractant protein (MCP)-1, …] by macrophages in a TLR-4-dependent manner, promoting attraction of T cells, neutrophils, and additional macrophages (58–60).

Alarmins

Large amounts of S100A8 and its binding partner S100A9 are released by neutrophils, monocytes, and activated macrophages. This heterodimer is highly expressed by synovial tissue in experimental OA models and involved in synovitis and cartilage destruction. Furthermore, high levels may predict joint destruction in humans (61). Recently, in human OA tissue, Schelbergen et al. (62) also demonstrated that S100A8/S100A9 levels were closely associated with cartilage loss and that they stimulate chondrocytes to produce more MMPs and cytokines (catabolic factors) but less type II collagen and aggrecan (anabolic factors). This effect was triggered by TLR-4. These authors also highlighted the role of S100A8/S100A9 in osteophyte formation and synovial activation in collagenase-induced OA and destabilized medial meniscus OA (62). In a study conducted in patients with knee OA, Ruan et al. (63) also demonstrated the association between serum levels S100A8/S100A9 and increased knee symptoms, cartilage defects, and MMP-3 serum levels. Finally, the canonical Wnt signaling pathway plays a key role in S100A8/S100A9 complex activity (64).

S100A10 forms with annexin II, a heterotetrameric complex called AIIt. This last activates human macrophages, which in turn secretes a number of inflammatory mediators including TNF *via* TLR-4 (65). Moreover, Song et al. (66) also demonstrated that the production of cytokines (TNF, IL-1β, and IL-10) in human chondrocytes was dependent on S100A10 through MAPK and NF-κB pathways.

Recently, S100A12 expression was found to be increased in OA cartilage and to contribute to the development of OA through an increase of MMP-13 and VEGF expression resulting from p38 MAPK and NF-κB pathway activation (67). Wang et al. (68) has also demonstrated that S100A12 levels in synovial fluid may correlate to clinical severity of patients with primary knee OA. In OA synovial fluid, S100A12 is significantly overexpressed, and Meijer et al. (69) highlighted this role in the innate and acquired inflammatory responses. This role in this innate immunity would be linked to RAGE receptors (70).

HMGB1 is released by necrotic cells or secreted by macrophages and other myeloid cells in response to inflammatory cytokines (IL-1β and TNF). Magna et al. (71) highlighted its role as alarmin binding to a lot of receptors, cytokines, and chemokines to stimulate the innate immune system. Since then, through cytokine production *via* TLR-4, HMGB1 promotes chemotaxis. HMGB1 was found overexpressed in the synovial fluid and cartilage of OA patients (72, 73). Thus, several authors reported that HMGB1 and RAGE are expressed in OA cartilage, and the activation of OA chondrocytes triggers ERK and NF-κB phosphorylation as well as MMP expression. García-Arnandis et al. (74) also reported that in OA synoviocytes, HMGB1 cooperates with IL-1β to amplify the inflammatory response resulting in the production of cytokines, chemokines, and MMPs. It can also trigger and prolong inflammatory responses *via* TLR-2,−4 but also RAGE.

Crystals

Microcrystals associated with joint diseases trigger inflammation and beyond innate immunity responses through both inflammasome-dependent and inflammasome-independent pathways (75, 76). Rosenthal (77) highlighted that calcium-containing crystals [calcium pyrophosphate dehydrate (CPPD) and basic calcium phosphate (BCP)] contribute to OA pathogenesis. Thus, these crystals exert direct effects both on synoviocytes and chondrocytes through the production of MMPs, prostaglandins, and inflammatory cytokines and this, *via* NF-κB, MAPK signals, and NO-dependent pathways. Furthermore, these crystals, combined with uric acid presence, are also able to interact with NLRP3 (78, 79) and subsequent IL-1β and IL-18 activation. Liu-Bryan et al. (80) showed also that CPPD crystals induced NO production in a TLR-2-dependent manner. Rosenthal (77) also report that these calcium-containing crystals directly affect inflammatory cells. For example, CPPD crystals can inhibit neutrophil apoptosis and extend the inflammatory response.

CELLULAR RECEPTORS INVOLVED IN DAMAGE-ASSOCIATED MOLECULAR PATTERNS ACTIVITY

DAMPs exert their biological activities through receptors TLR, NLR, and RAGE. Actually, 10 functional TLRs were identified in humans numbered TLR1–10. TLR-1,−2,−4,−5,−6, and−10 are located at the cell surface, while TLR-3,−7,−8, and−9 are present at the endolysosomal membrane (81). The signaling pathways activated by TLR involve the recruitment of adapter proteins such as MyD88, TIR domain-containing adaptor-inducing interferon (TRIF), TRIF-related adaptor molecule (TRAM), MyD88-adaptor like (Mal), and the activation of nuclear factors among which NF-κB. TLR also initiates distinct parallel signaling pathways leading to MAPK and PI3K activation (82). These latter regulate the transcription, mRNA stability, and translation of pro-inflammatory cytokine genes (TNF-α, IL-1β, or IL-6) and cell membrane-bound co-stimulatory molecules [intercellular adhesion molecule (ICAM)-1]. TLR-2 and−4 play a key role in OA pathogenesis since their expressions were demonstrated to be increased particularly at sites of cartilage

lesions and inflammatory synovial membranes (83, 84). TLR-4 is expressed by numerous cell types in the joint including immune cells, chondrocytes, osteoblasts, and synoviocytes (83). Activation of TLR-4 leads to upregulation of IL-1β, MMP expression, NO release, and PGE_2 synthesis, as well as downregulation of aggrecan core protein and type II collagen synthsesis (84, 85). Recently, comparing human cartilage from carpometacarpal (CMC)-I and knee joints, Barreto et al. (86) have observed that TLRs, and specially TLR-4, are differentially expressed depending on cartilage origin. Soluble forms of TLR-2 and−4 were also detected in the OA synovial fluid with sTLR-4 being elevated in OA knee comparing to healthy knee. Studies also highlighted that TLR1–7 and−9 expression was upregulated in the synovium of OA patients. Increased concentrations of several DAMPs (Fn, HA, Tn-C, PRG4, biglycan, or S100 family) are found in the OA synovial joint fluids and tissues and are able to activate TLRs; among them, Fn, HA, Tn-C, PRG4, biglycan, or S100 family.

NLRs are intracellular sensors of pathogen-associated or endogenous danger-associated molecular patterns (87). NLR system counts 22 cytoplasmic proteins including the nucleotide-binding oligomerization domains (NOD) and Nacht domain-containing, leucine-rich repeat-containing and pyrin domain-containing protein (NALP) subfamilies. The best characterized NLR is NLRP3, highly expressed in macrophages, chondrocytes, synoviocytes, and osteoblasts (76). Once activated, NLRP3 forms an oligomer able to interact with adapter proteins, C-terminal caspase recruitment domain (ASC), and Cardinal, creating a complex able to recruit procaspase-1. In turn, it is activated and the result is a multimeric structure named "the inflammasome," which is capable of inducing maturation and secretion of pro-inflammatory cytokines (such as IL-1β, IL-1α, IL-18) (88, 89). In OA, NLRP3 has been associated with crystal-induced inflammation triggered by uric acid, calcium pyrophosphate, and hydroxyapatite crystals (76). These microcrystals are interpreted as DAMPs by the innate immune system and cause inflammation (75).

RAGE, a transmembrane receptor, which belongs to the immunoglobulin gene superfamily (90), is also bound by DAMPs. RAGE is composed of three distinct regions including an extracellular region responsible for ligand interaction through its V domain, a transmembrane domain, and a cytoplasmic domain responsible for downstream signaling. Activation of RAGE leads to the activation of NF-κB and MAPK pathways, which themselves induce the expression of pro-inflammatory and catabolic genes. Initially identified as a receptor for advanced glycation end-products (AGEs), it can also be bound by several DAMPs including HMGB1, S100 proteins, or amyloid-β protein (90, 91).

DAMAGE-ASSOCIATED MOLECULAR PATTERNS, PERSPECTIVES, AND TARGET THERAPEUTICS

Several strategies have been suggested especially to control TLR-4 signaling. TLR-4 signaling activities may be downregulated by agonist blockers, activators of antagonist pathways, or new molecules. Among the agonist blockers, high-molecular-weight hyaluronic acid acts as a dressing blocking TLR access to short HA oligosaccharides (HA 4-mers) (92). Another agonist is the blocking peptide, Pep-1. The latter, a 12-mer peptide, inhibits low-molecular-weight HA binding to TLR-4. In a mouse chondrocyte model, Campo et al. (93) hypothesize that hydrophobic and/or polar residues of Pep-1 function as primary binding sites to HA, therefore reducing its binding to TLR-4 and subsequently the pro-inflammatory responses associated with TLR-4 activation.

Another strategy is the activation of antagonist pathways. Among these, peroxisome proliferator-activated receptor γ (PPARγ), PGD_2, vasoactive intestinal peptide (VIP), adenosine 2A receptor (A2AR), and bone morphogenic protein 7 (BMP-7) are reported to be the most promising targets. PPARγ has been well characterized as intracellular receptor and transcription factor with anti-inflammatory functions in cartilage. In this context, molecules such as rosiglitazone and pioglitazone, defined as PPARγ agonists, have been proposed to block TLR-4 signaling pathway. Thus, the stimulation of human chondrocytes and synovial fibroblasts by rosiglitazone inhibits TLR4 activation, leading to inhibition of TLR-4-induced catabolism and inflammation mediated by serum amyloid A. Serum amyloid proteins are major acute-phase proteins, detected in OA serum and able to trigger *via* TLR-2 and−4 stimulating cytokines (IL-6, IL-8, CXCL-1) and metalloproteinase expression (94). Pioglitazone inhibits TLR-4-mediated effects of AGEs including the induction of cyclooxygenase (Cox-2), HMGB1, IL-6, and MMP-13 (95). Besides PPARγ, PGD_2 is another candidate pathway and innate immune inhibitor. It inhibits PGE_2-dependent induction of TLR-4 and, subsequently, the IL-6 synthesis by chondrocytes (96). Finally, VIP, a neuropeptide produced by immune cells, is also able to inhibit in OA synoviocytes TLR-4-mediated effects including pro-inflammatory responses and TLR-4 expression (97).

Among the new compounds developed to target TLR-4 in joint tissues, we can cite the promising 6-Shogoal that was demonstrated to reduce both TLR-4-mediated innate immune responses and the catabolic TLR-4 signaling pathway in mouse and human chondrocytes (98).

Among the other receptors implicated in innate immunity, the TLR-2 is another potential therapeutic target. In the collagen-induced arthritis model in mice, TLR-2 monoclonal antibody (mAb) reduced the pro-inflammatory cytokine production (IL-12 and TNF-α) as well as the development of clinical parameters (99). Alquraini et al. (100) also evaluated the binding of PRG4 with TLR-2 and−4. It appears that PRG4 binds to these two receptors, highlighting an anti-inflammatory role for PRG4 in OA synovial fluid. With promising *in vivo* effects, we can also cite RAGE and its soluble receptor, sRAGE. This last acts as a competitive inhibitor of RAGE, inhibiting downstream signaling and integrin binding (101).

Complement system can also be a therapeutic target. So, eculizumab, a humanized monoclonal antibody, is an inhibitor of terminal complement pathway (102). It binds specifically to the complement C5 protein, inhibiting the

terminal complex, MAC. The effects of methylprednisolone on complement activation in patients undergoing total knee arthroplasty are currently clinically evaluated (ClinicalTrials.Gov Identifier: NCT02332616).

Another approach is to block the biological activity of DAMPs using a specific ligand. Promising examples are found in the literature. In mouse models, blockage of the pro-inflammatory effects of S100A8/A9 using an anti-carboxylate glycan antibody has also been concluding (12). Neutralizing HMGB1 antibodies or truncated HMGB1-derived A-box proteins are currently evaluated in collagen-induced arthritis rodent models (103). Targeting NLRP3 also looks promising (76). MCC950, a small-molecule chemical inhibitor, selectively inhibits activation of NLRP3 and IL-1β production by preventing NLRP3-induced ASC oligomerization (104). Finally, within our research unit, we demonstrate that Coll2-1, a synthetic peptide, is an actor of synovitis (47). Neutralized Coll2-1 with a humanized mAb may also represent an original approach in the control of OA progression.

In addition to the therapeutic aspect, the question arises as to the clinical utility of DAMPs. A lot of authors suggest the possibility that these DAMPs could be used as diagnostic and prognostic biomarkers of OA. Thus, soluble biglycan in inflammatory renal diseases, HMGB1 in systemic lupus erythematosus, or S100 proteins in several inflammatory conditions are some examples (105, 106).

CONCLUSION

Numerous pieces of evidence highlight the close link between immune response and the inflammation in OA process. The DAMPs are key actors. The list of these is constantly growing and represents interesting targets for future immunotherapy by blocking DAMP activities or their receptors. A better of understanding of DAMPs, their receptors, and associated pathological mechanisms represents an issue for degenerative joint diseases such as OA.

AUTHOR CONTRIBUTIONS

CL, JZ, and YH contributed to drafting the manuscript. CL, CS, AF, and YH contributed to revising the manuscript content. CL, JZ, CS, AF, J-ED, and YH contributed to approving the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 22 March 2021
doi: 10.3389/fmed.2021.628843

Effects of Diet Induced Weight Reduction on Cartilage Pathology and Inflammatory Mediators in the Joint Tissues

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Edited by:
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Specialty section:
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

Received: *13 November 2020*
Accepted: *25 February 2021*
Published: *22 March 2021*

Citation:
Sun AR, Wu X, Crawford R, Li H, Mei L, Luo Y, Xiao Y, Mao X and Prasadam I (2021) Effects of Diet Induced Weight Reduction on Cartilage Pathology and Inflammatory Mediators in the Joint Tissues. Front. Med. 8:628843. doi: 10.3389/fmed.2021.628843

Obesogenic diets contribute to the pathology of osteoarthritis (OA) by altering systemic and local metabolic inflammation. Yet, it remains unclear how quickly and reproducibly the body responds to weight loss strategies and improve OA. In this study we tested whether switching obese diet to a normal chow diet can mitigate the detrimental effects of inflammatory pathways that contribute to OA pathology. Male C57BL/6 mice were first fed with obesogenic diet (high fat diet) and switched to normal chow diet (obese diet → normal diet) or continued obese diet or normal diet throughout the experiment. A mouse model of OA was induced by surgical destabilization of the medial meniscus (DMM) model into the knee joint. Outcome measures included changes in metabolic factors such as glucose, insulin, lipid, and serum cytokines levels. Inflammation in synovial biopsies was scored and inflammation was determined using FACs sorted macrophages. Cartilage degeneration was monitored using histopathology. Our results indicate, dietary switching (obese diet → normal diet) reduced body weight and restored metabolic parameters and showed less synovial tissue inflammation. Systemic blood concentrations of pro-inflammatory cytokines IL-1α, IL-6, IL-12p40, and IL-17 were decreased, and anti-inflammatory cytokines IL-4 and IL-13 were increased in dietary switch group compared to mice that were fed with obesogenic diet continuously. Although obese diet worsens the cartilage degeneration in DMM OA model, weight loss induced by dietary switch does not promote the histopathological changes of OA during this study period. Collectively, these data demonstrate that switching obesogenic diet to normal improved metabolic syndrome symptoms and can modulate both systemic and synovium inflammation levels.

Keywords: osteoarthritis, diet induced obesity, infrapatellar fat pad, synovium, inflammation, cartilage

INTRODUCTION

Obesity and its associated metabolic disorders are an important contributor to the progression of OA (1–5). It is well-accepted that obesity is mainly caused by an imbalance between energy intake and expenditure that promotes storage of nutrient oversupply in white adipose tissue (6). Although the underlying mechanisms that link OA with obesity remain to be elucidated, the state of chronic low-grade inflammation in obesity, which is caused by an increased accumulation of macrophages in the adipose tissue, has been proposed (7). As the links between obesogenic diet, inflammatory responses and OA have been established, it is critical to investigate whether these changes are reversible by weight loss following nutritional changes, as obesity may be the only risk factor that is modifiable. In mice and monkeys, a 20–40% reduction of dietary caloric intake can reduce body weight, extending lifespan, improving metabolic disturbances and decreasing the systemic inflammatory status through changes in inflammatory gene expression, reduction of oxidative stress, decreased metabolism and increased capacity of DNA repair (8–10). Substantial evidence also supported that weight loss of 20% in patients with obesity and OA by gastric surgery led to an improvement in pain and physical function (11). Moreover, there was an inverse dose-response relationship between weight loss and progression of cartilage abnormalities in obese patients with knee OA (12, 13). Although weight loss has been extensively studied regarding its short-term benefits on major symptoms in obese patients with chronic diseases including OA (13, 14), the effect of weight loss on the progression of morphologic abnormalities of the knee joints remains a key area of ongoing research.

We have previously reported that consumption of a high-fat, high-carbohydrate diet promoted systemic and local synovial inflammation and contributed to development of OA (5). We have further reported that dietary intake of long-chain saturated fatty acids was associated with the development and acceleration of OA (15, 16). This study used diet-induced obese rodents with OA to investigate whether switching to normal chow effect cartilage pathology and synovial inflammation. The aim of this study is to test if dietary-induced reversal of OA would be concurrent with reversal of the systemic and local synovial inflammatory state, metabolic syndrome characteristics and cartilage degradation.

METHODS AND MATERIALS

Animals

Animal experimental protocols were approved by the Institutional Animal Care and Use Committees and Institutional Biosafety Committees of Central-South University (CSU; 2013-05), China. Female C57BL/6 mice were purchased from the Animal Center of Central-South University (Changsha, Hunan, China). Mice had free access to food and water during the experimental protocols. At the start of the experimental protocols, mice were 6 weeks old.

Dietary Interventions

A total of 60 C57BL/6 mice were included in this study. Mice were randomly divided into six groups. Group 1: Control diet (CD), Group 2: High fat diet (HFD), Group 3: Dietary switch group (HFD diet → CD diet), Group 4: Sham surgery + fed with CD, Group 5: OA surgery + fed with HFD diet, Group 6: OA surgery + dietary switch HFD diet → CD diet). C57BL/6 mice were initially placed on a standard chow diet or control diet composed of 10 kcal% Fat (D12450B, Research Diets) and an obesogenic diet or HFD composed of 60 kcal% fat (D12492, Research Diets, Chengdu, China). After 11 weeks of feeding, some mice underwent surgery for destabilization of the medial meniscus (DMM) to induce knee OA in the right knee joint or a sham operation that did not dissect the medial meniscal ligament as described previously (17). Mice were randomized for the remainder of the study to either continue the CD diet; continue the HFD; or HFD switch to CD (**Figure 1A**). Mice were group-housed in a temperature-controlled room on 12-h light/dark cycles with routine veterinary assessment. Body weight and food consumption measures were recorded weekly. After euthanasia, blood was withdrawn by cardiac puncture, and the serum was collected for cytokine analysis. Serum was used for biochemical analyses according to previous studies (15).

Biochemical and Metabolic Parameters

Blood samples collected during the terminal experiments were analyzed to determine the concentrations of insulin and lipids. Serum insulin was measured using enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's instructions (Abcam, Changsha, Hunan, China). Serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) were using a Cholesterol Assay Kit (Abcam, Changsha, Hunan, China). The concentration of serum triglyceride was determined using a Triglyceride Quantification Kit (BioVision, Dakewe Biotech, Beijing, China). Serum of mice were obtained by centrifuging at 1,500 × g for 10 min, and levels of cytokines were measured with a Bio-Plex™ Mouse Cytokine 23-Plex Panel (#M60009RDPD, BioRad, Life Science, China) by using a BioRad Bio-Plex 200 System according to the manufacturer's instruction. These measured cytokines were as follows: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p (40), IL-12 p (70), IL-13, IL-17A, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNFα.

Isolation of Synoviocytes and Sorting Macrophages

Isolation of synovium was performed according to published methods (18–20). Briefly, immediately after euthanasia, the synovial tissues around the knee joints were collected and pooled from 3 animals per group. The synovium was minced and digested in a 1 mg/ml collagenase type I (Sigma-Aldrich) for 1 h at 37°C and rinsed through a 70-μm filter (BD Biosciences). The isolated synoviocytes were suspended in phosphate-buffered saline (PBS) containing 20 μg/ml of antibody cocktail. Brilliant Violet 510- and phycoerythrin-conjugated antibodies against mouse CD45.2 and F4/80 were obtained from Biolegend

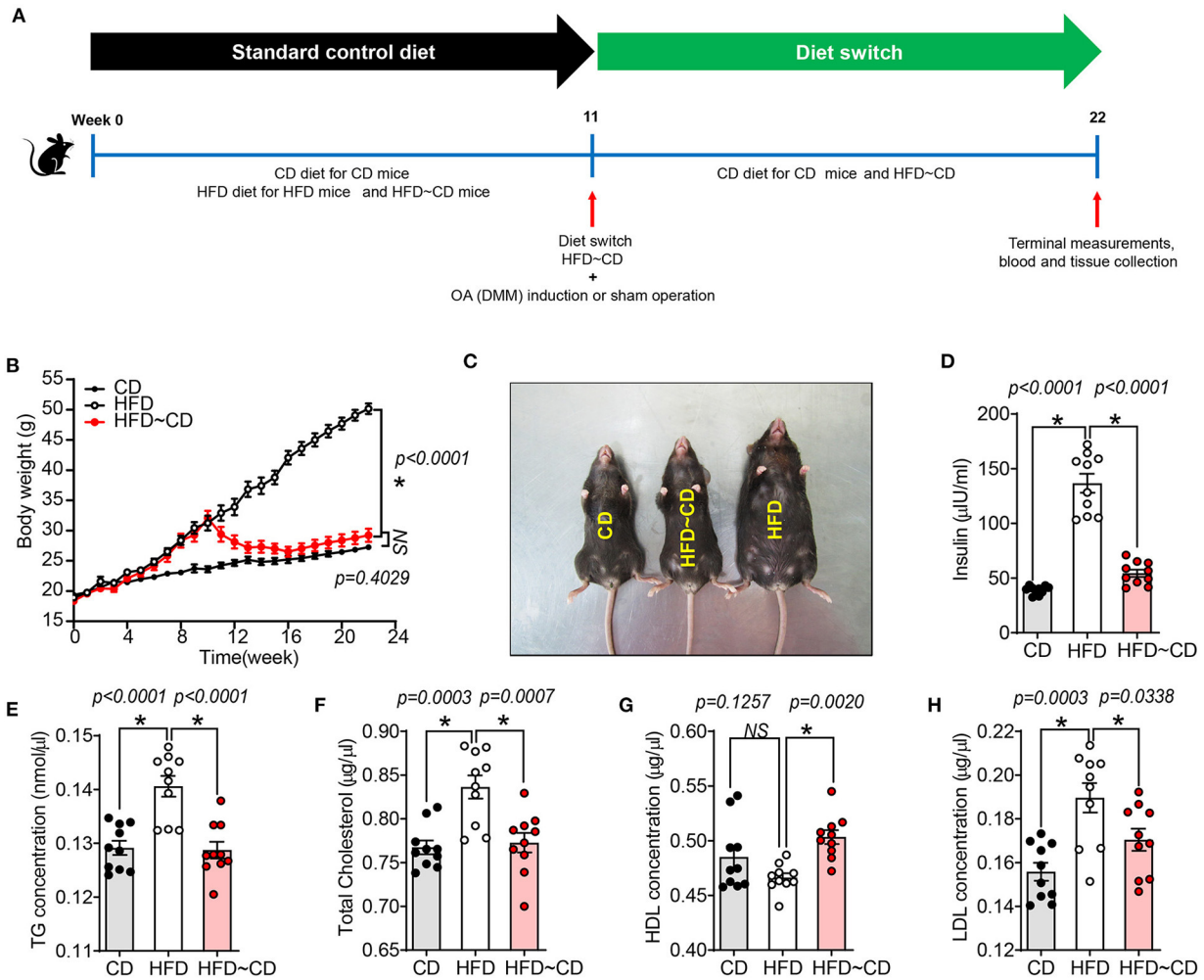

FIGURE 1 | High fat diet -induced obesity and altered metabolic parameters are reversed by diet-initiated weight loss. **(A)** Schematic diagram showing the experimental procedure. Six-week-old female C57BL/6 J mice were fed a CD or HFD for 11 weeks where half of the HFD-mice were switched to or continued a CD or HFD for additional 11 weeks, respectively. CD-fed mice were maintained on the same diet. After 11 weeks of feeding, mice either underwent surgery for destabilization of the medial meniscus (DMM) to induce knee OA in the left knee joint or a sham operation that did not dissect the medial meniscal ligament. CD, control diet; HFD, high fat diet; HFD~CD, high fat diet switched to control diet. **(B)** Body weight of CD, HFD and HFD~CD mice were monitored over 22 weeks. **(C)** Dorsal view of the mice showing the changes caused by the diet after 22 weeks. **(D–H)** Effect of diets on metabolic parameters in mice. Measurement of serum insulin **(D)**, total triglyceride **(E)**, total cholesterol **(F)**, HDL **(G)**, LDL **(H)**. Graphs represent mean ± SD ($N = 10$ per group). *Significant differences between results in different group (i.e., $p < 0.05$).

(Chaoyang, Beijing, China). For isotype control, Brilliant Violet 510- or phycoerythrin-conjugated non-specific mouse or rat IgG2a were substituted for the primary antibody, respectively. After incubating with antibody cocktails for 30 min at 4°C, the cells were washed with PBS and resuspended in PBS and macrophages were sorted using FACS and RNA was isolated from these sored cells.

RNA Extraction, Reverse Transcription, and Gene Expression Profiling by Real-Time PCR

Immediately after tissue digestion, isolation of total RNA from sorted CD45.2+F4/80+ synovial macrophages were performed using TRIzol. RNA quantity and quality were assessed in a NanoDrop-100 spectrophotometer. cDNA synthesis from total RNA was performed using TaKaRa PrimeScript 1st strand cDNA Synthesis Kit according to the manufacturer's protocol. For quantification of gene expression by real-time PCR, SYBR Green detection chemistry was used on the Roche LightCycler 96 System. Quantitative measurements of all primers used in this study were determined using (2−ΔΔCt) method, and β-actin/GAPDH expression were used as the internal control (21–23).

Histological Assessment of OA Development

The knee joints from seven mice per group were fixed overnight at 4% paraformaldehyde in 1X PBS, decalcified in 10%

ethylenediaminetetraacetic acid (EDTA), embedded in paraffin and cut with a rotary microtome to generate 5-μm-thick sections. Sagittal sections were stained with safranin-O/fast green and haematoxylin & eosin to evaluate the disease severity by 2 observers under blinded conditions to dietary groups (24). The severity of OA was assessed in the medial compartment of the knee using the Mankin scoring system from 0 to 14 (**Table 1**) (21, 25). The degree of synovitis was scored using a 0–6 scoring system that measured the thickness of the synovial lining cell layer on a scale of 0–3 and cellular density in the synovial stroma on a scale of 0–3 as previously described (**Table 2**) (26). The sum of both parameters was used for analysis (26). Immunohistochemistry was performed (5, 15, 27) to determine the population of synovial macrophages and cartilage degradation products, respectively: anti-F4/80 (Abcam; Cat No: ab6640, Melbourne, VIC, Australia; dilution 1:100), anti-CD169 (Bioss Antibodies; Cat No: bs-10751R, Sapphire Bioscience, Redfern, NSW, Australia; dilution 1:100), anti-aggrecan NITEGE epitope (dilution 1:950) and anti-collagen DIPEN neoepitope (kind gift from Professor Amanda Fosang, Murdoch Children Research Institute, Melbourne, VIC, Australia; dilution 1:1240). The sections were incubated with corresponding secondary antibodies and the antibody complexes visualized using a diaminobenzidine (DAB) substrate and counterstained with Mayer's hematoxylin. Images were captured to perform histomorphometric measurements on the entire area of the knee synovial joint with a Leica SCN400 slide scanner (Leica Biosystems, Australia). Images were analyzed using Image J (National Institute of Health, Bethesda, BA, USA) for semi-quantitative data analysis. The positive cells within cartilage/synovium in each field (40 × objective lens) of observation were counted and normalized to the cell number per 100/total cells in each group. The number of positively stained cells in the medial compartment of the entire tibia above the tidemark per specimen in three sequential sections per mouse in each group. The total number of immuno-positive cells appearing within the synovium was estimated quantitatively in three sequential sections per animal in each group. Assessment of the histopathology and immunostaining was performed by two independent observers in a blind random manner.

TABLE 1 | Mankin score grading system for osteoarthritic articular cartilage.

Category	Subcategory	Score
Structure	Normal	0
	Surface irregularities	1
	Pannus and surface irregularities	2
	Clefts to transitional zone	3
	Clefts to radical zone	4
	Clefts to calcified zone	5
	Complete disorganization	6
Cells	Normal	0
	Diffuse hypercellularity	1
	Cloning	2
	Hypocellularity	3
Safranin O staining	Normal	0
	Slight reduction	1
	Moderate reduction	2
	Severe reduction	3
	No dye noted	4
Tidemark integrity	Intact	0
	Crossed by blood vessels	1
Total		0–14

TABLE 2 | Histopathological assessment of synovitis.

Category	Subcategory	Score
Enlargement of the synovial lining cell layer	Thickness 1–2 cells	0
	Thickness 2–4 cells	1
	Thickness 4–9 cells	2
	Thickness ≥ 10 cells	3
Cellular density in the synovial stroma	Normal cellularity	0
	slightly increased	1
	Moderately increased	2
	Greatly increased, pannus formation and rheumatoid-like granulomas might occur	3
Total		0–6

Statistics

The minimum animal number and/or biological replicates for the primary outcome were determined by previous data (power = 0.8, $p < 0.05$, two-sided) (15, 16, 27). Briefly, $n = 7$/group for histology analysis; $n = 10$/group for metabolic parameters analysis; and $n = 7$/group for cytokine analysis. One-way ANOVA with Tukey's *post-hoc* analysis was used to determine whether parameters from the three groups of mice were significantly different using GraphPad Prism 7.0 for Windows (San Diego, CA, USA). Non-parametric data was evaluated using Friedman tests (Prism, GraphPad). Values are presented as mean ± SD for all variables unless indicated otherwise. Results were significantly different with $p < 0.05$.

RESULTS

Changes in Obesogenic Diet-Induced Obesity Parameters Are Reversed by Weight Loss

HFD mice showed a significant increase in body weight compared to CD mice (50.17 ± 2.1 g vs. 27.26 ± 2.9 g) (**Figures 1B**,**C**). HFD-mice showed increased serum insulin concentrations compared to CD mice (**Figure 1D**). As shown in **Figures 1E**–**H**, total triglyceride (TG), cholesterol and low-density lipoprotein (LDL) concentrations in serum were markedly higher in HFD-mice, but no difference was observed in serum high-density lipoprotein (HDL) concentration compared to CD mice. Following weight loss of the HFD∼CD group, serum

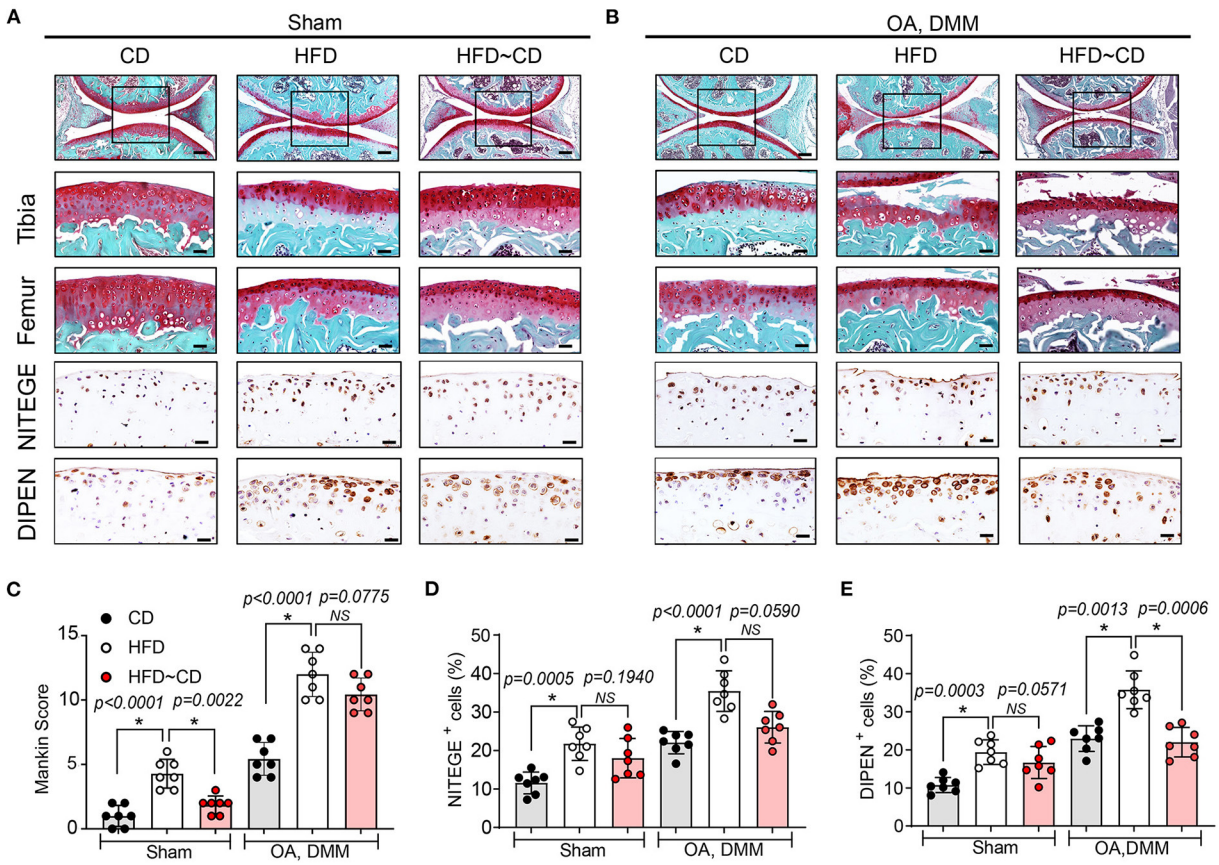

FIGURE 2 | Effects of Diet switching on cartilage destruction in mouse model of OA. **(A)** Top panel: Representative Safranin O and fast green stained sagittal sections of sham knee regions in mice fed a CD, HFD, or HFD∼CD. Scale bars, 100 μm. The inset boxes in upper panels are shown at higher resolution in lower panels. Scale bars, 20 μm. Bottom panel: Similar sections were stained with DIPEN and NITEGE. Scale bars, 20 μm. **(B)** Top panel: Representative Safranin O and fast green stained sagittal sections of OA-operated knee regions in mice fed a CD, HFD, or HFD∼CD. Scale bars, 100 μm. The inset boxes in upper panels are shown at higher resolution in lower panels. Scale bars, 20 μm. Bottom panel: Consecutive sections were stained with DIPEN and NITEGE. Scale bars, 20 μm. $N = 7$ per group **(C)** Severity of articular cartilage degradation was graded using Mankin scoring system. **(D,E)** The percentage of DIPEN **(D)** and NITEGE **(E)**—positive cells per knee section were counted. Graphs represent mean ± SD (Data represents $n = 7$ mice per each group). *Significant differences between results in different group (i.e., $p < 0.05$).

insulin, TG, cholesterol, and LDL concentrations were decreased, and HDL concentrations were increased as compared to HFD-mice (**Figures 1D–H**). These parameters in the HFD∼CD group returned to similar concentrations as in CD (**Figures 1D–H**).

Effects of Diet Switching on Cartilage Pathology in Mouse Model of OA

HFD-mice is characterized by decreased intensity of safranin-O staining indicating proteoglycan degradation and increased Mankin scores mainly in tibia (**Figures 2A,C**). In DMM-operated mice, HFD administration led to more severe OA symptoms compared with DMM-operated CD mice (**Figures 2B,C**). IHC was performed to assess neoepitopes generated at the aggrecan cleavage sites produced by aggrecanase ADAMTS-4,5 (NITEGE) or matrix metalloproteinases (DIPEN) in murine articular cartilage. DIPEN and NITEGE are two commonly used matrix degradative markers to revaluate cartilage degeneration in OA (28, 29). As shown in **Figure 2**, HFD-mice showed an increased expression of DIPEN- and NITEGE-positive articular chondrocytes in knee joints compared to CD mice. DMM-operated mice fed with HFD also exhibited increase in production of the aggrecan neoepitopes, NITEGE and DIPEN compared with the DMM-treated CD mice (**Figures 2A,D,E**). Compared with HFD-mice, HFD∼CD mice in sham group showed decreased Mankin score an indicative of OA cartilage pathology. However, the dietary switch from HFD∼CD does not show any significant effect in the OA development induced by DMM surgery (**Figures 2A–C**). However, the upregulation of NITEGE and DIPEN was quantitatively reduced in HFD∼CD mice compared to HFD group alone in OA DMM group (**Figures 2B,D,E**).

Effects of Diet Switching on Synovial Pathology and Macrophage Expression in Mouse Model of OA

Next, we observed that mice fed HFD showed detectable synovial lining hyperplasia and an increment in fibrosis, and

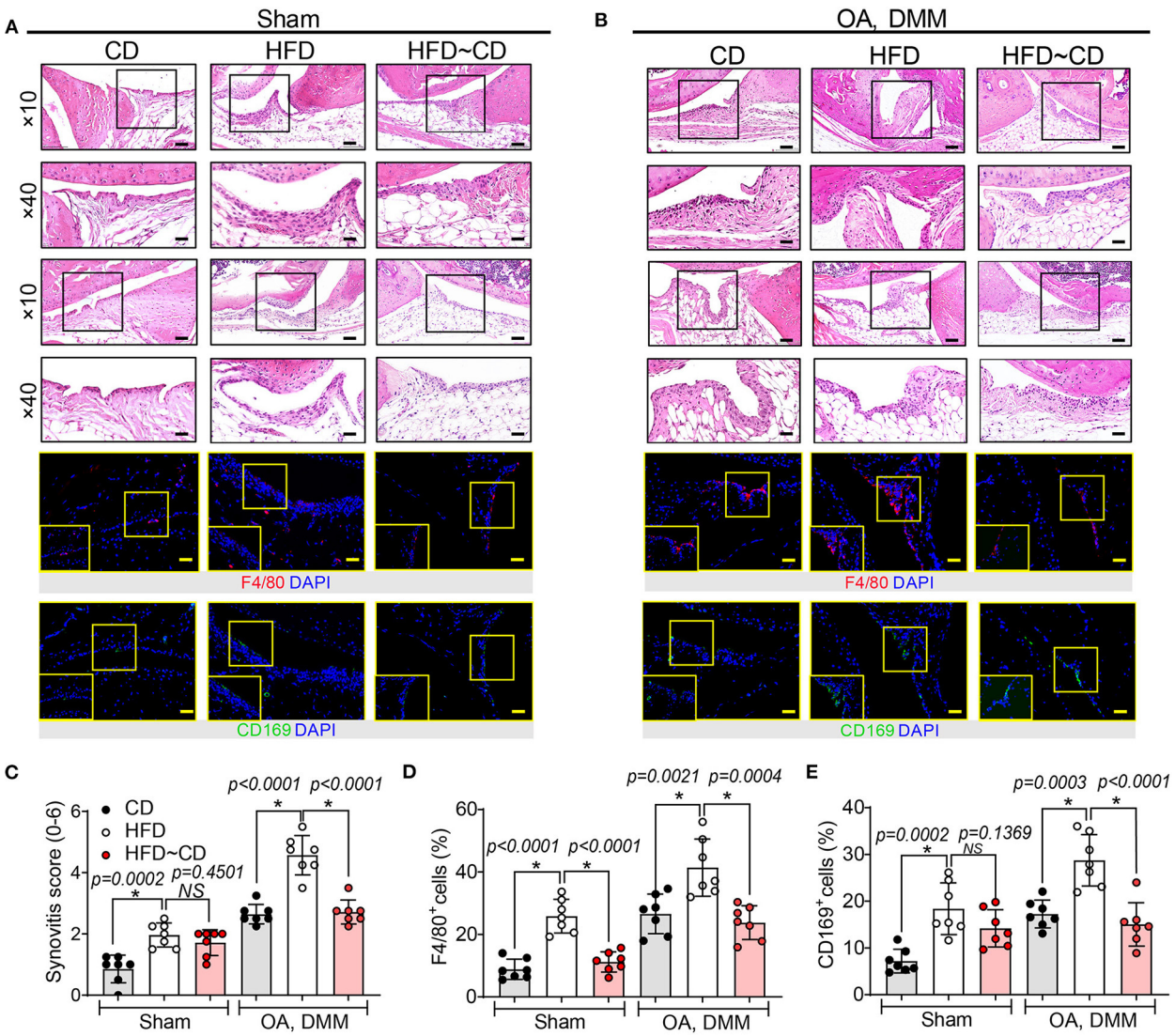

FIGURE 3 | The presence of macrophage-associated synovitis in mouse model of osteoarthritis was reversible by diet intervention. **(A)** Top panel: Representative H&E stained sagittal sections of sham knee regions in mice fed a CD, HFD, or HFD∼CD. Scale bars, 100 μm. The inset boxes in upper panels are shown at higher resolution in lower panels. Scale bars, 20 μm. Bottom panel: Similar sections were stained with F4/80 and CD169; the inset boxes are shown at higher resolution. Scale bars, 20 μm. **(B)** Top panel: Representative H&E stained sagittal sections of OA-operated knee regions in mice fed a CD, HFD or HFD∼CD. Scale bars, 100 μm. The inset boxes in upper panels are shown at higher resolution in lower panels. Scale bars, 20 μm. Bottom panel: Similar sections were stained with F4/80 and CD169; the inset boxes are shown at higher resolution. Scale bars, 20 μm. $N = 7$ per group. **(C)** Synovial inflammation was assessed using synovitis scoring based on degree of cell thickness in the synovial lining layer and cell density of the synovial stroma. **(D,E)** The percentage of F4/80 **(D)** and CD169 **(E)**—positive cells per knee section were counted. Graphs represent mean ± SD ($N = 7$ per group). *Significant differences between results in different group (i.e., $p < 0.05$).

thus the synovitis score was higher than that observed in CD mice (**Figures 3A**,**C**). HFD administration also increased the number of F4/80+ macrophages in inflamed synovium, especially localized in the lining layer (**Figures 3A**,**D**). The OA-HFD group exhibited more severe synovitis correlated with extensive infiltration of F4/80-positive cell than in the OA-CD groups (**Figures 3B**,**D**). We examined whether HFD modulates the expression of inflammatory CD169+ macrophages in synovium. HFD mice manifested stronger immunoreactivity to CD169 in the synovium in comparison to CD mice (**Figures 3A**,**E**). The number of CD169+ macrophages further increased in the synovium in the OA-HFD group in comparison to the OA-CD group (**Figures 3B**,**E**). However, in the HFD∼CD dietary switch group with OA, the population of $F4/80^+$ macrophages in the synovium returned to similar levels as in CD group (**Figures 3A**,**D**). A clear diminution in the percentage of $F4/80^+$ macrophages in the synovium of HFD∼CD mice with OA surgery was observed in comparison to the OA-HFD group (**Figures 3B**,**D**).

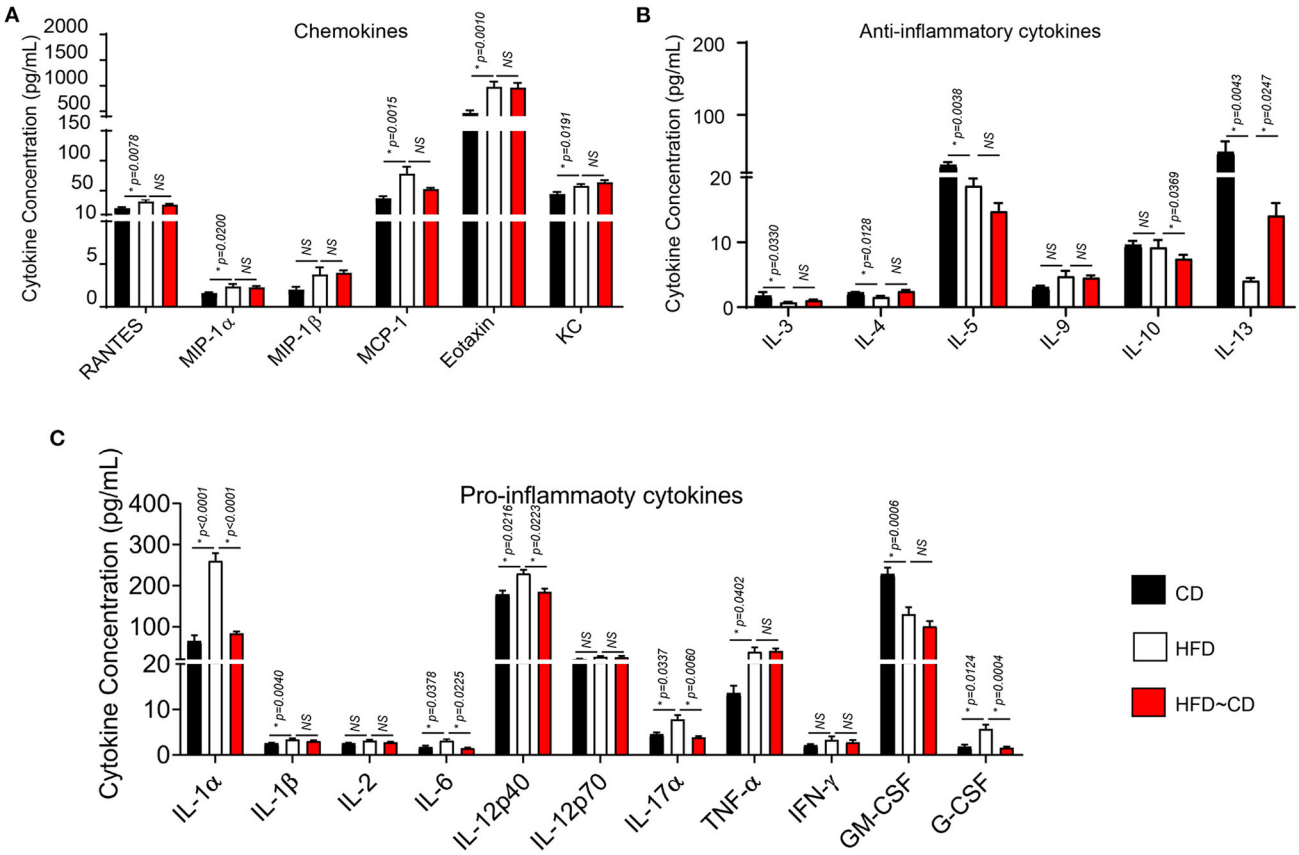

FIGURE 4 | Effect of diet intervention-induced weight loss on systemic inflammation. **(A)** Measurement of serum chemokines in mice fed a CD, HFD, or HFD∼CD. **(B)** Measurement of serum anti-inflammatory cytokines in mice fed a CD, HFD, or HFD∼CD. **(C)** Measurement of serum pro-inflammatory cytokines in mice fed a CD, HFD, or HFD∼CD. Graphs represent mean ± SD ($n = 7$ per group). $p < 0.05$ is considered significant.

Weight Loss Leads to an Alteration in Systemic and Local Inflammation

We determined the serum inflammatory cytokine changes to test the effects of HFD followed by weight loss on the development of obesity-induced OA in mice. After 22 weeks of HFD, the chemokines RANTES, MIP-1α, MCP-1, EOTAXIN, and KC were increased in HFD subjects relative to CD controls while there were no differences in MIP-1β concentrations (**Figure 4A**). Moreover, HFD led to lower concentrations of anti-inflammatory cytokines including IL-3, IL4, IL-5, and IL-13 with no changes of IL-9 and IL-10 (**Figure 4B**). Additionally, concentrations of serum pro-inflammatory cytokines for IL-1α, IL-1β, IL-6, IL-12p40, IL-17α, and TNF-α were increased in HFD-mice, but there were no changes in IL-2, IL-12p70, G-CSFor IFN-γ (**Figure 4C**) compared with CD group. Notably, the concentration of GM-CSF in HFD-fed mice was markedly decreased (**Figure 4C**). Following dietary switch, HFD∼CD mice had lower concentrations of pro-inflammatory cytokines IL-1α, IL-6, IL-12p40, and IL-17 compared to HFD-fed mice among the tested markers. Anti-inflammatory cytokines IL-4 and IL-13 were elevated, while circulating concentrations of IL-10 were decreased in the HFD∼CD group compared with HFD-mice. Unlike alteration in cytokine expression, which changed in HFD∼CD mice, there were no differences in chemokines that including MIP-1α, MIP-1β, RANTES, KC, and EOTAXIN (**Figures 4A–C**).

To test whether HFD diet induces pro-inflammatory conditions in the synovium, the gene expression in FACS-sorted CD45.2+F4/80+ macrophage-like synoviocytes was verified by qRT-PCR. Mice in the HFD group had a clear increase in pro-inflammatory markers including IL-1β, CD11c, and TNF in the synovium in comparison to controls (**Figure 5A**). However, anti-inflammatory markers such as IL-4, IL-10, Mrc1 were not affected by HFD in synovial macrophages (**Figure 5B**). We further examined the effect of diet intervention-induced weight loss on HFD-induced synovitis. Gene expression of pro-inflammatory markers IL-1β and CD11c were inhibited by diet reversal, but the expression of IL-6 mRNA in HFD∼CD mice was increased compared with HFD-fed mice (**Figure 5A**). On the other hand, HFD∼CD mice showed higher gene expression of anti-inflammatory cytokines IL-4 and IL-10. However, expression of Mgl2 was returned to normal levels in HFD∼CD mice in comparison to HFD group (**Figure 5B**).

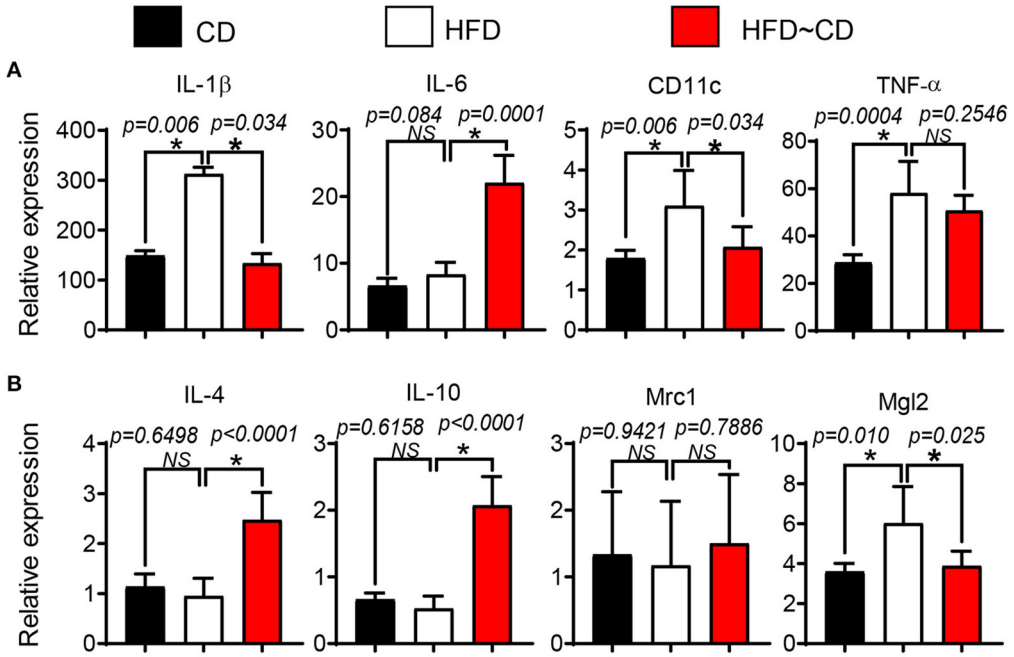

FIGURE 5 | Effect of diet intervention-induced weight loss on local inflammation. **(A)** qPCR analysis of IL-1β, IL-6, CD11c, and TNF (pro-inflammatory markers) mRNA in synovial macrophages from CD-, HFD-, or HFD∼CD- mice. **(B)** qPCR analysis of IL-4, IL-10, Mrc1, and Mgl2 (anti-inflammatory markers) mRNA in synovial macrophages from CD-, HFD-, or HFD∼CD- mice. Data presented as mean ± SD of two independent experiments (each a pool of 3 mice) performed in triplicates. $p < 0.05$ is considered significant.

DISCUSSION

Diet and exercise are widely accepted as useful strategies for losing weight in obese adults (14). However, in obese patients with limited mobility and pain induced by OA, long-term maintenance of weight loss through exercise is challenging. Despite this limitation, weight loss has been recognized as an important approach to reduce OA symptoms in obese patients (13, 14). However, whether there are any benefits in joint tissue metabolism or structural progression is unknown. We determined whether weight loss induced by diet can effectively prevent OA development in rodent models of obesity and OA. We report 4 key findings: (1) obesogenic diets lead to OA changes in the knee joint in C57BL/6J mice; (2) obesogenic diets induce an accumulation of pro-inflammatory macrophages in the synovium and fat pad tissues; (3) replacement of the obesogenic diet with a control diet decreased systemic metabolic syndrome symptoms, circulating concentrations of inflammatory cytokines and inflammatory signaling activities; and (4) replacement of obesogenic diets with a control diet somewhat reduced synovial pro-inflammatory gene expression, and mitigated the effects of obesity-associated OA development.

Our results showed diet switching resulted in lowering of both body weight and serum inflammatory cytokine concentrations. Notably, we found that HFD∼CD disrupted the clustering of cytokine expression which are mainly associated with increased adiposity (30, 31) such as IL-6 and IL-12. Our findings are consistent with the clinical findings that obese OA patients on a calorie-restricted diet without exercise showed weight loss and reduced plasma inflammatory biomarkers (32). Substantial evidence also supports that weight loss of 20% in patients with obesity and OA by gastric surgery led to an improvement in pain and physical function, and attenuation in systemic inflammation resulting in a structural improvement of cartilage (11). Moreover, systemic blockade of IL-6 improved cartilage structure in mouse OA models (33). Taken together, these results suggest that reduced dietary energy intakes reduce the systemic inflammatory status in obese rodents and humans. It is well-documented that the systemic production of inflammatory mediators contributes to cartilage degradation and activation of synoviocytes. Scanzello et al. has discussed that obesity-triggered low-grade inflammation consists of components such as inflammatory cytokines, abnormal metabolites that contribute to OA pathophysiology leading to cartilage matrix impairment, synovitis and subchondral bone remodeling (34). However, local synovial inflammation can be reflected outside the joint in the blood of patients with OA (35, 36). In this current study, we found that the dietary switch can modify some of the obesogenic diet-induced alterations in the immune response of synovium in mice along with improved systemic inflammatory status. These findings are in agreement with previous studies which showed that, in obese adults with knee OA, weight loss improved synovitis-induced pain (37). We have previously demonstrated that synovial macrophages in HFD-mice are predominantly polarized to the M1 pro-inflammatory phenotype, while M2 activated macrophages are also present (15, 16). Therefore, we

hypothesized that improvement of systemic inflammation by dietary switch reduced obesity-associated synovitis by reversing the inflammatory status in the synovium. The effect of dietary switch on synovial macrophage phenotype in obesity-related OA remains unknown, although the cytokine presence in the OA synovial macrophages has been extensively studied and a close relationship between cytokine expression and progression of OA was shown in previous studies (15, 38).

It is interesting to note that there is no differences between HFD (DMM) and HFD∼CD (DMM) on cartilage destruction assessment. However, further longitudinal investigations in future could shed light on the influence of diet control on the onset and development of OA at various time points. Although we did not observe significant differences between these two groups on cartilage destruction assessment, macrophage-associated synovitis was significantly improved after diet intervention along with decreased systemic inflammatory conditions in the animals. Our previous study has shown that an increase of synovial inflammation halfway through the 16-week-diet (i.e., week 8), when the cartilage still appeared normal (39). Larranga-Vera et al. also suggested that the aggravation in synovial inflammation induced by HFD is not a secondary event resulting from a pathological change in the cartilage (40). This indicated the immune response in the synovium preceded cartilage degeneration which became more apparent at the experimental endpoint. As such, therapeutic strategies targeting the inflammatory synovium can be pivotal to attenuate the severity of OA, but also prevent the onset of disease. In this study, we observed that weight loss with HFD∼CD dietary switch group resulted in change of synovial macrophages from a pro-inflammatory phenotype with high expression of CD11c to an anti-inflammatory or resolving state with a reduction in CD11c expression. Considering the pro-inflammatory properties of saturated fatty acids in obese individuals (38), this suggests that the low-fat content in the diet might play an important role in the conversion of synovial macrophage phenotype and resolution of synovial inflammation in HFD∼CD-induced weight loss.

Our results show that mice on HFD∼CD increased concentrations of the pro-inflammatory cytokine IL-6. Although IL-6 is commonly known as a pro-inflammatory cytokine, it is a multifunctional cytokine with anti-inflammatory activity as it increases the production of anti-inflammatory mediators IL-10 and IL-4 by the suppression of IFNγ signaling (41–43). Mgl2 mRNA encoding the CD301b protein is highly expressed in the M2-like macrophage. In this study, we observed that the obese mice on a HFD∼CD exhibited decreased expression of Mgl2 mRNA in macrophage-like synoviocytes, consistent with the reduced expression of Mgl2 after weight loss in obese patients (44). The decreased Mgl2 expression in synovial macrophages sorted from HFD∼CD mice might be due to its important roles in maintaining positive energy balance and glucose metabolism. HFD-fed Mgl2-DTR mice treated with diphtheria toxin showed weight loss, enhanced insulin sensitivity and gluconeogenesis, accompanied by a marked reduction in circulation of RELM α, a multifunctional cytokine produced by mononuclear phagocytes with roles in promoting insulin resistance and obesity (45). Taken together, these results suggest that the beneficial effect of HFD∼CD on knee joint may be due to the upregulation of IL-6 and downregulation of Mgl2 in the synovial macrophage. Thus, HFD∼CD diet switching improves systemic metabolism, modifies inflammatory status and supports the important association between obesogenic diet-induced obesity (DIO) and the development of OA. However, an overall profile of cytokines, chemokines and other signaling proteins of the synovium from the two rodent groups is required to clarify the effect of dietary switch in local inflammation of obesogenic diet-induced obesity-associated OA.

The application of dietary intervention and protein augments as a therapeutic strategy on obesity-induced diseases are widely reported. The obesity-associated hyperinsulinemia and liver dysfunction are reversible by diet-mediated weight loss in DIO mouse model (46). Further investigation on these non-pharmacological interventions could shed light on the influence of diet on practical pathological changes.

CONCLUSION

In conclusion, our data demonstrate that obesogenic diets induce systemic and synovial inflammation. Further long-term studies are required to assess if weight loss through dietary switching is an intervention that remodels the systemic and local inflammatory status and slows down the progression of OA cartilage degeneration.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committees of Central-South University (CSU), China.

AUTHOR CONTRIBUTIONS

AS, XW, RC, YX, XM, and IP conceived the study conception and design. XW, HL, LM, YL, and XM performed the animal experiments. AS, XW, YX, and IP conducted the histology and/or analyzed the data. RC provided advice on osteoarthritis experiments. All authors have read and approved the final manuscript.

FUNDING

This work was supported by funding The National Natural Science Foundation of China for XM (81371997) and Hunan Provincial Science and Technology Department, China (2018JJ2579 and 2015JC3034), IP received Prince Charles Hospital Research Foundation fellowship (RF-01).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 29 April 2021
doi: 10.3389/fmed.2021.612370

Decreased Sulfate Content and Zeta Potential Distinguish Glycosaminoglycans of the Extracellular Matrix of Osteoarthritis Cartilage

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Specialty section:
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

Received: *30 September 2020*
Accepted: *03 March 2021*
Published: *29 April 2021*

Citation:
Nunes RdM, Girão VCC, Cunha PLR, Feitosa JPA, Pinto ACMD and Rocha FAC (2021) Decreased Sulfate Content and Zeta Potential Distinguish Glycosaminoglycans of the Extracellular Matrix of Osteoarthritis Cartilage. Front. Med. 8:612370. doi: 10.3389/fmed.2021.612370

We aimed to determine the characteristics that distinguish glycosaminoglycans (GAGs) from osteoarthritis (OA) and normal cartilage and from men and women. Cartilage samples from 30 patients subjected to total joint arthroplasty secondary to OA or fracture (control) were evaluated, and the GAG content (μg/mg dry cartilage) after proteolysis was determined by densitometry, using agarose-gel electrophoresis. Relative percentages of carbon (C), nitrogen (N), and sulfur (S) in GAGs were determined by elemental microanalysis, as well as the zeta potential. Seventeen samples (56.6%) were from patients >70 years old, with 20 (66.6%) from women, and most [20 (66.6%)] were from the hip. The GAG content was similar regardless of patients being >/≤70 years old with 96.5 ± 63.5 and 78.5 ± 38.5 μg/mg ($P = 0.1917$), respectively. GAG content was higher in women as compared to men, with 89.5 ± 34.3 and 51.8 ± 13.3 μg/mg, respectively ($P = 0.0022$), as well as in OA than fracture samples, with 98.4 ± 63.5 and 63.6 ± 19.6 μg/mg, respectively ($P = 0.0355$). The GAG extracted from the cartilage of patients >70 years old had increase in N, and there were no gender differences regarding GAG elemental analysis. GAG from OA had a highly significant ($P = 0.0005$) decrease in S% (1.79% ± 0.25%), as compared to fracture samples (2.3% ± 0.19%), with an associated and significant ($P = 0.0001$) reduction of the zeta potential in the OA group. This is the first report of a reduced S content in GAG from OA patients, which is associated with a reduced zeta potential.

Keywords: cartilage, osteoarthritis, zeta potential, sulfate, glycosaminoglycans

INTRODUCTION

There is a great need for accurate, reproducible biomarkers to be used in the management of osteoarthritis (OA). Without adequate parameters, it is virtually impossible to evaluate interventions to treat OA patients. Numerous attempts to quantitate soluble biomarkers or to use imaging techniques have failed to provide those most needed biomarkers (1). When

subjected to OA damage, inadequate proliferation and osteophyte formation in the joint are followed by complete disruption and erosion of the cartilage leading to bare areas exposing the underlying subchondral bone. Proliferative rather than degenerative changes occurring in OA lead to osteophyte formation (2). It thus might well be that qualitative rather than quantitative changes will help distinguish OA from non-OA (normal) cartilage. Type II collagen and chondroitin sulfate (CS)–rich proteoglycans represent the major organic components of the extracellular joint cartilage matrix. Assembly of type II collagen and proteoglycans is essential for cartilage function, a highly hydrated structure that is frequently subjected to reversible deformation. Also, the number of glycosaminoglycan (GAG) chains attached to the protein backbone of proteoglycans and their aggregating pattern are relevant to cartilage homeostasis (3, 4). Modifications of GAG have been found in the cartilage obtained from OA patients. The molar mass (MM) of hyaluronan, which is the main GAG in joint cartilage, was shown to be altered in areas of more severely damaged cartilage of knee OA patients (5, 6). Also, CS extracted from the cartilage of areas most severely affected by OA was shown to display reduced MM (7, 8).

In addition to the biological and biochemical characteristics, the electric charge of extracellular components of matrix cartilage is also relevant to homeostasis (9). The presence of both carboxylate (COO–) and sulfate (OSO3–) groups linked to the GAG attached to the protein core of proteoglycans provides a negative surface charge that is crucial to cartilage function, particularly during weight-bearing deformation (10). The zeta potential is a parameter that measures the surface charge of nanoparticles/macromolecules that can be either negative or positive, depending on the predominant electrical charge. The repulsion between surfaces of similar charge is responsible for the stability of a dispersion. When the zeta potential is reduced, compounds dispersed in a matrix have a tendency to aggregate (11). In this regard, the negatively charged groups present in GAG from joint cartilage account for its negative zeta potential. It has been shown that a zeta potential of 0 ± 10 mV compromises the stability of a polysaccharide in a solution, which then exhibits a tendency to flocculate (12). Maintenance of GAG stability may impact cell function as it was shown that *in vitro* chondrocyte growth is optimized in negatively charged rather than in neutral hydrogel matrices (13). We performed an elemental microanalysis of GAG extracted from the cartilage of patients subjected to arthroplasty either secondary to OA or that sustained a fracture (control) in an attempt to detect differences between those groups.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all reagents were purchased from Sigma–Aldrich do Brasil S.A., São Paulo, Brazil.

Collection of Human Cartilage Samples

Thirty cartilage samples from patients subjected to total joint arthroplasty in the Hospital Universitário Walter Cantídio, Fortaleza-CE, Brazil, secondary to OA or fracture (control) were collected.

Inclusion Criteria

Fifty- to 80-year-old patients subjected to total hip or shoulder arthroplasty secondary to OA or fracture, according to the clinical files, with body mass index <35 kg/m^2; a written informed consent was signed prior to collection of the material, as per the Brazilian rules of human experimentation.

Exclusion Criteria

refusal of the patient to participate at any time, presence of diabetes or a specific acute or chronic inflammatory arthropathy but OA. Patients who died following remote postsurgical period had the material discarded.

Prior to collecting samples, the clinical history of each patient and radiographies of the joint that would undergo arthroplasty were reevaluated by a senior rheumatologist (F.A.C.R.) both to confirm OA diagnosis and to exclude OA or other arthropathy in patients with a fracture diagnosis. GAGs were extracted within < 3 h postsurgery and analyzed as described above. The protocol was approved by our local Ethics Committee on Human Research (protocol 090.12.08) that follows the rules of the Comitê Nacional de Ética em Pesquisa, which is the Brazilian Official Committee for Ethics in Human Research. All patients signed a written informed consent prior to any procedure.

GAG Extraction

Cartilage samples were weighed after overnight drying (80°C) and stored in acetone. Proteolysis of this material was done by incubating 1 mg with 20 μL of a 0.4% wt/vol suspension of PROLAV 750™ (Prozyn, São Paulo, Brazil) in Tris–HCl/NaCl 50/150 mmol/L buffer (pH 8.0) for 48 h, at 56°C. Subsequently, the NaCl concentration was corrected to 1.0 mol/L, and the mixture was kept at 37°C during 30 min. The remaining proteins were precipitated with trichloroacetic acid to a final concentration of 10% wt/vol and centrifuged (10,000 *g* for 15 min at 25°C). GAG was precipitated from the supernatant with two volumes of ethanol, followed by an overnight incubation at 4°C and centrifugation (10,000 *g* for 15 min at 15°C). The precipitated material was dissolved in 20 μL of distilled water. Protein content in the debris was considered negligible as it was undetected even using a NanoDrop apparatus.

GAG Quantification

The GAG extract was separated on a 0.6% wt/vol agarose-gel electrophoresis in diaminopropane–acetate buffer (50 mmol/L, pH 9.0). The GAG was fixed in the gel through immersion in a 0.1% wt/vol cetyl-trimethylammoniun bromide solution for 2 h. The gel was dried and stained with 0.1% wt/vol toluidine blue (in acetic acid:water:ethanol 1:49:50). For comparison, C4S, C6S, and heparan sulfate standards were subjected to the same protocol (14). Quantification was made by densitometry (525 nm). Data are expressed as μg CS/mg of dried cartilage.

Elemental Analysis of GAG

The relative percentages of carbon (C), nitrogen (N), and sulfur (S) were determined by elemental microanalysis in a Carlo Erba

TABLE 1 | Clinical features of patients subjected to arthroplasty secondary to Osteoarthritis (OA) or fracture (control).

	Groups	Fracture	OA
Age (Mean± SD)		67 ± 5	67 ± 4
Age range	≤ 70	6	7
	> 70	10	7
Gender	Female	10	10
	Male	6	4
Joint	Hip	10	10
	Shoulder	6	4

EA 1108 micro. The sulfate content was calculated from S% by a previously proposed equation (15). This strategy subjects the sample to combustion in pure oxygen atmosphere, and the expelled gases are detected and semiquantified using a thermal conductivity detector. Thus, the oxygen content cannot be determined.

Determination of the Zeta Potential (Pζ)

Zeta potential and conductivity were measured by a Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd., Worcestershire, United Kingdom) with an $\lambda = 633$-nm laser detector with a 17° detection angle, at 25°C. GAG samples (50 μg/mL in deionized water) were swollen in deionized water to the equilibrium state and ground into small particles. After drying in a vacuum oven for 24 h, the particles were weighed, diluted in 1 mL deionized water, and measured.

Statistics

Results are presented as means ± SD for GAG concentration and medians [interquartile range (IQR)] for percentage of elements and evaluated using Student *t*-test and Kruskal–Wallis test, respectively; $P < 0.05$ was considered as significant.

RESULTS

Clinical Demographics

A total of 30 cartilage samples were collected. There were 17 samples (56.6%) from patients >70 years old, with 20 (66.6%) collected from women, and most [20 (66.6%)] were hip samples (**Table 1**). According to the clinical history, all fractures occurred after falls with minimal trauma.

Quantification of the GAG Extracted From the Articular Cartilage

The relative GAG content in analyzed samples was in the range of 50–85 wt%. **Figure 1** illustrates that the GAG content relative to the dried cartilage weight was similar regardless of patients being >/≤70 years old (a); regarding gender, the relative GAG content was significantly higher in samples from women (b); there was also a significantly higher increase in the relative GAG content in samples from OA patients as compared to samples from patients who sustained a fracture (c).

Elemental Analysis of GAG

Using CS as reference (chemical formula $C_{13}H_{21}NO_{15}S$), the relative C, N, and S percentages are roughly 34, 3, and 7 wt%, respectively. However, the observed percentage values of C (circa 23 wt%) and S (circa 2 wt%) were smaller than the predicted theoretical values, particularly regarding S content. Elemental analysis showed that GAG from the cartilage of patients >70 years old had a significant decrease in N, as compared to patients ≤70 years old (**Figure 2**). There were no differences in C, N, or S relative content regarding gender (**Figure 3**). Remarkably, the GAG extracted from the cartilage of patients with OA had a highly significant decrease in the relative S content, as compared to samples obtained from patients who sustained a fracture (**Figure 4**). There was also a slightly higher N relative content in GAG samples from OA patients, which reached statistical significance, probably secondary to the relative reduction of the S content in that group (**Figure 4**).

Analysis of the Zeta Potential (Pζ) of GAG

Figure 5 illustrates the analysis of the zeta potential considering age, gender, and disease variation. As expected, all samples had a negative zeta potential, which varied within a −19 to −26 mV range. The zeta potential of GAG samples from the articular cartilage of patients >/≤ 70 years old was similar (**Figure 5A**); regarding gender, there was a slight variation as cartilage samples obtained from women had a trend toward a higher modulus (−26 mV), meaning being more negative, although not reaching statistical significance, when compared to GAG samples from men (**Figure 5B**); finally, there was a remarkable significant reduction of the zeta potential in GAG samples collected from the cartilage of OA patients as compared to those from patients who sustained a fracture (**Figure 5C**).

DISCUSSION

The present data describe a decrease in the sulfate content and a correspondent decrease in the zeta potential of GAG extracted from the cartilage of joints affected by OA. There is also an increase in the relative GAG content in samples from OA patients, as compared to those from fracture (control) patients.

Both GAG content and integrity are crucial to the aggrecan role in cartilage physiology. It was reported that CS obtained from OA cartilage exhibits structural alterations, meaning different length and sulfation patterns, which may impact cartilage function (7, 8). Our data reinforce those findings to suggest that qualitative changes reflect cartilage damage in OA joints. Integrity of GAG molecules is crucial to provide deformability of the cartilage particularly during weight-bearing. Additionally, GAGs are able to specifically bind to cytokines and growth factors, triggering intracellular signaling. Thus, structural modifications of the GAG structure may impact cellular responses, thus altering the function of cartilage and synovial cells (16–18).

Increased GAG content in OA cartilage has been previously shown and may illustrate the initial proliferative response of chondrocytes, as part of a repairing process. However, subjected to an OA inflammatory milieu, the "osteoarthritic chondrocytes"

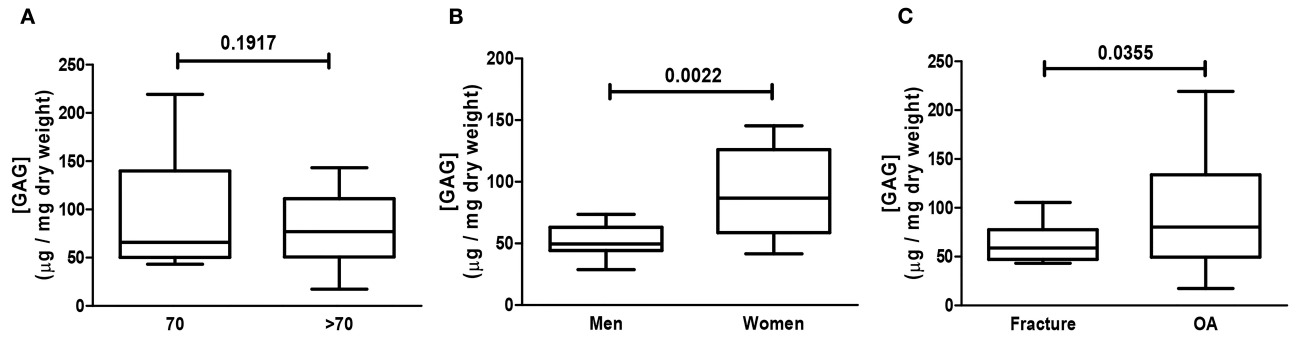

FIGURE 1 | Assessment of glycosaminoglycan (GAG) content. Human cartilage samples from patients subjected to arthroplasty secondary to OA or fracture were assessed for GAG content. Data represent mean ± SEM of GAG content (µg/mg) of dried cartilage as follows: patients >/≤70 years old **(A)**, gender **(B)**, and OA and fracture **(C)** of at least $n = 6$/group; Student *t*-test.

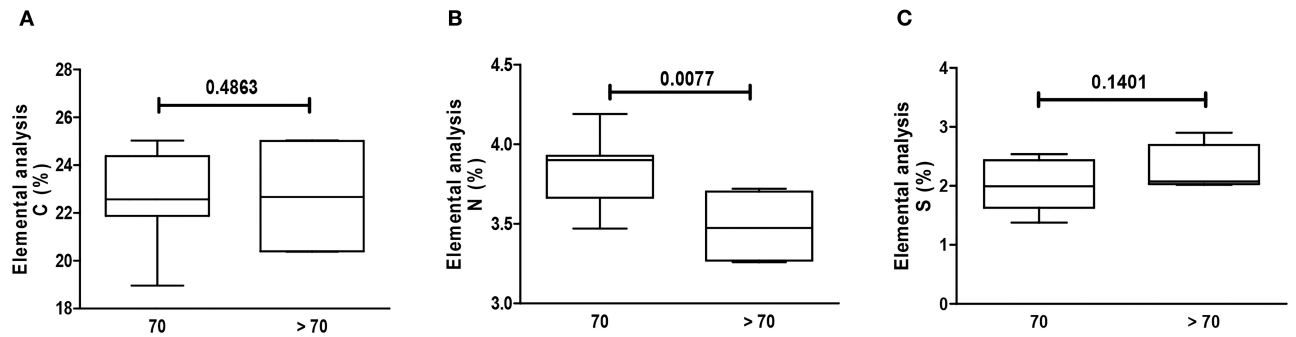

FIGURE 2 | Elemental analysis of GAG. Elemental analysis of GAG from human cartilage samples of patients >/≤70 years old subjected to arthroplasty secondary to OA or fracture. Data represent medians (IQR) of relative percentage of carbon (C), nitrogen (N), hydrogen (H), and sulfate (S) of at least $n = 6$/group; Kruskal–Wallis test.

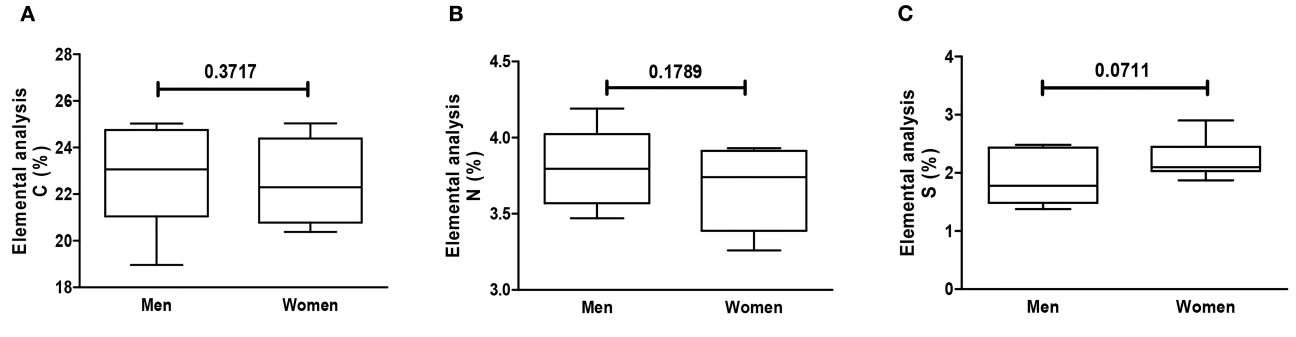

FIGURE 3 | Elemental analysis of GAG per gender. Elemental analysis of GAG from human cartilage samples of patients subjected to arthroplasty secondary to OA or fracture patients. Data represent medians (IQR) of relative percentage of carbon (C), nitrogen (N), hydrogen (H), and sulfate (S) of at least $n = 6$/group; Kruskal–Wallis test.

not only lose capacity to synthesize normal GAG but also fail to replace normal cartilage. In later stages, joint erosion occurs, with areas of denuded cartilage and exposure of the subchondral bone (4, 5). In fact, our OA samples were from patients with end-stage disease, and all possible remaining cartilage was collected. Gross macroscopic evaluation does not always allow discriminating normal from damaged cartilage. Although we cannot rule out some remaining areas of normal cartilage in the OA samples, data were treated as one sample for each patient. Cartilage from dogs subjected to an experimental OA model had increased amount of proteoglycans, as compared to controls (6). Also, joint cartilage collected from humans subjected to hip arthroplasty secondary to OA was shown to have an increase in GAG content, as compared to cartilage collected from patients with fracture of that

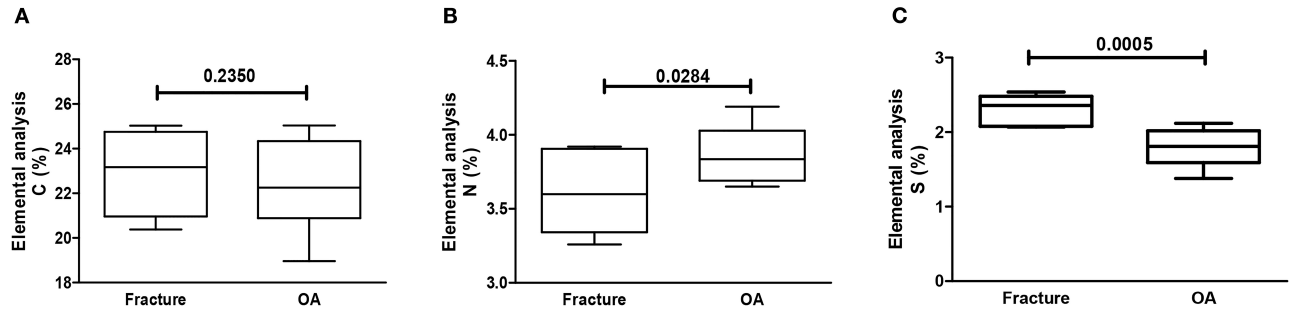

FIGURE 4 | Elemental analysis of GAG. Elemental analysis of GAG from human cartilage samples of patients subjected to arthroplasty secondary to OA or fracture. Data represent medians (IQR) of relative percentage of carbon (C), nitrogen (N), hydrogen (H), and sulfate (S) of at least $n = 6$/group; Kruskal–Wallis test.

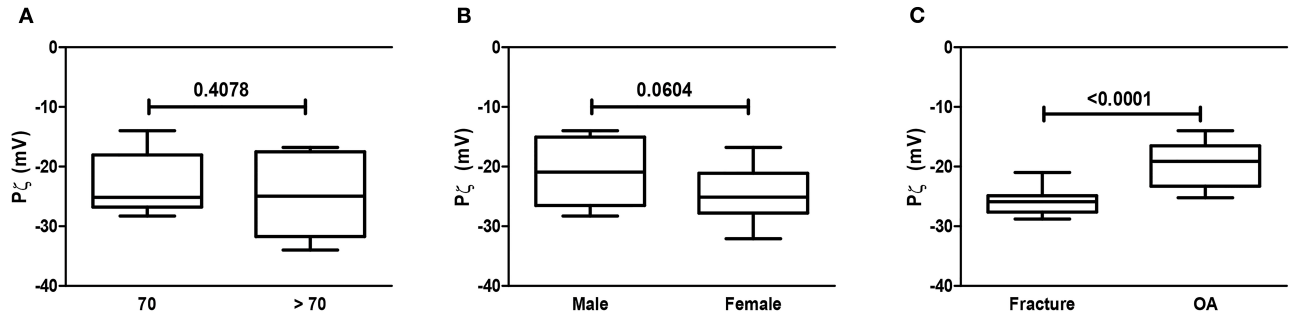

FIGURE 5 | Analysis of the zeta potential (Pz). Analysis of the Pz of GAG from human cartilage samples of patients subjected to arthroplasty secondary to OA or fracture. Data are expressed as mean ± SEM of zeta potential (mV) considering age (>/≤70 years old) **(A)**, gender **(B)**, and **(C)** OA/fracture of at least $n = 6$/group; Student *t*-test.

joint, used as control (19). Notwithstanding, analysis of magnetic resonance imaging of OA joints revealed an increase in matrix production in patients with recently developed OA, an aspect that was regarded as part of a repair mechanism (20, 21). Although still seen as a degenerative disease, characteristic imaging findings in the OA joint reveal sclerosis of the subchondral bone and osteophyte formation, which gives an impression of *de novo* remodeling. In keeping with those data, the relative increase in the GAG content found in OA samples may be secondary to a proliferative, although inadequate, process happening in the affected joint.

We are not aware of previous studies showing elemental analysis of GAG isolated from the cartilage of human joints. However, at least regarding S content, a similar order of relative percentage (0.7–1.3%) was found in cartilage obtained from dogs (22). Our samples were processed just after surgical removal, aiming to avoid any possible alterations that could be due to postmortem modifications or freeze-thawing issues. There was an increase in the relative GAG content in dried cartilage seen in samples from women, which could be linked to a higher number of women in the OA group. Samples from OA cartilage had a remarkable significant decrease in the S% content as compared to control samples of patients with similar age range. Indeed, all but one of the GAG samples from the OA group had a relative S% content in the lowest value found in fracture patients. Increased thickness of OA cartilage using delayed gadolinium-enhanced magnetic resonance was associated with increased swelling secondary to a decrease in GAG content (23). In keeping with our present data, using micro–X-ray fluorescence, it was shown that the deep zones of OA cartilage have a decrease in elemental S, which was associated with a decrease in GAG staining (24). The modulus of the zeta potential of the GAG from OA samples was significantly reduced, meaning a reduction in the negative charge of the polysaccharides probably secondary to the reduced S% relative content. We are not aware of previous studies focusing on the relevance of GAG charge to cartilage physiology. Sulfation of GAG is responsible for the negative charge of those molecules. After a compressive force applied to the cartilage, the repulsion between adjacent negatively charged GAG molecules allows the entry of water providing adequate cartilage hydration, which is crucial to a healthy joint (25). Although there is a positive association of the zeta potential and the stability of small particles, reducing its tendency to aggregate (13, 26, 27), there are no previous studies on the stability of GAG, let alone the relevance of the zeta potential of those molecules. However, it is reasonable to admit that a normal sulfation pattern contributes to the physiology of polysaccharides in the extracellular cartilage matrix. In this case, a reduced charge does also compromise hydration of the cartilage (28, 29).

Biomarkers to be used in clinical practice are an unmet need in OA (1). Our data show that a decrease in sulfation is associated with a correspondent reduction in the zeta potential of GAG collected from OA cartilage. Current imaging studies can be designed to deliver markers able to quantitate the sulfate content or the charge of GAG in the joint cartilage. Mapping those alterations may provide semiquantitative imaging useful to evaluate interventions to modify the disease course in OA patients.

There are some limitations to our study, including time sampling. However, as mentioned previously, all material was processed within < 3 h postsurgery. Additionally, GAGs are very stable and probably would not be affected by processing. One may also argue that our samples represent solely end-stage OA joint disease. Collecting enough material from living humans is very hard, and ethical rules do apply. Considering that we analyzed the whole joint, we probably saw the predominant parameter in all remaining cartilage. However, it remains to be shown if such data are reproduced in less severely affected joints. Another limitation is the low number of samples, particularly those from men, limiting gender analysis. We also restricted our samples to the hip and shoulders, and the low numbers did not allow us to compare possible differences regarding specific joints. Although knee OA is more prevalent than hip OA (30), knee fractures that lead to joint replacement are rare, making it difficult to have a suitable non-OA knee control. We also cannot completely rule out subclinical OA changes in fracture (control) samples. However, our combined clinical and imaging rheumatologic and orthopedic evaluations suggest that an OA diagnosis in the fracture group is unlikely.

In summary, we demonstrate that cartilage from OA samples displays a relative increase in the CS content. We also show that GAGs from the extracellular matrix of joints affected by OA have a decrease in sulfate content, which is associated with a decrease of the zeta potential of those polysaccharides. The possible relevance to the pathophysiology of this disease, as well as utility as a biomarker, warrants further investigation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Conselho de Ética do HUWC-UFC (Protocol number 090.12.08). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FR and JF conceived the protocol. RN, AP, VG, PC, and FR performed experiments and sample collection. RN, JF, and FR performed data analysis. All authors wrote, revised, and approved final manuscript.

FUNDING

This work was partially supported by Grants from CNPq (405661/2018-5; 308429/2018-4) and INOMAT.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer JF is currently organizing a Research Topic with the author FR.

frontiers in Medicine

REVIEW
published: 26 May 2021
doi: 10.3389/fmed.2021.684389

Synovial Fibrosis Involvement in Osteoarthritis

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Bone changes have always been the focus of research on osteoarthritis, but the number of studies on synovitis has increased only over the last 10 years. Our current understanding is that the mechanism of osteoarthritis involves all the tissues that make up the joints, including nerve sprouting, pannus formation, and extracellular matrix environmental changes in the synovium. These factors together determine synovial fibrosis and may be closely associated with the clinical symptoms of pain, hyperalgesia, and stiffness in osteoarthritis. In this review, we summarize the consensus of clinical work, the potential pathological mechanisms, the possible therapeutic targets, and the available therapeutic strategies for synovial fibrosis in osteoarthritis to gain insight and provide a foundation for further study.

Keywords: osteoarthritis, fibrosis, synovitis, fibroblast-like synoviocytes, extracellular matrix

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Specialty section:
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

Received: *23 March 2021*
Accepted: *03 May 2021*
Published: *26 May 2021*

Citation:
Zhang L, Xing R, Huang Z, Ding L, Zhang L, Li M, Li X, Wang P and Mao J (2021) Synovial Fibrosis Involvement in Osteoarthritis. Front. Med. 8:684389. doi: 10.3389/fmed.2021.684389

RHEUMATOLOGY KEY MESSAGES

Synovial fibrosis is closely associated with joint pain, hyperalgesia, and stiffness in osteoarthritis.

Sounder diagnostic criteria should be established for OA-related synovial fibrosis.

The mechanism of synovial fibrosis is being investigated, and available therapeutic strategies require further study.

INTRODUCTION

Osteoarthritis (OA) is the most common degenerative joint disease and is characterized by pain, stiffness, and limited function in the clinic (1). In 2017, OA affected nearly 303 million people worldwide, including ∼263 million people with knee OA and 40 million people with hip OA (2). Bone changes, such as the progressive loss and destruction of articular cartilage, thickening of the subchondral bone, and the formation of osteophytes, reflect the pathogenesis of OA, so the study of cartilage and subchondral bone in OA has always been a priority (3). Such studies are highly consistent with the etiology of OA, which involves aging, mechanical stress, and environmental changes in the joints. Bone changes may be the determining factor for the eventual use of surgical treatment for OA; unexpectedly, the consistency of the bone structure with clinical symptoms remains unclear, at least in terms of pain (4, 5). This implies that further efforts are needed to discover the pathological mechanisms of OA, especially those related to OA symptoms.

As OA involves chronic low-grade inflammation, the presence of an inflammatory microenvironment is likely to affect all tissues constituting the joint (6). It is widely accepted that synovitis can occur in the early stage of OA, promoting the development of OA throughout the whole pathologic process. Therefore, non-steroidal anti-inflammatory drugs (NSAIDs) are

strongly recommended for clinical treatment, and OA is considered to be a highly prevalent rheumatic musculoskeletal disorder (7, 8). As cartilage destruction partly depends on the effect of inflammation, which disrupts the balance between synthesis and degradation in the extracellular matrix (ECM) (7), it may also be valuable to evaluate the damage caused by inflammation in the synovium. Overall, one major outcome of inflammation or inflammatory exudation is fibrosis, especially in the lung, liver, and kidney. In OA, synovial fibrosis (SF) is an imbalance caused by fibroblast proliferation and the disturbance of collagen synthesis and degradation, ultimately leading to excessive collagen deposition in the ECM (9, 10).

Recent research has also revealed that the ECM plays multiple roles in OA (11, 12). This may indicate that SF is not only a pathological outcome but also a likely pathogenic factor. SF is often accompanied by angiogenesis in both OA and rheumatoid arthritis (RA) (13). Recent studies have also found evidence for increased sensory innervation in the synovium in knee OA, but there is still no direct evidence on whether SF is associated with pain (14, 15). Does joint stiffness due to fibrosis associate with OA pain? Is SF associated with increased sensory innervation? Can the progression of SF be blocked when synovitis is alleviated? Obtaining a narrative review of SF in OA is an interesting research direction; thus, we searched PubMed with the keywords "fibrosis," "OA," and "RA." We reviewed the pertinent literature to answer the following questions: What do we know about SF in osteoarthritis?

Synovial Fibrosis and Synovitis

Synovitis is a typical chronic aseptic inflammation. Common symptoms caused by synovitis include pain, local temperature rise, swelling, joint movement limitation, and the severity of these symptoms is related to the degree of joint effusion (16). Synovitis is also known to produce a large number of pro-inflammatory factors, such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), IL-6, IL-8, IL-15, IL-17, IL-21, inflammatory mediators, including PGE2, NO, adipokines, and matrix metalloproteinases (MMP-1, MMP-3, MMP-9, MMP-13), which lead to cartilage destruction, amplifying synovitis and ultimately creating a vicious cycle (17, 18). Besides, synovitis promotes the production of pain neurotransmitters, such as nerve growth factor and bradykinin (19). At the same time, synovitis promotes synovial angiogenesis, which in turn accelerates inflammation and leads to SF directly (20).

Usually, SF appears in the later stages of OA, which is different from synovitis. But in a study examining the effects of the intra-articular application of bupivacaine and levobupivacaine, inflammation and late fibrosis were found shortly after injection, suggesting that synovitis promotes fibrosis (21). On the other hand, as an aseptic chronic inflammatory disease, SF may be the inevitable outcome of "damage-repair," and thus it can be emphasized that synovial inflammation drives the development of fibrosis. Notably, current studies cannot conclude that SF can cause synovitis independently, and whether synovial fibrosis can exist independently of synovitis, remains a topic of great interest to OA research. As for the relationship between synovitis and SF, maybe it is not well-understood what is the hen and what the egg, but this question is the one to inspire researchers' in-depth research.

Synovial Fibrosis in Osteoarthritis

Clinical Status of Synovial Fibrosis

Much evidence has shown that SF is one of the most important causes of joint stiffness, synovial hyperplasia, and limited function, which are common symptoms in moderate and severe OA; other evidence also confirmed that a higher SF score is correlated with lower scores for KL grade, which indicates that SF may be negatively associated with clinical symptoms of OA (22, 23). This is because generalized pain is a major claim in OA patients, while independent joint stiffness does not occur very often. When joint stiffness begins to bother OA patients and joint movement is limited, loss of function becomes a reality.

Surgical treatments for OA, such as total knee arthroplasty (TKA), can cause arthrofibrosis, a fibrosing pathology of the synovial membrane, and the infrapatellar fat pad (24). In contrast to moderate and severe OA, TKA eliminates the effects on cartilage and the meniscus, so post-operative pain and dysfunction derived from SF can be observed more easily. Kalson et al. attempted to establish criteria for the diagnosis, classification, and severity grading of soft-tissue fibrosis after TKA and suggested that the diagnosis of fibrosis after TKA should be based on the exclusion of other causes of stiffness, the range of movement of the knee, the pathological anatomy and histopathology (**Table 1**) (25). These recommendations may also be adapted for the diagnosis of SF associated with primary OA.

Recent developments in MRI and ultrasound have made it possible to investigate SF, but there is currently not enough evidence for routine use (26, 27). The degree of synovial thickening, not the volume, has been proven to be correlated with the level of SF on MRI, but others reported that the correlations between these factors were very weak (28, 29). This is likely because the MRI evaluation is based on the synovitis score, which fluctuates at different stages of OA, while the extent of fibrosis is relatively fixed. Ultrasound has also been shown to be useful in detecting and quantifying synovial abnormalities, especially for synovitis, as Doppler signals indicate active inflammation and vascularization in synovial arthritis but not fibrosis (30, 31). Laboratory tests of the synovium may be more advantageous for SF assessment than imaging evidence, and methods such as identification of cell phenotypes, quantitative detection of profibrotic markers, immunohistochemistry of collagen, and even HE staining can provide some guidance (32). According to the criteria established by Ruppert et al. for HE staining of sections, SF can be divided into three levels according to the ratio of the fibroblast-like synoviocyte (FLSs) length to the distance between FLSs (33). It seems that the "gold standard" evaluation for SF in OA is histology, although this requires an invasive biopsy that may not be applicable or acceptable to all patients.

Pathological Characteristics of Synovial Fibrosis

The normal synovium can be divided into the intima (synovial lining) and the subintima (outer layer). The intima comprises one to three layers of specialized columnar FLSs, which are interspersed with macrophages, while the subintima consists

TABLE 1 | Criteria for the diagnosis, classification and severity grading of soft-tissue fibrosis after TKA established by Kalson et al.

	Category	Criteria	Exclude
Main diagnostic criteria	Restricted ROM	① Soft-tissue fibrosis that was not present preoperation. ② Loss of movement on extension>5°. ③ Flexion range≤100°	Problems with implant (malpositioning, cement, ectopic bone formation, loosening, malalignment); ligament reconstruction, infection, pain, CRPS or other specific causes; wound issues, incorrect surgical indication
Secondary diagnostic criteria	Stiffness		
	Pain		
	Inflammatory markers	CRP, WBCs	
	Aspiration of the joint	Microbiological culture and cell count	
Auxiliary diagnosis	X-ray, CT		Component malalignment; heterotopic ossification; patella infera
	MRI	Measurement of perisynovial thickness or quantification of fibrotic tissue in the parapatellar gutters	Focal fibroses; scar tissue
	Open or arthroscopic surgery	Direct visualization of fibrosis	
	Pathological anatomy and histopathology	Supply evidence of fibrosis, not essential; characterized by a varying degree of cellularity of fibroblasts.	

of multiple types of connective tissues, such as fibrous dense collagen, adipose tissue, or loose collagens. This layer is rich in type I and III collagen and microvascular blood supply, accompanied by lymphatic vessels and nerve fibers, but is relatively acellular (34, 35). From histological patterns, the synovium in OA patients is characterized by intima hyperplasia, subintima fibrosis, and stromal vascularization (36). In the latest report, scholars demonstrated increased innervation of the medial synovium after KOA surgical modeling, and the medial compartment of OA knees exhibited striking changes in $Na_V1.8^+$ innervation (7). Oehler et al. subtyped osteoarthritic synoviopathy and identified four patterns of OA-associated synoviopathy: hyperplastic, fibrotic, detritus-rich, and inflammatory synoviopathy (37). Interestingly, excluding hyperplastic synoviopathy, the remaining three subtypes are nearly all overlapping, with different emphases and degrees of inflammation. The fibrotic type has most of the characteristics of the other three types, but there is no macromolecular cartilage and bone debris, which is one of the typical features of detritus-rich cartilage. This indicates that OA induced by different factors could involve different subtypes of synoviopathy with varied features, and at least the fibrotic type originates more from inflammation than from cartilage debris. In summary, SF associated with OA should be identified as the accumulation of collagen under pathological conditions, dominated by abnormal remodeling of collagen types I and III in the subintima, together with angiogenesis and nerve invasion. Therefore, in the study of SF, angiogenesis and nerve invasion should also be considered.

The function of the normal synovium is mainly reflected by FLSs, as they are involved in the production of hyaluronan, collagens, and fibronectin in the intima and synovial fluid. This is essential for joint movement and cartilage nutrition (38). Macrophages make up a minority of cells in the normal intima, but their numbers increase dramatically in inflammatory arthritis (36, 38). Both types of cells are involved in SF associated with osteoarthritis. Surprisingly, these cells not only determine ECM changes but also dictate the functions of resident cells within tissues. The ECM supplies cells with proper chemical and mechanical signals to regulate cell proliferation, migration, and differentiation to maintain tissue homeostasis (36, 39). In SF, collagen I exhibit a disorganized structure and enhanced cross-linking, while collagen III is crucial for appropriate collagen I fibrillogenesis and tissue functionality (39). Petersen et al. believed that markers of type I or III collagen turnover may reflect the severity of synovitis and SF, which is highly correlated with OA pain sensitivity. Fragments of type I, II, and III collagens were then investigated in blood from OA patients compared with blood from control individuals, revealing increased degeneration of type I and II collagen and decreased degeneration of type III collagen, which was highly correlated with localized hyperalgesia in response to pressure stimulation (40). In conclusion, abnormal secretion of synovial cells forms the pathological basis of SF due to the inherent effects of the ECM as a pathogenic factor and biomechanical stimuli involved in OA. The subsequent collagen environment presents a fibrotic state with changes in synovial permeability and mechanical properties, which may cause pain and stiffness in OA joints.

Potential Etiology of Synovial Fibrosis

Fibrosis typically originates from abnormal tissue repair in response to wound healing. Fibroblasts activated by multiple diverse signals play a central role in this process, differentiate into

TABLE 2 | Fibrogenic factors in OA.

Protein (encoding gene)	Risk factors	Function	Notes	References
TGF-β and TGFβR	Aging	Receptors and ligands, signaling	Senescence-associated secretory phenotype	(41)
	Hypoxia		Positive feedback cycle between NLRP3 inflammasome activation and TGF-β1 induction	(42)
	ECM changes		Promotion of terminal differentiation of fibroblasts and the secretion of ECM components	(43)
	Mechanical stress		activation and release of TGF-β1	(44)
VEGF (*VEGF*)	Hypoxia	Growth factor	Modulated by HIF-1α at transcriptional level	(45)
IGF2 (*IGF*)	Hypoxia	Growth factor	Modulated by HIF-1α at transcriptional level	(45)
Angiotensin II	Hypoxia	Signaling	Modulated by HIF-2α at transcriptional level	(45)
NLRP3	Hypoxia	Signaling	Positive feedback cycle between NLRP3 inflammasome activation and TGF-β1 induction	(42)
IL-1β	Hypoxia	Cytokine	Increases TGF-β1 induction	(42)
LOXs and LOXL	ECM changes	Amine oxidases and LOX like proteins	Regulation of phosphorylation of Smad2/3 or p65 orERK1/2	(46–48)
LH2 (*PLOD2*)	ECM changes	Protease	PI3K/Akt signaling transduction; regulated by HIF or TGF	(49, 50)
CTGF	ECM changes	Growth factor	Reduction of Smad7 and promotion of TGF-β signaling	(51)

myofibroblasts, and secrete matrix molecules to rebuild the ECM structure. Any risk factor for primary OA, such as aging, hypoxia, changes in the ECM environment, and mechanical stress, may play a similar role in SF (**Table 2** and **Figure 1**). Although existing studies have not always targeted the synovium, evidence related to the balance of cartilage matrix degradation or fibrosis of other tissues can also provide some guidance for the study of SF.

Aging

Directly relevant to aging is the study of cellular senescence, which refers to a state of cell cycle arrest, increased expression of cell cycle inhibitors, and enhanced production of proinflammatory cytokines, chemokines, and growth factors. A variety of stimuli and stresses, including telomere shortening, epigenetic changes, metabolic stresses, and mitochondrial dysfunction, can cause senescence. Markers for cellular senescence, including p16INK4A and p21, are upregulated in OA tissues, including cartilage, subchondral bone, and the synovium (41, 52, 53), suggesting cellular senescence in the FLSs of OA. Besides, senescence-associated secretory phenotype (SASP) is a pro-inflammatory secretory phenotype associated with cell senescence, including pro-inflammatory cytokines (such as IL-1α, IL-1β, IL-6, and IL-8), Tissue growth factors (TGF-β), MMPs, tissue inhibitors of metalloproteinases (TIMPs), and all these cytokines play important roles in SF. As cells in the synovium become proliferative and activated during SF, they may themselves become more susceptible to undergoing senescence. Thus, despite the lack of direct evidence, FLSs senescence is likely to promote the development of SF during aging.

Hypoxia

Hypoxia refers to a decrease in oxygen tension in tissues, and the central effector of the hypoxia response is the transcription factor hypoxia-inducible factor (HIF). In the hypoxic state, the alpha subunit in HIF is no longer hydroxylated but accumulates and translocates to the nucleus, where it binds to the beta subunit of HIF and exerts its function as a transcription factor (54). The genes encoding VEGF, TGF-β, and IGF-2, which are regulated by HIF-1α, and angiotensin II, which is modulated by HIF-2α, are all important profibrotic factors (45). Clinical studies have shown that HIF-1α levels in the serum, synovial fluid, and articular cartilage of knee OA patients are associated with progressive joint damage (55, 56). Hypoxic TGF-β1 induction increased succinate accumulation due to the reversal of succinate dehydrogenase activation and induced NLRP3 inflammasome activation in a manner dependent on HIF-1α induction. In response to NLRP3 inflammasome activation, the released IL-1β further increased TGF-β1 induction, suggesting the existence of a positive feedback cycle between inflammation and fibrosis in myofibroblast activation; this highlights the importance of studying SF associated with OA from the perspective of hypoxia (42).

Extracellular Matrix Changes

Remst et al. analyzed gene expression in TGF-β-stimulated human OA synovial fibroblasts and the synovium of mice with TGF-β-induced fibrosis, mice with experimental OA, and humans with end-stage OA. The genes encoding lysyl oxidase (LOX), pro-collagen-lysine, 2-oxoglutarate 5-dioxygenase 2 [PLOD2, also known as lysyl hydroxylase 2b (LH2b)], tissue inhibitor of metalloproteinase 1 (TIMP-1), collagen type I α1 chain (COL1A1), and collagen type V α1 chain (COL5A1) were upregulated under TGF-β stimulation, indicating that the signaling cascades of these key fibrotic factors were activated (57). Moreover, due to the vital role of matrix metalloproteinases,

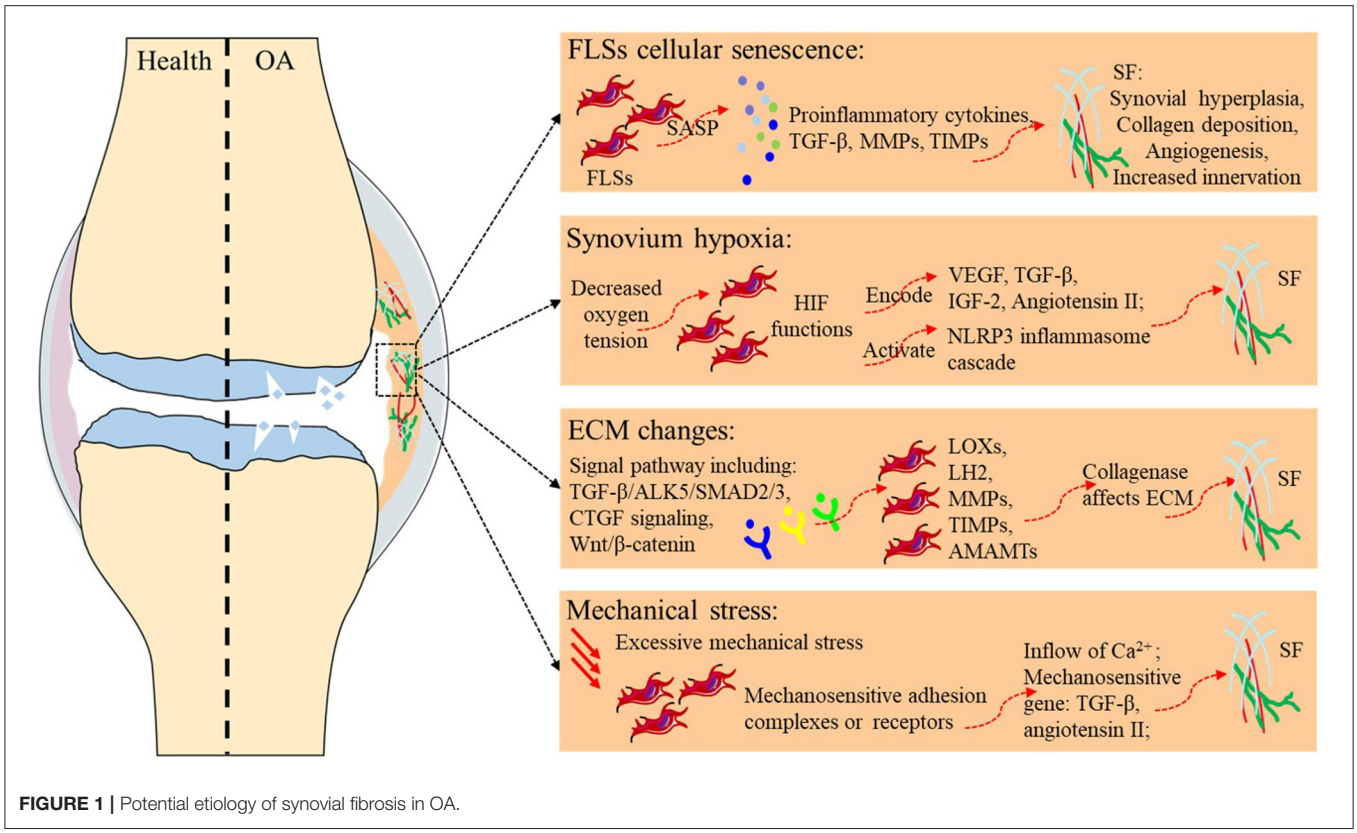

FIGURE 1 | Potential etiology of synovial fibrosis in OA.

a disintegrin and metalloprotease (ADAMS) and a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS) in ECM remodeling, the contributions of specific members in this family to SF should also be considered.

TGF-β plays a central role in the fibrotic cascade and is present as three isoforms (TGFβ1-3), all of which are elevated in OA patients and positively correlated with pain, loss of function, and radiographic staging (58). TGF-β signaling is initiated by binding to the TGF receptor, a heterodimer composed of TGFβR1 and TGFβR2. Further signal transduction is usually divided into SMAD-dependent classical pathways and non-classical pathways that are independent of SMAD. In the classical pathways, a phosphorylated TGFβR1, typically ALK5, can then transduce the TGF-β signal intracellularly to activate SMAD2/SMAD3, which complexes with SMAD4 to regulate gene expression. In contrast, the non-canonical pathway signals *via* other kinases, such as extracellular signal-regulated kinase, mitogen-activated protein kinase, nuclear factor-κB, and JUN amino-terminal kinase (43). TGF-β pathways promote the terminal differentiation of fibroblasts and the secretion of ECM components, especially collagen, fibronectin, and proteoglycans. A detailed description of the role of the TGF-β signaling pathway in OA is beyond the scope of this paper, but it is remarkable that TGF-β and its subfamily, bone morphogenetic proteins, play multiple roles in maintaining homeostasis of the cartilage and subchondral bone in OA. The TGF-β-mediated protective effects on cartilage matrix turnover rely not only on the production of ECM proteins such as type II collagen and aggrecan but also on the blockade of ECM protein degradation *via* increased production of protease inhibitors such as TIMP. Broeren et al. developed a 3-dimensional synovial membrane model involving micromasses made of either human primary synovial cell suspensions or a mixture of primary FLSs and CD14+ mononuclear cells. To recreate the synovial membrane in OA, the micromasses were exposed to TGF-β, which led to fibrosis-like changes in the membrane, including increased alpha smooth muscle actin (α-SMA) and increased expression of the fibrosis-related genes PLOD2 and COL1A1 (59). These results provide a detailed analysis of SF and show the suitability of this setup as a synovial membrane model for further research on RA and OA. Consistent with Broeren, Remst et al found that TGF-β induced PLOD2 expression in human FLSs *via* the ALK5/SMAD2/3 signaling pathway, thus aggravating SF in OA (60). In summary, high expression of TGF-β in the OA synovium accelerates OA progression, and inhibition of TGF-β in the synovium seems to be a favorable therapeutic strategy for SF. However, further research on TGF-β is still urgently needed due to its possible protective effects on cartilage.

The LOX family enzymes LOX and four lysyl oxidase-like proteins (LOXL1-4) are copper-dependent amine oxidases that catalyze the covalent cross-linking of collagen by oxidatively deaminating specific lysine and hydroxylysine residues in the telopeptide domains; this cross-linking increases collagen stiffness, which stiffens the ECM and promotes tissue fibrosis in the lung, myocardium, and liver. LOX may be induced

by TGF-β1/Smad2/3 signaling, and knockdown of LOXL1 suppressed cell proliferation and fibrogenesis in TGF-β1-stimulated HSCs by regulating the phosphorylation of Smad2/3 (46, 47). Some research suggests that LOX expression was markedly elevated in OA-damaged regions of human cartilage and mouse OA cartilage induced by destabilization of the medial meniscus (DMM) surgery, and this elevated transcription caused cartilage destruction (61). Others have suggested that LOXL2 expression may be a protective response due to the inhibition of IL-1β-induced phospho-NF-κB/p65 and TGF-β1-induced ERK1/2 phosphorylation, although LOXL2 is upregulated in OA cartilage (48). These different results may be due to the varied expression of TGF-β and its receptors during different pathological stages of OA. Signal transduction in different environments may be a determinant of TGF-β and LOX function. Therefore, in the study of the OA synovium, researchers have observed that IL-1β simultaneously promotes LOX expression but has a depressing effect combined with TNF-α, while overexpressing LOX in the synovium exacerbates OA-related fibrosis (62, 63). Overall, LOX is closely related to tissue fibrosis through TGF signaling pathways, and the potential association with HIF-2α, mechanical conduction, and other OA-related factors may be a further direction for the study of SF.

PLOD2 encodes lysyl hydroxylase 2 (LH2), which catalyzes the hydroxylation of lysine intracellularly before the collagen is secreted. Then, LOX binds to hydroxylysine residues in the extracellular collagen fibers and induces cross-linking, the final step in the maturation of collagen, which is essential for the physical and mechanical properties of collagen fibrils (64). Aberrant lysyl hydroxylation and collagen cross-linking contribute to the progression of many collagen-related diseases, such as cancer and fibrosis. Wan et al demonstrated that PLOD2 expression was increased in endometrial carcinoma cells under hypoxic conditions and modulated the migration, invasion, and epithelial-mesenchymal transition of endometrial carcinoma cells *via* PI3K/Akt signaling (49). Other tumor diseases have also been reported to have similar pathological processes. In addition, PLOD2 is regulated by HIF-1 or TGF1 and mediates ECM remodeling, alignment, and mechanical properties through a transcriptionally mediated mechanism. Mia and Bank identified a selective inhibitor of IκB kinase, suppressed the expression of PLODs in dermal fibroblasts, and inhibited the TGFβ1-induced transition of fibroblasts into myofibroblasts, thus relieving excessive ECM synthesis (50). Gilkes et al. proved that HIF-1 activity in hypoxic fibroblasts promotes ECM remodeling by inducing the expression of the collagen hydroxylases P4HA1, P4HA2, and PLOD2 (65). In our most recent study, we explored the effect of inflammatory cascade amplification mediated by synovial macrophage pyroptosis on SF. High expression of TGF-β and PLOD2 in OA animals and FLSs was positively correlated with the degree of SF. Interestingly, TGFβ1, TGFβR1, LOX, PLOD1, and PLOD2 in the glenohumeral capsule of patients with shoulder instability may play a role in shoulder instability. We speculate that this correlation is closely related to SF of the shoulder joint, although the specific pathological mechanism still needs further study.

MMP1, which is also known as fibroblast collagenase, has mainly been implicated in mediating the degradation of type I collagen, which is most often mentioned in fibrosis as the major constituent of the fibrotic ECM. MMP1 cleaves collagen only between amino acids 775 and 776; thus, it is possible that hydroxylysylpyridinoline collagen cross-linked through aberrant PLOD2 and LOX is more difficult to degrade (66). It has been proven that ∼0.1 Schiff base of LOX-mediated cross-linking per collagen molecule results in 2-3-fold higher resistance to human collagenase compared with that of un-cross-linked collagen (67). Therefore, despite the upregulation of both MMP1 and TIMP1 in the synovium in OA, the pathological changes of the synovial membrane continue to promote fibrosis, as indicated by not only the quantity of collagen but also the quality of collagen, as determined by its post-translational modifications, which actively drive the progression of fibrosis. In addition, MMP13 and ADAMTS-5 were also validated as drug targets that participate in the regulation of the ECM in OA, and ADAMTS-5 inhibitors were shown to reduce synovial joint damage in OA animal models.

Connective tissue growth factor (CTGF) is a well-known fibrogenic factor that has been shown to induce synovial fibrosis (60). It has been observed that both FLSs and chondrocytes were strongly induced to express CTGF after stimulation by TGF-β (60, 68). The main function of CTGF is to regulate proteoglycans on the cell surface, which can affect fibroblast proliferation, chemotaxis and accelerate ECM deposition (69). CTGF is thought to coordinate some fibrogenic effects through the TGF-β response element, but CTGF may also act independently of TGF-β (69, 70). Smad7, the inhibitory smad of TGF-β signaling, is reduced by CTGF, which in turn promotes TGF-β signaling, but the mechanism by which CTGF regulates Smad7 has not been fully elucidated (51). Therefore, it is valuable to further elucidate the induction effect of CTGF on synovial fibrosis in OA.

Wnt/β-catenin is closely associated with embryonic skeletal formation, tissue repair, fibrosis, and joint homeostasis (71). Wnt mediates several signaling cascades, especially the β-catenin–dependent (canonical) pathway (72), and β-catenin, as a transcriptional regulator, its stabilization or degradation is a central event in the Wnt signaling pathway. Existing studies show that the Wnt/β-catenin classical pathway has long been proven to be over-activated in the pathogenesis of OA (73). To be specific, increased expression of Wnt ligands and target genes was observed in both articular cartilage and synovium after injury, indicating Wnt signaling activation (74, 75). A recent study showed that XAV-939, a Wnt inhibitor, may reduce the proliferation of synovial fibroblasts and type I collagen levels by inhibiting the Wnt pathway, ultimately exerting a protective effect on synovial fibrosis (73). In conclusion, the Wnt/β-catenin signaling pathway may be a key molecular mechanism in the treatment of synovial fibrosis in OA, which may provide new ideas for the treatment of OA.

Mechanical Stress

Physical activity is one of the most frequently recommended non-pharmacological therapies for OA, but the duration and intensity of exercise vary widely. Moderate mechanical stress may reduce sensitization to the inflammatory response in the articular cartilage and chondrocytes and be beneficial for OA (76). However, excessive mechanical stress exacerbates OA progression by inducing chondrocyte apoptosis and osteophyte formation (77, 78). This indicates that mechanical stimulation can regulate the balance of synthesis-degradation in cartilage and osteogenesis-osteoclastogenesis in the subchondral bone. *In vivo*, mechanical stress is transduced into the cell from the sites at which the cells attach to the ECM. The cells may engage their ECM both *via* mechanosensitive adhesion complexes and *via* other surface receptors, including those for growth factors and inflammatory mediators, which cannot act as adhesive anchors but may modify the mechanical signals transduced at the cell/ECM interface (79). Under these conditions, activated mechanosensitive plasma membrane channels allow the inflow of Ca^{2+} that can act as a second messenger to regulate gene expression. TGF-β1 is a typical mechanosensitive gene, and previous studies have suggested that mechanical stretching activates and releases latent TGF-β1 in living tissues from fibrotic lungs (44). In cardiac fibrosis, mechanical stress is a major factor for cardiac hypertrophy in response to pressure or volume overload, and angiotensin II seems to be another mechanosensitive gene that promotes fibrosis (80). As the FLS response to mechanical stress is critical during the initial stages of OA, SF caused by excessive mechanical stimulation is likely to occur (81), and subsequent ECM stiffness may affect tissue delivery of mechanical signals and exacerbate OA progression.

Existing and Potential Treatments for Synovial Fibrosis

Open surgery for SF is undoubtedly the most direct and effective treatment, but it often requires large incisions with extensive exploration of the joint and surrounding extracapsular soft tissues. Unfortunately, the surgery itself induces a fibrotic process, and the outcomes of post-traumatic surgery are poor, with most patients unable to return to their presurgery level of function. Even if satisfactory results are achieved during the operation, SF is likely to recur within a certain period.

Recently, SF research has made some progress in conservative drug treatment. Numerous studies have consistently reported that PRG4 and HA attenuate profibrotic responses to TGF-β in OA animals or FLSs. Interestingly, FLSs themselves can synthesize and secrete PRG4 and HA. Correcting the pathological state of FLSs in OA seems to be of great significance for the treatment of SF. In this context, Qadri et al concluded that increasing intracellular cAMP levels in FLSs mitigates SF through enhanced production of HA and PRG4 (82). Plaas et al. proved that HA injection blocked all gait changes and protected joints from femoral cartilage erosion and tissue fibrosis in KOA mice, and they deduced that HA injection could mimic the protective effects of ADAMTS-5 ablation (83). Their further findings supported this hypothesis and demonstrated that ADAMTS-5 was blocked by a CD44-dependent mechanism (84). As PRG4 is a ligand of the CD44 receptor, Qadri et al examined the role of the PRG4-CD44 interaction in regulating SF in OA and demonstrated that PRG4 inhibited fibroblast-to-myofibroblast transition, thus downregulating the expression of fibrotic genes in the OA synovium (85).

Furthermore, regarding the balance between MMP and TIMP expression in FLSs from KOA with flexion contracture using adenovirus-mediated relaxin gene therapy, relaxin could serve as an alternative therapeutic agent during the initial stage of OA with flexion contracture by exerting antifibrogenic effects (86). In addition, methylene blue, NSAIDs, and salmon calcitonin were also reported to have therapeutic effects on SF, although their specific mechanisms are still unclear (87–89). The Wnt/β-catenin signaling pathway and senescent cells are potential targets for antifibrosis therapy, but the intervention procedure needs further exploration (73, 90).

CONCLUSION

Evidence from direct research on SF in OA and related studies suggests the following. (I) Fibrosis is the outcome of inflammation. It is not clear whether the ongoing process of SF has a pathogenic effect in OA, especially in relation to pain. If so, effective intervention to slow the progression of fibrosis is necessary even if fibrosis is irreversible, as the greatest advantage is the improvement of joint function and the relief of OA symptoms. (II) Although we have some understanding of ECM environmental changes and the mechanism by which they are involved in the pathological process of SF associated with OA, our knowledge of this pathological mechanism is still insufficient. Angiogenesis and nerve invasion are likely to have a strong correlation with the pathological process of SF and may be involved in the development of SF, which deserves further exploration. (III) Existing research on the pharmacodynamic targets and intervention effects of SF is not sufficient, and further exploration is still needed in the future. Notably, the combined use of antifibrotic drugs has high potential during anti-inflammatory therapy for OA.

AUTHOR CONTRIBUTIONS

PW and JM conceptualized the current study. LZ (1st author) and RX drafted and revised the paper. ZH, LD, and LZ (5th author) provided the relevant literature. XL and ML were responsible for the proofreading. PW provided final approval of the version to be submitted. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 81804123, No.81774334); the Leading Talents of Traditional Chinese Medicine Project (SLJ0207); and the Social Development Fund of Jiangsu Province (BE2017774).

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