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New trends in osteoarthritis treatment

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Published in
Frontiers in Medicine
Frontiers in Bioengineering and Biotechnology

ISSN 1664-8714
ISBN 978-2-8325-4450-1
DOI 10.3389/978-2-8325-4450-1

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New trends in osteoarthritis treatment

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Citation
Belluzzi, E., Pozzuoli, A., eds. (2024). *New trends in osteoarthritis treatment*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-4450-1

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frontiers | Frontiers in Medicine

TYPE Editorial
PUBLISHED 31 January 2024
DOI 10.3389/fmed.2024.1372052

OPEN ACCESS

EDITED AND REVIEWED BY
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RECEIVED 17 January 2024
ACCEPTED 19 January 2024
PUBLISHED 31 January 2024

CITATION
Pozzuoli A, Belluzzi E and Ruggieri P (2024) Editorial: New trends in osteoarthritis treatment. *Front. Med.* 11:1372052. doi: 10.3389/fmed.2024.1372052

Editorial: New trends in osteoarthritis treatment

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KEYWORDS

osteoarthritis, knee, treatment, extracellular vesicles, articular cartilage, regenerative rehabilitation, autophagy, mitochondria

Editorial on the Research Topic
New trends in osteoarthritis treatment

Osteoarthritis (OA) is the most common type of arthritis affecting millions of persons worldwide (1, 2). It is a complex and multifactorial disease that could affect any joint, but particularly the knee, hip and hands. All the joint tissues are involved, including synovial membrane, subchondral bone, infrapatellar fat pad, subchondral bone, and especially cartilage, which undergoes several changes impacting its biomechanical behavior (3–5). These changes lead to swelling, pain, and difficulty in joint movement, thus impacting quality of life (6).

Several risk factors have been identified such as joint injury, comorbidities, female gender, genetic predisposition, obesity, metabolic diseases, and age (7, 8).

Despite the high prevalence of OA, there is still no treatment to cure or delay the progression of OA.

Currently, medical treatment focuses on symptoms relief with painkillers and anti-inflammatory drugs to improve patient's quality of life (9, 10). This Research Topic aimed to address new trends and updates in OA treatment, including pharmaceutical, non-pharmaceutical, and surgical treatments. A total of 11 articles were published: 2 reviews, 1 systematic review, 1 scoping review, 1 clinical trial, and 6 original research articles on OA treatments.

Dyslipidaemia represents a risk factor for OA onset and progression. It is usually treated with statins, drugs generally safe and well tolerated. While statins efficacy in reducing cardiovascular diseases is well documented, their effect on skeletal muscles is poor investigated. As statin-induced muscle symptoms have been reported as a cause of statin discontinuation, Lim et al. performed a *post-hoc* analysis of a placebo-controlled trial to evaluate the effect of atorvastatin on skeletal muscles of patients with knee OA. Only a tendency for increased myalgia was reported not clearly related to atorvastatin.

Recently, new lipid-lowering drugs are used in the secondary prevention of atherosclerosis but their effects on OA have not been reported. Wang et al. estimated the casual effects of blood lipids and lipid-lowering agents on knee and hip OA risk, performing a Mendelian randomization study. A genetic predisposition to higher blood LDL-C levels may decrease the risk of knee and hip OA, independently of HDL-C and TG levels, and body mass index (BMI). Moreover, genetically proxied LDL-C-lowering effects of statins increased the risk of knee but not hip OA.

Female gender and obesity are well-documented risk factors for OA. OA incidence increases in women after menopause due to oestrogens decrease, weight gain, and BMI increase. Based on this evidence, Abshirini et al. performed a placebo-controlled trial enrolling 55 overweight/obese postmenopausal women with joint discomfort at risk or at early-stage OA. They evaluated the effect of whole greenshell mussel (GSM) powder, supplemented for 12 weeks, on biomarkers of cartilage metabolism, inflammatory cytokines, and joint symptoms and functions. Oral GSM supplementation was effective in improving overall joint pain and it might slow down type II collagen degradation but did not impact on knee-related symptoms and on the level of inflammatory cytokines, suggesting that GSM may act within the joint microenvironment rather than at the systemic level.

Genetic predisposition plays a role as a risk factor for OA along with ethnic heritage and geographic localization (11). Several genome-wide association studies investigated the relationship between fat mass and obesity-related (FTO) gene variation and OA risk but with inconclusive results. Therefore, Zhao et al. conducted an integrated meta-analysis with bioinformatics to better elucidate the role of the FTO gene in the development of OA, confirming that FTO gene polymorphism increased OA risk especially through obesity in the Caucasian population.

Several treatments have been proposed especially in early-stages of OA, including cells and extracellular vesicles (EVs) therapies (12, 13). Colombini et al. applied a bioinformatics approach to study the miRNA composition of EVs secreted by cartilage cells (CCs), adipose tissue-derived (ASCs), and bone marrow-derived stem cells (BMSCs), isolated from hip OA patients. Moreover, the authors co-cultured CCs, ASCs, and BMSCs with T cells and macrophages showing immunomodulatory ability, supporting the rationale behind the use of cell-based therapy for OA treatment.

Regenerative rehabilitation, which involves both regenerative and rehabilitation medicine, is a new approach for OA treatment (14). Popov et al. applied a regenerative rehabilitation mathematical model of local articular cartilage defects based on the features of cartilage tissue and the responses of chondrocytes and progenitor chondrocytes observed in *in vitro* experiments applying different mechanical stimuli. Tissue micro and macro environment, restored after mechanical stimulation, had a significant effect on ECM formation of cartilage.

The use of a disease modifying treatment for OA is a growing area of interest. Lin et al. used a comprehensive 3D contrast enhanced μCT to evaluate the effect of intra-articular injection of a micronized dehydrated human amnion/chorion membrane (mdHACM) on joint tissues in a preclinical post-traumatic OA rat model. mdHACM was delivered intra-articularly 24 h (acute treatment) or 3 weeks (delayed treatment). Delayed treatment improved joint health, slowing the degeneration of cartilage, subchondral bone, and marginal osteophytes. This study supports the suitability of mdHACM to treat symptomatic OA.

Autophagy has a protective role against microenvironment changes in knee OA and its failure could worse cartilage degradation (15). Wu et al. showed the activation of autophagy in human chondrocytes and in cartilage of an OA rabbit model after intra-articular injection of clioquinol. Moreover, clioquinol had a protective effect increasing the expression of ECM components, suppressing inflammatory mediators and decreasing chondrocyte apoptosis.

Gene therapy is a growing Research Topic in OA treatment allowing local production of target therapeutic proteins (16). Uebelhoer et al. conducted a scoping review on the current knowledge about gene therapies in preclinical and clinical settings. Studies about *in vitro*, *in vivo*, or *ex vivo* gene therapies were analyzed. The results showed that gene therapy could be a highly promising treatment for OA.

Mitochondrial function could represent a target for OA treatment (17). Mao et al. reviewed mitochondrial dysfunctions in OA chondrocytes reported as decreased ATP production, increased oxidative stress, calcium dysregulation, increased permeability of the mitochondrial membrane, mtDNA alternations, and activation of mitochondrial apoptotic pathway resulting in cartilage degeneration. Endogenous mitochondrial molecular targets, exogenous drugs, stem cells and exosomes, that could improve mitochondrial function, were reviewed.

OA has been described as a "wound that does not heal" because of the dysregulation of the immune response, the inflammation, and the normal healing and repair process (18). Huston reviewed the positive effects of Tai Chi in OA treatment. Tai Chi can improve knee alignment, optimize knee biomechanical forces, strengthen the lower limbs, and importantly can decrease systemic inflammation. Moreover, Tai Chi is able to decrease the risk of falls and further injury of patients affected by OA.

Collectively, this Research Topic focused on the effects of new treatments for OA also discussing new possible targets. Different strategies were used, starting from clinical trials to *in-silico* models. Further studies are needed to find new treatments and test their efficacy and safety in controlled randomized clinical trials. In this context, research unraveling OA pathophysiological mechanisms is essential in order to better elucidate the complexity of this disease.

Author contributions

AP: Writing – original draft, Writing – review & editing. EB: Writing – original draft, Writing – review & editing. PR: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The guest editors would like to thank all authors for their valuable contribution to this Research Topic.

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 author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers in Medicine

REVIEW
published: 26 November 2020
doi: 10.3389/fmed.2020.581402

Mitochondria: Potential Targets for Osteoarthritis

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Osteoarthritis (OA) is a common and disabling joint disorder that is mainly characterized by cartilage degeneration and narrow joint spaces. The role of mitochondrial dysfunction in promoting the development of OA has gained much attention. Targeting endogenous molecules to improve mitochondrial function is a potential treatment for OA. Moreover, research on exogenous drugs to improve mitochondrial function in OA based on endogenous molecular targets has been accomplished. In addition, stem cells and exosomes have been deeply researched in the context of cartilage regeneration, and these factors both reverse mitochondrial dysfunctions. Thus, we hypothesize that biomedical approaches will be applied to the treatment of OA. Furthermore, we have summarized the global status of mitochondria and osteoarthritis research in the past two decades, which will contribute to the research field and the development of novel treatment strategies for OA.

Keywords: osteoarthritis, mitochondria, mitochondrial dynamics mtDNA, biomedical therapy, bibliometrics

OPEN ACCESS

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

Received: *08 July 2020*
Accepted: *19 October 2020*
Published: *26 November 2020*

Citation:
Mao X, Fu P, Wang L and Xiang C (2020) Mitochondria: Potential Targets for Osteoarthritis. Front. Med. 7:581402. doi: 10.3389/fmed.2020.581402

INTRODUCTION

Osteoarthritis (OA), a chronic and progressive cartilage degeneration disease (1) with a high morbidity and disability rate (2), is characterized by cartilage degeneration, osteophyte formation, thickening of subchondral bone, synovial inflammation, and meniscal injuries (3). As the global population ages and the proportion of obese people increases, the morbidity of OA continues to rise. At present, ∼250 million people suffering from OA worldwide bear a tremendous economic burden as does society (4). OA tends to occur in the elderly population; cellular senescence is a contributor to age-related diseases (5), and studies have shown that OA is typical representatives of age-related diseases (6). Alleviating pain is the main purpose of non-surgical treatment, but this treatment does not alleviate the progression of OA (7).

Chondrocytes are the only cell type present in mature cartilage and change pathologically when OA occurs (8). Multiple factors can lead to OA, including inflammatory cytokines, mechanical stress, ageing, metabolic factors, and other pathological changes, which could increase reactive oxygen species (ROS) (9), induce oxidative stress in mitochondria, cause mitochondrial DNA (mtDNA) damage, result in mitochondrial damage, and shorten the life span of chondrocytes (10). The loss of mitochondrial membrane potential (MMP) leads to a reduction in energy production, an increase in the permeability of the mitochondrial membrane (11), and the release of apoptotic factors such as cytochrome C (Cyt-C), apoptosis-inducing factor, and procaspases from the mitochondria into the cytoplasm. Obvious changes in the morphology and function of mitochondria have been shown in ageing cells, and mitochondrial dysfunction is a key factor in cellular senescence (5, 12), demonstrating that mitochondria may be a therapeutic target

for anti-ageing treatment and reduce the morbidity of OA in the elderly population (13). In addition, mitochondrial genetics are indispensable in the pathogenesis of OA. The accumulation of somatic mutations in mtDNA is a major contributor to human ageing and degenerative diseases (14). Reducing mtDNA damage, including the integrity of mtDNA4977, could optimize mitochondrial function, and maintain the homeostasis of chondrocytes. Furthermore, the mitochondrial apoptotic pathway has been implicated in chondrocyte apoptosis in OA (15). More specific therapeutic strategies on the basis of an in-depth molecular understanding of OA are thus essential (16).

With the research and application of stem cells and exosomes in cartilage repair, biomedical approaches to optimize mitochondrial function will be the preferred method for the thorough treatment of OA. Furthermore, gene therapy is also booming, and we therefore think that biological measures to modify the disease will be the major approach for OA treatment. In the present article, we have reviewed mitochondrial dysfunction mainly in the context of OA chondrocytes and summarized the endogenous molecular targets related to mitochondrial function. Moreover, research progress on exogenous drugs for the treatment of OA by restoring mitochondrial function in chondrocytes has been reviewed. In addition, we have described the global status of mitochondrial and OA research, which may contribute to predicting the trend in mitochondrial research regarding the treatment of OA. Furthermore, these findings will be instructive for mechanistic research on mitochondrial functions in OA, contributing to fundamental research on the treatment of OA through the mitochondrial pathway and providing novel strategies for the clinical treatment of OA.

BIOLOGICAL FUNCTION OF MITOCHONDRIA

Mitochondria, encapsulated by bilayer membranes, are remarkably dynamic organelles and considered as the "powerhouse" of eukaryote cells. Mitochondria not only generate the energy required for cellular metabolism by oxidative phosphorylation (OXPHOS), but they also produce heat in certain specialized cell types, such as brown adipocytes (17). Approximately 2,000 mitochondria within a eukaryotic cell occupy ∼20% of the cell volume (12). There are protein complexes in the inner mitochondrial membrane that transfer and pump protons through the mitochondrial respiratory chain (MRC) for ATP production, such as NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), Cyt-C reductase (complex III), and Cyt-C oxidase (complex IV). Pyruvate and fatty acids could be converted to acetyl CoA by mitochondria, and CoA is metabolized by the citric acid cycle to produce NADH (18) where energy electrons are used to produce ATP (19). In addition to ATP production, intermediate metabolites for biosynthesis, protein modifications, signal transduction, programmed cell death, bioenergetic metabolism, the redox state, calcium homeostasis, innate immunity, stem cell reprogramming, and ageing-related responses (20–22) occur in the mitochondria (17) (**Figure 1**).

Recently, more research has focused on mitochondrial dynamics. The dynamic characteristics consist of mitochondrial fusion, mitochondrial fission and mitophagy (36), which are crucial for normal mitochondrial function and are critically associated with mitochondrial biogenesis and mitophagy (37). Mitofusins 1 (Mfn1) and Mitofusins 2 (Mfn2) mediate the fusion of the outer membrane, and optic atrophy 1 (OPA1) mediates the fusion of the inner membrane (38). Dynamin-related protein 1 (Drp1) and classical dynamin 2 (Dnm2) are the main mediators of mitochondrial fission (39) (**Figure 2**). When mitochondrial fission becomes increasingly dominant, damaged mitochondria undergo mitophagy in chondrocytes in the context of OA (40, 41), which could cause mitochondria to fail to produce sufficient bioenergy, regulate calcium and maintain the redox state. In contrast, mitochondrial fusion could enhance the biological function of mitochondria, which could make chondrocytes energetic and inhibit apoptosis.

In normal chondrocytes, mitochondria play a role in regulating signaling by modulating the redox state, supplying cofactors for biochemical reactions, such as molecular chaperones to facilitate protein folding, and generating ligands for signal transduction, such as AMPK signaling and calcium signaling (17, 42, 43). Calcium stored in mitochondria is helpful for maintaining calcium homeostasis in cells, and mitochondria are dedicated to transport extracellular matrix (ECM) calcium (12, 44). The mineralization of cartilage has been confirmed to involve calcium phosphate-containing granules, which are known as "matrix vesicles" (45). Moreover, Professor Alexandra E. Porter and colleagues found that mitochondrial granules contribute to the transport of clusters of calcium and phosphate ions to the ECM to facilitate mineralization, and Professor Lehninger AL suggested that mitochondria could release calcium phosphate to the ECM to take part in bone formation (46, 47). In addition, Professor Brian Glancy and colleagues showed that calcium activated nearly every step within the electron transport chain (ETC) (48) and activated enzymes, such as NADH, Cyt-C, complex III, and complex IV, in the pathways of oxidative metabolism in mitochondria (49, 50). Furthermore, mitochondria could regulate and balance the apoptosis by initiating cell death (17).

MITOCHONDRIAL DYSFUNCTION IN OSTEOARTHRITIS

Mitochondrial dysfunction mainly manifests as decreased ATP production, increased oxidative stress, calcium dysregulation, increased permeability of the mitochondrial membrane, and mtDNA alternations, which result in cartilage degeneration. Chondrocyte damage occurs and is mainly reflected in the increases in MMP-3, MMP-13, NO, and inflammatory injury with an imbalance between catabolism and anabolism of extracellular matrix (51), including reductions in aggrecan and collagen II, which eventually induce OA (**Figure 3**).

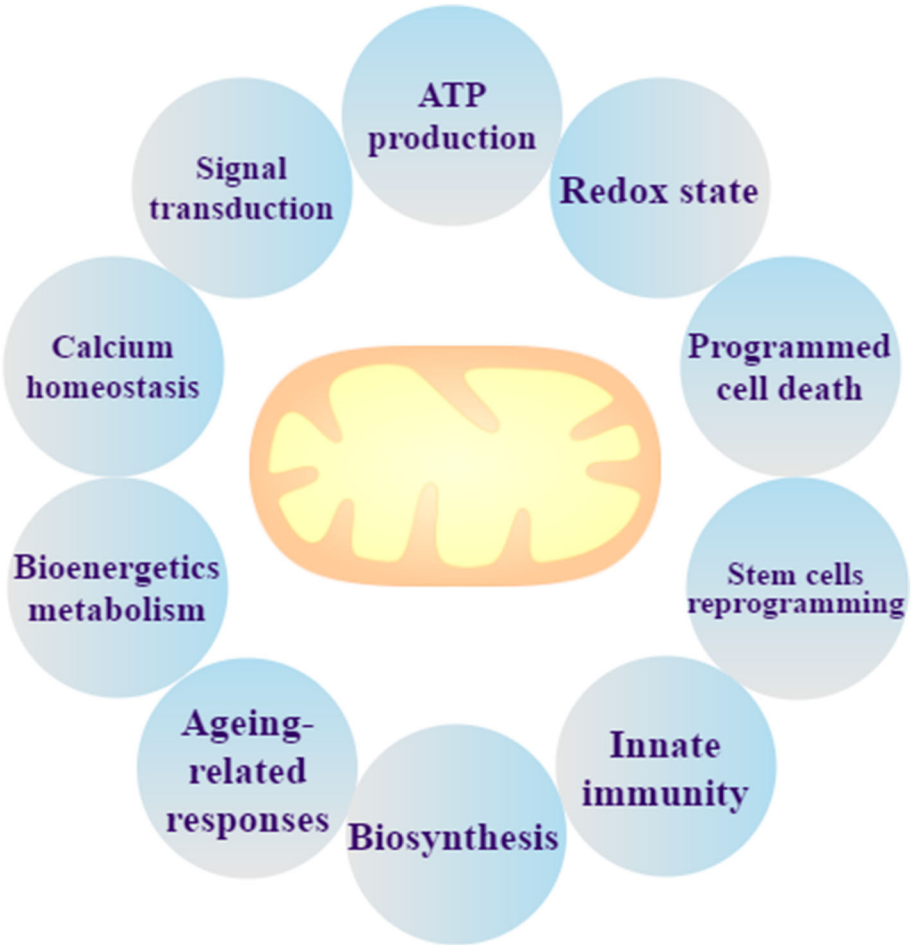

FIGURE 1 | Mitochondrial biological function. Mitochondria are not only the organelle for ATP production and signal transduction, but they can also maintain the redox state and calcium homeostasis, regulate programmed cell death, and perform bioenergetics metabolism, stem cells reprogramming, ageing-related responses, innate immunity, and biosynthesis.

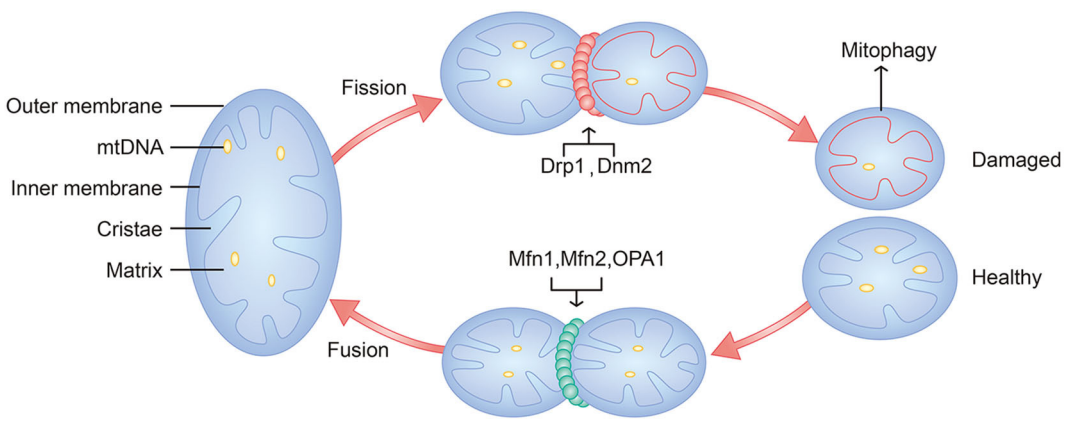

FIGURE 2 | Schematic diagram of mitochondria and mitochondrial dynamics. Major components of mitochondria include outer membrane, inner membrane, cristae, matrix, and mtDNA. Mitochondrial fusion is mediated by Mfn1, Mfn2, and OPA1. Mitochondrial fission is mediated by Drp1, Dnm2. Damaged mitochondria will undergo mitophagy.

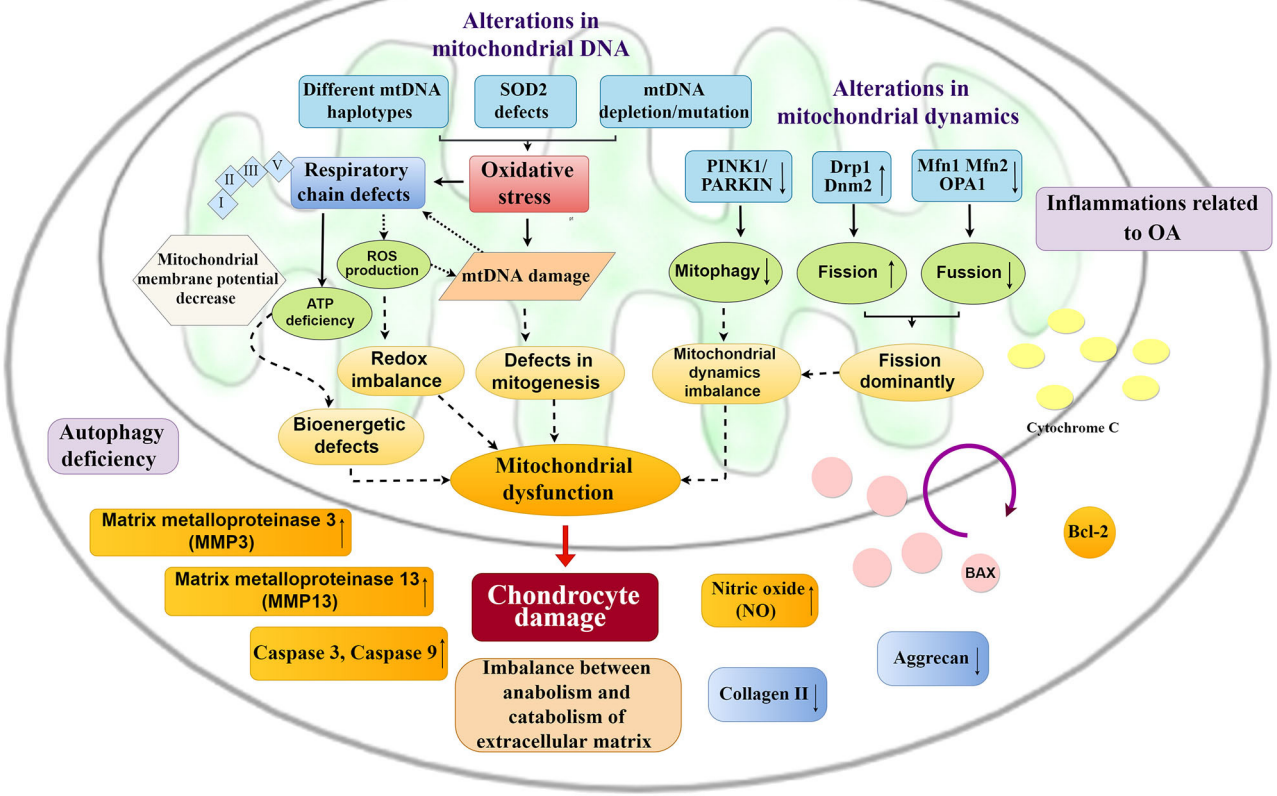

FIGURE 3 | Mitochondrial changes and chondrocyte damage in OA. Alternations in mitochondrial DNA and mitochondrial dynamics cause a series of mitochondrial changes. Excessive oxidative stress, respiratory chain defects and imbalance of mitochondrial dynamics result in mitochondrial dysfunction, which could promote chondrocyte damage, including inflammatory injury, imbalance between anabolism and catabolism of the extracellular matrix, and an increase in apoptosis of chondrocytes.

Decreased ATP Production

Mitochondrial dysfunction can lead to a decrease in the activity of respiratory chain complexes I, II, III, and V, the loss of MMP, and decreases in OXPHOS in OA chondrocytes (52), which could induce chondrocytes to release interleukin-1β (IL-1β) and lead to inflammation (12). Two primary mechanisms of ATP production include substrate phosphorylation in the glycolytic pathway and in the tricarboxylic acid (TCA) cycle and OXPHOS occur at the inner membrane (53). ATP production is driven by the transmembrane proton gradient. An inflammatory response in chondrocytes with the upregulation of cyclooxygenase 2 (COX-2) and prostaglandin E2 (PGE2) production could be generated by ETC dysfunction (54). Both TNF-α and IL-1β inhibit the activity of ETC complex I (55), which induces decreases in ATP production and MMP. Inhibiting the ETC thus could decrease ATP synthesis (56).

Increased Oxidative Stress

Mitochondrial dysfunction maintains a positive regeneration circle with oxidative stress, increased ROS, and mtDNA damage, which are regarded as hallmarks of chronic degenerative diseases (57). The accumulation of ROS and mtDNA damage can activate the nuclear factor-κB (NF-κB) pathway, which is the main regulator of inflammation (57). Avascular and hypoxic tissue are always used to describe cartilage, and chondrocytes are the only cell types in articular cartilage that maintain the balance of extracellular matrix (ECM) synthesis and degradation (58). ROS, as by-products of oxidation-reduction reactions, are generated in the MRC (59). A lower level of ROS is beneficial for maintaining chondrocyte homeostasis, and a higher level of ROS induces the depolarization of mitochondrial membrane, which could lead to sustained ROS production (60). An initial theory suggested that ROS have deleterious effects on ageing and degenerative diseases (61). Accumulating evidence has demonstrated that increased oxidative stress and the overproduction of ROS, including superoxide anion, hydrogen peroxide (H_2O_2), and nitric oxide (NO), play pivotal roles in the pathogenesis of OA (10, 62). The overproduction and accumulation of ROS and ATP deficiency decrease mitogenesis and break the redox balance. DNA and especially mtDNA could be injured (63). Oxidative stress could damage the mitochondrial

respiratory chain protein complexes in chondrocytes (12). Due to the accumulation of ROS in chondrocytes, the decrease in collagen and glycosaminoglycan synthesis and the enhancement of metalloproteinases and aggrecanases induce chondrocytes to undergo a switch from anabolic to a catabolic gene expression, which results in cartilage breakdown (34). Furthermore, the depletion of superoxide dismutase 2 (SOD2), the major mitochondrial antioxidant protein, occurs in early cartilage degradation and could exacerbate inflammation and enhance ROS, contributing to OA progression (64, 65). Mitochondria are the dominant intracellular organelles in charge of the generation of ROS (66). ROS overload induced by oxidative stress results in the loss of MMP by stimulating the mitochondrial permeability transition pore (PTP) (67). High levels of cholesterol are naturally present in the cell membrane of chondrocytes, and chondrocytes could produce their own cholesterol and synthesize all the indispensable proteins for cholesterol biosynthesis (68, 69). Hypercholesterolemia animal models with changes in cartilage have been studied by Mao et al. (69), and the researchers demonstrated the direct effect of high cholesterol on cartilage degeneration and chondrocyte hypertrophy. When exposed to the synovial fluid with raised cholesterol levels, chondrocytes could be damaged because of the changes in the fluidity of the cell membrane and activation of membrane lipid signaling pathways (70). There is a close relationship between increased cholesterol oxidation products and mitochondria-derived oxidative stress, which leads to increased production of mitochondrial ROS (69), and Mao et al. showed that the cholesterol-lowering drug and the mitochondria-specific antioxidant have protective effects on attenuating OA symptoms caused by high cholesterol, such as atorvastatin and Mito-TEMPO.

Calcium Dysregulation

Calcium, a ubiquitous intracellular second messenger, is involved in numerous cellular processes (71, 72). Calcium overload can lead to ROS overproduction, mitochondrial depolarization, MMP damage, and apoptosis (73). The maintenance of intracellular calcium homeostasis is achieved by mitochondrial uptake of calcium through a uniport transporter and the release of calcium through the inositol-1,4,5-trisphosphate receptor (IP_3R), the sodium/calcium exchanger, or through the PTP, which is stimulated by excessive calcium in the mitochondrial matrix (72, 74). The PTP is a large conductance channel in the inner membrane of mitochondria (75), and both high levels of calcium and ROS can activate the PTP opening (76). The PTP makes the membrane non-specifically permeable to any molecule up to 1.5 kDa, including protons, and the mitochondria cannot maintain a pH gradient or MMP any longer (77, 78). The PTP leads to the collapse of MMP, leading to mitochondrial swelling and release of calcium and Cyt-C, ultimately stimulating apoptosis (8, 72). Calpains are calcium-activated proteases that could destroy the sodium/calcium exchanger and result in calcium overload and cell death (79). Furthermore, calcium overload and the activation of BAX by calpains lead to mitochondrial depolarization (72, 80).

Increased Permeability of the Mitochondrial Membrane

Chondrocyte apoptosis induced by inflammation, oxidative stress, and increased mitochondrial membrane permeability (81) is positively associated with the degree of cartilage damage (82, 83). The collapse of the MMP leads to mitochondrial depolarization (8), which causes mitochondrial swelling, outer mitochondrial membrane collapse, and release of Cyt-C (84, 85). The BAX/mitochondrial Cyt-C/Caspase signaling pathway is shown to be associated with chondrocyte apoptosis (31). The downregulation of Bcl-2, the increase in expression of BAX, Caspase-3, and Caspase-9, and the increase in permeability of mitochondrial membrane can promote the outflow of Cyt-C from mitochondria into the cytoplasm and the inflow of BAX from the cytoplasm into mitochondria, increasing chondrocyte apoptosis. When damaged by various oxidative stimuli, the initiation of chondrocyte apoptosis induced by increased ROS is promoted (10, 15, 86). Studies have shown that mitochondrial dysfunction with reduced MMP and increased mitochondrial membrane permeability could promote the migration of Cyt-C from the mitochondrial matrix to the cytoplasm (87), which could induce apoptosis due to the activation of caspases and increase the BAX/Bcl-2 ratio (88). Moreover, the level of ROS in mitochondria is significantly increased (89), which could induce oxidative stress, destroy cartilage homeostasis, and increase chondrocyte apoptosis (60). The balance of mitochondrial dynamics could inhibit the apoptosis induced by oxidative stress (90, 91).

mtDNA Mutation

In addition to mitochondrial dysfunction, the inheritance of mitochondria also acts as a pivotal role in the process of OA (92). mtDNA, a 16,569 bp circular and double-stranded molecule, encodes 13 protein subunits for the respiratory chain and 24 RNA components (22 tRNAs and 2 rRNAs) for mitochondrial protein synthesis (93). Chondrocytes from OA patients exhibit higher levels of mtDNA damage than chondrocytes from normal individuals (94). mtDNA damage could be caused by the increased ROS burden of aged chondrocytes (63, 95). At the same time, the accumulation of mtDNA mutations above a critical level could lead to dysfunction of the respiratory chain and increased ROS production, which could promote excessive chondrocyte apoptosis and enhance inflammatory responses (8). mtDNA haplogroups modulate crucial functions such as ATP production, oxygen consumption, ROS generation, and the expression of mitochondrial and nuclear genes (96). mtDNA haplotype J is associated with a lower risk of knee osteoarthritis (KOA) compared to that of mitochondrial mtDNA haplotype H (97). The mtDNA haplotype may be a biomarker for OA diagnosis and prognosis, and be closely involved in the OA phenotype (98). Therefore, the pattern of latent drugs that mimic the physiological effects of mtDNA haplotype J may be a potential treatment strategy for OA (99).

ENDOGENOUS MOLECULAR TARGETS TO REVERSE MITOCHONDRIAL DYSFUNCTION

The vital role of mitochondrial changes in the development of OA has been demonstrated (8, 12, 52, 100), and endogenous molecular targets that optimize mitochondrial dynamics and morphology will turn into potential targets for OA treatment (**Figure 4**). AMPK, Sirtuin, PGC-1α, PINK1, PARKIN, and Nrf2 are endogenous molecules, and the activation of AMPK/SIRT1/3/PGC-1α, AMPK/SIRT3/SOD2, and AMPK/SIRT3/Parkin/PINK1 signaling could promote mitochondrial biogenesis and reduce oxidative stress, contributing to balancing mitochondrial dynamics and improving MMP. Moreover, upregulating OPA1, Mfn1, and Mfn2 and downregulating Drp1 and Dnm2 through GPS2 could promote mitochondrial fusion to enhance mitochondrial biological functions.

AMPK

Adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK), the serine/threonine kinase, is a key regulator to adapt to changes in energy demand (101). When in a hypoxic state, AMPK can be activated and phosphorylate multiple downstream targets, promoting the inhibition of ATP-consuming pathways and the activation of the ATP-producing pathway (101, 102). Dysregulation of AMPK has been associated with a variety of age-related diseases related to mitochondrial dysfunction and imbalance of cellular energy, including diabetes, atherosclerosis, cardiovascular disease, cancer, neurodegenerative diseases, and OA (103, 104), suggesting the translational potential of pharmacological AMPK activators to limit OA progression (52, 102). In chondrocytes, activation of AMPK suppresses NF-κB activation, oxidative stress, and multiple inflammatory and catabolic responses (104). Moreover, AMPK could regulate both mitochondrial biogenesis and mitophagy to balance mitochondrial dynamics (52).

Sirtuin

AMPK activity regulates energy metabolism via downstream mediators, including the nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases Sirtuin1 and Sirtuin3 (SIRT1 and SIRT3, respectively). The key role of AMPK in the treatment of OA through the mitochondrial pathway and mitochondrial

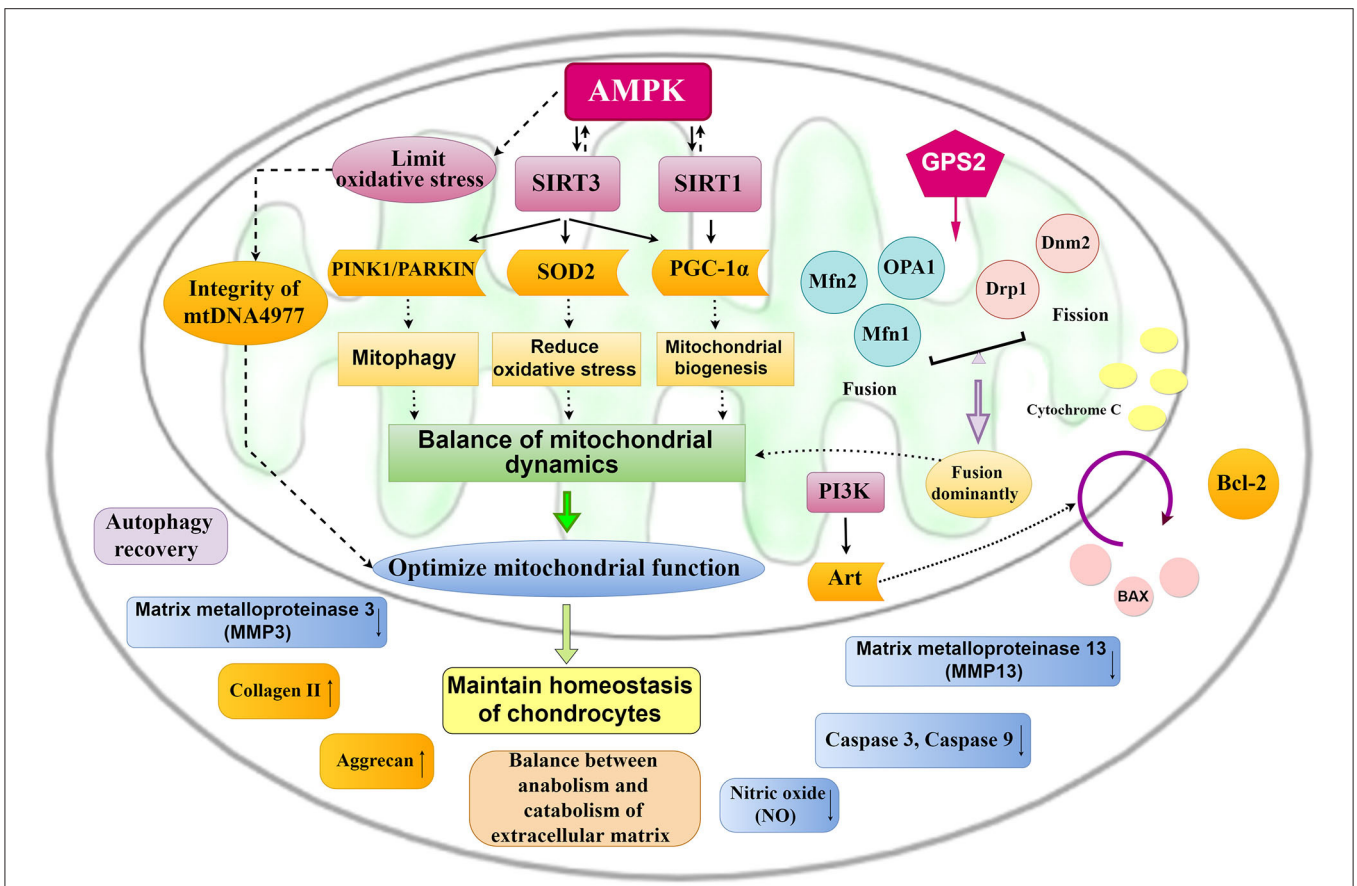

FIGURE 4 | Mitochondrial pathways for the treatment of OA. The activation of AMPK-SIRT-3/ SIRT1-PGC-1α, AMPK-SIRT-3-SOD2, and AMPK-SIRT-3-PINK1/PARKIN signaling could promote mitochondrial biogenesis and mitochondrial fusion and reduce oxidative stress, which contribute to optimize mitochondrial function and then maintain chondrocyte homeostasis.

acetylation-induced OA has been identified, while SIRT3 is the main deacetylase in mitochondria, and SIRT3 activation can protect cells by regulating mitochondrial dynamics and mitophagy. SIRT1/3 and AMPK regulate each other (105). Increasing evidence shows that SIRT1 is significant in promoting mitochondrial dysfunction and OA progression (83). It has been proven that SIRT1 enzymatic activity is necessary for cartilage homeostasis (64, 65). The loss of SIRT1 in chondrocytes also leads to increases in MMP-13, apoptotic markers, and NF-κB, resulting in the accelerated OA development (83, 106). Upregulation of SIRT1 can inhibit the activation of COX-2, MMP-13, and NF-κB-induced TNF-α and decrease the upregulation of MMP-13 and acetylation of NF-κB p65 induced by IL-1β (83, 107). SIRT1 is a strong inducer of autophagy (108), which is reduced in OA, and therapeutic enhancement of autophagy is chondroprotective *in vitro* and *in vivo* (52, 109, 110). The NAD+-dependent deacetylase Sirtuin3 is the major deacetylase in mitochondria (111), contributing to the regulation of the mitochondrial antioxidant system and adenosine-triphosphate (ATP) production (112). Depletion of the mitochondrially localized antioxidant superoxide dismutase 2 (SOD2) promotes mitochondrial dysfunction and increased production of ROS (64, 65). A study showed that mitochondrial acetylation could promote the development of OA, while SIRT3 could enhance the antioxidant capacity of chondrocytes by enhancing the activity of SOD2 (113). Moreover, SIRT3 could activate and enhance the activity of AMPK in chondrocytes, which could reduce the loss of mtDNA4977 and maintain mtDNA integrity, thereby improving the function of mitochondria and protecting chondrocytes (28). Studies have shown that mitophagy can eliminate damaged mitochondria isolated by mitochondrial fission, which is a cytoprotective mechanism to maintain mitochondrial stability and quality (114). Moreover, the relationship between mitophagy and OA has been confirmed (60, 115). SIRT3 depletion can reduce mitophagy (116) and SIRT3 activation protects cells by regulating mitochondrial dynamics and mitophagy (117). Drugs that can activate SIRT3 may therefore be potential treatments for OA through the mitochondrial pathway.

PGC-1 α

The mitochondrial biogenesis master regulator peroxisome proliferator–activated receptor γ coactivator 1α (PGC-1α) acts by inducing the transcription of nuclear respiratory factors (NRFs) (e.g., NRF-1 and NRF-2) (52), thereby increasing the expression of mitochondrial transcription factor A (TFAM) and other nuclear-encoded mitochondrial respiratory complex subunits (118, 119). TFAM is induced to translocate to mitochondria, which stimulates mitochondrial DNA replication and mitochondrial gene expression, thus stimulating the biogenesis of mitochondria (52, 118). It is well-known that SIRT1 and its substrate PGC-1α regulate aspects of energy metabolism through mitochondria (83). PGC-1α activity is regulated by phosphorylation and NAD 1-dependent deacetylation via metabolic biosensors AMPK, SIRT1, and SIRT3 (52, 120). Furthermore, Zhao et al. showed that PGC-1α is essential for mediating AMPK activity to block catabolic responses and suppress oxidative stress in chondrocytes (118).

Parkin/PINK1

Autophagy is closely related to apoptosis in the pathogenesis of numerous degenerative diseases, and studies have shown that autophagy is inhibited in OA chondrocytes (121). Autophagy is a mechanism of intracellular catabolism through which cells can remove dysfunctional organelles and macromolecules to prevent the occurrence of cell stress, preventing mitochondrial dysfunction (122). Lotz et al. called the process mitophagy, which eliminates damaged mitochondria and prevents oxidative stress (123). Parkin, an E3 ubiquitin ligase and mitochondrial outer membrane (OMM) protein, operates in conjunction with PTEN-induced kinase 1 (PINK1), and phosphorylation of Parkin by PINK1 transforms it into an active phospho-ubiquitin-dependent E3 ligase, which can respond to the loss of MMP ($\Delta\Psi M$) to eliminate damaged mitochondria (124). The evidence that Parkin-mediated clearance of damaged mitochondria limits the generation of ROS and prevents the induction of oxidative stress in OA chondrocytes was first demonstrated by Mohammad et al. (60).

Nrf2

Nuclear transcription factor erythroid-2-like factor 2 (Nrf2) plays a chondroprotective role in OA and can suppress metalloproteinase expression induced by IL-1β (125). Nrf2 is a redox-sensitive transcription factor that positively regulates the expression of antioxidant and cytoprotective enzymes, including HO-1, NQO1, GST, SOD, GPx, and CAT (35, 126). Nrf2/antioxidant response element (ARE) signal transduction is one of the crucial antioxidant systems to maintain the redox state and has been regarded as a strategy to eliminate the damage caused by excessive ROS production (99, 127). Heme oxygenase-1 (HO-1), a ARE regulated by Nrf2, has been reported to prevent diseases caused by oxidative stress as a major therapeutic target of Nrf2 (128).

EXOGENOUS DRUGS TO OPTIMIZE MITOCHONDRIA IN OA

The presence of the antioxidant defense system to avoid mitochondrial dysfunction and excessive chondrocyte apoptosis is extremely limited (129). Research on exogenous drugs to improve mitochondrial function in OA based on endogenous molecular targets is thus necessary (**Table 1**).

Antioxidants

Appropriate antioxidant strategies and the discovery of antioxidants are essential to protect chondrocytes against oxidative stress (86, 130, 131). Recent studies have shown that melatonin, dihydromyricetin, quercetin, taurine, and diallyl disulfide all act as antioxidants and are potential drugs for the treatment of OA.

Melatonin

Melatonin (N-acetyl-5-methoxytryptamine), an amine hormone produced by the pineal gland of mammals, is a broad-spectrum antioxidant and free radical scavenger (132). Melatonin and its metabolites can remove ROS by radical scavenging and improve the activation of antioxidant enzymes,

TABLE 1 | Mitochondrial pathways for the treatment of OA.

Potential drugs	Cells	Methods	Mechanism	Effects	References
Melatonin	Chondrocytes (CHON-001)	*In vitro*: Co-culture *In vivo*: Histological evaluation	Inhibit PI3K/Akt, JNK, ERK, p38 and MAPK	Inos↓, COX-2↓, NO↓, PGE_2↓	(23)
Resveratrol	Chondrocytes	*In vitro*: Co-culture	BAX/mitochondrial Cyt-C/Caspase	COX-2↓, NO↓, PGE_2↓	(24)
DHM	TNF-α-treated chondrocyte and rats	*In vitro*: Co-culture *In vivo*: Histological evaluation	AMPK/SIRT3/PGC-1α	Mitochondrial fusion↑, antioxidant capacity ↑, ECM balance↑	(25)
Apple procyanidins	Primary chondrocytes and chondrocyte-specific $Sod2^{-/-}$ mice	*In vitro*: Co-culture *In vivo*: Histological evaluation	AMPK/SIRT1/PGC-1α	Integrity of mtDNA↑, mitochondrial biogenesis↑ and proteoglycan biosynthesis↑	(26)
25 μM Zinc	MIA-treated SW1353 chondrocytes	*In vitro*: Co-culture	PINK1-Mitophagy PI3K/Akt/Nrf2	Mitophagy↑, oxidative stress ↓	(27)
SIRT3 activator	Human and mouse chondrocytes; C57BL/6 male mice	*In vitro*: Co-culture *In vivo*: Histological evaluation	AMPK/SIRT3/SOD2	Integrity of mtDNA4977↑	(28)
Quercetin	Chondrocytes from 1-week-old Sprague Dawley rats; OA rats.	*In vitro*: Co-culture *In vivo*: Histological evaluation	AMPK/SIRT1; Inhibit caspase-3	NO↓, MMP-3↓, MMP-13↓ and apoptosis↓	(29)
Puerarin	MIA-treated OA rats	*In vivo*: Histological evaluation	AMPK/PGC-1α	Mitochondrial biogenesis↑	(30)
LRWXG	ACLT-treated rats	*In vivo*: Histological evaluation	BAX/mitochondrial Cyt-C/Caspase	Bcl-2↑, MMP-3↓ and MMP-13↓	(31)
Ginsenoside Rg1	IL-1β-treated chondrocytes	*In vitro*: Co-culture	PI3K/Art	Caspase-3↓, TIMP-1↑, MMP-13↓ and Bcl-2↑	(32)
CS	H_2O_2-treated chondrocytes	*In vitro*: Co-culture	Increase MMP	MMP↑, Caspase-3↓ and Caspase-9↓	(33)
200 μM taurine	H_2O_2-induced chondrocytes	*In vitro*: Co-culture	Regulate Nrf2, miR-146a and miR-34a	Bcl-2↑, BAX↓	(34)
DADS	C2812 chondrocytes	*In vitro*: Co-culture	Enhance Nrf2	GPx1↑, GPx3↑, GPx4↑, CAT↑, SOD1↑, BAX/Bcl-2↓ and Caspase-3↓	(35)

thus regulating inflammation, proliferation, apoptosis and metastasis (133). Various experiments have demonstrated that melatonin can inhibit the phosphorylation of PI3K/Akt and MAPKs (23) and inhibit the loss of MMP and the release of mitochondrial Cyt-C (134). Kim et al. (23) demonstrated that melatonin acts as a potent inhibitor of H_2O_2-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expression while also suppressing the production of NO and PGE_2 in human chondrocytes, and the researchers thought that the inhibitory effect of melatonin on cartilage degeneration may be associated with the SIRT1 pathway.

Dihydromyricetin

Dihydromyricetin (DHM), which is mainly composed of flavonoids, can scavenge free radicals and has anti-inflammatory and antioxidative effects (135). SIRT3 can be activated by DHM through the AMPK/SIRT3/PGC-1α signaling pathway and can enhance mitochondrial fusion, maintain mitochondrial function and the homeostasis of chondrocytes, improve the antioxidant capacity of chondrocytes, and increase aggrecan and collagen II levels (25). DHM can also promote mitophagy to protect chondrocytes by activating SIRT3, which provides a new treatment strategy for OA.

Quercetin

Quercetin, a flavonoid compound, is widely found in vegetables and fruits and possesses antioxidant properties. Studies have revealed that quercetin is a potent anti-atherosclerotic drug as a result of its anti-inflammatory and antioxidative capacities (136). A study showed that quercetin could be used for the treatment of OA rats and demonstrated that quercetin could reverse mitochondrial dysfunction, improving MMP, oxygen consumption, and ATP production. The induction of glutathione (GSH) and glutathione peroxidase (GPX) by quercetin eliminated excessive ROS, which reduced or even abolished oxidative stress (29). Moreover, quercetin inhibited the accumulation of nitric oxide (NO), matrix metalloproteinase 3 (MMP-3), and MMP-13 produced by inflammation through AMPK/SIRT1 signaling, playing a key role in the inhibition of extracellular matrix degeneration. Quercetin also decreased chondrocyte apoptosis by inhibiting the caspase-3 signaling pathway (7). Therefore, quercetin is a potential therapeutic drug for OA that acts through the mitochondrial pathway.

Taurine

Taurine (2-aminoethane sulfonic acid), another antioxidant that is highly effective in attenuating free radical toxicity, has been identified (137). Taurine can ameliorate ROS-induced chondrocyte damage and exert chondroprotective properties, including the deposition of extracellular matrix components and proliferation of chondrocyte (138). Sara et al. (34) showed that 200 μM taurine could reduce mitochondrial superoxide anion production by activating Nrf2 and promote an increase in anti-apoptotic Bcl-2 and a reduction in proapoptotic BAX to inhibit chondrocyte apoptosis (139). In addition, the regulation of miR-146a and miR-34a expression in OA chondrocytes was first demonstrated. Taurine may be a potential drug for OA.

Diallyl Disulfide

Diallyl disulfide (DADS), a main component of garlic with antioxidant and anti-inflammatory properties (35, 140), could reduce pro-inflammatory cytokines expression, such as TNF-α, IL-1β, inducible nitric oxide synthase (iNOS), and COX-2 (35), by inhibiting the nuclear factor-κB (NF-κB) signaling pathway (141). Moreover, the pivotal etiological role of apoptosis in cartilage degeneration and the antioxidant and anti-apoptotic properties of DADS were considered (126, 140), the mechanism of DADS in oxidative stress and consequent apoptosis induced by IL-1β in C2812 human chondrocytes was studied by Hosseinzadeh et al. (35). The findings demonstrated that DADS protected C2812 chondrocytes against oxidative stress and reduced ROS and NO production by enhancing Nrf2 nuclear translocation. In addition, DADS markedly enhanced the expression of GPx1, GPx3, GPx4, CAT, and SOD1 and decreased the ratio of BAX/Bcl-2 and Caspase-3 activation to inhibit apoptosis (35). DADS could therefore be extracted and developed a potential drug for OA, and an interesting perspective emerged that a diet rich in garlic might be beneficial to reduce both the incidence and progression of OA.

Inhibiting the Mitochondrial Apoptotic Pathway

Resveratrol

The natural polyphenolic compound resveratrol (polystilbene, $C_{14}H_{12}O_3$), a non-flavonoid polyphenol compound with anti-inflammatory and antioxidative properties, is mainly derived from grape leaves, grape skin, and various fruits (142). Mitochondrial dysfunction increased the inflammatory response to cytokines in human chondrocytes and resveratrol significantly reduced the inflammatory response (143). Resveratrol alleviated the chondrocyte damage induced by interleukin-1β (IL-1β) through the NF-κB signaling pathway (144). Moreover, resveratrol has been regarded as a potent activator of SIRT1, which can prevent human chondrocyte apoptosis under cellular stresses, including nutritional stress, catabolic stress, and mechanical shear stress, by promoting Bcl-2 translocation to mitochondria and inhibiting BAX translocation to mitochondria (145). The optimization of mitochondrial function in animal models and protection against IL-1β-induced chondrocyte apoptosis can be achieved by resveratrol (24).

Xanthan Gum

Xanthan gum (XG), an extracellular acidic polysaccharide, is released by the fermentation of Xanthomonas (146, 147). Studies have shown that the BCL2-associated X protein (BAX)/Cyt-C/Caspase signaling pathway contributes to cartilage degeneration (88). A low range of molecular weights of XG (LRWXG) has been applied for rabbit OA treatment (31). In this study, the inhibition of cartilage matrix destruction and the protection of subchondral bone were demonstrated. In addition, LRWXG could inhibit the formation of small pores in the mitochondrial inner membrane and inhibit the swelling and rupture of the mitochondrial outer membrane, which could stabilize membrane potential and the permeability of the mitochondrial membrane. Moreover, activation of Bcl-2 and inhibition of BAX activity were achieved by LRWXG. Both of these factors could reduce the translocation of Cyt-C from mitochondria to the cytoplasm (31). The decrease in Cyt-C in the cytoplasm downregulated Caspase-3 and Caspase-9 in chondrocytes, which reduced the formation of apoptotic bodies and decreased chondrocyte apoptosis. Xintian Shao, the author of the study, therefore thought that LRWXG could inhibit chondrocyte apoptosis by conditioning the BAX/mitochondrial Cyt-C/Caspase signaling pathway and protect chondrocytes from degeneration.

Chondroitin Sulfate

Chondroitin sulfate (CS), a glycosaminoglycan that is widely extracted from animal and fish cartilage, is an essential component of the extracellular matrix (148). A study indicated that carp chondroitin sulfate increased MMP and inhibited the levels of Caspase-3 and Caspase-9 by reducing mitochondrial fission, which decreased chondrocyte apoptosis (33). It appears that chondroitin sulfate also has the potential to treat OA through the mitochondrial pathway.

Ginsenoside Rg1

Ginsenoside Rg1 (Rg1) is one of the most active components in ginseng along with steroidal saponin (149). The therapeutic effect of Rg1 on nervous system diseases and cardiovascular diseases has been reported, which inspired Huang et al. to investigate whether Rg1 protected chondrocytes (32). Their findings showed that Rg1 could enhance Bcl-2 expression, advance tissue inhibitor of metalloproteinase-1 (TIMP-1) expression, inhibit Bax activity, inhibit MMP-13 synthesis, and inhibit Cyt C release from mitochondria to the cytosol through enhancing phosphatidylinositol 3-kinase (PI3K)/Akt signaling, which inhibited Caspase-3. The inhibition of Caspase-3 led to the inhibition of chondrocyte apoptosis and protected chondrocytes. Rg1 may thus be a potential treatment for OA treatment through the PI3K/Akt/mitochondrial signaling pathway.

Enhanced Mitochondrial Dynamics

Apple Polyphenols

Apple polyphenols from immature apples, compounds composed of several polyphenols, exert anti-allergy, anti-fatigue and life-extending effects (26, 150). Masuda et al. investigated the role of apple polyphenols in protecting

chondrocytes and improving OA (26). Their findings showed that apple polyphenols could enhance mitochondrial biogenesis by promoting the integrity of mtDNA and mitochondrial fusion through AMPK/SIRT1/PGC-1α signaling. Moreover, apple polyphenols could promote proteoglycan biosynthesis. In an *in vivo* study, apple procyanidins protected against articular cartilage degeneration and prevented the development of knee OA in chondrocyte-specific Sod2-/- mice (26). Based on these results, we can conclude that apple polyphenols may be potential drugs for treating OA.

Puerarin

Puerarin, an isoflavone derivative, is isolated from the Chinese medicine Pueraria and possesses antioxidative, anti-inflammatory, anticancer and vasodilating effects (151). The ability of puerarin to restore mitochondrial dysfunction has been confirmed (152). Furthermore, puerarin could reduce mitochondrial dysfunction and damage to chondrocytes by increasing mitochondrial biogenesis and restoring mitochondrial function through the upregulation of AMPK/PGC-1α signaling, which protected chondrocytes in OA (30).

Zinc (25 μM)

In a study of metformin for the treatment of OA, Chenzhong Wang found that metformin could improve the expression of SIRT3 in chondrocytes and activate the PINK1 (PTEN induced putative kinase 1)/Parkin signaling pathway and could ameliorate mitochondrial function and protect chondrocytes from OA by promoting mitochondrial fusion and eliminating dysfunctional mitochondria through mitophagy (89). Huang et al. showed that 25 μM zinc could protect chondrocytes injured by monosodium iodoacetate (MIA) through the PINK1-dependent selective mitophagy pathway, which indicated that 25 μM zinc was protective against OA (27).

GLOBAL STATUS OF MITOCHONDRIAL AND OSTEOARTHRITIS RESEARCH

We collected 361 papers, and the dataset from Jan. 2000 to Dec. 2019 was derived from the Web of Science (WOS) Core Collection, which is regarded as the optimum database (153). The search terms were as follows: [(TS = (mitochondria* AND osteoarthritis)] OR [TS = (mitochondrion* AND osteoarthritis))] AND (Language = English) AND (Document type = Article AND Review). The logistic growth model $f(x) = a/[1 + e^{b-cx}]$, where x is the year and f(x) represents the cumulative quantity of papers by year, was used to model the cumulative volume of documentation because of its great fitness and ability to predict future trends (154). VOS viewer (Leiden University, Leiden, Netherlands) were tools used to develop the co-occurrence analysis map (155).

Global Status

Variations in the quantity of academic publications in a certain research field are a significant indicator of the development trend (155). Determining the number of papers within a period of time and guiding multivariate statistical analysis are conducive to the research level and future trends (155). The global status of mitochondrial and osteoarthritis research has demonstrated that research on mitochondria and OA has been a popular topic in the field. An total of 361 papers from 2000 to 2019 were obtained from the WOS database on the basis of the search formula. Over the past 20 years, there has been a growing trend in global publications, which showed that the relative research on mitochondria and OA increased and the total number of publications significantly increased (**Figure 5C**). Moreover, **Figure 5D** shows the logistic regression model meeting curves $f(x) = 330/[1 + e^{328.2543-0.1618x}]$ of the quantity of papers on mitochondria and OA research in the future per year. The top 20 productive countries are listed in **Figure 5B** due to the total quantity of papers per country. China was the largest contributor with the highest number, and **Figure 5A** shows the top 25 countries that made the greatest contributions to mitochondrial and OA research globally. The darker the color, the greater the quantity of papers.

Co-occurrence Analysis

The purpose of co-occurrence analysis is to determine the relevance of items according to the quantity of projects that appear together and describe the internal relationships and structure of an academic field, and reveal the research frontiers (156). The development of scientific research and programs could be monitored and followed closely as popular topics and directions were identified through co-occurrence analysis (155, 157). Keywords were analyzed by VOS viewer, and 277 identified keywords are shown in **Figure 6**. The larger the spheres, the greater the frequency. It was obvious that "Apoptosis," "Chondrocytes," "Oxidative," "Nitric-oxide," and "Autophagy" had the highest frequency and may be the main research themes in the past two decades. In addition, the blue color means that the keywords occurred early, and red colored keywords occurred later. We found that "Phenotype," "SIRT3," "PCG-1α," "AMPK," "FOXO transcription factors," "Mitophagy," "Acetylation," "Nrf2," and "Repair," which were red colored, occurred recently, which may mean that research on mitochondria and osteoarthritis will focus on mechanistic studies and cartilage repair.

CONCLUSION AND PERSPECTIVES

Research on mitochondria and OA is currently a popular topic. Mechanistic research on the relationship between mitochondria and OA has been launched, and corresponding research on the treatment of OA has also made excellent progress. Although articular cartilage deterioration is the main pathological characteristic of OA, it is now widely accepted that the entire joint, including the synovium, is involved (158). The synovium contributes to the general physiological function of joints and the regulation of the joint microenvironment by secreting synovial fluid to supply nutrients and lubricate the cartilage (159). Fibroblast-like synoviocytes (FLSs) are highly sensitive to hypoxia and reoxygenation (H/R), and IGFBP-3 is overexpressed in cartilage and synovial fluid under H/R

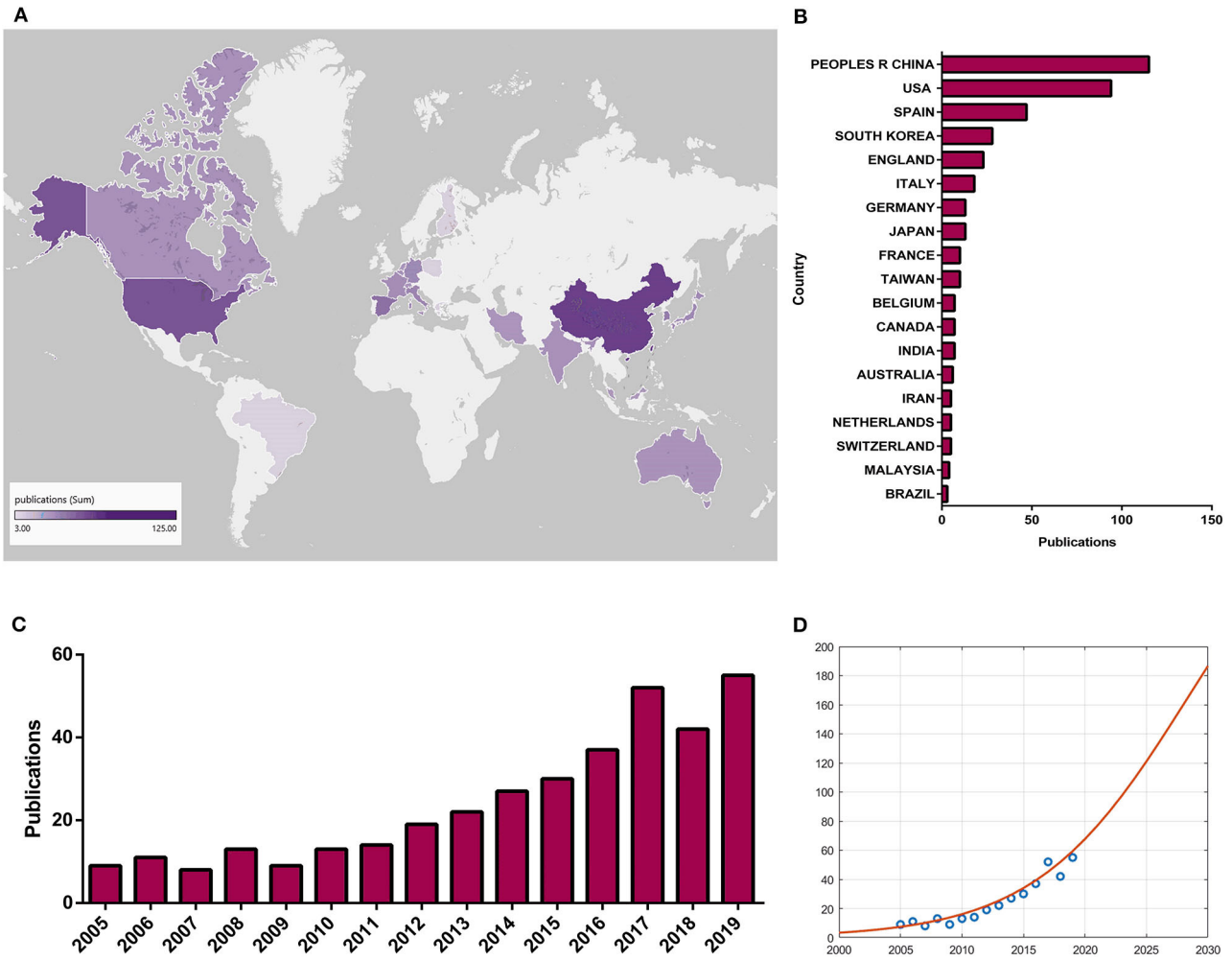

FIGURE 5 | Global trends and contributed countries on mitochondria and OA research. **(A)** World map showing the distribution of mitochondria and OA research, in which the different color depths represent the different numbers of publications in different countries. **(B)** The sum of publications related to mitochondria and OA research from 20 countries or regions. **(C)** The annual number of publications related to mitochondria and OA research in the past 20 years. **(D)** Model fitting curves of growth trends of accumulated number of publications on mitochondria and OA research.

conditions (160). The induction of nerve growth factor-induced gene B (NGFI-B, Nur77) by IGFBP-3 has been confirmed (161). The mitochondrial membrane permeability could be enhanced by Nur77, which results in the translocation of Cyt-C from the mitochondrial matrix to the cytoplasm and initiates an intrinsic and classic apoptosis pathway: the caspase pathway (162). Therefore, improving synovitis through the mitochondrial pathway may be a potential strategy for OA treatment (163).

Strategies for OA treatment are tiered, and non-pharmacological methods, including education and self-management, exercise, weight loss if overweight or obese, and walking aids as indicated, are widely recommended and regarded as first-line treatments (3, 164). The most commonly recommended pharmacological methods in the guidelines include paracetamol and NSAIDs (3, 165). In the context of surgery, joint replacement surgery, knee osteotomy, knee joint distraction and arthroscopic knee surgery (3), and autologous chondrocyte transplantation are currently the most effective treatments (166). The current pharmacological methods used to OA treatment are largely palliative (3), thus modifying OA progression, including slowing, halting, and reversing progression, are critical.

Biotherapy and gene therapy are current research trends in disease treatment. Stem cells, including mesenchymal stem cells (BMSCs), umbilical cord stem cells, embryonic stem cells and induced pluripotent stem cells, are regarded as exceptional donor cells for mitochondrial transfer, and numerous studies have confirmed the significance of mitochondrial transfer in stem cell therapy (167), especially BMSCs (168). Moreover, transplantation of stem cells has recently become a research hotspot in treating tissue injury. Whether stem cell transplantation can optimize mitochondrial

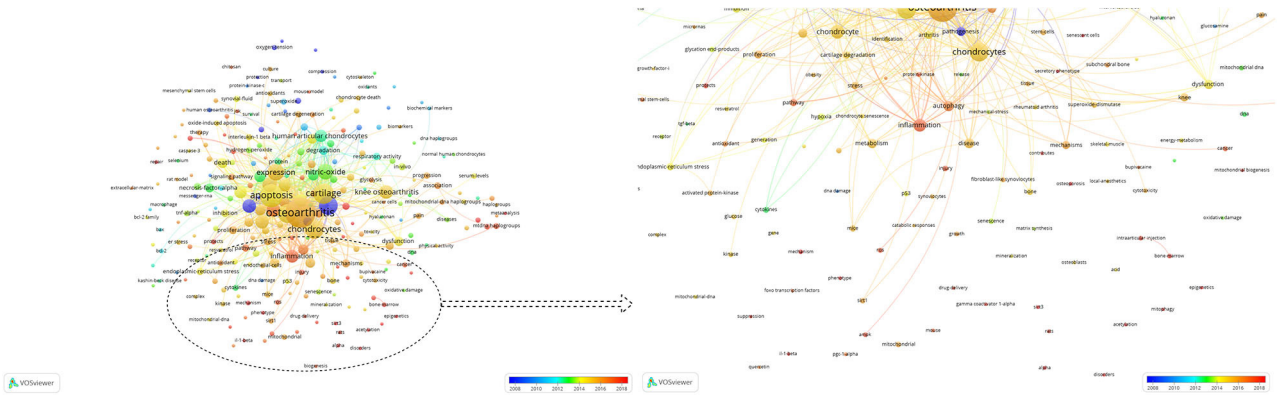

FIGURE 6 | Co-occurrence analysis of global research about mitochondria and OA. Mapping of keywords in the research on mitochondria and OA. The size of the points represents the frequency. Distribution of keywords according to the mean frequency of appearance. Keywords in blue appeared earlier than those in yellow and red colored keywords appeared later.

function in OA is therefore worth exploring. Exosomes, which are extracellular vesicles 30–150 nm in diameter, have similar functions as those of derived cells without apparent side effects in both healthy and diseased cells (169), and studies have shown that the therapeutic effects of mesenchymal stem cells (MSCs) can be replicated by their secreted exosomes (52, 170). MSC-derived exosomes possess the biochemical potential to restore homeostasis in bioenergetics, cell number and immunomodulation (52, 171, 172). Exosomes contain mitochondrial membrane components and mtDNA (173). Zheng et al. investigated the ability of primary chondrocyte-derived exosomes to abrogate mitochondrial dysfunction in degenerated chondrocytes (170). The results indicated that exosomes from chondrocytes could reduce the expression of inflammatory cytokines, restore mitochondrial dysfunction, and reduce macrophage polarization toward an M2 phenotype, resulting in the repair of injured chondrocytes. This finding is in accordance with the treatment of a mouse OA model with chondrocyte exosomes. Collectively, primary chondrocyte exosomes are potential disease-modifying therapeutic agents for OA. We therefore thought biomedical measures would be efficient for the treatment of OA based on optimizing mitochondrial function. CRISPR/Cas9 is the most convenient gene-editing tool so far, widely used in human embryonic stem cells (hESCs) and their derivatives for basic and clinical research (16, 174). Deng et al. showed that MSCs without DiGeorge syndrome critical region 8 (DGCR8) could alleviate human MSC senescence and mouse osteoarthritis (16, 175). More efficient and targeted gene-editing tools need to be developed, which contribute to precise genetic and epigenetic regulation, such as activation or inhibition of target genes *in vivo* (16). We thus predict that gene therapy will be a radical therapeutic strategy for OA treatment.

There are still numerous mechanisms that need to be further explored. Pain is the main symptom of OA patients and a major driver of clinical decisions (3, 176); therefore, whether the new strategy targeting the mitochondrial pathway for OA has an effect on pain relief is still unclear in the current study. Microvesicles are popular for research on the mechanism of OA treatment and whether microvesicles can promote mitochondrial fusion and biosynthesis to reduce chondrocyte apoptosis is not known. At the same time, how drugs that affect the mitochondrial pathway in OA work in mitochondria, which are subcellular organelles, is unclear. It is widely accepted that mitochondria and the nucleus are in two-way communication, and the way mitochondria conduct signal transduction with the nucleus after exposure to a drug effects to inhibit cell apoptosis and protect cells is worth studying. With the continuous investment in mitochondrial and OA research worldwide, a new strategy targeting the mitochondrial pathway in OA will have great breakthroughs and will make a great contribution to the treatment of OA. The day is coming when we will provide subcellular, cellular, and tissue-level mechanistic and clinical evidence for the treatment of OA to provide a more comprehensive and efficient treatment for OA patients.

AUTHOR CONTRIBUTIONS

XM, LW, and CX made the review article structure. CX and LW are responsible for reviewing. XM and PF finished writing. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a grant from the National Natural Science Foundation of China (No. 81972075) and the Shanxi Province Science and Technology Research Project (201803D421050).

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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 | Frontiers in Medicine

TYPE Original Research
PUBLISHED 25 August 2022
DOI 10.3389/fmed.2022.939800

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Family Medicine and Primary Care, a section of the journal Frontiers in Medicine

RECEIVED 09 May 2022
ACCEPTED 05 August 2022
PUBLISHED 25 August 2022

CITATION
Lim YZ, Cicuttini FM, Wluka AE, Jones G, Hill CL, Forbes AB, Tonkin A, Berezovskaya S, Tan L, Ding C and Wang Y (2022) Effect of atorvastatin on skeletal muscles of patients with knee osteoarthritis: *Post-hoc* analysis of a randomised controlled trial. *Front. Med.* 9:939800. doi: 10.3389/fmed.2022.939800

Effect of atorvastatin on skeletal muscles of patients with knee osteoarthritis: *Post-hoc* analysis of a randomised controlled trial

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Objective: Populations with knee osteoarthritis (KOA) are at increased risk of cardiovascular disease, due to higher prevalence of risk factors including dyslipidaemia, where statins are commonly prescribed. However, the effect of statins on muscles and symptoms in this population is unknown. Thus, this study examined the effect of atorvastatin on muscle properties in patients with symptomatic KOA.

Design: *Post-hoc* analysis of a 2-year multicentre randomised, double-blind, placebo-controlled trial.

Setting: Australian community.

Participants: Participants aged 40–70 years (mean age 55.7 years, 55.6% female) with KOA who met the American College of Rheumatology clinical criteria received atorvastatin 40 mg daily (n = 151) or placebo (n = 153).

Main outcome measures: Levels of creatinine kinase (CK), aspartate transaminase (AST), and alanine transaminase (ALT) at 1, 6, 12, and 24 months; muscle strength (by dynamometry) at 12 and 24 months; vastus medialis cross-sectional area (CSA) on magnetic resonance imaging at 24 months; and self-reported myalgia.

Results: There were no significant between-group differences in CK and AST at all timespoints. The atorvastatin group had higher ALT than placebo group at 1 (median 26 vs. 21, p = 0.004) and 6 (25 vs. 22, p = 0.007) months without significant between-group differences at 12 and 24 months. Muscle strength increased in both groups at 24 months without between-group differences [mean 8.2 (95% CI 3.5, 12.9) vs. 5.9 (1.3, 10.4), p = 0.49]. Change in vastus medialis CSA at 24 months favoured the atorvastatin group [0.11 (−0.10, 0.31) vs. −0.23 (−0.43, −0.03), p = 0.02] but of uncertain clinical significance. There was a trend for more myalgia in the atorvastatin group (8/151 vs. 2/153,

p = 0.06) over 2 years, mostly occurring within 6 months (7/151 vs. 1/153, p = 0.04).

Conclusions: In those with symptomatic KOA, despite a trend for more myalgia, there was no clear evidence of an adverse effect of atorvastatin on muscles, including those most relevant to knee joint health.

KEYWORDS

statins, osteoarthritis, knee, muscles, myalgia

Introduction

Osteoarthritis (OA) is a common cause of pain and disability. However, generally overlooked is the fact that people with OA die of cardiovascular disease (CVD) at approximately twice the rate of the general population (1, 2). This relates to the increased prevalence of CVD risk factors among those with OA, including dyslipidaemia (3).

Statins, one of the most widely prescribed drug classes worldwide, have the well documented benefit of reducing coronary heart disease events and stroke, by lowering the levels of low-density lipoprotein cholesterol (4). Statins have been the cornerstone of pharmacotherapy for the management of dyslipidaemia virtually since their development (5). They are generally safe and well tolerated (6). Nevertheless, statin-associated muscle symptoms, present most commonly as myalgia and rarely as myopathy, myositis or rhabdomyolysis, have been cited as the most common reason for statin discontinuation (7, 8). In a survey of 10138 statin users, while most patients (62%) discontinued statin therapy due to side effects, nearly 1/3 stopped their statin therapy due to muscle related side effects without consulting their clinicians (7), possibly due to distortion of the risk-benefit ratio and hence unduly concerns about potential harms of statins from non-clinician sources (9). The prevalence of statin-induced muscle symptoms varies, depending on how it is defined and assessed. There is a huge discrepancy in the incidence of myalgia, ranging from 1 to 5% in clinical trials to 11–29% in observational cohort studies (10). The National Lipid Association Task Force on Statin Safety 2014 update highlighted the limitation of using current evidence of safety from randomised controlled trials because such populations are typically very restricted in their study entry characteristics, excluding patients with multiple comorbidities, previous statin intolerance, and people with active musculoskeletal conditions (10). In addition, varying definitions for statin-associated muscle symptoms have been used (8, 10).

Muscles play an important role in the prevention and management of knee OA (11). Muscle weakness has been associated with the development and progression of knee OA. In patients without radiographic knee OA, weak knee extensor strength has been associated with increased risk of developing symptomatic knee OA (12) while in patients with established radiographic and symptomatic knee OA, weak knee extensor is associated with increased risk of symptomatic and functional deterioration (13). There is evidence that statin use may exacerbate the age-related decline in muscle performance and increase the risk of falls despite no reduction in muscle mass in community-dwelling older adults (14). Hence, it is possible that statin-associated muscle symptoms may worsen the tolerability of statin in patients with OA. Conversely, individuals with OA are at twice the risk of CVD mortality (1) and therefore at greater need for statin. As those with symptomatic OA are excluded from clinical trial of statins, the effect of statin on skeletal muscles in populations with symptomatic OA is unknown. Thus, the aim of this study was to examine the effect of atorvastatin on skeletal muscle properties (biochemistry, strength, size, and myalgia) in a *post-hoc* analysis of a randomised controlled trial examining the effect of atorvastatin on progression of knee OA (15).

Materials and methods

Study design and participants

The Osteoarthritis of the Knee Statin (OAKS) study was a 2-year multicentre randomised, double-blind, placebo-controlled trial evaluating whether atorvastatin had a disease-modifying effect in patients with symptomatic knee OA (15, 16). In brief, eligible participants aged 40–70 years with symptomatic knee OA for ≥6 months with a pain score of >20 mm on a 100 mm visual analog scale, and who met the American College of Rheumatology clinical criteria for knee OA (17) were enrolled. Exclusion criteria were severe radiographic knee OA [grade 3 joint space narrowing according to Altman's atlas (18)]; severe knee pain (on standing >80 mm on 100 mm visual analog scale); inflammatory arthritis; accepted indications for statin therapy, including familial hypercholesterolaemia, known atherosclerotic cardiovascular disease, and diabetes mellitus; current use of lipid-lowering therapy, or previous adverse reaction to statins; absolute cardiovascular risk estimated using the Framingham Risk Equation of >15% within the next 5 years; fasting total cholesterol level >7.5 mmol/L; clinically significant

renal disease or abnormal liver function. Ethics approval was obtained from Alfred Hospital Ethics Committee, Monash University Human Research Ethics Committee, Tasmania Health and Medical Human Research Ethics Committee, and The Queen Elizabeth Hospital Human Research Ethics Committee. All participants provided written informed consent. The trial was registered with Australian New Zealand Clinical Trials Registry (ACTRN12613000190707).

Study protocol

Participants were randomly assigned in 1:1 ratio to receive either 40 mg atorvastatin once daily or inactive matching placebo once daily. Details concerning randomisation and masking have been reported previously (15, 16). All participants were provided usual care by their treating health practitioners. At screening, participants completed questionnaires, had a knee X-ray, and underwent biochemical testing including liver function tests, creatine kinase (CK) and renal function tests, to ensure inclusion criteria were met. Height and weight were measured at baseline. Subsequent study visits were scheduled at 6, 12, and 24 months. Adverse events were monitored throughout the trial. Participants were requested to report any adverse event at each study visit and by phone calls outside the scheduled study visits. Serious adverse events were determined by a rheumatologist who was blinded to treatment allocation. Details of the adverse event and its relationship with the intervention were recorded and reported to the Ethics Committees. The primary outcome of the OAKS study was the annual percentage change in tibial cartilage volume, measured by magnetic resonance imaging (MRI) (15).

Muscle biochemistry

Biochemical testing including CK and liver function tests [alanine transaminase (ALT), aspartate transaminase (AST)] were performed at screening, 4 weeks, 6, 12, and 24 months for safety monitoring, according to the manufacturer's instruction in accredited commercial laboratories. All abnormal biochemistry results were reviewed by a rheumatologist to determine the clinical significance, relevance, and appropriate management.

Muscle strength

Muscle strength was measured by dynamometry to the nearest kilogramme in both legs simultaneously at baseline, 12 and 24 months (14). The muscles measured in this technique are mainly quadriceps and hip flexors. The technique has been previously described (14). Three readings were recorded, and the highest score was used. The devices were calibrated by suspending known weights at regular intervals. Repeatability estimates (Cronbach's) were 0.91 (19).

Muscle size

Magnetic resonance imaging of the study knee was performed at baseline and 24 months using 1.5T or 3T whole-body MRI units with a commercial transmit-receive knee coil. Details of MRI units, sequences and parameters have been published (16). Cross-sectional area (CSA) of vastus medialis, a central muscle responsible for knee joint stability and function (11, 13), was measured on axial MRI images (11, 20). The CSA of vastus medialis was measured specifically at the MRI slice 37.5 mm superior to the quadriceps tendon insertion at the proximal pole of the patella, orthogonal to the long axis of the leg. The muscle boundary was manually traced using the OsiriX Software. Baseline and follow-up MRIs were read paired by one trained observer, blinded to group allocation, participant characteristics, and time sequence of MRI. The intraobserver reproducibility (intraclass correlation coefficient, ICC) of the measurement was 0.95.

Muscle symptoms

Participants who self-reported myalgia through adverse events monitoring were assessed by a rheumatologist and managed on a case-by-case basis.

TABLE 1 Baseline characteristics of study participants.

	Total population $N = 304$	Atorvastatin $N = 151$	Placebo $N = 153$	P
Age, years	55.7 (7.6)	55.7 (7.3)	55.8 (7.9)	0.89
Female, *n* (%)	169 (55.6)	92 (60.9)	77 (50.3)	0.06
Body mass index, kg/m^2	29.4 (5.8)	29.4 (5.7)	29.5 (5.8)	0.85
Joint space narrowing[a], *n* (%)				0.97
Grade 0	131 (44.3)	64 (43.8)	67 (44.7)	
Grade 1	102 (34.5)	52 (35.6)	50 (33.3)	
Grade 2	63 (21.3)	30 (20.6)	33 (22.0)	
Muscle strength[b], kg	84.5 (49.7)	81.0 (46.3)	88.0 (52.9)	0.23
Vastus medialis cross-sectional area[c], cm^2	11.0 (3.4)	10.8 (3.2)	11.2 (3.6)	0.29
CK[d], U/L	93.5 (70, 130)	91 (70, 126)	96 (70, 133)	0.37
ALT[e], U/L	21 (16, 28)	19 (14, 26)	21 (16, 29.5)	0.04
AST[f], U/L	20 (17, 24)	19.5 (16, 23)	21 (18, 25)	0.04

Data presented as mean (standard deviation), no (%), or median (interquartile range).
[a] $n = 296$; [b] $n = 290$; [c] $n = 301$; [d] $n = 298$; [e] $n = 303$; [f] $n = 302$.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatinine kinase.

Statistical analysis

Participant characteristics at baseline were tabulated and compared between the atorvastatin and placebo groups using independent samples *t*-test, Mann-Whitney *U*-test, or chi square test, as appropriate. Per protocol analyses of all the outcome measures were performed according to the participants' randomised treatment group restricted to those with available outcome measures at different timespoints. Independent samples *t*-test and Mann-Whitney *U*-test was used to compare muscle measures between the two groups at each time point, when appropriate. Muscle biochemistry biomarkers, strength, and CSA were compared between the atorvastatin and placebo groups by using a repeated measures mixed-effects linear regression model with terms of treatment, time, sex and corresponding baseline values as covariates. The correlations within the repeated measures were addressed by using the participants' randomisation identification as a random effect. The effect of treatment at baseline and week 4, months 6, 12, and 24 was evaluated by adding an intervention-by-time interaction to the regression models. The linear mixed-effects model incorporates all the study participants and assumes that data are missing at random. Chi square test or Fisher's exact test was used to compare the incidence of myalgia between the two groups. With 248 participants completing the 2-year follow-up, the study had 80% power to detect a difference of 5 kg in muscle strength change, a difference of 0.5 cm^2 in muscle CSA change, and a difference of 6 units in muscle biochemistry biomarker change between the atorvastatin and placebo groups (alpha 0.05, 2-sided significance). A two-sided *p*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using Stata 16.0 (StataCorp LP., College Station, TX, United States).

Results

Of the 304 participants randomised to receive atorvastatin (n = 151) or placebo (n = 153), 248 (81.6%) participants completed the study (**Supplementary Figure 1**) (15). Participant characteristics at baseline are shown in **Table 1**. The mean age was 55.7 (SD 7.6) years, and 169 (55.6%) were women. There were no significant between-group differences for age, body mass index, severity of radiographic knee OA, muscle

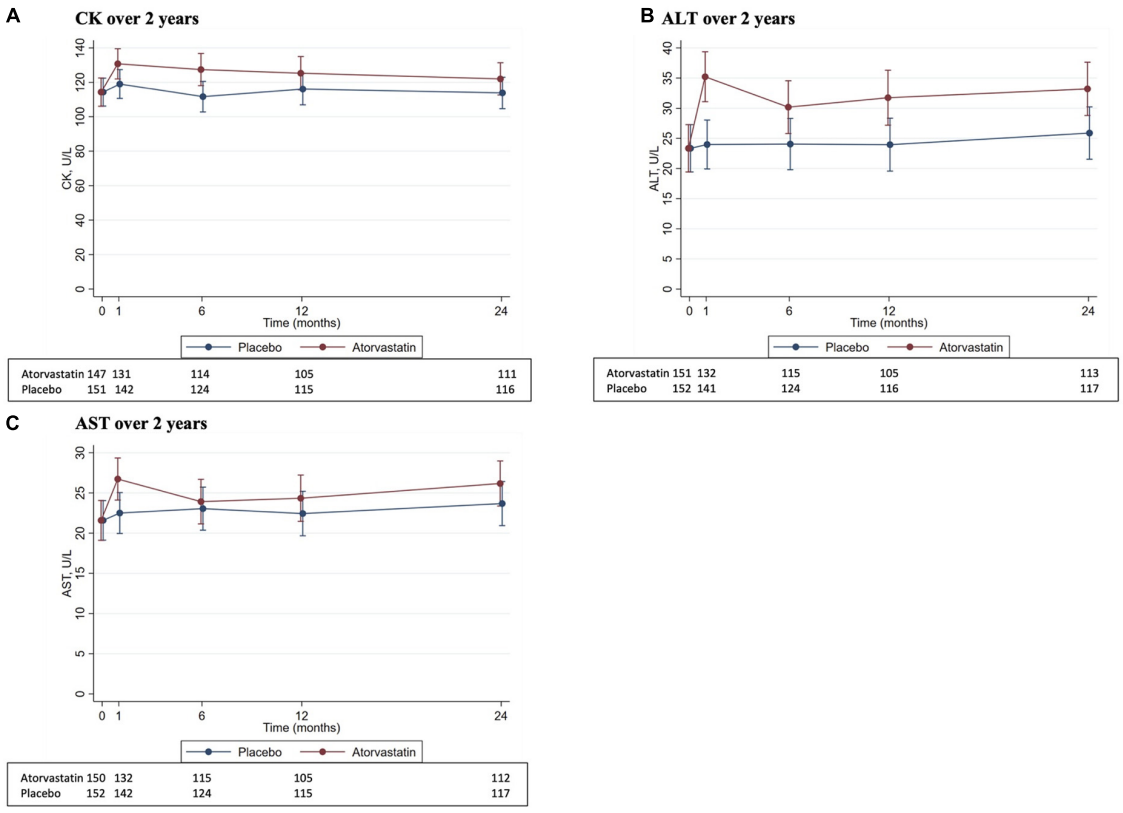

FIGURE 1
Muscle biochemistry biomarkers (mean and 95% confidence interval) at each time point over 2 years. Data were estimated from linear mixed-effects models. ALT, alanine aminotransferase; CK, creatinine kinase.

strength, vastus medialis CSA, or CK levels. The atorvastatin group had a higher proportion of females (p = 0.06), lower ALT (p = 0.04) and AST (p = 0.04) levels than the placebo group. Baseline characteristics of participants who completed the study and those who dropped out are presented in Supplementary Table 1. Participants who dropped out in the atorvastatin group were significantly younger than those who completed the study (p < 0.001). Participants who dropped out in the placebo group had higher ALT (p = 0.04) and AST (p = 0.02) levels than those who completed the study. Reasons for dropouts are presented in Supplementary Table 2.

Muscle biochemistry

Figure 1 and Table 2 show the effect of atorvastatin on muscle biochemistry biomarkers and their changes over 2 years. There were no significant between-group differences in CK levels at all timespoints (Figure 1A and Table 2). The change in CK levels at 6 months from baseline was higher in the atorvastatin group compared with the placebo group (p = 0.04) with no significant between-group differences at other timespoints (Table 2). Although ALT levels were lower in the atorvastatin group compared with the placebo group at baseline, the atorvastatin group had higher ALT levels than the placebo group at 4 weeks (p = 0.004) and 6 months (p = 0.007). The between-group differences in ALT levels were not statistically significant at 12 and 24 months (Figure 1B and Table 2). The change in ALT levels from baseline was higher in the atorvastatin group compared with the placebo group at 4 weeks (p = 0.001), 12 (p = 0.03) and 24 (p = 0.03) months (Table 2). Despite higher AST levels in the placebo group than the atorvastatin group at baseline, there were no significant between-group differences in AST levels at

TABLE 2 Muscle biochemistry biomarkers over 2 years.

	CK, U/L			ALT, U/L			AST, U/L		
	Atorvastatin	**Placebo**	***P***	**Atorvastatin**	**Placebo**	***P***	**Atorvastatin**	**Placebo**	***P***
Baseline Median (IQR)	91 (70, 126)	96 (70, 133)	0.37[a]	19 (14, 26)	21 (16, 29.5)	0.04[b]	19.5 (16, 23)	21 (18, 25)	0.04[c]
4 weeks Median (IQR)	107 (74, 157)	110 (75, 142)	0.76[d]	26 (19.5, 35)	21 (17, 29)	0.004[d]	22 (18.5, 26.5)	21 (17, 26)	0.14[e]
6 months Median (IQR)	109 (76, 149)	101.5 (72, 136)	0.37[f]	25 (19, 34)	22 (15, 30)	0.007[g]	21 (18, 26)	20 (18, 26)	0.45[g]
12 months Median (IQR)	103 (78, 140)	103 (72, 148)	0.93[h]	24 (19, 30)	21 (16, 30)	0.08[i]	22 (17, 26)	21 (17, 27)	0.99[h]
24 months Median (IQR)	103 (78, 139)	93.5 (62, 136)	0.17[j]	24 (19, 32)	21 (16, 29)	0.053[k]	22 (19, 26)	21 (17, 26)	0.34[l]
Change from baseline to 4 weeks Mean (95% CI)*	16.4 (6.3, 26.6)	4.7 (−5.1, 14.5)	0.10	11.9 (7.3, 16.4)	0.6 (−3.8, 5.1)	0.001	5.1 (2.2, 8.0)	0.9 (−1.9, 3.7)	0.04
Change from baseline to 6 months Mean (95% CI)*	13.1 (2.5, 23.7)	−2.6 (−12.8, 7.5)	0.04	6.8 (2.1, 11.6)	0.7 (−3.9, 5.3)	0.07	2.3 (−0.7, 5.4)	1.5 (−1.5, 4.4)	0.69
Change from baseline to 12 months Mean (95% CI)*	11.0 (0.1, 21.9)	1.8 (−8.7, 12.2)	0.23	8.4 (3.5, 13.3)	0.6 (−4.2, 5.4)	0.03	2.8 (−0.4, 5.9)	0.9 (−2.2, 3.9)	0.40
Change from baseline to 24 months Mean (95% CI)*	7.7 (−2.9, 18.4)	−0.5 (−10.9, 9.9)	0.28	9.9 (5.1, 14.7)	2.5 (−2.2, 7.3)	0.03	4.6 (1.5, 7.6)	2.1 (−0.9, 5.1)	0.26

[a]n = 298; [b]n = 303; [c]n = 302; [d]n = 273; [e]n = 274; [f]n = 238; [g]n = 239; [h]n = 220; [I]n = 221; [j]n = 227; [k]n = 230; [l]n = 229.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatinine kinase; IQR, interquartile range; CI, confidence interval.
*Linear mixed-effects models adjusted for sex and respective baseline value.

TABLE 3 Muscle strength and size over 2 years.

	Muscle strength, kg			Muscle CSA, cm^2		
	Atorvastatin	**Placebo**	***P***	**Atorvastatin**	**Placebo**	***P***
Baseline Mean (SD)	81.0 (46.3)	88.0 (52.9)	0.23[a]	10.8 (3.2)	11.2 (3.6)	0.29[d]
12 months Mean (SD)	87.6 (38.6)	91.1 (51.1)	0.55[b]	–	–	–
24 months Mean (SD)	90.5 (40.0)	91.9 (48.7)	0.81[c]	10.8 (3.4)	10.8 (3.3)	0.95[e]
Change from baseline to 12 months Mean (95% CI)*	6.1 (1.4, 10.8)	2.1 (−2.4, 6.7)	0.23	–	–	–
Change from baseline to 24 months Mean (95% CI)*	8.2 (3.5, 12.9)	5.9 (1.3, 10.4)	0.49	0.11 (−0.10, 0.31)	−0.23 (−0.43, −0.03)	0.02

[a]n = 290; [b]n = 238; [c]n = 237; [d]n = 301; [e]n = 244.
CSA, cross-sectional area; SD, standard deviation; CI, confidence interval.
*Linear mixed-effects models adjusted for sex and respective baseline value.

all follow-up timespoints (Figure 1C and Table 2). The change in AST levels from baseline was higher in the atorvastatin group compared with the placebo group at 4 weeks ($p = 0.04$) with no significant between-group differences at other timespoints (Table 2).

Muscle strength and size

The effect of atorvastatin on muscle strength, size, and their changes over 2 years are shown in Table 3. There was a significant increase in muscle strength in the atorvastatin group over 12 and 24 months and in the placebo group over 24 months. However, the change in muscle strength was not significantly different between the two groups. Although no significant change in vastus medialis CSA was observed over 24 months in either group, there was significant between-group difference in the change of vastus medialis CSA (0.11 vs. −0.23, $p = 0.02$).

Incidence of myalgia

Table 4 shows the incidence of myalgia at different timespoints throughout the study. The incidence of myalgia was slightly higher in the atorvastatin group than the placebo group over 2 years (8/151 vs. 2/153, $p = 0.06$). Most of the myalgia occurred within the first 6 months after drug commencement (7/151 vs. 1/153, $p = 0.04$).

Relationship between creatinine kinase levels and myalgia

Characteristics of participants who developed myalgia are presented in Supplementary Table 3. There was no relationship between the incidence of myalgia and CK levels. Of the 10 participants (8 in atorvastatin group and 2 in placebo group), the majority had normal levels of CK, AST, and ALT. Only 2 participants in the atorvastatin group had mildly elevated levels of CK, <1.5 times the upper limit of normal. Participants described their symptoms as muscle cramps, aches, unilateral calf pain, or severe muscle pain with weakness. CK levels were within normal limit in the participant who reported severe muscle pain with weakness. Two participants (one in each group) had underlying thyroid disease.

TABLE 4 Incidence of myalgia.

	Atorvastatin, $n = 151$	Placebo, $n = 153$	P
Myalgia within 4 weeks, n (%)	2 (1.3)	0	0.25
Myalgia 4 weeks–6 months, n (%)	5 (3.3)	1 (0.7)	0.12
Myalgia 6–12 months, n (%)	1 (0.6)	1 (0.7)	1.00
Total, n (%)	8 (5.3)	2 (1.3)	0.06

Discussion

We showed in this *post-hoc* analysis of a randomised placebo-controlled trial, that in participants with symptomatic knee OA, atorvastatin 40 mg daily had no adverse effect on muscle biochemistry, strength or size, despite a slightly higher incidence of myalgia over 2 years that usually occurred within 6 months of drug commencement. As such, given the OA population has twice the risk of cardiovascular death than the general population, clinicians should not withhold the substantial benefit of statins in OA populations, especially when dealing with mild statin-associated muscle symptoms.

Muscle biochemistry biomarkers, including CK and AST, muscle strength and size were not affected by high-intensity atorvastatin dose (40 mg daily) in people with symptomatic knee OA. No participants developed myopathy or myositis. Our study found no significant between-group differences in CK levels at all timespoints. This is in contrast to the Effects of Statins on Skeletal Muscle Function and Performance (STOMP) trial that showed a small (∼20 U/L) but significant ($p < 0.01$) increase in CK levels at 6 months with atorvastatin 80 mg among healthy, statin-naïve participants (21). Among our eight participants assigned to atorvastatin who developed myalgia, there was no significant increase in CK levels. There is evidence for a dose-dependent effect of statins on statin-induced muscle symptoms, such that high dose statins produce a 10-fold higher rate of myopathy development than a low dose statin (8, 22). This may explain the differences in CK findings between our study and the STOMP trial. In our study despite a statistically significant higher ALT levels in the atorvastatin group at 4 weeks and 6 months compared with the placebo group which diminished after 6 months, these changes were not clinically significant. Of those who had abnormal ALT levels at 4 weeks (24/151 in atorvastatin group vs. 15/153 in placebo group), only 2 participants in the atorvastatin group had ALT levels of 3 times the upper limit of normal. These ALT abnormalities were transient and were all resolved by 6 months, with no participants having ALT levels of 3 times the upper limit of normal at 6 months after drug commencement. Although ALT is usually present in the liver at a much higher concentration, it can also be found in skeletal muscles (23). Its levels tend to stabilise despite continuation of treatment, as seen in our study, and most likely represent adaptation of the liver to the lower serum cholesterol, rather than direct hepatotoxicity (24, 25).

The evidence regarding the effect of statins on muscle strength, function and performance is conflicting (14). Our

study showed high-intensity atorvastatin dose had no adverse effect on muscle strength and size. In fact, we found increased muscle strength at 12 and 24 months in the atorvastatin group, while in the placebo group increased muscle strength was observed at 24 months but not 12 months. In contrast, a previous study showed that self-reported statin use in older adults (mean age 62 years) was associated with significantly reduced leg strength, and that those remaining on statin use at baseline and follow-up demonstrated significantly lower leg strength than those who ceased statin therapy (14). The participants in the previous study were older and had more comorbidities (63.9% of statin users had CVD and 12.9% had diabetes) than in our study and the dosage of statin was unknown. It may be that other factors such as age-related neuromuscular decline, may explain the lower leg strength in statin users. In support of our findings, the STOMP trial which examined healthy people without OA, also showed no detrimental effect on muscle strength or exercise performance with high dose 80 mg atorvastatin (21).

Consistent with evidence from previous clinical trials (10, 21), we found that high-intensity atorvastatin dose was associated with a trend to a higher incidence of myalgia over 2 years, usually occurring within 6 months of drug commencement. Within the statin drug class, atorvastatin has been associated with higher incidence of myalgia compared to placebo (8, 21). However, most of the concerns arise from significantly higher incidence of myalgia noted in observational studies rather than that reported in randomised controlled trials (10, 25). Additionally, most clinical trials excluded participants with chronic pain, such as those with symptomatic OA. Encouragingly, we showed the incidence of myalgia from high-intensity dose of atorvastatin in patients with symptomatic knee OA was 5.3% (8/151), which was similar to other non-OA clinical trials (8, 10). In real-life clinical practice, statins are often discontinued because of their "perceived" side effects (7), in particular related to skeletal muscle. Although we did not show any significant relationship between CK levels and myalgia in our analysis, of those who developed myalgia, 50% (3 of 8 in the atorvastatin group and 2 of 2 in the placebo group) discontinued therapy. The magnitude of this potential nocebo effect was elegantly evaluated in the N-of-1 trial that showed 90% of the symptom burden caused by statin was also elicited by placebo and 50% of them were able to successfully restart statins (26).

People with OA are twice more likely to die from CVD than the age-matched general population (2, 27), owing to the high prevalence of shared traditional CVD risk factors, including dyslipidaemia (1). The benefit of reduction in low-density lipoprotein cholesterol on CVD events is well documented, such that for every 1 mmol/L reduction, there is a significant 22% reduction in the risk of major vascular and coronary events, regardless of the baseline level (5, 28). Given the increased risk of CVD death in those with OA, there is a need to target CVD risk factors in those with OA. This study provides reassuring data of the safety of high-intensity atorvastatin on skeletal muscles in those with symptomatic knee OA, despite a slightly increased incidence of myalgia symptoms. In our group of participants with low-to-medium CVD risk, we found no adverse effects of atorvastatin on muscle properties including muscle biochemistry, strength, or size, particularly when we focused on lower limb muscles that are significantly affected in those with symptomatic knee OA. Hence, the substantial benefit of statin in people with OA should not be held back.

This study has limitations. As it was a *post-hoc* analysis of a randomised controlled trial, the inherent issue of statin-induced myalgia incidence discrepancy between observational studies and randomised controlled trials remains as participants in this study were highly selected. However, in our study, we targetted a population with symptomatic knee OA, hence addressing a significant clinical gap on statin safety in a group with high CVD risk. Additionally, we showed no differences in baseline muscle properties between those who dropped out and those who completed the study (**Supplementary Table 1**). Our study population was limited to those without a valid indication for statin use, as it would be unethical to withhold statin with a clinical indication, for example those with estimated high cardiovascular risk, currently on lipid-lowering therapy, or with fasting total cholesterol level >7.5 mmol/L (who often have familial hypercholesterolaemia) were excluded. It is likely that those with knee OA who were excluded from this study, are the population at greatest need for statins. At the same time, this population also generally has more comorbidities requiring other concomitant drugs, and thus is at an increased risk of statin toxicity (8). Therefore, our study may have underestimated the potential muscle-related adverse effect of statins in people with OA. However, we showed that high-intensity atorvastatin dose had no adverse effects on skeletal muscle in people with symptomatic knee OA with low-to-medium CVD risk. One of the strengths of this study is that we recruited participants from the community. Since knee OA is common, with 48% of community-based adults (mean age 63 years, range 50–79 years) having knee pain (29), this increases the generalisability of our findings to the broad population with symptomatic knee OA. Apart from self-reported myalgia, all other muscle measures were objectively assessed, including using laboratory tests for muscle biochemistry, MRI for muscle size, and dynamometer for muscle strength.

In conclusion, we showed that high-intensity atorvastatin at a dose of 40 mg once daily had no adverse effect on muscle biochemistry, strength and size among participants with symptomatic knee OA, apart from a slightly higher incidence of myalgia over 2 years, usually occurring within 6 months of drug commencement. Given the OA population is known to be at higher risk of cardiovascular morbidities and mortality

than the general population, with the findings of this study, the substantial benefit of statins in OA populations should not be withheld (3, 5).

Data availability statement

The datasets presented in this article are not readily available because the data generated from this study will not be deposited in a public repository due to privacy and consent restrictions. De-identified data can be made available from the corresponding author on reasonable request, subject to a data sharing agreement. Requests to access the datasets should be directed to YW, yuanyuan.wang@monash.edu.

Ethics statement

The studies involving human participants were reviewed and approved by the Alfred Hospital Ethics Committee, Monash University Human Research Ethics Committee, Tasmania Health and Medical Human Research Ethics Committee, and The Queen Elizabeth Hospital Human Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YL: analysis and interpretation of the data, drafting of the manuscript, and final approval of the manuscript. FC and YW: conception and design, analysis and interpretation of the data, critical revision of the manuscript for important intellectual content, and final approval of the manuscript. AW, GJ, CH, AT, and CD: interpretation of the data, critical revision of the manuscript for important intellectual content, and final approval of the manuscript. AF: analysis and interpretation of the data, critical revision of the manuscript for important intellectual content, and final approval of the manuscript. SB: acquisition of data, interpretation of the data, critical revision of the manuscript for important intellectual content, and final approval of the manuscript. LT: conception, critical revision of the manuscript for important intellectual content, and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

The study was funded by a project grant from the National Health and Medical Research Council of Australia (NHMRC, APP1048581). YL was the recipient of NHMRC Clinical Postgraduate Scholarship (APP1133903) and Royal Australasian College of Physicians Woolcock Scholarship. FC was the recipient of NHMRC Investigator Grant (APP1194829). AW was the recipient of the Royal Australian College of Physicians Fellows Career Development Fellowship. GJ was the recipient of NHMRC Practitioner Fellowship. YW was the recipient of NHMRC Translating Research into Practice Fellowship (APP1168185).

Conflict of interest

GJ received honoraria for talks from BMS, Roche, AbbVie, Amgen, Lilly, Novartis, and Janssen, and grant for a clinical trial from Covance. AT received honoraria for lectures from Pfizer, honoraria for lectures and advisory board participation from Amgen, honoraria for data and safety monitoring board participation from Merck, and honoraria for data and safety monitoring board participation from Novartis.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.939800/full#supplementary-material

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frontiers | Frontiers in Medicine

TYPE Systematic Review
PUBLISHED 23 September 2022
DOI 10.3389/fmed.2022.1024750

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

RECEIVED 22 August 2022
ACCEPTED 02 September 2022
PUBLISHED 23 September 2022

CITATION
Zhao K, Nie L, Chin GMJ, Ye X and Sun P (2022) Association between fat mass and obesity-related variant and osteoarthritis risk: Integrated meta-analysis with bioinformatics. *Front. Med.* 9:1024750. doi: 10.3389/fmed.2022.1024750

Association between fat mass and obesity-related variant and osteoarthritis risk: Integrated meta-analysis with bioinformatics

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Objective: The association of fat mass and obesity-related (*FTO*) gene with osteoarthritis (OA) risk has been investigated in multiple genome-wide association studies but showed inconsistent results. Our study aimed to assess *FTO* expression in different OA sequencing datasets and to meta-analyze whether *FTO* polymorphism was associated with the risk of osteoarthritis.

Method: Gene expression profiles were obtained from ArrayExpress, Gene Expression Omnibus (GEO), and BioProject databases. Three electronic databases including PubMed and EMBASE were systematically retrieved to identify articles exploring the association between *FTO* polymorphisms and OA risk published before September 2022. Summary odds ratios (ORs) and corresponding 95% confidence intervals (95% CIs) were calculated to perform the result. Stata software was utilized to conduct analyses on predetermined ethnicity and gender subgroups and sensitivity.

Results: *FTO* gene was differentially expressed in the datasets from the UK. This systematic review and meta-analysis encompasses eight studies that revealed a significant association between *FTO* polymorphisms and OA risk [OR 1.07, 95% CI (1.03, 1.11), $P < 0.001$] in the overall population. In subgroup analysis, a marked association was observed in European Caucasian [OR 1.08, 95% CI (1.04–1.12), $P < 0.001$] and North American Caucasian with the Asian subgroups [OR 0.98, 95% CI (0.83–1. 6), $P = 0.83$] as an exception. Among the studies, four of them demonstrated attenuation in their OA risk after body mass index (BMI) adjustment in Caucasian populations.

Conclusion: *FTO* significant differential expression was associated with the increased risk of OA in Caucasian populations. Nevertheless, the causality

between *FTO* polymorphisms and OA risk remains largely elusive. Hence, further studies with larger sample size are necessary to validate whether *FTO* gene polymorphism contributes to OA susceptibility.

KEYWORDS

osteoarthritis, *FTO*, polymorphism, meta-analysis, systematic review

Introduction

Osteoarthritis (OA) is the most prevailing form of whole joint degenerative disease characterized by the degeneration of articular cartilage, bone remodeling, synovial inflammation, osteophyte formation, subchondral sclerosis, infrapatellar fat pad, and meniscus injuries, etc. (1). Its prevalence does not cease to escalate due to population aging, prolonged life expectancy and obesity, making the disease a major healthcare problem and socioeconomic burden affecting millions of people worldwide (1). OA is a multifactorial disease as its pathogenesis is an amalgamative effect of environmental factors such as traumatic joint injury and chronic mechanical overloading alongside genetic risk factors such as aging, gender, genetic predisposition, obesity, and inflammation (2). Previous studies frequently associate obesity with an augmented risk of OA, but how it contributes to the onset and progression of OA has not been well-established (3). On the other hand, it has been demonstrated the presence of OA in non-weight-bearing joints of obese subjects and obesity determines a low-grade inflammatory systemic inflammatory status. Thus, it is suggested that other factors other than mechanical loading contribute to the disease (4, 5).

The fat mass and obesity-associated (*FTO*) gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase which is the first well-established obesity-susceptibility gene (6). Recently, several genome-wide association studies (GWAS) explored the relationship between *FTO* gene variation and OA risk (7–9). However, these studies presented incongruent and inconclusive results attributed to the clinical heterogeneity of patients and various single nucleotide polymorphisms (SNP), different ethnic populations, and small sample sizes. In addition, the microarray and RNA-sequencing data provide us the possibility to investigate whether *FTO* is a candidate gene for OA susceptibility. To precisely elucidate the role of the *FTO* gene in the development of OA, we firstly detected the *FTO* expression between OA and normally followed by a comprehensive meta-analysis to determine the association between *FTO* polymorphisms and OA risk.

Method

Search strategy

Microarray and RNA-sequencing data from cartilage samples in OA patients were obtained from ArrayExpress, Gene Expression Omnibus (GEO), and BioProject databases using the search terms "osteoarthritis," and "cartilage." We conducted literature searches of databases which include PubMed, EMBASE to retrieve relevant articles that underlined the associations between *FTO* polymorphisms and OA up to September 1, 2022 with "FTO" AND ("OA" OR "osteoarthritis" OR "arthrosis") as keywords. The search strategy in detail that we performed is illustrated in **Supplementary Tables 1**, **2**. Additionally, the references of related studies were also screened to identify potentially relevant studies. This systematic review and meta-analysis was conducted by adhering to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) reporting guideline (10).

Inclusion and exclusion criteria

Two investigators assessed the retrieved studies independently according to the pre-specified inclusion criteria as follows: studies that (1) case-control or cohort design; (2) evaluated the association between *FTO* gene polymorphism and knee or hip OA, no limitation in single-nucleotide polymorphisms (SNPs) sites; (3) contained genotype data for the calculation of odds ratios (ORs) and 95% confidence intervals (CIs); (4) were written in English. If several articles reported findings for repeated study populations, we only selected the most recent study or the one with the largest sample size. Any disagreements will be solved by discussion to decide for inclusion or exclusion of the study for the meta-analysis.

Data extraction and quality assessment

Two investigators extracted the following information from each eligible study independently: first author, year of

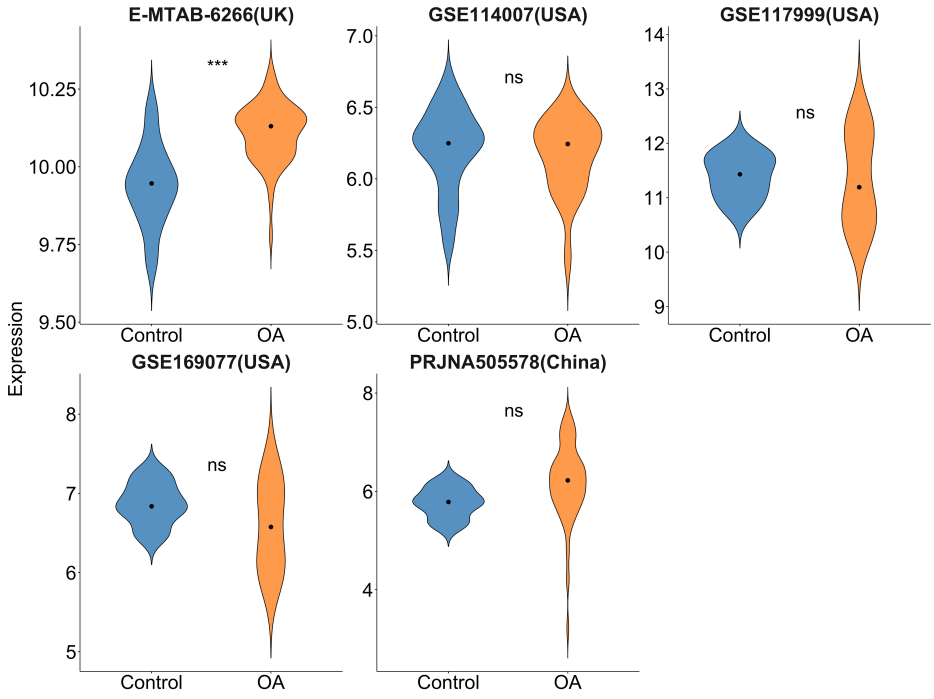

FIGURE 1
Violin plot of *FTO* gene expression in microarray and RNA-sequencing data. Dots mean median. ***$P < 0.001$.

publication, country, ethnic origin of the study population, names of SNPs, type of OA and sample size, age, female proportion of cases and controls.

Two investigators analyzed the methodological quality of each study by applying the Newcastle–Ottawa Scale (NOS), in terms of the selection of study participants, comparability of outcome groups and outcome measures.

Any disagreements will be resolved by discussion until consensus is reached.

Statistical analysis

Microarray datasets were obtained using the "GEOquery" R package (11), and after probe id conversion, the "edgR" R package was used to normalize the data with the CPM (computes counts per million) function (12). RNA sequencing datasets were normalized by applying the variance stabilizing transformation (VST) function from the "DESeq2" R package (13). Mann–Whitney *U* test was utilized to compare the *FTO* expression between the OA group and controls. These computational and statistical analyses were performed using the R software.[1]

The odd ratios (ORs) and 95% confidence intervals (CIs) were estimated by the random effects model (DerSimonian and Laird methods) to evaluate the strength of correlation between *FTO* gene polymorphism and OA risk. Stratification analyses were carried out by ethnicity and gender. $P < 0.05$ was considered statistically significant. Sensitivity analysis was performed by repeating analysis after omitting one study each time to estimate the impact on the overall effects. Heterogeneity was assessed by Q statistic with *P*-value and I^2 statistic (14). Potential publication bias will be examined by Egger's test if more than 10 studies were included (15). These data analyses were performed in Stata 16.0 (Stata Corp, College Station, TX, USA).

Results

Fat mass and obesity-related expression between osteoarthritis and control

A total of 208 records were derived after incipient search. GSE169077 (USA), GSE117999 (USA), GSE11400 (USA), E-MTAB-6266 (UK), and PRJNA505578 (China) datasets were included. Our results (**Figure 1**) revealed that *FTO* demonstrated a significantly increased differential expression ($P < 0.001$) in the UK OA population but not for the USA or Chinese population ($P > 0.05$).

1 https://www.r-project.org/

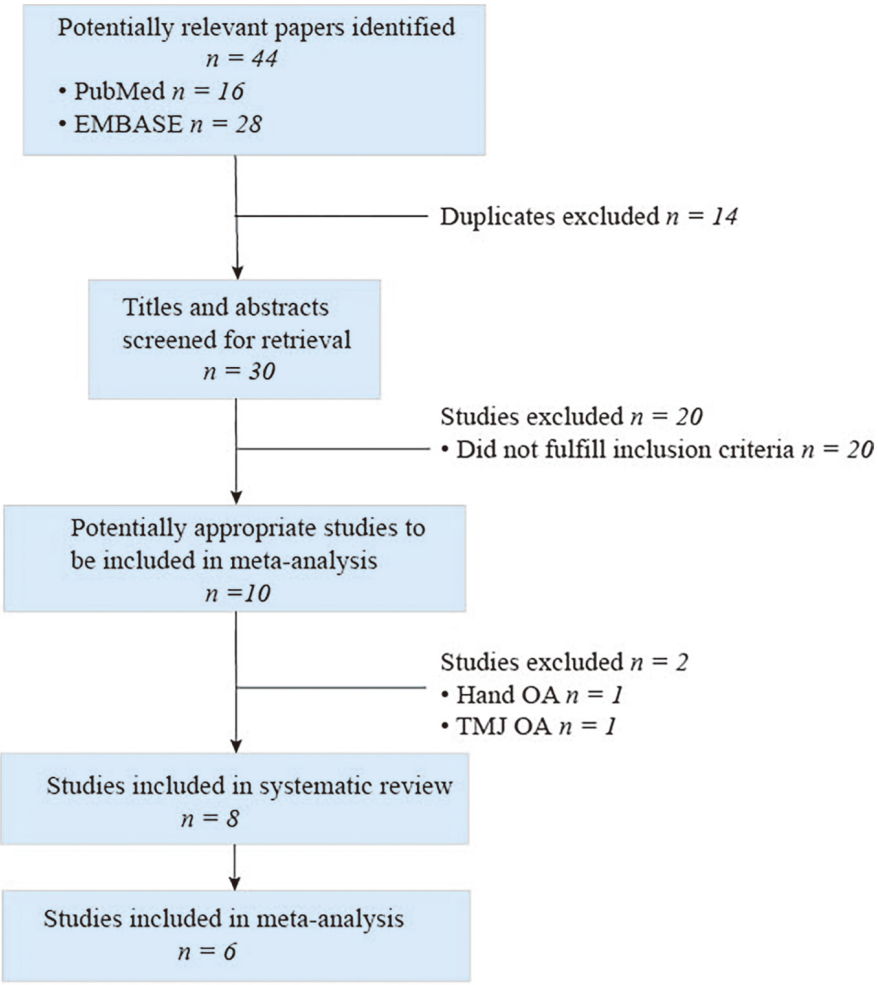

FIGURE 2
Selection for eligible citations included in this systematic review and meta-analysis.

Characteristics of the included studies for meta-analysis

Selection for qualified studies was presented in **Figure 2**. Our initial computerized literature search identified a total of 44 citations. Among these results, 14 records were duplication, and 20 records did not meet our inclusion criteria following a thorough review of the titles and abstracts. Ten citations were retrieved for further full-text review; two out of the 10 studies investigated the association of *FTO* polymorphism with hand or temporomandibular joint (TMJ) OA, respectively. Eventually, we identified eight eligible citations for systematic review (7–9, 16–20) and six studies for meta-analysis (7–9, 16–18). The characteristics and quality of these included studies are summarized in **Table 1**. These available cohort studies were conducted in three countries (number of studies): the UK (2), Finland (1); and China (3) for meta-analysis, and the other two studies synthesized rs8044769 SNP and OA risk in different independent study cohorts (19, 20). Four *FTO* polymorphisms rs8044769 (7, 9, 16, 19, 20), rs12149832 (8), rs9939609 (18), rs1558902 (17) were investigated in this meta-analysis and systematic review. These results of quality assessment were not performed as one studies was from abstract (18) and two studies from several cohorts (19, 20).

Meta-analysis of fat mass and obesity-related gene polymorphism and osteoarthritis risk in all population

In the general analysis, we found that *FTO* gene polymorphism increased OA risk [OR and 95% CI, 1.07 (1.03, 1.11), $P < 0.001$, **Figure 3**] with accept heterogeneity ($I^2 = 48.42\%$). Stratified analysis of ethnicity showed that the risk of OA was considerably elevated by *FTO* polymorphism thein European Caucasian (OR 1.08 [95% CI 1.04–1.12], $P < 0.001$,

TABLE 1 Main characteristics of included studies.

References	Country	Ethnicity	SNP	OA status	Sample size	Case			Sample size	Control			NOS
						Age	BMI	Female		Age	BMI	Female	
Zeggini et al. (7)	UK	Caucasian	rs8044769	Hip, Knee	7,410	/	/	60.4%	11,009	/	/	50.1%	8
Elliott et al. (8)	UK	Caucasian	rs12149832	Hip, Knee, K/L grade ≥ 2	/	/	/	/	/	/	/	/	8
Welling et al. (18)	Finland	Caucasian	rs9939609	Knee	402	/	/	/	5,348	/	/	/	/
Panoutsopoulou et al. (19)	UK and Australia	Caucasian	rs8044769	Hip, Knee, K/L grade ≥ 2	9,764	/	/	/	5,362	/	/	/	/
Wang et al. (9)	China	Asian	rs8044769	Knee, K/L grade ≥ 2	196	62.19 ± 8.76	/	76%	442	57.17 ± 9.19	/	69%	8
Yau et al. (20)	USA	Caucasian	rs8044769	Hip, Knee, K/L grade ≥ 2	3,898	/	/	/	3,168	/	/	/	/
Dai et al. (16)	China	Asian	rs8044769	Knee	890	62.51 ± 11.43	25.76 ± 3.69	75%	844	54.07 ± 11.60	24.91 ± 3.04	20%	8
Li et al. (17)	China	Asian	rs1558902	Knee K/L grade ≥ 1	532	58.1 ± 7.2	23.9 ± 4.1	60%	927	57.5 ± 8.9	23.4 ± 6.3	63%	7

BMI, body mass index (kg/m^2); K/L grade, Kellgren–lawrence (K/l) grading system.

I^2 = 51.67%, Figure 3) but did not reveal a statistically significant rise in Asian (OR 0.98 [95% CI 0.83–1.16] P = 0.83, Figure 3) with low heterogeneity (I^2 = 13.03%). Meanwhile, Yau et al. documented that *FTO* polymorphism (rs8044769) increased OA risk in North American Caucasian (OR 1.10 [95% CI 1.03–1.19], P = 0.00613) (20). Four studies investigated the effect of body mass index (BMI) covariate on the OA outcome, consistent herewith, we observed an attenuation of the OA risk after BMI adjustment in the Caucasian population (7, 8, 18, 19). Nonetheless, we did not discover any solid association between *FTO* polymorphism (rs8044769) and higher BMI in the Chinese population (9, 16).

Meta-analysis of fat mass and obesity-related gene polymorphism and osteoarthritis risk in female population

Four studies investigated the association of *FTO* polymorphism and OA risk in female population, all of which reported results that ascribed the increase in OA risk to *FTO* gene polymorphism [OR and 95% CI, 1.10 (1.04, 1.16), $P < 0.01$, Figure 4]. The ethnic-stratified analysis demonstrated that FTO polymorphisms significantly augmented the OA risk in European Caucasian, with Asian as the exception, which was consistent with the overall population (Figure 4).

Sensitivity analysis and publication bias

The leave-one-out analysis in all populations revealed that no single study changed pooled ORs (Supplementary Table 3), indicating the statistical robustness of our results. Since only six studies were included, we would not carry out the publication bias.

Discussion

To our best knowledge, this is the first meta-analysis that incorporated *FTO* gene expression data to evaluate the association between *FTO* gene polymorphism and OA susceptibility. Our results revealed that *FTO* polymorphism-induced OA risk increase was significant in European Caucasian but not in Asian populations, which is consistent with the results of *FTO* exhibiting significant differential expressions in the UK population but not in Chinese population. The association strength of *FTO* polymorphism and risk of OA attenuated after BMI adjustment in Caucasian population.

Fat mass and obesity-related polymorphism manifests differences between Asian and Caucasian populations. This is possibly owing to the fact that ethnic heritage and geographic

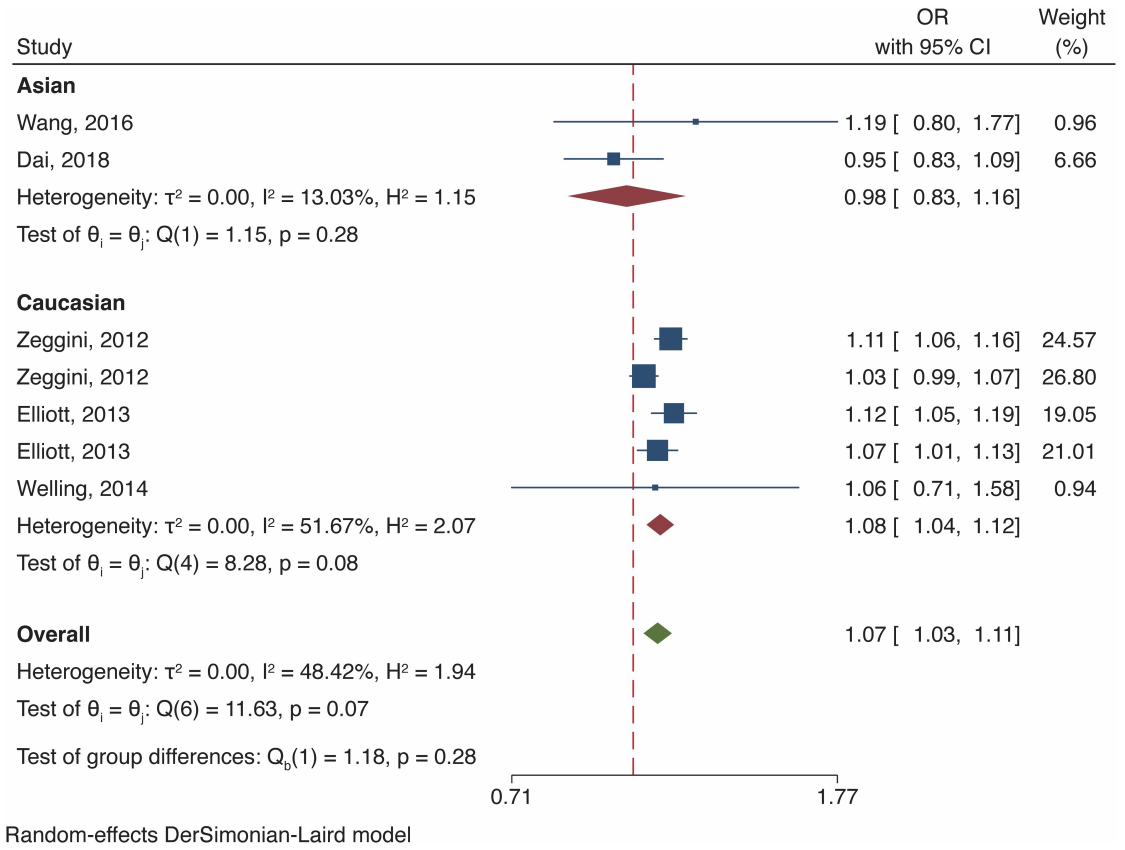

FIGURE 3
Meta-analysis of *FTO* gene polymorphism and OA risk in all population.

localization are major influential factors contributing to genetic polymorphisms, which could render a difference in allele frequency (21). However, this result may also be affected by other confounding factors. The sample size of the Chinese population might not be statistically large enough to reach a convincing conclusion. On the other hand, selection bias in patient enrolment and differences in OA-occurring joint sites could potentially undermine the robustness of the findings. Panoutsopoulou et al. examined the strength of association of rs8044769 with knee or hip OA (adjusted for gender) and detected a distinct association of the FTO variant with knee OA (OR 1.08 [95% CI 1.02–1.14], P = 0.009) rather than hip OA (OR 1.04 [95% CI 0.98–1.11], P = 0.17) in Caucasian populations. For non-weight-bearing joints such as hand and TMJ OA, *FTO* polymorphism also increased the OA risk (22, 23). These findings require further validation in the future with larger-scale observational studies.

Fat mass and obesity-related is an obesity susceptibility gene, but its mechanism on OA is still controversial. Our results showed that the association signal was fully attenuated after BMI adjustment, insinuating the possibility that the *FTO* gene exerts its effect on OA through obesity in the Caucasian population. However, in the Asian population, the relationship between *FTO* gene polymorphism and obesity remained ambiguous. Our results illustrated that there is no solid association between *FTO* polymorphism and higher BMI in the Chinese population, which is contrary to the result of Chang et al. (24). Consistent herewith, the Japanese studies also failed to demonstrate the association of *FTO* polymorphism with obesity or BMI in their population (25–27). Since the Asian population, generally, is lighter than the UK and even more the USA one; it is possible the presence of a bias due to this difference in BMI of the different populations. On the other hand, these may be due to the sample selection bias for study subjects or genetic variants in *FTO*, and further studies are necessary to contemplate the association of *FTO* with BMI and the risk of OA in the Asian population. Meanwhile, more research needs to fill the gaps in the association between *FTO* polymorphism and OA risk in the African population. In addition, FTO plays an important role in N^6-methyladenosine (m^6A) modification. m^6A modification affects the stability and function of RNAs through the "writers," "erasers," and "readers" proteins (28). Several studies reinforced this concept by proving that METTL3

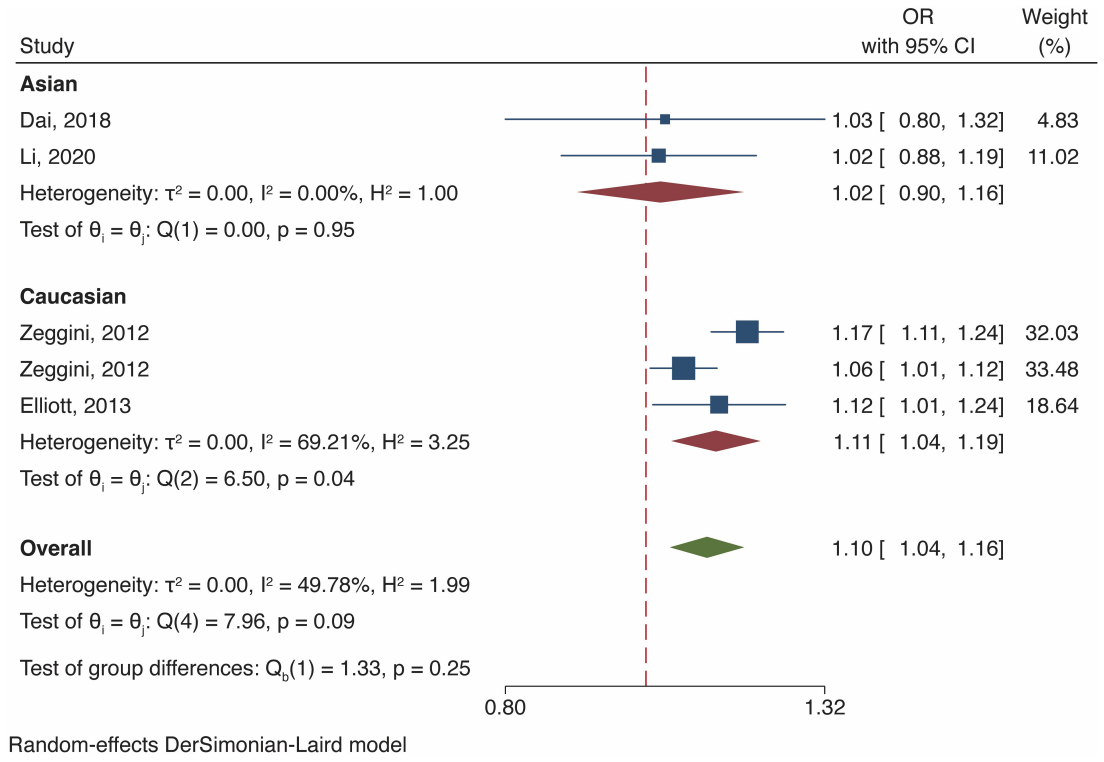

FIGURE 4
Meta-analysis of *FTO* gene polymorphism and OA risk in female population.

which is the "writer" of m^{6}A, could limit OA progression by inhibiting m^{6}A expression (29). Herein, FTO, as the "eraser" of m^{6}A, has the ability to remove the m^{6}A modification. As such, FTO should therefore be fully investigated for its role in the onset and progression of OA.

Nevertheless, there are some limitations in this meta-analysis. First, due to limited data, we were unable to conduct further stratification analyses of other potential risk factors, such as age, type of SNPs, BMI, and OA site. On top of that, we could not perform a meta-analysis using a dominant model or recessive model. Second, some studies shared the study subjects of control group, which may lead to bias in the final results albeit the fact that a sensitivity analysis was conducted. Third, our results were predominantly based on unadjusted estimates for confounding factors, which might have affected the final results. Fourth, the exclusive inclusion of articles written in English but no other languages in this study might have introduced selection bias.

In conclusion, this meta-analysis confirms that *FTO* gene polymorphism increased OA risk. Stratification analysis of ethnicity revealed that the augmented risk of OA due to *FTO* polymorphism may exert its effect through obesity in the Caucasian population. Further studies with larger sample size are necessary to validate whether *FTO* gene polymorphisms contribute to OA susceptibility with an emphasis on studying Asian and African populations.

Data availability statement

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

Author contributions

KZ and PS conceived and designed the meta-analysis. KZ, LN, and PS performed the literature search and analyzed the data. KZ wrote the manuscript and XY revised it. GC polished the language. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Natural Science Foundation of Zhejiang Province, China (Grant No. LQ23H060024) and the Medical Science and Technology Project of Zhejiang Provincial (Grant No. 2022KY555).

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.1024750/full#supplementary-material

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frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 12 October 2022
DOI 10.3389/fmed.2022.992386

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

RECEIVED 12 July 2022
ACCEPTED 16 September 2022
PUBLISHED 12 October 2022

CITATION
Colombini A, Libonati F, Lopa S, Ragni E, De Luca P, Zagra L, Sinigaglia F, Moretti M and de Girolamo L (2022) Immunomodulatory potential of secretome from cartilage cells and mesenchymal stromal cells in an arthritic context: From predictive fiction toward reality. *Front. Med.* 9:992386. doi: 10.3389/fmed.2022.992386

Immunomodulatory potential of secretome from cartilage cells and mesenchymal stromal cells in an arthritic context: From predictive fiction toward reality

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The purpose of the present study is to predict by bioinformatics the activity of the extracellular vesicle (EV)-embedded micro RNA (miRNAs) secreted by cartilage cells (CCs), adipose tissue-derived- (ASCs), and bone marrow-derived stem cells (BMSCs) and verify their immunomodulatory potential supporting our bioinformatics findings to optimize the autologous cell-based therapeutic strategies for osteoarthritis (OA) management. Cells were isolated from surgical waste tissues of three patients who underwent total hip replacement, expanded and the EVs were collected. The expression of EV-embedded miRNA was evaluated with the QuantStudio 12 K Flex OpenArray® platform. Mientournet and ingenuity pathway analysis (IPA) were used for validated target prediction analysis and to identify miRNAs involved in OA and inflammation. Cells shared the expression of 325 miRNAs embedded in EVs and differed for the expression of a small number of them. Mienturnet revealed no results for miRNAs selectively expressed by ASCs, whereas miRNA expressed by CCs and BMSCs were putatively involved in the modulation of cell cycle, senescence, apoptosis, Wingless and Int-1 (Wnt), transforming growth factor beta (TGFβ), vascular endothelial growth factor (VEGF), Notch, Hippo, tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL-1β), insulin like growth factor 1 (IGF-1), RUNX family transcription factor 2 (RUNX2), and endochondral ossification pathways. Cartilage homeostasis, macrophages and T cells activity and inflammatory mediators were identified by IPA as

targets of the miRNAs found in all the cell populations. Co-culture tests on macrophages and T cells confirmed the immuno-modulatory ability of CCs, ASCs, and BMSCs. The study findings support the rationale behind the use of cell-based therapy for the treatment of OA.

KEYWORDS

adipose stem cells, bone marrow stem cells, secretome, miRNAs, early osteoarthritis, immunomodulation, cartilage cells

Introduction

Cell therapy for the treatment of early osteoarthritis (OA) is essentially based on mesenchymal stromal cells (MSCs), mainly adipose tissue-derived (ASCs), and bone marrow-derived stem cells (BMSCs). These cell sources represent the elective choice because adipose tissue and bone marrow are easily harvestable and allow an adequate cell number (1) to be obtained.

The rationale for the use of these cells lies in the fact that they are able to respond to the environment in which they are placed, acting as protagonists of immunomodulation if they have to face antagonists or a hostile microenvironment (2).

Autologous chondrocytes also represent a therapeutic option for the treatment of joint conditions with specific reference to focal chondral lesions (3). However, apart from their regenerative potential, chondrocytes show immunomodulatory abilities (4), likely explaining their clinical effectiveness also in patients with early OA (5–9).

Donor matched cartilage cells (CCs), ASCs and BMSCs were previously compared, in a published paper of our research group, in term of their phenotype and secretory features (10). All the analyzed cell types shared a similar immunophenotype, negative for hematopoietic markers and positive for mesenchymal stromal cell markers, and were able to differentiate into the osteogenic and chondrogenic lineages. Moreover, an exhaustive multiplex-based analysis of the cell secretome revealed that CCs exhibited the largest amount of secreted growth factors overall, with a special presence of chondrogenic, angiogenic, and pro-mitogenic molecules (10).

Characterizing the ideal candidates for cartilage cell therapy in osteoarthritic patients is fundamental to face in the best way the degenerative processes leading to tissue loss, as well as to counteract the inflammatory infiltration that represents a severe issue in an arthritic joint (11). In this regard, immune cells, particularly macrophages (65% of the infiltrate) (12–14) followed by T cells (22% of the infiltrate) (14–17), are recruited from the bloodstream and infiltrate into the synovium, participating to the chronicization of the inflammatory and catabolic processes leading to early OA (18, 19). In particular, macrophages have a multifaceted role in OA and their phenotypic alterations were observed during the pathology development, with M1 (pro-inflammatory) macrophages elevated in synovium and M2 (anti-inflammatory/remodeling) macrophages decreased (20). With respect to T cells, most of those found in the synovial membrane of patients with OA are helper T cells ($CD4^+$), whereas cytotoxic T cells ($CD8^+$) occur sparsely (21–23).

Using cell secretome and in particular extracellular vesicles (EVs) has been also indicated as a potential strategy to counteract OA (24). These vesicles carry proteins, nucleic acids and lipids playing important roles in the intercellular communication and representing useful biomarkers for physio-pathological conditions (25). EV-embedded micro RNAs (miRNAs) are small non-coding RNAs playing important roles in post-transcriptional regulation of biological processes even in cartilage (26). The expression profile of miRNA molecules is exploitable as a tool to have picture of normal and pathological tissues, searching for biomarkers of disease and therapy also in the OA context (27).

Considering the clinical relevance of cell-based therapies in OA treatment, with the aim of identifying the best cell candidate, the objective of the present study is to predict by bioinformatics the activity of the EV-embedded miRNAs secreted by CCs, ASCs, and BMSCs, and involved in OA and verify the immunomodulatory potential of these cells to support our bioinformatics findings. The knowledge of the interaction of these cells and immune cells will help to optimize the autologous cell-based therapeutic strategies for OA management.

Materials and methods

Isolation and expansion of cartilage cells, adipose tissue-derived and bone marrow-derived stem cells

This study was approved by the local Institutional Review Board (M-SPER-015). After patients' informed written consent, articular cartilage harvested from superficial areas of femoral head/neck, bone marrow from femoral channel and subcutaneous adipose tissue from hip fat deposit were

collected from 2 females (53 and 56 y/o) and 1 male (41 y/o) having OA (Kellgren–Lawrence III–IV), who underwent total hip replacement.

Cartilage cells and ASCs were isolated by enzymatic digestion, whereas BMSCs were selected for plastic adherence. All these cell types were characterized, as previously reported (10). Cells were seeded at a density of 5,000 cells/cm^2, detached after 7 days and expanded for 14 days (2 passages). At passage 2 cells were frozen in liquid nitrogen using heat-inactivated FBS added with 10% (v/v) DMSO at concentration of 3–5 × 10^6 cells/vial. After thawing, cells were cultured for other 7 days until passage 3.

Cartilage cells were cultured in high glucose DMEM supplemented with 10% FBS, 200 mM glutamine L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 1 mM sodium pyruvate (all reagents from Thermo Fisher Scientific Waltham, MA, USA). ASCs and BMSCs were cultured in α-MEM supplemented as described above, adding 5 ng/mL fibroblast growth factor 2 (FGF-2) (PeproTech, Rocky Hill, NJ, USA), to preserve their stemness features and proliferative potential (28, 29). Cells were maintained at 37°C, 5% CO_2, and 95% humidity.

Isolation of extracellular vesicles

Cartilage cells, ASCs, and BMSCs at passage 3 and at 90% confluence were washed with phosphate buffered saline (PBS) and serum free medium was added for 48 h. The culture supernatants (30 mL) were collected and differentially centrifuged at 4°C with the following steps: 376 × *g* for 15 min, 1,000 × *g* for 15 min, 2,000 × *g* for 15 min, 4,000 × *g* for 15 min, 4,000 × *g* for 15 min. The cleared supernatants were ultra-centrifuged at 100,000 × *g* for 3 h at 4°C in a 70 Ti rotor (Beckman Coulter, Pasadena, CA, USA) to obtain EVs. EV pellets were suspended in 100 μL PBS, counted and characterized in term of size, shape, and surface marker expression, as previously reported (30–32). Cell viability after 48 h in serum free medium was checked with a NucleoCounter NC-3000 (ChemoMetec, Allerød, Denmark) to verify that culture in serum free medium did not compromise cell viability. CCs showed a viability of 95.1 ± 0.5%, BMSCs of 96.5 ± 2.0%, and ASCs of 97.2 ± 1.4%.

Expression of extracellular vesicles-embedded micro RNA

The EV pellets were dissolved by Trizol and low molecular weight nucleic acids (<200 nt) were obtained with miRNeasy Kit and RNeasy CleanUp Kit (Qiagen, Hilden, Germany). During the extraction, synthetic ath-miR-159a was added as a spike-in to each sample as quality control for the process.

Reverse transcription and pre-amplification were performed to obtain cDNAs to be used as template for Real-Time PCR with the QuantStudio 12 K Flex OpenArray® Platform (QS12KFlex). The Open Array covered 754 human miRNA sequences from the Sanger miRBase v21 Gene, divided into A and B panels.

micro RNA data normalization

The miRNA expression was analyzed by Expression Suite Software (Life Technologies, Carlsbad, CA, USA). The spike-in was used to equalize A and B panels of the Open Array and to balance any technical difference during the process (33). C_{RT} of 27 was considered as a threshold for the presence/absence of amplification. Global mean, calculated from miRNAs amplified in all samples, was the normalization method (34). The relative quantification $2^{-\Delta CRT}$ was used to determine the miRNA expression.

micro RNA target prediction analysis

The bioinformatics Mientournet tool (35)[1] was used for validated target prediction analysis, considering miRTarBase database for experimentally validated miRNA-target interactions. The analysis specifically focused on miRNA embedded in the EVs from CCs, ASCs, or BMSCs.

Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, Redwood City, CA, USA)[2] was used to identify, among all the analyzed miRNAs, the ones experimentally observed as involved in OA. To achieve this, the miRNA Target Filter tool was applied as follows: "experimentally observed" and disease "skeletal and muscular disorders," especially involved in "osteoarthritis pathway," or "inflammatory response."

Relevant pathways for immune response and OA were identified by the IPA tool using the target genes of miRNA of the three cell populations.

Cell modulation of macrophages switch

Human peripheral blood mononuclear cells were isolated by Ficoll (GE Healthcare, Chicago, IL, USA) density gradient separation from 16 buffy coats of healthy donors obtained from the local blood bank. Monocytes were then isolated using CD14 magnetic microbeads (MACS, Miltenyi, Bergisch Gladbach,

1 http://userver.bio.uniroma1.it/apps/mienturnet/

2 www.ingenuity.com

Germany) (36). After isolation, monocytes were counted and frozen in liquid nitrogen using heat-inactivated FBS added with 10% (v/v) DMSO at concentration of 10×10^6 cells/vial.

After thawing, 90×10^6 pooled monocytes were seeded at a density of 2×10^5 cells/cm^2 in RPMI 1640 (Gibco, St. Louis, MO, USA) added with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 200 mM glutamine (Thermo Fisher Scientific, Waltham, MA, USA). The strategy to pool monocytes was used to achieve a suitable cell number for the following tests. To differentiate monocytes into M0 macrophages, 20 ng/mL of macrophage colony-stimulating factor (M-CSF, Peprotech Inc., Rocky Hill, NJ, USA) was added to the medium (37–39). The medium was refreshed every 2/3 days until day 9. In parallel, at day 2, CCs, ASCs, and BMSCs from the three matched donors were thawed and plated on polycarbonate membrane of *trans*-wells (Merck, Darmstadt, Germany) at a density of 0.7×10^5 cells/*trans*-well and left in appropriate expansion medium to favor cell adhesion. At day 7, *trans*-wells seeded with CCs, ASCs, or BMSC were transferred to the macrophage plates. During the co-culture phase, which lasted 2 days, macrophage culture medium and the appropriate expansion medium of CCs, ASCs, and BMSC were combined in a 1:1 ratio. Non-co-cultured M0 macrophages were used as control.

After 2 days of co-culture, the macrophage immunophenotype was analyzed by flow cytometry. Briefly, macrophages were washed with PBS, detached with non-enzymatic cell dissociation buffer (Thermo Fisher, Frankfurt, Germany) and centrifuged at $500 \times g$ for 5 min to collect them.

Macrophages were then suspended in MACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany), treated with FcR Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 min at 4°C to block unwanted binding of antibodies to human Fc receptor and counted. Afterward, 10^5 cells were stained to evaluate the expression of cell surface markers with the following antibodies: anti-human CD80-APC (Clone REA661, Miltenyi Biotec, Bergisch Gladbach, Germany) and CCR7-APC/Fire-750 (Clone G043H7, Biolegend, San Diego, CA, USA) for M1 phenotype, anti-human CD206-FITC (Clone 15–2, Biolegend, San Diego, CA, USA) for M2a phenotype, and anti-human CD163-PE (Clone GHI/61, Biolegend, San Diego, CA, USA) for M2c phenotype. Unstained cells were used as negative control. All the stains were performed at 4°C for 20 min in the dark. Data were acquired using a Cytoflex flow cytometer (Beckman Coulter, Brea, CA, USA) acquiring a minimum of 10,000 events.

Characterization of T cells after co-culture

After isolation by Ficoll, 2×10^5 human peripheral blood cells (PBMCs) were co-cultured with 1×10^5 or 2×10^4 CCs, ASCs, and BMSCs, plated 2 days before. After 4 days of co-culture, PBMCs were collected and stained with monoclonal anti-human CD3-APC antibody (Clone UCHT1, Biolegend, San Diego, CA, USA) for gaiting lymphocytes and with monoclonal anti-human CD4-PE/Cy7 antibody (Clone RPA-T4, Biolegend, San Diego, CA, USA), and monoclonal anti-human CD8-PerCP antibody (Clone SK1, Biolegend, San Diego, CA, USA) to evaluate the ability of cells to modify the $CD4^+/CD8^+$ T cells ratio. Cells were analyzed on a Cytoflex flow cytometer acquiring 10,000 events.

Statistical analysis

To analyze the data obtained from tests on macrophages and T cells, the normality of data distribution was assessed by Kolmogorov–Smirnov test. Unpaired Student's *t*-test was used to compare control cells and co-cultured cells. Significance difference was considered for $p \leq 0.05$. Statistical analysis was performed using GraphPad software (GraphPad Prism v5.00, La Jolla, CA, USA).

Results

Cartilage cells, adipose tissue-derived and bone marrow-derived stem cells share the expression of most micro RNAs

Among all the 428 detected miRNAs, 325 were embedded in the EVs from all the 3 cell populations, 26 were embedded in the EVs from CCs and ASCs, 21 were embedded in the EVs from CCs and BMSCs, and 17 were embedded in the EVs from ASCs and BMSCs. The miRNAs selectively embedded in the EVs from only one cell population were 16 from CCs, 12 from ASCs, and 11 from BMSCs. The list of all the analyzed miRNAs and their C_{RT} in each single cell population is showed in **Supplementary Table 1**.

The lists of miRNAs embedded only in the EVs from each single cell population with target genes were retrieved by setting at least two gene-miRNA interactions (**Supplementary Tables 2–4**) as a threshold. Functional enrichment analysis was conducted by the open-source tools KEGG pathway, Reactome, and Wikipathway (**Figures 1**, **2**). Significant pathways overrepresented within the targets of selected miRNAs are listed and indicated by circles, starting with those that are common to at least two miRNAs. Circles are colored according to the significance of the enrichment and their size is proportional to the number of involved targets. Bioinformatics analysis evidenced that no relevant pathways were modulated by miRNAs embedded only in the EVs from ASCs, whereas some relevant pathways were modulated by miRNAs embedded

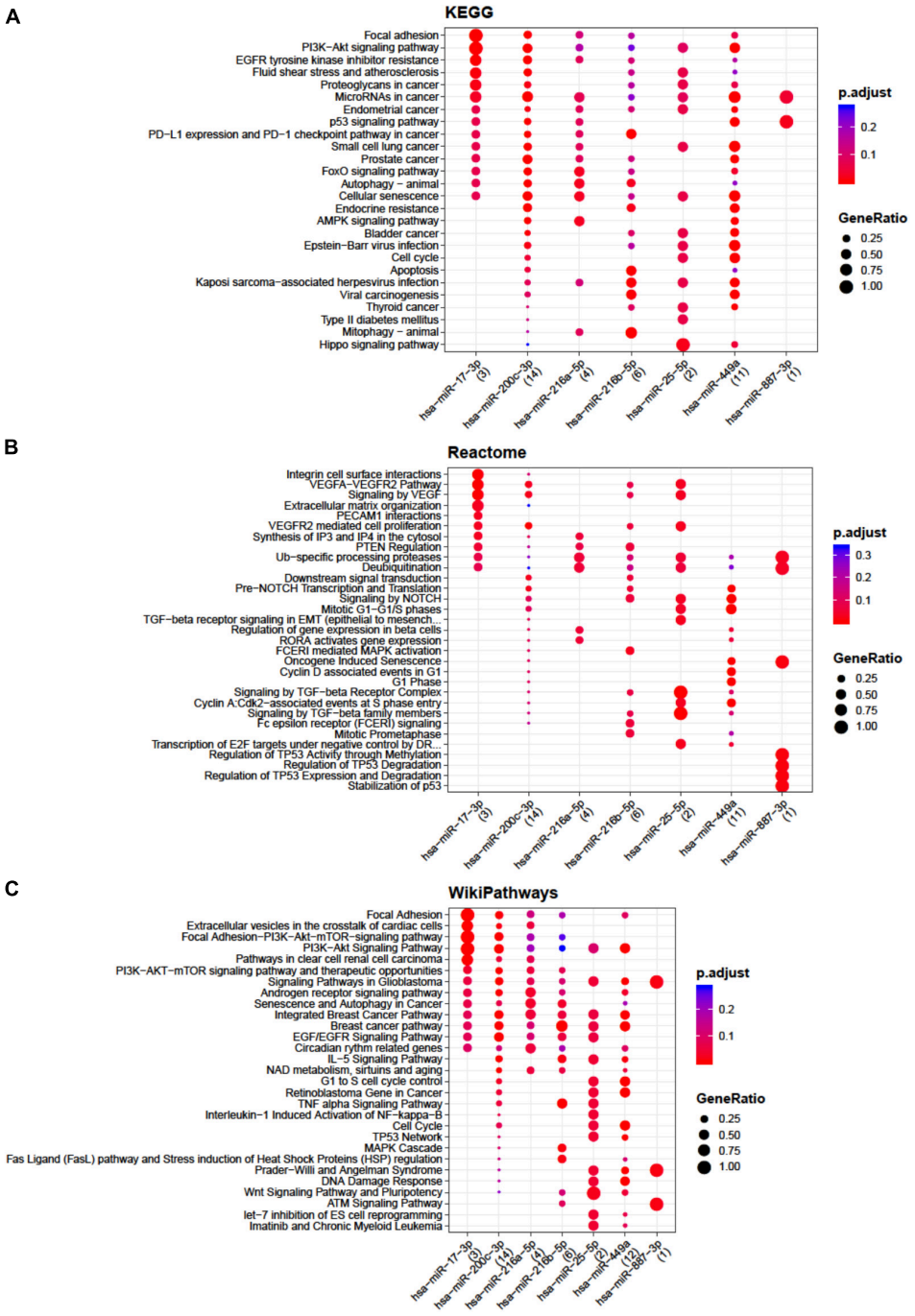

FIGURE 1
Functional enrichment analysis on micro RNAs (miRNAs) selectively expressed by cartilage cells (CCs). This analysis was conducted by KEGG pathway **(A)**, Reactome **(B)**, and Wikipathway **(C)**. Significant pathways are listed and represented by circles colored according to the significance of the enrichment and their size is proportional to the number of target genes regulating the described signaling pathways.

in the EVs from CCs and BMSCs. In particular, for CCs miR-17-3p, miR-25-5p, miR-200c-3p, and miR-449a showed the highest number of interactions (Supplementary Table 2). These miRNAs putatively modulate cell cycle, senescence, apoptosis, Wingless and Int-1 (Wnt), transforming growth factor beta (TGFβ), vascular endothelial growth factor (VEGF), Notch, Hippo, tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β) signaling (Figure 1), potentially related to OA.

Concerning BMSCs, the highest number of interactions was showed by miR-141-3p, miR-143-5p, miR-363-3p, miR-205-5p,

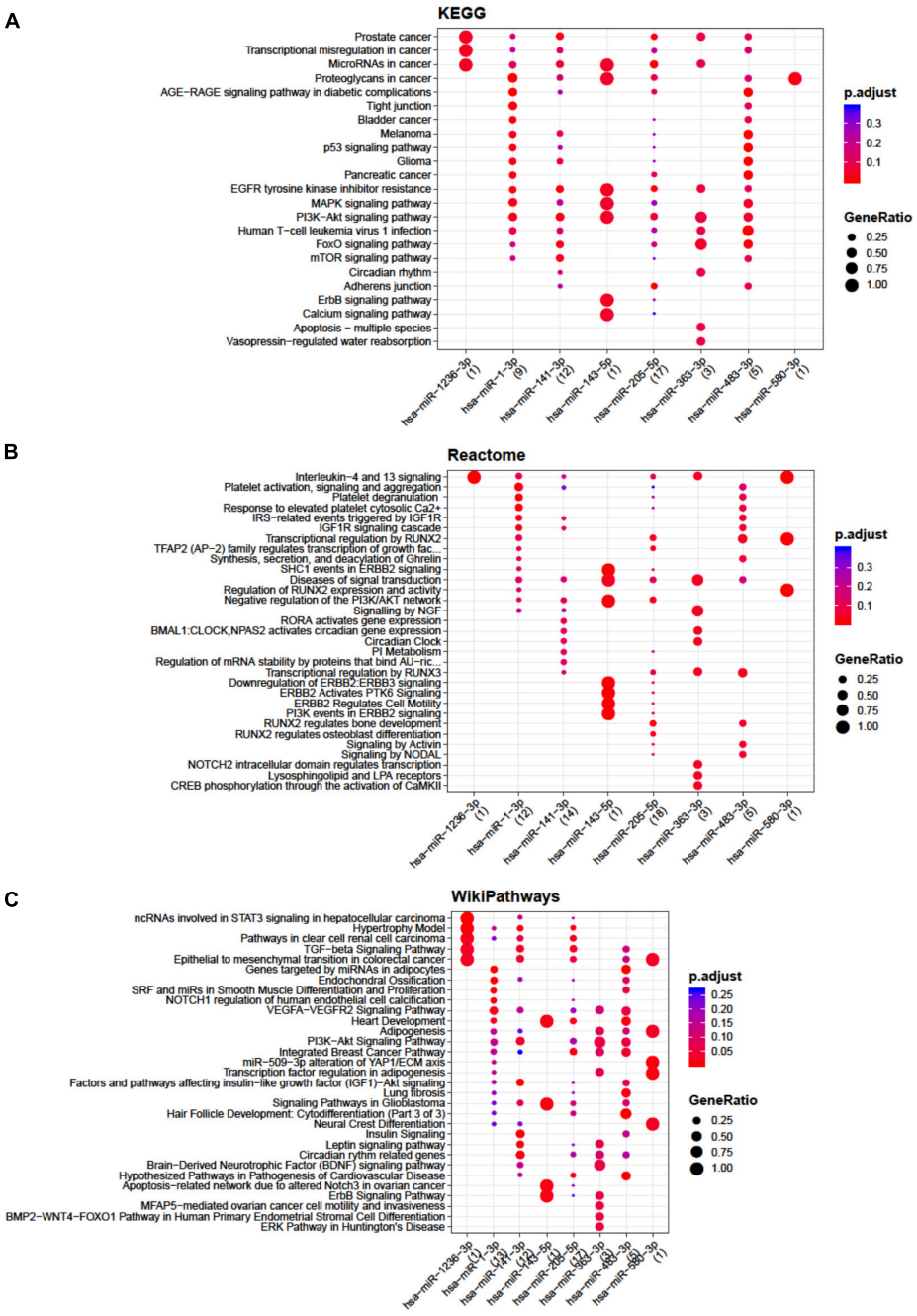

FIGURE 2
Functional enrichment analysis on micro RNAs (miRNAs) selectively expressed by bone marrow-derived stem cells (BMSCs). The analysis was conducted by KEGG pathway **(A)**, Reactome **(B)**, and Wikipathway **(C)**. Significant pathways are listed and represented by circles colored according to the significance of the enrichment and their size is proportional to the number of target genes regulating the described signaling pathways.

and miR-483-3p (Supplementary Table 4) mainly involved in apoptosis, TGFβ, insulin like growth factor 1 (IGF-1), RUNX family transcription factor 2 (RUNX2), and endochondral ossification pathways (Figure 2).

All the miRNAs embedded in the EVs from all the three populations of cells were analyzed by IPA tool to identify their involvement in OA and inflammation (Table 1). None of the miRNAs embedded only in the EVs from CCs was involved

TABLE 1 micro RNAs (miRNAs) in the extracellular vesicle (EVs) from cartilage cells (CCs), adipose tissue-derived (ASCs), and bone marrow-derived stem cells (BMSCs) and their target genes identified as involved in inflammation or osteoarthritis (OA) (target genes in blue) by ingenuity pathway analysis (IPA).

miRNA	Fc vs. ASCs	Fc vs. BMSCs	Target genes
miR-140-5p	**36.6**	**5.4**	**ADAMTS5**, **HDAC4**, IGFBP5, **SMAD3**, and **VEGFA**
miR-302a-3p	**8.4**	**4.0**	**DKK1**, PRKACB, **RELA**, and **VEGFA**
miR-101-3p	**4.0**	**3.7**	**PTGS2**
miR-138-5p	**3.5**	**3.2**	ROCK2
miR-24-3p	**2.1**	**2.0**	ACVR1B, MAP2K4, MAPK14, **NOTCH1**, **SMAD3**, **SMAD4**, and **SMAD5**
miR-126-3p	**2.0**	**2.5**	IRS1, PIK3R2, and **VEGFA**
miR-210	**10.8**	1.7	ACVR1B
miR-532-5p	**9.8**	1.0	RUNX3
miR-196b-5p	**8.9**	0.7	**S100A9**
miR-335-5p	**7.8**	1.0	PTPN11, PXN, RASA1, and SRF
miR-203	**6.9**	1.7	**CREB1**, PRKCA, **RUNX2**, SOCS3, and SRC
miR-21-5p	**4.5**	1.7	ACTA2, **BMPR2**, **JAG1**, PIK3R1, SOCS5, **TGFBR2**, **TIMP3**, and **TNF**
miR-100-5p	**4.3**	1.0	**FGFR3**, IGF1R, and **MTOR**
miR-615-5p	**3.1**	1.3	IGF1R
miR-185-5p	**2.6**	1.0	AKT1, CDC42
miR-186-5p	**2.3**	1.0	FOXO1
miR-130a-3p	**2.0**	1.0	**SMAD4**
miR-1285-3p	0.6	**8.2**	AKT2
miR-487b	1.4	**3.2**	MAP2K4
miR-155-5p	1.9	**2.3**	CCN1, **CEBPB**, **CTNNB1**, **FADD**, MYD88, PRKCI, **SMAD1**, **SMAD2**, SOCS1, and TCF7L2
miR-191-5p	1.9	1.4	IL6
miR-31-5p	1.3	1.6	**CASR**, **HIF1A**
miR-214-3p	1.0	1.0	**ATF4**
miR-34a-5p	0.9	0.6	BCL2, **CREB1**, HDAC1, **JAG1**, MAP2K1, **NOTCH1**, **SIRT1**, and **VEGFA**
miR-29a-3p	1.6	1.3	ACVR2A, CDC42, **HDAC4**, PIK3R1, **SP1**, TGFB3, **TGFBR1**, and **TGFBR2**
miR-26a-5p	1.5	1.4	**PTGS2**, **SMAD1**, and **TGFBR2**
miR-27a-3p	1.8	1.4	**FADD**, FOXO1, GRB2, IGF1, **MEF2C**, **MMP13**, **NOTCH1**, PDPK1, **PPARG**, PXN, **SMAD3**, **SMAD4**, and **SMAD5**
miR-132-3p	0.8	0.7	**MMP9**
miR-22-3p	1.6	1.1	BMP7, SRF
miR-25-3p	1.7	1.2	**BMPR2**, **ITGA5**, **ITGB3**, and MAP2K4
miR-23a-3p	1.8	1.2	**HES1**, **NOTCH1**, **SMAD3**, **SMAD4**, and **SMAD5**
miR-19a-3p	1.1	0.8	**BMPR2**, CCN2
miR-125a-5p	0.6	0.6	BMPR1B, **CASP6**, **CASP7**, ELAVL1, IGFBP3, IL1RN, MYD88, and **SMO**
miR-128	1.7	1.0	**TGFBR1**
miR-491-5p	1.6	1.4	BCL2L1
miR-222-5p	0.8	0.7	ACTA2, ROCK2
miR-18a-5p	1.2	0.6	CCN2, **HIF1A**
miR-199a-5p	0.9	0.7	**HIF1A**, **SIRT1**
miR-15a-5p	1.7	0.9	BCL2, **FGF2**, **FGFR1**, GRB10, GRB2, IFNG, IGF1, IGF1R, **ITGA2**, JUN, MAP2K1, MAP2K4, MAPK3, PANX1, **PTGS2**, RAF1,**VEGFA**, and **WNT3A**
miR-1271-5p	1.4	1.8	FOXO1, IRS1
miR-335-3p	1.5	0.8	**TGFBR1**, **TGFBR2**
miR-139-5p	1.3	0.8	FOXO1, IGF1R, and SHC1
miR-150-5p	1.0	1.0	AKT, **CEBPB**, and **VEGFA**
miR-184	1.2	1.2	AKT2
miR-223-3p	0.8	0.7	IRS1, **MEF2C**

(Continued)

TABLE 1 (Continued)

miRNA	Fc vs. ASCs	Fc vs. BMSCs	Target genes
miR-296-3p	0.8	1.3	**CREB1**
miR-193a-3p	0.8	0.9	PTK2, RPS6KB2
miR-221-3p	**0.4**	**0.3**	**DDIT4**, FOS, **FOXO3**, **MMP1**, PIK3R1, and **TIMP3**
miR-149-5p	**0.3**	**0.5**	RAP1A, RAP1B
miR-218-5p	**0.1**	**0.1**	PIK3C2A, PLCG1, **RUNX2**, and **SP1**
miR-143-3p	**0.5**	**0.1**	BCL2, IGFBP5, KRAS, and MAPK12
miR-181a-5p	**0.2**	**0.1**	BCL2, KRAS, **TIMP3**
miR-197-3p	**0.4**	**0.5**	ACVR1
miR-296-5p	**0.5**	**0.3**	BCL2
miR-503	**0.1**	**0.4**	**FGF2**, **FGFR1**
miR-542-3p	**0.4**	**0.5**	**PTGS2**
miR-145-5p	**0.4**	**0.1**	IGF1R, IRS1, **MMP1**, RASA1, and **SOX9**
miR-124-3p	**0.02**	0.9	CCN2, **GLI3**, **HDAC4**, **HES1**, **ITGB1**, **JAG1**, MAPK14, **PGF**, **RELA**, **SMAD5**, **SOX9**, **SP1**, and STAT3
miR-204-5p	**0.2**	0.6	**ITGB4**, **MMP3**, **MMP9**, SHC1, and **TGFBR2**
miR-146a-5p	**0.3**	0.6	**CHUK**, **CXCL8**, **FADD**, IFNA1/IFNA13, IFNB1, IL10, IL1F10, **IL1R1**, **IL1RAP**, **IL1RAPL2**, **IL1RL2**, IL36A, IL36B, IL36G, IL36RN, IL37, **NOS2**, **TLR4**, and TRAF6
miR-422a	**0.4**	0.6	CASP9, IGF1R, and PDPK1
miR-34b-5p	**0.5**	0.7	**CREB1**, **VEGFA**
miR-142-3p	0.6	**0.3**	BCL2L1, PRKCA
miR-7-5p	1.1	**0.3**	FOS, IRS1, IRS2, p70 S6k, and RAF1
miR-106a-5p	0.7	**0.5**	BCL2, **BMPR2**, **CREB1**, **CXCL8**, JAK1, **MMP3**, **PPARG**, STAT3, **TGFBR2**, **TNF**, and **VEGFA**
miR-193a-5p	0.6	**0.5**	IL10, **MTOR**, and PIK3R3
miR-324-5p	1.2	**0.5**	**GLI1**, **SMO**, and SRF
miR-30a-3p	1.1	**0.5**	CCN1, PIK3C2A
let-7a-5p	**2.6**	**0.5**	BCL2L1, **CASP3**, **ITGB3**, KRAS, NEDD4, NRAS, **PTGS2**, RAS, **TGFBR1**, **TGFBR2**, and **TLR4**
miR-9-5p	**0.2**	**2.3**	FOXO1, JAK1, JAK2, and **NFKB1**
miR-133a	**2.3**	/	CCN2, IGF1R, **RUNX2**, and SRF
miR-486-5p	1.1	/	FOXO1
miR-122-5p	1.7	/	AKT3 MAPK11
miR-200b-3p	0.8	/	PLCG1
miR-135b-5p	/	0.9	JAK2, **RUNX2**, and SMAD5
miRNA	**CRT ASCs**	**CRT BMSCs**	**Target genes**
miR-125b-1-3p	20.9	21.3	IL13, **IL1B**, and **TNF**
miR-129-5p	22.7	22.5	**BMPR2**
miR-141-3p	22.5		**CTNNB1**, **DLX5**, MAP2K4, PITX2, **RAC1**, and TGFB2
miR-18a-3p	25.9	26.2	KRAS
miR-205-5p	18.8		**VEGFA**
miR-219-5p		24.5	PLCG2, **TNFRSF1B**
miR-375	23.8	24.9	JAK2, PDPK1
miR-483-3p	19.3		IGF1, **SMAD4**, and SOCS3

In bold fold change Fc ≥ 2 or ≤0.5 of expression in CCs in comparison with ASCs and BMSCs considered of interest.

in OA. Six miRNAs (miR-140-5p, 302a-3p, 101-3p, 138-5p, 24-3p, and 126-3p) appeared up-regulated in CCs in comparison with both ASCs and BMSCs. Additional 13 miRNAs (miR-210, miR-532-5p, miR-196b-5p, miR-335-5p, miR-203, miR-21-5p, miR-100-5p, miR-615-5p, miR-185-5p, miR-186-5p, miR-130a-3p, let-7a-5p, and miR-133a) were up-regulated in the EVs from CCs in comparison with ASCs, whereas only 4 miRNAs (miR-1285-3p, miR-487b, miR-155-5p, and miR-9-5p) were up-regulated in the EVs from CCs in comparison with BMSCs.

Ten miRNAs (miR-221-3p, miR-149-5p, miR-218-5p, miR-143-3p, miR-181a-5p, miR-197-3p, miR-296-5p, miR-503, miR-542-3p, and miR-145-5p) were down-regulated in the EVs from CCs in comparison with both ASCs and BMSCs. Additional six miRNAs (miR-124-3p, miR-204-5p, miR-146a-5p, miR-422a,

miR-34b-5p, and miR-9-5p) were down-regulated in the EVs from CCs in comparison with ASCs, whereas seven miRNAs (miR-142-3p, miR-7-5p, miR-106a-5p, miR-193a-5p, miR-324-5p, miR-30a-3p, and let-7a-5p) were down-regulated in the EVs from CCs in comparison with BMSCs.

Finally, four miRNAs (miR-125b-1-3p, miR-129-5p, miR-18a-3p, and miR-375) in the EVs from ASCs and BMSCs, three in the EVs from ASCs (miR-141-3p, miR-205-5p, and miR-483-3p), and one in the EVs from BMSCs (miR-219-5p) were involved in OA and not expressed in the EVs from CCs.

Micro RNAs in the EVs from CCs, ASCs, and BMSCs and their target genes identified as involved in inflammation or OA by IPA are reported in Table 1. Starting from target genes retrieved, relevant pathways for immune response and OA were identified by IPA and reported in Figure 3. Briefly, macrophages and T cells as actors and pro- and anti-inflammatory cytokines and mediators were identified as modulated by target genes of miRNAs of interest for what concerns immune response and signaling. Finally, cartilage homeostasis and cell proliferation-related pathways involved in OA modulated by the identified target genes were observed.

Cartilage cells, adipose tissue-derived and bone marrow-derived stem cells potential in macrophage polarization

Cartilage cells, ASCs, and BMSCs co-cultured with macrophages shared the ability to promote the increase of the $CD206^+$ M2a anti-inflammatory macrophages (1.6, 1.2, and 1.4-fold increase, respectively; $p < 0.0005$ for CCs and $p \leq 0.05$ for MSCs), Figure 4A. ASCs showed a decrease ($p < 0.05$) of $CD163^+$ M2c remodeling macrophages (Figure 4B) and of $CD80^+$ M1 inflammatory macrophages (Figure 4C).

Cartilage cells, adipose tissue-derived and bone marrow-derived stem cells modulation of T cell survival and phenotypes

The co-culture of all the three cell types and PBMCs showed a decrease in the $CD3^+$ T lymphocytes survival (Figure 5A, $p < 0.01$ for CCs and ASCs and $p < 0.05$ for BMSCs). When looking at T cell phenotype, a decrease in $CD4^+$ T cells survival was observed in CCs and ASCs ($p < 0.05$) (Figure 5B). On the contrary, $CD8^+$ T cells percentage increased in presence of CCs and ASCs ($p < 0.01$) (Figure 5C).

Discussion

The main findings of the present investigation show that there was a similar basal expression of EV-embedded miRNAs in the CCs and both types of MSCs. In general, cartilage homeostasis-related pathways are identified as targets of the miRNAs found in all the cell populations. Interestingly, macrophages and T cells are actors potentially modulated by

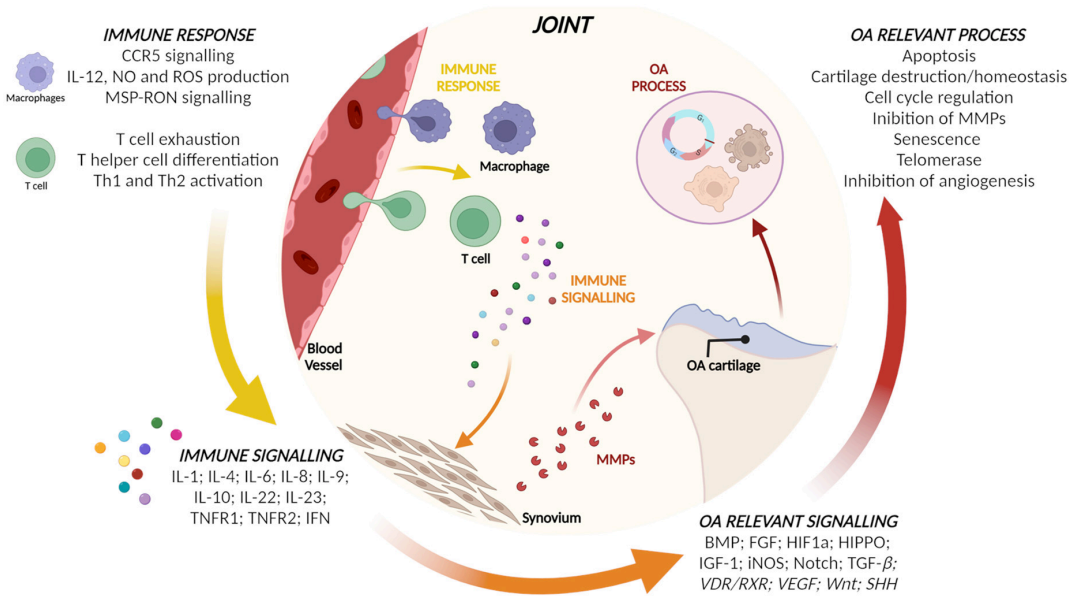

FIGURE 3
Relevant pathways for immune response and osteoarthritis (OA) identified by ingenuity pathway analysis (IPA) using the target genes of micro RNAs (miRNAs) of the three populations.

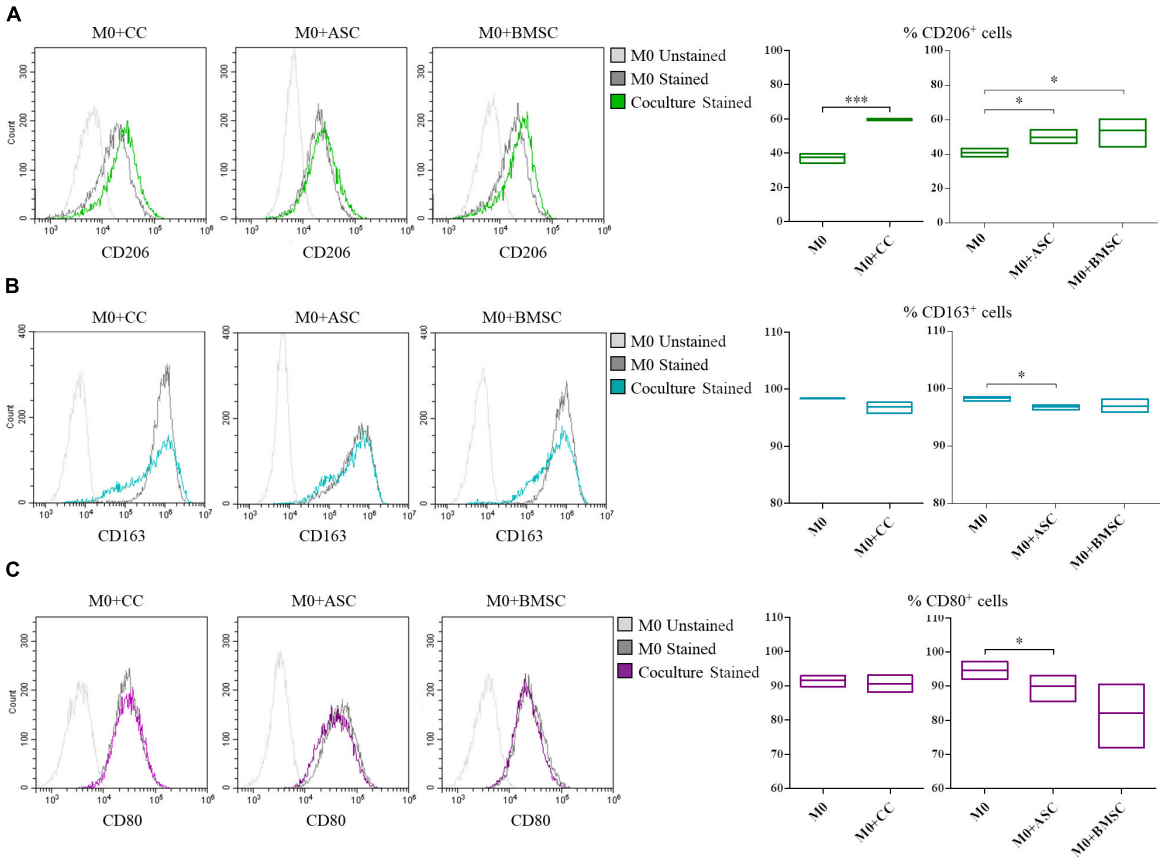

FIGURE 4
Modulation of M0 macrophages polarization by cartilage cells (CCs), adipose tissue-derived (ASCs), and bone marrow-derived stem cells (BMSCs). For $CD206^+$ **(A)**, $CD163^+$ **(B)**, and $CD80^+$ **(C)** cells representative plots overlay show M0 co-cultured with the different cell types. Floating bars represent mean to max percentages and mean values of cells positive for the different analyzed markers in M0 and co-cultured M0 with CCs, ASCs, and BMSCs. $N = 3$; $^*p < 0.05$, $^{***}p < 0.0001$.

miRNAs in the EVs from all the three cell types. Donor-matched CCs and MSCs behave similarly in the modulation of the anti-inflammatory macrophages polarization and of the T cell survival and phenotypes. Each cell population presents specific properties to be exploited to treat early OA. Of the 3 cell populations, ASCs seem to be the most anti-inflammatory, but the least pro-remodeling ones, whereas CCs and BMSCs show a more prominent ability of inflammatory modulation and remodeling, toward the restoration of tissue homeostasis. This aspect is reasonable also considering the affinity of the CCs with the cartilage and of the BMSCs with the subchondral bone. On the other side, ASCs would be more useful to counteract the immune cells when placed in an inflammatory microenvironment. Finally, because these cells showed immunomodulatory ability, their potential use to facilitate the resolution of OA induced inflammation and cartilage regeneration should be advised.

Our data are in line with those reported by previous publications. In co-culture with non-polarized macrophages, CCs have already shown an enhancement in maturation (19, 40–43). Furthermore, CCs were able to shift the M1 macrophage phenotype toward the M2-like phenotype (43) and to modulate pro-inflammatory macrophage activity by reducing MHC-II expression and TNF-α secretion (44). The anti-inflammatory and pro-regenerative interaction of CCs and macrophages is particularly clinically relevant for the achievement of the joint homeostasis in a patient who undergoes cell therapy in an OA context.

In this study, we also observed the MSC ability to modulate macrophage polarization. Other scientific reports analyzed the ability of ASCs in co-culture with synovial cells to interact with macrophages, showing that the amount of synovial macrophages differently induced or down-modulated inflammatory and degradative factors (45). BMSCs previously showed immunomodulatory ability in co-culture with macrophages. These cells promoted an anti-inflammatory phenotype of macrophages, assessed in term of decrease in M1-cytokines (TNFα and IL-1β) (46, 47) and increase of

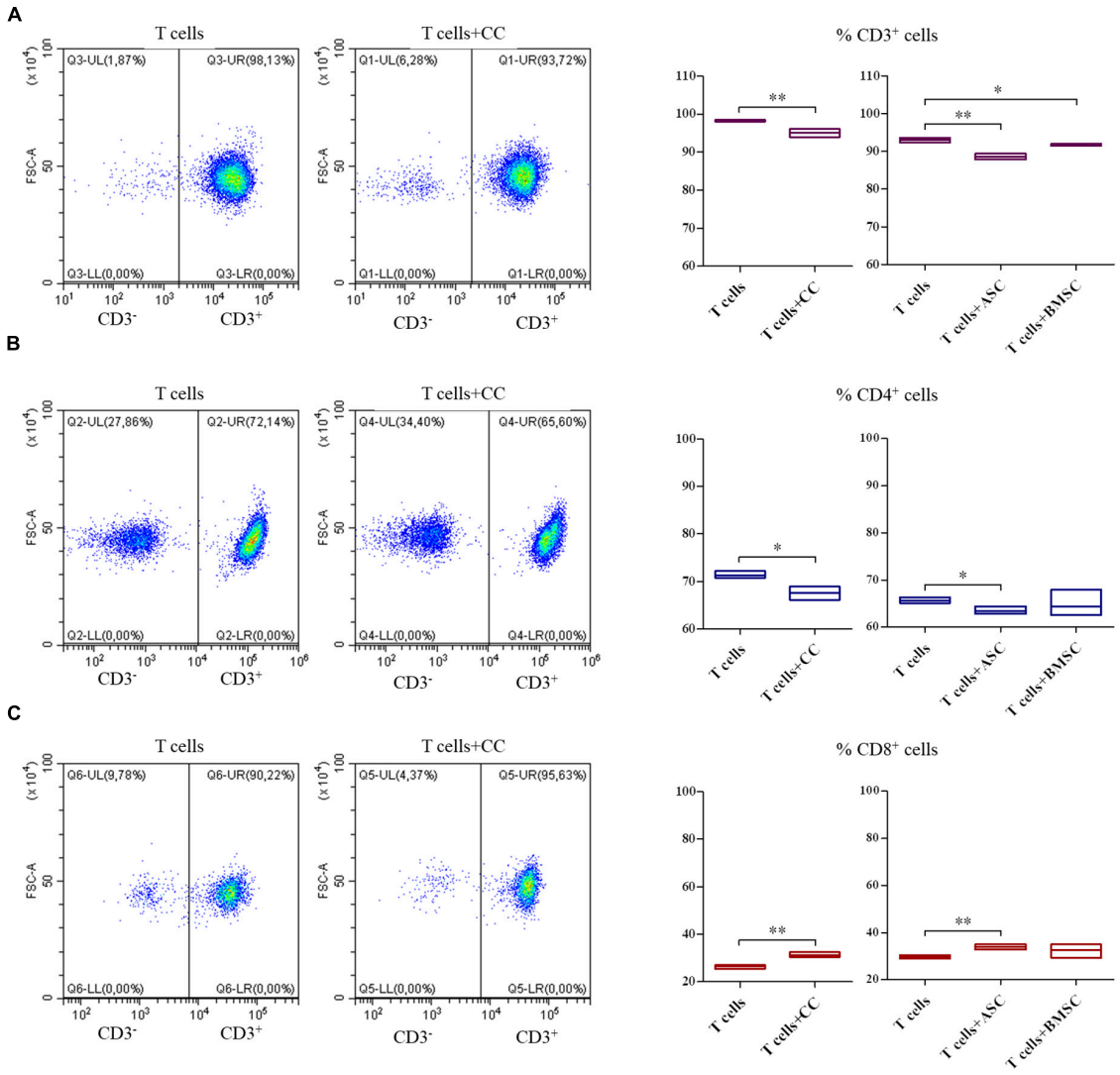

FIGURE 5
Modulation of T cell survival and phenotype by cartilage cells (CCs), adipose tissue-derived (ASCs), and bone marrow-derived stem cells (BMSCs). For $CD3^+$ **(A)**, $CD4^+$ **(B)**, and $CD8^+$ **(C)** cell representative density plots show T cells co-cultured with CCs. Floating bars represent mean to max percentages and mean values of cells positive for the different analyzed markers in T cells and co-cultured T cells with CCs, ASCs, and BMSCs. $N = 3$; $^*p < 0.05$, $^{**}p < 0.001$.

M2-cytokines (CCL17, CCL22) secretion (47) or of increase in the frequency of M2-macrophages (48).

With respect to T cells, CCs already showed a reduced induction of proliferation and activation in allogeneic T cells and a potent ability to suppress allogeneic T cell proliferation, which was dependent on nitric oxide production (44).

In our study, $CD4^+$ helper T cells did not proliferate, whereas $CD8^+$ cytotoxic T cells showed higher survival when co-cultured with CCs and ASCs. A possible reason for this response could be the differential expression on CCs and ASCs of MHC class II and I, presenting to helper and cytotoxic T cells, respectively. In fact, these cells showed the expression of MHC class I, but not of MHC class II (49–51).

These results are interesting since lymphoid cell aggregates, containing primarily $CD3^+$ T lymphocytes, were found in the synovial membrane of the 65% of patients with OA (52) and the most prevalent T cells type found in the synovium were T helper cells ($CD3^+$, $CD4^+$, and $CD8^-$) (23, 53), acknowledged as having a pivotal role in the pathogenesis of OA (54). Cytotoxic/suppressor T cells occur sparsely and are not the predominant T cell type in the synovial aggregates of OA patients (21). Nevertheless, these cells likely shape the pathogenesis of OA, although they do not play the most important role in this disease (55). Considering these observations, the lack of promotion of $CD4^+$ and the slight promotion of the $CD8^+$ T cell survival mediated by CCs and

ASCs reflects a non-immunogenicity of these cells. This is particularly interesting for $CD4^+$ cells, which are considered predominant and active in OA infiltrates, whereas the data reported for $CD8^+$ cells should be better evaluated when their role in OA pathophysiology will be better elucidated.

The main limitation of the present study resides in the fact that, as with all the co-culture *in vitro* tests, these present the intrinsic limitation of being based on pre-established models. These models are unable to faithfully represent the dynamic OA pathophysiology. Nevertheless, if compared with *in vitro* models exploiting the conditioned medium to mimic the inflammatory status, the use of co-culture tests with more cell types allows at least in part overcoming this limitation. In fact, co-culture tests add complexity and dynamicity to classical *in vitro* tests, allowing for exploration of crosstalk between cells sharing the same environment. Another limitation is the number of donors used in this study. The results need to be confirmed in a wider population, but are useful to restrict the biological fields of investigation in the OA context.

Conclusion

In conclusion, this study is a proof-of-concept to support the idea that bioinformatics data need to be validated by means of co-culture tests, establishing in reality which pathways are activated by cell crosstalk and how much these pathways contribute in the overall outcome in the context of a biological process. In fact, although data derived by gene, miRNA and protein arrays are a precious mine of information, they can only provide an idea of the processes modulated by the cells. But, when these cells are placed in specific contexts, being competent, they respond to the stimuli they find in the environment, in turn producing mediators that stimulate both the cells themselves and those with which they interact. As a consequence, co-culture tests represent an essential step in the investigation of the effect of cell therapy in the modulation of a biological process, while the analysis of the expression of genes, miRNAs and proteins should be the tool that allows defining a modulation in a pro- or anti-inflammatory sense. Using this approach, we found that CCs, ASCs, and BMSCs are able to modulate macrophage phenotype and *T* cell survival, by promoting a general anti-inflammatory environment without inducing an inflammatory response mediated by immune cells, which usually infiltrate the synovial membrane of OA patients.

Data availability statement

The data presented in this study are deposited in the OSF repository, accession number: https://osf.io/y96rc/?view_only=e96afba950ec469bb7cfea1162aa5672.

Author contributions

AC: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing. FL, ER, PD, and FS: collection and/or assembly of data and final approval of the manuscript. SL: collection and/or assembly of data, data analysis and interpretation, and final approval of the manuscript. LZ: provision of the study material or patients and final approval of the manuscript. MM and LG: financial support and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work reported in this publication was funded by the Italian Ministry of Health, RCR-2021-23671217 project, under the Italian Musculoskeletal Apparatus Network RAMS.

Acknowledgments

We are grateful to Giovanna Chiorino for the support in the bioinformatics analysis of the data and Joshua Harliono for language editing.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.992386/full#supplementary-material

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TYPE Original Research
PUBLISHED 11 November 2022
DOI 10.3389/fmed.2022.990569

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

RECEIVED 15 July 2022
ACCEPTED 25 October 2022
PUBLISHED 11 November 2022

CITATION
Wang Z, Liu M, Zhou Y, Shao H, Yang D, Huang Y and Deng W (2022) Effect of blood lipids and lipid-lowering therapies on osteoarthritis risk: A Mendelian randomization study. *Front. Med.* 9:990569. doi: 10.3389/fmed.2022.990569

Effect of blood lipids and lipid-lowering therapies on osteoarthritis risk: A Mendelian randomization study

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Background: We aimed to investigate the effects of blood lipids and lipid-lowering agents on osteoarthritis (OA) risk.

Materials and methods: We performed Mendelian randomization (MR) analyses to estimate the causal effect of blood low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) levels on knee and hip OA. Single nucleotide polymorphisms (SNPs) were selected from large genome-wide association studies (GWASs) of individuals of European ancestry as genetic instruments for blood lipid levels. The associations of selected genetic instruments with knee and hip OA were estimated in a recent GWAS of the UK Biobank and arcOGEN datasets. Univariate and multivariate MR analyses were performed to detect and adjust for potential pleiotropy. Furthermore, genetic instruments in *HMGCR*, *NPC1L1*, and *PCSK9* regions were used to mimic LDL-C-lowering effects of statin, ezetimibe, and evolocumab, respectively.

Results: Genetically determined LDL-C increments led to reduced risks of both knee OA (OR = 0.91 per 1-SD increment, 95% CI: 0.86–0.95, $P = 6.3 \times 10^{-5}$) and hip OA (OR = 0.92, 95% CI: 0.85–0.99, $P = 0.027$). Multivariate MR analysis proved that the effect was independent of HDL-C, TG, and body mass index. TG increment was associated with reduced risks of hip OA in the univariate MR analysis; however, this was not supported by the multivariate MR analysis. Genetically proxied LDL-C-lowering effects of statins are related to increased risks of knee OA but not hip OA.

Conclusions: The findings suggested that LDL-C increments have independent protective effects on both knee and hip OA. LDL-C-lowering effects of statins may increase the risk of knee OA.

KEYWORDS

osteoarthritis, statins, Mendelian randomization, ezetimibe, blood lipid

Introduction

Osteoarthritis (OA) is the most common form of arthritis; it affects more than 5% of people worldwide, and its prevalence is growing (1). OA is characterized by articular cartilage degeneration, chronic pain, joint deformities, and eventual disability (2). Although the etiology of OA is not well-understood, it is considered a metabolic syndrome-associated disease rather than a purely age- or weight-related disease (3). Experimental studies and a recent meta-analysis of observational studies showed that dyslipidemia is involved in OA pathophysiology (4, 5). However, the causal effect of blood lipid profile, particularly low-density lipoprotein cholesterol (LDL-C), on the risk of developing OA remains unclear.

Statins, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), are the most frequently prescribed cholesterol-lowering drugs. These drugs are recommended as first-line therapy to reduce the risk of atherosclerotic cardiovascular disease (ASCVD) (6). To date, the effects of statins on OA have aroused great interest from researchers; however, results are conflicting and vary from reduced risk to no effect or even an increased risk of OA (3, 7). Nevertheless, the current evidence is limited to observational studies and is inevitably affected by confounding factors, making it difficult to clarify the causal relationship. In recent years, in addition to statins, newer lipid-lowering agents acting on different mechanisms, including ezetimibe or anti-proprotein convertase subtilisin/kexin type 9 (PCSK9) monoclonal antibodies (evolocumab), have been widely used to achieve LDL-C targets in the secondary prevention of ASCVD (8). However, the effects of these drugs on OA have not been reported.

Therefore, we aimed to use a Mendelian randomization (MR) approach to investigate the effect of blood lipid profiles and lipid-lowering agents on OA risk. Because MR employs genetic variants associated with the target of cholesterol-lowering agents, which are random with respect to potential confounding factors, our study will help clarify the causal relationship among blood lipids, lipid-lowering agents, and OA.

Materials and methods

This study was performed according to the guidelines of the Strengthening the Reporting of Observational Studies in Epidemiology using Mendelian Randomization (STROBE-MR) (9).

Genetic instruments for blood lipids

Single nucleotide polymorphisms (SNPs) for plasma LDL-C, high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) were selected as instrumental variables. Data on these variables were obtained from the genome-wide association studies (GWASs) of the Global Lipids Genetics Consortium (10), which included 188,578 individuals with European ancestry and excluded those receiving lipid-lowering treatment. The included SNPs need to be significantly associated with the trait at the genome-wide level ($P < 5 \times 10^{-8}$) and independent of each other ($r^2 < 0.001$). Among the included SNPs, 81, 89, and 55 were associated with LDL-C, HDL-C, and TG, respectively (Supplementary Tables S1–S3).

Assessment of knee and hip OA

The associations between the selected genetic instruments and knee and hip OA were estimated in a recent GWAS meta-analysis of the UK Biobank and Arthritis Research UK Osteoarthritis Genetics (arcOGEN) datasets (77,052 cases and 378,169 controls) (11), which also included individuals with European ancestry. The UK Biobank is a cohort based on 22 assessment centers in the UK that includes 500,000 participants aged 40–69 years and recruited from 2006 to 2010 (12). The diagnosis of hip and knee OA was based on self-reports and hospital records in the UK Biobank. The arcOGEN dataset includes unrelated UK-based knee and hip OA cases from the ArcOGEN Consortium (13). Knee and hip OA were diagnosed if the individual underwent total joint replacement or had radiographic evidence of OA (Kellgren–Lawrence grade $\geq$2).

Two-sample MR

We conducted two-sample MR analysis using the "TwoSampleMR" package in the R software (version 4.1.2). First, we performed a harmonization process to ensure that the effect alleles of SNPs were the same for exposure and outcome. Palindromic SNPs were aligned if the minor allele frequency was <0.3. As a result, two LDL-C SNPs and three HDL-C SNPs were excluded because they were palindromic with intermediate allele frequencies. To estimate the individual effect of each SNP, the Wald ratio was calculated by dividing the SNP-outcome association by the SNP-exposure association. We primarily estimated the causal effect of blood lipids on OA using the random-effect inverse variance-weighted (IVW) method. Estimates of causal effects were reported as odds ratios (ORs) per one standard deviation (SD) increase in LDL-C, HDL-C, and TG. The weighted median, MR-Egger regression, weighted mode, and Mendelian Randomization Pleiotropy RESidual Sum and Outlier (MR-PRESSO) outlier-corrected methods were used for additional sensitivity analyses. The weighted median method can provide an unbiased estimate

TABLE 1 Univariate Mendelian randomization analysis results.

Exposure	Outcome	Methods	Number of SNPs	OR (95% CI)	*P*-value
LDL-C	Knee OA	Inverse variance weighted	79	0.91 (0.86–0.95)	6.3×10^{-5}
		MR Egger	79	0.92 (0.86–0.99)	0.022
		Weighted median	79	0.91 (0.85–0.97)	0.003
		Weighted mode	79	0.90 (0.85–0.96)	9.4×10^{-4}
		MR-PRESSO outlier-corrected	79 (1 outlier SNP)	0.91 (0.87–0.95)	1.3×10^{-4}
LDL-C	Hip OA	Inverse variance weighted	79	0.92 (0.85–0.99)	0.027
		MR Egger	79	0.89 (0.79–0.99)	0.038
		Weighted median	79	0.90 (0.83–0.97)	0.006
		Weighted mode	79	0.90 (0.84–0.96)	0.003
		MR-PRESSO outlier-corrected	79 (3 outlier SNPs)	0.91 (0.85–0.97)	0.006
HDL-C	Knee OA	Inverse variance weighted	86	0.99 (0.90–1.09)	0.835
		MR Egger	86	1.10 (0.93–1.30)	0.277
		Weighted median	86	0.99 (0.90–1.08)	0.793
		Weighted mode	86	1.05 (0.96–1.14)	0.309
		MR-PRESSO outlier-corrected	86 (6 outlier SNPs)	1.01 (0.94–1.09)	0.771
HDL-C	Hip OA	Inverse variance weighted	86	1.00 (0.92–1.08)	0.968
		MR Egger	86	1.08 (0.93–1.26)	0.313
		Weighted median	86	1.13 (1.02–1.25)	0.025
		Weighted mode	86	1.09 (0.98–1.22)	0.112
		MR-PRESSO outlier-corrected	86 (2 outlier SNPs)	1.02 (0.95–1.10)	0.585
TG	Knee OA	Inverse variance weighted	55	0.94 (0.86–1.02)	0.135
		MR Egger	55	0.96 (0.84–1.11)	0.584
		Weighted median	55	0.88 (0.80–0.96)	0.005
		Weighted mode	55	0.93 (0.86–1.02)	0.136
		MR-PRESSO outlier-corrected	55 (3 outlier SNPs)	0.93 (0.87–1.00)	0.072
TG	Hip OA	Inverse variance weighted	55	0.91 (0.84–0.98)	0.017
		MR Egger	55	0.91 (0.80–1.03)	0.149
		Weighted median	55	0.91 (0.81–1.02)	0.106
		Weighted mode	55	0.89 (0.80–1.00)	0.053
		MR-PRESSO outlier-corrected	55 (0 outlier SNPs)	0.91 (0.84–0.98)	0.021

OA, Osteoarthritis; SNP, single nucleotide polymorphisms; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; MR-PRESSO, Mendelian Randomization Pleiotropy RESidual Sum and Outlier.

of the causal effect even if half of the SNPs exhibit pleiotropy (14). The MR-Egger method adds a non-zero intercept to allow directional horizontal pleiotropy (15). It makes the assumption that horizontal pleiotropic effects are independent of SNP-exposure effects, which is also known as the InSIDE assumption. In addition, the MR-Egger regression intercept value was used to estimate the degree of horizontal pleiotropic effects. The weighted mode method groups SNPs according to the similarity of their effects and estimates the causal effect based on the largest cluster of SNPs. Therefore, it can provide an unbiased causal effect estimate as long as the largest cluster of SNPs is valid (16). The MR-PRESSO method can reduce the heterogeneity of the estimate by correcting the effects of outlier SNPs (17). We conducted MR-PRESSO analysis using the MR-PRESSO R package and set the number of distributions to 10,000 and the significance threshold to 0.05. For additional sensitivity analyses, we used forest plots for visual inspection of potential pleiotropy. Cochran's Q statistics were calculated to assess the extent of heterogeneity, with *P*-values < 0.05 indicating significant heterogeneity. The causal direction between exposure and outcome was determined using the Steiger test, which compares the extent of outcome variance and exposure variance explained by instrumental variables (16).

Multivariable MR

As the included instrument SNPs may be associated with multiple lipid fractions and body mass index (BMI), we

performed multivariable MR to estimate the independent effect of each lipid. Multivariable MR analysis was conducted using the IVW method. Instrumental SNPs for BMI were selected from a GWAS meta-analysis of European ancestry conducted by the Genetic Investigation of ANthropometric Traits (GIANT) Consortium (18) (Supplementary Table S4).

Estimating the effect of lipid-lowering therapy on OA risk

Three sets of SNPs within the *HMGCR*, Niemann-Pick C1-Like 1 (*NPC1L1*), and *PCSK9* genes were used to mimic the LDL-C-lowering effect of statins, ezetimibe, and evolocumab, respectively, as used in previous studies (10, 19–23) (Supplementary Table S5). Because some SNPs were not completely independent (r^2 value for linkage disequilibrium <0.3), we estimated the causal effect of lipid-lowering therapy on OA using the random-effect IVW method that accounted for the correlation among variants, provided by the Mendelian Randomization R package (24). The linkage disequilibrium matrix for SNPs was extracted from the European 1,000 genome data (25).

Results

Causal effects of blood LDL-C on OA risk

IVW MR suggested that LDL-C increment was associated with reduced risks of knee OA (OR = 0.91, 95% CI: 0.86–0.95, $P = 6.3 \times 10^{-5}$) and hip OA (OR = 0.92, 95% CI: 0.85–0.99, $P = 0.027$). The MR-Egger, weighted median, weighted mode, and MR-PRESSO outlier-corrected methods yielded similar results (Table 1). The Cochrane Q statistic suggested significant heterogeneity (knee OA, Q = 113.77, $P = 0.005$; hip OA, Q = 180.24, $P < 0.001$). The MR-Egger regression showed no evidence of horizontal pleiotropy for knee OA (Egger intercept = −0.001, $P = 0.589$) or hip OA (Egger intercept = 0.003, $P = 0.410$) (Figures 1A,D). Visual inspection of the funnel plots revealed no signs of horizontal pleiotropy (Supplementary Figures S1, S2). The MR-PRESSO analysis identified one outlier and three outliers for knee and hip OA, respectively. However, these outliers did not influence the effect estimates for knee OA (MR-PRESSO distortion test *P*-value = 0.846) or hip OA ($P = 0.708$). In addition, the Steiger test demonstrated a causal relationship between exposure and outcome ($P < 0.001$).

Causal effects of blood HDL-C on OA risk

IVW MR revealed no evidence of the association between HDL-C levels and OA risk (Table 1). The Cochrane Q statistic suggested significant heterogeneity (knee OA: Q = 273.34, P < 0.001; hip OA: Q = 142.05, P < 0.001). The MR-Egger regression showed no evidence of horizontal pleiotropy for knee OA (Egger intercept = −0.006, $P = 0.157$) or hip OA (Egger intercept = −0.004, $P = 0.223$) (Figures 1B,E). Visual inspection of funnel plots revealed no horizontal pleiotropy (Supplementary Figures S3, S4). The MR-PRESSO analysis revealed six outliers and two outliers for knee and hip OA, respectively. However, these outliers did not influence the effect estimates for knee OA ($P = 0.132$) and hip OA ($P = 0.201$).

Causal effects of blood TG on OA risk

IVW MR revealed that TG increment was associated with reduced risks of hip OA (OR = 0.91, 95% CI: 0.84–0.98, $P = 0.017$) but not knee OA (OR = 0.94, 95% CI: 0.86–1.02, $P = 0.135$) (Table 1). The effect of TG on hip OA was reproduced using the MR-PRESSO method (OR = 0.91, 95% CI: 0.84–0.98, $P = 0.021$). Weighted mode MR also showed a trend toward reduced risks of hip OA (OR = 0.89, 95% CI: 0.80–1.00, $P = 0.053$). However, the MR-Egger regression and weighted median MR did not show any association. The Cochrane Q statistic suggested significant heterogeneity in the association between TG and knee OA (Q = 125.11, $P < 0.001$) but not between TG and hip OA (Q = 64.85, $P = 0.148$). The MR-Egger regression showed no evidence of horizontal pleiotropy for knee OA (Egger intercept = −0.002, $P = 0.635$) and hip OA (Egger intercept=0, $P = 0.994$) (Figures 1C,F). Visual inspection of funnel plots revealed no horizontal pleiotropy (Supplementary Figures S5, S6). The MR-PRESSO analysis identified three outliers for knee OA. However, these outliers did not influence the effect estimates ($P = 0.930$). In addition, the Steiger test demonstrated a causal relationship between exposure and outcome ($P < 0.001$).

Multivariable MR

Multivariable MR revealed a protective effect of LDL-C increment on the risk of knee OA (OR = 0.93, 95% CI: 0.87–0.99, $P = 0.021$) and hip OA (OR = 0.91, 95% CI: 0.84–0.98, $P = 0.009$) independent of HDL-C, TG, and BMI (Table 2). The estimated OR was comparable to that obtained using univariate MR analyses (Figure 2). Multivariable MR analysis revealed that neither HDL-C nor TG level was associated with OA risk.

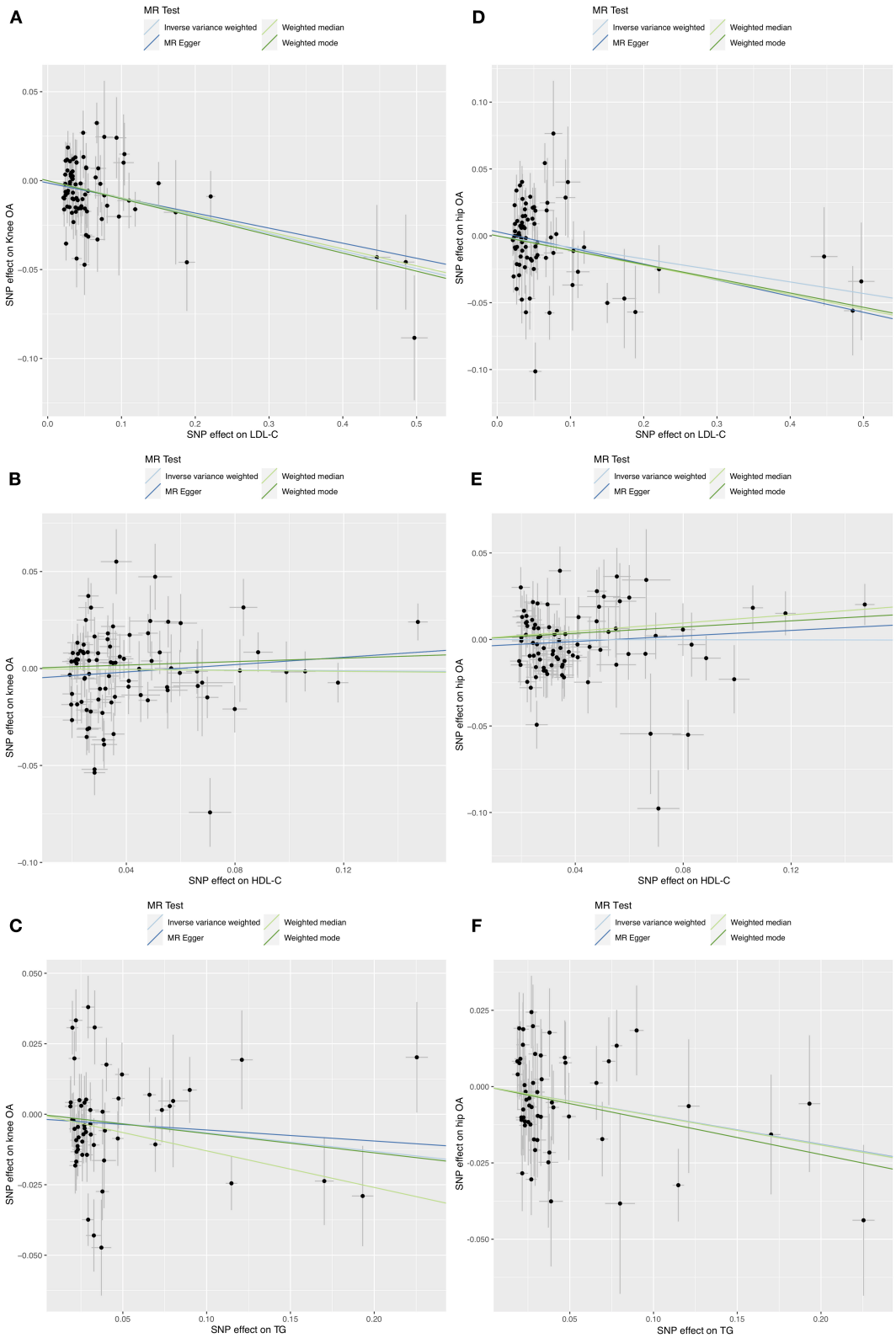

FIGURE 1
Scatter plots of MR analyses for the causal effect of LDL-C, HDL-C, and TG on knee **(A–C)** and hip **(D–F)** OA risk.

TABLE 2 Multivariate Mendelian randomization analysis results.

Exposure	Outcome	Number of SNPs	OR (95% CI)	*P*-value
LDL-C	Knee OA	38	0.93 (0.87–0.99)	0.021
HDL-C	Knee OA	50	1.03 (0.95–1.11)	0.532
TG	Knee OA	27	0.99 (0.90–1.08)	0.755
BMI	Knee OA	360	2.14 (1.95–2.34)	6.4×10^{-61}
LDL-C	Hip OA	38	0.91 (0.84–0.98)	0.009
HDL-C	Hip OA	50	1.02 (0.92–1.12)	0.717
TG	Hip OA	27	1.00 (0.90–1.12)	0.942
BMI	Hip OA	360	1.54 (1.38–1.72)	5.3×10^{-15}

OA, Osteoarthritis; SNP, single nucleotide polymorphisms; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; BMI, body mass index.

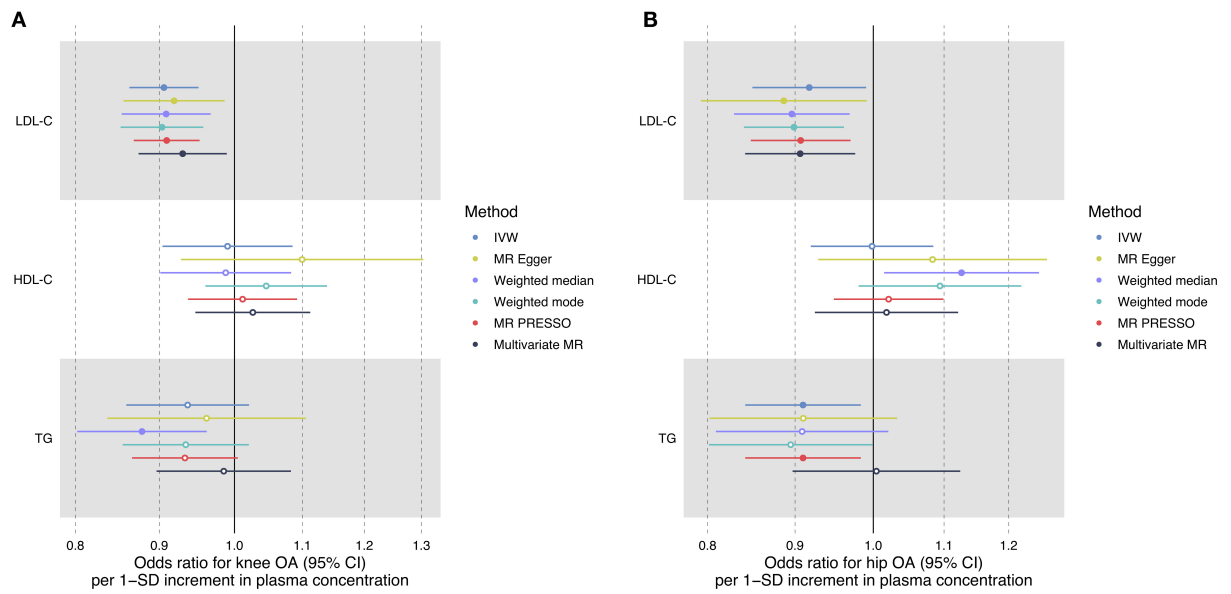

FIGURE 2
Forest plot comparing causal effect estimates of serum lipid levels on knee **(A)** and hip **(B)** OA risk using univariate and multivariable MR analyses. Odds ratios (ORs) with 95% confidence intervals (CIs) are scaled to 1-SD increment in blood lipid level.

Causal effects of lipid-lowering therapy on OA risk

LDL-C increment determined by six SNPs in the *HMGCR* region was significantly associated with a reduced risk of knee OA (OR = 0.76, 95% CI: 0.60–0.96, $P = 0.024$) but not hip OA (OR = 1.00, 95% CI: 0.75–1.34, $P = 0.994$) (Table 3, Figure 3), suggesting that the LDL-C-lowering effect of statins is related to increased risks of knee OA. In contrast, the LDL-C-lowering effect of ezetimibe and evolocumab had no influence on OA risk (Table 3). There was some evidence of heterogeneity across the effects of SNPs in the *NPC1L1* region ($P = 0.013$).

Discussion

We performed this two-sample MR study to investigate the effects of blood lipids and cholesterol-lowering agents on the risk of knee and hip OA. We found that an increase in LDL-C levels was associated with reduced risks of both knee and hip OA. Multivariate MR analysis proved that this effect was independent of HDL-C level, TG level, and BMI. There was some evidence (from IVW and MR-PRESSO method) that TG increment was associated with reduced risks of hip OA; however, this was not reproduced in the multivariate MR. Another important finding was that the genetically proxied LDL-C-lowering effect of statins was related to increased risks of knee OA but not hip OA.

TABLE 3 Estimates of the effect of LDL-C on OA risk using SNPs in specific genes.

Gene	Outcome	Number of SNPs	OR (95% CI)	*P*-value	*P* for heterogeneity
HMGCR	Knee OA	6	0.76 (0.60–0.96)	0.024	0.610
NPC1L1	Knee OA	5	1.44 (0.81–2.56)	0.219	0.091
PCSK9	Knee OA	7	0.94 (0.78–1.13)	0.512	0.665
HMGCR	Hip OA	6	1.00 (0.75–1.34)	0.994	0.621
NPC1L1	Hip OA	5	1.47 (0.59–3.67)	0.411	0.013
PCSK9	Hip OA	7	0.83 (0.63–1.09)	0.184	0.226

OA, Osteoarthritis; SNP, single nucleotide polymorphisms; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; NPC1L1, Niemann-Pick C1-Like 1; PCSK9, proprotein convertase subtilisin/kexin type 9.

In contrast, the genetically proxied effects of ezetimibe and evolocumab had no influence on OA risk.

In the current study, we performed two-sample MR analyses using different methods under different assumptions, including MR-PRESSO and multivariate MR, which yielded consistent causal effect estimates. Previous studies also investigated the effect of increased LDL-C levels on OA risk using MR analyses (26–28). However, several major differences existed between these existing studies and the current study in terms of analyses and results. Hindy et al. (26) conducted one- and two-sample MR studies based on the Malmö Diet and Cancer Study (MDCS) cohort, which included ∼30,000 adults. They found that increased LDL-C levels were associated with reduced overall OA risk (OR = 0.83). Gene-specific subgroup analysis revealed a trend toward reduced OA risk using SNPs within the *HMGCR* gene, but this did not reach statistical significance. However, their sample size was lower than that of the current study. In addition, they did not evaluate site-specific OA risk (26) unlike the present study, wherein we showed the different effects of statin on knee and hip OA. Gill et al. (27) performed a two-sample MR analysis and reported OR estimate for OA risk per 1-SD increment of LDL-C similar to our study (OR = 0.94); however, they did not adjust for other lipids, which could be potential sources of pleiotropy. In addition, they did not investigate site- and gene-specific OA risk. Recently, Meng et al. (28) conducted a two-sample MR analysis and demonstrated that LDL-C increment was associated with reduced risks of both knee OA (OR = 0.899) and hip OA (OR = 0.870). However, they used the same database to estimate SNP-exposure and SNP-outcome association, which could introduce bias in the two-sample MR owing to significant sample overlap (29). Therefore, we believe that our study provides a more robust and specific estimate of the causal effect than previous studies.

Although LDL-C plays a critical role in the pathogenesis of atherosclerosis, its role in OA has received relatively little attention. Since both obesity and hyperlipidemia are manifestations of metabolic syndrome and obesity is a well-recognized risk factor for OA, it is natural to assume that increased LDL-C is also a risk factor for OA (30). Evidence from animal experiments also supports this assumption. In a hyperlipidemic mouse model, Gierman et al. (31) found that a high-cholesterol diet could lead to the development of both OA and atherosclerosis. Interestingly, administration of atorvastatin can suppress the development of both OA and atherosclerosis, whereas ezetimibe only has an effect on atherosclerosis. It was found that lipid deposits in osteoarthritic cartilage and chondrocytes at an early stage of OA, which may trigger the development of OA (32). In addition, oxidized LDL participates in cartilage destruction by activating synovial cells, thereby promoting the release of growth factors and proinflammatory cytokines (30). Nevertheless, our MR results provide an alternative hypothesis that genetically predicted lower LDL-C levels are associated with increased risks of OA. Further research is warranted to explain the discrepancies between animal and human genetic studies.

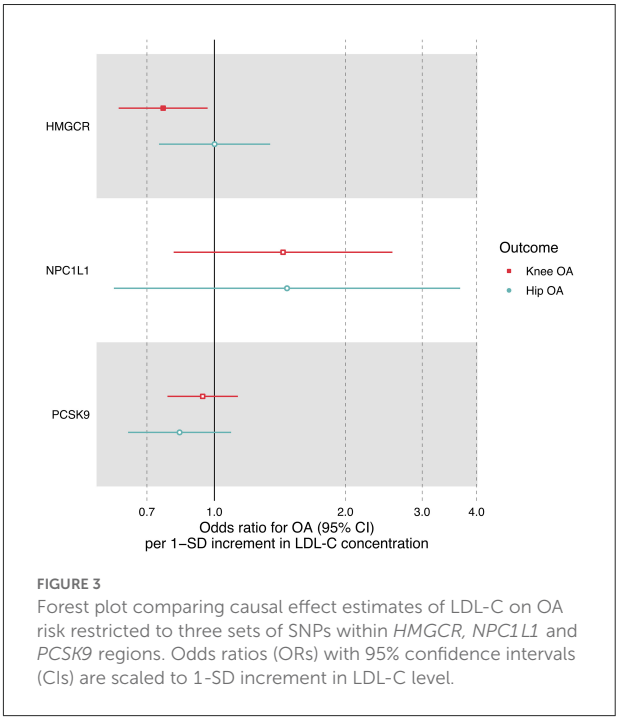

FIGURE 3
Forest plot comparing causal effect estimates of LDL-C on OA risk restricted to three sets of SNPs within *HMGCR, NPC1L1* and *PCSK9* regions. Odds ratios (ORs) with 95% confidence intervals (CIs) are scaled to 1-SD increment in LDL-C level.

In line with the effect of LDL-C on OA risk, our MR results suggested that the LDL-C-lowering effect of statins increased the

risk of knee OA. Many observational studies have investigated the association between statin use and OA risk; however, conflicting results have been reported (33–40). In a prospective cohort study of 5,674 women, Beattie et al. (33) found that statin use was associated with increased risks of incidental hip OA but not with the progression of hip OA. Eymard et al. (34) performed a *post-hoc* analysis of 336 patients from the SEKOIA trial and found an independent association between statin use and radiological progression of knee OA (OR = 1.49, $P = 0.010$) after adjusting for potential confounding factors. Makris et al. (35) conducted a 1:1 propensity score matching study that included 6,728 statin users and 6,728 non-users. They concluded that statin use led to an increased risk of non-traumatic arthritis (OR = 1.17, 95% CI: 1.09–1.25). In contrast, Clockaerts et al. (36) conducted a prospective cohort study of 2,921 participants and revealed that statin use led to a 50% reduction in overall knee OA progression, as assessed using the Kellgren and Lawrence score. Haj-Mirzaian et al. (37) conducted a retrospective cohort study stratifying participants based on the existence of Heberden nodes (HNs) and found a protective effect of statin use on the progression of radiographic knee OA in HN-positive participants. Other studies have found no effect of statin use on the risk or progression (38–40). A recent meta-analysis of observational studies found high heterogeneity among studies on the effect of statins on the progression of OA (7). Nevertheless, observational studies are inevitably affected by confounding factors, and more importantly, by indication bias (41), which has been well-discussed in studies evaluating statin use and colorectal cancer risk (42). Because of indication bias, observational studies may falsely show a protective effect of statins if hyperlipidemia is related to a lower risk of the disease, which is exactly the current situation since we proved that LDL-C increment was associated with reduced risks of knee and hip OA. According to Mendel's law of inheritance, alleles obtained by individuals in an SNP are random with respect to potential confounding factors. Using SNPs as instrumental variables, MR studies can mimic the effect of randomized controlled trials (43) and can thus provide causal effect estimation closer to the real situation. In addition to statins, ezetimibe and evolocumab are commonly used LDL-C-lowering drugs. However, to the best of our knowledge, no previous study has reported its effects on OA. Further studies need to compare the effects of statins, ezetimibe, and evolocumab on OA, which may overcome the potential indication bias of previous observational studies (44).

The limitations of this study are as follows. First, the MR methodology requires the absence of horizontal pleiotropy, and instrumental variables affect outcomes only through their effect on exposure. In the current study, potential pleiotropy may be due to the effect of instrumental SNPs on other lipids and body weight. Nevertheless, we conducted sensitivity MR analyses using different methods under different assumptions, including MR-Egger regression, which showed no evidence of directional horizontal pleiotropy. We also performed multivariate MR wherein all the analyses yielded similar causal effect estimates. Second, the analyses were conducted based on European ancestry. Therefore, these results may not be applicable to other populations as well. Thirdly, estimates of causal effects were reported as OR per one SD increase in LDL-C, therefore, we could not assess the actual dose-response effect of increasing LDL-C levels and risk of OA. Fourthly, sex may modify the correlation between blood lipid and OA. Future study may stratify male and female individuals to identify this effect. Finally, we used SNPs within *HMGCR*, *NPC1L1*, and *PCSK9* to mimic the LDL-lowering effect of statins, ezetimibe, and evolocumab. There may be differences between the genetically proxied effect and the real drug effect. In addition, we could not compare the effects of different types of statins.

Conclusions

In conclusion, our MR study suggests that genetic predisposition to higher blood LDL-C levels may decrease the risk of both knee and hip OA. This effect was independent of HDL-C level, TG level, and BMI. The genetically proxied LDL-C-lowering effects of statins may increase the risk of knee OA but not hip OA. Further studies are needed to reveal the mechanisms underlying the effect of LDL-C and statin on OA and its potential role in treating and preventing OA.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.mrbase.org/.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the Local Legislation and Institutional requirements. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZW and YZ designed the study. ZW and ML performed statistical analyses and wrote the manuscript. All authors were involved in results interpreting and were involved in revising the article and approved the final version to be published.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.990569/full#supplementary-material

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frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 17 November 2022
DOI 10.3389/fmed.2022.1028575

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

RECEIVED 30 August 2022
ACCEPTED 01 November 2022
PUBLISHED 17 November 2022

CITATION
Wu X, Xu P, Shi X, Shang J, Chen X, Guo A, Wang F and Yin Z (2022) Intra-articular injection of clioquinol ameliorates osteoarthritis in a rabbit model.
Front. Med. 9:1028575.
doi: 10.3389/fmed.2022.1028575

Intra-articular injection of clioquinol ameliorates osteoarthritis in a rabbit model

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Osteoarthritis (OA) is characterized by the degeneration of articular cartilage. Decreased autophagy is tightly associated with chondrocyte death, which contributes to the progression of OA. Thus, pharmacological activation of autophagy may be a promising therapeutic approach for OA. Here, we discovered that clioquinol, an antibiotic, significantly induces autophagy in OA chondrocytes from human tissue and rabbit model. Meanwhile, clioquinol can also augment the expression of extracellular matrix (ECM) components and suppress inflammatory mediators to improve OA microenvironment. Intra-articular injection of clioquinol can greatly prevent or slow down the development of this disease in a trauma-induced rabbit model of osteoarthritis. Such protective effect induced by clioquinol was at least in part explained by decreasing chondrocyte apoptosis and increasing autophagy. This study reveals the therapeutic potential of clioquinol in OA treatment.

KEYWORDS

osteoarthritis, clioquinol, intra-articular injection, autophagy, therapeutic potential

Introduction

Osteoarthritis (OA) is the most common degenerative disease worldwide characterized by the loss of chondrocytes, degradation of articular matrix, and synovial inflammation (1). The etiology of OA is multifactorial and complex, with a variety of risk factors contributing to the progression, such as mechanical stress, aging, obesity, inflammation, and genetic susceptibility (2). Articular cartilage is composed of the extracellular matrix (ECM), mainly containing collagen type II and the cell type—chondrocytes. Cartilage architecture and biochemical composition are regulated by chondrocytes in response to the alterations from the surroundings of cartilage matrix (1). The ability of adult articular chondrocytes to maintain the normal cartilage matrix architecture is limited and declines with age (3). Therefore, keeping the chondrocytes in a healthy state may be an important way for maintaining the integrity of the entire cartilage.

Autophagy is a highly conserved physiological process that degrades long-lived or impaired organelles and proteins via the lysosomal system (4). Increasing evidence has shown that autophagy plays an important role in cell survival, aging, and homeostasis (5). In addition, it has been revealed that autophagy is associated with the pathogenesis of OA (6). During the development of OA, autophagy may function as an adaptive response to exert protective effect against various environmental changes, while the failure of the adaptation may lead to the progression in cartilage degradation (7). Previous studies found that the intra-articular injection of rapamycin, an autophagy inducer, facilitates the postponement of cartilage degeneration in the OA mouse model (8). Thus, it can be concluded that pharmacological activation of autophagy may be a promising therapeutic strategy for OA.

Herein, we attempt to explore new autophagy-inducing pharmacophores. Previous research revealed that some antibiotics can be utilized to induce autophagy (9, 10). Clioquinol (5-chloro-7-iodo-8-quinolinol), an antimicrobial agent against common pathogenic microbes, functions as a novel autophagy activator in multiple cells (11–13). Clioquinol can trigger pro-death autophagy via interrupting mTOR signaling pathway in leukemia and myeloma cells, which not only inhibits enzymatic activity of mTOR (a critical modulator of autophagy) as rapamycin, but also suppresses the expression of mTOR (12). Clioquinol also induces autophagy in a zinc-dependent manner and contributes to the clearance of aggregated proteins in astrocytes and neurons (11). The protective effects of clioquinol have been reported in various neurodegenerative diseases, such as Alzheimer's disease (14), Parkinson's disease (15), and Huntington's disease (16), but not yet in OA. Therefore, exploring the potential of clioquinol as autophagy inducer in OA treatment may be helpful for the therapy.

In this study, we investigated the effects of clioquinol on autophagy process in chondrocytes. Furthermore, we also evaluated the therapeutic potential of clioquinol in the rabbit OA model of anterior cruciate ligament transection with partial medial meniscectomy (ACLT + PMM) and explored the underlying mechanism.

Materials and methods

Isolation and culture of chondrocytes

Human cartilage tissue was obtained from the knees of OA patients who had undergone Total Knee Arthroplasty (TKA). This study was approved by the human research ethics committee. All patients' OA satisfy the American College of Rheumatology's criteria for OA (17), and the patients' consent was obtained. The Clinical characteristics of OA patients are in Table 1. The isolation and culture of chondrocytes were applied according to the previous study (10). A non-weightbearing area of cartilage without any macroscopically visible abnormalities was harvested and washed in sterilized saline. Next, the tissue was cut into smaller sections of 1 mm^3 and digested by trypsin (2.5 mg/ml) (Sigma Co., St. Louis, MO, USA) at 37°C for 40 min, and then treated for 8 h with Type II collagenase (2 mg/ml) (Sigma Co., St. Louis, MO, USA) in a DMEM/F12 medium (Thermo Scientific, Waltham, MA, USA). The isolated chondrocytes were placed in DMEM/F12 with 10% FBS and 100 units/ml of penicillin and 0.1 mg/ml of streptomycin, incubated at 37°C. Following the above procedure, we applied the second-passage chondrocytes for further studies.

Cell viability assay

The cell viability of chondrocytes was detected by the CCK-8 Reagent (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Chondrocytes were treated with clioquinol for 24 and 48 h, respectively. The cells were washed with PBS, and then added CCK-8 solution. The 450 nm absorbance was detected by micro-plate reader. All experiments were repeated at least five times.

Establishment of osteoarthritis model and intra-articular injection of clioquinol

Male white New Zealand rabbits (2.5–2.8 kg, 3-month-old) were used with the approval from the institutional animal care and use committee. Anterior cruciate ligament transection with partial medial meniscectomy (ACLT + PMM) were performed on rabbits as described previously (18). Briefly, under general anesthesia, the anterior horn of the medial meniscus was dissected and the anterior cruciate ligament of the right knee was transected. In the control group, a sham operation was carried out on the contralateral knee with no meniscus dissection and no ligament transection and then treated by the vehicle only. Eighteen rabbits were randomly assigned to three groups (n = 6/group): the sham group (sham operation plus the vehicle treatment as control), ACLT + PMM plus the vehicle treatment group, ACLT + PMM plus the clioquinol (5 mg kg^{-1}) treatment group (10). Rabbits were given clioquinol (100 μl) were intra-articular injected into rabbits once a week following surgery for 8 weeks. All rabbits were kept in individual cages at 22 ± 3°C with 55 ± 20% humidity and a 12-h light-dark cycle. After 8 weeks of surgery, the tibial plateaus of the rabbits' hind legs were harvested. Animal studies were based on ARRIVE guidelines.

Autophagosome formation detection

Chondrocyte autophagosome formation was detected by Cyto-IDTM autophagy detection kit (Enzo Life Sciences, Farmingdale, NY, USA), following the manufacturer's recommendations. Articular cartilage cells of humans with OA were treated with or without 5 μM clioquinol for 48 h and then stained with a dual detection reagent for 30 min in the dark at 37°C. The green dot fluorescence (autophagosome) was observed under microscope.

RNA extraction and RT-qPCR analysis

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Real-time RT-qPCR was

TABLE 1 Clinical characteristics of osteoarthritis (OA) patients.

Sample	Sex	Age (year)	Disease duration (year)	CRP (μg/ml)	ESR (IU/ml)	RF (IU/ml)	Sample-obtained time
OA1	Male	65	16	7.31	18	10.44	February 2021
OA2	Male	72	13	5.23	24	16.32	February 2021
OA3	Male	71	17	2.45	19	4.75	March 2021
OA4	Male	63	18	5.78	7	9.87	March 2021
OA5	Male	69	13	2.12	16	15.27	March 2021
OA6	Female	64	20	7.98	27	17.21	March 2021
OA7	Female	55	9	9.12	25	13.56	April 2021
OA8	Female	63	15	11.13	28	12.98	April 2021
OA9	Male	64	7	7.63	15	9.45	May 2021
OA10	Male	72	10	2.78	14	5.32	May 2021

CRP, C-reactive protein; ESR, erythrocyte sedimentation; RF, rtheumatoid factor.

TABLE 2 Details of the primers used in RT-qPCR.

Gene name	Sense (5′–3′)	Antisense (5′–3′)
Col2a1	GGCAATAGCAGGTTCACGTACA	CGATAACAGTCTTGCCCCACTT
Acan	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA
MMP-1	GGGGCTTTGATGTACCCTAGC	TGTCACACGCTTTTGGGGTTT
MMP-13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
IL-6	CCACTCACCTCTTCAGAACGAAT	GGCAAGTCTCCTCATTGAATCCA
IL-1ß	AACAGGCTGCTCTGGGATTC	GGTCGGAGATTCGTAGCTGG
LC3	CCACACCCAAAGTCCTCACT	CACTGCTGCTTTCCGTAACA
Beclin1	AAATGCTGCTTGGGGTCAGA	CGGAATCCACCAGACCCATA
ATG5	AAGCAACTCTGGATGGGATT	GCAGCCACAGGACGA AAC
ATG7	CAGTCCGTTGAA GTCCTC	TCAGTGTCCTAGCCACATTAC
GAPDH	AGGTCGGTG TGAACGGATTTG	GGGGTCGTTGATGGC AACA
DRAM1	TCAAATATCACCATTGATTTCTGT	GCCACATACGGATGGTCATCTCTG

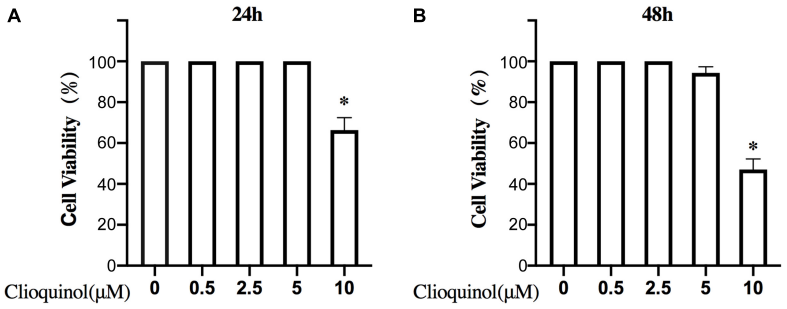

FIGURE 1
Effects of clioquinol on the cell cytotoxicity *in vitro*. The cytotoxic effects of clioquinol on chondrocytes were determined with increasing concentrations (0, 0.5, 2.5, 5, and 10 μM) for 24 and 48 h using a CCK8 assay **(A,B)**. All experiments were repeated five times. All data are shown as the mean ± SD. *$p < 0.05$.

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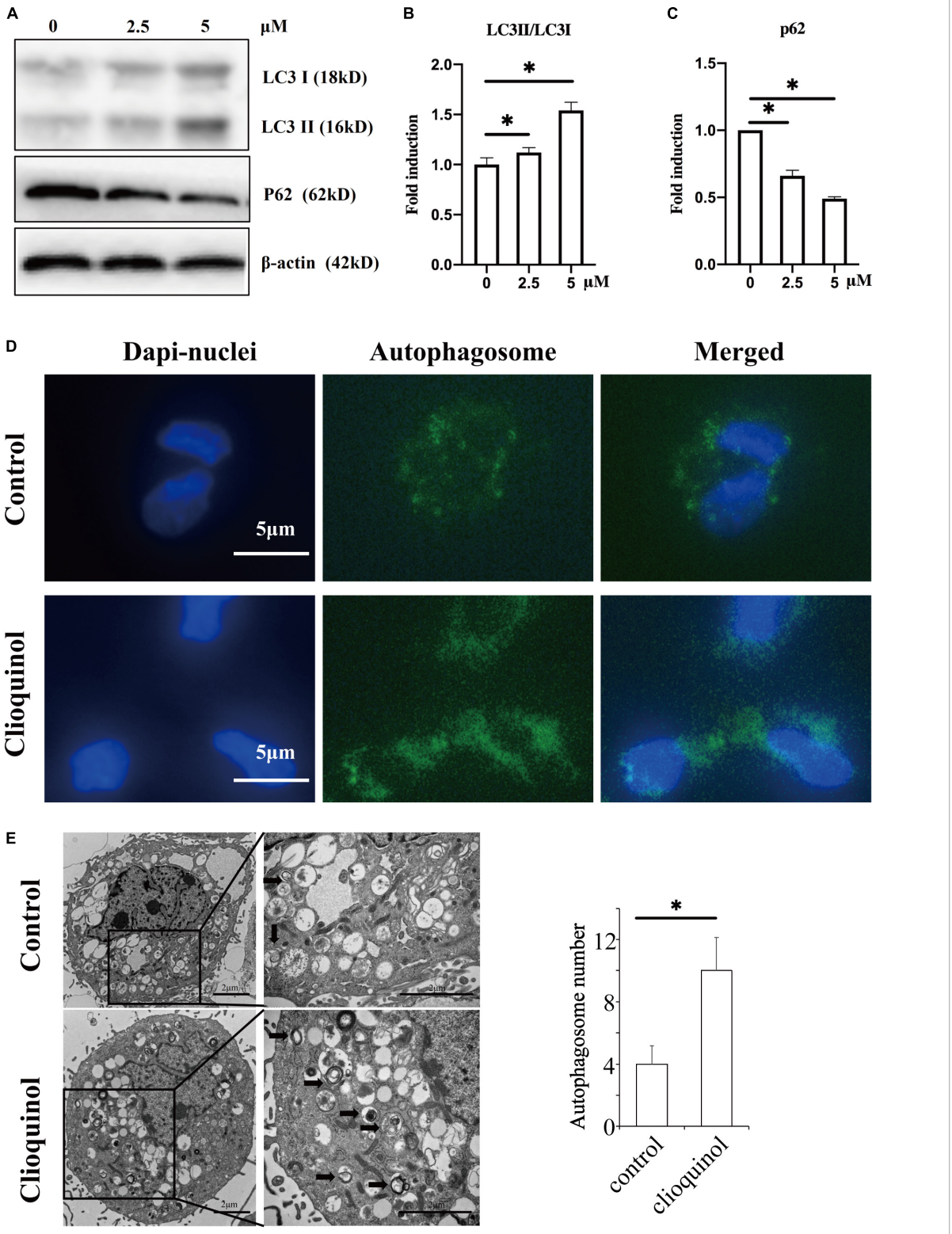

FIGURE 2

Clioquinol induces autophagy in human osteoarthritis (OA) chondrocytes. The expression of LC3, p62, and β-actin were detected with Western blotting **(A–C)**. Human OA chondrocytes were treated or not treated with 5 μM of clioquinol for 48 h, and autophagosome formation (green dots) was detected using an autophagy detection kit **(D)** and transmission electron microscopy **(E)**. All data are shown as the mean ± SD. *$p < 0.05$.

performed with the Applied Biosystems StepOnePlus Real-Time PCR System. Information on the primer sequences was provided in Table 2. All samples were performed in triplicate.

Western blot

The treated cells were collected and lysed for protein sample preparation. We loaded 30 mg of lane protein into the well of SDS page gel and electrophoretically transferred the protein to NC membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 1 h and incubated in primary antibodies against microtubule associated protein 1 LC3 (1:1,000, Sigma Co., St. Louis, MO, USA), p62 (1:8,000, Abcam, Cambridge, UK), and β-actin (1:1,000, Bioss, Beijing, China). The membranes were then washed and incubated with secondary antibodies (1:10,000, Bioss, Beijing, China) for 2 h. Afterward, the membrane was then washed and exposed with an electrochemiluminescence system. Relative densitometric analysis (a semi-quantitative analysis) was performed following densitometric scanning.

Immunohistochemistry

After the tissues were dewaxed, 3% H_2O_2 was applied to suppress the levels of endogenous peroxide on the histologic slices, after which microwave heating was used to retrieve antigen. The slices were blocked for 0.5 h with goat serum (Beyotime Institute of Biotechnology, Shanghai, China). The slices were then incubated in the solution of MMP-13 primary antibodies (1:100, Abcam, Cambridge, UK), Col-2a1 antibodies (1:100, Abcam, Cambridge, UK), Cleaved-caspase3 antibody (1:200, Abcam, Cambridge, UK). After the incubation, a horseradish peroxidase-conjugated secondary antibody (Abcam) was used for 0.5 h, and cells were stained with 3,3′- diaminobenzidine (Beyotime Institute of Biotechnology) and mounted. The microscope was used for detecting the percentage of MMP-13, Cleaved-caspase3 positive cells (brown cells).

Transmission electron microscopy

Chondrocytes or cartilage tissue were fixed in ice-cold 2% glutaraldehyde/0.1 M PBS and post-fixed in 1% osmium tetroxide. After being washed and dehydrated with a series of graded ethanol (30–100%), the samples were embedded in propylene oxide or embedding resin (1:1). Resin blocks were cut into thin sections, and the sections were placed on copper grids and stained with uranyl acetate and lead citrate. An H-7650 transmission electron microscope was used for detecting the autophagic vesicles (double membrane-enclosed vesicles containing engulfed organelles or other cell components).

Histological assessment

The tibial plateaus of hind legs from rabbits were fixed in 4% paraformaldehyde and decalcified for 2 months with 10% EDTA. The tissues were then dehydrated, infiltrated with paraffin, and embedded in paraffin wax. The paraffin blocks were sectioned into 5 μm slices along the sagittal plane using a microtome. Safranin O-fast green and alcian blue staining was performed. Select three slices from each medial tibial plateau, and two observers who were blinded to animal study, respectively, utilized a semi-quantitative scoring system (OARSI's histopathology grading system of cartilage OA) to assess articular cartilage degeneration.

Statistical analysis

All the data are presented as mean ± SD. Differences between two groups were analyzed by the unpaired *t*-test

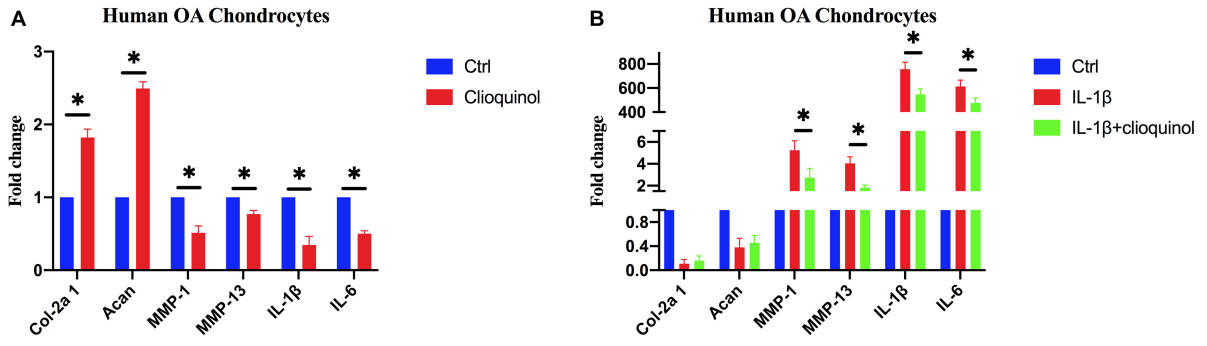

FIGURE 3
The expression of osteoarthritic markers in human osteoarthritis (OA) chondrocytes after incubating with clioquinol (5 μM) **(A)**, or IL-1ß (10 ng/ml), IL-1ß (10 ng/ml) + clioquinol (5 μM) **(B)**, determined by PCR. All data are shown as the mean ± SD. *$p < 0.05$.

or the Mann-Whitney U test. Differences between different groups were analyzed by ANOVA. *P*-values less than 0.05 were statistically significant.

Results

Clioquinol induces autophagy in human osteoarthritis chondrocytes

First, we investigated the cytotoxic effect of clioquinol on human OA chondrocytes. The cells were incubated with clioquinol at different concentrations (0, 2.5, 5, and 10 μM) for 24 and 48 h. Finally, CCK-8 analysis showed that no apparent cytotoxic effects on chondrocytes were observed at low concentrations of clioquinol (0–5 μM) (**Figures 1A,B**). However, at 10 μM, clioquinol induced a modest level of cell death. Based on this result, we chose 5 μM as the concentration for following studies. To determine whether clioquinol could induce autophagy, western blot was performed to detect the marker of autophagy, LC3 and P62. The clioquinol-treated chondrocytes show an increased level of LC3-I/II, and a decreased P62 level, in a concentration-dependent manner (**Figures 2A–C**), indicating the augment of autophagy. Other

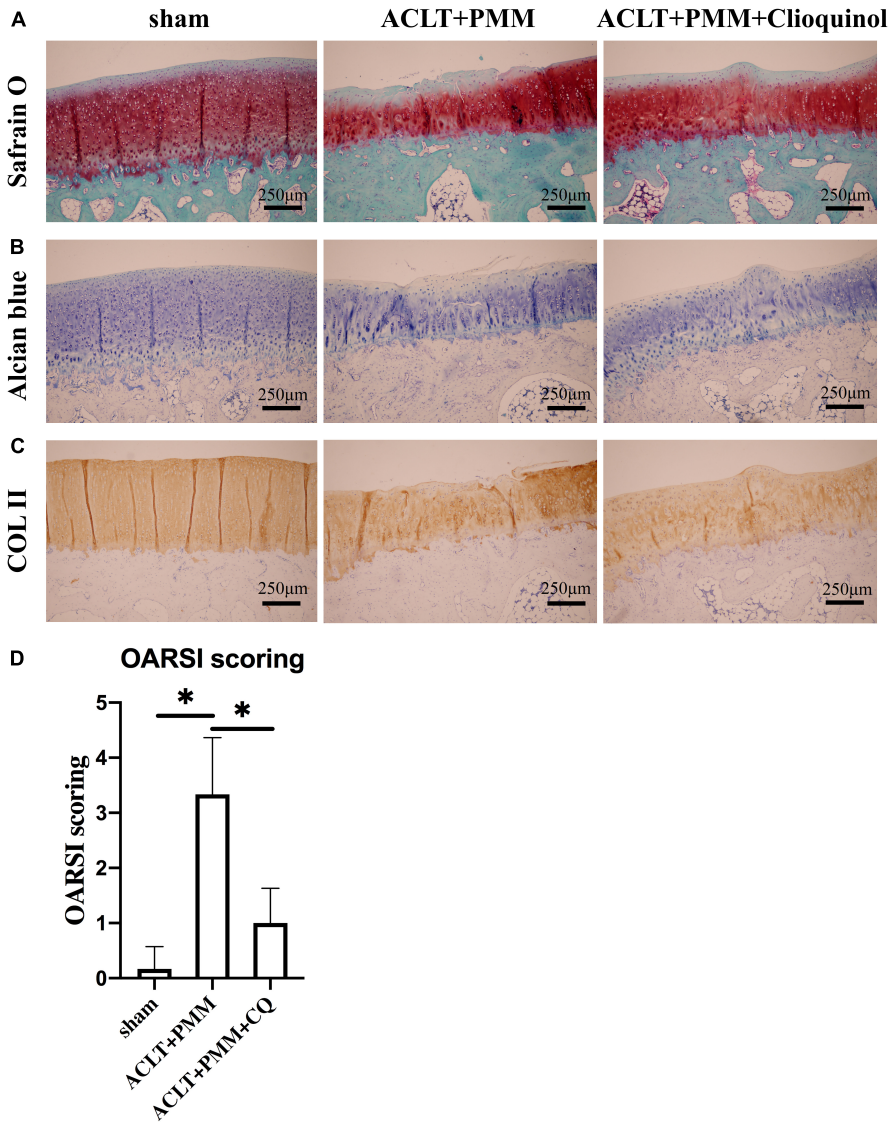

FIGURE 4
Clioquinol ameliorates osteoarthritis (OA) development in rabbit model. **(A,B)** Representative Safranin O-Fast green and Alcian Blue staining of cartilage in three groups (n = 6) at the 8th weeks post-surgery. **(C)** Representative IHC staining showing the expression and distribution of Col-2a1 in cartilage after treatment. **(D)** Cartilage degeneration evaluated by OARSI scoring system. All data are shown as the mean ± SD. $^{*}p < 0.05$.

autophagy-associated genes, DRAM1, ATG5, and ATG7, were also dose-dependently increased (Supplementary Figure 1). Furthermore, we also used immunofluorescence staining (Figure 2D) and transmission electron microscopy (Figure 2E) to visualize the autophagosome formation. In consistent with the results of western blot, autophagosome formation was enhanced in the clioquinol-treated chondrocytes, compared to the control. Together, these results indicated that clioquinol exposure could facilitate autophagy in human OA chondrocytes.

Clioquinol enhances chondrogenic markers and reduces inflammatory markers in human osteoarthritis chondrocytes

We next sought to characterize the osteoarthritic microenvironment after clioquinol treatment. RT-qPCR results showed that the mRNA levels of chondrogenic marker Col-2a1 and Acan were significantly elevated after the exposure to clioquinol (Figure 3A). Meanwhile, clioquinol treatment can also reduce the levels of inflammatory marker MMP-1, MMP-13, IL-1ß, and IL-6 (Figure 3A). Additionally, clioquinol can also exert preventive effects on the function of IL-1ß, an important inflammatory factor. Although clioquinol failed to rescue the IL-1ß-induced inhibitory effects on Col-2a1 and Acan, it correspondingly repressed the IL-1ß-mediated upregulated expression of MMP-1, MMP-13, IL-1ß, and IL-6 (Figure 3B).

Clioquinol ameliorates osteoarthritis development in a rabbit model

We further investigated the therapeutic efficacy of clioquinol on ACLT + PMM-induced OA *in vivo*. Clioquinol was administered by intra-articular injection once a week for 8 weeks beginning the day after surgery. Histological analysis by Safranin O and Alcian Blue staining respectively showed osteoarthritic changes with cartilage abrasion and hypocellularity in the ACLT + PMM group, whereas no OA-like changes were observed in the sham group (Figures 4A,B). However, the ACLT + PMM + clioquinol group showed less cartilage erosion and richer proteoglycan (Figures 4A,B), suggesting clioquinol ameliorates ACLT + PMM-induced impairment. The contrast in different groups of Col-2a1 expression was not obvious, yet the abrasion in the control group was profound (Figure 4C). Consistent with staining, OARSI score of the ACLT + PMM group was significantly higher than that of the sham group, while the clioquinol treatment resulted in the decrease of OARSI scores (Figure 4D).

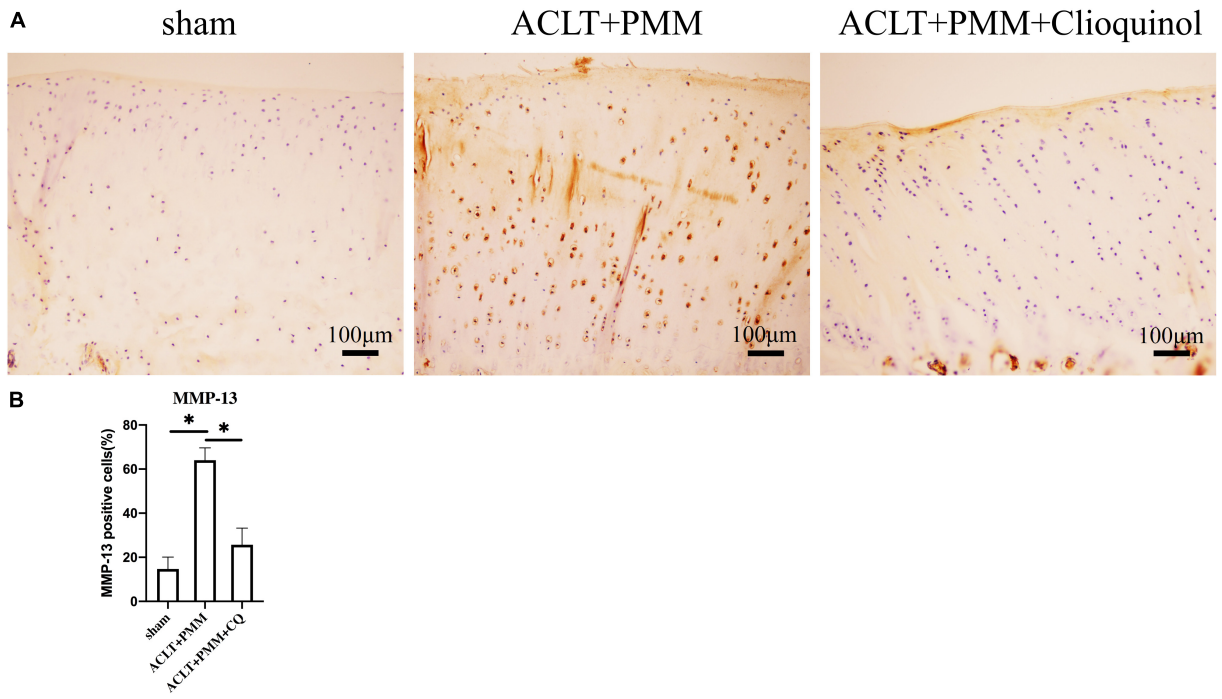

FIGURE 5
The change of MMP-13 expression in cartilage after intra-articular injection of clioquinol. **(A)** Representative IHC staining showing the expression and distribution of MMP-13 in cartilage. **(B)** Quantitation of ICH staining. All data are shown as the mean ± SD. *$p < 0.05$.

Clioquinol reduces the expression of inflammatory markers in the rabbit model

Immunohistochemistry (IHC) as performed for MMP-13, a main protease responsible for collagen degradation in articular cartilage. The results showed that chondrocytes with high MMP-13 expression were increased in the ACLT + PMM group compared to the sham group. However, in the ACLT + PMM + clioquinol group, MMP-13 positive cells in the articular cartilage was significantly reduced (**Figures 5A,B**).

Clioquinol enhances autophagosome formation and attenuates apoptosis of chondrocytes in the rabbit model

We examined the expression of autophagy markers to clarify if clioquinol treatment can facilitate autophagy in the rabbit OA model. RT-qPCR results showed that the mRNA expression of LC3 and beclin-1 was significantly ($p < 0.05$) reduced in rabbit OA chondrocytes compared with the sham surgery chondrocytes, while clioquinol treatment markedly rescued the inhibited expression of these autophagy markers (**Figures 6A,B**). Consistent with the expression of autophagy markers, TEM also showed that autophagosome formation was significantly augmented in the ACLT + PMM + clioquinol group, compared with the ACLT + PMM group (**Figures 6C,E**). Apoptosis in chondrocytes is positively associated with OA progression. Herein, we also detected the level of apoptosis marker cleaved-caspase 3 by immunohistochemical staining. The cartilage of ACLT + PMM group showed an increased expression of cleaved caspase 3 compared to the sham group, while clioquinol administration significantly repressed the ACLT + PMM surgery-induced elevation of cleaved caspase 3 (**Figures 6D,F**), which indicates clioquinol can also protect chondrocytes from the apoptosis in OA progression.

Discussion

Osteoarthritis has been regarded as a degenerative disease with a high prevalence, which is the primary cause of disability and burden for the elderly (19, 20). There is a clear and urgent need to develop new drugs for OA treatment. In this study, we reported that clioquinol can significantly promote autophagy, enhance chondrogenic markers and inhibit inflammatory markers in OA chondrocytes. Furthermore, we demonstrated that the intra-articular clioquinol can ameliorate OA damages in the ACLT-induced OA rabbit model, and clioquinol can also block chondrocyte apoptosis, which may be achieved by enhancing autophagy.

Autophagy plays an essential role in maintaining cellular metabolism and homeostasis (21). Recent studies suggest that the imbalance of autophagy is a key factor in the pathogenesis of OA (22). Studies have demonstrated that the level of autophagy in OA cartilage is reduced (23) and autophagy can protect chondrocytes from the degradation (24). Activation of autophagy in chondrocytes by intra-articular injection of resveratrol, an autophagy inducer, can significantly delay articular cartilage degeneration in a destabilized medial meniscus OA mouse model (25). Our previous study found that an intra-articular injection of chloramphenicol attenuates the severity of cartilage degradation in a type II collagen-induced rabbit model of OA, which may be associated with the induction of autophagy (10). Therefore, pharmacological induction of autophagy may be an appropriate therapeutic approach for OA. The development of safe and effective drugs that can enhance autophagic activities or restore autophagy flux is a promising strategy for the treatment of OA. This study, therefore, was planned to assess the potential of clioquinol as autophagy inducer in primary chondrocytes and rabbits with ACLT + PMM surgery-induced OA. Clioquinol is a quinoline derivative used as an antibiotic for the treatment of diarrhea and soft tissue infections (26, 27). Recently, clioquinol has been proven to induce autophagy of a variety of cells, including astrocytes, neurons, leukemia and myeloma cells (11–13). However, there is no previous study to explore the application of clioquinol against OA development, whereas our study revealed the potential of clioquinol as an autophagy inducer for the treatment of OA. From a mechanistic standpoint, previous studies indicated that clioquinol acts as a zinc ionophore and increases intracellular free zinc levels in the cytosol and in lysosomes, which augments autophagic flux (11, 28). However, whether clioquinol would induce autophagy in OA through a similar or the same pathway requires further exploration. In order to investigate if the autophagy induction property of clioquinol can protect chondrocytes, we incubated primary chondrocyte cells with or without clioquinol. Consistent with the previous studies, autophagy is a self-protective process in OA in response to the stimulation by clioquinol. In our study, clioquinol exerts chondroprotective effects on human OA chondrocytes, which was manifested by the increased chondrogenic markers Col-2a1 and Acan and the suppressed expression of genes encoding inflammatory cytokines IL-1β and IL-6, as well as the cartilage-degrading enzymes from the MMP family, including MMP-1 and MMP-13 (**Figure 3**).

According to *in vitro* experiments, we also sought to investigate whether intra-articular injection of clioquinol could block OA progression *in vivo*, and ACLT + PMM surgery-induced OA rabbit model was constructed for further studies. Our study shows that intra-articular injection of clioquinol can distinctly repress articular cartilage erosion and rescue the proteoglycan content. Increasing evidences show that MMP-13, a zinc-dependent protein, plays a vital role by degrading type II

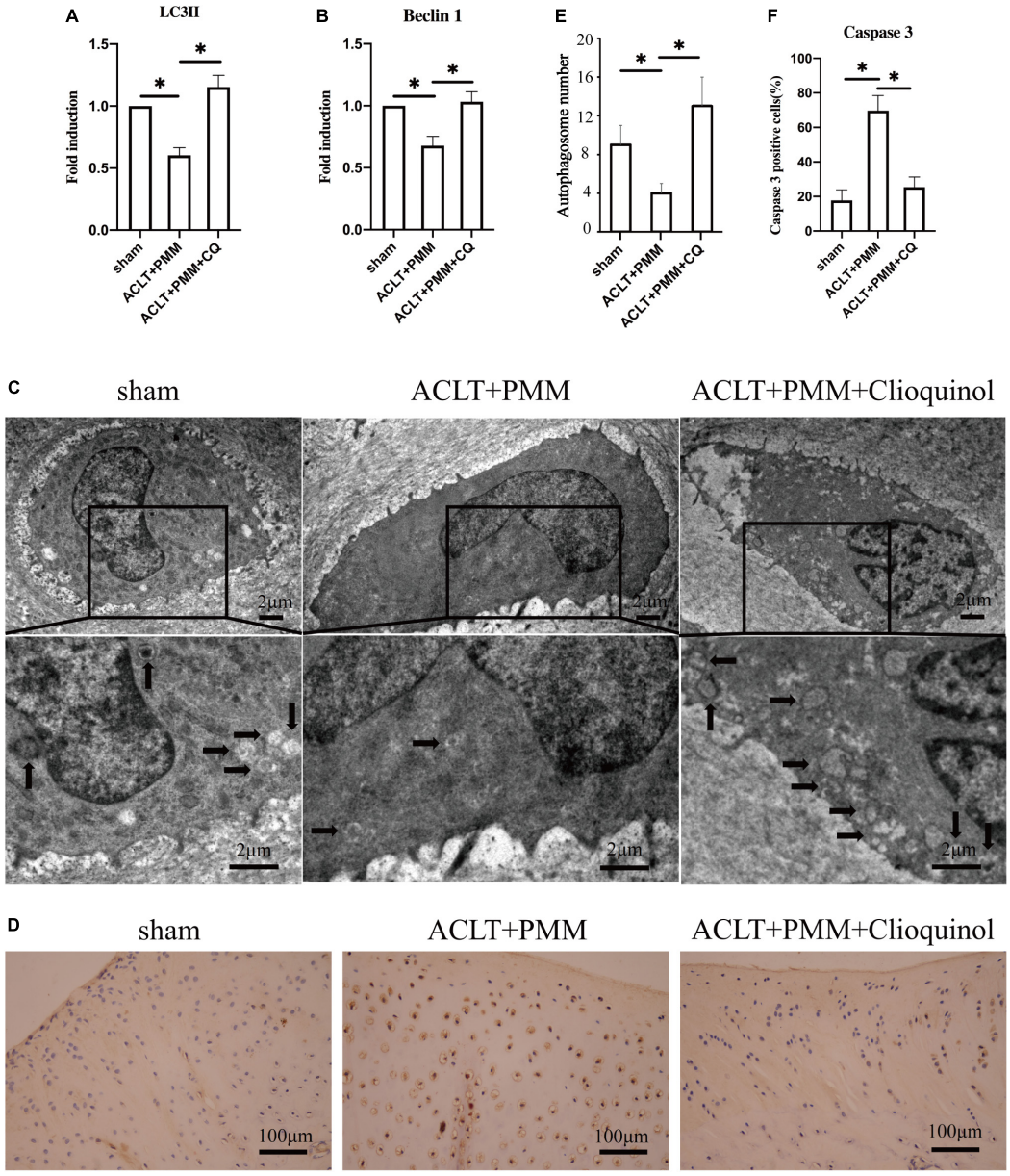

FIGURE 6
(A,B) The expression of LC3 and Beclin-1 evaluated by PCR. **(C,E)** Autophagosome formation (arrows) detected using transmission electron microscopy. **(D)** Representative IHC staining showing the expression and distribution of cleaved caspase 3 in cartilage. **(E,F)** Quantitative analysis of autophagosome formation and cleaved caspase 3. All data are shown as the mean ± SD. *$p < 0.05$.

collagen in articular cartilage in OA (29), which indicates that the level of MMP-13 is positively associated with OA severity (30–32). Our findings shows that intra-articular injection of clioquinol can inhibit MMP-13 expression, which was consistent to our *in vitro* study. However, there was no significant degradation of Col-2a1 in the ACLT + PMM group, which may be owing to the long half-life of Col-2a1 and activated metabolism of chondrocytes after the surgery that confound the effect of clioquinol administration on the expression of Col-2a1 (33, 34).

We also investigated the role of an intra-articular injection of clioquinol on autophagy in rabbits with OA and detected that clioquinol can increase the expression of autophagy-related factors, including LC3 and Beclin1. More autophagosomes were observed by transmission electron microscopy, consistent with the alterations of autophagy markers. In addition, clioquinol reduced the level of cleaved caspase 3, a primary executioner for apoptosis, which indicated that the increase of autophagy with a subsequent decrease of apoptosis may be a part of the mechanism of clioquinol-mediated amelioration for OA.

There were also some limitations to our study. First, this is a preliminary result, and it is expected that details of the interaction between the clioquinol-induced autophagy and OA require further research. Second, the animal model we used in this paper was ACLT + PMM surgery-induced OA model, which is a classical method for establishing OA models; thus, it may not be broadly representative of all OA conditions. Third, although clioquinol is promising for the treatment for OA, owing to the side effect–termed subacute myelo-optic neuropathy (SMON), it still need more efforts to explore its appropriate utilization in human.

Taken together, our results suggest that intra-articular injection of clioquinol can alleviate ACLT + PMM surgery-induced OA progression. The underlying mechanisms may include reducing MMP-13, increasing autophagy and decreasing chondrocyte apoptosis. Intra-articular administration of clioquinol may be a promising treatment for OA, and additional comprehensive studies examining the clinical potential of clioquinol for OA therapy are still required.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Human Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study. This animal study was reviewed and approved by the Institutional Animal Care and Use Committee.

Author contributions

XW, XS, and ZY: conception and design. XW, JS, and XC: analysis and interpretation of the data. XW, XS, AG, PX, and ZY: drafting of the manuscript. PX, JS, FW, and ZY: critical revision of the manuscript for important intellectual content. XW, PX, JS, and ZY: final approval of the manuscript. PX: provision of the study material or patients. XW, XS, AG, and ZY: statistical expertise. AG, PX, and ZY: obtaining of funding. XW, XS, XC, FW, and ZY: administrative, technical, and logistic support. XW, XC, and FW: collection and assembly of data. All authors drafting the manuscript or revising it critically for intellectual content and approved the final version to be published.

Funding

This work was supported by Foundation for Distinguished Young Talents in Higher Education of Guangdong Province (2019KQNCX124).

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.1028575/full#supplementary-material

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frontiers | Frontiers in Medicine

TYPE Clinical Trial
PUBLISHED 05 December 2022
DOI 10.3389/fmed.2022.1063336

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

RECEIVED 07 October 2022
ACCEPTED 17 November 2022
PUBLISHED 05 December 2022

CITATION
Abshirini M, Coad J, Wolber FM, von Hurst P, Miller MR, Tian HS and Kruger MC (2022) Effects of Greenshell™ mussel intervention on biomarkers of cartilage metabolism, inflammatory markers and joint symptoms in overweight/obese postmenopausal women: A randomized, double-blind, and placebo-controlled trial. *Front. Med.* 9:1063336. doi: 10.3389/fmed.2022.1063336

Effects of Greenshell™ mussel intervention on biomarkers of cartilage metabolism, inflammatory markers and joint symptoms in overweight/obese postmenopausal women: A randomized, double-blind, and placebo-controlled trial

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Objective: To investigate the effect of whole greenshell mussel (GSM) powder on biomarkers of cartilage metabolism, inflammatory cytokines, and joint symptoms in postmenopausal women with overweight/obesity and joint discomfort.

Design: Fifty-five postmenopausal women with overweight/obesity were randomly assigned to receive 3 g/day whole GSM powder or placebo for 12 weeks. Cartilage turnover biomarkers urinary C-telopeptide of type II collagen (CTX-II) and serum cartilage oligomeric matrix protein (COMP) were measured at baseline, week 6 and 12. Plasma cytokines were measured at baseline and week 12. Joint pain and knee-related problems were assessed at baseline and week 12 using a 100 mm Visual Analogue Scale (VAS) and the Knee injury and Osteoarthritis Outcome Score (KOOS) questionnaire, respectively.

Results: Forty-nine participants completed the study (GSM n = 25, placebo n = 24). After 12 weeks, urinary CTX-II showed no significant change over time or between the groups (interaction effect P = 0.1). However, in women with symptomatic knees, a significant difference was noted between the group (treatment effect P = 0.04), as it was lower in the GSM group compared to placebo group at week 6 (P = 0.04) and week 12 (P = 0.03). Serum COMP and plasma cytokines were not affected. GSM supplementation showed

greater reduction in the VAS pain score than placebo (−13.2 ± 20.3 vs. −2.9 ± 15.9; $P = 0.04$). No significant change in KOOS domains between the two groups was observed.

Conclusion: Oral supplementation of whole GSM powder at 3 g/day may slow down the degradation of type II collagen in postmenopausal women with symptomatic knees. GSM treatment conferred clinical benefit on overall joint pain. No significant effect was noted for inflammatory cytokines, suggesting that GSM may act within the joint microenvironment rather than at the systemic level.

Clinical trial registration: [www.australianclinicaltrials.gov.au/clinical-trial-registries], identifier [ACTRN12620000413921p].

KEYWORDS

osteoarthritis, biomarker, inflammation, greenshell mussel, joint pain

Introduction

Osteoarthritis (OA) is characterized by progressive degradation of articular cartilage and loss of joint function and is considered the most common type of joint disease and leading cause of disability among the elderly (1). OA prevalence is higher among women compared to men and its incidence rises following menopause (2). Women also tend to have a greater severity of knee OA (3). This drastic increase in OA incidence among postmenopausal women is linked to estrogen, which declines after menopause. The presence of both alpha and beta estrogen receptors (ERα and ERβ) in cartilage indicates that the chondrocytes may respond to estrogen and thereby reduction in estrogen would influence the metabolism of chondrocytes (4). Furthermore, menopause is associated with weight gain and increased body mass index (BMI) which is highly correlated with risk of knee and hip OA (5, 6).

A report on the association between obesity and OA incidence for non-weight bearing joints indicates the involvement of obesity-related metabolic factors such as adipokines and pro-inflammatory cytokines (7). Interestingly, the roles of mechanical loading and inflammation in development of radiographic knee OA were found to be more relevant in overweight and obese women than men (8). Excessive fat tissue induces production and release of the adipokines and pro-inflammatory cytokines resulting in low-grade systemic inflammation (9).

In addition, it is well-documented that inflammatory immune cells are recruited into the synovial joint and are involved in initiation of pathological changes in the synovial joint and initiation of obesity-associated OA (10). Tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) are involved in cartilage degradation and bone resorption. These cytokines stimulate the expression of the cartilage-degrading enzymes, matrix metalloproteinases (MMPs), while inhibiting the formation of type II collagen and other cartilage matrix components (11).

Molecules derived from synovial joint tissue, particularly cartilage, have been used as biochemical markers of OA to detect the early change in metabolic and chemical properties of cartilage or predict disease progression and treatment monitoring (12). Type II collagen is the main component of cartilage and makes up 90–95% of the total collagen in cartilage (13). Proteolysis of type II collagen results in fragments of C-terminal telopeptides of type II collagen (CTX-II), which is measured as a biomarker of cartilage degradation. Cartilage oligomeric matrix protein (COMP) is a non-collagen structural protein involved in stabilization of extracellular matrix through interaction with collagen fibrils (14). Urine CTX-II and serum COMP are the most frequently studied biomarkers and have shown the best performance across all available biomarkers for OA. Both markers have been shown to be elevated in patients with OA and are correlated with radiographic severity of OA (15, 16). Despite extensive research and development of various markers, no single gold standard biomarker that is specific and sensitive to the damaged tissue and OA progression has been identified; therefore measuring a panel of biomarkers

Abbreviations: GSM, greenshell mussel; CTX-II, C-telopeptide of type II collagen; COMP, cartilage oligomeric matrix protein; VAS, visual analogue scale; KOOS, knee injury and osteoarthritis outcome score; OA, osteoarthritis; ER, estrogen receptors; BMI, body mass index; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1 beta; IL-6, interleukin-6; MMPs, matrix metalloproteinases; HFHS, high-fat/high-sugar; OVX, ovariectomized; RA, rheumatoid arthritis; NZPAQ-SF, New Zealand Physical Activity Questionnaire – Short Form; IPAQ, International Physical Activity Questionnaire; CV, co-efficient of variation; Cr, creatinine; CTX-1, C-terminal telopeptide of type I collagen; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ALA, alpha-linolenic acid; SPMs, specialized pro-resolving mediators; SDA, stearidonic acid; ETA, eicosatetraenoic acid; KLG, Kellgren-Lawrence grading; WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index.

is necessary to provide an accurate picture on joint tissue metabolism (12).

An extract from New Zealand green-lipped mussel (*Perna canaliculus*) known as greenshell mussel^{TM} (GSM) was found to be beneficial for joint health and symptom-relieving of OA in animal (17) and human clinical trials (18). The inhibitory effect of omega-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) present in GSM on cyclooxygenase-2 (COX-2) and the 5-lipoxygenase (5-LOX) cascade suppress synthesis of Prostaglandin E2 (PGE2) and reduce the inflammatory response (19). Furthermore, these fatty acids can resolve the existing inflammation through specialized pro-resolving mediators (SPMs) or derived compounds from EPA and DHA metabolized by LOXs. These novel anti-inflammatory molecules promote resolution of inflammation, tissue healing and relief of the chronic pain in rheumatic diseases (20, 21).

Recently, the cartilage protective activity of GSM has been demonstrated in a rat model of metabolic OA (22). The short-term pre-clinical study revealed that feeding with whole GSM powder decreased plasma levels of CTX-II, in rats fed a high-fat/high-sugar (HFHS) diet, indicating a preventive effect of GSM on cartilage degradation (22). Interestingly, in a matching long-term trial, rats were ovariectomized (OVX) to establish a model of post-menopausal OA combined with diet-induced obesity. Histopathological assessment of cartilage in knee joints demonstrated the Mankin score, a standard indicator of cartilage damage severity, was reduced in GSM-fed rats (23). Based on these observations, whole GSM powder has the potential to reduce type II collagen degradation and thereby attenuate the progression of OA in human subjects.

Previous studies have mainly focused on symptom-modifying effects of GSM extracts among OA patients and there is a lack of clinical studies using cartilage metabolism biomarkers to measure the GSM chondroprotective efficacy.

The clinical diagnosis of OA is usually made once disease is at late stage and most likely irreversible. Thus, this study targeted postmenopausal women with overweight/obesity with joint discomfort who are at risk or with early stage of OA, when an intervention is more likely to be beneficial. The current study a randomized, assessor and patient blinded, placebo-controlled trial aimed to investigate whether 12 weeks of supplementation with whole meat GSM powder supplementation affects the levels of cartilage metabolism biomarkers (primary outcome) and inflammatory cytokines along with joint pain and knee-related symptoms and function (secondary outcome) in overweight/obese postmenopausal women with joint discomfort. The placebo group was included for control. This study hypothesized that supplementation with GSM powder will decrease cartilage degradation biomarkers; urinary CTX-II and serum COMP and result in reduction of inflammatory cytokine levels, joint pain score and knee-related symptoms compared to placebo.

Materials and methods

Study participants

A total of 55 New Zealand women aged 55–75, ≥5 years post-menopause (based on the natural cessation of menstruation), with self-reported body mass index (BMI) between 25 and 35 kg/m^2 (weight status was evaluated according to definition provided by Centre of Disease Control and Prevention: BMI 25 to <30 and 30–35 fell under overweight and obese, respectively) (24), and living in the Manawatū-Whanganui area were included. Participants reporting joint pain or discomfort within ≥3 months prior to study commencement without daily use of analgesic medicine were included. Subjects were excluded if they had a formal diagnosis of clinical OA, inflammatory arthritis or rheumatoid arthritis (RA), diabetes mellitus, or atherosclerosis, having chronic liver or renal disorder detected based on the screening blood test, having allergy to mussels or seafood, history of recent joint injury or trauma, smoking or having alcohol intake of more than two units per day, being on hormone replacement therapy for <6 months prior to the beginning of the trial, or taking anti-inflammatory drugs (glucocorticoids or NSAIDs) on a daily basis.

Study design

A 12-week randomized; blinded, placebo-controlled study design was conducted. Women who met the initial inclusion criteria were screened by a routine non-fasted blood test for liver and kidney function, blood glucose (HbA1c), and lipid profile including triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol at MedLab Central, Palmerston North, New Zealand. The purpose of routine blood tests was to screen the potential participants for liver and kidney disease and were repeated at the end of study to assess the safety of supplement. Prior to the baseline visit, participants who were regularly consuming oily fish (more than one meal per week) or taking fish oil or other joint health supplements were required to undergo a 4-week washout period. Then participants were randomly allocated into two groups, each consuming six capsules per day for 12 weeks: whole meat GSM powder (3 g/day) or placebo (sunflower seed protein). The subjects were instructed to consume the capsules with or after their meals.

The flash-dried whole meat GSM powder used in this study was comprised of 41.4% protein, 30.8% carbohydrate, 10.1% fat (EPA and DHA was 20.7 and 8% total fatty acids, respectively), 10.7% ash, and 7% moisture. The dose of 3 g/day was selected as it is achievable through diet (equivalent to 1–2 mussels) and this dose and duration were comparable to previous studies using whole GSM extracts in knee OA patients which resulted in pain improvement without any major adverse side effects

(18). Flash dried whole meat GSM powder was produced by Sanford Ltd (PernaUltra^{TM}, Sanford, Blenheim, New Zealand) using standard manufacturing processes. Sunflower seed protein (BP Bulk powders, Braeside, Melbourne, Australia) was used as placebo as a neutral source of protein and was selected to be relatively similar to GSM powder in respect to macronutrient composition (66.6% carbohydrate, 24.3% protein, 3% fat, 3.4% moisture, and 2.7% ash) and to be as inert and non-bioactive as possible. Both GSM powder and placebo were encapsulated in hard-shell capsules by a commercial facility (Alaron, Nelson NZ) and stored under nitrogen in the dark at room temperature or lower until use. The GSM and placebo capsules were matched in the shape, size, and color of hard-shell encapsulant. Activated carbon sachets for absorbing moisture and odor were put in bottles to conceal any "fishy" odor. The nutritional composition and fatty acids profile of GSM powder and placebo used in the study is presented in **Supplementary Table 1**.

A randomization list was generated by Excel and maintained by project's supervising investigator, who did not interact with the study subjects or conduct the primary data analysis. Randomization was stratified based on BMI (overweight: 25–29.9 kg/m^2 and obese: 30–35 kg/m^2) and age (55–64, 65–75 years) distribution. The primary researcher who was blinded to treatments code allocated participants to two supplements (A and B). Participants were blinded to treatment group until all analyses were completed. Data were collected during participants' visit at baseline, follow-up (week 6) and end of the study (week 12) as shown in **Figure 1**.

Recruitment, screening, and data collection took place at the Human Nutrition Research Unit (HNRU) at Massey University, Palmerston North, New Zealand from August 2020 to September 2021.

Demographic, anthropometric, and physical activity measurement

At baseline, participants completed a demographic questionnaire as well as anthropometric measurements including body weight and standing height measured using a beam balance to the nearest 0.2 kg and stadiometer to the nearest 0.1 cm, respectively. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Physical activity was assessed by the New Zealand Physical Activity Questionnaire – Short Form (NZPAQ-SF) (25). The NZPAQ has been validated by Boon et al. (26), and physical activities were computed by metabolic equivalent of task (METs)-min/week, which was calculated by the scoring protocol of International Physical Activity Questionnaire (IPAQ) for continuous score (27).

Metabolic equivalent of task values and formula for calculation of MET-minutes were assessed and used as below:

- Walking MET-minutes/week at work = 3.3 × walking minutes × walking days at work.
- Moderate MET-minutes/week at work = 4.0 × moderate-intensity activity minutes × moderate intensity days at work.
- Vigorous MET-minutes/week at work = 8.0 × vigorous-intensity activity minutes × vigorous.
- Total Work MET-minutes/week = sum of Walking + Moderate + Vigorous MET-minutes/week scores at work.

Dietary intake assessment

Each participant's daily nutrient intake from the diet was measured using a 3-day food record including 2 weekdays and 1 weekend day at the midpoint of the trial. The 3-day food record has been recommended and considered as the "gold standard" for dietary assessment. Instructions on how to accurately complete the food record was provided (28). The brand name of food products, recipes, and food preparation were recorded. Each participant's nutrient intake was calculated using Foodworks 9 Professional, Xyris Software.

Biochemical analyses

Second void morning urine specimens were collected after overnight fasting at baseline, weeks 6 and 12 for assessment of CTX-II. Overnight fasting blood samples were collected by a certified phlebotomist at baseline, weeks 6 and 12 to measure COMP. Blood samples were collected into serum and EDTA-anticoagulated plasma vacutainer tubes. The blood sample tubes for serum collection, were incubated for 1 h at room temperature, followed by centrifugation at 2,264 g for 10 min at 4°C (Gyrozen 1248R Multi-Purpose High-Speed, Korea) to isolate the serum. The EDTA tubes were centrifuged immediately after blood drawing in the same manner to collect the plasma. Serum, plasma and urine samples were aliquoted and stored at −80°C until use.

Measurement of serum biomarkers was performed using commercially available enzyme linked immunoassay (ELISA) kits. Assays for serum cartilage oligomeric matrix protein (COMP) were performed with BioVendor Research and Diagnostic Products (Karasek, Czechia). The detection limit was 0.4 ng/ml. The intra-assay precision co-efficient of variation (CV) was 4.0–8.0% and the inter-assay precision CV was 3.1–6.6%.

Urinary CTX-II concentrations were determined using an enzyme immunoassay (EIA) kit (Urine CartiLaps® EIA; Immunodiagnostic systems, Herlev, Denmark). Urinary creatinine (Cr) was measured by colorimetric method (RX Daytona+; Randox Laboratories Ltd.). Concentrations of

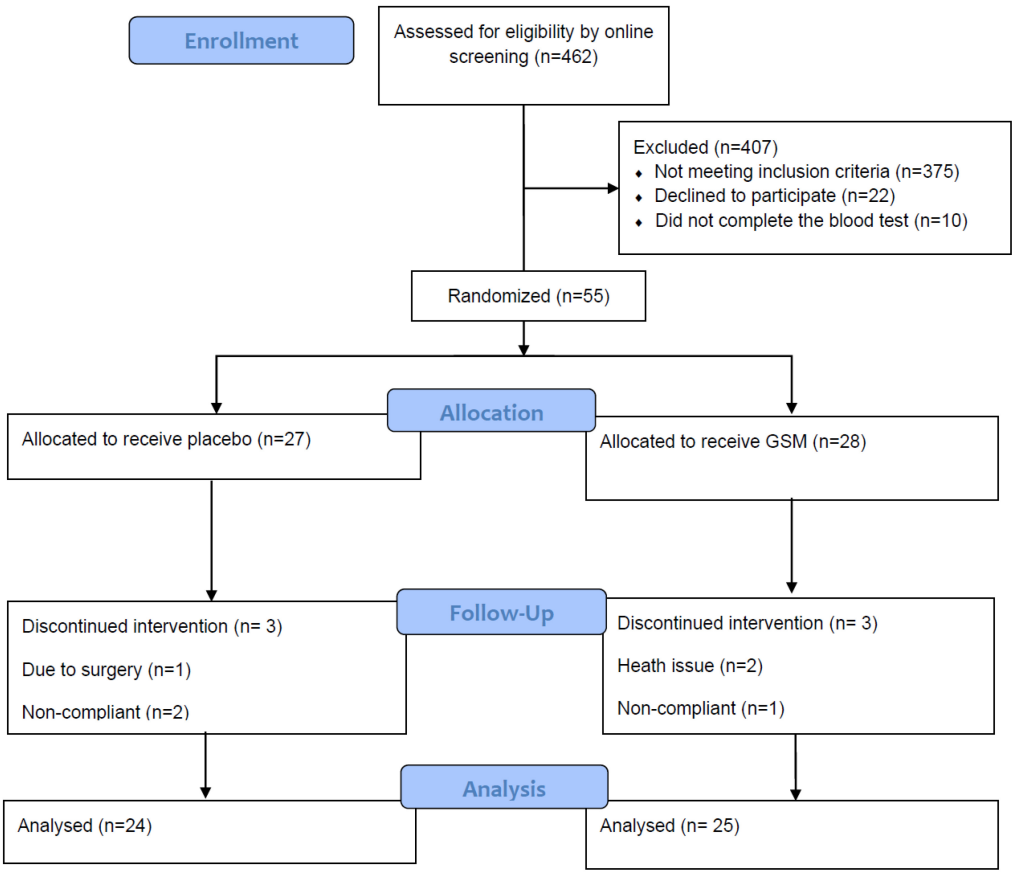

FIGURE 1
Schematic diagram of study design.

urinary CTX-II were corrected by urinary Cr using the following formula: corrected CTX-II value (ng/mmol Cr) = 1,000 × urine CartiLaps (μg/L)/creatinine (mmol/L). The detection limit was 0.2 ng/ml. The intra-assay precision CV were 5.2, 4.6, and 7.8% for high, medium and low ranges of measurement. The inter-assay precision CV were 6.9, 10.8, and 12.2% for high, medium and low ranges of measurement. Serum COMP and urinary CTX-II were assessed in duplicate.

Plasma C-terminal telopeptide of type I collagen (CTX-1) and parathyroid hormone (PTH) at baseline were analyzed by electrochemiluminescence immunoassays using the Roche COBAS® e411 system (Roche Diagnostics, Indianapolis, IN, USA). Cytokine assays were performed using BioLegend® LEGENDplex Multi-Analyte Flow Assay following the kit instructions and measured using a Beckman Coulter Gallios flow cytometer. Levels of cytokines including TNF-α, IL-1β, IL-6, IL-4, IL-10, IL-15, and IL-18 were quantified in plasma at baseline and end of the study. Baseline level of plasma 25(OH) vitamin D were analyzed using isotope-dilution liquid chromatography-tandem mass spectrometry (ID-LC-MSMS) by Canterbury Health, Christchurch, New Zealand. Serum 25(OH)D ≥ 50 nmol/L at the end of winter, and 10–20 nmol/L higher at the end of summer to allow for seasonal variation, has been considered optimal for musculoskeletal health for people residing in Australia and New Zealand (29). Vitamin D insufficiency or deficiency in this study was considered as plasma 25(OH)D < 50 nmol/L.

Self-assessment of pain visual analogue scale and knee injury and osteoarthritis outcome score

Secondary outcome measures including pain visual analogue scale (VAS) and knee injury and osteoarthritis outcome score (KOOS) were recorded at baseline and week 12. The pain levels were reported by participants using a 100 mm linear measure of pain status scored from 0 to 100 mm where 0 was defined as having no pain and 100 the worst pain ever experienced within the past week. Pain rated at ≥30 was regarded as having a moderate to high level of pain. This cut-off was selected based on the required entry criteria of a previous

clinical trial (30). Participants with more than one joint site with pain completed the VAS for overall joint pain.

Knee Injury and Osteoarthritis Outcome Score is commonly utilized in research and clinical practice to measure short- and long-term consequences of knee problems (31). The previous week is the period included when answering the questions about the knee problem. It consists of 42 items which cover five domains: knee pain (Pain), other symptoms (Symptoms), activities of daily living (ADL), function in sport and recreation (Sport/Rec) and knee related quality of life (QOL). All items are scored on a 5-point Likert scale (0–4), and each domain is scored separately as the sum of all corresponding items. A total score has not been validated and is not recommended. Scores are then converted to a 0–100 scale (percentage of total possible score obtained), where 0 represents extreme knee problems and 100 represents no knee problems (32). This questionnaire was completed by those who reported knee pain and established cut-off score of ≤86 for any of the domains is used to classify individuals with symptomatic knees (33). The validity of KOOS has previously been demonstrated by construct and content and good to excellent test-retest reliability (34, 35).

Compliance assessment

To assess subjects' compliance, diaries were provided to participants at baseline to record their daily intake of study supplement and analgesic medications. Participants were allowed to continue taking paracetamol or any supplements that did not contain omega-3 fatty acids or chondroprotective bioactive compounds. Compliance assessment was performed using cumulative capsule counts at the completion of the study, and adherence was measured as a percentage: [(number of capsules provided minus number of unused capsules)/number of capsules provided] × 100. Adherence below 80% was considered a protocol violation.

Moreover, at the baseline and end of the study, the plasma and red blood cell membrane *n*-3 PUFA, EPA, DHA, and total *n*-3 L-C PUFA (including alpha-linolenic acid (ALA, 18:3 *n*-3), stearidonic acid (SDA, 18:4 *n*-3), eicosatetraenoic acid (ETA 20:4 *n*-3), EPA, docosapentaenoic acid (DPA,22:5 *n*-3) and DHA) were measured to assess the adherence to study protocol. The plasma and red blood cell *n*-3 PUFA were analyzed by gas chromatography (GC, Agilent Technologies Australia, VIC, Australia). Fatty acids were identified to an external commercial fatty acid standard. The analysis were done at the Cawthron Institute, Nelson, New Zealand and methodologies are published elsewhere (36).

Safety assessment

Any adverse side effect was recorded by participants in their diaries. Participants documented the events by rating the severity (mild, moderate, and severe) and medications required to treat the events. Moreover, routine laboratory blood test including liver and kidney function tests, blood glucose (HbA1c) and lipid profile (triglyceride, total cholesterol, HDL-cholesterol, and LDL-cholesterol) were assessed from non-fasted venous blood samples at baseline and end of trial at MedLab Central Palmerston North, New Zealand.

Statistical methods

The sample size was based on urine CTX-II/ creatinine and serum COMP as the primary outcomes of the study. Sample size was calculated to detect 20% difference between the groups using the standard deviation from an unpublished report. For urine CTX-II/ creatinine a sample size of 24 was required to detect a 20% relative difference from baseline with 80% power. For serum COMP a sample size of 17 was required to detect a 20% difference between groups with a power of 95%. The sample size of 48 (n = 24 per group) was required as a manageable sample size. Finally, a total sample size of 55 was needed to allow for at ∼10% potential dropout rate (n = 27–28 per group).

Statistical analysis was performed using IBM SPSS version 26.0 (Armonk, NY, USA). Analysis was conducted on the dataset from participants who completed assessment at both timepoints (baseline and endpoint). Variables were checked for normality using the Kolmogorov-Smirnov, Shapiro-Wilk tests and data that were not normally distributed were log-transformed. The data were reported as mean ± standard deviation (SD) for normally distributed data, and as median (25th, 75th percentiles) for non-normally distributed data, and as frequencies for categorical data. The baseline characteristics of subjects between two treatment groups were compared using Student's t-test for parametric, and the Mann-Whitney U test for nonparametric data. Regarding the categorical variables, the distribution of participants was analyzed using the Chi-square tests or Fisher's exact test where more than 20% of data cells had expected count below 5.

Missing data points were imputed with mean values of each group (the mean value of each group was assigned to those with missing data) to include all the data in the analysis. Outcome analyses were conducted on data with and without imputed missing values. Two-way repeated measures ANOVA was used to examine differences within each group over time (pre- vs. post-intervention) and between the groups (GSM vs. placebo). In case of significant effect, analysis was followed by post hoc comparison using the Tukey test. For analysis of cartilage markers, the data were analyzed on both mean value and value relative to baseline in order to reduce the variability and achieve the normal distribution. In order to control the effect of the main potential confounding factors, age and BMI on outcome measures, particularly cartilage markers, the treatment groups were stratified for these factors (37).

The interactions between treatments and time indicate differences in efficacy. For VAS pain and KOOS score analysis, covariates including baseline level of VAS pain or KOOS domain score, compliance, paracetamol use, and season of enrolment were adjusted in models. The relationships between cartilage degradation markers, plasma 25(OH)D, VAS, and KOOS domain score were assessed using Pearson correlation. Statistical significance was considered by two-sided $P < 0.05$.

Results

Baseline characteristics of participants

The flow diagram of study is presented in Figure 1. Initially 462 women filled a pre-screening online questionnaire, and the majority of them were excluded due to distance from the location of research or being diagnosed with health condition mentioned in the exclusion criteria. Finally, 66 women passed the online screening and were phone interviewed and invited for a blood test screen. From this group, 55 completed the blood test and were eligible for trial entry. Of the 55 enrolled participants, six subjects dropped out from the trial. Finally, a total of 49 participants (GSM, n = 25 and placebo, n = 24) completed the study. The baseline characteristics of the participants who completed the study are shown in Table 1. It is important to note that due to COVID-19 restrictions, one subject from the GSM group missed a follow-up visit. For blood markers analysis, blood samples were not available from two participants at week 6 from GSM group (one due to missing the visit due to COVID-19 lockdown and one due to phlebotomy issues). Urine samples were provided by all participants from both groups at all time points, except for one participant from the GSM group due to missing the visit at week 6.

TABLE 1 General characteristics of participants who completed the study.

General characteristics	Overall population (n = 49)	Placebo (n = 24)	GSM (n = 25)	P-value
Age (years), mean ± SD	63.5 ± 5.4	62.9 ± 5.4	64.2 ± 5.1	0.3
Height (cm), mean ± SD	164.8+6.7	164.7 ± 6.4	164.8 ± 7.1	0.8
Weight (kg), median (25th, 75th percentiles)	75.7 (68.3, 86.2)	73.8 (68.2, 88.6)	77.2 (68.5, 86.2)	0.9
BMI categories [n (%)]				0.6
Overweight	33 (67.4)	17 (70.8)	16 (64)	
Obese	16 (32.6)	7 (29.2)	9 (36)	
Physical activity (MET-minutes/week), median (25th, 75th percentiles)	764 (307.5, 1794)	751 (318, 2373.7)	764 (287, 1483)	0.1
Ethnicity [n (%)]				0.1
NZ European	44 (89.1)	20 (83.3)	24 (96)	
Māori/Other	5 (10.2)	4 (16.7)	1 (4)	
Season of enrolment [n (%)]				
Spring	7 (14.3)	6 (25)	1 (4)	
Summer	17 (34.7)	6 (25)	11 (44)	0.1
Autumn	22 (44.9)	10 (41.7)	12 (48)	
Winter	3 (6.1)	2 (8.3)	1 (4)	
Whole body T-score ≤2.5 [n (%)]	12 (24.5)	6 (25)	6 (24)	0.9
VAS pain score ≥ 30 [n (%)]	21 (42.9)	7 (29.2)	14 (56)	**0.05**
KOOS domain score ≤ 86 [n (%)]	39 (79.6)	18 (75)	21 (84)	0.4
Paracetamol use [n (%)]				0.2
Yes	14 (28.5)	5 (20.8)	9 (36)	
No	35 (71.4)	19 (79.2)	16 (64)	
Biochemical markers				
Plasma CTX-I (μg/L), mean ± SD	0.44 ± 0.14	0.46 ± 0.13	0.43 ± 0.16	0.4
Plasma 25(OH)D (nmol/L), median (25th, 75th percentiles)	73 (56, 83)	69.5 (46, 79.7)	78 (64, 87)	**0.007**
Plasma PTH (picomol/L), median (25th, 75th percentiles)	4.3 (3.5, 5.3)	4.7 (4.2, 6.4)	3.8 (3.5, 4.8)	**0.007**

BMI, body mass index (kg/m^2); MET, metabolic equivalent of task; VAS, visual analogue scale; KOOS, knee injury and osteoarthritis outcome score; CTX-I, C-terminal telopeptides of type I collagen. Values are presented as mean ± standard deviation or median (25th and 75th percentile) for normally distributed and non-normally distributed variables, and n (%) for categorical variables for which the percentage within each treatment group is reported. Significance level ($P < 0.05$) is indicated in bold.

The two groups were similar at baseline with respect to most demographic characteristics. For the overall study population, the mean age was 63.5 ± 5.4 years; 67.4% of women were overweight (BMI between 25 and 29.9 kg/m^2) and 32.6% were obese (BMI ≥ 30 kg/m^2).

The median (25th, 75th percentile) of physical activity level was 751 (318, 2373.7) MET-minutes/week and 764 (287, 1483) MET-minutes/week in placebo and GSM group, respectively, with no significant difference between the groups.

The majority of participants (89.1%) were of European-New Zealand ethnicity. Paracetamol use during the study was reported by 14 (28.5%) of the participants and did not differ between groups.

Some significant differences were observed. Out of 49 women, 21 (42.9%) were characterized as having moderate to high levels of joint pain (VAS pain score ≥ 30) and this was significantly different between the groups with a higher proportion in the GSM group (56 vs. 29.2%, $P = 0.05$). In term of knee related problems, 39 (79.6%) women had knee symptoms (KOOS domain score ≤ 86). With respect to joint pain location, 18 (36.7%) had only knee pain, 21 (42.8%) had pain at knee and hip or other joints, and 10 (20.4%) reported pain at hand and/or back or shoulder.

The baseline level of plasma CTX-I was comparable between the two groups, while the baseline level of 25(OH)D and PTH were significantly different between the groups, as vitamin D level was higher and PTH was lower in the GSM group compared to the placebo group ($P = 0.007$). However, the percentage of participants with vitamin D insufficiency or deficiency [25(OH)D below 50 nmol/L] was not significantly different between the groups with 2 (8%) in the GSM group and 6 (25%) in the placebo having vitamin D levels below the normal range ($P = 0.1$).

Daily energy and nutrient intake of participants are presented in **Supplementary Table 2**. The daily nutrient intake of participants showed no differences between the groups. The average consumption of fish and seafood among the participants prior to enrolment to the study was less than once a week (60%), once a week (35%) and more than once a week (5%), and none were a regular mussel eater.

Evaluation of treatment on cartilage degradation markers

At baseline, the mean urinary CTX-II level was 560.4 ± 428 ng/mmol Cr in GSM group and 583.3 ± 411 ng/mmol Cr in placebo group and there was no significant difference between the groups ($P = 0.8$). As demonstrated in **Figure 2A**, the urinary CTX-II level slightly decreased from the baseline during intervention in the GSM group, while it notably increased from baseline and peaked at week 6 and then slightly reduced at week 12 in placebo group, however, the overall change was not significant between the groups (interaction effect $P = 0.3$).

The baseline level of serum COMP was 972.3 ± 272 and 1,040.4 ± 402 ng/ml in the GSM and placebo groups, respectively. As shown in **Figure 2B**, serum COMP trended to slightly decrease in placebo and increase in GSM group. Overall, it remained stable and did not change meaningfully over the study period or between group (interaction effect $P = 0.1$).

To further evaluate the effect of GSM supplementation on urinary CTX-II level, subjects with a KOOS domain score 86 or below were included in a further analysis (GSM, $n = 21$ and placebo, $n = 18$). The baseline characteristics of these subjects and level of cartilage degradation biomarkers were not statistically significant between the treatment groups (data not shown). As shown in **Figure 3**, in subjects with symptomatic knees, the urinary CTX-II showed similar pattern of change as overall population. The result of analysis on participants with KOOS below 86 showed urine CTX-II level were significantly different among the treatment groups during the intervention (treatment effect $P = 0.04$) with significantly lower levels in the GSM group compared to placebo at week 6 (534.6 ± 255.4 vs. 824.7 ± 570.4 ng/mmol Cr, $P = 0.04$) and end of the study (496.6 ± 204.2 vs. 757.4 ± 493.2 ng/mmol Cr, $P = 0.03$). However, there was no significant change over time within groups (time effect $P = 0.9$) and between groups (interaction effect $P = 0.3$).

The analysis for urine CTX-II and serum COMP was conducted on the data corrected for baseline as shown in **Supplementary Figure 1**. There was a significant change overtime (time effect $P = 0.03$), although the overall change was not significant between the groups (interaction effect $P = 0.1$; **Supplementary Figure 1A**). The result for serum COMP corrected for the baseline was similar to uncorrected as no significant effect was noted (**Supplementary Figure 1B**).

The result of analysis for data without imputing missing values were similar to the imputed data.

Evaluation of treatment on visual analogue scale pain and knee injury and osteoarthritis outcome score domains

The baseline VAS pain score in the GSM group was 21.6 ± 15.9 and in the placebo group was 29.4 ± 21 and there was no significant difference between the two groups ($P = 0.07$).

The pattern of change in VAS pain score over the study period is presented in **Figure 4**. There was a significant change in VAS pain score between the groups. Both unadjusted and adjusted analysis of the VAS pain score showed a greater reduction from baseline in the GSM group compared with placebo (−13.2 ± 20.3 vs. −2.9 ± 15.9, $P = 0.03$ unadjusted, and $P = 0.04$ adjusted for covariates). A significant time effect

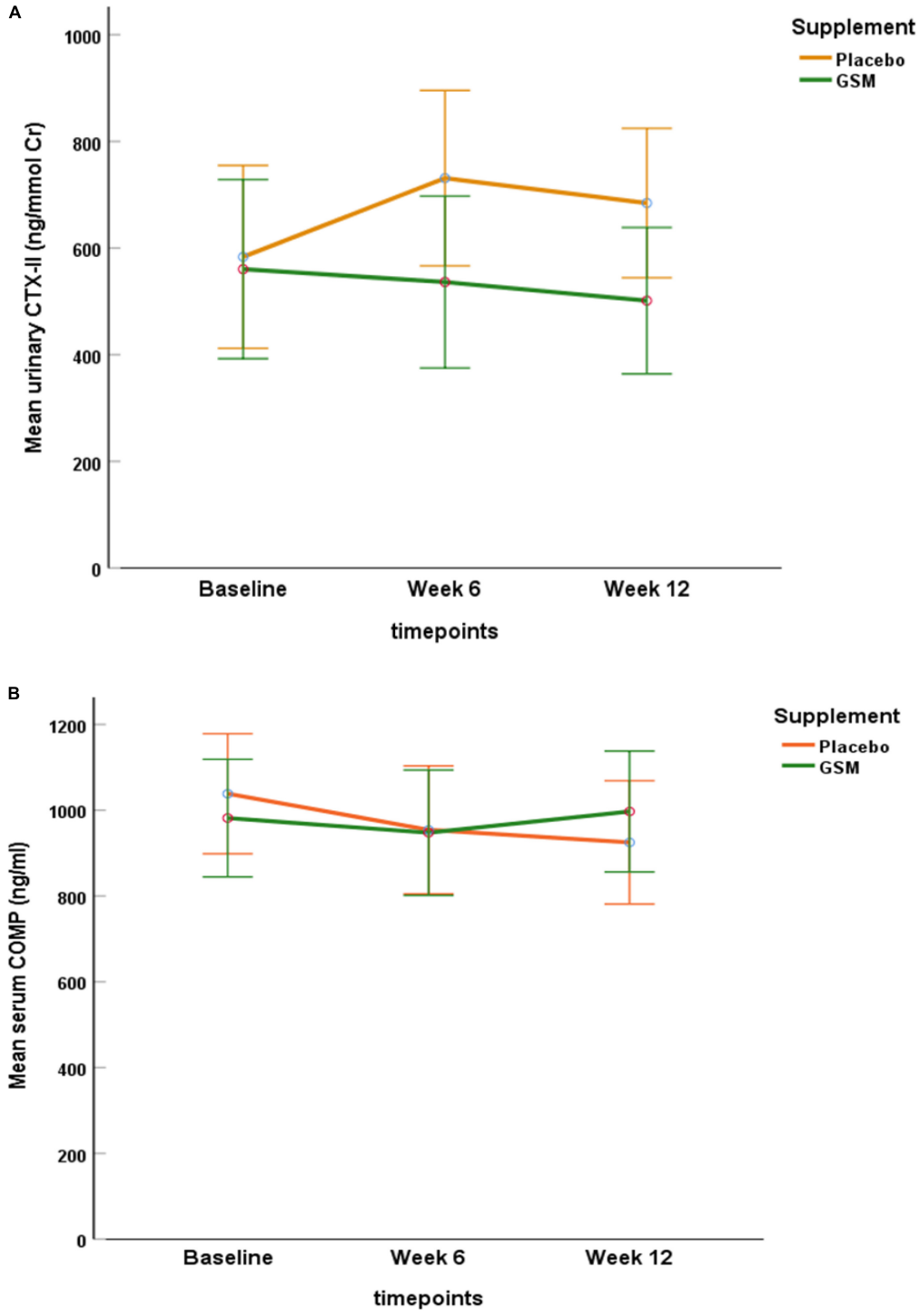

FIGURE 2
Pattern of change in urinary C-telopeptide of type II collagen (CTX-II) level **(A)**, and serum level of cartilage oligomeric matrix protein (COMP) **(B)** over the study period (baseline, follow-up, and endpoint) within each of the treatment groups. No significant neither over time nor between the groups for urine CTX-II and serum COMP (interaction effect $P = 0.3$, $P = 0.1$, respectively). Data are expressed as the mean ± standard error.

(the difference between baseline and endpoint) was found for the VAS pain score ($P = 0.002$ unadjusted). The rate of positive response or minimal clinical improvement in VAS pain score (at least 10 mm reduction) was 56% in GSM group as compared with 29% in placebo group ($P = 0.05$), as shown in Figure 5.

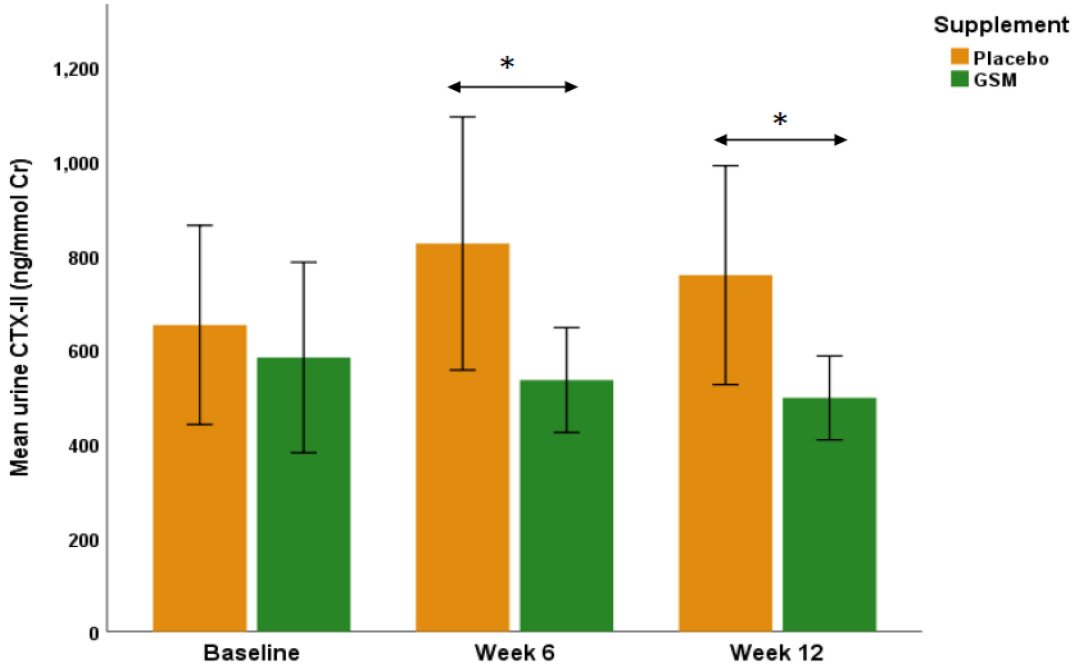

FIGURE 3
The urinary C-telopeptide of type II collagen (CTX-II) levels in those subjects with knee injury and osteoarthritis outcome score (KOOS) domain score of 86 or below in GSM group (n = 21) and placebo (n = 18). There was a significant difference between groups as urinary CTX-II was significantly lower in GSM compared to placebo at week 6 (P = 0.04) and week 12 (P = 0.03). The values are expressed at mean ± standard error and compared by Student's t-test at each time point. *Indicates the significance ($P < 0.05$).

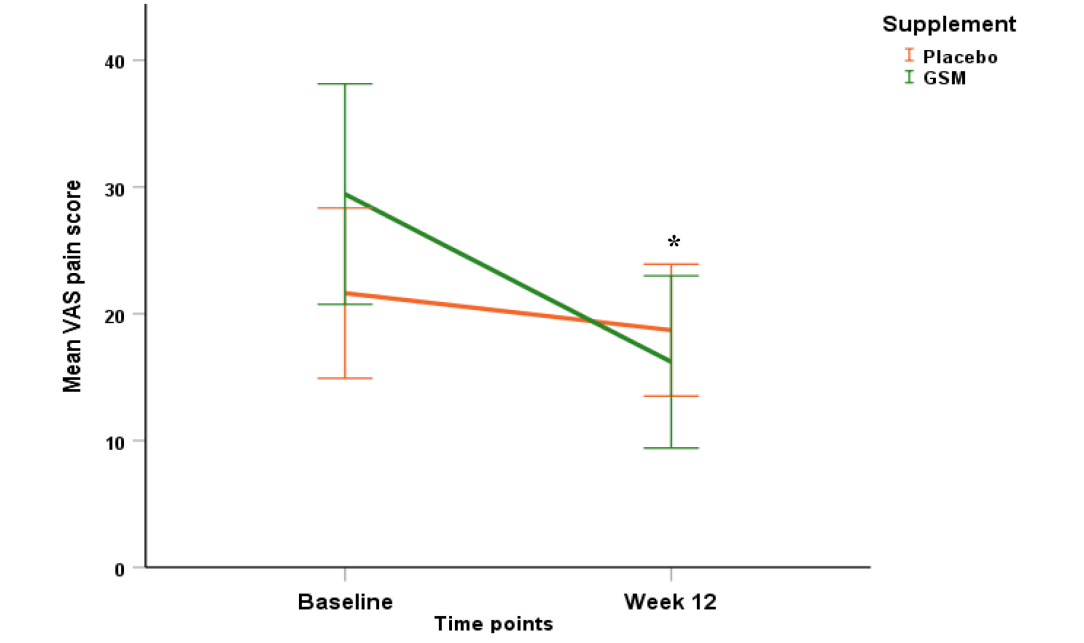

FIGURE 4
Pattern of change in visual analogue scale (VAS) pain score over the study period (baseline and endpoint) within each of the treatment groups. Significant effect of time (P = 0.002), and greater reduction in VAS pain score in GSM supplement compared to placebo (interaction effect P = 0.03 unadjusted and P = 0.04 adjusted for baseline level, compliance, use of paracetamol and season of enrolment). Placebo = orange, GSM = green. Values are expressed as mean (95% confidence interval). *Indicates the significance ($P < 0.05$).

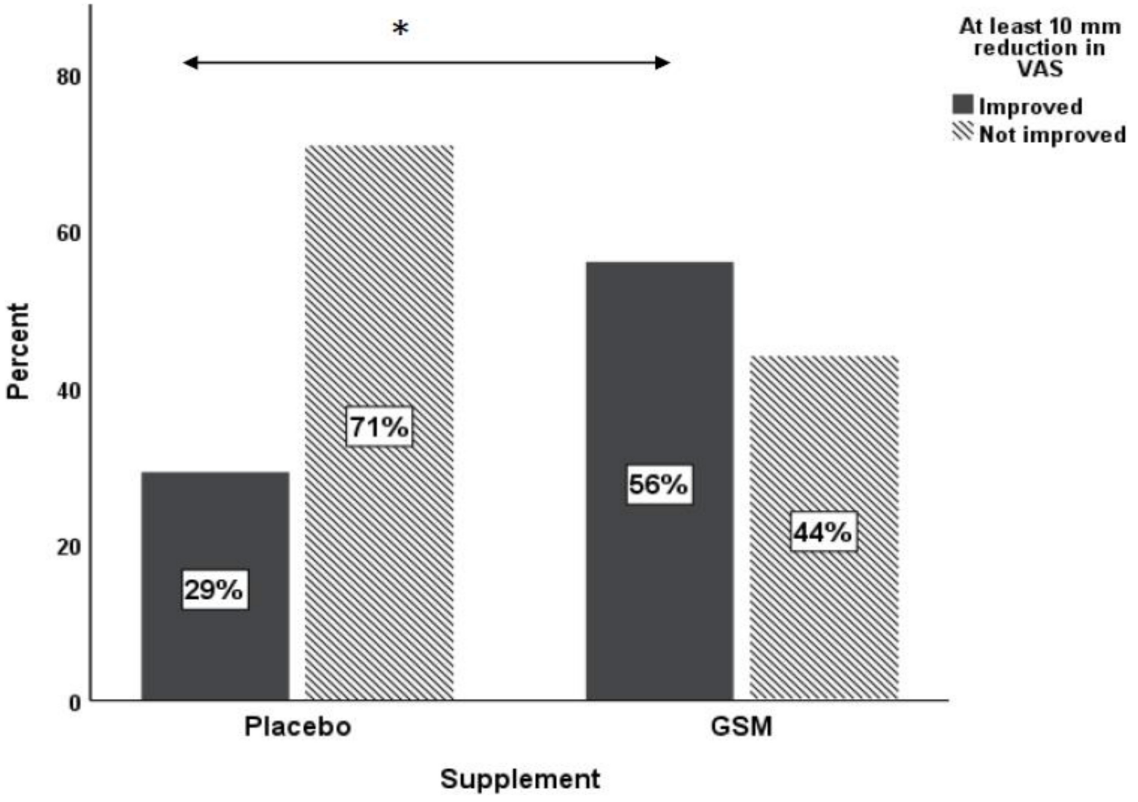

FIGURE 5
Proportion of responders [those who had ≥10 mm reduction in baseline visual analogue scale (VAS)] versus non-responders in placebo and GSM supplement groups. *Pearson's chi-square test $P = 0.05$.

Further assessments of the KOOS domains focused on subjects with symptomatic knees (those with a score ≤ 86). The mean ± SD of baseline, endpoint, change in KOOS domains score over time, and difference in change between the treatment groups are presented in Table 2. The baseline level of KOOS for any of domains was not different between the groups. There was no significant change between the two groups for any KOOS domains. However, a significant time effect was found for the KOOS pain domain ($P = 0.002$ unadjusted); however, it lost its significance after adjustment for covariates ($P = 0.3$ adjusted). Both unadjusted and adjusted analysis of KOOS symptoms domain revealed a significant time effect ($P = 0.02$ unadjusted, and $P = 0.03$ adjusted) with greater improvement from baseline in the GSM group, although this change was not statistically significant between the groups ($P = 0.6$).

Evaluation of treatment on plasma cytokines

The plasma cytokines measured were present at low concentrations (pg/mL) and in some of the samples were not detectable, which were not replaced with the limit of quantification (LOQ) in order to avoid overestimating the cytokine level. The levels of cytokines showed high variability and distribution was skewed. The medians (25th and 75th percentile) of plasma cytokines at baseline and end of the study and mean ± SD of % change from baseline is presented in Table 3. There were no significant differences between the groups at each time point (baseline and endpoint) or over the time within the groups for any of the cytokines assessed by Mann-Whitney *U* test and paired *t*-test, respectively.

Correlation between the cartilage degradation markers, plasma 25(OH)D, and self-reported knee injury and osteoarthritis outcome score outcomes

As shown in Table 4, no correlation could be found between serum COMP and urinary CTX-II levels ($r = 0.103$, $P = 0.4$). However, a significant negative correlation was found between urinary CTX-II levels and KOOS pain score ($r = -0.292$, $P = 0.02$), symptoms score ($r = -0.276$, $P = 0.01$), and ADL

TABLE 2 Mean ± SD of knee injury and osteoarthritis outcome score (KOOS) domain scores over the 12 weeks of study across treatment groups[1].

KOOS domains	Placebo (n = 18)	GSM (n = 21)	P-value*		
			Time effect	Treatment effect	Interaction effect
Pain					
Baseline	73.0 ± 14.2	79.5 ± 16.1	0.3	0.1	0.8
Endpoint	79.0 ± 16.1	86.4 ± 12.7			
Change	6.5 ± 14.6	7.4 ± 11.7			
Difference in change		0.912 (−4.4, 6.2)			
Symptoms					
Baseline	67.4 ± 14.7	74.0 ± 15.8	**0.03**	0.09	0.6
Endpoint	71.4 ± 17.8	81.2 ± 17.9			
Change	4.0 ± 18.7	7.2 ± 11.6			
Difference in change		3.2 (−2.0, 8.4)			
Activities of daily living					
Baseline	77.2 ± 13.6	81.8 ± 17.2	0.2	0.3	0.8
Endpoint	84.5 ± 15.0	88.6 ± 15.1			
Change	7.2 ± 13.1	6.8 ± 14.8			
Difference in change		−0.33 (−7.0, 6.3)			
Sport/recreation					
Baseline	59.8 ± 27.8	70.3 ± 26.5			
Endpoint	67.8 ± 24.7	69.0 ± 32.5	0.7	0.3	0.1
Change	8.0 ± 25.6	−1.2 ± 31.0			
Difference in change		−9.2 (−23.4, 4.8)			
Quality of life					
Baseline	49.8 ± 15.2	59.8 ± 22.7			
Endpoint	61.9 ± 22.3	70.3 ± 22.2	0.1	0.1	0.8
Change	12.1 ± 22.1	10.4 ± 18.3			
Difference in change		−1.6 (−9.9, 6.7)			

Standardized scores for each of KOOS domain ranged from 0 to 100, with higher scores representing lower pain levels and a better KOOS response.
[1]Participants with cut-off scores of 86 or below were included in analysis. Mean (95% confidence interval) for difference in change between the groups (GSM vs. placebo). *The two-way repeated measure ANOVA analyses were adjusted for baseline level, compliance, paracetamol use, and season of enrolment. Significance level ($P < 0.05$) is indicated in bold.

score (r = −0.443, P = 0.002). Serum COMP did not show a correlation with any of the KOOS domain scores.

Correlations between baseline plasma 25(OH)D and cartilage markers and KOOS domain scores were also evaluated. Plasma 25(OH)D levels did not show any significant correlation with urinary CTX-II or serum COMP levels. However, a weak and insignificant positive correlation appeared between plasma 25(OH)D and KOOS pain (r = 0.240, P = 0.09), ADL score (r = 0.253, P = 0.07) and quality of life (r = 0.252, P = 0.08).

As expected, the baseline plasma CTX-I level were shown to be negatively correlated with whole body mass density (r = −0.355 P = 0.01) and not with any of cartilage markers.

Medication and analgesic use over the study period

Participants continued their current medications prescribed to them by their physician for management of chronic diseases throughout the trial. The type of medications included cholesterol-lowering agents, anti-hypertensive medications, proton pump inhibitors, anti-depressants, and thyroid medications. The majority of subjects received COVID-19 vaccinations during the study. In the GSM group, 36% (n = 9) of subjects used analgesic medication (Paracetamol) for joint symptoms, compared with 20.8% (n = 5) in the placebo group. During the study, 8% (n = 2) of subjects in the GSM and 16.6% (n = 4) in the placebo group took NSAIDs (diclofenac sodium and ibuprofen) for headaches or migraine.

Safety and adverse events

Baseline blood analyses indicated that total cholesterol and LDL were above the normal range and elevated in both groups. HbA1c was also close to the upper cut-off of the normal range. Cholesterol and blood glucose may be elevated with obesity and menopause. There was no difference between the groups at the

TABLE 3 Median (25th, 75th percentile) and mean ± SD of plasma cytokines at baseline, end of the study and % change from baseline and number of participants with measurement above the detection limit in each group.

Cytokines (pg/mL)	Placebo		GSM		*P*-value*
TNF-α		*N* = 20		*N* = 20	
Baseline	26.8 (16.0, 98.2)		30.6 (9.0, 66.0)		0.4
Endpoint	37.0 (8.7, 104.3)		26.4 (14.9, 51.8)		0.5
% Change	32.6 ± 158		40.3 ± 131		0.8
IL-1β		*N* = 20		*N* = 17	
Baseline	15.3 (7.0, 49.6)		9.0 (4.1, 24.2)		0.3
Endpoint	8.5 (6.4, 92)		9.3 (4.9, 14.4)		0.3
% Change	70.7 ± 268		46.6 ± 162.2		0.7
IL-6		*N* = 22		*N* = 25	
Baseline	7.0 (3.9, 15.8)		4.6 (2.3, 10.8)		0.2
Endpoint	7.9 (4.2, 18.7)		5.1 (3.4, 9.7)		0.1
% Change	59.5 ± 183		45.8 ± 129.9		0.7
IL-15		*N* = 19		*N* = 18	
Baseline	344.9 (298.8, 706.5)		336.5 (241.4, 443.3)		0.2
Endpoint	376.3 (274.3, 822.7)		382.8 (288.6, 425.2)		0.5
% Change	7.7 ± 289.8		18.9 ± 231.6		0.3
IL-18		*N* = 22		*N* = 23	
Baseline	152.4 (88.7, 168.7)		114.8 (68.6, 199.9)		0.6
Endpoint	116.4 (104.2, 185.6)		105.0 (78.6, 159.7)		0.2
% Change	10.0 ± 54.4		6.6 ± 52.1		0.8
IL-4		*N* = 21		*N* = 24	
Baseline	47.6 (29.9, 86.0)		45.8 (20.2, 83.9)		0.4
Endpoint	41.4 (23.2, 149.7)		46.7 (20.2, 97.0)		0.4
% Change	49.2 ± 167.2		38.5 ± 137.0		0.8
IL-10		*N* = 20		*N* = 19	
Baseline	4.7 (2.5, 12.2)		3.4 (2.1, 7.3)		0.4
Endpoint	4.9 (2.3, 13.5)		4.3 (1.9, 6.3)		0.4
% Change	23.9 ± 91.2		17.4 ± 81.9		0.8

*No significant difference between GSM vs. placebo at baseline and endpoint using Mann-Whitney *U* test. No significant difference was observed overtime (baseline vs. endpoint) within the group using paired *t*-test.

end of the study for the lipid profile other than HDL, liver enzymes and kidney function tests (**Supplementary Table 3**). Of all 49 subjects who completed the study, 20% (n = 5) of participants in the GSM group and 8.3% (n = 2) in the placebo group reported adverse events that occurred on a few occasions during the intervention. The most frequent adverse event reported was mild to moderate indigestion and reflux (GSM, n = 3 and placebo, n = 1) for which two participants took omeprazole. Other adverse events include mild abdominal pain (GSM, n = 1 and placebo n = 1), and nausea (GSM, n = 1).

Compliance and adherence to study supplement

A generally high adherence was observed (98%) in both groups. Two participants (one in GSM, and one in placebo) were not able to complete the final visit on week 12 due to COVID-19 restrictions and their final visit was postponed to week 16. Compliance was also confirmed by the analysis of n-3 PUFA concentration in plasma and RBC membranes. The mean plasma and RBC concentrations of EPA, DHA and total n-3 L-C PUFA at baseline, end of the study and change from baseline are presented at **Table 5**. Plasma EPA and total n-3 L-C PUFA (g/L) increased by 0.57 ± 1.4 and 0.32 ± 5.0 g/L, respectively, in the GSM group while they decreased in the placebo group by −0.31 ± 1.2 and −2.07 ± 5.1 g/L ($P \leq 0.05$). The plasma DHA concentration decreased in both groups although the decrease was greater in the placebo group compared to GSM (−1.6 ± 2.3 vs. −0.27 ± 1.8 g/L, P = 0.03). Regarding the n-3 PUFA in RBC, at the end of the study a higher level of DHA was shown in the GSM group compared to placebo (0.81 ± 0.38 vs. 0.62 ± 0.32 g/L, P = 0.07). In addition, RBC omega-3 index tended to increase in the GSM group while reduced in placebo

TABLE 4 Correlation between urinary C-telopeptide of type II collagen (CTX-II), serum cartilage oligomeric matrix protein (COMP), and plasma 25(OH)D with visual analogue scale (VAS) pain and knee injury and osteoarthritis outcome score (KOOS) domain scores at baseline (n = 49).

	Urine CTX-II	Serum COMP	Plasma 25(OH)D
VAS pain score	0.132	0.01	0.150
KOOS			
Pain	−0.292*	−0.053	0.240
Symptoms	−0.276*	−0.006	0.201
ADL	−0.390*	−0.04	0.253
Sport/recreation	−0.222	−0.240	0.136
Quality of life	−0.318*	0.078	0.252
Urine CTX-II	–	0.103	−0.236
Serum COMP	0.103	–	0.101
Plasma 25(OH)D	−0.236	0.101	–

Values represent Pearson correlation coefficients.
*Indicates significance at $P \leq 0.05$.

(0.13 ± 1.5 vs. −0.02 ± 2.8). However, there was no significant change between the groups or over the study period. Overall, the measurement of n-3 PUFA in plasma and RBC indicated a good compliance rate and confirmed the capsule count.

Discussion

This study was the first to evaluate the effect of whole meat GSM powder on cartilage metabolism in overweight/obese postmenopausal women with joint pain and discomfort using the biomarkers of type II collagen degradation (CTX-II) and non-collagen cartilage degradation (COMP). The present study revealed the change in the urinary CTX-II/Cr was not significant between the two treatment groups. The result showed it was moderately decreased following 12 weeks of GSM treatment but notably elevated in the placebo group. This effect was observed in subjects with symptomatic knees, as urinary CTX-II levels were significantly different between the treatment groups at week 6 and end of study. This study also showed benefits of GSM supplement over placebo for secondary outcomes of VAS pain. The improvement for VAS pain in GSM group was 13 mm which is considered as clinically meaningful. However, GSM supplementation did not influence the level of circulating inflammatory cytokines.

The lack of effect of GSM on urinary CTX-II could be due to high levels of urinary CTX-II at baseline (571.6 ± 415.6 ng/mmol Cr), which were higher than the values from a previous study (511.92 ± 486.21 ng/mmol Cr) using the same ELISA kit in elderly females with knee OA (age 64.45 ± 10.6 years) (38). Thus, due to high levels of urinary CTX-II, a notable reduction may not have been detected after

TABLE 5 Mean ± SD in plasma and red blood cell (RBC) of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and total n-3 long chain-polyunsaturated fatty acids (n-3 LC-PUFA) at baseline, end of study and change from baseline.

Plasma fatty acid (g/L)	Placebo (n = 24)	GSM (n = 25)	P-value*
Eicosapentaenoic acid (EPA)			
Baseline	3.1 ± 1.1	3.2 ± 1.0	0.9
Endpoint	2.8 ± 0.9	3.8 ± 1.3	**0.008**
Change	−0.31 ± 1.2	0.57 ± 1.4	**0.02**
Docosahexaenoic acid (DHA)			
Baseline	7.2 ± 2.9	6.5 ± 2.0	0.3
Endpoint	5.6 ± 1.6	6.2 ± 2.0	0.2
Change	−1.6 ± 2.3	−0.27 ± 1.8	**0.03**
Total n-3 LC- PUFA			
Baseline	16.4+5.9	15.1 ± 4.3	0.3
Endpoint	13.7 ± 3.9	15.4 ± 4.3	0.1
Change	−2.07 ± 5.1	0.32 ± 5.0	**0.03**
RBC fatty acid (g/L)	**Placebo (n = 24)**	**GSM (n = 23)**	***P*-value***
Eicosapentaenoic acid (EPA)			
Baseline	0.39 ± 0.3	0.51 ± 0.34	0.2
Endpoint	0.62 ± 0.32	0.81 ± 0.38	0.07
Change	0.32 ± 0.38	0.30 ± 0.42	0.5
Docosahexaenoic acid (DHA)			
Baseline	1.9 ± 1.1	2.31 ± 2.2	0.3
Endpoint	2.7 ± 1.1	3.1 ± 1.6	0.3
Change	0.7 ± 1.4	0.8 ± 1.8	0.8
Total n-3 LC- PUFA			
Baseline	3.7 ± 2.3	4.5 ± 2.2	0.2
Endpoint	5.2 ± 2.0	6.1 ± 2.9	0.2
Change	1.4 ± 3.0	1.6 ± 3.3	0.8
Omega-3 index (%)			
Baseline	5.17 ± 2.5	5.8 ± 1.7	0.2
Endpoint	5.14 ± 2.0	5.9 ± 1.9	0.1
Change	−0.02 ± 2.8	0.13 ± 1.5	0.8

Values are reported as mean ± SD. The total n-3 PUFA including alpha-linolenic acid (ALA, 18:3 n-3), stearidonic acid (SDA, 18:4 n-3), eicosatetraenoic acid (ETA 20:4 n-3), EPA, docosapentaenoic acid (DPA,22:5 n-3), and DHA. Omega-3 index is content of EPA + DHA in RBC membranes expressed as a percent of total fatty acids. *The difference between group at baseline, endpoint and change from baseline were determined by Student's t-test. $P \leq 0.05$ is indicated in bold.

12 weeks of GSM treatment, and longer duration may result in a more significant effect. Of note, high levels of urinary CTX-II could be due to its high variability as a recent meta-analysis reported the mean levels were between 129 and 345 ng/mmol Cr in healthy adults (39). A significant difference in levels of urinary CTX-II between groups at follow-up and end of trial in participants with symptomatic knees was found, which may suggest these groups within the population obtain a larger cartilage-protective effect by GSM assessed through reduction of type II collagen degradation. However, the reason for elevation

in level of urinary CTX-II observed in the placebo group during the intervention is not clear. This might be due to a withdrawal effect of chondroprotective supplements and dietary restrictions for omega-3 rich foods during the study by these participants. It worth mentioning that urine samples were collected following overnight fasting, although it is possible that it does not reflect the acute chondroprotective effect of GSM. However, it can be proposed that the chondroprotective effect of GSM is not acute when consumed over a long period of time and could be reflected in general body fluids.

The effect of GSM on urinary CTX-II is consistent with a previous rat study which revealed a lower concentrations of serum CTX-II in diet-induced obese rats fed with GSM powder (22). The concentration of serum CTX-II is in line with urine CTX-II in rats (40); however, the serum CTX-II assay it is not the same as urine level in humans. Although the serum level has less analytical and biological variation than urine, we applied urine CTX-II in this study because it is known to have better clinical relevance than serum. Urine CTX-II has been used to discriminate OA patients from non-OA, and is strongly associated with clinical variables such as Kellgren-Lawrence grading (KLG) grade and knee OA symptoms (41).

In contrast, GSM supplementation did not affect the levels of serum COMP, a degradation marker from non-collagen components of cartilage. This lack of effect could be partly explained by the insignificant correlation between urinary CTX-II and serum COMP, suggesting that these markers are unlikely to change in parallel within the body after treatment. The levels of both urine CTX-II and serum COMP have shown a significant increase post-menopause; however, the increase tended to be less apparent for serum COMP and its level was generally lower in women than men within a similar age range (42).

This study showed a clinically significant reduction of pain on VAS (over 10 mm reduction) in favor of GSM. To our knowledge there are only two recently published clinical trials of whole GSM powder [16, 37]; both showed improvement in pain and knee OA symptoms measured by VAS pain and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC). These studies used the freeze-dried whole GSM powder product GlycOmega^{TM} PLUS, which was administrated at the same dose (3 g/day) and duration as our study. The first trial was a single arm with duration of 8 weeks resulting in a significant improvement in WOMAC total and sub-scores (pain, stiffness, and physical function) in knee OA patients (43). In the second trial, GSM powder was compared to glucosamine sulfate in a 12 week intervention where both supplements showed equal effectiveness on the aforementioned outcome measures (18). Our recent systematic review on the existing clinical trials concluded that both GSM lipid extract or whole meat powder products provide a clinically meaningful improvement in VAS pain for OA symptoms (44). In terms of knee related symptoms, the GSM group showed a greater improvement in KOOS symptoms domain overtime, although the change was not significant and did not reach the suggested minimal clinical improvement (at least eight points improvement) (32).

In our study, the urinary CTX-II levels showed an inverse correlation with some of the KOOS domains scores, showing that a higher level of knee-related problems was reflected in a higher level of urinary CTX-II. This is in accordance with previous studies using the patient-reported outcome of WOMAC index (38). This observed correlation may explain the beneficial effect of GSM on both urinary CTX-II and the KOOS domains, while COMP levels were not affected.

The level of plasma CTX-I at baseline was comparable between the two groups and within the range reported by a previous study (0.45 ± 0.1 μg/L) (45).

The main compounds with bioactive properties in GSM are lipids PUFA, EPA, and DHA that are known for their anti-inflammatory effect by inhibiting the COX enzyme, which most likely explain the analgesic and pain-reducing effect of GSM (19). Matrix metalloproteinases (MMPs), particularly MMP-13, are primary enzymes involved in the degradation of type II collagen and inhibition of MMP-13 has been a target in OA treatment (46). There is *in vitro* evidence showing that omega-3 from GSM oil extract down-regulates the expression of catabolic genes MMP-1, MMP-3, and MMP-13, while up-regulating the expression of anabolic genes that encode aggrecan and collagen type II-alpha (AGG and COL2A1) (47). It must be noted that whole meat GSM powder also contains cartilage protective and glycosaminoglycan such as glucosamine and chondroitin (3% of whole GSM powder extract) (48). These compounds been shown to have inhibitory effects on MMP production *in vitro* (49). Three months supplementation with glucosamine (1.5 and 3 g/day) has been shown to reduce the urinary levels of CTX-II in athletes (50). Although the glycosaminoglycan content in the dose provided in this study was ∼90 mg/day which is less than the effective dose reported by the previously mentioned study (50), whole GSM powder is a blend of omega-3 PUFA, glucosamine and chondroitin and several other bioactive components; therefore, it can be speculated that GSM powder can provide additive chondroprotective effects through regulation of MMPs which result in suppression of type II collagen degradation. Further *in vitro* studies are required to elucidate detailed molecular mechanisms.

Greenshell mussel supplementation did not significantly affect the inflammatory or anti-inflammatory cytokines as compared with placebo. Similarly, no significant change in circulating cytokine levels were observed in the previous study of obese rats fed with a GSM-enriched diet (22). Changes in circulating markers of inflammation such as TNF-α, IL-6, CRP, and adhesion molecules have not been observed in previous studies among healthy elders (51), or healthy obese postmenopausal women supplemented with omega-3 PUFA or fish oil supplements (52).

One obvious strength of the current study is its novelty, assessing the effect of GSM supplementation on cartilage degradation markers in human subjects for the first time. Secondly, this study assessed EPA, DHA, and total omega-3 PUFA in plasma and RBC to confirm compliance of the study participants.

Some limitations of our study must be acknowledged. Firstly, participants were not screened by radiographic evidence to detect OA due to resource limitation. Observing the high level of urinary CTX-II raised the possibility of participants having established OA which would not be unexpected as most participants were older women with moderate to severe pain. Stratifying based on age and adjusting the baseline VAS pain score helped to negate this effect to some extent. Secondly, higher levels of urinary CTX-II in women than men were reported in a previous study (38), which was partly explained by the effects of menopause, and thus a male population may respond better to GSM supplementation. The effects of diurnal variation on biomarker levels should be taken into consideration. The urinary CTX-II has the highest level in the morning which decreases 4 h after arising from bed and then remains stable till after 12 h (53). The timing of urine sample collection in our study was in the morning between 8 and 9 a.m. which was most convenient for participants but is considered the highest phase of diurnal variation. Moreover, the urine sample was collected according to standard methods for CTX-II assessment, although a 24-h urine sample collection was proposed to fully monitor the chondroprotective effect of active agents (54). However, in this study 24-h samples were not collected due to the potential burden on participants.

No minimum level of pain was set as inclusion criteria, and thus half of the study participants (53%) had mild symptoms (below 30 mm) at baseline. The study subjects were predominantly of New Zealand-European ethnicity. Previous research has reported significant ethnicity-based differences in the experience of pain and treatment response among OA patients (55). Whether our findings are applicable to individuals with only severe symptoms or from other ethnicities is unclear and requires further research. This study used KOOS questionnaire for outcome measure which is specifically for the knee joint because the knee OA is dominant among postmenopausal women (56), and majority of this study participants were experiencing knee pain. This study was not limited to individuals with only knee pain in order to generalize the findings to individuals with pain at other joint sites. This also allowed to have the study population that represent the population of postmenopausal women with affected joints at different site. Finally, this study focused on a limited number of cartilage degradation markers and lacked assessment of a cartilage synthesis marker. It was originally proposed to determine the level of C-terminal propeptide type II collagen (C-propeptide, also referred as CPII), a commonly measured collagen type II synthesis biomarker (57). However, delivery of the assay kits was severely delayed due to COVID-19 impacts on global transport systems, and the kits when received, proved to be unusable. We acknowledge that the current results identifying effects of GSM supplement on urine CTX-II should be interpreted cautiously and need to be evaluated against other cartilage markers, specifically the ratio of CTX-II/CPII. However, these limitations do not negate our overall conclusions.

Conclusion

In summary, the present study revealed that whole meat GSM powder did not change the cartilage metabolism evaluated by urinary CTX-II and serum COMP levels in overweight/obese postmenopausal women, however, in those with knee symptoms, urinary CTX-II was decreased. GSM supplementation was effective in clinically improving joint pain; however, it did not impact knee-related symptoms and the level of inflammatory cytokines.

Data availability statement

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

Ethics statement

Massey University Human Ethics Committee approved this study: Southern A, Application 20/03. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MK, JC, and FW: conceptualization, methodology, and supervision. MA: chief investigator and writing—original draft preparation. JC, FW, and PH: writing, reviewing, and editing the manuscript. MM and HT: funding acquisition, reviewing, and editing the manuscript. All authors read and approved the final manuscript.

Funding

This study was funded by the National Science Challenge, High Value Nutrition (HVN) "Musseling-up 2.0: Greenshell™

mussels for inflammation, metabolism and muscular skeletal function (HVN1904)", as a collaboration between Massey University, Cawthron Institute, Plant and Food Research Ltd., AgResearch and Sanford Ltd. HVN was underwritten by the Ministry of Business, Innovation and Employment (MBIE). The funder has no role or ultimate authority over any of the trial-related management, analysis, writing of the report, or the decision to submit the report for publication.

Acknowledgments

The authors thank Louise Shaw and Chris Kendrick (Massey University) for phlebotomy and Shampa De (Massey University) for supporting in blood processing and sample collection.

Conflict of interest

Author HT was employed by New Zealand fisheries that produces and sells GSM (Sanford Ltd.).

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.1063336/full#supplementary-material

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frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 07 March 2023
DOI 10.3389/fmed.2023.1134786

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

RECEIVED 30 December 2022
ACCEPTED 16 February 2023
PUBLISHED 07 March 2023

CITATION
Popov VL, Poliakov AM and Pakhaliuk VI (2023) *In silico* evaluation of the mechanical stimulation effect on the regenerative rehabilitation for the articular cartilage local defects.
Front. Med. 10:1134786.
doi: 10.3389/fmed.2023.1134786

In silico evaluation of the mechanical stimulation effect on the regenerative rehabilitation for the articular cartilage local defects

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Osteoarthritis is one of the most severe diseases of the human musculoskeletal system, and therefore, for many years, special attention has been paid to the search for effective methods of its treatment. However, even the most modern methods only in a limited number of cases in the early or intermediate stages of osteoarthritis lead to positive treatment results. In the later stages of development, osteoarthritis is practically incurable and most often ends with disability or the need for joint replacement for a large number of people. One of the main reasons hindering the development of osteoarthritis treatment methods is the peculiarities of articular cartilage, in which there is practically no vascular network and tissue homeostasis is carried out mainly due to the diffusion of nutrients present in the synovial fluid. In modern medicine, for the treatment of osteoarthritis, tissue engineering strategies have been developed based on the implantation of scaffolds populated with chondrogenic cells into the area of the defect. *In vitro* studies have established that these cells are highly mechanosensitive and, under the influence of mechanical stimuli of a certain type and intensity, their ability to proliferate and chondrogenesis increases. This property can be used to improve the efficiency of regenerative rehabilitation technologies based on the synergistic combination of cellular technologies, tissue engineering strategies, and mechanical tissue stimulation. In this work, using a regenerative rehabilitation mathematical model of local articular cartilage defects, numerical experiments were performed, the results of which indicate that the micro-and macro environment of the restored tissue, which changes during mechanical stimulation, has a significant effect on the formation of the extracellular matrix, and, consequently, cartilage tissue generally. The results obtained can be used to plan strategies for mechanical stimulation, based on the analysis of the results of cell proliferation experimental assessment after each stimulation procedure *in vivo*.

KEYWORDS

articular cartilage, osteoarthritis, articular stem cell implantation, autologous chondrocyte implantation, mechanical tissue stimulation, articular cartilage regenerative rehabilitation

1. Introduction

In the process of life in its natural environment, a person is constantly under the influence of external forces and adapts to them. When the environment changes as a result of homeostasis, his body adapts to the changed conditions, which is accompanied by a change in the properties of tissues and organs. Thus, for example, under conditions of weightlessness, a local loss of bone mass occurs due to the activation of resorption as a result of reactions to the disappearance of mechanical stress and rearrangement in the hierarchy of ion and volume regulation (1). Therefore, it is quite reasonable to assume that these changes are predetermined by the evolution of the musculoskeletal system of terrestrial vertebrates in the earthly gravity field and are determined biomechanically. But, as is known, a decrease in the load on the bone is accompanied not only by a decrease in bone mass, but also by a change in the relationship of the entire cellular aggregate and the extracellular matrix (ECM) of the tissue (2). Similar processes occur not only in bone, but also in other tissues, and not only when the force of gravity changes. It has been established that various kinds of physical influences affect the physiological and reparative regeneration of tissue defects resulting from injuries or diseases. Moreover, the course of these processes depends not only on the nature of the external influence, but also on many other factors, including the physical condition of the patient, his gender, age and even race, as well as the type and size of the tissue defect, the strategy of pharmacotherapy, etc. In this regard, it is quite natural for specialists in the area of regenerative medicine to understand how and why rehabilitation medicine, which uses physical influences in its practice, can help restore damaged tissues of a particular patient. In recent years, this desire has led to the creation of a new direction in medical science-regenerative rehabilitation, the essence of which is to find and practically implement the best conditions for the restoration of damaged tissues through the parallel use of advanced methods of regenerative and rehabilitation medicine. However, despite a number of optimistic results obtained using regenerative and rehabilitation approaches, there are still many questions and problems that need to be addressed for the development of this area of science and the creation on its basis of effective technologies for the treatment of diseases associated with various types of damage to tissues and organs.

One of the main problems hindering the introduction of regenerative rehabilitation technologies into medical practice is the lack of a complete understanding of the cells and tissues response to physiological effects and the lack of theoretical and experimental data for their systematization. Such technologies should take into account not only the type and biophysical state of the restored tissue, but also the features of its interaction with surrounding tissues, which requires considering many factors that affect the intensity and quality of restoration. In this regard, of particular relevance is the mathematical simulation of the regenerative rehabilitation processes, which is necessary to assess the significance of the parameters that determine their course and use the results obtained in planning experimental studies *in vivo*.

In this paper, we study a mathematical model of regenerative rehabilitation the local articular cartilage defects, taking into account the features of this type of skeletal connective tissue, which are well studied and described in detail in many literature sources (3–7). It also takes into account the responses of chondrocytes and progenitor chondrocytes observed in experiments *in vitro* to a wide range of mechanical stimuli, including tensile, compressive, shear deformations, fluid flow, hydrostatic and osmotic pressure (8–10). Mesenchymal stem cells (MSCs), capable of chondrogenic differentiation, also respond to these stimuli, and therefore represent a potential source of chondroblasts, from which, in turn, chondrocytes are formed, the main function of which in cartilage tissue homeostasis is the synthesis and release of intercellular substance components consisting of water, proteoglycan aggregates, glycoproteins and minerals (11–13). As a result of this activity, chondrocytes wall themselves up in specific areas of the ECM–lacunae, thereby providing interstitial cartilage growth and its potential ability to regenerate.

It is known that the responses of cells to mechanical influences are different and cause many changes and sensations, the study of which has received much attention for a long period of time (14–19). However, it is still not fully understood how exactly mechanical signals are transmitted to individual cells, how versatile the mechanisms of mechanotransduction are, and whether there is redundancy between possible signal transduction pathways. The mathematical model studied in this work takes into account the experimentally observed effect of chondrogenic cells physical stimulation on their proliferation, differentiation, viability, and ECM formation, both with and without regard to the biochemical processes that determine these phenomena. At the same time, the model itself is built considering the following conditions (20, 21):

- healthy cartilage in the process of life is subjected to a complex load that ensures its stress–strain state;
- an important factor determining the viability and regeneration of articular cartilage under *in vivo* conditions is its dynamic loading;
- when an external load is applied to the cartilage, due to mechanotransduction, biochemical signals are activated in it that regulate both anabolic and catabolic processes, including the synthesis of matrix proteins, transcription factors, growth factors, proteases and protease inhibitors;
- the balance between these processes is largely achieved due to the external load perceived by the joint and depends on its type and intensity.
- The model also does not contradict the assessments currently accepted in the scientific community of the influence of various factors on homeostasis and the function of articular cartilage (22):
- excessive mechanical stress on the articular cartilage leads to mitochondrial dysfunction, hypertrophy of chondrocytes, degradation of collagen, a decrease in the level of adenosine triphosphate and the formation of reactive oxygen species;
- proper mechanical stimulation of MSCs increases viability and enhances chondrogenesis of cells, promotes collagen synthesis, increased ECM formation and organization of a network of fibers in tissue-engineered cartilage structures;
- growth factors (BMP, TGF, IGF, etc.) maintain the integrity of the articular cartilage, promote the secretion of glycosaminoglycans, the expression of chondrogenic genes, the proliferation and differentiation of MSCs into chondrocytes;
- pro-inflammatory cytokines (Il-1β, TNF-α) inhibit the expression of genes responsible for the formation of cartilage ECM and chondrocyte phenotype, as well as the differentiation of MSCs into chondrocytes.

It is clear that it is practically impossible to simultaneously take into account in the mathematical model all the many factors listed

above, considering among other things, their possible mutual influence. In addition, even considering these factors conditionally independent, it is very difficult to formulate their mathematical representations and determine the corresponding numerical values. For example, it is possible to experimentally determine quantitative indicators of the increase in viability, cell proliferation, the rate of chondrogenesis, collagen synthesis and ECM depending on the location, type and intensity of mechanical stimulation of MSCs, the mathematical representation of which, obviously, should be based on a synergistic combination of heterogeneous natural phenomena (mechanical, chemical, biological) at different levels of detail. These phenomena are of a random nature, and their occurrence essentially depends on the state of the medium. Therefore, taking into account the understanding of their essence, the mathematical model of tissue regenerative rehabilitation can be reasonably simplified by using the average values of the parameters corresponding to the experimental data. At the same time, the nature of the occurrence of phenomena and the mechanisms that determine the change in the parameters of the medium state remain important, but are not considered directly in the process of their determination.

The main goal of this work is evaluating the effect of mechanical stimulation on the effectiveness of regenerative rehabilitation for local articular cartilage defects using various cell technologies and tissue engineering strategies as a result of studying a mathematical model with parameters determined as a result of experimental studies available in the literature.

2. Materials and methods

2.1. Basic strategies for articular cartilage defect repair

Articular cartilage covers the surfaces of bones in diarthrotic joints, ensuring their relative movement with low energy consumption for friction and acting as a shock absorber for external loads. At the same time, it is able to deform with an increase in the area of the contact surface, which helps to reduce pressure on the bones that form the joint. Articular cartilage has a two-phase structure and possesses viscoelastic properties that provide stress relaxation during compression and resistance to damage from external loads (4).

The thickness of the cartilage on the surfaces of the bones that form human joints ranges from 1 mm to 4 mm and its properties vary depending on the depth, forming four pronounced zones: superficial, intermediate (middle), deep and calcified, in which the shape of chondrocytes changes from flat to spherical (6). Collagen fibers in the superficial zone are parallel to the articular surface, in the intermediate zone they are randomly oriented in different directions, and in the deep zone they are organized perpendicular to the articular surface so that they penetrate into the calcified zone, thereby ensuring the structural stability of the articular cartilage on the subchondral bone.

Articular cartilage degradation can occur because of injury, disease, or constant mechanical stress and is classified into three main types: superficial destruction (damage to the ECM), partial thickness defects (does not extend into subchondral bone), and full-thickness defects (penetrate deep into subchondral bone) (23). Only with superficial destruction of the articular cartilage, viable chondrocytes can form clusters and are potentially capable of independently synthesizing a new matrix. With deep defects of all types, cartilage self-healing is practically excluded, therefore, for the purpose of their therapeutic or surgical restoration, a number of strategies have been developed, which, however, in most cases also do not guarantee positive results (22, 24, 25).

Modern strategies focused on the restoration or regeneration of articular cartilage, including in osteoarthritis, involve the implantation of chondrocytes or MSCs, biodegradable scaffolds, and signaling molecules (cytokines and growth factors) into the defect area. Scaffolds in this triad are used to potentially provide biological signals that regulate cell behavior or as scaffolds in which cells must synthesize ECM, and signaling molecules to stimulate recruitment, differentiation of progenitor cells, and also to direct the synthesis of the desired tissue phenotype (26).

2.1.1. Autologous chondrocyte implantation technology and matrix-induced autologous chondrocyte implantation technology

Autologous Chondrocyte Implantation Technology (ACIT) is used to treat certain symptomatic articular cartilage defects in synovial joints, usually implemented in three stages (27–29). At the first of these, arthroscopy of the patient's joint is performed with a sampling of 200–300 milligrams of cartilage, usually from the least loaded area. ECM is enzymatically removed from the harvested tissue and chondrocytes are isolated. At the second stage, these cells are grown in a specialized bioreactor under *in vitro* conditions until their number is sufficient for implantation into the defect area, which takes approximately 1.0–1.5 months. In recent years, ACIT has been improved and at this stage, modern biodegradable scaffolds or hydrogels have been used to promote the formation of a three-dimensional tissue ECM. This ACIT modification, called Matrix-Induced Autologous Chondrocyte Implantation Technology (MACIT), is becoming increasingly popular due to its cost-effectiveness compared to first-generation ACIT, as well as better cell maturation *in vitro* and better cell growth *in vivo* (30). And, finally, at the third stage, the cells grown in the bioreactor are implanted into the defect area, adapt to the new environment and form a new cartilage.

2.1.2. Articular stem cell implantation technology

Relatively recently, it was found that cartilage can be restored if there are sufficient resources of MSCs in the area of the defect (31, 32). Undifferentiated MSCs, like other cells, are mechanosensitive; therefore, not only biochemical but also biomechanical factors play an important role in their chondrogenic differentiation. This is an important feature of MSCs, which are able to differentiate into different cell types during the process of committing to chondrocytes. Since the committing mechanism is a persistent repression of some and de-repression of other genes, the spectrum of functionally active genes gradually changes in cells as they develop, which determines an increasingly specific direction for their future fate. At a certain stage, the commitment leads to the fact that cells become determined with genetic programming for only one developmental path. That is, under certain conditions, these cells have the ability to differentiate along different mesenchymal lines, including cartilage (33–35). At the same time, as noted above, proteins such as fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), etc., are involved in the regulation of MSCs chondrogenesis (36, 37). In healthy cartilage, the metabolism and renewal of chondrocytes are primarily provided by the growth factors FGF-1 and BMP-2 (38–40).

MSCs used to repair cartilage defects are obtained from various autologous tissues, including bone marrow, adipose tissue, and peripheral blood (41) and, depending on the specific pathology, are either surgically implanted into the defect or injected into the joint. This process is called Articular Stem Cell Implantation Technology (ASIT).

The area of a local articular cartilage defect replaced by a tissue-engineered construct supported by a collagen plate placed on the subchondral bone and the surgical procedures required for ACIT/ASIT are schematically shown in Figure 1.

2.2. Mechanical stimulation of chondrogenesis

In the process of life, the joints of the human lower limb during normal locomotion are subjected to cyclic compression *in vivo* with a frequency of about 1 Hz. In this case, chondrocytes are cyclically loaded with uniform pressure ranging from 3 MPa to 10 MPa (9). It is also known that certain movement and load patterns are required for the normal development of joints and articular cartilage *in vivo* (42). In this regard, the opinion has been formulated in the scientific community that mechanically generated signals play a critical role in the proliferation, differentiation, and maturation of progenitor chondrocytes and MSCs to the chondrogenic phenotype.

It has been established that compressive loading causing compression deformation of scaffolds seeded with MSCs induces a prochondrogenic and biosynthetic response, useful in the creation of implants for cartilage regeneration and repair using ASIT (14). And in more advanced bioreactors, which allow, in addition to compression, to realize shear and other components of the load, the chondrogenic response of MSCs to mechanical load not only increases, but also better mimics the *in vivo* environment, which contributes to better differentiation of chondrocytes, leads to an increase in the formation, composition and location of the ECM during cartilage regeneration (43).

But not only has a certain one-dimensional or complex stimulation contributed to the emergence of a prochondrogenic reaction of cartilage tissue. It was shown in (44) that cartilage formation *in vitro* increases under the influence of any "correct" physical stimuli that promote proliferation, differentiation of chondrogenic cells, and ECM production. For example, signals generated by oscillatory fluid flow (OFF) regulate the expression of transcription factors involved in multiple differentiation pathways (45) and promote an increase in the proliferation rate of MSCs (46). In this case, the RhoA and ROCKII proteins are activated, which ultimately also regulates the differentiation of MSCs (47). The authors of (48) demonstrated that chondrogenesis of bone marrow-derived MSCs under the action of cyclic compressive load is induced similarly to that under treatment with growth factors, and both stimuli use similar pathways for this (49). But when MSCs are subjected to the combined action of cyclic contraction and treatment with growth factors, chondrogenesis is a much more complex process. With the simultaneous action of these factors, the expression levels of aggrecan decrease compared to the action of only the last of them (50); cartilage ECM synthesis in agarose hydrogels is reduced when mechanical stimulation is initiated at the onset of growth factor-induced chondrogenesis (50, 51); cyclic compression enhances the accumulation of proteoglycans and collagen for MSCs seeded in a gelatin scaffold (52), etc. Such contradictions indicate that the nature

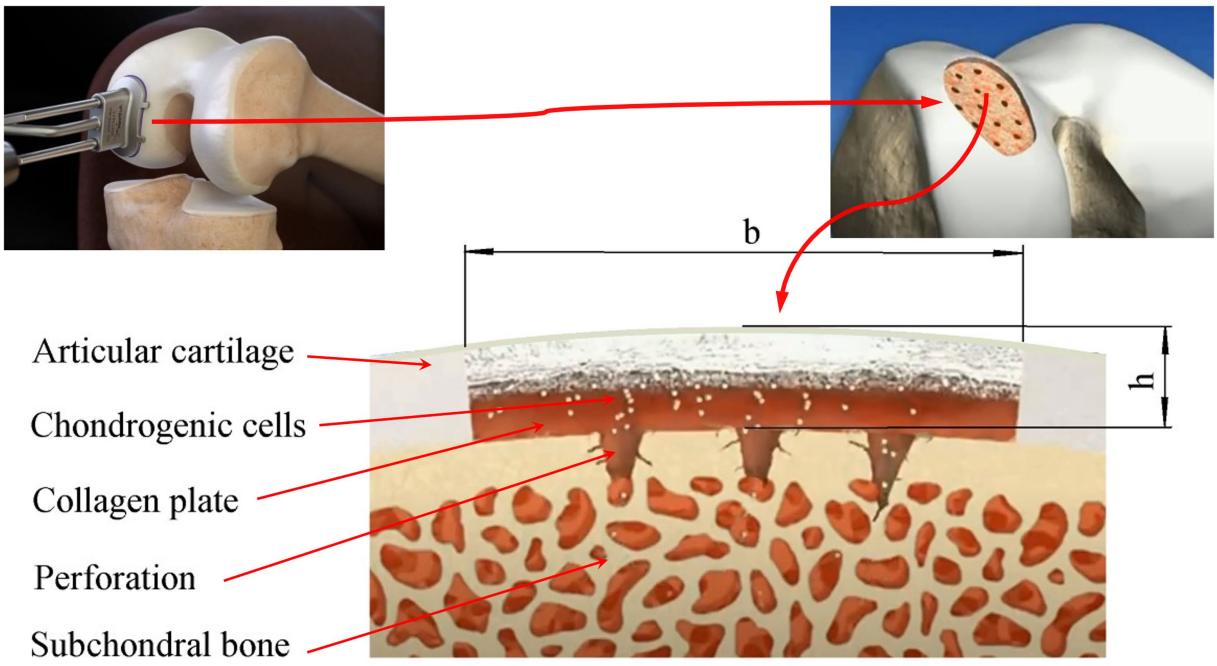

FIGURE 1
Scheme of the damaged area of the articular cartilage filled with a tissue-engineered structure based on a collagen plate placed on the subchondral bone: *b*, *h*-the maximum size and depth of the defect, respectively.

of their interaction with the surrounding ECM is of decisive importance on the response of MSCs to the load. This, in particular, explains the experimentally observed effect that the response of MSCs to dynamic compression in the presence of growth factors depends on when the load is initiated. Mouw et al. showed that early mechanical stimulation (on day 8) reduces the expression of the aggrecan gene, and at a later date (on day 16) increases the expression of the chondrogenic gene (53). That is, the results of *in vitro* experiments indicate that the mechanoreactivity of MSCs varies depending on the stage of chondrogenesis and the development of the ECM. In addition, it was found that the maintenance of the chondrogenic phenotype of MSCs by cyclic compression depends on the concentration of growth factors, but does not disappear after the exclusion of this type of stimulation (54, 55).

Chondrogenesis in scaffolds seeded with MSCs also increases under cyclic hydrostatic pressure, as evidenced by the observed *in vitro* increase in the content of proteoglycan and collagen in the chondrogenic culture medium (56). In (57), the authors note that this type of mechanical stimulation also enhances Sox9 mRNA expression, as well as type II collagen and aggrecan mRNA expression in MSCs aggregates maintained in chondrogenic conditions compared to unloaded cells (57). In addition, they found that different values of hydrostatic pressure (0.1 MPa, 1.0 MPa, 10.0 MPa) had different effects on the regulation of chondrogenesis of MSC aggregates, with greater expression of collagen type II mRNA and accumulation of collagen observed at 10 MPa (58). At the same time, it was demonstrated in (59) that hydrostatic pressure has practically no effect on the expression of chondrogenic genes or the accumulation of ECM in MSC aggregates, both in the presence and in the absence of growth factors FGF-1 or BMP-2.

The above information about the reactions of cartilage tissue to mechanical stimulation under various conditions is not systematic and does not give a complete picture of the transformations occurring in the tissue. However, it indicates that biological processes in a tissue are determined not only by its biochemical environment, but also by its biomechanical one. At the same time, the regulation of the biomechanical environment has a significant effect on the course of both anabolic and catabolic processes. In this regard, it is quite reasonable to assume that there is such a state of damaged tissue, induced biomechanically, in which its self-healing is possible. The most striking example here is the state of the tissue, in which its physiological regeneration occurs in the natural habitat of a healthy biological object. Therefore, one of the main tasks of regenerative rehabilitation is to establish such a biomechanical environment of the damaged tissue, in which the processes of its physiological regeneration are initiated. This is a very complex problem, the solution of which depends not only on a large number of parameters, but also on the range and nature of their change when exposed to tissue stimuli of different nature and intensity. In addition, there is a high probability that mechanical and other stimuli are synergistically related to each other and their contribution to the course of tissue and cellular processes is characterized by a high degree of uncertainty, and most modern studies in the field of mechanobiology consider cell responses to each stimulus separately (60). Along with the incompletely understood molecular mechanisms that determine mechano transduction, this leads to a difficult understanding of regenerative processes in tissues subjected to stimulation and their use in medicine. Nevertheless, even the currently known results of research in the field of mechanobiology make it possible to build and study mathematical models of various degrees of detail, representing changes in tissues and cells during various types of their stimulation.

2.3. Mathematical model of regenerative rehabilitation for local articular cartilage defects

A mathematical model of regenerative rehabilitation for local articular cartilage defects used by authors in this work is based on a system of differential equations of the "diffusion–reaction" type (61), similar to the model used by A. Bailón-Plaza and M.C. van der Meulen to study the healing of bone fractures (62). Sufficiently realistic results in the study of such a model were obtained by M. Lutianov and colleagues who studied the processes of cartilage tissue regeneration using cell therapy (63), as well as by K. Campbell and colleagues when studying ACIT and ASIT in the presence of growth factors FGF-1 and BMP-2 (64, 65).

ACIT and ASIT, as well as their combinations, suggest the presence of a scaffold populated by chondrocytes and/or chondrogenic cells (MSCs) in the area of the cartilage defect. If we denote the density of MSCs by C_S, then the mathematical model of its change in time, taking into account the fact that C_{S0} is the threshold density, can be represented by the differential equation (63):

$$\frac{\partial C_S}{\partial t} = \underbrace{\nabla\left[D_S \nabla C_S\right]}_{diffusion} + \underbrace{p_1 C_S \frac{n}{n+n_0} H\left(n-n_1\right)}_{proliferation} - \underbrace{p_2 C_S H\left(C_S - C_{S0}\right)}_{differentiation} - \underbrace{p_3 C_S H\left(n_1 - n\right)}_{death}, \quad (1)$$

where $\nabla = \frac{\partial}{\partial x}\vec{i} + \frac{\partial}{\partial y}\vec{j} + \frac{\partial}{\partial z}\vec{k}$ is the Laplace operator;

$$\nabla C_S = \frac{\partial C_S}{\partial x}\vec{i} + \frac{\partial C_S}{\partial y}\vec{j} + \frac{\partial C_S}{\partial z}\vec{k} = \overrightarrow{grad} C_S; \nabla \overrightarrow{grad} C_S = div \overrightarrow{grad} C_S = \frac{\partial^2 C_S}{\partial x^2} + \frac{\partial^2 C_S}{\partial y^2} + \frac{\partial^2 C_S}{\partial z^2};$$

D_S is the probability diffusion coefficient of MSCs; n is the concentration of nutrients that provide tissue homeostasis (n_0, n_1 are the threshold and critical concentrations, respectively); p_1, p_2, p_3 are the coefficients that determine the proliferation, differentiation and death of MSCs, respectively;

$$H\left(C_S - C_{S_0}\right) = \begin{cases} 0, C_S \le C_{S_0} \\ 1, C_S > C_{S_0} \end{cases}, H\left(n-n_1\right) = \begin{cases} 0, n \le n_1 \\ 1, n > n_1 \end{cases}, H\left(n_1 - n\right) = \begin{cases} 0, n_1 \le n \\ 1, n_1 > n \end{cases}$$

are the Heaviside step functions.

Here and below, we use the parameters designations and state variables of the model adopted earlier by K. Campbell et al. in (64, 65).

It follows from Equation (1) that the change in C_S in the region of the defect is determined by four terms on the right side, which describe the processes of diffusion, proliferation, differentiation, and death of MSCs. Indeed, the increase in cell density depends on the number of MSCs implanted in the defect area using ASIT and their entry into this area as a result of diffusion from the subchondral bone. If, in this case, the concentration of nutrients in the area of the defect

is greater than the critical one ($n > n_1$), MSCs proliferate, which also leads to an increase in their density. Otherwise, due to a lack of nutrients ($n \le n_1$) a certain number of MSCs die, which leads to a decrease in their density. In addition, the decrease in C_S occurs due to the fact that, when the threshold density is exceeded, some MSCs differentiate into chondrocytes as a result of commitment.

Similarly, a mathematical model of changes in the density of C_C chondrocytes in the area of a cartilage defect can be presented (63):

$$\frac{\partial C_C}{\partial t} = \underbrace{\nabla\left[D_C \nabla C_C\right]}_{diffusion} + \underbrace{p_4 C_C \frac{n}{n+n_0} H\left(n-n_1\right)}_{proliferation} + \underbrace{p_2 C_S H\left(C_S - C_{S0}\right)}_{differentiation} - \underbrace{p_5 C_C H\left(n_1 - n\right)}_{death}, \quad (2)$$

where D_C is the probability coefficient of chondrocytes diffusion; p_4, p_5 are the coefficients of proliferation and death of chondrocytes, respectively.

The fundamental difference between Formulas (1), (2) from each other is that the differentiation of MSCs leads to a decrease in C_S and, simultaneously, to an increase in C_C .

Taking into account the meaning of the elements of the structure of Equations (1), (2), the mathematical model of the change in the concentration of nutrients can be represented as follows:

$$\frac{\partial n}{\partial t} = \underbrace{\nabla\left[D_n \nabla n\right]}_{diffusion} - \underbrace{\frac{n}{n+n_0}\left(p_6 C_S + p_7 C_C\right)}_{reaction}, \quad (3)$$

where D_n is the probability coefficient of nutrients diffusion into the defect area from the synovial cavity; p_6, p_7 are the nutrient consumption constants of MSCs and chondrocytes, respectively.

That is, the nutrients that enter the defect as a result of diffusion through the surface of the articular cartilage are used to maintain the viability of MSCs and chondrocytes.

It is well known that articular cartilage is a collection of cells–chondrocytes occluded in the ECM containing collagen II, glycosaminoglycans, glycoproteins and proteoglycans (aggrecan) that bind large amounts of water. ECM elements, as well as MSCs, penetrate into the cartilage defect from the subchondral bone as a result of diffusion, which contributes to an increase in the density of the matrix *m*. In addition, the ECM density increases due to chondrocytes diffusing into the defect and differentiated from MSCs, secreting it and promoting the growth of the collagen network in the scaffold. Therefore, the mathematical model for changing the density of the matrix *m* can be represented by the equation (63):

$$\frac{\partial m}{\partial t} = \underbrace{\nabla\left[D_m \nabla m\right]}_{diffusion} + \underbrace{p_8 \frac{n}{n+n_0} C_C}_{reaction}, \quad (4)$$

given that $m \le m_{\max}$, where D_m is the probability coefficient of ECM elements diffusion; p_8 is the ECM secretion rate; $m_{\max}$ is the maximum ECM density.

Considering the fact that chondrocytes are relatively evenly distributed in the ECM in each layer of healthy cartilage, the time to reach a certain density threshold value $m_0 \le m_{\max}$ can serve as a conditional criterion for the quality of the regenerative rehabilitation process for an articular cartilage defect. If it is impossible to reach the threshold value of density due to a complex of possible reasons related to the biomechanical environment of the tissue being restored, the maximum achievable ECM density in a certain period of time can be taken as a criterion for the quality of various processes.

Growth factors play an important role in maintaining the balance and regeneration of articular cartilage. But their influence on changes in the density of MSCs, chondrocytes and ECM in mechanobiological models is taken into account in an implicit form. Nevertheless, such an influence can be quite noticeable, since the change in the state variables of the regenerative rehabilitation model largely depends on the ability of chondrogenic cells to proliferate and differentiate into chondrocytes, which, as shown above, is also stimulated by growth factors. Therefore, models of changes in the concentrations of growth factors in the regenerative rehabilitation process are necessary to consider their influence on cellular processes. They can be represented in the following form (63):

$$\frac{\partial g}{\partial t} = \underbrace{\nabla\left[D_g \nabla g\right]}_{diffusion} + \underbrace{p_9 C_S - p_{11} g}_{reaction}, \quad (5)$$

$$\frac{\partial b}{\partial t} = \underbrace{\nabla\left[D_b \nabla b\right]}_{diffusion} + \underbrace{p_{12} C_C - p_{13} b}_{reaction}, \quad (6)$$

where g, b are the concentrations of growth factors FGF-1 and BMP-2, respectively; $\left(D_g, D_b\right), \left(p_9, p_{12}\right), \left(p_{11}, p_{13}\right)$ are probabilistic diffusion coefficients, production and degradation constants of growth factors FGF-1 and BMP-2, respectively.

The mathematical model represented by the system of differential equations (1–6), justified earlier and described in detail in (63), makes it possible to investigate the changes occurring in the articular cartilage defect area since the beginning of ASIT/ACIT use. However, rehabilitation procedures provided by regenerative rehabilitation protocols and including physical stimulation of the tissue are usually applied with a certain time delay in order to achieve the best results, which, as shown for example in (53), is highly desirable, because allows you to achieve the maximum effect of tissue restoration. Therefore, in the mathematical model of regenerative rehabilitation with the same delay, changes in the values of parameters determined by the nature of physical stimulation should be taken into account.

Let us assume that stimulation of a scaffold populated with chondrogenic cells according to the ASIT/ACIT protocols and placed at the site of a local cartilage defect begins at time $t = t_1$. Then Equations (1), (2) can be represented in the following form:

$$\begin{aligned}\frac{\partial C_S}{\partial t} = {} & \nabla\left[D_S \nabla C_S\right] + C_S \frac{n}{n+n_0} H\left(n-n_1\right)\left[p_1 + \left(p_1^* - p_1\right) H\left(t-t_1\right)\right] \\ & - C_S H\left(C_S - C_{S0}\right)\left[p_2 + \left(p_2^* - p_1\right) H\left(t-t_1\right)\right] \\ & - C_S H\left(n_1 - n\right)\left[p_3 + \left(p_3^* - p_3\right) H\left(t-t_1\right)\right], \end{aligned} \quad (1^*)$$

$$\frac{\partial C_C}{\partial t} = \nabla\left[D_C \nabla C_C\right] + C_C \frac{n}{n+n_0} H(n-m_1)\left[p_4 + \left(p_4^* - p_4\right) H(t-t_1)\right] + C_S H(C_S - C_{S0})\left[p_2 + \left(p_2^* - p_1\right) H(t-t_1)\right] - C_C H(n_1 - n)\left[p_5 + \left(p_5^* - p_5\right) H(t-t_1)\right], \quad (2^*)$$

where $H(t-t_1)$ is the Heaviside step function.

Thus, the mathematical model of regenerative rehabilitation of a local articular cartilage defect is represented by a system of partial differential equations (1*, 2*, 3, 4, 5, 6).

3. Results and discussion

In general, the mathematical model described above can be used to study state variables that change over time in three-dimensional space. However, due to the fact that the main goal of research in this work is to study the generalized reaction of cartilage tissue in response to stimulating effects, it is sufficient to study a one-dimensional model that allows studying the change in state variables only with respect to the depth of the defect *h*. This limitation is also admissible from a geometric point of view, provided that the defect dimensions (*h* and *b*) are small and the articular surface is curvature in the area of the defect.

A number of limitations of the mathematical model are determined by the nature of the interaction between subchondral bone, chondrogenic cells, nutrients, growth factors, and ECM. In this work, it is assumed that the subchondral bone is permeable and MSCs can diffuse from it into the defect area, the flow of which is given as a function of time *f*(*t*). In practice, in order to increase the intensity of this flow, the subchondral bone is usually perforated and covered with a thin permeable collagen sheet, as shown in Figure 1. At the same time, it is assumed that the flow from the subchondral bone of chondrocytes, growth factors, nutrients and ECM elements is zero. However, if necessary, the model allows you to set them in the form of certain functions of time.

Similarly, plausible model constraints on the defect surface can be represented. It can be assumed that the fluxes of MSCs, chondrocytes, and ECM elements on the surface of the defect are equal to zero, and nutrients with a constant concentration N_0 enter the defect from the synovial fluid. In this paper, it is assumed that the fluxes of growth factors are proportional to their concentrations with proportionality factors γ and χ, respectively.

Taking into account the above restrictions, the boundary conditions of the mathematical model have the form:

(a) on the surface of the collagen plate resting on the subchondral bone, i.e., for $x = 0$:

$$-D_S \frac{\partial C_S}{\partial x} = f(t), D_C \frac{\partial C_C}{\partial x} = 0, D_n \frac{\partial n}{\partial x} = 0, D_m \frac{\partial m}{\partial x} = 0, D_g \frac{\partial g}{\partial x} = 0, D_b \frac{\partial b}{\partial x} = 0, \quad (7)$$

(b) on the surface of the defect, geometrically coinciding with the surface of the articular cartilage in the area of the defect, i.e., for $x = d$:

$$D_S \frac{\partial C_S}{\partial x} = 0, D_C \frac{\partial C_C}{\partial x} = 0, n = N_0, D_m \frac{\partial m}{\partial x} = 0, D_g \frac{\partial g}{\partial x} = -\gamma g, D_b \frac{\partial b}{\partial x} = -\chi b. \quad (8)$$

The initial conditions of the mathematical model are formulated in accordance with the technology of cell therapy used in the process of regenerative rehabilitation. For example, when implementing the ASIT strategy, it is assumed that MSCs are implanted into a defect, and the cells are arranged according to a certain law $C_S(0) = C_S^{(0)} h(x)$ according to the height of the defect. If a scaffold is implanted into the defect, then the initial density of ECM is assumed to be $(0) = m_3 + m_s$, where m_3 is the initial ECM density, and m_s is the scaffold density. In this case, given the nutrient density $n(0) = N_0$ and zero values of other state variables at the initial time, the initial conditions are as follows:

$$C_S(0) = C_S^{(0)} h(x), C_C(0) = 0, n(0) = N_0, m(0) = m_3 + m_s, g(0) = 0, b(0) = 0, \quad (9)$$

where $C_S^{(0)}$ is the initial MSC density.

Similarly, the initial conditions for the implementation of ACIT or the combination of ASIT+ACIT can be formulated.

The equations of the mathematical model studied in this work only to a small extent characterize the relationship of state variables. To a greater extent, these connections are manifested in the disclosure of the variable parameters of the model. In this paper, the content and structure of the model parameters were adopted according to (63). So, for example, it is assumed that the probability coefficients of diffusion of MSCs and chondrocytes depend on the density of the ECM and are determined by the following expressions:

$$D_S = D_{S0} \frac{m}{m^2 + m_1^2}, D_C = D_{C0} \frac{m}{m^2 + m_1^2} \quad (10)$$

where m_1 is the intermediate ECM density, and $D_{S0} = 2m_1 D_S^*$ and $D_{C0} = 2m_1 D_C^*$ are the diffusion constants of MSCs and chondrocytes, calculated taking into account the maximum possible diffusion coefficients D_S^* and $D_!^*$. That is, at $m = 0$, the diffusion coefficients D_S and D_C also tend to zero, and their maximum values D_S^* and $D_!^*$ are reached at $m = m_1$.

The proliferation coefficients of MSCs p_1 and chondrocytes p_4 also depend on state variables and are presented as

$$p_1 = p_{1_0} \frac{m}{m^2 + m_2^2}\left[1 - \frac{C_S}{C_{Smax}}\right], \quad (11)$$

$$p_4 = \left(p_{4_0} \frac{m}{m^2 + m_2^2} + p_{4_{00}} \frac{g}{g + g_0}\right)\left[1 - \frac{C_C}{C_{Cmax}}\right], \quad (12)$$

where p_{1_0} and p_{4_0} are the proliferation constants of MSCs and chondrocytes; $p_{4_{00}}$ are the degree of chondrocytes proliferation due to growth factor FGF-1; g_0 is the reference concentration of FGF-1. At the same time, the rate of synthesis of ECM p_8 decreases as its density increases and can be represented by a linear dependence (62).

$$p_8 = p_{8_0} - p_{8_1} m, \quad (13)$$

where p_{8_0} is the ECM expression constant; p_{8_1} is the rate of its degradation.

Formulas (11) and (12) take into account the fact that in the absence of growth factors at $p_{1_0} = 0$ and $p_{4_0} = 0$, the values of p_1 and p_4, respectively, tend to zero and reach maxima at some intermediate ECM density $m = m_2$. In addition, if we assume that the maximum possible cell densities decrease linearly with increasing m and are represented by dependencies: $C_{Smax} = C_{Smax0}\left(1 - \frac{m}{m_{\max}}\right)$ and $C_{Cmax} = C_{Cmax0}\left(1 - \frac{m}{m_{\max}}\right)$, where $m_{\max}$ is the maximum possible ECM density, then these formulas correspond to a logistic growth model, according to which the cell proliferation rate decreases as their densities approach their maximum values C_{Smax0} and C_{Cmax0}. That is, the maximum space available for cell proliferation at any location is modulated by the ECM density at that location.

In addition, if we assume that the maximum possible cell densities decrease linearly with increasing m and are represented by dependencies:

It should also be noted that the proliferation of MSCs and chondrocytes is possible only when the concentration of nutrients n becomes greater than the critical n_1 which in Equations (1*), (2*) is taken into account by introducing the Heaviside function $H(n - n_1) = \begin{cases} 0, n \le n_1 \\ 1, n > n_1 \end{cases}$. In contrast, at $n_1 > n$ MSCs and chondrocytes begin to die at rates p_3 and p_5, respectively.

The differentiation of MSCs into chondrocytes also does not occur constantly, but only when the condition

$$C_S > C_{S_0}, \tag{14}$$

where C_{S_0} is the threshold density of MSCs, determined by the expression

$$C_{S_0} = \left(C_{S_{0\max}} - C_{S_{0\min}}\right)e^{-\alpha b} + C_{S_{0\min}},$$

where $C_{S_{0\min}}$ and $C_{S_{0\max}}$ are the minimum and maximum boundaries of the MSCs density; α is the threshold stem cell density reduction factor (66).

Condition (14) in Equations (1*), (2*) is conceded by introducing the Heaviside function

$$H\left(C_S - C_{S_0}\right) = \begin{cases} 0, C_S \le C_{S_0} \\ 1, C_S > C_{S_0} \end{cases}.$$

Thus, it is not possible to directly take into account changes in the rates of proliferation, differentiation, and death of chondrogenic cells, as well as other significant parameters observed during physical stimulation of the tissue during regenerative rehabilitation, because these changes are characterized by an excessive number of degrees of freedom, due to the fact that they occur under the influence of many interrelated factors. At the same time, the results of our earlier numerical simulation indicate that low-amplitude high-frequency mechanical stimulation promotes the intensification of chondrogenesis and can be used for regenerative rehabilitation of local articular cartilage defects (61). This, as shown above, is also evidenced by the results of numerous *in vitro* studies. The question remains–how does mechanical stimulation *in vivo* contribute to the intensification of chondrogenesis? The answer to this question can be the hypothesis that mechanical stimulation changes not only the micro, but also the macro environment of the cartilage tissue, which contributes to an increase in the proliferation, differentiation, and viability of chondrogenic cells. Therefore, in the first approximation, it can be assumed that in response to mechanical stimulation, the rates of proliferation p_1, p_4 and differentiation p_2 of chondrogenic cells increase to a certain extent, as well as the rates of their death p_3 and p_5 decrease, which corresponds to an increase in viability.

Thus, when studying the mathematical model, we will assume that low-amplitude high-frequency mechanical stimulation contributes to a change in the macroenvironment of cartilage tissue. This change occurs continuously and at a certain point in time a certain equilibrium state is reached, described by parameters corresponding to the nature and intensity of stimulation. At the same time, as noted above, *in vitro* cartilage formation increases under the influence of any "correct" physical stimuli that promote proliferation, differentiation of chondrogenic cells, and ECM production. Therefore, it can be assumed that there is a high probability that, as a result of experimental studies, an optimal stimulation method from a practical point of view can be found that contributes to the achievement of the desired macroenvironment of cartilage tissue *in vivo*, which increases the proliferation, differentiation, and viability of chondrogenic cells. Obviously, the parameters of the mathematical model p_1, p_2 and p_4 corresponding to such a macroenvironment will be increased relative to the unstimulated ones, and p_3 and p_5 will be reduced, acquiring the values $p_1^* > p_1, p_2^* > p_2, p_4^* > p_4$ and $p_3^* < p_3, p_5^* < p_5$, respectively (14, 43–45). At the same time, the diffusion coefficients of cells, nutrients, growth factors, and ECM elements depend indirectly on the nature of stimulation, since they are dependent on the state variables of the model and change in the process of regenerative rehabilitation along with them.

Figure 2 schematically shows graphs of the change in the conditional stimulating effect, the corresponding law of change in the conditional parameter of the mathematical model and its average value. It is assumed that the mechanical stimulation of the tissue in the area of the defect begins after a certain period of time t_1 and ends at time t_2. During the time "$t_{12} = t_2 - t_1$ the value of the parameter reaches the maximum/minimum value, and during the time "$t_{23} = t_3 - t_2$ it returns to the minimum/maximum value. In the future, this process is repeated and, to a certain extent, it can be considered close to a process with a certain constant average value of the parameter: p_1^*, p_2^*, etc.

In the future, this process is repeated and, to a certain extent, it can be considered close to a process with a certain constant average value of the parameter: p_1^*, p_2^*, etc.

Numerical experiments by studying the mathematical model of regenerative rehabilitation for local articular cartilage defects (1*, 2*, 3, 4, 5, 6) were performed by the finite element method in the Matlab environment using the built-in m-function "pdepe," designed to solve systems of parabolic and elliptic partial differential equations with one space variable *x* and time *t*. All solutions were obtained on a 100×100 finite element spatiotemporal grid using the Supercomputer cluster "Afalina" in Sevastopol State University.

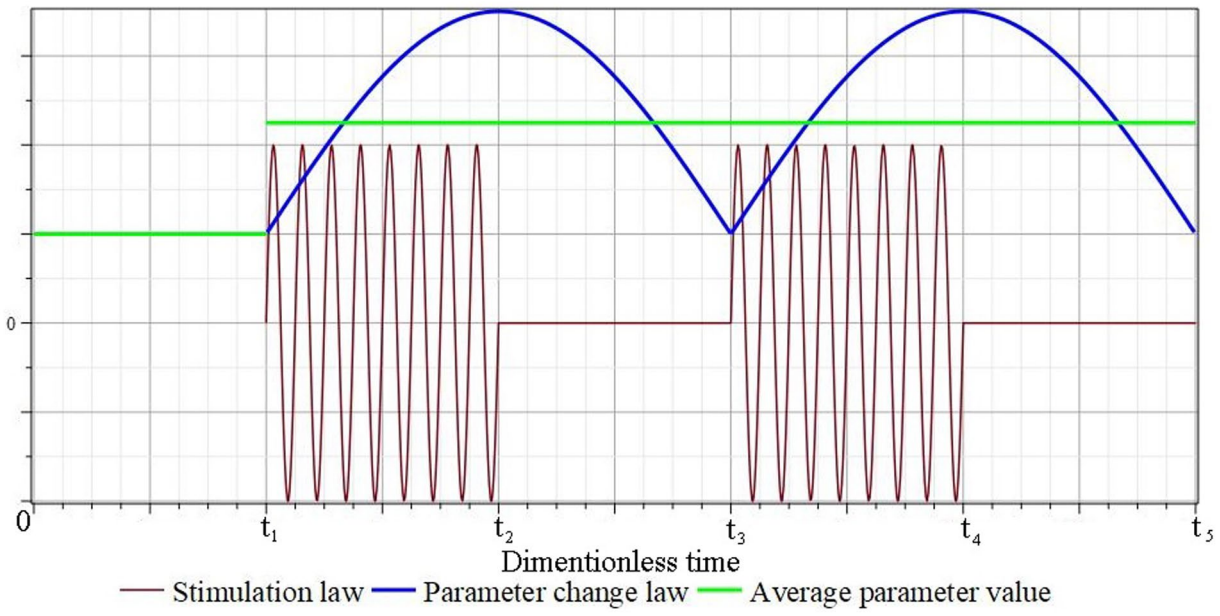

FIGURE 2
Schematic representation of the mathematical model conditional parameters corresponding to the law of periodic short-term tissue stimulation.

The first series of numerical experiments was carried out taking into account the implementation of ASIT/ACIT/ASIT+ACIT with the parameters, the values of which are borrowed in (63, 64) and are given in Appendix 1. In this case, the following were studied:

1. ASIT with boundary Conditions (7), (8) and initial conditions:

$$C_S = 0.25 \cdot \frac{1 - \tanh\left(10^4 (x - 0.1)\right)}{2},$$
$$C_C = 0, n = N_0, m = m_3 + m_s, g = 0.01, b = 0.01;$$

2. ACIT with boundary Conditions (7), (8) and initial conditions:

$$C_S = 0, C_C = 0.0001 \cdot \frac{1 - \tanh\left(10^4 (x - 0.1)\right)}{2},$$
$$n = N_0, m = m_3 + m_s, g = 0.01, b = 0.01;$$

3. ASIT+ACIT with boundary conditions (7), (8) and initial conditions:

$$C_S = 0.25 \cdot \frac{1 - \tanh\left(10^4 (x - 0.1)\right)}{2},$$
$$C_C = 0.0001 \cdot \frac{1 - \tanh(10^4 (x - 0.1)}{2},$$
$$n = N_0, m = m_3 + m_s, g = 0.01, b = 0.01.$$

Figure 3 shows plots of ECM density changes at different time points during ASIT implementation. Similar results were also obtained for other variants of cell therapy.

The general view of the plots of ECM density changes at the same time points is practically the same in the study of all variants of cellular technologies, but their numerical values at the nodes of the finite element grid are different. These differences in the form of maximum ECM density values are shown in Table 1 in dimensionless and real time parameters.

The second series of numerical experiments was performed taking into account the implementation of ASIT/ACIT/ASIT+ACIT with parameters, most of which were also borrowed in (63, 64), show that the proliferation and differentiation of chondrogenic cells under mechanical stimulation increases by (10–30%) (14, 21, 43–45, 67, 68). Two options were considered:

(1) $p_{10} = 15.6, p_2 = 1.3, p_3 = 0.7, p_{40} = p_{400} = 0.0156, p_5 = 0.7;$

(2) $p_{10} = 14.4, p_2 = 1.2, p_3 = 0.8, p_{40} = p_{400} = 0.0144, p_5 = 0.8.$

The assumption we have formulated may not be fully implemented in rehabilitation practice, but it can always be verified as a result of future experimental studies, because the rates of proliferation and differentiation of chondrogenic cells after appropriate rehabilitation procedures can be easily measured in the laboratory. Thus, we create a scientific basis for further research in this direction, which is necessary to confirm the adequacy of the mathematical model we use and improve the technologies for regenerative rehabilitation of articular cartilage defects.

Figure 4 shows graphs of changes in ECM density at different time points during the implementation of ACIT with mechanical stimulation with a time delay $t_1 = 2$ (~ 22 days) using the first option of the parameters described above. It is easy to see that they are similar in form to the corresponding graphs obtained in the implementation of ASIT without stimulation. In addition, as in the first series of numerical experiments, their general form at the same time points is almost the same in the study of all options of cellular technologies, with different values at the nodes of the finite element grid. Similar

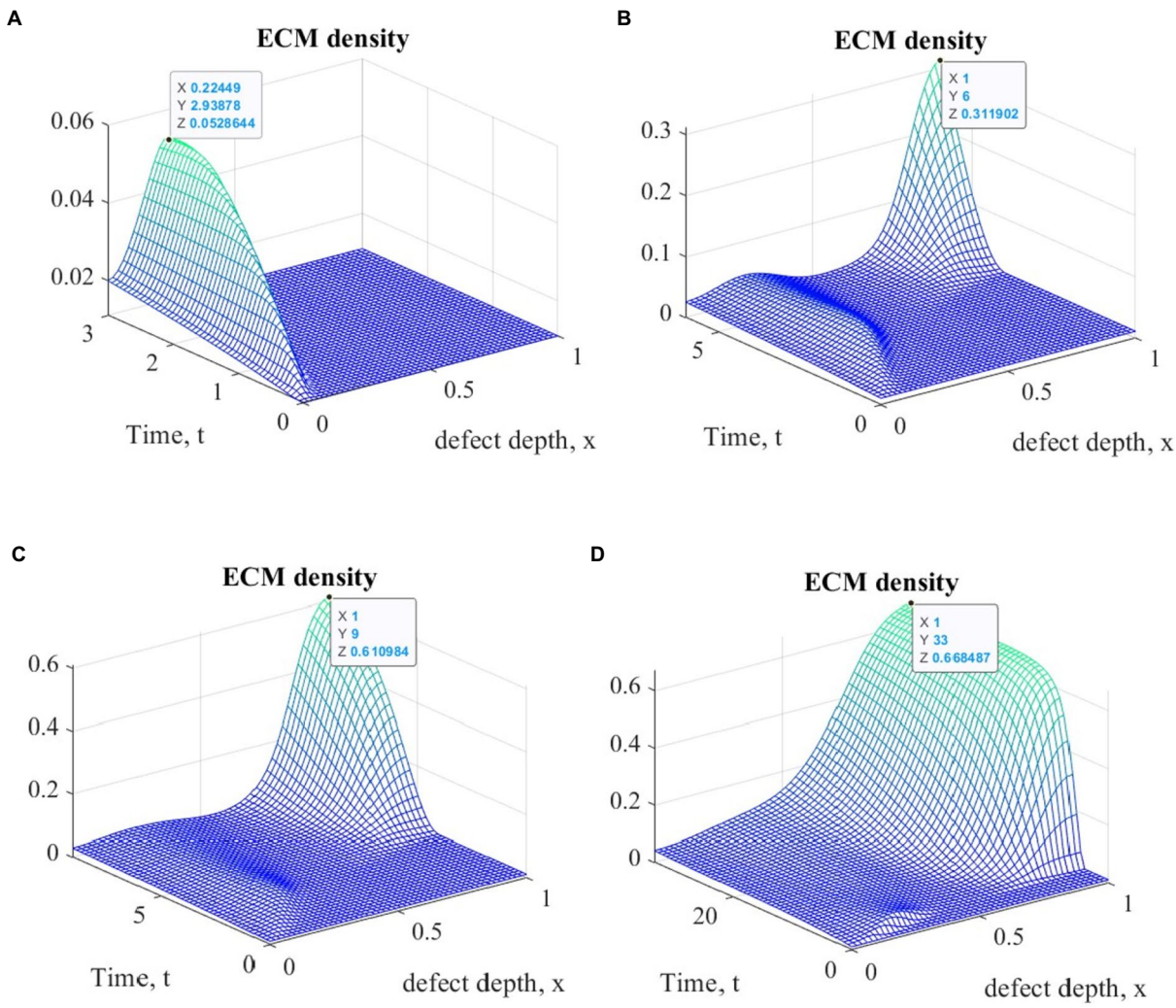

FIGURE 3
Graphs of ECM density changes at different time points during ASIT implementation: **(A)** t=3 (~1month); **(B)** t=6 (~ 2months); **(C)** t=9 (~ 3months); **(D)** t=33 (~ 12months).

TABLE 1 Maximum values of ECM density at different time points with different options of cell therapy.

Cell therapy option	Maximum ECM density, dimensionless			
	Time elapsed since start of cell therapy			
	Dim.less/Months	Dim.less/Months	Dim.less/Months	Dim.less/Months
	3/1	6/2	9/3	33/12
ASIT	0.0528644	0.311902	0.610984	0.668487
ACIT	0.0571913	0.271928	0.603764	0.668896
ASIT_ACIT	0.0527203	0.312082	0.611128	0.668575

results were also obtained using the second version of the parameters. The maximum values of the densities of the formed ECM at various points in time are given in Table 2 for the two options of the parameters given above.

An elementary analysis of the data presented in Tables 1, 2 allows us to notice that with the parameters of the model adopted on the basis of the cell therapy conditions for articular cartilage with no stimulation, they correspond to the slowest formation of the ECM among other conditions. In this case, the best conditions for the formation of the ECM are achieved with more intense tissue stimulation. At the same time, it should be noted that after a sufficient period of regenerative rehabilitation, the ECM density on the surface of the formed tissue is determined mainly by the restrictions imposed on the values of the model parameters and practically does not depend on the type of cell therapy and the nature of mechanical stimulation.

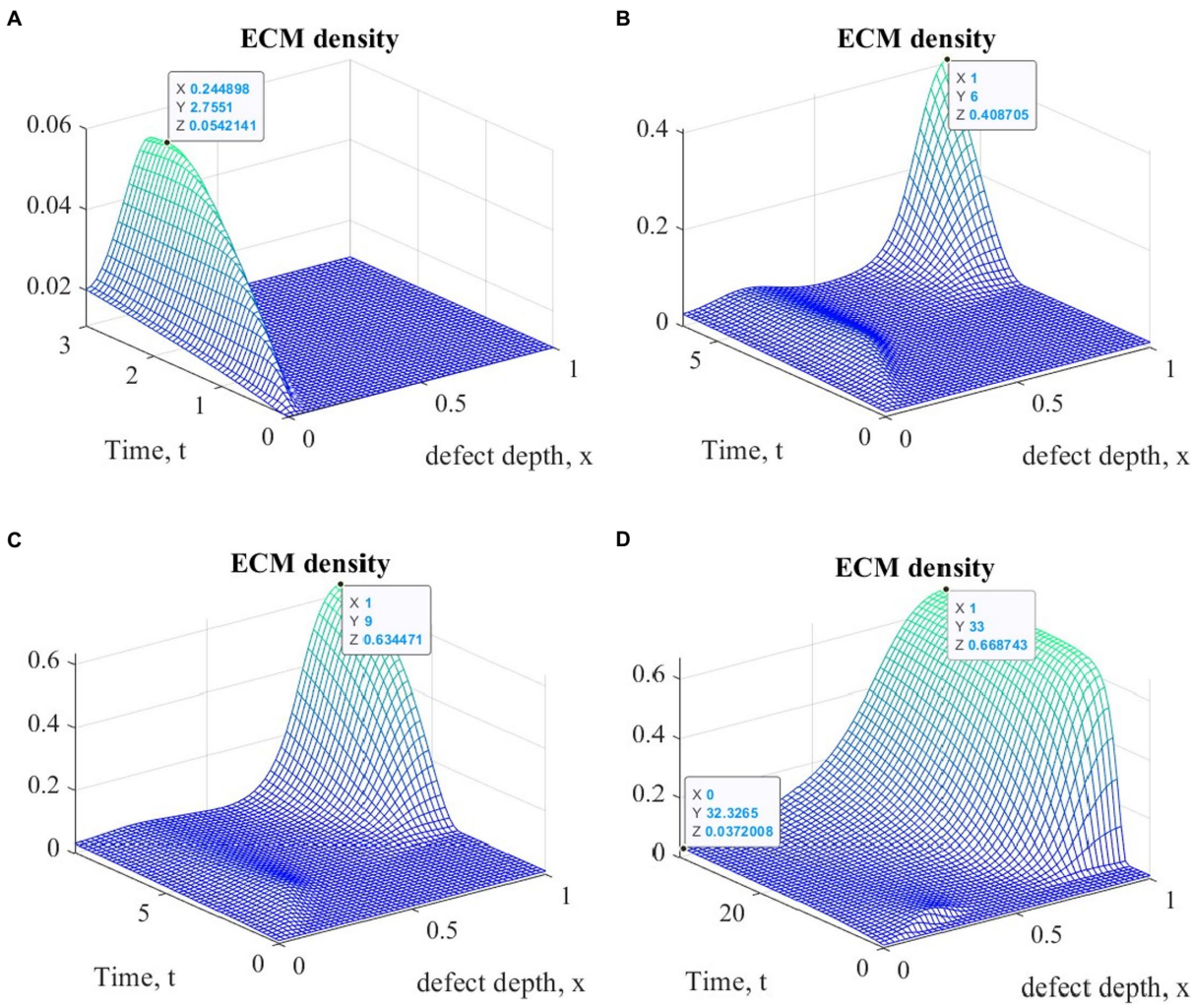

FIGURE 4
Graphs of changes in ECM density at different time points in the implementation of ASIT with mechanical stimulation with a time delay t_1=2 (~ 22days): **(A)** t=3 (~1month); **(B)** t=6 (~ 2months); **(C)** t=9 (~ 3months); **(D)** t=33 (~ 12months).

TABLE 2 Maximum values of ECM density at different time points with different cell therapy options under conditions of tissue mechanical stimulation in the area of the defect.

Cell therapy option	Maximum ECM density, dimensionless			
	Time elapsed since start of cell therapy			
	Dim.less/Months	Dim.less/Months	Dim.less/Months	Dim.less/Months
	3/1	6/2	9/3	33/12
ASIT 1	0.051351	0.436539	0.637694	0.668728
ASIT 2	0.0509713	0.401257	0.630414	0.668703
ACIT 1	0.0542141	0.408795	0.634471	0. 668,743
ACIT 2	0.0549617	0.36955	0.626309	0.668748
ASIT+ACIT 1	0.0515903	0.436741	0.637714	0.668798
ASIT+ACIT 2	0.051433	0.401377	0.630437	0.668706

Of particular interest is also the nature of the dynamics of the cartilage tissue regenerative rehabilitation process, which is observed in all types of cell therapy, regardless of the mechanical stimulation intensity. It can be estimated from the results of the other state variables analysis, the graphs of which at various points in time are shown in Figures 5–7.

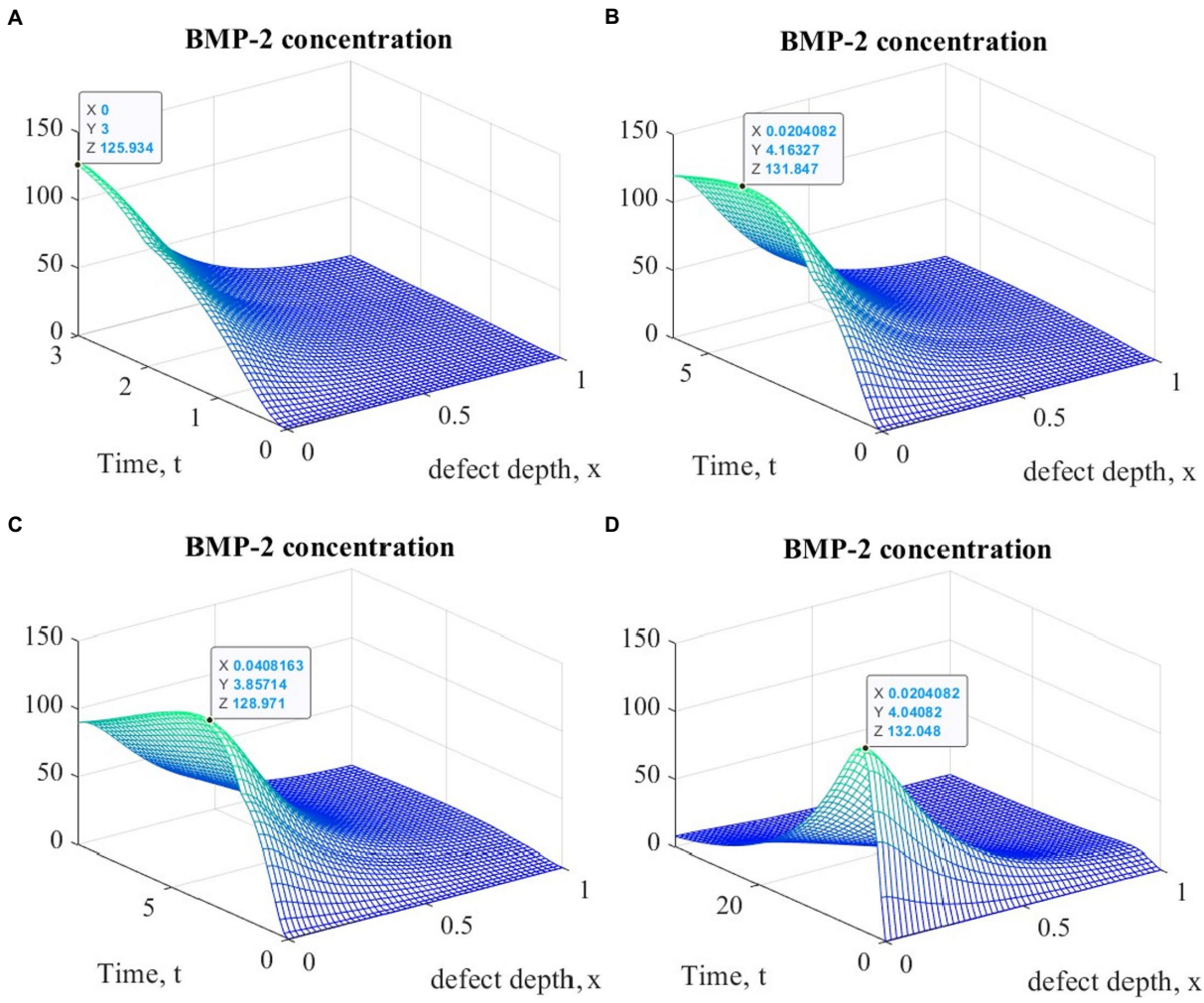

FIGURE 5
Graphs of changes in BMP-2 concentration at different points in time during the implementation of ACIT with mechanical stimulation with a time delay t_1=2 (~ 22days): **(A)** t=3 (~1month); **(B)** t=6 (~ 2months); **(C)** t=9 (~ 3months); **(D)** t=33 (~ 12months).

It is easy to see that the process of matrix formation begins from the side of the subchondral part, while growth factors BMP 2 and FGF 1 play a significant role, the concentrations of which at a certain point in time reach maximum values. These proteins continue to promote the formation of the ECM in the future, but their concentrations significantly decrease and practically tend to zero on the surface of the formed cartilage.

After a certain period of time, more intensive formation of the ECM begins to occur on the surface of the articular cartilage, which is explained by the accumulation in space the necessary amount of nutrients that promote cell proliferation and maintain their viability. Because the mathematical model under study implies a constant replenishment of nutrients from the synovial fluid, this leads to a further increase in the density of the ECM throughout the entire depth of the defect. It can be assumed that in the long term, the ECM will have a gradient structure with a density decreasing towards the subchondral bone.

It should be noted that in this study it was assumed that the maximum diffusion coefficients of all state variables remain constant throughout the entire process of regenerative rehabilitation, since there are no data indicating their change depending on changes in the macroenvironment of the tissue. At the same time, it was assumed that the probabilistic diffusion coefficients change as the ECM density increases. However, given the two-phase structure of the cartilage, it can be assumed that the intensity of diffusion of nutrients under conditions of mechanical stimulation can be significantly increased. Our estimates of this situation show that with an increase in the maximum diffusion coefficient D_n^*, the density of the formed ECM also tends to increase in all types of cell therapy under conditions of mechanical stimulation.

In this work, for the first time, the process of regenerative rehabilitation for cartilage tissue with mechanical stimulation, which provides for some time delay, was studied. The results of the numerical experiments analysis showed that a certain effect associated with this delay is observed and noticeable in the results obtained. However, it should be noted here that delayed rehabilitation procedures for ASIT and ACIT are envisaged in order to achieve the best macroenvironment of the restored tissue, which is not taken into account in the

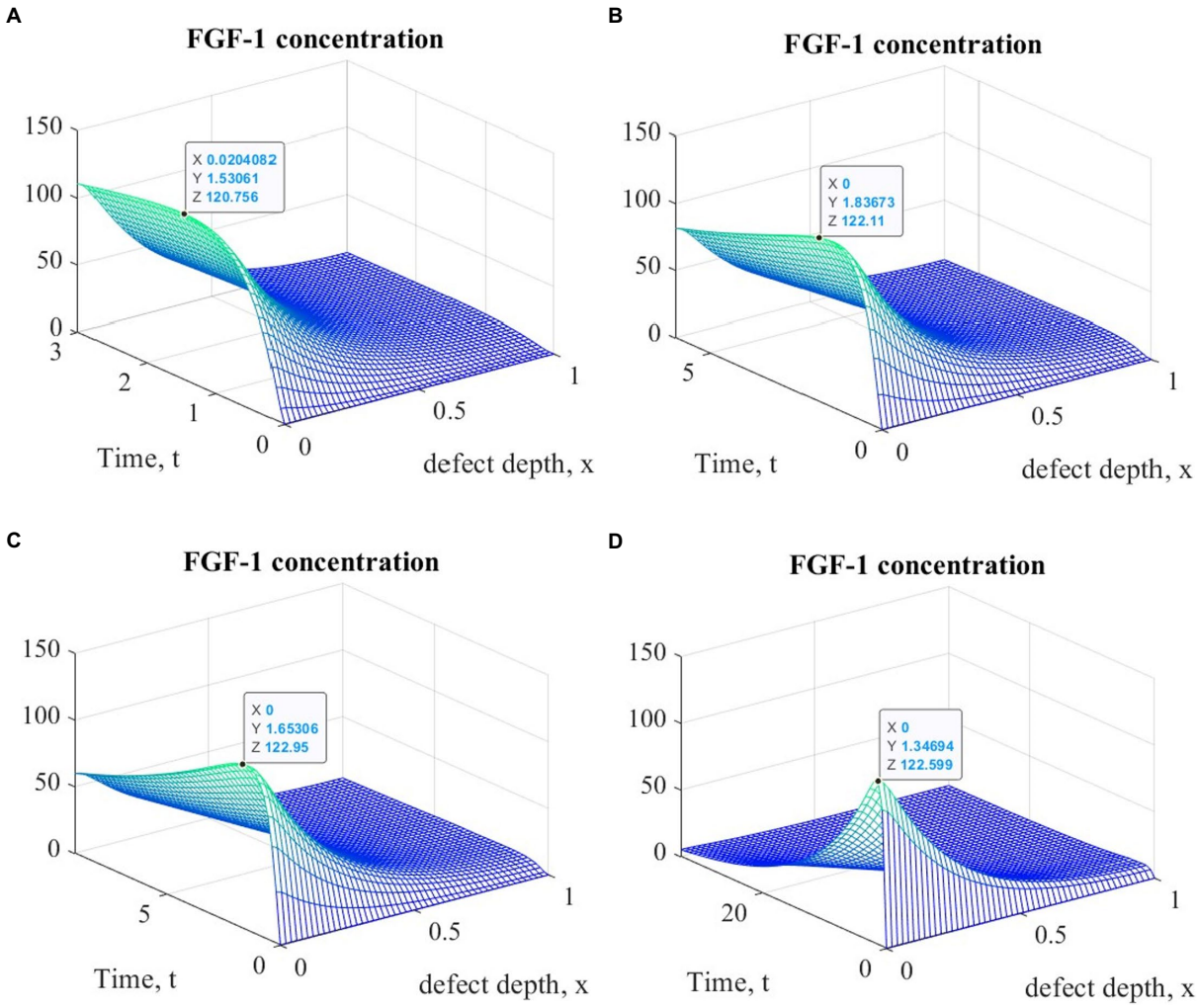

FIGURE 6
Graphs of changes in FGF-1 concentration at different time points during the implementation of ACIT with mechanical stimulation with a time delay t_1=2 (~ 22days): **(A)** t=3 (~1month); **(B)** t=6 (~ 2months); **(C)** t=9 (~ 3months); **(D)** t=33 (~ 12months).

mathematical model used in this study. Therefore, the beginning of mechanical stimulation in a delayed period of time led only to an additive result and practically did not consider the changes that occurred in the tissue during this period of time. Nevertheless, the problem of the mechanical stimulation beginning in the process of cartilage regenerative rehabilitation remains relevant and requires a deeper analysis.

4. Conclusion

Modern methods of treating deep articular cartilage lesions are based on the use of various ASIT/ACIT options and are potentially able to provide the formation of new tissue in the area of the defect. However, due to the extremely low regenerative capacity of cartilage due to its morphology, these methods and the technologies underlying them need to be improved. One of the directions that allow eliminating a number of disadvantages inherent in tissue regeneration technologies, including articular cartilage, is called regenerative rehabilitation, which involves the parallel use of regenerative and rehabilitation medicine technologies. Since chondrogenic cells (chondroblasts, young chondrocytes, MSCs) are highly mechanosensitive and proper mechanical stimulation can ensure their differentiation to the phenotype of the main cartilage tissue cells–chondrocytes, it is assumed that this can enhance the regenerative capacity of cartilage tissue and ensure the restoration of its defects. The theories underlying these assumptions are supported by the results of numerous *in vitro* studies. However, it is still not possible to achieve reliable results in the restoration of deep articular cartilage defects *in vivo* using regenerative rehabilitation technologies. One of the reasons is that it is not clear exactly how to stimulate the cartilage tissue in the area of a defect or a tissue-engineered structure populated with chondrogenic cells in order to achieve an adequate chondrogenic response and stimulate the regeneration of new tissue. The answer to this question, or at least the direction in which this answer should be sought, can be obtained as a result of the mathematical models study the regenerative rehabilitation process for articular cartilage. Such models are quite complex, and attempts

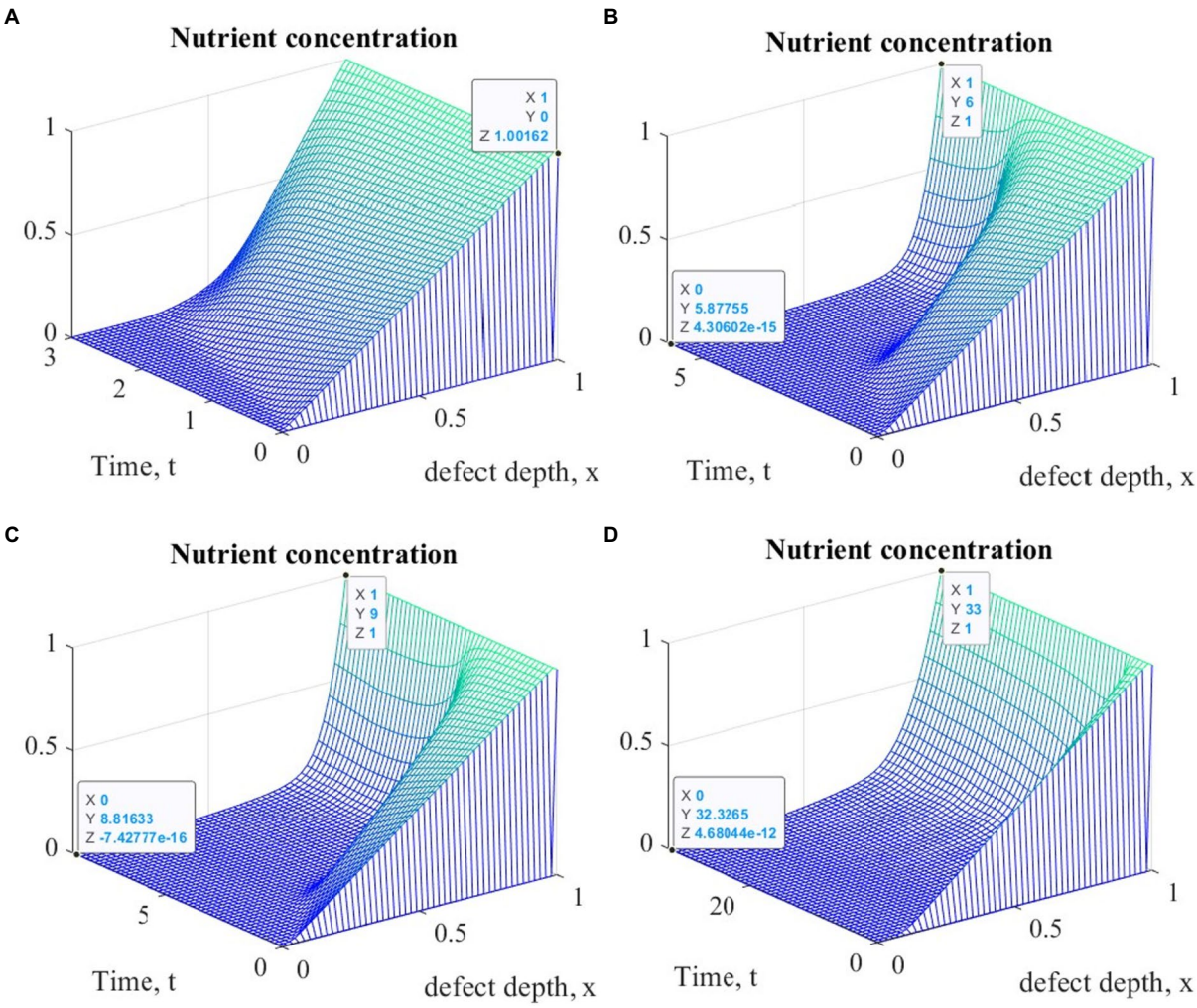

FIGURE 7
Graphs of changes in nutrient concentration at different points in time during the implementation of ACIT with mechanical stimulation with a time delay t_1=2 (~ 22days): **(A)** t=3 (~1month); **(B)** t=6 (~ 2months); **(C)** t=9 (~ 3months); **(D)** t=33 (~ 12months).

to take into account all the nuances inherent in tissue regeneration in them can lead to the impossibility of studying them. On the other hand, the results obtained in the study of simplified models may turn out to be far from the true ones. Quite adequate models describing the changes that occur in the tissue during regenerative rehabilitation, the study of which is currently available using finite element methods, are models of the "diffusion–reaction" type. One of the options of such a model was used in this work to study the dynamics of changes in state variables that indirectly characterize the dynamics of the new cartilage tissue formation under conditions of cell therapy and tissue engineering strategies. In particular, we studied the regenerative rehabilitation processes of a local articular cartilage defect using ASIT, ACIT, and ASIT+ACIT both without tissue stimulation and under conditions of delayed mechanical stimulation of varying intensity. The results obtained at the same time indicate that an increase in the proliferation rate of chondrogenic cells seeded in a scaffold placed in the area of the defect leads to a noticeable change in the process of ECM formation. In addition, as a result of numerical experiments, it was found that with an increase in the intensity of mechanical stimulation, accompanied by an increase in the supply of nutrients to the defect area, the process of ECM formation also noticeably intensifies.

The results obtained are of great practical importance, since the rates of proliferation and differentiation of chondrogenic cells after appropriate rehabilitation procedures can be measured in the laboratory. Therefore, such measurements can be used to plan rehabilitation procedures that provide the best tissue repair process.

In further studies, it is planned to study a mathematical model of regenerative rehabilitation with delayed rehabilitation procedures in the short and long term, taking into account all possible options for cellular technologies. Important attention will be paid to the structure and properties of the biodegradable scaffolds used in this case. In addition, in order to obtain modeling results that are of great practical relevance, it is necessary to determine the optimal time delay for the onset of mechanical tissue stimulation in order to ensure its best effect on the process of regenerative rehabilitation.

Data availability statement

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

Author contributions

VLP, AP, and VIP contributed to conception and design of the study. VLP and VIP performed an analysis of literary sources on the research problem. AP performed numerical experiments. All authors contributed to manuscript revision, read, and approved the submitted version.

Acknowledgments

The computer simulations were carried out using the Supercomputer cluster "Afalina" in Sevastopol State University. The authors acknowledge support by the Open Access Publication Fund of TU Berlin.

Conflict of interest

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1134786/full#supplementary-material

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frontiers | Frontiers in Medicine

TYPE Systematic Review
PUBLISHED 03 April 2023
DOI 10.3389/fmed.2023.1148623

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Rheumatology, a section of the journal Frontiers in Medicine

RECEIVED 20 January 2023
ACCEPTED 15 March 2023
PUBLISHED 03 April 2023

CITATION
Uebelhoer M, Lambert C, Grisart J, Guse K, Plutizki S and Henrotin Y (2023) Interleukins, growth factors, and transcription factors are key targets for gene therapy in osteoarthritis: A scoping review. *Front. Med.* 10:1148623. doi: 10.3389/fmed.2023.1148623

Interleukins, growth factors, and transcription factors are key targets for gene therapy in osteoarthritis: A scoping review

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Objective: Osteoarthritis (OA) is the most common degenerative joint disease, characterized by a progressive loss of cartilage associated with synovitis and subchondral bone remodeling. There is however no treatment to cure or delay the progression of OA. The objective of this manuscript was to provide a scoping review of the preclinical and clinical studies reporting the effect of gene therapies for OA.

Method: This review followed the JBI methodology and was reported in accordance with the PRISMA-ScR checklist. All research studies that explore *in vitro*, *in vivo*, or *ex vivo* gene therapies that follow a viral or non-viral gene therapy approach were considered. Only studies published in English were included in this review. There were no limitations to their date of publication, country of origin, or setting. Relevant publications were searched in Medline ALL (Ovid), Embase (Elsevier), and Scopus (Elsevier) in March 2023. Study selection and data charting were performed by two independent reviewers.

Results: We found a total of 29 different targets for OA gene therapy, including studies examining interleukins, growth factors and receptors, transcription factors and other key targets. Most articles were on preclinical *in vitro* studies (32 articles) or *in vivo* animal models (39 articles), while four articles were on clinical trials related to the development of TissueGene-C (TG-C).

Conclusion: In the absence of any DMOAD, gene therapy could be a highly promising treatment for OA, even though further development is required to bring more targets to the clinical stage.

KEYWORDS

genetic therapy, osteoarthritis, gene transfer techniques, interleukins, growth factors, transcription factors

1. Introduction

Osteoarthritis (OA) is the most common degenerative joint disease (1). Traditionally considered as a disease of "wear and tear", OA is now considered as a complex disorder affecting the whole joint and involving pro-inflammatory immune pathways (2, 3). For the patients, it is associated with a significant handicap and alteration of their quality of life. Currently, this pathology affects 500 million people worldwide and therefore contributes highly to the costs of the health and social care (4, 5).

Despite the high prevalence of OA, there is no treatment to cure or delay OA. Currently, medical treatment focuses on symptoms relief with painkillers and anti-inflammatory drugs (6, 7). Recommendations for OA non-surgical treatments are divided into non-pharmacological and pharmacological interventions with the purpose to reduce pain and joint stiffness and maintaining and improving mobility. The pharmacological treatments are dependent on patients' preferences, its phenotype, the severity of the diseases, and the presence of co-morbidities (8). In the absence of a disease-modifying OA drug (DMOAD), clinical guidelines suggest physical therapy, education, and weight management as a core treatment with pharmacological intervention if needed (9, 10).

The development of an effective treatment for OA is highly challenging. Recent advances have been made in the development of a range of biological drugs that position gene therapy as a promising option to overcome the limitations of traditional therapeutics in OA (11, 12). Gene therapy has the advantage of local delivery and, therefore, local production of therapeutic proteins for targeted, local treatment of the joint, along with a potentially reduced risk of systemic adverse events and drug-drug interactions (13). In addition, relatively long-term expression of the target gene can be achieved, thus avoiding repeated intra-articular (IA) injections.

While viral gene transfer is more efficient, non-viral gene delivery is considered safer (14, 15). It can involve lipid-based systems, polymers, nanoparticles, natural components, or simple plasmids (14, 15). Viral therapy, on the other hand, involves the administration of viral vectors, such as adenovirus (Ad), helper-dependent adenovirus (HDAd or HDV), adeno-associated virus (AAV), and retroviruses (RV) including lentivirus (LV) (13). Both non-viral and viral gene delivery systems can be used for direct *in vivo* administration or for cell-based approaches in which cells are genetically modified *ex vivo* and then infused into the patient. In cell-based approaches, different cell types can be used such as synovial fibroblasts, primary chondrocytes, mesenchymal stem cells (MSCs) or pluripotent stem cells. The investigated transgenes include both secreted proteins such as growth factors and anti-inflammatory proteins, as well as transcription factors, components of signaling pathways and small regulatory nucleic acids (miRNAs).

Gene therapy for OA treatment is a booming research topic, as evidenced by an explosion in the volume and in the quality of genomic studies, a growing number of preclinical and clinical-stage gene therapy drug candidates, and the first gene therapies being tested in clinical trials (11, 12). While a significant number of reviews of the literature exist about gene therapy in OA, no scoping reviews have thus far been published on this subject. We, therefore, propose this scoping review to map the relevant literature related to both non-viral and viral gene therapy in the management of OA.

The objective of this scoping review was to provide a comprehensive view on the current knowledge about gene therapies developed in the context of preclinical and clinical studies targeting OA. Four sub-questions were developed and will reflect the objectives of the scoping review:

(1) What are the delivery methods for gene therapies in OA?
(2) Which models have been developed to assess gene therapies in OA?
(3) What are the target genes in gene therapy approaches for OA?
(4) What are the effects observed for gene therapies in OA?

2. Methods

2.1. Eligibility criteria

Population: All research studies that address OA disease were included.

Concept: All research studies that explore *in vitro*, *in vivo,* or *ex vivo* gene therapies as well as clinical trials on gene therapies that follow a viral or non-viral gene therapy approach were considered. Gene therapies using siRNA approaches, or targeting miRNA, circRNA, as well as germline gene therapies including CRISPR/Cas9, were not taken into consideration.

Context: All research studies that include models of experimental OA, *in vitro* and *in vivo* models of OA, as well as clinical trials and studies on humans were included.

Types of sources: This scoping review considered all peer-reviewed published research studies that address the use of gene therapy in OA. Only studies published in English were considered for inclusion in this review. There were no limitations to their date of publication, country of origin, or setting.

The protocol for this review has not been registered.

2.2. Information sources

The following electronic databases were searched: Medline ALL Ovid, Embase, and Scopus. The most recent search was executed on March 7, 2023.

2.3. Search

The research team (MU and CL) undertook a preliminary search to identify controlled terms and keywords in titles and abstracts from relevant literature. Then, an extensive literature search was conducted.

The search strategies (see below for an example of Ovid MEDLINE and Appendix) were performed with the help of an information specialist experienced in evidence synthesis and adapted for each database. The search strategies focused on two concepts—gene therapy and osteoarthritis—and used a set of keywords and controlled terms.

Database: Ovid MEDLINE (R) ALL <1946 to March 02, 2023>
Search Strategy:

1 Genetic Therapy/ (52544)
2 Targeted gene repair/ (201)
3 ((gene or genes or genetic) adj3 (therap* or repair* or correction*)).ti,ab,kf. (82728)
4 DNA therap*.ti,ab,kf. (100)
5 1 or 2 or 3 or 4 (104404)
6 exp Osteoarthritis/ (75797)
7 osteoarthr*.ti,ab,kf. (92344)
8 osteo-arthr*.ti,ab,kf. (635)

9 arthros*.ti,ab,kf. (46096)
10 (degenerative adj3 arthriti*).ti,ab,kf. (1748)
11 (degenerative adj3 joint* adj3 disease*).ti,ab,kf. (3757)
12 coxarthros*.ti,ab,kf. (1705)
13 gonarthros*.ti,ab,kf. (1221)
14 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 (155956).
15 5 and 14 (450).

2.4. Selection of sources of evidence

All identified relevant records were uploaded into Covidence (https://www.covidence.org) and duplicates were removed. Following a pilot test, titles and abstracts were screened by two independent reviewers (MU & CL) for assessment according to the inclusion criteria for the scoping review. Then, the full text of selected papers was assessed according to the inclusion criteria by the same two independent reviewers. For both stages, discrepancies between the reviewers were resolved by discussion or by consulting a third reviewer (YH).

2.5. Data charting process

A data-charting form was jointly developed by two reviewers (MU & CL) to determine which variables to extract (experimental models, delivery methods, targets, and effects). This data charting form was used to extract data from selected studies. The same two reviewers independently charted the data, discussed the results, and updated, if necessary, the data-charting form to enable the capture of all relevant data to answer the review question.

2.6. Data items

Data on the population, concept, context, and key findings relevant to the review question was extracted. The data-charting form included the following items:

- Author and year of publication
- Objectives
- Participants (for clinical trials, only)
- Study design
- Disease models
- Delivery methods
- Targets
- Effects.

2.7. Synthesis of results

Data were analyzed and summarized quantitatively through numerical counts as well as descriptively. Data were presented graphically or in tabular form. A narrative summary accompanied the charted and/or tabulated results and describes how the results relate to the objectives and questions of the review.

3. Results

3.1. Selection of sources of evidence

The PRISMA flowchart outlining study selection is shown in Figure 1. An initial search identified 2,182 studies, 1,335 of which remained after the removal of duplicates. All but 274 of these studies were excluded at the stage of abstract review. Furthermore, 208 additional studies were excluded after reviewing the full manuscript. The reasons for exclusion at each stage are detailed in Figure 1. Overall, 66 studies responded to the inclusion criteria and constituted the study data.

3.2. Characteristics of sources of evidence

Most articles were on preclinical *in vitro* studies (32 articles) or *in vivo* animal models (39 articles), while four articles were on clinical trials.

3.3. Results of individual sources of evidence

We found a total of 29 different targets for gene therapy in the context of OA. These included studies examining interleukins (IL-1Ra alone or in combination with another target, TNF-RI, IL-1RII, IL-10, IL-4, TSG6, CrmA), growth factors, and receptors (IGF-1, relaxin, TGF-β1, BMP2 and 4, follistatin, GDF-5, FGF-2/bFGF), transcription factors (SOX9 alone or in combination with another target, KLF2 and 4, and ATF-4) and other key targets such as PRG4 (alone or in combination with another target), LOXL2, GlcAT-1, GGCX, kallistatin, RHEB, HSP70, PUM1, sCCR2 E3 and LRP3 (Figure 2).

4. Discussion

4.1. Summary of evidence

4.1.1. Interleukins

The activation of the immune system is closely linked to the initiation and perpetuation of low-grade systemic inflammation in OA (3). Interleukin-1 (IL-1) is considered among the most powerful molecules of the innate immune system and is linked to the pathogenesis of OA. It therefore appears to be a natural target for gene therapy. The IL-1 family is constituted of seven agonists including IL-1α and β, and four antagonists including the IL-1 receptor antagonist (IL-1Ra). The effect of IL-1 on joint tissues can be controlled by inhibiting its receptors using gene therapy-mediated overexpression of IL-1Ra (Table 1).

4.1.1.1. IL-1Ra

The therapeutic effect of IL-1Ra on the progression of OA lesions was evaluated by Pelletier et al. in a surgical dog OA model (ACLT model) (16). Interestingly, they observed a reduction in macroscopic lesion severity in animals that were injected with synovial cells retrovirally transduced with the IL-1Ra gene compared with the lac Z (control) group. Similarly,

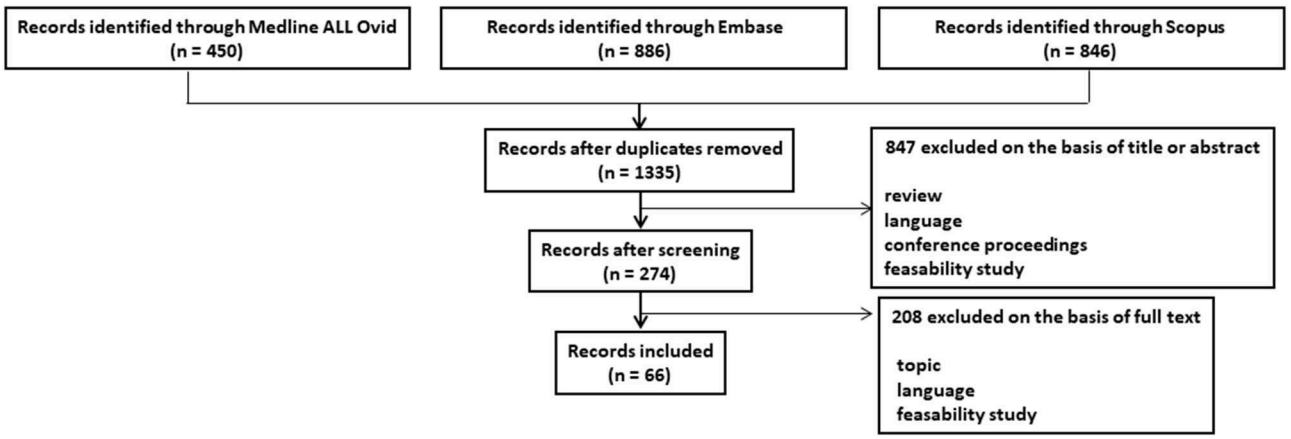

FIGURE 1
PRISMA flowchart outlining study selection procedure.

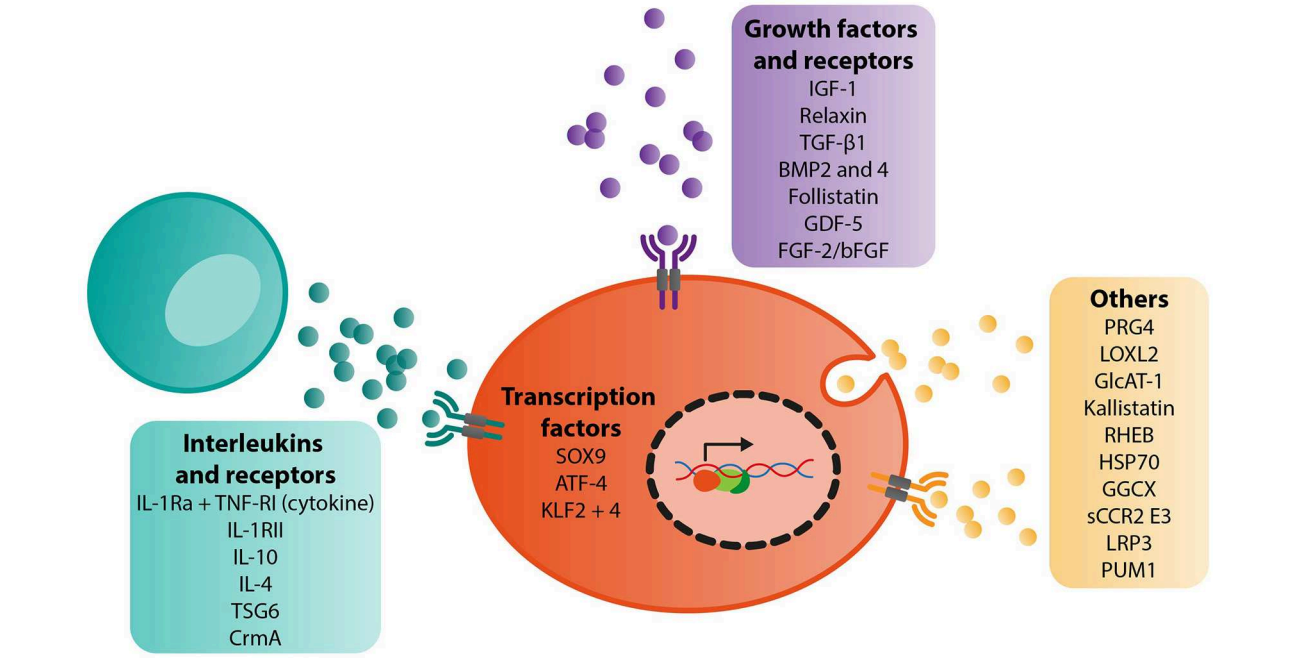

FIGURE 2
Summary of target genes identified for gene therapy of OA and their biological functions.

Baragi et al. observed a positive effect on human OA articular chondrocytes and OA cartilage explants transduced with Ad.RSV hIL-1ra cDNA (17). Nixon et al. investigated the disease-modifying properties of IL-1Ra gene therapy with an adenoviral-mediated overexpression of IL-1Ra under the control of the inflammation-inducible NF-kB promoter (HDAd-IL-1Ra) (18). In a anterior cruciate ligament transection (ACLT) mouse model of OA, they highlighted an improvement in histological scores, a decrease in osteophytes, and an increase in the volume and surface area of the cartilage. HDAd-IL-1Ra treatment also protected osteoarthritic mice against increased thermal hyperalgesia. Moreover, they confirmed these results in an osteochondral fragment horse OA model, observing an improvement in pain, claudication, and amplitude of movement, together with improved macroscopic and histological cartilage and synovium status. Deng et al. evaluated the potential of loaded nanomicelles to treat articular inflammation in a rat temporomandibular joint OA model (MIA model) (19). In this one, IL-1Ra mRNA reduced pain behavior and attenuated cartilage degradation as evidenced by the decrease of Mankin score. In addition, authors observed a downregulation of pro-inflammatory cytokines (IL-6 and TNF-α) blocking the MIA-induced inflammatory cascade. Finally, Senter et al. evaluated efficacy, biodistribution and safety of FX201 (HDAd expressing human IL-1Ra) and its ratIL-1Ra expressing ortholog vector in the

rat ACLT OA model (20). IL-1Ra gene therapy significantly reduced joint damage in arthritic animals, and the gene therapy vectors were shown to be well-tolerated and to remain predominantly at the injection site after IA injection. Following these investigational new drug (IND)-enabling studies, a Phase 1 clinical trial with FX201 was conducted in 72 knee OA patients (NCT04119687).

Fernandes et al. developed a direct method using a non-viral vector, named "lipoplex" (21). It is a plasmid complexed to a lipid injected IA into the knees of rabbits. In a rabbit menisectomy model, they observed a dose-dependent reduction of lesion size associated with a reduction of the width of osteophytes. Another group, Zhang et al., evaluated a different non-viral strategy, in a rabbit surgery OA model (medial collateral ligament excision and medial menisectomy) (22). They demonstrated that the transfection efficiency of chitosan–DNA nanoparticles containing IL-1Ra or IL-10, was closely related to the gene product, and observed less severe cartilaginous lesions after treatment with IL-1Ra. Deng et al., employed a non-viral strategy using nanoparticles consisting of chitosan (CS)/hyaluronic acid (HA)/plasmidDNA (pDNA) encoding IL-1Ra to transfect primary synoviocytes (23). This strategy reduced MMP-3,−13, COX-2, and iNOS expression in IL-1β-stimulated synoviocytes. These results constitute a promising therapeutic approach for synovitis. Frisbie et al. investigated the effect of an Ad vector expressing IL-1Ra (Ad-EqIL-1Ra) in an equine osteochondral fragment OA model (24). They demonstrated that in addition to the conservation of the articular cartilage and the synovial membrane, the IA injection of the Ad-EqIL-1Ra vector significantly improved pain- and inflammation-related parameters. Goodrich et al. worked with an equine model, with the aim of developing the self-complementary AAV (scAAV) to produce higher levels of protein more quickly than with single stranded AAV (25). They proposed a dosing/alternative serotype redosing protocol, and examined the neutralizing antibody (Nab) response to the capsid. They did not observe any IA toxicity but the development of Nab against AAV capsid, which did however not decrease protein expression. Watson Levings et al. investigated the therapeutic capacity of an scAAV (sc-AAV.eqIL-1Ra) in two equine OA models (26, 27). On the first spontaneous model, they confirmed the safety. On the second, a surgically induced osteochondral fragmentation model of early OA, the results showed both a reduction in forelimb lameness and a reduction in inflammation. The authors also observed an improvement in the repair of osteochondral lesions, a reduction in joint effusion, and synovial proliferation. These data confirmed the results of previous studies.

Besides the "traditional" methods, Glass et al. investigated the possibility to combine gene therapy and tissue engineering by inducing IL-1Ra overexpression in human MSCs *via* scaffold-mediated lentiviral gene delivery (28). The results were quite promising since they demonstrated that the constructs could produce IL-1Ra in an inducible manner and that they protected against the effects of IL-1α. Gabner et al. also investigated the combination of tissue engineering methods employing bone marrow-derived equine MSCs transduced with a lentiviral vector expressing IL-1Ra gene under the control of the inducible NF-kB promoter (pSEWNFKBIL-1Ra) (29). They observed an increase of aggrecan, collagen IIA1 expression and a decrease of IL-6,−8, MMP-1, and−13 expression in equine chondrocytes stimulated with IL-1β or TNF-α.

Based on the assumption that the therapeutic effects of a single gene administration are limited, several teams have also evaluated the combinatorial effect of different targets. For example, Chen et al. investigated the combination of basic fibroblast growth factor (bFGF)/IL-1Ra and/or insulin-like growth factor 1 (IGF-1) (ADbFGF/ADIL-1Ra and/or ADIGF-1) (30). Transfection of human OA chondrocytes with two or three genes in different combinations resulted in increased proliferation of chondrocytes along with an increased synthesis of glycosaminoglycans, type II collagen, and TIMP-1, and a decreased expression of MMP-3,−13 and ADAMTS-5. In a rabbit ACLT model, the same team observed similar effects for type II collagen as well as a lower cartilage Mankin score. For his part, Zhang et al. evaluated the effect of IL-1Ra/IL-10 using retroviruses PLXRN-IL-1Ra and PLXRN-IL-10 in a rabbit OA model (medial collateral ligament excision and medial menisectomy) (31). While they observed no effect on synovitis, results on cartilage damage and glycosaminoglycan (GAG) content were promising. Haupt et al. tested the combinatorial effect of adenovirus-mediated overexpression of IL-1Ra with IGF-1 (equine AdIL-1Ra and equine AdIGF-1) to control cartilage degradation (32). *In vitro*, they demonstrated that the gene transfer promoted GAG and type II collagen synthesis and reduced IL-1β, IL-1α, and matrix metalloproteinases expression. Zang et al. evaluated the combined injection of IL-1Ra and TGFβ1 with liposomes *in vivo* (33). In a rabbit OA model (medial collateral excision and medial menisectomy), they observed a significant inhibition of cartilage matrix degradation as well as a prevention of osteophyte formation. The Mankin score was decreased in the transfected groups, and the expression of IL-1Ra and TGF-β1 was correlated to an increase of type II collagen expression and an extracellular matrix deposition. Finally, Wang et al. considered IL-1Ra with the soluble tumor necrosis factor-α receptor type I (sTNF-RI) (34). In a rabbit surgery OA model (Medial collateral ligament excision and medial menisectomy), IA injection of the combination Ad-IL-1Ra and Ad-sTNF-RI resulted in a decrease in cartilage lesions and synovitis whereas injection of sTNF-RI alone had no significant effect on these parameters. Multiple target combinations therefore seem to have beneficial effects in the context of gene therapy in OA.

Taken together, IL-1Ra gene therapy has been reported to improve clinical parameters such as pain and disease activity. In addition, beneficial effects on histological parameters of the synovial membrane and articular cartilage have been observed by different study groups.

4.1.1.2. IL-1RII

Type II IL-1β receptor (IL-1RII) serves as a "decoy" target for IL-1β. Previous data showed that it significantly inhibited IL-1-induced production of NO and/or PGE_2 in synovial cells, chondrocytes, and epithelial cells. Under OA conditions, these same cells lack detectable amounts of IL-1Ra and IL-1RII, two molecules that normally antagonize IL-1 (44). In this context, and in order to reconstitute the functional expression of IL-1RII, Attur et al. transduced human articular OA chondrocytes and synoviocytes (IL-1RII$^-$ cells) with an Ad vector expressing IL-1RII, AdRSVRII (35). They observed a dose-dependent decrease

TABLE 1 Interleukins.

References	Objectives	Study design	Disease models	Delivery methods	Targets	Effects
Pelletier et al. (16)	Evaluate the therapeutic effect of IL-1Ra on progression of OA lesions	Preclinical *in vivo*	Dog ACLT section and partial synovectomy of the knee	Retrovirally transduced synovial fibroblasts	IL-1Ra	Reduced progression of experimentally induced OA lesions after intraarticular injection of transduced synovial cells
Baragi et al. (17)	Demonstrate the chondroprotective effect of IL-1ra	Preclinical *in vitro* and *ex vivo*	Human articular OA chondrocytes and OA cartilage explants	Adenovirus: Ad.RSV hIL-1ra	IL-1Ra	Adherence and viability of transduced chondrocytes on surface of hyaline cartilage; protection of OA cartilage from Il-1β-induced matrix degradation
Nixon et al. (18)	Investigate the disease-modifying properties of IL-1Ra gene therapy	Preclinical *in vivo*	Mouse ACLT model	Helper-dependent adenovirus: HdAd-mIL-1Ra	IL-1Ra	Prevention and treatment of surgically induced OA: improved histologic scores, fewer osteophytes; higher cartilage volume and surface
			Horse osteochondral fragment model	Helper-dependent adenovirus: HdAd-eqIL-1Ra		Improvement in pain: improved lameness, range of motion and effusion; better cartilage status; improved synovial membrane status; fewer osteophytes
Deng et al. (19)	Evaluate the potential of loaded nanomicelles to treat articlular inflammation in *in vivo* TMJOA model	Preclinical *in vivo*	Rat MIA model	Polyplex nanomicelles	IL-1Ra	Reduced pain behavior; less cartilage degradation; lower Mankin score; reduced surface fibrosis; reduced OA progression; downregulation of pro-inflammatory cytokines
Senter et al. (20)	Assess efficacy, biodistribution and safety of HDAd-ratIL-1Ra as well as biodistribution of FX201 (human equivalent)	Preclinical *in vivo*	Rat ACLT model	Helper-dependent adenovirus: HDAd-ratIL-1Ra and FX201 (HDAd-hIL-1Ra)	IL-1Ra	HDAd-ratIL-1Ra decreased OA-induced joint damage; HDAd-ratIL-1Ra and FX201 mainly localized to knee joint; HDAd-ratIL-1Ra well-tolerated
Fernandes et al. (21)	Determine the effect of IL-1Ra through a lipoplex on structural changes in *in vivo* OA model	Preclinical *in vivo*	Rabbit menisectomy model	IL-1Ra plasmid (Lipoplex)	IL-1Ra	Reduced width of osteophytes and size of macroscopic lesions (dose-dependent); reduced severity of histologic cartilage lesions; presence of IL-1Ra in the synovium and cartilage of injected rabbits
Zhang et al. (22)	Evaluate the efficiency of chitosan-EGFP nanoparticles for gene therapy of OA	Preclinical *in vivo*	Rabbit medial collateral ligament excision and medial menisectomy model of OA	Chitosan-EGFP nanoparticles	IL-1Ra or IL-10	Less severe lesions after treatment with IL-1Ra; (no expression of IL-10, therefore effect was not studied)
Deng et al. (23)	Development of a new nanoparticle made of chitosan (CS)/hyaluronic acid (HA)/plasmid-DNA	Preclinical *in vitro*	Rat IL-1β-treated synoviocytes	CS/HA/pDNA nanoparticles	IL-1Ra	Increased IL-1Ra expression and decreased MMP-3, MMP-13, COX-2 and iNOS expression in IL-1β-induced synoviocytes
Frisbie et al. (24)	Evaluate the utility of the equine IL-1Ra gene therapy in a equine OA model	Preclinical *in vitro*	Equine synoviocytes	Adenovirus: Ad-EqIL-1a	IL-1Ra	Dose dependent increase of IL-1Ra after transduction of equine synoviocytes; inhibit PGE2 production in response to human IL-1α
		Preclinical *in vivo*	Equine OA model			Improvement in clinical parameters of pain, disease activity, preservation of articular cartilage, beneficial effects on histologic parameters of synovial membrane and articular cartilage
Goodrich et al. (25)	scAAVIL-1Ra dosing trial in an equine model	Preclinical *in vivo*	Skeletally mature horses	Adenoassociated virus: scAAV2IL-1ra	IL-1Ra	Transduction of the scAAV vector both in the synovial and cartilage tissues; no evidence of intra-articular toxicity; neutralizing ABs within 2 weeks of administration which persisted for the duration of the study but did not lower protein expression intra-articularly

(Continued)

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TABLE 1 (Continued)

References	Objectives	Study design	Disease models	Delivery methods	Targets	Effects
Watson Levings et al. (26)	Generate pharmacokinetic profile of homologous gene delivery of scAAV.IL-1Ra	Preclinical *in vivo*	Naturally occurring OA in horses	Adenoassociated virus: sc-AAV.eqIL-1Ra	IL-1Ra	Safe and sustained drug delivery to joints
Watson Levings et al. (27)	Efficacy of local treatment with scAAV.IL-1Ra	Preclinical *in vivo*	Horse surgically induced osteochondral fragmentation (OCF) model	Adenoassociated virus: sc-AAV.eqIL-1Ra	IL-1Ra	Reduced forelimb lameness; reduced inflammation; enhanced repair of osteochondral injury; reduced joint effusion; reduced synovial proliferation
Glass et al. (28)	Evaluate the possibility to combine gene therapy and functional tissue engineering to develop engineered cartilage with inductible immunomodulatory properties	Preclinical *in vitro*	IL-1β-stimulated MSCs from human bone marrow	IL-1Ra lentivirus *via* scaffold	IL-1Ra	Engineered cartilage constructs are capable of inducible and tunable IL-1Ra production at therapeutically relevant concentrations; these constructs protect from the effects of IL-1
Gabner et al. (29)	Evaluate IL-1Ra expression in equine MSCs	Preclinical *in vitro*	Equine OA chondrocytes co-culture	Lentivirus: pSEWNFKBIL-1Ra	IL-1Ra	Protective ability of the IL-1Ra protein (increased ACAN and COL2A1 and decreased IL-6, MMP-1 and MMP-13); upon TNF-α, a dose-dependent increase in IL-1Ra expression in MSC/IL-1Ra cells
Chen et al. (30)	Investigate the combinatorial effect of adenovirus-mediated overexpression of bFGF vs. IL-1Ra vs. IGF-1 on OA	Preclinical *in vitro*	Human articular OA chondrocytes	Adenovirus: AdbFGF; AdIL-1Ra; AdIGF-1	bFGF • IL-1Ra • IGF-1	Increased chondrocyte proliferation; increased GAG and type II collagen synthesis
		Preclinical *in vivo*	Rabbit ACLT model			Protects from cartilage degradation: lower Mankin score; increased type II collagen and proteoglycan synthesis; better results with combinations
Zhang et al. (31)	Evaluate the effect of using IL-1Ra and IL-10 together as gene therapy for OA	Preclinical *in vivo*	Rabbit medial collateral ligament excision and medial menisectomy model of OA	Retrovirus: PLXRN-IL-1Ra and PLXRN-IL-10	IL-1Ra and IL-10	Reduced cartilage lesions and decreased loss of proteoglycans after combined injection; no effect on synovitis
Haupt et al. (32)	Evaluate the combinatorial effect of adenovirus-mediated overexpression of IGF-1 and IL-1Ra in an OA culture model	Preclinical *in vitro*	IL-1β-stimulated horse cartilage explants and synovial membrane	Adenovirus: equine AdIGF-1; equine AdIL-1Ra	IGF-1 and IL-1Ra	Matrix synthesis stimulation and catabolics blockers, prevention matrix degradation by IL-1, protection and partial restoration of cartilage matrix
Zhang et al. (33)	Evaluate feasibility of gene therapy by co-injecting IL-1Ra and TGF-beta1 genes into joints together with liposomes	Preclinical *in vivo*	Rabbit medial collateral ligament excision and medial menisectomy model of OA	Lipofectamine transfection	IL-1Ra and TGF-β1	Inhibited cartilage damage and prevention of osteophyte formation; increased Mankin score; normalization of choncdrocyte number and order; increased type II collagen expression and ECM deposition
Wang et al. (34)	Determine the efficacy of local expression of IL-1Ra and sTNF-RI	Preclinical *in vivo*	Rabbit medial collateral ligament excision plus medial menisectomy OA model	Adenovirus: Ad-IL-1Ra and Ad-sTNF-RI	IL-1Ra and TNF-RI	Reduced cartilage lesions after IL-1Ra injection and combination, but not after sTNF-RI injection alone; reduced synovitis after combinatorial injection
Attur et al. (35)	Determine the effect of IL-1RII expression on modulating effects of IL-1beta	Preclinical *in vitro*	Human articular OA chondrocytes and synoviocytes	Adenovirus: AdRSVRII	IL-1RII	Dose-dependent decrease in response of OA chondrocytes and synoviocytes to IL-1beta (induction of NO, PGE2, IL-6, IL-8; production of IL-1beta and proteoglycan) protection of other cells in co-culture and transplant from effect of IL-1beta *via* sIL1-RII

(Continued)

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TABLE 1 (Continued)

References	Objectives	Study design	Disease models	Delivery methods	Targets	Effects
Broeren et al. (36)	Determine the therapeutic potential of CXCL10p-IL10 in 3D micromass synovial membrane model that mimics early stage OA	Preclinical *in vitro*	Human OA synovial tissue	Lentivirus: CXCL10p-IL10	IL-10	Reduced IL-1β-induced secretion of IL-1β and IL-6
Farrell et al. (37)	Evaluate the ability of hMSCS overexpressing vIL-10 to modulate the inflammation and alter OA disease progression	Preclinical *in vivo*	CIOA mouse model	Adenovirus: AdIL-10	vIL-10	A trend toward more damage in animals treated with hMSCs; reduced CD4 and CD8 T cells in the vIL-10-expressing hMSC group
Cameron et al. (38)	Investigate combinatorial effect of BM-MSCs and IL-10 overexpression	Preclinical *in vitro*	IL-1β/TNF-α-stimulated horse BM-MSCs and cartilage explant co-cultures	Adenoassociated virus: AAV-IL10	IL-10	Decreased T cell proliferation; decreased expression of inflammatory markers (IL-1beta, IL-6 and TNF-alpha) in stimulated cartilage explant co-cultures; no protection from ECM degradation
Watkins et al. (39)	Toxicology study of intra-articular hIL-10var pDNA in dogs	Preclinical *in vivo*	Healthy naive dogs	hIL-10var pDNA (transfection with Fugene 6)	IL-10	Well-tolerated without toxicologic effects for up to 1.5 mg of plasmid
	Efficacy of intra-articular hIL-10var pDNA in companion dogs		Naturally occurring OA in companion dogs			No adverse changes; decreased pain scores
Lang et al. (40)	Optimization of a non-viral transfection system to evaluate Cox-2 controlled IL-4 expression for OA gene therapy	Preclinical *in vitro*	Equine chondrocytes	pN3.Cox2.IL-4 (different transfection agents)	IL-4	Exogenous stimulation of chondrocytes transfected with pN3.Cox-2.IL-4 led to increased IL-4 expression and decreased IL-1β,−6,−8, MMP-1 and−3 expression
Song et al. (41)	Investigate whether IL-4 transfection and spheroid formation potentiates therapeutic effect of MSCs for OA	Preclinical *in vitro*	Rat IL-1β stimulated primary chondrocytes	IL-4 MSC spheroids (delivered *via* cationic liposomes)	IL-4	Reduced IL-1beta induced apoptosis; lower production of osteoarthritic factors; higher production of cartilage ECM
		Preclinical *in vivo*	Rat ACLT-MMx model			Enhanced attenuation of tissue regeneration; improved chondroprotective and anti-inflammatory effects; higher pain relief
Broeren et al. (42)	Determine the effect of viral overexpression of TSG-6 in experimental OA	Preclinical *in vitro*	BM-derived cells differentiated into osteoclasts	Adenovirus: pShuttle-CMV-TSG-6	TSG-6	Inhibited osteoclast activity
		Preclinical *in vivo*	Mouse CIOA model			No difference in protease activity or cartilage damage; increased ectopic bone formation
Qiu et al. (43)	Investigate the effect of HA/CS/pCrmA on OA synoviocytes	Preclinical *in vitro*	IL-1β stimulated primary rat synoviocytes	Hyaluronic acid/chitosan (HA/CS) nanoparticles	pCrmA	Attenuated IL-1β mediated inflammation: normalization of increased MMP-3 and MMP-13 expression caused by IL-1β stimulation

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of NO, PGE_2, IL-6, IL-8, IL-1, and proteoglycan production in response to IL-1β. Another consequence was the release of sIL-1RII from transduced cells, thus protecting the other cells in co-culture and in transplants from the effect of IL-1β *via* sIL1-RII.

4.1.1.3. IL-10

Another interesting target for gene therapy is the cytokine IL-10. Produced by cells of innate and adaptive immunity, it is known for its anti-inflammatory and immunosuppressive properties. Thus, several gene therapies targeting this pleiotropic cytokine have been reported. In a rabbit OA model (medial collateral ligament excision and medial menisectomy), Zhang et al. were the first to evaluate the effect of retroviral IL-1Ra and IL-10 gene delivery on rabbit knee joints during the early inflammatory phase of OA (31). Thus, IA retroviral administration of IL-1Ra and IL-10 (PLXRN-IL-1Ra and PLXRN-IL-10) was able to impede an acute inflammatory reaction. IL-1Ra was more effective than IL-10 and most importantly, the co-injection of IL-1Ra and IL-10 was found to have significantly greater chondroprotective effects. Cartilage degradation and loss of proteoglycans was significantly more reduced by the combination than either one alone. In a 3D micromass synovial membrane model that mimics early-stage OA, Broeren et al. showed that adequate amounts of IL-10 transgene (lentivirus named CXCL10p-IL-10) reduced the synovial production of IL-1β and IL-6 and consequently, the inflammatory response (36). Farrell et al. assessed whether hMSCs overexpressing vIL-10 (*via* AdIL-10) were able to modify inflammation and adjust OA progression in a collagenase induced osteoarthritis model (CIOA) mouse model (37). Interestingly, the amount of activated CD4 and CD8 T-cells was significantly reduced in the vIL-10-expressing hMSCs group. A subsequent study conducted by Cameron et al. supports their findings (38). Indeed, in a stimulated, co-culture OA model, T-cell proliferation was significantly reduced by BM-MSCs overexpressing IL-10 (AAV-IL-10). This was accompanied by a reduced expression of inflammatory markers (IL-1β, IL-6, and TNF-α). Finally, considering the short half-life of IL-10 as well as its poor joint permeability, Watkins et al. used a plasmid DNA-based therapy for the production of a long-acting human IL-10 variant called hIL-10var (39). The first results of the 6-month GLP toxicology study looked promising. Bilateral IA injections of up to 1.5 mg of hIL-10var pDNA into canine stifle joints were well-tolerated and without pathologic findings. In addition, they have also conducted a small double-blinded, placebo-controlled study to assess the effect of IA hIL-10var pDNA on pain in companion dogs with naturally occurring OA, and observed a decrease of pain parameters without any adverse findings.

In summary, IL-10 gene therapy mainly reduces the expression of inflammatory markers. Protection from ECM degradation has only been reported in combination with other target genes.

4.1.1.4. IL-4

Among the anti-inflammatory cytokines, IL-4 is considered to have a strong therapeutic potential due to its inhibitory effect on IL-1β, the main mediator of inflammation leading to cartilage degradation. In this context, Lang et al. developed a non-viral transfection model to assess Cox-2 regulated IL-4 expression (pN3.Cox2.IL-4) (40). In an equine chondrocyte model, transfection with pN3.Cox-2.IL-4 of IL-1β or LPS-stimulated cells resulted in increased IL-4 expression and decreased expression of the inflammatory cytokines IL-1β,−6,−8 and the matrix degrading-enzymes MMP-1 and−3. Recently, Song et al. investigated the therapeutic potential of IL-4 overexpressing mesenchymal stem cells in spheroids (IL-4 MSC spheroid) (41). MSCs in spheroids are less prone to cell death after IA injection than naïve MSCs. In IL-1βstimulated primary rat chondrocytes *in vitro*, IL-4 MSC spheroids led to a reduction of IL-1β-induced apoptosis and a decrease in the production of OA factors (i.e., NO, iNOS, MMP-13), as well as an increase in the synthesis of cartilage extracellular matrix (ECM) (i.e., Col2). *In vivo*, in a rat anterior cruciate ligament transection (ACLT) with partial medial meniscectomy (MMx) (ACLT-MMx) model, an increased attenuation of tissue regeneration was observed after IA implantation of IL-4 MSC spheroids, along with improved chondroprotective and anti-inflammatory effects and better pain relief. Interestingly, these effects were higher in IL-4 MSC spheroids than in either IL-4 naïve MSCs or MSC spheroids without IL-4 suggesting that IL-4 MSC spheroids may increase the therapeutic efficacy of MSCs.

4.1.1.5. TSG6

Tumor necrosis factor-inducible gene 6 (TSG6) is an HA-binding protein associated with inflammatory processes. However, several studies report its protective effects in experimental arthritis. Inflammation and cartilage damage being two components of OA, Broeren et al. first demonstrated the functionality of TSG6 gene therapy *in vitro* by evaluating its effects on osteoclasts (42). They observed that overexpression of TSG6 using pShuttle-CMV-TSG-6, inhibited the resorption activity of osteoclasts. They then evaluated the effect of TSG6 therapy *in vivo*, in a CIOA mouse model. While no difference in protease activity or cartilage damage was observed, there was an increase in ectopic bone formation. Taken together, these data suggest that IA gene therapy with TSG6 does not seem to be a promising treatment for OA.

4.1.1.6. CrmA

The cytokine response modifier A (CrmA) is an inhibitor of caspases and IL-1β converting enzyme proteases and plays a role in attenuating IL-1β-induced inflammation and apoptosis in OA chondrocytes. Previously, due to their good biocompatibility, biodegradability and high stability, HA/CS microspheres have been shown to be a secure vehicle for the release of drugs. In this context, Qiu et al. investigated the use of these microspheres as vectors to deliver CrmA pDNA into OA synoviocytes (43). They demonstrated a decrease in IL-1β-mediated inflammation *via* a significant reduction of MMPs (MMP-3 and−13) in the HA/CS/pCrmA group compared to the control group.

4.1.2. Growth factors and receptors

In OA pathophysiology, the balance between catabolic and anabolic processes is crucial for the phenotype of chondrocytes. Thus, a promising approach in the context of gene therapy would be to either inhibit degradation or stimulate the synthesis of the

TABLE 2 Growth factors and receptors.

References	Objectives	Study design	Disease models	Delivery methods	Targets	Effects
Manning et al. (45)	Evaluate co-expression of IGF-1 and IL-4 in an *in vitro* inflammatory model	Preclinical *in vitro*	IL-1β /TNF-α-stimulated canine chondrocytes	pVitro2-IGF-1; pVitro2-IGF-1/IL-4 (transfection with Fugene6)	IGF-1 and IL-4	Reduced pro-inflammatory mediators and IGF-binding proteins; increased type II collagen and proteoglycans
Weimer et al. (46)	Investigate efficient and prolonged IGF-I overexpression *via* rAAV transfection and its effect on restoring OA cartilage	Preclinical *in vitro* and *in situ*	Human OA chondrocyte monolayer cultures and alginate spheres; human OA explant cultures	Adenoassociated virus: rAAV-hIGF-I	IGF-I	Increased proliferation; decreased apoptosis; increased levels of proteoglycan and type II collagen; increased cell proliferation *in situ*; decreased apoptosis *in situ*; increased proteoglycan and type II collagen content *in situ*
Aguilar et al. (47)	Determine efficiency of pAAV/IGF-I transfection of chondrocytes and determine effect of endogenous *vs.* exogenous IGF-I delivery	Preclinical *in vitro*	Mature vs. neonatal articular bovine chondrocyte culture (carpal joints vs. stifle condyles)	Adenoassociated virus: pAAV/IGF-I transfection vs. exogenous IGF-I stimulation	IGF-I	Dose-dependent increase of IGF-I production after transfection with pAAV/IGF-I; mature chondrocytes respond better than neonate chondrocytes; exogenous delivery into cell culture medium showed lower results
Aguilar et al. (48)	Development of new peptide-based material with high affinity to IGF-I	Preclinical *in vitro*	Neonatal articular bovine chondrocyte culture (stifle condyles)	Hydrogels; alginate (transfection with Fugene 6)	IGF-I	Enhanced binding affinity of IGF-I; extended IGF-I availability; increased GAG and HYPRO synthesis
Ko et al. (49)	Evaluate the effects of relaxin expression on fibrosis inhibition in OA synovial fibroblasts	Preclinical *in vitro*	Human OA synovial fibroblasts	Adenovirus: Ad-RLN	Relaxin	Anti-fibrogenic effects on OA synovial fibroblasts *via* inhibition collagen synthesis and collagenolytic pathways such as MMP-1,-13, TIMP-1 and -2
Ulrich-Vinther et al. (50)	Investigate potential of TGF-β1 overexpression to restore cartilage anabolism	Preclinical *in vitro*	Human primary OA chondrocytes	Adenoassociated virus: AAV-TGF-beta1-IRES-eGFP	TGFβ1	Increased expression of type II collagen, aggrecan; decreased expression of MMP3
Venkatesan et al. (51)	Investigate potential of TGF-β1 overexpression to restructure OA cartilage	Preclinical *in vitro* and *in situ*	Human primary OA chondrocytes and OA cartilage explants	Adenoassociated virus: rAAV-hTGF-beta	TGFβ1	Increased cell proliferation; reduced apoptosis; increased proteoglycan and type-II collagen deposition; decreased type-X collagen content; decreased hypertrophic differentiation players (MMP13, PTHrP and beta-catenin); increased protective TIMP-1 and TIMP-3 expression
Noh et al. (52)	Evaluate potential of TGF-β1-secreting human chondrocytes (TG-C) to regenerate cartilage	Preclinical *in vivo*	Rabbit surgically induced single partial cartilage defect model	Retrovirally induced human chondrocytes	TGFβ1	Dose-dependent effect on cartilage regeneration
			Goat surgically induced single full-thickness cartilage defect model	TG-C		Increased proliferation of new chondrocytes; positive effect on joint cartilage at 6 months
Lee et al. (53)	Evaluate the effects of TissueGeneC on pain and cartilage structure *via* the polarization of M2 macrophages	Preclinical *in vivo*	Rat MIA model	TissueGeneC	TGFβ1	Pain relief and cartilage structural improvement; increased IL-10 in the synovial fluid; induction of arginase 1 expression (M2 macrophages marker) and decreased CD86 (M1 macrophages marker) → Polarization of M2 macrophages

(Continued)

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TABLE 2 (Continued)

References	Objectives	Study design	Disease models	Delivery methods	Targets	Effects
Gao et al. (54)	Compare BMP2 delivery by coacervation and lentiviral delivery on cartilage repair	Preclinical *in vitro*	hMDSCs	Lentivirally (LBMP2/GFP) transduced hMDSCs; coacervate sustain release technology	BMP-2	LBMP2/GFP transduction increases chondrogenic differentiation of hMDSCs
		Preclinical *in vivo*	Rat MIA model			hMDSC-LBMP2/GFP improves cartilage repair and of cartilage erosion; coacervate delivery of BMP2 similar articular cartilage regeneration than with hMDSC-LBMP2/GFP
Matsumoto et al. (55)	Evaluate the effect of BMP-4 and Flt-1-transduced MDSCs on cartilage repair in a rat OA model	Preclinical *in vivo*	Rat MIA model	Retrovirally transduced MDSCs	BMP-4 • Flt-1	BMP-4-transduced MDSCs lead to good cartilage repair, but with osteophyte formation; exacerbated effect without osteophyte formation with the combination of sFlt-1 and BMP-4-transduced MDSCs; higher levels of chondrocyte differentiation and proliferation; lower levels of chondrocyte apoptosis
Tang et al. (56)	Assess the effect of follistatin delivery on metabolic inflammation and knee OA caused by a high-fat diet	Preclinical *in vivo*	Mouse DMM model	Adenoassociated virus: AAV9-FST	Follistatin (FST)	Reduced cartilage degeneration; decreased joint synovitis; lower levels of pro-inflammatory cytokines; normalization of obesity-induced increased heat withdrawal latency; enhanced muscle growth and muscle performance; protection from injury-mediated trabecular and cortical bone structure changes
Chen et al. (57)	Evaluate the effects of nanomicrosphere-delivered GDF-5 on OA	Preclinical *in vitro*	Rabbit chondrocytes	Nanomicrospheres	GDF-5	Increased expression of collagen II and aggrecan
		Preclinical *in vivo*	Rabbit ACLT and menisectomy model			Improved cartilage morphology and joint structure

10.3389/fmed.2023.1148623

ECM. Previously, it has been shown that insulin-like growth factor 1 IGF-1 stimulates matrix synthesis by stimulating type II collagen and aggrecan synthesis and promotes chondrocyte proliferation (Table 2).

4.1.2.1. IGF-1

Manning et al. evaluated the co-expression of IGF-1 and IL-4 using a dual promoter plasmid pVitro2, in an *in vitro* inflammatory model (45). In canine chondrocytes stimulated with IL-1β or TNF-α, the authors observed a decrease of pro-inflammatory mediators (i.e., IL-1β, TNFα, and IL-6) in the presence of IGF-1/IL-4 (pVitro2-IGF-1/IL-4), contrary to cells transfected with IGF-1 alone (pVitro2-IGF-1). They also observed a decrease in IGF-binding proteins and an increase in the key cartilage matrix proteins type II collagen and proteoglycans. These data suggested that the combination of genes could provide a better perspective in the context of gene therapy. Weimer et al. studied the overexpression of IGF-1 by rAAV transfection (rAAV-hIGF-1) and its effect on OA cartilage restoration in primary human OA chondrocytes *in vitro* and in explant cultures *in situ* (46). Prolonged IGF-1 secretion increased proliferation levels of chondrocytes and decreased apoptosis. Proteoglycan and type II collagen levels were also increased. Aguilar et al. compared the efficacy of AAV-mediated upregulation of IGF-1 (pAAV/IGF-1) (47). In articular bovine chondrocyte cultures and after transfection with pAAV/IGF-1, they observed a dose-dependent increase of IGF-1 and GAG production (58). However, IGF-1 added to media was less effective than endogenously produced IGF-1. In 2017, Aguilar et al. continued their studies on IGF-1 and developed a new material based on peptides (IGFBP-5) with a high affinity for IGF-1 and grafted a binding peptide sequence from IGFBP-5 onto alginate (48). Results demonstrated an increased binding affinity and availability of IGF-I as well as an increased synthesis of GAGs and hydroxyproline. According to the authors, "these data demonstrate the coordinated engineering of cell behavior and material chemistry to greatly enhance extracellular matrix synthesis and tissue assembly and can serve as a template for the enhanced performance of other therapeutic proteins" (48). Taken together, all these studies suggest that IGF-1 is a promising candidate for OA gene therapy.

Besides IGF-1, another target was studied, namely relaxin (RLN) (Table 2). This hormone belonging to the insulin superfamily, down-regulates TGF-β1-mediated collagen production and is important for matrix turnover by regulating MMP expression in the cartilage of synovial joints. In this context, Ko et al., investigated i*n vitro* the effects of Ad-mediated RLN (Ad-RLN) expression on fibrosis inhibition in human OA synovial fibroblasts (49). Compared to control cultures, authors observed in Ad-RLN transfected synoviocytes, an increase of MMP-1 and conversely, a decrease of collagen IV, TIMP-1, and−2 protein expression suggesting that RLN exerts anti-fibrogenic effects on OA synovial fibroblasts *via* inhibition of collagen synthesis and collagenolytic pathways.

Taken together, IGF-1 gene therapy increased the production of major matrix components, such as proteoglycans and type II collagen, while also reducing pro-inflammatory mediators.

4.1.2.2. TGF-β1

The transforming growth factor-β (TGF-β) family has more than 35 members, including TGF-β, activins, and bone morphogenetic proteins (BMPs). Three isoforms of TGF-β are known (TGF-β1, -β2, and -β3), TGF-β1 being amongst the most prevalent growth factors involved in cartilage repair. Indeed, it directly stimulates the synthesis of proteoglycans and collagen, and antagonizes the effects of IL-1 on metalloproteinases in both normal and OA chondrocytes. In this context, Ulrich-Vinther et al. were among the first to evaluate the effect of overexpression of TGF-β1 on cartilage anabolism in human OA chondrocytes (Table 2) (50). They demonstrated that after AAV-TGF-β1 transduction, chondrocytes expressed higher levels of type II collagen and aggrecan, while the expression of MMP-3 was decreased. Venkatesan et al. also investigated the overexpression of TGF-β1 (rAAV-hTGF-beta) in human primary OA chondrocytes and OA cartilage explants (51). They observed an increase in proliferation and cell survival compared to the control vector. They highlighted a decrease of key markers of chondrocyte hypertrophy such as type-X collagen, MMP-13, the parathyroid hormone-related protein (PTHrP) and β-catenin and, on the contrary, an increased expression of protective TIMP-1 and−3. The development of TissueGene-C (TG-C), a mix of human allogeneic chondrocytes and irradiated cells overexpressing TGF-β1, represented a key step for the advancement of OA gene therapy. Thus, Noh et al. evaluated the potential of TG-C *in vivo*, in a rabbit surgically induced cartilage defect OA model. In the presence of TG-C, they observed a dose-dependent effect on cartilage regeneration (52). It was also studied in goats in a surgically induced single full-thickness cartilage defect model. They observed an increase in the proliferation of new chondrocytes and a positive effect on articular cartilage at 6 months. After a demonstration of safety and efficacy, the authors proceeded with a phase I clinical study of TG-C (Table 3). Ha et al. evaluated the dose-response of this cell therapy in OA patients (59). This was a single center, open-label, dose escalation study on 12 adults, to assess the dose-response of three different doses of TG-C. No serious treatment-related adverse events were observed. Swelling, effusion, and minor local reactions were dose dependent. Knee evaluation scores (KSCRS, WOMAC, and VAS) seemed to point toward a dose-dependent trend of efficacy. Cherian et al. led the development of a clinical phase II (60). The objective was to evaluate the efficacy of TG-C in patients with grade III OA (n = 102). The characteristics of the study were as follows: multi-center, double-blinded, placebo-controlled, and randomized. The authors observed improved knee function and pain as assessed by the International Knee Documentation Committee (IKDC) and the Visual Analog Scale (VAS) and in parallel, reduced analgesics use by patients. A phase IIa study evaluated the efficacy and safety of TG-C in patients who had late-stage knee OA (grade IV) (n = 27) (61). This was a multi-center and single-blinded study. There was an improvement in both symptoms and activity level and function of the knee; stiffness, motor function and pain improved significantly, as evidenced by improved IKDC, WOMAC, and VAS scores. Of note, approval of TG-C as gene therapy for OA under the name of *Invossa* was revoked in Korea when it became apparent that the transgenic cells were mainly

HEK293 cells instead of chondrocytes, and its status is currently being investigated. In the US, on the other hand, trials are ongoing as HEK293 cells had been used from the beginning [reviewed in (64)].

Thus, Kim et al. conducted a phase III (NCT03291470), multi-center and double-blinded clinical trial including a total of 163 Kellgren–Lawrence grade III patients (62). Results highlighted a decrease in serum CTX-I and urine CTX-II levels, as well as significant improvements in function and pain in OA patients. Recently, Lee et al. investigated the effects of TG-C on pain and cartilage structure *via* the polarization of M2 macrophages (53). In an OA rat monosodium iodoacetate (MIA) model, pain relief and improvement of cartilage structure were demonstrated; an increase in IL-10 and TGF-β1 in synovial fluid was observed followed by induction of arginase 1 expression (M2 macrophage marker) and decrease of CD86 (M1 macrophage marker). The effect was therefore favorable for the induction of M2 macrophages implicated in tissue repair.

In summary, TGF-β1 gene therapy mainly restored synthesis of proteoglycan and type II collagen, while also reducing markers of hypertrophic differentiation.

4.1.2.3. BMPs

Bone morphogenic proteins (BMPs) are important players in the formation of functional joints and in the maintenance of cartilage homeostasis. Both BMP2 and BMP4 are involved in chondrogenesis, cartilage growth, and chondrocyte proliferation (23) and are therefore very interesting targets for OA gene therapy (Table 2). Muscle-derived stem cells (MDSCs) are a promising cell type for tissue engineering to allowre musculoskeletal regeneration. Gao et al. showed that hMDSCs lentivirally transduced to overexpress BMP2 have an increased chondrogenic differentiation capacity (54). In addition, these lentivirally-transduced hMDSCs improved cartilage repair and erosion in a rat MIA model *in vivo*. Interestingly, BMP2 delivery by coacervation resulted in similar articular cartilage repair as lenti-BMP2/GFP-mediated delivery. Matsumoto et al. showed that hMDSCs retrovirally transduced to overexpress BMP4 and transplanted into a rat MIA model of OA, are able to repair articular cartilage; osteophyte formation was however observed in some areas of the joint (55). A combination of soluble Fms-like Tyrosine Kinase 1 (sFLT-1) and BMP4-transduced hMDSCs exacerbated this effect without osteocyte formation. Moreover, increased differentiation and proliferation and decreased apoptosis was observed in chondrocytes in the animals transplanted with sFLT-1 and BMP4-transduced hMDSCs.

4.1.2.4. FST

Recently, Tang et al. were interested in the effect of follistatin (FST) delivery on metabolic inflammation (Table 2) (56). In the OA pathology, the authors assume that "FST delivery using a gene therapy approach has multifactorial therapeutic potential through its influence on muscle growth via inhibition of myostatin activity as well as other members of the TGFβ family" (56). In a mouse destabilization of the medial meniscus (DMM) OA model, FST gene therapy (AAV9-FST) reduced cartilage degeneration and decreased joint synovitis. The overexpression decreased the inflammatory cytokine, IL-1β and restored muscle performance by enhancing muscle growth and muscle performance. Finally, this therapy protected injury-mediated trabecular and cortical bone structure changes. This systemically delivered therapy is very promising especially for OA, but also for associated metabolic conditions, such as diseases of muscle wasting.

4.1.2.5. GDF-5

Like Deng et al. for IL-Ra, Chen et al. investigated a non-viral gene therapy vector to evaluate the therapeutic potential of the growth and differentiation factor-5 (GDF-5) (Table 2) (57). This factor belongs to the TGF-β and BMP superfamilies that regulate cell growth and differentiation. They generated nano-microspheres (NMPs) consisting of chitosan, HA, and chondroitin sulfate, and containing a GDF-5 plasmid. In rabbit chondrocytes, these GDF-5 containing NMPs increased the expression of collagen II and aggrecan, two key ECM proteins. In a rabbit ACLT and meniscectomy OA model, the authors demonstrated that these NMPs improved cartilage morphology and joint structure compared to the saline control group.

4.1.3. Transcription factors

Targeting transcription factors for OA gene therapy, such as sex-determining region Y-type high mobility group box 9 (SOX9), krüppel-like factor 2 or 4 (KLF2/KLF4), or activating transcription factor 4 (ATF-4), has the benefit of correcting gene expression profiles altered in OA toward the expression of genes implicated in the production of ECM compounds.

4.1.3.1. SOX9

SOX 9 is of particular interest because of its role in cartilage formation and chondrocyte differentiation. Furthermore, its expression is notably downregulated in OA (Table 4). The combinatorial effect of SOX9 and FGF2 overexpression after rAAV-mediated gene delivery to human OA chondrocytes or OA cartilage explants was evaluated by Cucchiarini et al. (66). They observed a higher production of proteoglycans and type II collagen, which was attributed to SOX9 rather than FGF2. The same effects were observed by Daniels et al. when transforming human articular OA chondrocytes in a 3D aggregate culture model after rAAV-mediated SOX9 overexpression (67). The combinatorial overexpression of SOX9 and TGF-β1 after rAAV-mediated gene delivery to human OA chondrocytes or OA cartilage explants as evaluated by Tao et al. showed the same effects, along with increased proliferation and cell density and enhanced levels of chondrogenic aggrecan (ACAN) and collagen type II alpha 1 chain (COL2A1) expression (68). The downside of rAAV-mediated gene delivery is however the widespread presence of neutralizing antibodies against the viral proteins in the host. Therefore, Urich et al. explored the potential of delivering rAAV-FLAG-hsox9 vectors *via* polymeric micelles into human primary OA chondrocytes. These micellar systems increased proliferation, type II collagen, and proteoglycan deposition in human primary OA chondrocytes stimulated with IL-1β and TNF-α (69).

TABLE 3 Clinical trials.

References	Objectives	NCT #	Participants	Study design	Disease models	Delivery methods	Targets	Effects
Ha C-et al. (59)	Evaluate the dose-response of TissueGeneC in OA patients	NCT02341391	Adults with KL grade IV knee OA	Clinical Phase I, single center, open-label, dose-escalation	Human OA patients	TissueGeneC	TGFβ1	No treatment-related serious adverse events; swelling, effusion and minor reactions are dose-dependent; a dose-dependent trend efficacy
Cherian et al. (60)	Evaluate efficacy of treating grade III OA patients with genetically engineered allogenic human chondrocytes expressing TGF-β1	NCT01221441	Adults with KL grade III knee OA	Clinical phase II: multi-center, double-blinded, placebo-controlled, randomized	Human OA patients	Retrovirally transduced allogenic human chondrocytes (TissueGene-C)	TGFβ1	Improved keen function and pain as assessed by IKDC and VAS; less analgesic use
Ha et al. (61)	Evaluate the efficacy and safety of a cell-mediated therapy in OA patients	NCT02341378	Adults with KL grade IV knee OA	Clinical phase IIa, multicenter, single -blind	Human OA patients	GEC-TGF-β1 (TissueGeneC)	TGFβ1	Improved symptoms, activity levels and knee function; Significant improvement in stiffness, motor function and pain demonstrated *via* the IKDC, WOMAC and VAS scores
Kim et al. (62)	Evaluate the efficacy and safety of a cell-mediated therapy in OA patients	NCT02072070	Adults with KL grade III knee OA	Clinical Phase III, multicenter, double-blind	Human OA patients	TissueGeneC	TGFβ1	Decreased serum CTX-1 and urine CTX-II levels over 1 year in TG-C than placebo-treated patients; significant improvements in function and pain in OA patients; frequent adverse events were edema, arthralgia, joint swelling and injection site pain
NA	Post Marketing Surveillance on Safety and Effectiveness Evaluation of INVOSSA	NCT03412864	Adults with KL grade III knee OA	Post marketing surveillance study	Human OA patients	INVOSSA	TGFβ1	No outcome data
NA	Preliminary Evaluation of Safety, Tolerability, and Efficacy of XT-150	NCT03282149 NCT03477487 NCT03769662	Adults for whom replacement knee surgery is recommended	Clinical Phase I, single center, dose escalation	Human OA patients	hIL-10var pDNA (XT-150)	IL-10	No outcome data
NA	Evaluate the efficacy and safety of XT-150 in patients experiencing moderate to severe pain due to OA of the knee	NCT04124042	Adults with WOMAC pain score $\geq$ 8	Clinical phase II: multi-center, double-blinded, placebo-controlled, randomized	Human OA patients	hIL-10var pDNA (XT-150)	IL-10	No outcome data
Sellon et al. (63) (reviewed by Evans C 2022) (64)	Evaluate safety of three different doses of sc-rAAV2.5IL-1Ra	NCT02790723	Adults with moderate OA of the knee	Clinical Phase I, single center, open-label, dose-escalation	Human OA patients	Sc-rAAV2.5IL-1Ra	IL-1Ra	No outcome data
Kelley et al. (65) (reviewed by Evans C 2022) (64)	Evaluate the Safety and Tolerability of FX201	NCT04119687	Adults with KL grade II, III or IV knee OA	Clinical Phase I, single center, open-label, dose-escalation	Human OA patients	HDAd-IL-1Ra (FX201)	IL-1Ra	No outcome data

10.3389/fmed.2023.1148623

TABLE 4 Transcription factors.

References	Objectives	Study design	Disease models	Delivery methods	Targets	Effects
Cucchiarini et al. (66)	Evaluate the combinatorial effect of FGF-2 and Sox9 *via* rAAV gene transfer upon the OA cartilage	Preclinical *in vitro* and *in situ*	Human articular OA chondrocytes (3D culture model) and OA cartilage explants	Adenoassociated virus: rAAV-hFGF-2; rAAV-FLAG-h*sox9*	FGF-2 SOX9	Improvement both survival and proliferation of chondrocytes with single overexpression of FGF-2; Combination of FGF-2 and Sox-9 ↑production of proteoglycans and type-II collagen, ↓ expression of type-X collagen
Daniels et al. (67)	Evaluate the effects of rAAV-mediated sox9 overexpression on the biological activities of human OA chondrocytes	Preclinical *in vitro*	Human articular OA chondrocytes (3D culture model)	Adenoassociated virus: rAAV-FLAG-h*sox9*	SOX9	Significant production of major matrix components (proteoglycans and type-II collagen)
Tao et al. (68)	Assess the effect of TGF-beta and SOX9 co-overexpression	Preclinical *in vitro* and *in situ*	Human OA chondrocyte monolayer cultures and alginate spheres; human OA explant cultures	Adenoassociated virus: rAAV-hTGF-beta; rAAV-FLAG-hsox9	TGF-beta SOX9	Increased proliferation and cell density; enhanced deposition of matrix proteoglycans; enhanced type-II collagen deposition; enhanced levels of chondrogenic SOX9, ACAN and COL2A1 expression; reduced type-X-collagen expression
Urich et al. (69)	Investigate the ability of polymeric micelles to deliver therapeutic rAAV-FLAG-hsox9 into human OA chondrocytes	Preclinical *in vitro*	Human primary OA chondrocytes in presence of pro-inflammatory cytokines	Adenoassociated virus: rAAV-FLAG-hsox9/Polymerci micelles	SOX9	Increased type-II collagen deposition; increased proliferation and proteoglycan deposition
Kawata et al. (70)	Evaluate the therapeutic effects of KLF4 and KLF2 for OA	Preclinical *in vitro*	Human OA chondrocytes, meniscal cells and BMSCs	Adenovirus: Ad-KLF2 and Ad-KLF4	KLF2 and KLF4	Upregulation of cartilage genes
		Preclinical *in vivo*	Mouse DMM model	Adenoassociated virus: AAV-KLF4	KLF4	Upregulation of cartilage genes; improved pain; improved OARSI score, meniscus histopathological score, synovitis score and bone score
Wang et al. (71)	Explore therapeutic effects of serum-derived exosomes from OA mice	Preclinical *in vivo*	Mouse anterior medial meniscus excision OA model	OA exosomes: ATF4-OA-Exo	ATF4	Alleviated cartilage damage of OA mice (proteoglycan loss, increased Mankin score and increased osteophytes); increased COL-II and decreased MMP13 and inflammatory cytokines; partial recovery of weakened autophagy; inhibited TM/TNF-alpha induced chondrocyte apoptosis

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4.1.3.2. KLF2 and 4

Interestingly, OA is associated with a decreased expression of KLF family members of transcription factors (72). Kawata et al. evaluated the therapeutic potential of KLF2 and 4 for OA. In human OA chondrocytes, meniscal cells or BMSCs, Ad-mediated overexpression of KLF2 or 4 induced an upregulation of cartilage genes, including COL2A1, COL11A2, COMP, PRG4, and SOX9 (70). Furthermore, AAV-mediated overexpression of KLF4 in a mouse DMM OA model not only upregulated above mentioned cartilage genes, but also resulted in reduced pain and an improved OARSI score, meniscus histopathological score, synovitis score, and bone score. The authors concluded "that KLF4 had therapeutic and protective effects against OA-associated tissue damage and pain" (70).

4.1.3.3. ATF4

Exosomes are small extracellular vesicles, which can contain proteins, RNA, and DNA, making them very interesting vehicles for gene delivery because of their high penetration and low immunogenicity. Wang et al. were the first to explore the therapeutic effects of serum-derived exosomes from meniscus-injury-induced OA mice overexpressing ATF4 (ATF4-OA-Exo) in a meniscus injury-induced mouse model of OA (Table 4) (71). These ATF-OA exosomes alleviated the cartilage damage and proteoglycan loss observed in these OA mice and decreased the number and size of osteophytes, as well as the cartilage Mankin score. In line with these observations, type II collagen was increased, while MMP-13 and inflammatory cytokines were decreased. In addition, ATF4-OA-exosome injection led to a partial recovery of the weakened autophagy in OA cartilage and inhibited apoptosis in tunicamycin (TM)- or TNF-α-treated chondrocytes, indicating that the effect ATF4-OA-Exo might be exhibited through the induction of autophagy.

4.1.4. Other key targets

4.1.4.1. PRG4

Proteoglycan 4 (PRG4), also known as lubricin, is a glycoprotein expressed by chondrocytes and synoviocytes and naturally secreted in synovial fluid. It is of particular interest for gene therapy, as it acts as a lubricant to reduce friction within articular cartilage, diminishes inflammation, and as an anabolic factor slows down OA progression (Table 5). In humans, Camptodactyly-Arthropathy-Coxa Vara-Pericarditis Syndrome, characterized by early onset OA, is caused by loss-of-function mutations in PRG4. Similarly, PRG4 knock-out mice also develop early OA. Ruan et al. reported that transgenic, joint-specific PRG4 overexpression protects mice against OA development and motor impairments in a mouse ACLT model and protects aging mice from natural OA development (73). Treatment with an HDV vector expressing PRG4 (HDV-PRG4) protected mice against OA development and improved structural joint status in a mouse ACLT model, as evidenced by improved histology scores, cartilage volume and bone area covered by cartilage. Later, Ruan et al. reported that in the mouse ACLT model, IA injection of HDV-PRG4 or a capsid-modified HDV (a10mabHDV-PRG4, which specifically targets chondrocytes), prior to surgical OA induction, protected animals from OA development (74). Cartilage volume and bone area covered by cartilage were preserved in both cases. The efficacy was, however, greater for the a10mabHDV-PRG4. When injecting viral vector 2 weeks after ACLT surgery, a 10-times lower effective dosage of a10mabHDV-PRG4 was needed to prevent post-ACTL OA compared with the untargeted HDV-PRG4. Using the low dose of a10mabHDV-PRG4, the preservation of cartilage volume was greater, and it covered a larger area of bone compared to the same dose of HDV-PRG4 vector. The effect of a10mabHDV-PRG4 was similar to the 10-fold higher dose of HDV-PRG4. Stone et al. explored the added benefits of a combinatorial gene therapy approach compared to targeting PRG4 alone (75). The IA co-injection of HDVs expressing IL-1Ra and PRG4 in a mouse ACLT model, resulted in better preservation of cartilage volume and bone area covered by cartilage than injection of either HDV alone. This was accompanied by the increase of anabolic and the decrease of catabolic and inflammatory gene expression. In the less severe DMM model, both PRG4 and IL-1Ra + PRG4 combinatorial gene therapy were able to maintain cartilage volume and covered surface area. While all treatment groups prevented OA-induced thermal hyperalgesia at the early timepoint, only the combined gene therapy showed significant protective effect at the late timepoint. The chondroprotective effect of PRG4 gene therapy was confirmed by Seol et al. in a rabbit ACLT model (76). IA injection of AAV-PRG4-GFP immediately after ACLT surgery inhibited cartilage damage and reduced the severity of post-traumatic OA (PTOA). In addition, more cartilage surface and superficial chondrocytes were covered by lubricin.

4.1.4.2. LOXL2

The copper-dependent amine oxidase lysis oxidase-like 2 (LOXL2) catalyzes the first step in the formation of crosslinks in collagens and elastin and has been shown to mediate endochondral ossification. Tashkandi et al. wanted to assess the potential of Ad-mediated overexpression of LOXL2 for OA treatment (Table 5) (77). In an IL-1β stimulated ATDC5 cartilage cell line, LOXL2 overexpression blunted the decrease of aggrecan and SOX9, and attenuated both the IL-1β mediated expression of ADAMTS4/5, and MMP-13, as well as the IL-1β induced activity of NF-κB. In chondrodysplasia (Cho/+) mice, LOXL2 overexpression protected these mice against progressive OA, as evidenced by increased proteoglycan deposition, increased aggrecan, type II collagen, and anabolic gene expression, and decreased MMP-13 and ADAMTS5 expression. Finally, in MIA-induced LOXL2 transgenic mice, LOXL2 overexpression protected against proteoglycan and aggrecan degradation and reduced MMP-13 levels. It protected these mice from MIA-induced OA-related decline in knee function. The authors, therefore, conclude that LOXL2 is a promising target for gene therapy for knee OA.

4.1.4.3. Proteoglycans

Proteoglycans (PG) are made of "core proteins" with covalently attached glycosaminoglycan (GAG) chains. Their depletion is a major hallmark in joint destruction, and factors that might accelerate PG synthesis and deposition are therefore of great interest for gene therapy. Venkatesan et al. investigated how GAG-synthesizing enzyme β1,3-glucuronosyltransferase-I (GlcAT-I) gene therapy might influence cartilage repair in IL-1β stimulated

TABLE 5 Other targets.

References	Objectives	Study design	Disease models	Delivery methods	Targets	Effects
Ruan et al. (73)	Evaluate functionality of HDV-PRG4 in OA mouse model	Preclinical *in vivo*	Mouse ACLT model	HDV-PRG4	PRG4	Improved histology scores, cartilage volume and coverage
Ruan et al. (74)	Development of a targeted vector for chondrocyte-specific delivery of target genes for OA therapy	Preclinical *in vivo*	Mouse ACLT model	(Modified) helper-dependent adenovirus: (a10mab)HDV-PRG4	PRG4	Prevention of OA development with early treatment with both HDV-PRG4 and a10mabHDV-PRG4; greater efficacy of a10mabHDV-PRG4; preserved cartilage volume and surface area; with late treatment, greater preservation of cartilage volume; larger bone area covered by cartilage with a10mabHDV-PRG4 compared to HDV-PRG4 vector, resulting in 10-fold reduction of effective dosage requirement for preventing post-ACTL OA
Stone et al. (75)	Evaluate the beneficial effects of a combinatorial gene therapy approach compared to monotherapy	Preclinical *in vivo*	Mouse DMM and mouse ACLT model	Helper-dependent adenovirus: HDV-NFκB-IL1ra and HDV-EF1-PRG4	IL-1Ra and PRG4	ACLT model: better preservation of cartilage volume and covered surface area in combined therapy; prevented decrease of anabolic gene expression and upregulation of inflammatory and catabolic pathways; DMM model: maintained cartilage volume and covered surface area of underlying bone in combined and PRG4 therapy; longer prevention of OA-induced thermal hyperalgesia with combined therapy
Seol et al. (76)	Evaluate functionality of recombinant PRG4-GFP fusion protein in delaying OA progression	Preclinical *in vivo*	Rabbit ACLT model	Adenoassociated virus: AAV-PRG4-GFP	PRG4	Reduced post-ACLT severity of PTOA; higher percentage of cartilage surface and superficial chondrocytes coated with lubricin
Tashkandi et al. (77)	Assess the potential of LOXL2 to be used for translational research and clinical applications in OA treatment	Preclinical *in vitro*	IL-1β stimulated ATDC5 cartilage cell line	Adenovirus: Adv-RFP-LOXL2	LOXL2	Blunted decrease of Acan and Sox9; attenuated expression of Adamts4/5 and MMP13; attenuated IL-1β induced NF-κB activity
		Preclinical *in vivo*	Chondrodysplasia (Cho/+) mice			Protection against progressive OA: increased proteoglycan deposition; increased expression of aggrecan and Col2; decreased expression of Mmp13 and Adamts5; increased expression of anabolic genes
			MIA-induced LOXL2 transgenic mice	LOXL2 transgenic mice (loxP-PGK-neo-stop-loxP-LOXL2-IRES-eGFP)		Protection against MIA-induced proteoglycan and aggrecan degradation and decreased Mmp13 expression; protection against MIA-induced OA-related decline in knee function
Venkatesan et al. (78)	Develop a non-viral gene transfer strategy to stimulate GAG synthesis to promote cartilage repair	Preclinical *in vitro*	IL-1β stimulated primary rat chondrocytes; IL-1β stimulated cartilage explants	Transfection of pShuttle-GlcAT-I using PEI	GlcAT-I	Inhibited IL-1β induced loss of PGs; increased GAG content but no influence on chain size; increased amount of CS chains; restored PG synthesis in IL-1β treated cartilage explants
Fu et al. (79)	Explore the effect of GGCX overexpression on ACLT-induced OA	Preclinical *in vivo*	Rabbit ACLT model	Lentivirus GGXC	GGCX	Reduced morphological changes caused by ACLT; increased cMPG to normal levels; decreased ACLT-induced inflammation (expression of TNFα and IL-1β); decreased collagen type X and MMP13 expression, increased collagen type II expression

(Continued)

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TABLE 5 (Continued)

References	Objectives	Study design	Disease models	Delivery methods	Targets	Effects
Hsieh et al. (80)	Evaluate the effects of Ad-mediated kallistatin overexpression in OA rat model	Preclinical *in vivo*	Rat ACLT model	Adenovirus: AdHKBP	Kallistatin	Reduced inflammatory response (IL-1β and TNF-α levels in joints); reduced OA severity and apoptosis; decreased macrophage infiltration; reduced hyperplasia and synovitis
Ashraf et al. (81)	Determine effect of Rheb on phenotype and function of OA chondrocytes	Preclinical *in vitro*	Human articular OA chondrocytes	Transfection of pEGFP-N1 vector using microporator	RHEB	Normalized morphology; reduced senescence; decreased oxidative stress
	Determine effect of Rheb expression on OA progression in mice	Preclinical *in vivo*	Mouse DMM model	Adenovirus: Ad-Rheb		Attenuated cartilage destruction; suppressed expression of Adamts5, Mmp13, Col10 and Col2a1; inhibited apoptosis
Grossin et al. (82)	Determine efficiency of gene transfer with HSP70 in rat patellar cartilage	Preclinical *in vivo*	Rat MIA model	Transfection of pcDNA3.1/CT-GFP-HSP70 by electroporation	HSP70	Inhibited endochondral ossification in the deep layer; reduced severity of OA-induced lesions
Yoon et al. (83)	Identify the role of PUM1 in OA progression	Preclinical *in vivo*	Mouse DMM model	Lentivirus: pLenti-GII-CMV-PUM1	PUM1	Reduced cartilage destruction; less chondrocyte loss; reduced OARSI score
Na et al. (84)	Asses the therapeutic potential of sCCR2 E3 for OA	Preclinical *in vivo*	Rat MIA model	sCCR2 E3 vector *via* electroporation	sCCR2 E3	Reduced pain; less bone loss and cartilage degradation; lower OARSI and Mankin score; inhibition of IL-1β, IL-6 and MMP-13 expression
Cao et al. (85)	Elucidate the role of cholesterol-LRP3 axis in OA	Preclinical *in vitro*	TNFa-induced rat OA chondrocytes	Lentivirus: Lv-Lrp3	LRP3	Increased expression of anabolic genes COL2A1, ACAN, SOX9; increased proteoglycan and GAG
		Preclinical *in vivo*	Rat ACLT model			Less cartilage degradation; rescued proteoglycan and type II collagen level; milder OA phenotype; increased expression of anabolic genes COL2A1, ACAN, SOX9; decreased expression of catabolic genes Adamts5 and Mmp13

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primary rat chondrocytes *in vitro* (Table 5) (78). Lipid-mediated overexpression of GlcAT-I inhibited IL-1β induced loss of PGs, and increased the GAG content and number of chains, rather than chain size. In addition, it restored PG synthesis in IL-1β treated cartilage explants.

4.1.4.4. γ-Glutamyl Carboxylase

γ-glutamyl carboxylase (GGCX) regulates the carboxylation of cartilage matrix Gla protein (MPG), a calcification inhibitor. Since the level of uncarboxylated, non-functional MPG (ucMPG) seems to be elevated, while GGCX seems to be reduced in OA patients, Fu et al. set out to investigate the effect of lentiviral-mediated GGCX overexpression in a rabbit ACLT model of OA (Table 5) (79). GGCX overexpression resulted in less ACLT-induced morphological changes and inflammation, which was accompanied by normalized levels of carboxylated MPG. MMP-13 and type X collagen were decreased, while type II collagen was increased to normal levels. GGCX, therefore, constitutes an interesting target for OA gene therapy.

4.1.4.5. Kallistatin

Kallistatin, a serine proteinase inhibitor and known inhibitor of angiogenesis, has been shown to protect cardiomyocytes from apoptosis and to prevent an inflammatory response after myocardial ischemia-reperfusion injury. Hsieh et al. investigated the effect of its Ad-mediated overexpression in a rat ACLT OA model after IA delivery (Table 5) (80). They observed a reduced inflammatory response as evidenced by reduced levels of IL-1β and TNF-α in the joints, as well as an overall reduced OA severity and a reduced number of apoptotic cells. In addition, there was less macrophage infiltration, hyperplasia, and synovitis in Adenoviral vector encoding human kallistatin (AdHKBP) animals, which was exacerbated when treated in combination with HA. Kallistatin gene therapy might therefore be an interesting alternative for OA treatment, especially when administered in combination with HA.

4.1.4.6. RHEB

Ras homolog enriched in the brain (RHEB) is part of the Ras family and is involved in cell growth, proliferation, and differentiation. Ashraf et al. were interested in its effect on OA chondrocytes *in vitro* and OA progression in a mouse DMM model (Table 5) (81). Human articular OA chondrocytes displayed normalized morphology, reduced senescence, and decreased oxidative stress when transfected with a vector containing RHEB cDNA. Ad-mediated overexpression of RHEB in a DMM mouse model resulted in attenuated cartilage destruction, suppressed expression of ADAMTS5, MMP-13, type 10 collagen, and COL2A1, and inhibited apoptosis. The authors, therefore, concluded that RHEB is important for maintaining the chondrogenic phenotype of chondrocytes and is likely involved in preventing the progression of OA *in vivo*.

4.1.4.7. HSP70

Heat shock protein 70 (HSP70) is expressed as a protective agent upon various types of stresses. Grossin et al. set out to investigate its cyto- and/or chondroprotective role in a rat MIA OA model (Table 5) (82). HSP70 overexpression was successfully achieved by electroporation and resulted in the inhibition of endochondral ossification in the deep layer and reduced severity of OA-induced lesions. The authors conclude that HSP70 might therefore be a novel chondroprotective target for gene therapy in OA.

4.1.4.8. PUM1

RNA-binding proteins (RBPs) have been shown to play a role in age-related degenerative diseases (86). Yoon et al. investigated the effect of lentiviral-mediated overexpression of the RBP Pumilio1 (PUM1) in a mouse DMM OA model (83). They observed that it prevented cartilage destruction and chondrocyte loss, and significantly improved the OARSI score. They therefore conclude that PUM1 might be an interesting target for OA therapy.

4.1.4.9. sCCR2 E3

The monocyte chemoattractant protein-1 (MCP-1/CCL2) - C-C chemokine receptor type 2 (CCR2) pathway is involved in OA progression, and ways to block this pathway are being explored (87). Na et al. investigated the effect of a fusion protein constituted of 20 amino acids of the third extracellular domain (E3) of the CCL2 receptor and a soluble CCL2 receptor (sCCR2) in a rat MIA OA model (84). Intra-articular injection of a sCCR2 E3 vector *via* electroporation resulted in reduced pain and less bone loss and cartilage degradation, as well as a lower OARSI and Mankin score. In addition, the expression of inflammatory cytokines IL-1β and IL-6 and catabolic factor MMP-13 was reduced. sCCR2-E3 therefore protects against cartilage damage and inhibits catabolic factors.

4.1.4.10. LRP3

Low-density lipoprotein (LDL) receptor-related proteins (LRPs) play an important part in the regulation of cholesterol metabolism and have recently been shown to be involved in the onset and progression of OA (88). Cao et al. investigated the potential role of LRP3 in OA pathogenesis and therapy (85). In TNF-α stimulated rat OA chondrocytes, lentiviral-mediated overexpression of Lrp3 induced the expression of the anabolic genes COL2A1, ACAN, and SOX9, and restored expression of proteoglycan and GAG. Furthermore, in a rat ACLT OA model, it inhibited cartilage degradation and restored proteoglycan and type II collagen levels. The expression of anabolic genes COL2A1, ACAN, and SOX9 was increased, while the expression of catabolic genes ADAMTS5 and MMP13 was decreased. The authors conclude that LRP3 overexpression may be a new therapeutic target for OA therapy by delaying the degeneration of cartilage.

5. Limitations

This scoping review took a rigorous approach based on the JBI framework to the conduct of scoping reviews and was reported following the PRISMA-ScR checklist. To our knowledge, it is the first scoping review of preclinical and clinical studies reporting the effect of gene therapies used to treat OA. It does however suffer from one minor limitation: unpublished work, and ongoing clinical trials were not included. We searched for ongoing clinical trials on gene therapy in OA on clinicaltrials.gov and discussed the outcome of our search in the conclusion and perspective section.

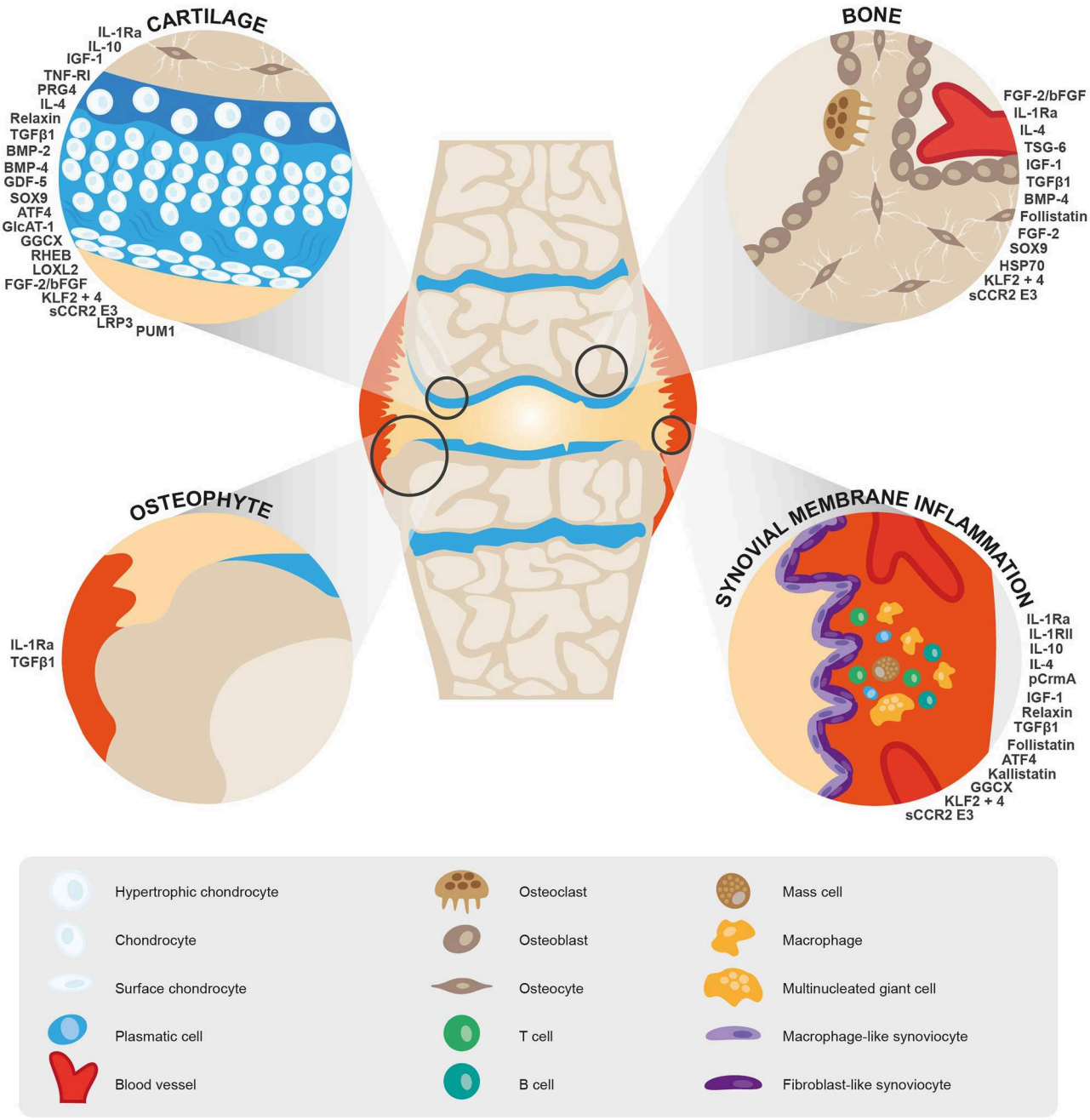

FIGURE 3
Schematic representation of targets identified for gene therapy of OA and their therapeutic potential.

6. Conclusions

In the last decade, research on gene therapy has been in full expansion, as evidenced by an increasing number of scientific articles on the subject. In the absence of any DMOAD, gene therapy is of special interest in the context of OA. Both viral and non-viral gene therapy approaches are highly promising treatment alternatives of OA in the future, and several targets have been explored and studied. Both technologies have, however, their own advantages and inconveniences. While viral gene therapy is highly efficient and allows for targeted and sustained delivery of genes, it might activate the innate immune system and cause local inflammation in the host. This is why more recently, several groups have started to explore the option of non-viral gene therapy approaches, such as NMPs or exosomes. While non-viral therapies have the handicap of relatively low transfection efficiency and transient gene expression, they are considered a safer option particularly for OA because of their reduced risk to cause an inflammatory response. CRISPR/Cas9-mediated gene editing, while not the subject of this review, is another alternative for the development of new gene therapies for OA. The technology has revolutionized the field of gene editing

and is widely considered an accurate and easy-to-use tool for genome editing. As an example, intra-articular injection of AAV expressing CRISPR/Cas9 components that target MMP-13, IL-1β, and/or nerve growth factor (NGF) suggest that combined deletion of these genes has beneficial effects on both pain management and joint integrity (89). The disadvantage of CRISPR/Cas9 is however the difficulty to deliver enough material to mature cells for efficient genome editing activity. Moreover, it requires the presence of a PAM sequence near the target site, thus limiting its effective targeting range. Most importantly, the relatively high frequency of off-target effects is a significant challenge for employing CRISPR/Cas9 for gene therapy, particularly in clinical applications.

Alternatively, several groups have explored the possibility of targeting multiple genes, so as to heighten and potentiate their effect. In OA with its complex physiopathology, targeting only one actor might not be sufficient to efficiently treat the disease long-term. This strategy seems very promising, and good pre-clinical results have been obtained for various combinations, for example, IL-1Ra and PRG4, FGF2 and SOX9, or IL1-Ra and IL-10.

The main targets identified for gene therapy of OA belong to the interleukin family (IL-1Ra, IL-10, IL-4, TSG6, and CrmA), growth factors and their receptors (IGF-1, relaxin, TGF-β1, BMP2 and 4, follistatin, GDF-5, and FGF2/bFGF), and transcription factors (SOX9, KLF2 and 4, and ATF-4). In addition, lubricin, LOXL2 and other enzymes and proteins are being explored for their therapeutic potential (Figure 3).

Three Phase I studies (NCT03282149, NCT03477487, and NCT03769662) have been successfully completed for XT-150, the locally injectable non-viral therapy expressing interleukin (IL)-10v described by Watkins et al. and discussed above (39). A multinational, double-blind, placebo-controlled Phase II study (NCT04124042) is currently evaluating the safety and efficacy of XT-150 in adults with moderate to severe pain due to knee OA. A Phase I, open-label study (NCT02790723) evaluating the safety of intra-articular injection of scAAV2.5IL-1Ra in nine subjects with moderate knee OA has recently been completed with encouraging results. A Phase I open-label, single ascending dose study (NCT04119687) assessing the safety and tolerability of FX201, the HDAd-IL-1Ra intra-articular gene therapy described by Senter et al., in 72 subjects with moderate to severe knee OA has been recently completed, and the sponsor reported "very compelling" results (no further details were given) (20). A post marketing surveillance study (NCT03412864) including 3,000 OA patients (Kellgren–Lawrence grade III) assessing adverse events as primary endpoints is currently underway for TG-C.

In conclusion, gene therapy is a highly promising treatment for OA, even though it still is in its early stages and further development is required to bring more targets to the clinical stage, as has been done with TG-C targeting TGF-β1, XT-150 targeting IL-10, FX201 and scAAV2.5-IL-1Ra. Overall, the optimal system (s) for safe and effective disease-specific therapy still need to be identified, including the vector type or non-viral delivery system, the therapeutic single gene or gene combination, and the levels of therapeutic gene expression. As more and more clinical trials for gene therapy in OA are being undertaken, we need guidelines for their design and conduct.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

MU, CL, and YH: drafting manuscript. MU, CL, JG, KG, SP, and YH: revising manuscript content and approving final version of manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors would like to thank Dr. Nancy Durieux from the University of Liège—Research Unit for a life-Course perspective on Health and Education, Faculty of Psychology, Speech and Language Therapy and Educational Sciences for her help in setting up research strategies and their implementation into Covidence.

Conflict of interest

YH is the founder and president of Artialis S.A. and received consulting fees from Laboratoires Expanscience, Tilman, Biose, Immubio, Naturex, and GeneQuine Biotherapeutics GmbH.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1148623/full#supplementary-material

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Glossary

OA, osteoarthritis; DMOAD, disease-modifying osteoarthritis drug; IA, intra-articular; Ad, Adenovirus; HDAd or HDV, helper-dependent adenovirus; AAV, adeno-associated virus; RV, retroviruses; LV, lentivirus; MSCs, mesenchymal stem cells; miRNAs, microRNAs; siRNA, small interfering RNA; circRNA, circular RNA; IL, interleukin; IL-1Ra, interleukin 1 receptor antagonist; ACLT, anterior cruciate ligament transection; IND, investigational new drug; CS, chitosan; HA, hyaluronic acid; pDNA, plasmidDNA; Sc AAV, self-complementary AAV; Nab, neutralizing antibodies; TNF-α, Tumor Necrosis Factor alpha; MMPs, matrix metalloproteinases; bFGF, basic fibroblast growth factor; IGF-1, insulin-like growth factor-1; TIMP-1, Tissue inhibitor matrix metalloproteinase 1; ADAMTS5, disintegrin and metalloproteinase with thrombospondin motifs 5; GAG, glycosaminoglycan; TGFβ1, transforming growth factor-β1; sTNF-RI, soluble tumor necrosis factor-α receptor type I; IL-1RII, type II IL-1β receptor; NO, nitric oxide; iNOS, inducible nitric oxide synthase; PGE_2, prostaglandin E2; CIOA, collagenase induced osteoarthritis; BM-MSCs, Bone marrow-derived mesenchymal stromal cells; Cox-2, cyclooxygenase-2; LPS, lipopolysaccharide; ECM, extracellular matrix; TSG6, tumor necrosis factor α-stimulated gene 6; CrmA, cytokine response modifier A; IKDC, International Knee Documentation Committee; RLN, relaxin; TIMP-1, tissue inhibitor of metalloprotease-1; BMPs, bone morphogenetic proteins; LAP, pro-peptide latency-associated peptide; LTBPs, latent TGF-β binding proteins;; PTHrP, parathyroid hormone-related peptide; TG-C, TissueGene-C; FDA, Food and Drug Administration; KSCRS, Knee Society Clinical Rating System; VAS, Visual Analog Scale; IKDC, International Knee Documentation Committee; MIA, monosodium iodoacetate; MDSCs, muscle-derived stem cells; sFLT-1, soluble Fms-like Tyrosine Kinase 1; FST, follistatin; DMM, destabilization of the medial meniscus; GDF-5, growth and differentiation factor-5; NMPs, nano-microspheres; SOX9, sex-determining region Y-type high mobility group box 9; KLF, krüppel-like factor; ATF-4, activating transcription factor 4; ACAN, aggrecan; COL2A1, collagen type II alpha 1 chain; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; PRG4, Proteoglycan 4; PTOA, post-traumatic OA; LOXL2, the copper-dependent amine oxidase lysis oxidase-like 2; PG, Proteoglycans; GlcAT-I, GAG-synthesizing enzyme β1,3-glucuronosyltransferase-I; GGCX, γ-glutamyl carboxylase; MPG, matrix Gla protein; ucMPG, non-functional MPG; RHEB, Ras homolog enriched in the brain; HSP70, Heat shock protein 70; RBP, RNA-binding protein; PUM1, Pumilio 1; MCP-1, monocyte chemoattractant protein-1; CCR2, C-C chemokine receptor type 2; LDL, Low-density lipoprotein; LRO, LDL receptor-related proteins; PRISMA-ScR, PRISMA Extension for Scoping Reviews; NGF, nerve growth factor.

frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Original Research
PUBLISHED 06 September 2023
DOI 10.3389/fbioe.2023.1224141

OPEN ACCESS

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RECEIVED 01 June 2023
ACCEPTED 28 August 2023
PUBLISHED 06 September 2023

CITATION
Lin ASP, Reece DS, Thote T, Sridaran S, Stevens HY, Willett NJ and Guldberg RE (2023), Intra-articular delivery of micronized dehydrated human amnion/chorion membrane reduces degenerative changes after onset of post-traumatic osteoarthritis. *Front. Bioeng. Biotechnol.* 11:1224141. doi: 10.3389/fbioe.2023.1224141

Intra-articular delivery of micronized dehydrated human amnion/chorion membrane reduces degenerative changes after onset of post-traumatic osteoarthritis

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Background: Micronized dehydrated human amnion/chorion membrane (mdHACM) has reduced short term post-traumatic osteoarthritis (PTOA) progression in rats when delivered 24 h after medial meniscal transection (MMT) and is being investigated for clinical use as a disease modifying therapy. Much remains to be assessed, including its potential for longer-term therapeutic benefit and treatment effects after onset of joint degeneration.

Objectives: Characterize longer-term effects of acute treatment with mdHACM and determine whether treatment administered to joints with established PTOA could slow or reverse degeneration. Hypotheses: Acute treatment effects will be sustained for 6 weeks, and delivery of mdHACM after onset of joint degeneration will attenuate structural osteoarthritic changes.

Methods: Rats underwent MMT or sham surgery (left leg). mdHACM was delivered intra-articularly 24 h or 3 weeks post-surgery (n = 5–7 per group). Six weeks post-surgery, animals were euthanized and left tibiae scanned using equilibrium partitioning of an ionic contrast agent microcomputed tomography (EPIC-μCT) to structurally quantify joint degeneration. Histology was performed to examine tibial plateau cartilage.

Results: Quantitative 3D μCT showed that cartilage structural metrics (thickness, X-ray attenuation, surface roughness, exposed bone area) for delayed mdHACM treatment limbs were significantly improved over saline treatment and not significantly different from shams. Subchondral bone mineral density and thickness for the delayed treatment group were significantly improved over acute treated, and subchondral bone thickness was not significantly different from sham. Marginal osteophyte degenerative changes were decreased with delayed mdHACM treatment compared to saline. Acute treatment (24 h post-surgery) did not reduce longer-term joint tissue degeneration compared to saline. Histology supported μCT findings and further revealed that while delayed treatment reduced cartilage damage, chondrocytes displayed qualitatively different morphologies and density compared to sham.

Conclusion: This study provides insight into effects of intra-articular delivery timing relative to PTOA progression and the duration of therapeutic benefit of mdHACM. Results suggest that mdHACM injection into already osteoarthritic joints can improve joint health, but a single, acute mdHACM injection post-injury does not prevent long term osteoarthritis associated with meniscal instability. Further work is needed to fully characterize the durability of therapeutic benefit in stable osteoarthritic joints and the effects of repeated injections.

KEYWORDS

osteoarthritis, amniotic membrane, intra-articular, delayed treatment, medial meniscal transection, 3D EPIC-μCT

Introduction

Osteoarthritis (OA) is a progressive, degenerative joint disease that is a leading cause of chronic disability and pain in the US, estimated to affect over 13% of the US adult population (Cisternas et al., 2016). There are no approved disease modifying or regenerative therapies to alter degenerative processes or eliminate the need for eventual total joint replacement. OA is a complex disease characterized by morphological and compositional changes in joint tissues including cartilage surface erosion, loss of proteoglycans, lesion formation, osteophyte formation, and synovial inflammation (Hunter, 2011). Growing evidence indicates that OA has diverse pathophysiology and etiology, meaning there are different origins and various ways to categorize subpopulations, and a patient's specific presentation of localized tissue degeneration, OA phenotype, and other biological and environmental factors may impact the efficacy of a therapeutic for that individual (Mobasheri and Batt, 2016; Roze et al., 2016; Salazar-Noratto et al., 2019a; Van Spil et al., 2019). Current standards of therapy only provide generalized symptomatic relief in the form of non-steroidal anti-inflammatory drugs, steroid injections, analgesics, and viscosupplementation (Clouet et al., 2009). A number of candidate drugs targeting disease modification are being evaluated in clinical trials, but none have clearly demonstrated modified disease progression for any OA phenotypes to date (Roze et al., 2016; Van Spil et al., 2019; Deveza et al., 2017; Ghouri and Conaghan, 2019; Karsdal et al., 2016; Martel-Pelletier et al., 2012; Waarsing et al., 2015; Wieland et al., 2005). These developments in OA characterization and treatments point toward the need for greater understanding of localized OA phenotypic changes and more complete assessments of how existing and new therapies affect different joint tissues and OA subtypes.

This study focuses on an extracellular matrix (ECM)-based approach using a placentally-derived amnion/chorion membrane material, which has been shown in other applications to possess anti-inflammatory properties, display low immunogenicity, and promote wound healing while inhibiting scar formation (Faulk et al., 1980; Hannon et al., 2019; Hao et al., 2000). The product utilized in this study is considered a human cellular and tissue-based product (HCT/P) by the U.S. Food and Drug Administration (FDA), and this umbrella category includes stem cell and ECM-based approaches. The regulatory processes covering these products range from clinicians being allowed to use them with no requirements to be licensed, approved, or cleared by FDA to being required to undergo premarket review of safety and efficacy data and postmarket reporting activities (GovInfo, 2019; FDA, 2007; FDA, 2017). The guidelines for and sub-categorization of developed products have evolved over time, which also means that some products may have shifted from being less to more regulated. This has encouraged a more systematic evidence-based approach towards translation that encourages collaboration between researchers, regulators, and industry to provide patients with safe products that have more clearly defined mechanisms of action, evidence to support use, and guidelines on treatment timing (Jones et al., 2019a; Jones et al., 2019b; Lamplot et al., 2020; LaPrade et al., 2016; Rodeo, 2018). Even for products that are already undergoing clinical trials, evolutions in regulatory oversight have put a higher value on researchers to continue elucidating effects of candidate biologics preclinically in order to identify which products may have higher efficacy and specificity for different joint tissues in various OA phenotypes as well as the appropriate timing for which therapeutics need to be administered (Hannon et al., 2019; Lamplot et al., 2020; Rodeo, 2018).

Several clinical case series have recently been published in support of the use of particulate amniotic membrane ECM-based materials (amnion, chorion, umbilical cord, amniotic fluid) to treat OA (Mead and Mead, 2020; Natali et al., 2022; Alden et al., 2021; Bennett, 2019; Castellanos and Tighe, 2019; Farr et al., 2019; Vines et al., 2016). Four of the studies specifically addressed knee OA, though they were relatively small preliminary studies (6, 20, 25, and 42 qualifying patients), and indicated that single injections with suspensions of either amniotic membrane, amniotic membrane combined with umbilical cord, or amniotic membrane combined with amniotic fluid may help reduce knee pain and improve function in moderate knee OA in the short term (Mead and Mead, 2020; Natali et al., 2022; Castellanos and Tighe, 2019; Vines et al., 2016). One large retrospective case series has been published on the use of injectable micronized dehydrated human amnion/chorion membrane (dHACM) alone, and it included review of 100 knees treated with the product. Patient-reported outcome scores (including pain, daily living, sports/recreation, and quality of life categories increased over time up to 6 months (Alden et al., 2021). One multi-center level I randomized controlled trial that enrolled 200 knee OA patients concluded that treatment with single injection of an amniotic membrane particulate/amniotic fluid suspension was superior to hyaluronic acid and saline at 3 and 6 months in terms of pain relief and improved function. Notably, significant improvements in patient-reported outcome categories of

pain and daily living at 3 and 6 months and sports/recreation and quality of life at 6 months were shown (Farr et al., 2019). At the time of writing, there were about 20 clinical trials of an interventional nature for osteoarthritis using amniotic materials listed at clinicaltrials.gov: Four had been withdrawn, two were not yet recruiting, five were recruiting, and eight were completed, but only one of the completed studies had results included on clinicaltrials.gov. Most of the clinical studies have been completed or are in progress, suggesting that those products are likely still viable candidates for OA disease modifying therapies. However, the limited available clinical trial results and number of withdrawn trials further highlight that variability in efficacy exists. This supports the continuing need for preclinical studies, such as this one, to improve scientific understanding of the effects being produced by such products.

In a previous study, we demonstrated the therapeutic potential of an injectable, micronized dHACM product (mdHACM; EpiFix® Injectable, MiMedx Group, Inc. Marietta, GA) to slow post-traumatic joint degeneration by testing its efficacy in a short-term preclinical rat post-traumatic OA model. A single intra-articular injection of mdHACM administered 24 h after surgical injury inhibited lesion formation and reduced cartilage sulfated glycosaminoglycan (sGAG) loss in the widely used rat medial meniscal transection (MMT) model at 3 weeks post-injury (Willett et al., 2014). 3D cartilage morphology and composition metrics were quantified using equilibrium partitioning of an ionic contrast agent with microcomputed tomography (EPIC-μCT). Results demonstrated ameliorated OA development at 3 weeks post-surgery with immediate delivery of mdHACM treatment, but critical issues such as the duration of therapeutic effect and the potential to treat joints with established OA were not evaluated.

In the present study, we evaluated both the longer-term effects (6 weeks post-surgery) of an acute (24 h) treatment of mdHACM and effects of delayed delivery, where mdHACM was injected 3 weeks post-MMT surgery when joint tissue degeneration has already begun. These conditions simulate two scenarios: (a) treatment immediately following acute injury that is known to induce post-traumatic OA and (b) treatment after symptoms have developed, which would be more consistent with a typical presentation of post-traumatic OA where the patient seeks medical care after becoming symptomatic.

The objectives of this study were to characterize the longer-term effects of immediate delivery (24 h post-surgery) of a micronized amnion ECM treatment and to determine whether this treatment could reduce the progression of cartilaginous and bony changes as evaluated by EPIC-μCT and histology. The hypotheses were that the duration of therapeutic effect of acute micronized amnion ECM treatment would extend to a 6 week endpoint and that delayed injection administered 3 weeks after induction of OA would attenuate further degenerative changes in cartilage and bone tissues in the rat MMT model.

Materials and methods

Preparation of mdHACM

mdHACM was manufactured using the proprietary PURION® process and is compliant with the American Association of Tissue Banks' regulations for donor tissues (MiMedx Group, Inc. Marietta, GA). This process produces a dehydrated, devitalized amnion and chorion tissue graft which is then sterilized and micronized.

Surgical methods–Medial meniscal transection

Weight matched adult male Lewis rats (Charles River, Wilmington, MA) weighing 300–325 g, were acclimated for 1 week after arrival, housed throughout the study at a 12 h light/dark cycle. Animals were dual-housed except immediately after surgeries when they were single-housed until wound clips were removed (10–14 days post-surgery). Animals were anesthetized with isoflurane, given subcutaneous sustained-release buprenorphine (ZooPharm) for analgesia, and the skin over the medial aspect of the left femoro-tibial joint was shaved and aseptically prepared. A skin incision was made by scalpel, 1–1.5 cm in length on the medial aspect of the knee joint. The medial collateral ligament was exposed by blunt dissection of the muscle and transected with microdissection scissors to access the joint space starting from the distal side. Clearance on tibial and femoral sides of the meniscus was carefully established, facilitated by external rotation of the limb and clearing of surrounding connective tissues, and a full thickness cut was made through the full thickness of the meniscus to ensure destabilization (Janusz et al., 2002). For sham surgeries (n = 7), the medial collateral ligament was exposed and transected to visualize the meniscus and joint spaces, but the meniscus was not transected. The muscle was closed with 4.0 Vicryl sutures and the skin stapled using wound clips.

MMT animals received an intra-articular injection to the left knee at 24 h (acute treatment; n = 7) or 3 weeks (delayed treatment; n = 5) post-surgery of mdHACM (EpiFix® Injectable, MiMedx Group Inc. Marietta GA). After resuspending mdHACM in 1 mL saline (80 mg/mL concentration), 50 μL solution was injected into the articular space (25 g needle, BD, Franklin Lakes, NJ). As a control, separate MMT animals received intra-articular injections of 50 μL saline at 24 h post-surgery (n = 6). All animals were euthanized at 6 weeks post-surgery via CO_2 inhalation. The Georgia Institute of Technology IACUC approved all experimental procedures for these *in vivo* studies (Protocol #A12018).

EPIC-μCT analysis of articular cartilage, subchondral bone, and osteophytes

Cartilage and subchondral bone morphology and composition were assessed using EPIC-μCT in the medial third of the medial tibial plateau as described previously (Thote et al., 2013; Willett et al., 2016; Xie et al., 2010; Xie et al., 2012; Xie et al., 2009). Dissected tibiae were immersion fixed in 10% neutral buffered formalin for 48 h then stored in 70% ethanol (v/v) until ready for scanning. Harvested hindlimbs were carefully microdissected to separate femora from tibiae, and in the process full transections of medial menisci were visually confirmed for each limb that received MMT surgery with intact menisci verified for sham surgeries. Immediately

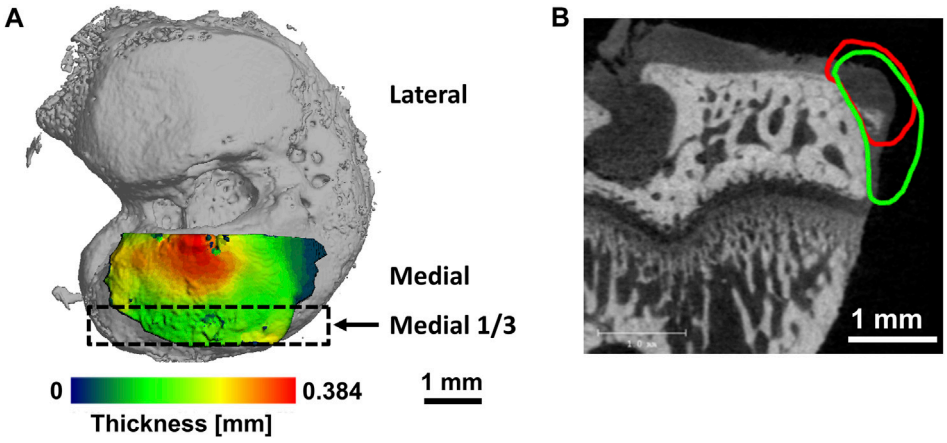

FIGURE 1
Representative figures illustrating evaluation areas. **(A)** Volumes of interest for cartilage on tibial plateau. Medial third of medial tibial plateau indicated by black rectangle. **(B)** Representative image of an osteophyte on the medial tibial margin in the rat medial meniscal tear model with contour for cartilage volume (red) and mineralized volume (green).

prior to scanning, tibiae were patted dry and then immersed in 2 mL of 30% Hexabrix™ 320 anionic contrast agent (Covidien, Hazelwood, MO) solution in phosphate buffered saline (without Ca^{2+} or Mg^{2+}) at 37°C for 30 min (Xie et al., 2009; Palmer et al., 2006). Tibiae were removed from solution, patted dry, secured in sample holders with water at the bottom, sealed to maintain humid environment to prevent drying/shrinkage, and each scanned in the same orientation using a μCT40 (Scanco Medical, Brüttisellen, Switzerland). Scan and reconstruction settings: 45 kVp, 177 μA, 200 m integration time, isotropic voxel size of 16 μm, pixel matrix dimensions 1024 × 1024, scan time ~26 min (Xie et al., 2009). Raw data were automatically reconstructed to axial 2D grayscale tomograms and orthogonally transposed to coronal and sagittal sections. Scanco evaluation software was used to assess local 3D morphologic and compositional measures, using semi-automated spatial segmentation and global thresholding parameters (expressed throughout the manuscript in mineral density units, mg hydroxyapatite (HA)/cm^3), for the following OA-affected tissue types and to output slice images for further image processing.

Articular cartilage: In sagittal sections, cartilage was contoured and thresholded with global segmentation parameters (Gauss sigma 1.0, support 1, lower and upper thresholds 179 and 740 mg HA/cm^3). Images regionally evaluated in the medial third of the medial tibial plateau (Figure 1A). Direct distance transformation algorithms were used to quantify 3D cartilage thickness measures (Xie et al., 2009; Hildebrand and Rüegsegger, 1997; Laib et al., 2000; Lin et al., 2015). Consistent volumes of cartilaginous tissue excluding margins were annotated for evaluation based on image processing standards previously established (Reece et al., 2018). For regions surrounding lesions on the medial tibial plateau in this model and at this timepoint, previous studies have shown that the remaining cartilage tissue swells, which results in higher average thickness measured by contrast enhanced μCT (Willett et al., 2014; Thote et al., 2013; Willett et al., 2016; Lin et al., 2015; Reece et al., 2018; Reece et al., 2020). Average X-ray attenuation of contrast enhanced cartilage (expressed in units of mg HA/cm^3) was also quantified and has previously been demonstrated to be inversely proportional to sulfated glycosaminoglycan (sGAG) content (Xie et al., 2010; Palmer et al., 2006). Degraded cartilage has a lower sGAG content and therefore, after equilibration in Hexabrix™, possesses higher contrast agent content and higher attenuation values. For cartilage surface roughness measurements, sequential 2D grayscale images of sagittal slices of the medial third of the tibial plateau were imported into MATLAB® (MathWorks, Natick, MA). A custom built algorithm, using a global threshold to separate the bone and cartilage surface pixels from each other and the surrounding air pixels, was used to automatically scan each image sequentially to create a 3D digital representation of the cartilage and bone surfaces (Reece et al., 2018). The cartilage surface was fit with a 3D polynomial surface that was fourth order along the ventral/dorsal axis and second order along the medial/lateral axis. The surface roughness was then calculated as the root mean square of the differences between the cartilage and polynomial surfaces. Exposed subchondral bone was calculated as a measure of lesion formation by summing the total area where the bone and cartilage surfaces were separated by less than 2 pixels (32 μm).

Subchondral bone: Quantifications of subchondral bone, the layer of bone underlying articular cartilage, in the medial third of the medial tibial plateau were also performed via sagittal section images using Scanco evaluation software. This analysis region was directly adjacent to the cartilage evaluation region described above and indicated in Figure 1A. Subchondral bone mineral density and thickness were calculated after spatially isolating the bone using contouring and a threshold range of 740–3,047 mg HA/cm^3.

Osteophyte: Defined as bony outgrowths with fibrocartilaginous tissue caps forming on the margins of articular weight bearing joints (van denBerg, 1999; van der Kraan and van den Berg, 2007) (Figure 1B), osteophytes in the medial tibial plateau were analyzed via coronal sections. Osteophyte cartilage volume was measured in volumes of interest that excluded peripheral soft tissue, and cartilage was segmented from air and bone using

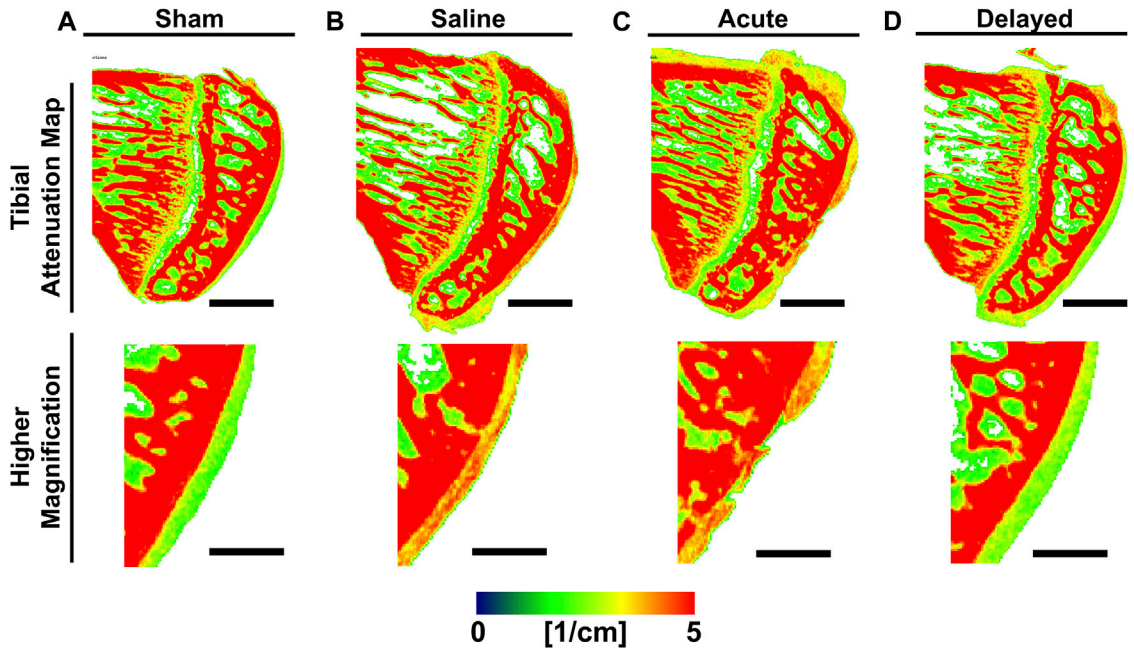

FIGURE 2
Representative medial tibial cartilage attenuation maps show lower attenuation in sham and delayed treatment samples. **(A)** Sham tibiae displayed smooth cartilage surface with green color indicating healthy sulfated glycosaminoglycan (sGAG) content. **(B)** Saline treated tibiae displayed high attenuation indicating lower sGAG content. **(C)** Tibiae receiving acute treatment (24 h post-surgery) of micronized dehydrated human amnion/chorion membrane (mdHACM) displayed high attenuation and initial lesion development. **(D)** Tibiae receiving delayed treatment (3 weeks post-surgery) of mdHACM displayed green central portion indicating healthy sGAG content. Pseudocolor X-ray attenuation bar (expressed in linear attenuation coefficient units [1/cm]): red = high attenuation/low sGAG content and green = low attenuation/high sGAG content. Scale bars = 1mm; higher magnification scale bars = 0.5 mm.

threshold range 179–670 mg HA/cm^3. Osteophyte mineralized volume measurements were made in volumes of interest that did include the peripheral soft tissue, segmented from air, cartilage, and soft tissue using threshold range 670–3,047 mg HA/cm^3.

Histology

Following EPIC-μCT scanning, tibiae and femora were decalcified in Cal-Ex II (Thermo Fisher Scientific, Waltham, MA) on a shaker plate for 7–8 days. Dehydrated samples were routinely processed and embedded into paraffin blocks. Sections parallel to the sagittal plane were cut at 5 μm thickness and stained with hematoxylin and eosin (H&E).

Statistical analysis

All quantitative data were expressed as mean + standard deviation (SD), with all data points displayed. All joint parameters between groups were evaluated using one factor (treatment) ANOVA with Tukey's test for *post hoc* analysis except for the exposed bone parameter which was analyzed using the nonparametric Kruskal–Wallis one-way analysis of variance with Dunn's *post hoc* analysis. Statistical significance was set at $p < 0.05$. All data were analyzed using GraphPad Prism software version 7.0 (GraphPad Software, Inc., La Jolla, CA).

Results

Representative sagittal sections from 3D contrast-enhanced cartilage X-ray attenuation maps showed qualitatively higher attenuation (i.e., lower sGAG content or more degenerated cartilage) at 6 weeks post-surgery for the saline and acute treatment groups compared to sham and delayed treatment groups (Figure 2, red = high attenuation/low sGAG content, green = low attenuation/high sGAG content).

Quantitative analysis of 3D EPIC-μCT images within the medial third of the medial tibial plateau revealed significantly lower cartilage X-ray attenuation in sham and delayed treatment groups compared to saline and acute treatment groups, indicating higher sGAG content in sham and delayed treatment groups (Figure 3A). Cartilage thickness was significantly lower in sham and delayed treatment groups compared to saline and acute treatment groups (Figure 3B). In contrast, no significant difference in cartilage attenuation or thickness was observed between the sham and delayed treatment groups.

Cartilage surface roughness and exposed bone area, additional 3D quantitative indicators of cartilage morphological change, were calculated using a custom MATLAB® program developed and validated in previous work[60]. Analysis of medial third of the medial tibial plateau showed an increase in surface roughness in saline and acute treatment groups compared to sham and delayed treatment (Figure 4A). Exposed bone area was calculated as a

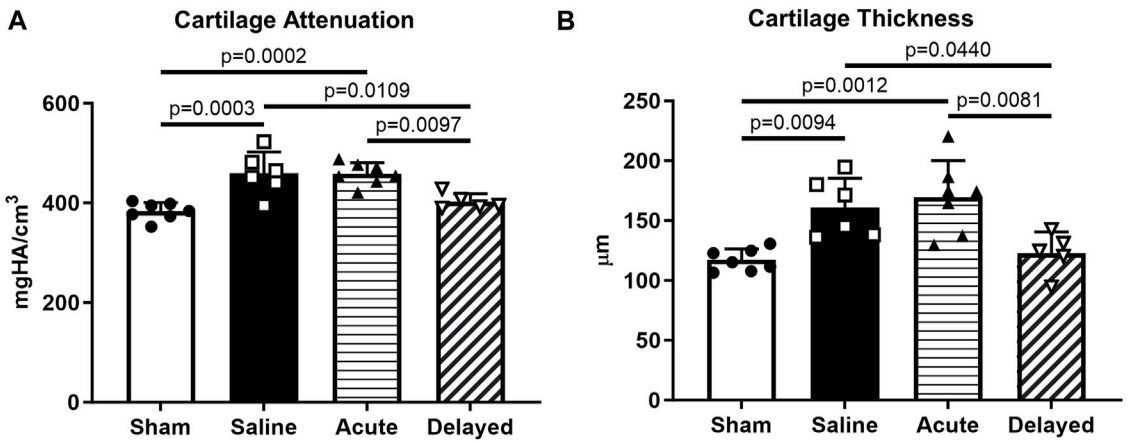

FIGURE 3
Delayed treatment (3 weeks post-surgery) of micronized dehydrated human amnion/chorion membrane (mdHACM) resulted in lower cartilage attenuation and thickness in the medial third of the medial tibial plateau compared to saline and acute mdHACM treatment (24 h post-surgery) groups. **(A)** Cartilage attenuation and **(B)** thickness were significantly higher than sham in the saline and acute mdHACM treatment groups, while both parameters in the delayed treatment group were significantly lower than saline and acute treatment groups. Data shown as mean + SD. Note that in this model and timepoint, degenerated cartilage has been shown to have higher X-ray attenuation (corresponding to lower sGAG content) and higher average thickness (more tissue swelling). n = 5-7.

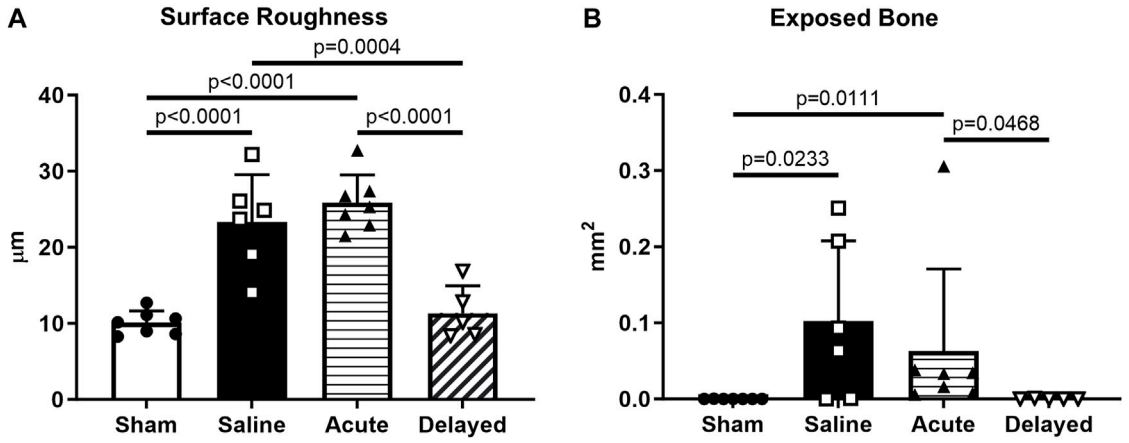

FIGURE 4
Articular cartilage surface roughness and exposed bone evaluated using custom program. **(A)** Cartilage surface roughness was significantly higher in the saline and acute (24 h post-surgery) micronized dehydrated human amnion/chorion membrane (mdHACM) treatment groups compared to sham, while surface roughness in the delayed mdHACM treatment (3 weeks post-surgery) group was significantly lower than saline and acute treatment groups. **(B)** Exposed bone/cartilage lesion area was significantly greater in saline and acute treatment groups compared to sham and significantly lower for delayed treatment compared to acute treatment. Data shown as mean + SD. n = 5-7.

measure of cartilage lesion size and was significantly higher in the saline and acute treatment groups compared to sham, and the delayed treatment group had significantly lower exposed bone area compared to the acute delivery group (Figure 4B).

Subchondral bone mineral density was significantly higher in saline, acute treatment, and delayed treatment groups compared to sham while the delayed treatment group had significantly lower mineral density compared to the acute treatment group (Figure 5A). Only the acute treatment group had significantly thicker subchondral bone compared to the sham group (Figure 5B).

Osteophyte cartilage volume was significantly higher in the saline and acute treatment groups compared to sham, and the delayed treatment group did not demonstrate significantly higher osteophyte cartilage volume compared to sham (Figure 6A). Similar results were observed for osteophyte mineralized volume with a significant increase for the saline and acute treatment groups compared to sham (Figure 6B). Osteophyte cartilage and mineralized volumes were not significantly increased in the delayed treatment group compared to the sham controls and osteophyte cartilage volume was significantly lower in the delayed treatment group compared to the acute treatment group.

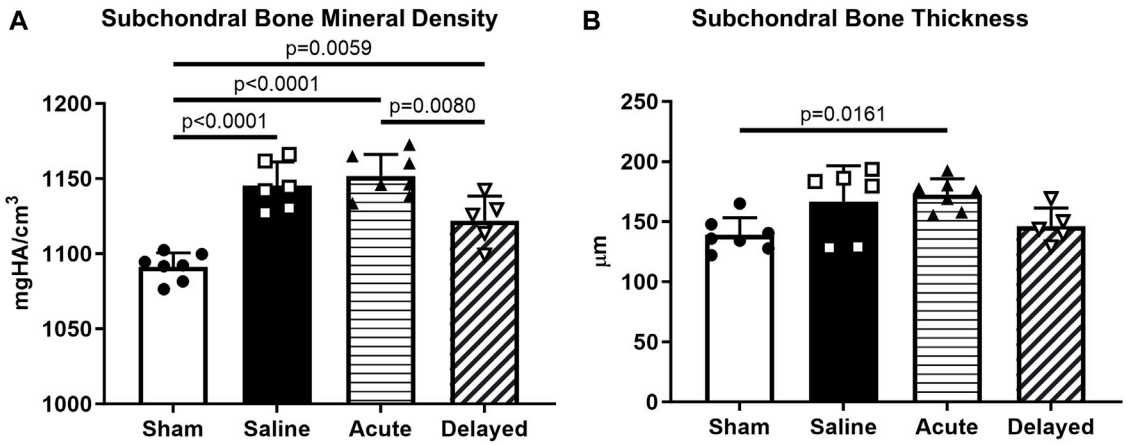

FIGURE 5
Subchondral bone quantifications. **(A)** Delayed treatment (3 weeks post-surgery) of micronized dehydrated human amnion/chorion membrane (mdHACM) resulted in lower subchondral bone mineral density in the medial third of the medial tibial plateau compared to acute mdHACM treatment (24 h post-surgery). Subchondral bone mineral density for the sham group was significantly lower than all other groups. **(B)** Acute dHACM treatment group demonstrated significantly greater subchondral bone thickness compared to sham. Data shown as mean + SD. n = 5-7.

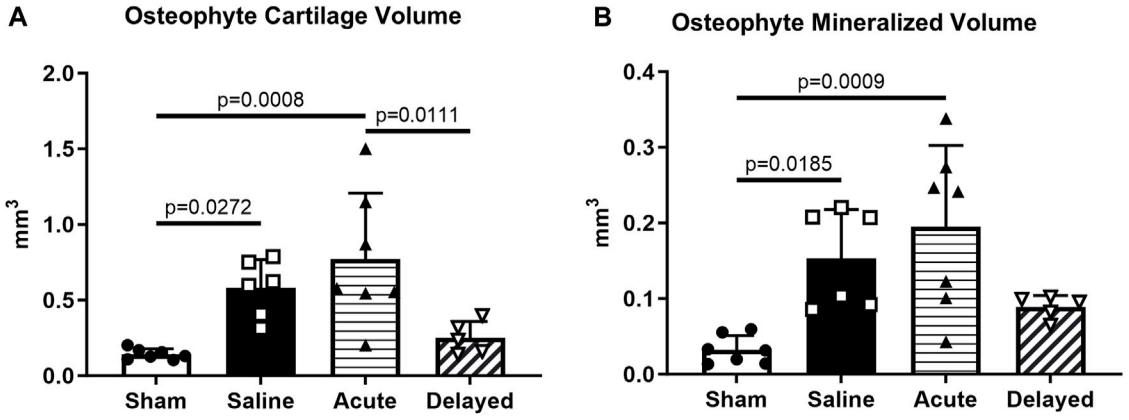

FIGURE 6
Saline and acute (24 h post-surgery) micronized dehydrated human amnion/chorion membrane (mdHACM) treatment groups display osteophyte progression compared to sham. **(A)** Osteophyte cartilage volume was significantly greater in saline and acute treatment groups compared to the sham group and significantly less in the delayed mdHACM treatment (3 weeks post-surgery) group compared to the acute treatment group. **(B)** Marginal mineralized volume was significantly greater in saline and acute treatment groups compared to the sham group. Data shown as mean + SD. n = 5-7.

Histology was performed on tibiae and femora (with synovium attached) at the 6 week time point. For femora, previous studies have shown evidence of mdHACM in the synovium at a 3 week end point (Willett et al., 2014), but in this study no mdHACM was visible in the synovium surrounding the femur (images not shown). Representative images of coronal tibial sections showed degradation of cartilage surface in the saline (Figures 7C, D) and acute treatment group (Figures 7E, F). The sham group demonstrated smooth cartilage surfaces with normal chondrocyte morphology (Figures 7A, B). The delayed treatment group (Figures 7G, H) demonstrated no fibrillations and relatively smooth cartilage surfaces. However, cell morphology suggests the tissue has not been restored to completely healthy state as 10X images show densely packed chondrocytes in columnar structures with potential presence of cloning/clustering chondrocytes.

Discussion

In this preclinical study, contrary to our hypothesis, the therapeutic benefit of a single intra-articular injection of mdHACM (from a single donor) at 24 h post-surgery was not sustained out to 6 weeks. Lack of protective effects of a single 24 h mdHACM injection may be a consequence of the unrepaired destabilization of the joint and subsequent advanced disease development throughout the 6 week study period. The acute treatment group in this study was intended to simulate therapeutic delivery directly after a traumatic injury known to produce downstream degenerative effects (e.g., clinically delivering a therapy immediately after a meniscus tear) and assess whether the single early injection prior to symptom onset could stave off degeneration. Delayed treatment was intended to be more

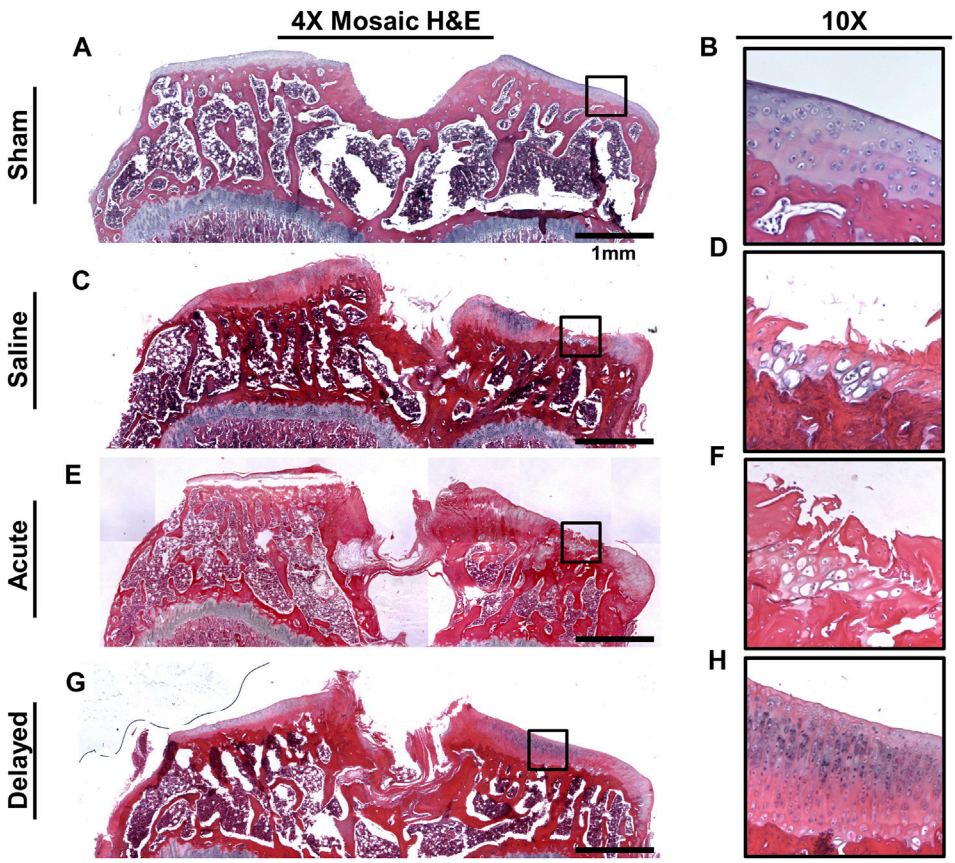

FIGURE 7
Representative H&E histology images. **(A, B)** Sham tibiae showed no cartilage damage and normal chondrocyte morphology. **(C, D)** Saline sample exhibited cartilage surface damage on the medial plateau indicated by fibrillations and lesions. **(E, F)** Acute micronized dehydrated human amnion/chorion membrane (mdHACM) treatment (24 h post-surgery) did not protect against cartilage damage as surface fibrillations were still observed. **(G, H)** Delayed mdHACM treatment provided some protection against cartilage damage as cartilage lesions and major fibrillations were not observed in this group. However, dense chondrocyte columns with elongated cell morphology and potential cloning chondrocytes were observed at 10× magnification in the delayed treatment group.

representative of the typical clinical scenario, where a patient seeks treatment after disease symptoms have already developed. In contrast to acute treatment, a delayed intra-articular injection of mdHACM delivered after joint tissues had already demonstrated osteoarthritic changes resulted in reduced disease progression at the 6 week endpoint compared to saline controls, and for some parameters (such as medial third cartilage X-ray attenuation, thickness, surface roughness, exposed bone, subchondral bone thickness, and osteophyte metrics) there were no significant differences compared to sham joints. Our data suggest that mdHACM could provide protection against continued articular cartilage, subchondral bone, and osteophyte changes, but bony tissues may be protected to a lesser degree.

In this study, we chose to perform 3D contrast-enhanced μCT analysis over semi-quantitative histomorphometry of all samples and provided representative histological depictions of structural and compositional effects as supporting data. A number of previous studies have shown combinations of μCT and histopathology data and demonstrated that 3D μCT quantifications are equally if not more powerful for sensitively characterizing structural changes due to disease and treatment compared to 2D semi-quantitative scoring methods (Willett et al., 2014; Thote et al., 2013; Willett et al., 2016; Lin et al., 2015; Reece et al., 2018; Reece et al., 2020; Thote, 2014). One such study compared and correlated various contrast-enhanced μCT 3D metrics with 2D histopathology analyses and demonstrated that μCT analyses provide fully quantitative and highly sensitive measures of joint degeneration with lower sample numbers compared to histopathology (Willett et al., 2016). Additional advantages of μCT analyses include mitigating scoring variability or bias and providing volumetric data instead of sampling 2D sections from a 3D region of interest. However, histology provides valuable information about cellular and molecular changes within joint tissues that are not available through μCT analyses. One limitation in the current study due to prioritizing μCT analysis over semi-quantitative histomorphometry scoring was that while femoral histology sections were examined for presence of mdHACM in synovium (images not shown), changes in synovial membrane tissues due to disease and therapeutic delivery could not be accurately assessed with the microdissected tissues (typically performed on intact joint histology).

It is important to note that several ECM-based therapeutics are currently being investigated in clinical trials for treating OA. However, there are very limited available clinical trial results published, and these therapeutics have shown inconsistent and

potentially unsustained effects. Specifically for the mdHACM material, a Phase 2B clinical trial was completed by MiMedx Group, Inc. in 2021 to assess efficacy of a single injection in patients with knee OA, with two primary efficacy endpoints (Visual Analog Scale (VAS) for Pain and Western Ontario and McMaster Universities (WOMAC) Osteoarthritis index). The study showed mixed results as the treatment group in one patient cohort demonstrated statistically significant improvement over placebo, whereas the second patient cohort and total patient cohort showed a positive response to both mdHACM and placebo with no significant difference between the groups (MiMedx Group and Inc, 2021). Results such as these highlight some of the complexities and potential sources of variability in clinical studies evaluating candidate therapeutics for effects on structural, pain, and functional aspects of knee OA.

One factor to consider in evaluating therapeutic efficacy is the timing of delivery as it relates to the degenerative state of joint tissues at the time treatment is administered, or in other words, how much the disease has already progressed by the time of delivery. A preclinical study by Raines et al., where MMT animals were treated 2 weeks after surgery with a particulate amniotic membrane/umbilical cord combination product, assessed disease progression at one and 4 weeks after treatment. The study showed potential dose- and time-dependent changes in disease progression metrics at 3 and 6 weeks post-surgery, or 1 and 4 weeks post-injection (Raines et al., 2016). Authors of this work delivered treatment at different post-surgery timepoints and assessed joint tissue changes at different post-treatment timepoints compared to our study, yet they also showed potential chondroprotective effects particularly at earlier post-injection timepoints for the higher dose. These preclinical results coupled with the mixed clinical study results suggest that therapeutic efficacy may depend on timing and disease progression for each joint tissue at the time of treatment.

Other factors to consider when attempting to understand the causes of variability in therapeutic efficacy include questions around donor differences, specificity of the *in vitro* assays used in characterizing potency/activity, changes in product manufacturing processes over time, and potency loss that might occur on the shelf as time passes. Two notable previous studies emphasize concerns that arise around donor-to-donor variability. Our work with mdHACM in this study utilized single donor material. Some studies have utilized pooled donor material as an approach to eliminate effects of donor variability. In one such study Reece et al., 2020, different formulations of mdHACM with varying particle size distributions were used for therapeutic assessment. One of the formulations was the traditional particle size of mdHACM–same size distribution used for this study as well as the prior Willett et al., 2014 shorter-term study (Thote, 2014). The Reece study (Reece et al., 2020) used pooled particulated material from 5 donors compared to use of single donor material in the Willett study (Willett et al., 2014; Thote, 2014). Contrary to Willett study, the Reece study showed that mdHACM did not reduce proteoglycan loss or cartilage tissue swelling in the medial third of the medial tibial plateau compared to saline treatment. There are several possible explanations for the differences in results. The single donor material in the Willett study could have been more potent for chondroprotection than the pooled material in the Reece study. Higher lesion volumes in the Reece study may have contributed to higher joint degeneration severity that could not be overcome by the therapeutic effects of mdHACM. These results taken together with our study's findings and the mixed results of the recent Phase 2B clinical trial illustrate that variability in outcomes can be caused by several possible factors that must be acknowledged and further explored.

In this study, we highlighted the importance of utilizing quantitative 3D μCT to comprehensively assess therapeutic efficacy in a disease that impacts all the tissues of the joint. A cautionary recent study by McKinney et al. indicated that while an encapsulated mesenchymal stem cell-based therapy provided chondroprotection in the rat MMT model of established OA (with the same delivery timing as this study), the therapy had no effect on subchondral bone changes and exacerbated osteophyte changes compared to saline controls (McKinney et al., 2019). While osteophyte development has been considered as highly associated with cartilage degradation in OA, degenerative changes for each tissue may manifest or be sequenced differently for different individuals (van denBerg, 1999; van der Kraan and van den Berg, 2007). This highlights the importance in preclinical OA research of reporting treatment timing and quantitative metrics for each joint tissue type and is aligned with recent work suggesting that specific pathophysiology and OA phenotype may play a role in treatment efficacy for patients (Mobasheri and Batt, 2016; Roze et al., 2016; Salazar-Noratto et al., 2019a; Van Spil et al., 2019). The Raines study and our study complement each other and independently suggest that amniotic membrane ECM-based particulate treatments could provide therapeutic benefit for cartilage, subchondral bone, and osteophyte tissues with a single injection in joints that are already experiencing osteoarthritic degeneration. However, more work to understand effect mechanisms, therapeutic timing, and causes of therapeutic variability for various placentally-derived materials in osteoarthritic environments is still needed.

While the mechanistic processes by which mdHACM may produce a therapeutic effect are not clearly understood, one contributing factor may be that mdHACM contains hundreds of factors, including basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), and tissue inhibitors of metalloproteinases (TIMPs) (Koob et al., 2014a; Koob et al., 2013; Koob et al., 2014b; Koob et al., 2015; Lei et al., 2017). Osteophyte formation results from endochondral ossification brought about by alterations in bone turnover (Gelse et al., 2003; Karsdal et al., 2014) and can be attenuated by bFGF (Sakano et al., 2002). FGF protein signaling, among others, has also been shown to determine the rate of chondrocyte proliferation (Goldring et al., 2006). TGF-β has been implicated in proliferation of perichondrium derived chondroprogenitor cells (Dounchis et al., 1997). TIMPs and other metalloproteinase inhibitors have been implicated in reducing subchondral bone sclerosis and cartilage damage in other osteoarthritis models (de Bri et al., 1998). Other potential mechanisms of action for mdHACM may involve its potential as a cell recruiter or cell substrate (Koob et al., 2013; Jin et al., 2007). Additionally, important previous work has provided further understanding of localized responses of joint tissues in the rat MMT model after intra-articular injection of mdHACM via gene expression analyses of harvested tissues from different sites within the joint (Salazar-Noratto et al., 2019a; Salazar-Noratto et al., 2019b). Results of this work demonstrated that

pro- and anti-inflammatory markers were upregulated in the medial synovial membrane due to treatment with mdHACM and suggested that immunomodulatory effects of mdHACM may help produce protective effects in the joint microenvironment through synovial membrane mediated processes. Further research toward more fully understanding the mechanisms of action of mdHACM are needed, as the complex composition of mdHACM could mean it would impact different PTOA disease microenvironments differently. One such future direction could be to develop suitable high throughput *in vitro* evaluation methods, such as co-culture or functional organoid models, to examine cellular and genetic responses when different cell types are exposed to mdHACM (Reece et al., 2020).

The use of micronized amnion ECM as a disease modifying treatment is a growing area of investigation in OA research. In this study, comprehensive 3D contrast enhanced μCT was utilized to evaluate the effects of mdHACM on joint tissues in a preclinical post-traumatic OA model. Acute treatment, which was shown previously in a shorter-term study to provide therapeutic benefit, was not shown to protect joint tissues out to a longer-term 6 week endpoint, but a single intra-articular injection of mdHACM at 3 weeks post MMT surgery, in an already osteoarthritic joint, slowed degenerative processes for articular cartilage, subchondral bone, and marginal osteophytes. This study supports the suitability of mdHACM as a clinical intervention, after symptom onset, for OA phenotypes where changes in articular cartilage, subchondral bone, and marginal osteophytes contribute to loss of function.

Data availability statement

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

Ethics statement

The animal study was approved by the Georgia Institute of Technology IACUC. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AL, TT, HS, NW, and RG: Conceptualization, development of study goals, experimental design work, and data interpretation. TT, AL, and NW: surgical and treatment procedures. TT, SS, DR, and HS: data/image acquisition, image processing, data analyses and statistics, data compilation, including all μCT and histology work. AL, TT, and DR: initial manuscript section writing, compiling, and editing. RG, NW, and HS: overall manuscript review and editing. All authors contributed to the article and approved the submitted version.

Funding

This study was provided by Department of Defense Award #W81XWH-14-2-0188 Proposal Log #OR130376.

Acknowledgments

Authors would like to thank MiMedx Group, Inc. for providing the micronized amnion/chorion material (mdHACM) for this study. Authors also acknowledge that portions of these data originally appeared in Thote's PhD dissertation document.

Conflict of interest

RG owns stock options in MiMedx Group, Inc. and serves on their Regenerative Medicine Scientific Advisory Board.

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.

Publisher's note

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frontiers | Frontiers in Medicine

TYPE Mini Review
PUBLISHED 27 November 2023
DOI 10.3389/fmed.2023.1208326

OPEN ACCESS

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RECEIVED 19 April 2023
ACCEPTED 10 October 2023
PUBLISHED 27 November 2023

CITATION
Huston P (2023) Why osteoarthritis of the knee is called "a wound that does not heal" and why Tai Chi is an effective treatment.
Front. Med. 10:1208326.
doi: 10.3389/fmed.2023.1208326

Why osteoarthritis of the knee is called "a wound that does not heal" and why Tai Chi is an effective treatment

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Context: Osteoarthritis (OA) of the knee is common and is associated with other chronic diseases and early mortality. OA is often described as a "wound that does not heal" because a local innate immune response gets dysregulated. Tai Chi is an aerobic mind-body practice that is recommended in national and international clinical practice guidelines as a treatment for OA of the knee. This review addressed two questions: What causes immune dysregulation in the knee? and Why is Tai Chi an effective treatment?

Recent findings: There is now a good understanding of what causes OA of the knee at the cellular level. OA begins in the synovium from a phenotypic shift in synovial macrophages in response to tissue damage. The synovial macrophages release inflammatory cytokines, as part of the first phase of the normal healing and repair process. Cytokines communicate to other cells that there has been damage. This stimulates chondrocytes, osteoblasts, and fibroblasts to release inflammatory cytokines as well. When tissue damage is repetitive, there is repetitive release of inflammatory cytokines, and the normal healing process stops. The most common cause of tissue damage is from abnormal biomechanical forces on the knee that arise from trauma, injury, and misalignment. Tissue damage is made worse when there is systemic low-grade inflammation associated with other chronic conditions. Pain and stiffness often result in decreased physical activity, which leads to muscle weakness, progressive instability of the joint, and an increased risk of falls, further injuring the knee. Tai Chi improves alignment, optimizes the biomechanical forces on the knee, strengthens the lower limbs, and decreases systemic inflammation. Tai Chi improves balance and decreases the risk of falls and further injury. There is clinical and experimental evidence to suggest that by removing the causes of cell dysregulation, Tai Chi enables the normal healing and repair process to resume.

Conclusion: Knee OA is a wound that does not heal primarily because repetitive adverse forces on the knee cause synovial macrophages and then local chondrocytes, osteocytes and fibroblasts to dysregulate and stop the normal healing and repair process. Tai Chi mitigates adverse forces on the knee and stabilizes the joint, creating the conditions whereby the normal healing and repair process can resume. Further research is needed.

KEYWORDS

Tai Chi, osteoarthritis, biomechanics, alignment, chronic low-grade inflammation, fibrosis, macrophages, innate immunity

Introduction

Arthritis is common. In the United States, almost one in four adults develop arthritis and it is the number one cause of work-related disability (1). Osteoarthritis (OA) is the most common form of arthritis. Compared to those without OA, people with OA are more sedentary, have more comorbidities (2), and have a 20% higher age-adjusted mortality rate (3, 4).

The understanding of OA and its recommended treatment has changed in the last 15 years. Traditionally OA was thought to arise from "normal wear and tear" of the cartilage with age, and the treatment was to rest the affected joint and take pain medications until a knee replacement could be offered. The current understanding of OA as "a wound that does not heal" was first identified in 2008. At that time, synovial inflammation had been increasingly recognized as an important process in OA pathology, but what caused it and how it was linked to cartilage loss were not well-defined. Scanzello and colleagues proposed that synovitis in the knee was caused by a persistent innate immune response leading to acute inflammation (5). Further research revealed this immune response is part of the normal healing and repair process but when it becomes dysregulated, it causes chronic inflammation and damage (6–10).

Since that time, treatment recommendations have gone from rest to aerobic activity, strength training and normalizing weight as first line therapies for OA of the knee (11–14). In light of both the new understanding that the innate immune system is involved in OA, and the absence of effective pharmacotherapies to address the underlying pathology of OA, there have been calls to "develop innovative ideas and approaches that go beyond conventional paradigms" (15).

Tai Chi is an aerobic mind–body practice that, based on systematic reviews of multiple randomized controlled trials (RCTs), is very effective for OA of the knee (16, 17). For example, in two head-to-head RCTs comparing Tai Chi with physiotherapy, both resulted in similar improvements of pain and function (18, 19). One trial documented a clear dose–response relationship. In both the Tai Chi and physiotherapy groups, median response time was 2 weeks for ≥20% improvement in pain and function and 4–5 weeks for ≥50% improvement (19). Tai Chi is now recommended for the treatment of OA of the knee in international guidelines (11) as well as national guidelines in the United States (12), and Canada (20). Tai Chi can begin before a child starts to attend school to 80 years and older. It involves slow movements, requires no special equipment, and is appropriate for those who may have lost their former level of fitness.

The goal of this article is to answer two questions: What causes immune dysregulation in the knee? and Why is Tai Chi an effective treatment?

Normal and dysregulated tissue repair

Some of the greatest gains in understanding knee OA pathology have arisen from the discovery that the immune cells in the knee are also involved in the normal healing and repair process; it is only when these cells are dysregulated does a pathological process ensue. These are complex processes involving multiple immune cells, signaling molecules, enzymes, metabolic processes, and epigenetics. The following description focuses on the main cells of the knee: synovial cells, cartilage and bone cells, as well as connective tissue and fat cells, and how they all interact in the process of repair as well as pathology.

Resident macrophages orchestrate normal tissue repair

Throughout the body, response to injury and hypoxia is orchestrated by resident macrophages, which are a part of the innate immune system (21). Unlike circulating macrophages, resident macrophages arise in all tissues of the body during embryonic development and remain there for the entire lifespan (22).

Resident macrophages are the body's first line of defense for threats and tissue damage of all types whether be it from infection, a wound, ischemia or injury (23). Throughout the body, resident macrophages undergo phenotypic shifts depending on local circumstances (24). In their steady-state, these macrophages are in a surveillance phenotype (called M0).

When macrophages discover tissue damage, they transition into an inflammatory phenotype (M1) and release pro-inflammatory cytokines (21). Cytokines are signaling molecules that communicate to other cells that there has been damage. Cytokines can attract other immune cells to help remove dead tissue and extravasated blood cells from the area. Once the "clean up" is complete, macrophages transition to their anti-inflammatory phenotype (M2), and release anti-inflammatory cytokines to facilitate tissue repair.

Resident macrophages are called by different names in different tissues. They are called microglia in the brain (25), Kupffer cells in the liver (26), alveolar macrophages in the lungs (27), and renal macrophages in the kidney (28). In the knee joint, resident macrophages are called synovial macrophages or synoviocytes A and B (29).

Synovial macrophages are also central to OA progression (30). How does a cell that orchestrates the normal healing and repair process cause disease?

Osteoarthritis arises from macrophage dysregulation

There are four stages in OA cellular pathology. The first stage is cytokine release by synovial macrophages in the synovial fluid. This is stimulated by micro cartilage fragments and an endogenous molecule called damage-associated molecular patterns (DAMPs) that are released in response to adverse biomechanical forces on the knee (31). Micro fragments and DAMPS are what stimulate synovial macrophages to shift into their M1 phenotype and release inflammatory cytokines (32). Cytokines in the synovial fluid lead to synovial inflammation, which persists throughout all stages of OA of the knee (33). This may help to explain why in the early stages of OA no radiological changes are seen. OA starts deep in the knee joint. It is only well into the second stage when the cartilage thins enough that the pathological changes of OA can be seen radiologically.

The second stage of OA of the knee is cytokine release by chondrocytes in the cartilage. Normal turnover of the cartilage is managed by chondrocytes. When synovial macrophages release inflammatory cytokines it signals to chondrocytes there has been tissue damage via a process called intracellular cross-talk (34). The

inflammatory cytokines stimulate chondrocytes to switch from an anabolic to a catabolic phenotype which then start to release their own inflammatory cytokines. Ongoing cytokine release by catabolic chondrocytes stimulate an enzymatic cascade that starts to degrade the cartilage matrix in a feed-forward loop (35).

The third stage of OA of the knee is cytokine release by osteocytes. As cartilage erodes, it can no longer equalize mechanical forces on the bone. When osteocytes are exposed to inflammatory cytokines, they start to release their own inflammatory cytokines. This stimulates a phenotypic shift in osteocytes toward osteoclast activity, resulting in subchondral bone absorption (36). Bone erosion further exacerbates the adverse mechanical load on the joint.

The fourth stage is cytokine release by fibroblasts and fat cells in the surrounding tissues and can begin during the other stages. Fibroblasts are interstitial connective tissue cells that are found in all tissues, including the synovium and infrapatellar fat pad, and are the main connective tissue cell found in tendons and ligaments. When exposed to inflammatory cytokines, fibroblasts release their own cytokines and cause damage. For example, synovial fibroblasts in the synovial membrane, release cytokines that increase synovitis, and extracellular matrix that leads to stiffness and eventually synovial fibrosis (37, 38). The increased stiffness, in turn, stimulates synovial macrophages into a new M1 mode, setting up a positive feedback loop of inflammation (35).

The infrapatellar fat pad is functionally linked with the synovial membrane (39). Macrophages are abundant in adipose tissue and respond to inflammatory cytokines released by synovial fibroblasts by releasing their own inflammatory cytokines called adipokines (40). This, in turn, stimulates fibrocytes within the collagen stroma to release increased matrix associated with thickening of the interlobular septa (41). Adipokines in the infrapatellar fat pad have been found to enter into the synovial fluid and accelerate the cellular senescence of chondrocytes (31) and may further contribute to synovial fibrosis (42).

Recurrent abnormal loading of the meniscus and anterior cruciate ligament is a mechanical stimuli for fibroblast dysregulation and, if chronic, will lead to chronic inflammation and progressive fibrosis of the ligaments (43).

In summary, adverse biomechanical forces on the joint lead to progressive changes in the cellular phenotypes of all the tissue cells of the joint. When this occurs repetitively, chronic inflammation ensues. Chronic inflammation prevents the different cells from going into their M2 anti-inflammatory phenotype, which is needed to complete the healing and repair process. This dysregulated cycle of inflammation is the major factor that explains why OA is called "a wound that does not heal" (Figure 1).

What causes immune dysregulation in the knee?

Traditionally, the usual risk factors for OA were identified as increasing age, being a woman, obesity, injuries, bone deformities, genetics and some metabolic diseases, such as diabetes (44, 45). It was then realized that most of these risk factors—obesity, acute and repeated stress injuries, and bone deformities—all result in adverse or abnormal forces on the knee.

Similar to coronary artery disease, physical activity can be either an OA prevention strategy or a precipitating factor for

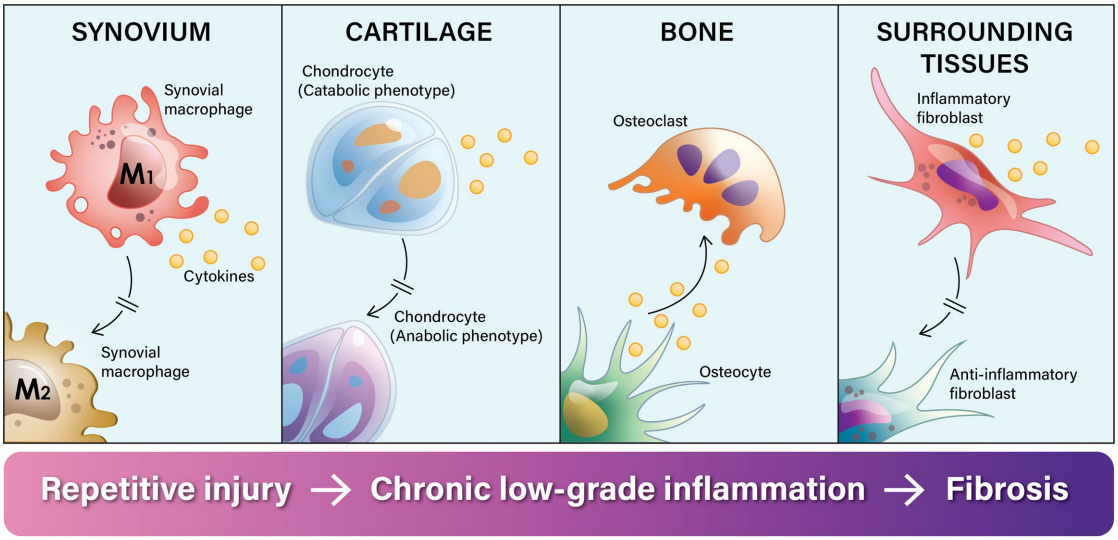

FIGURE 1
Knee osteoarthritis is called a "a wound that does not heal" because the normal tissue healing and repair process gets stalled in the inflammatory phase. Chronic adverse biomechanical forces on the knee joint disrupt and dysregulate the normal repair cycle of tissues in the knee by repeatedly stimulating synovial macrophages to transition into their inflammatory phenotype (M1), which prevents them from going into their anti-inflammatory phenotype (M2) and repairing the tissue. In response to injury and micro-fragments in the synovial fluid, synovial macrophages release inflammatory cytokines. Through a process of intracellular cross-talk, this inflammatory process spreads. First it stimulates chondrocytes to switch from an anabolic phenotype to a predominantly catabolic phenotype, leading to cartilage erosion. Inflammatory cytokines stimulate a phenotypic shift in osteoblasts toward osteoclast activity and leads to subchondral bone absorption. This progressively erodes the bone, and the inflammatory process further spreads to the surrounding fat pad and connective tissue. Intracellular cross-talk stimulates fibroblasts into an inflammatory phenotype and the release of inflammatory cytokines as well as excess extra-cellular matrix. The end result of this dysregulated process is destruction of the joint and surrounding fibrosis.

OA progression. When done within someone's physiologic and biomechanical limits, it has beneficial effects. But when it exceeds someone's physiologic/biomechanical limits, it can do harm. Specifically, physical activities that involve normal physiologic loads on the knee promote cartilage anabolism, but traumatic or hyper-physiologic loads trigger cartilage catabolism (46, 47).

A recent meta-analysis established that adverse biomechanics were associated with an increased risk of OA in over 90% of studies (8). In addition, systemic low-grade inflammation and other exacerbating factors also contribute to arthritic changes. So, what are the sources of adverse biomechanics, systemic low-grade inflammation, and other exacerbating factors?

Adverse biomechanical forces

Trauma is a common cause of adverse biomechanical forces on the knee, which can be acute (caused by falls, sports injuries, and motor vehicle accidents) or chronic, arising from repetitive low-grade trauma to the knee. A past history of knee injury in young adults is associated with a 6-fold increased risk of subsequent OA of the knee (48, 49). Knee injuries commonly involve the anterior cruciate ligament, the meniscus, or both (50–52). Repetitive or overuse injuries often arise from common activities, such as running and bicycling (53, 54), or may be linked to an underlying musculoskeletal deformity that repetitive use reveals, such as varus and valgus deformities (55). Patello-femoral syndrome is common in adolescents (56) and is associated with OA in adulthood (57). Patello-femoral syndrome has recently been linked with deficits in hip abduction, extension and external rotation, which are also associated with OA of the knee (58–60).

Misalignment of the ankle is related to OA of the knee (61), especially when the foot is pronated (62). This makes sense when one considers the role of the foot in receiving and distributing the ground reaction force that arises when the foot strikes the ground. Mechanical loading from increased ground reaction force directed to the knee has been implicated in the pathogenesis of OA of the knee (63). The use of high-heeled shoes has clearly been associated with OA of the knee (64) and may explain why OA is more common in women.

Increased systemic inflammation

It is thought that systemic low-grade inflammation—that is characterized as increased levels of inflammatory cytokines circulating in the bloodstream—either exacerbates OA or decreases the local threshold for its development (65). Obesity is a common source of systemic low-grade inflammation (66). This occurs through the release of adipokines—a type of pro-inflammatory cytokine—that enter the bloodstream. Circulating adipokines have been directly linked to disrupting cartilage homeostasis (67). A systematic review of prospective studies found the risk of knee OA increases by 35% with every 5 kg/m^2 increase in BMI (68).

Systemic low-grade inflammation was initially associated with aging (69), but now it is more clearly associated with chronic disease, including diabetes (70), heart disease (71), chronic lung disease (72), and more. In a recent international study, almost two thirds of people with knee OA (62%) had at least one co-morbidity, with hypertension, heart disease and diabetes being the most common (73).

Chronic disease progression has also been associated with social isolation, which often occurs among older adults with pain and decreased mobility (74). The adverse effects of stress and social isolation appear to be mediated through a chronic sympathetic response, which in turn amplifies systemic low-grade inflammation (75, 76). Thus, as people age with OA of the knee, multiple factors conspire to increase both local and systemic inflammation and advance osteoarthritic pathology.

Exacerbating factors

There are several exacerbating factors that compound adverse forces on the knee: obesity, aging, sedentariness, and progressive muscle weakness. Over 70% of Americans are either overweight or obese (77). Obesity is a well-known risk factor for OA of the knee (11–13, 78), in part because the increased weight alters the biomechanical forces on the knee (79, 80).

People with knee OA often decrease their physical activity because of pain, leading to progressive quadricep weakness, which destabilizes the joint and is linked to a loss of proprioception (81). A recent systematic review concluded that quadriceps weakness was a better predictor of OA of the knee than joint space narrowing on x-ray (82).

Unfortunately, there is a compounding nature to these factors. A systematic review found that people with OA of the knee were more likely to have misalignment, muscle weakness, joint laxity, and proprioception deficits (53), putting them at an increased risk of falls (83). And falls once again risks trauma to the knee. So, how can Tai Chi change this process?

Why is Tai Chi an effective treatment?

Tai Chi is an aerobic mind–body practice that involves mindful concentration, a series of biomechanically sound movements, and abdominal breathing. The center of gravity is low. The ankles, knees, and hips are often in flexion, and the foot moves before weight is transferred between the feet. Weight transfers occur through co-contraction of the agonist (movement) muscles of one leg supported by the antagonist (stabilizer) muscles of the other leg. When the body is alignment, and arm and leg movements are synchronized and coordinated with mindful breathing, the body is able to move with precision and ease.

Given that physical activity is now consistently recommended for OA of the knee (11–14, 78), one might conclude that Tai Chi is simply one of many options. However, there are six different types of physical activity that have been recommended for OA of the knee. All current guidelines recommend aerobics and strength training (11, 12, 14, 78, 84), two guidelines mention balance exercises (11, 12) and this may involve exercises to improve gait and postural control (58). And two recent trends are exercises to improve proprioception (85, 86) and neuromuscular training (87, 88).

TABLE 1 Comparison of exercise recommendations for knee osteoarthritis and Tai Chi.

Exercise recommendations for knee osteoarthritis	Tai Chi
Aerobics (11–14, 78)	Tai Chi is an aerobic activity (89, 90)
Strength training (11–14, 78)	Tai Chi increases lower limb strength (91–93)
Balance training (11, 12)	Tai Chi improves balance (91, 94, 95)
Gait and postural control (58)	Tai Chi improves gait and postural control (96, 97)
Proprioception exercises (85, 86)	Tai Chi improves proprioception (98, 99)
Neuromuscular training (87, 88)	Tai Chi has similarities with neuromuscular training (100, 101)

Current rehabilitation for OA of the knee may involve all six types of exercise. Once rehabilitation is completed, however, keeping up with all of them could be challenging.

Tai Chi is an "all in one" option. It is an aerobic activity (89, 90), that increases lower extremity strength (91–93), balance (91, 92, 94, 95), gait and postural control (96, 97), improves proprioception (98, 99), and has similarities with neuromuscular training (100, 101). And, unlike many sports, Tai Chi has a very low risk of injury (102, 103) (Table 1). Tai Chi is not just another type of physical activity; it is a comprehensive form of physical re-education (104). Tai Chi improves knee OA in three ways.

Optimal biomechanical forces

Tai Chi fosters optimal alignment of the hip, knee, and ankle joints (105). Good alignment helps to improve balance (94), proprioception (98), as well as gait and postural control (96) especially in the elderly (91).

Decreased systemic inflammation

Tai Chi is not just about biomechanics. It is also known to decrease systemic inflammation. It is now well-established that elevated serum levels of inflammatory cytokines, such as IL-6 and TNF-α, are associated with knee cartilage loss in older adults (106). A meta-analysis found that Tai Chi significantly reduced serum TNF-α and decreased IL-6 in those who attended most of the Tai Chi classes (107).

Physical activity in general is known to increase myokines, an anti-inflammatory cytokine released from muscle with physical activity, and this can moderate the effects of inflammatory cytokines (108). Tai Chi also increases myokine levels (109).

It is increasingly recognized that the immune system interacts with the autonomic nervous system (110, 111). Exercise is known to dampen the sympathetic response and protect against the upregulation of inflammatory cytokines (112). In addition, mind-body exercises, such as yoga and Tai Chi, are known for their parasympathetic or relaxation response thought to be due to mindfulness and abdominal breathing (113). Tai Chi also mitigates the inflammatory effect of social isolation. Offered in community-based classes, Tai Chi has been shown to decrease social isolation in the elderly (114–116).

Stabilizing factors

There are other factors that help restore and maintain a healthy knee joint. Two recent systematic reviews have highlighted that Tai Chi improves strength, especially in the lower limbs (91–93) likely through co-contraction of the lower limb muscles with movement. This helps to stabilize the knee and prevent joint laxity. Two biomechanical studies found Tai Chi produces less of a load on the knee joint than walking (100, 101) and improves the plantar load on the feet (117) which is often abnormal in individuals with knee OA (118). Tai Chi is associated with a decreased ground reaction force from gentle weight transfers and an even weight distribution on the feet (119). Tai Chi significantly improves proprioception of the lower limbs (98, 99).

Finally, Tai Chi is well-known to decrease the risk of falls. The evidence has been so compelling, Tai Chi has long been recommended as an effective fall prevention strategy in older adults (120, 121). A decrease in the risk of falls will, in turn, decrease the risk of further trauma to the knee.

In summary, the pathophysiology of OA of the knee and the therapeutic effects of Tai Chi are both self-perpetuating cycles. With OA of the knee there is a cycle of dysregulated cytokine release arising from repetitive tissue damage which stops the normal healing process. With Tai Chi, conditions are fostered that helps the normal healing process to resume. However, stopping Tai Chi means the cycle could revert again to osteoarthritic progression. These cycles are summarized in Figure 2.

Tai Chi and cellular phenotypes

Does Tai Chi actually change the phenotype of cells? To date it appears no study has specifically assessed this. It has been suggested that moderate physical activity in general helps to maintain or re-establish the physiological function of synovial macrophages (122). And in an animal study of experimentally induced OA, those who had been physically active beforehand were found to have more anti-inflammatory cytokines reflective of the M2 synovial macrophages needed to complete the repair process (123). It would not be surprising if people with OA of the knee, were found to have more M2 synovial macrophages after taking Tai Chi classes for 8 weeks than before they began—especially if they had improvements in their knee pain and function over that time.

Other evidence to support this idea comes from a recent clinical study of high tibial osteotomies, done to improve biomechanical forces on the knee. Following the procedure, there was less synovial inflammation and more M2 macrophages facilitating the completion of the normal tissue repair cycle (124). Since Tai Chi improves biomechanical forces on the knee, it would be reasonable to conjecture that Tai Chi optimizes cell phenotypes to enable the normal healing process to resume.

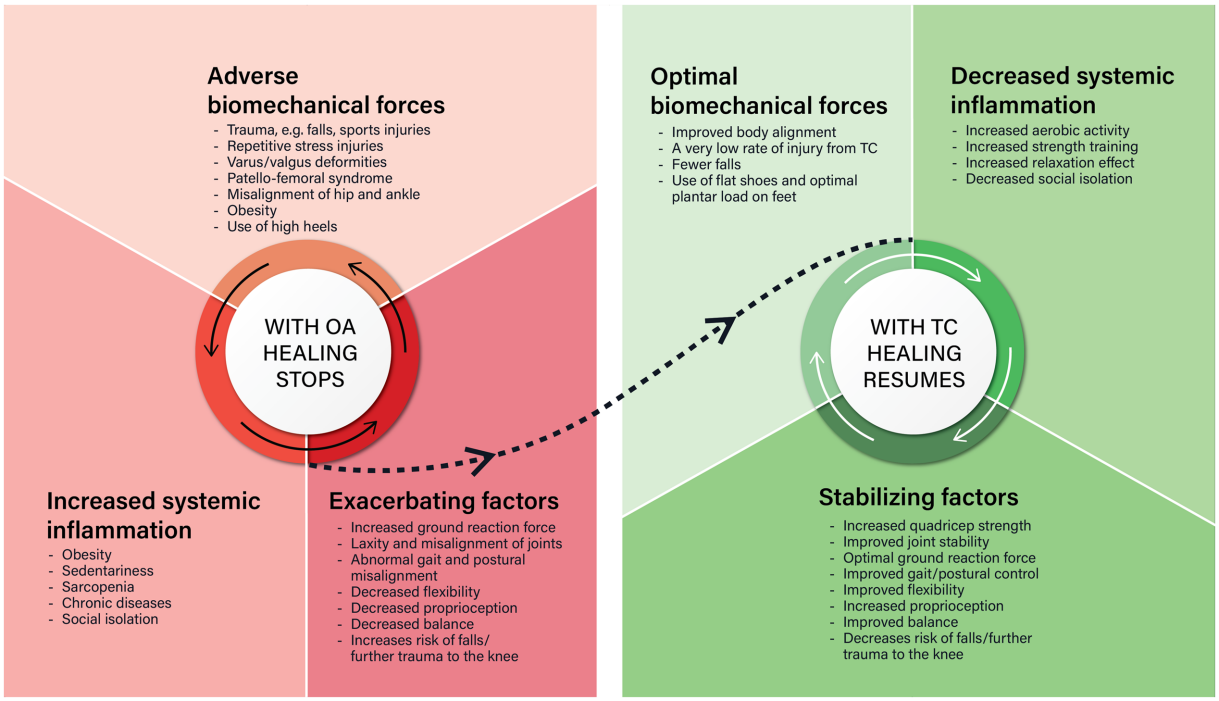

FIGURE 2
There are striking similarities and differences between what happens in osteoarthritis (OA) and Tai Chi (TC). The similarities are that both are self-perpetuating cycles that involve biomechanical forces on the knee, systemic low-grade inflammation, and other factors. In OA of the knee, adverse biomechanical forces on the knee causes phenotypic shifts in the key cells of the knee, that cause local inflammation which stops the normal tissue repair process. This is made worse by increased systemic low-grade inflammation and other exacerbating factors. Tai Chi improves body alignment, optimizes the biomechanical forces on the knee, decreases systemic inflammation, and stabilizes the knee joint so the normal tissue repair process can resume.

Discussion

This is the first time that Tai Chi as a treatment for OA of the knee has been linked to correcting the conditions that cause immune dysregulation in the knee. The wound does not heal in OA primarily because repetitive adverse biomechanical forces on the knee cause phenotypic shifts in synovial macrophages and other key cells of the joint causing chronic local inflammation that stops the normal healing process. This is made worse if there is systemic inflammation and other exacerbating factors. Tai Chi improves body alignment, strengthens the lower limbs, stabilizes the knee and decreases both local and systemic inflammation, enabling the normal healing and repair process to resume.

There are some limitations to consider. Much more is known on the pathology of OA than described. Other immune cells are involved, there are many phenotypes of macrophages, many cytokines and different enzymatic cascades they evoke, and all this is linked with epigenetic changes. Although the interaction between the immune and autonomic nervous system was briefly described, there are also metabolic, hormonal, and other influences, such as mitochondrial dysfunction and oxidative stress, that have a role in OA pathology.

Likewise, although a lot is known on the therapeutic effects of Tai Chi for OA of the knee, Tai Chi has many other therapeutic effects that were not described here (125). And Tai Chi has other mechanisms of action, such as increasing the functional connectivity of the brain (126). Tai Chi may well have additional mechanisms of action that have yet to be discovered.

Clearly, more research is needed. To date, research on the phenotypic shifts of cells in knee OA has focused on the potential to develop new therapeutic interventions. There have been calls to "develop a drug that skews inflammation toward a pro-chondrogenic microenvironment" (127) and stem cell transplants are still under evaluation for their potential to increase chondrocytes and improve cartilage recovery (128, 129). However, the phenotypic effects of normalizing weight, getting regular physical activity and optimizing biomechanical forces on the knee should not be overlooked. It is likely that not only Tai Chi, but any aerobic exercise (that is biomechanically sound) will optimize the phenotypes of cells in the knee. Research assessing the relative importance of strength training and other exercise types is also indicated.

There are clinical implications to consider. For example, when OA patients come to get physician clearance to start a new exercise program, it would be useful to have a summary of the adverse biomechanical forces on the knee for different types of sports and physical activities (130). And consideration could be given to recommending a biomechanical assessment of the knee (131).

One of the most positive implications of this new understanding for knee OA, is that osteoarthritis is starting to be seen as a reversible disease. OA can be reversed if there is intervention early in the course of disease (32). Understanding what is needed to re-establish the normal healing and repair process in knee OA may be a new motivation for lifestyle change. Much like cardiac rehabilitation supports lifestlye change for those with heart disease,

secondary prevention programs could support lifestyle change for those with OA. This would include biomechanical assessment, education, coaching and progressive patient self-management that is well-linked with both clinical care and exercise classes in the community (132, 133). OA of the knee is a common reason for older people to become sedentary. Recent international physical activity guidelines for older adults identify the importance of people regaining and maintaining regular physical activity: it can mitigate most chronic diseases, improve mental health and quality of life, and prevent premature mortality (134).

Conclusion

Synovial macrophages are key to understanding both knee OA pathology and the effectiveness of Tai Chi. Under adverse biomechanical conditions, synovial macrophages become dysregulated and this dysregulation spreads to the other cells in the joint and leads to OA pathology. When local biomechanical conditions are optimized with an activity such as Tai Chi, synovial macrophages can resume orchestrating the normal healing process. More research is needed on different types of exercise for knee OA and on how to help people make lifestyle changes more effectively. Some clinicians have hesitated to prescribe Tai Chi as it is a mind-body practice whose therapeutic effects are not yet widely known (135). However, mind-body practices are increasingly mainstream (136) and, based on RCTs, guideline recommendations and mechanism of action studies, clinicians can now be confident in recommending Tai Chi so that the wound of knee OA can heal.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Acknowledgments

The author thanks Chenchen Wang for suggesting that I write this manuscript, Adam Krajewski, the graphic designer who created the figures from my rough sketches, and Monica Fuijkschot who provided constructive feedback on an earlier draft of this manuscript.

Conflict of interest

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

Publisher's note

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