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Sex-specific reduction in inflammation of osteoarthritic human chondrocytes and nutraceutical-dependent extracellular matrix formation

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ABSTRACT

Introduction: The aim of this study was to investigate the ability of osteoarthritic human chondrocytes to produce articular cartilage (AC) tissues with a reduced inflammatory environment in response to 4 anti-inflammatory nutraceuticals: alpha-tocopherol (Alpha), gallic acid (G), ascorbic acid (AA), and catechin hydrate (C).

Methods: Chondrocytes isolated from patients who underwent total knee arthroplasty surgeries were divided into groups (9 male; mean age, 66.2 ± 3.5 years and 11 female; mean age, 64.2 ± 3.1 years). Cells were cultured based on sex and supplemented with either a negative control (NC) medium or NC plus one of the nutraceuticals at a concentration of 50 μM. At day 21, cultures were characterized histologically, biochemically, and for gene expression of vital markers.

Results: At day 21, 62.3% and 66.2% reduction in nitric oxide (NO) content was evident for female and male cells, respectively. G-treatment of female cells resulted in the lowest expression of nitric oxide synthase-2 (NOS2), matrix metalloproteinase-13 (MMP13), and collagen type-10 (COL10). Alpha-treatment of male cells resulted in the lowest expression of NOS2, bone morphogenic protein-2, MMP13, COL10 and tumor necrosis factor alpha induced protein-6 (TNFAIP6) relative to NC. AA and Alpha treatment resulted in the highest glycosaminoglycan (GAG) content for female and male cultures, respectively.

Conclusion: A sex-dependent response of osteoarthritic chondrocytes to nutraceutical treatment was evident. Our results suggest the use of G for female cells and Alpha for male cells in OA applications seems to be favorable in reducing inflammation and enhancing chondrocytes' ability to form AC tissues.

1. Introduction

Osteoarthritis (OA) is marked by synovial inflammation and degradation of articular cartilage (AC).[1–3] Inflammatory cytokines play a major role in OA's progression, triggering a cycle of pain and contributing to further tissue's degradation.[1,4] OA affects at least 10% of men and 13% of women aged 60 or older.[3,5] The risk of developing OA increases with systemic factors such as age, race, genetics, and local factors such as obesity, sex and injury.[3] Sex-differences in OA development have been recognized since the 1950s[6]; however *in-vitro* and animal model studies investigating OA typically do not specify sex used.[7] Without studies detailing the fundamental mechanisms by which sex influences OA's development or treatment, our ability to control OA is limited.[8]

There is currently no therapy that halts or reverses OA damage.[9] Existing treatments focus on medications and lifestyle changes until a total knee arthroplasty (TKA) surgery is needed; which is invasive, costly and requires significant recovery time.[2,10] A TKA's likelihood increases with age and is higher for females than males.[11] As such, there is a

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growing interest in seeking sustainable, health-conscious approaches to OA's management. Alternative treatments that may halt OA's progression, maintain AC, and reduce inflammation[12] are of considerable significance given that 47% of patients use them.[13] These include the use of nutraceuticals, which are vitamins, plants or extracts that provide health benefits.[13]

For this study, the following nutraceuticals were investigated for their anti-inflammatory and antioxidant properties. Ascorbic acid (AA), known as vitamin C, is an antioxidant found in many fruits and has shown a reduced risk of cartilage loss and OA's progression.[13] Alpha-tocopherol (Alpha), or vitamin E, is commonly used as an antioxidant, and was shown to relive OA's related pain.[13] Gallic acid (G), which is typically found in tea and grapes, has strong anti-inflammatory and chondroprotective abilities.[14] Finally, catechin hydrate (C), an extract found in green tea, is known for anti-inflammatory and antioxidant properties.[15] As potential treatments, these nutraceuticals can be orally supplemented. The bioavailability of nutraceuticals investigated here has been investigated in various studies in the literature[16–19] proving their therapeutic potential. As bioavailability depends on the exact type of supplement, encapsulation and the nano-delivery techniques were utilized, findings should not be generalized.[20] When nutraceuticals' bioavailability is concerned, positive associations between uptake of nutraceuticals and changes to the gut microbiome were suggested as evidence of their bioavailability *in-vivo*. A recent review emphasized the possible mechanism of action of nutraceuticals through the gut microbiome and the connections of gut dysbiosis to inflammatory diseases including OA.[21–23] In a study, it has been found that oral supplementation with a type of nutraceutical can lead to less pro-inflammatory bacterial metabolites in the gut that corresponded to a less severe OA grade compared to control's vehicle supplementation.[21]

Human clinical trials have suggested that nutraceuticals investigated orally are associated with a decline in OA reported pain,[4] however the mechanistic means whereby nutraceuticals achieve this at molecular and cellular levels in a sex-dependent manner were not examined. This lack of mechanistic studies calls the field to perform studies that explore the mechanistic pathway of nutraceutical action. While with limitations, our study is a step towards unravelling sex-dependent differences of nutraceutical treatments on chondrocytes. Specifically, we hypothesize that exposing human osteoarthritic chondrocytes to individual nutraceuticals with antioxidant and anti-inflammatory properties will reduce inflammation and reactive oxide species (ROS) resulting in enhanced extracellular matrix (ECM). We further hypothesize that responses will be sex-dependent.

Our findings as described in this paper are expected to aid in elucidating differences in how nutraceuticals affect our ability to engineer functional AC tissues with reduced inflammation starting with donor-isolated chondrocytes. By addressing the prominent cycle of inflammation, we expect that nutraceuticals may aid in the maintenance of AC and mitigate the effects of inflammation on OA's progression.

2. Materials and methods

Cell culture reagents and supplements are all Gibco, and all chemicals and nutraceuticals are from Millipore Sigma, unless otherwise specified.

2.1. Isolation of human articular chondrocytes (HACs)

HACs were isolated from AC from 20 consenting participants, undergoing TKA surgeries. Caucasian patients, with OA-grade of 4 based on the International Cartilage Repair Society, were divided into groups based on sex (9 male; mean age, 66.2 ± 3.5 years and 11 female; mean age, 64.2 ± 3.1 years). The means of ages for the two groups were similar ($P = 0.2242$). Washington State University's Institutional Review Board approved all studies. AC tissues were minced and washed with dissection-medium (phosphate buffered saline (PBS) with 1% penicillin-streptomycin (Pen-Strep)). Samples were then digested overnight in digestion-medium (0.1% collagenase type-1 in Dulbecco's Modified Eagle's Medium (DMEM), 2% v/v fetal bovine serum (FBS), 2% v/v Pen-Step, and 1% v/v Amphotericin-B) at 37 °C and 125-rpm. To isolate HACs, the solution was passed through a 40-μm filter. The filtrate was centrifuged, and the resulting pellet underwent two washes and resuspension in DMEM for cell counts. Cell-counting was performed using 0.4% trypan blue (TB) exclusion at a 1:10 dilution. HACs were resuspended in cryopreservation-medium, made up of culture-media (CM, (DMEM, 10% FBS, 1% pen-strep, and 1% Amphotericin-B)) with 10% v/v dimethyl sulfoxide (DMSO) and frozen under controlled decreased thermal conditions of 1 °C/min.[24] Cells from different donors were preserved separately.

2.2. Cell culture and nutraceutical treatment

For cell culture, primary HACs were thawed at 37 °C and added to CM and washed to remove residual cryopreservation media. HACs were pooled from different donors to yield a representative mixture of chondrocytes for the female and male groups. This was done for several reasons. First, pooling of cells is a common practice to reduce variability amongst donors.[25–27] Second, OA HACs dedifferentiate upon expansion; limiting the ability to increase cell numbers to cell densities desired *via* expansion.[28] Finally, the aim of this study was to investigate responses of primary cells and not expanded cells. Viability of pooled cells was assessed using 0.4% TB exclusion. HACs suspensions were seeded at random in 24-wellplates. Cultures were maintained in a humidified 5% CO_2 incubator at 37 °C in a negative control (NC) medium (DMEM/F-12 GlutaMAX (Gibco), 1% v/v Pen-Strep, 10% v/v FBS and 1% v/v Amphotericin-B). The antimicrobial additives were added to all media as recommended for mammalian cell culture and their effects are expected to be constant for all treatments compared.[29] After 24 h, culture media was changed to media supplemented with either a nutraceutical or the NC for the rest of the duration of the culture period. Group 1 was NC as described. Groups 2–5 were NC-medium supplemented with one nutraceutical (C, G, Alpha, and AA) at a final concentration of 50 μM.[30–32] The 50 μm concentration was the lowest concentration investigated in the literature such as, upon success, a low dose of the supplement is utilized. Cultures were maintained for 21 days, and the medium was changed every other day.

2.3. Histology

Total collagen and glycosaminoglycans (GAGs) formation was observed using aniline-blue (Masson's Trichrome-Kit) and toluidine blue (0.1% toluidine blue in 5% acetic acid) dyes, respectively. Further information can be found in supporting information S.1.

2.4. Colorimetric quantification of collagen, GAG and DNA

Cultures were analyzed for ECM formation, represented by GAG and collagen content that was normalized by DNA content. For DNA, samples were digested overnight at 65 °C in papain extraction reagent (0.1 mg/mL papain (≥16 units/mg protein) in 0.2M sodium-phosphate /ethylenediaminetetraacetic acid (EDTA) aqueous buffer suspension). DNA was quantified using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen), following manufacturer's instructions. Fluorescence was read at an excitation and emission wavelengths of 480 nm and 520 nm respectively using Cytation-5 Microplate-Reader (Biotek instruments, Vermont, USA). Total GAG and collagen were quantified with Blyscan and Sircol assays (Biocolor Ltd., UK), respectively, per manufacturer's protocols with the slight modification of adding dyes directly to wells. GAG and collagen absorbances were measured at 656 and 555 nm respec-

tively using a microplate-reader. GAG and collagen contents in μg were normalized by DNA for reported values.

2.5. Gene expression

Quantitative real time polymerase chain reaction (qRT-PCR) was used to quantify gene expression of collagen type II (COL2A1), collagen type IX (COL9A1), aggrecan (ACAN), SRY-box transcription factor-9 (SOX9), collagen type X (COL10A1), bone morphogenic protein-2 (BMP2), nitric oxide synthase-2 (NOS2), tumor necrosis factor alpha induced protein-6 (TNFAIP6), and matrix metalloproteinases (MMP13), as previously described.[33] Briefly, total RNA was isolated with TRIzol Reagent (Invitrogen) by adding 250 μL of the reagent to each well. After addition of the reagent, the solution with the lysate was pipetted up and down with a pipette to ensure lysis of the cells and isolation of the total RNA. This lysate was collected into tubes and frozen at −80 °C. Samples were sent to Regeneron Pharmaceuticals (Tarrytown, NY) for processing. Briefly, after lysis, chloroform was used for phase separation where the mRNA-containing aqueous phase was purified using the MagMAX™-96 for Microarrays Total RNA Isolation Kit (Life Technologies) according to manufacturer's specifications. The Qiagen CNase buffer and TURBODNase from the Mag-MAX kit were used to remove genomic DNA. For each treatment group, triplicates were analyzed and compared to a day zero sample as a reference for each group. Total mRNA (up to 2.5 μg) was reverse transcribed into core DNA (cDNA), which was then amplified with the Sensifast (Bioline) on an ABI 7900HT Sequence Detection System (Applied Biosystems) and probes that are specific for the housekeeping gene, human beta actin. The relative gene expression was calculated using the $\Delta\Delta C_T$ method, where fold difference was determined using the expression $2^{\Delta\Delta C_T}$.[34]

2.6. Quantification of nitric oxide (NO) content

NO content was measured on day 1 as the baseline for inflammation of HACs prior to nutraceutical treatments, and compared to NO content at day 21. Griess reagent assay was used to quantify NO in cell media.[35] Briefly, a 50 μL culture media sample was taken to a 96-wellplate. While protecting the sample from light, sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) was added for a 5 min incubation period followed by addition of NED (0.1% N-1-napthylethylenediamine dihydrochloride in water) and incubated for five additional minutes. Absorbance was then read at 540 nm. Values were reported in terms of sodium nitrite concentration in μM.

2.7. Statistical analysis

All experiments were performed in triplicates (n = 3). Statistical tests were performed using GraphPad Prism 8 (San Diego, USA). Two-way ANOVA was used to compare the two-group responses with Tukey post-hoc test to compare treatments within a group. Data were deemed significantly different with $P \leq 0.05$. In addition, comparisons between treatments and trends observed that are not statistically significant were occasionally discussed. When that was the case, the P-value was not included. All data were reported as mean ± standard error of the mean (SEM).

3. Results

3.1. Gene expression of inflammatory markers

A downregulation in NOS2 and TNFAIP6 expressions on day 21 relative to day 0 untreated cells was evident for both groups, irrespective of treatment. Nutraceuticals' treatment of cells resulted in an average downregulation of NOS2 relative to NC by 1.6-fold ($P \leq 0.0001$) in females and 3.1-fold in males ($P \geq 0.05$) (Fig. 1a and b). NOS2 expression in male cells was significantly lower relative to females by an average 5.2-fold in all treatments ($P \leq 0.0001$, Fig. 1c). For female cells, G-treatment had 2.3-fold less NOS2 expression compared to NC ($P \leq 0.0001$, Fig. 1a). Though insignificant, Alpha-treated male cells resulted in the most reduction in NOS2 compared to NC by 1.9-fold (Fig. 1b). Compared to other nutraceuticals, Alpha-treatment decreased TNFAIP6's expression in both groups significantly in some comparisons (Fig. 1d and e). Specifically, Alpha-treated female cells had an average 1.6-fold decrease versus G, AA and C ($P = 0.0193$, $P = 0.0032$, $P = 0.0064$, Fig. 1d). NC had similar TNFAIP6's expression as Alpha in female cells (Fig. 1d). Alpha-treated male cells had a significant average 2-fold decrease versus G and AA ($P = 0.0002$, $P \leq 0.0001$, Fig. 1e). Female cells had lower TNFAIP6's expression relative to male cells by an average 1.29-fold and significantly so in NC, G and AA ($P = 0.0186$, $P = 0.0220$, $P = 0.0114$, Fig. 1f).

3.2. NO content and viability of cells as a function of nutraceutical treatment

On day 1, the means of NO contents for both sexes were not different with an average 7.7 μM ($P = 0.5086$, Fig. 2a and b). In contrast, from day 1, the NO content significantly decreased across all treatments for both sexes on day 21 ($P \leq 0.0001$, Fig. 2a and b) and on average, had values of 2.9 μM and 2.6 μM respectively for female and male groups. On day 21 the female group had an average 1.12-fold higher NO relative to males in all treatments except Alpha. Though insignificant, AA-treated female and male cells had the least NO by an average 1.37-fold (Fig. 2a) and average 1.22-fold respectively (Fig. 2b). Viability of HACs was measured as in supporting information S.2. Nutraceutical-treatments had similar viability relative to NC on day 21 (Supporting information S.3, Fig. S1).

3.3. Gene expression of chondrogenic markers

Chondrogenic markers, COL2A1, COL9A1, SOX9, FOXO1 and ACAN are indicators of forming a robust ECM. In general, there was a downregulation of chondrogenic markers for cells on day 21 relative to day 0 cells, which is typical in 2D cultures.[36]

COL2A1 (Fig. 3a and b) and COL9A1 (Fig. 3d and e) expressions followed the same trend in response to the 5 treatments in the female and male groups. Alpha-treatment resulted in significantly higher COL2A1 expression than AA, G and C-treated cells in the female group ($P \leq 0.0001$, Fig. 3a) and slightly higher than NC ($P > 0.05$). Similarly, Alpha-treatment resulted in significantly higher COL9A1 expression versus all nutraceuticals ($P \leq 0.0001$, Fig. 3d). In the male group, Alpha-treatment resulted in significantly higher COL2A1 expression vs. G and C ($P = 0.0063$, $P = 0.0266$, Fig. 3b). Similarly, Alpha-treatment for COL9A1 was highest among nutraceuticals (Fig. 3e, $P \leq 0.0001$). Female cells expressed significantly higher COL2A1 and COL9A1 at 1.79-fold ($P = 0.0112$) and 1.81-fold ($P \leq 0.0001$), respectively when Alpha-treated, relative to male cells (Fig. 3c and f). Alpha-treated female cells had significantly higher SOX9 expression among treatments by an average 1.24-fold ($P \leq 0.001$, Fig. 3g). Similarly, Alpha-treated male cells had the highest SOX9 expression among treatments and significantly higher vs. C and G ($P = 0.0063$, $P = 0.0280$, Fig. 3h). Female cells had an average 1.87-fold higher SOX9 expression compared to male cells (Fig. 3i). Interestingly, there is a strong trend observed between SOX9 and COL2A1 across the 5 treatments for both the female (Fig. 3a and g), and male groups (Fig. 3b and h). Alpha and NC-treated female cells had similarly higher ACAN expression compared to other treatments and significantly higher than G-treated cells ($P = 0.013$, $P = 0.0121$, Fig. 3j). Males had comparable ACAN expression (Fig. 3k). Overall, female cells had significantly an average 2.69-fold higher ACAN expression than male cells ($P \leq 0.0001$, Fig. 3l).

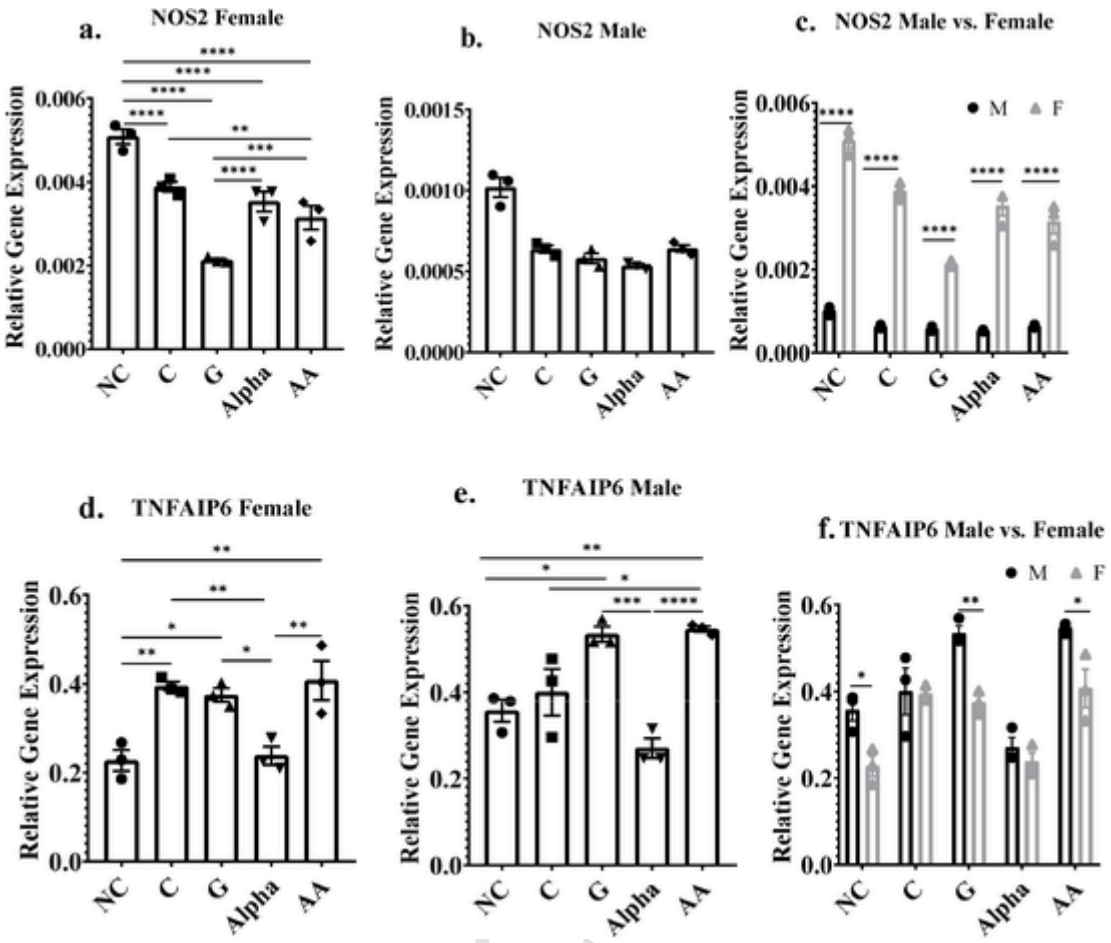

Fig. 1. Relative gene expression of inflammatory markers NOS2 and TNFAIP6 for cultures of male (M) and female (F) groups of cells as a function of nutraceutical treatments. (a–c) NOS2 gene expression on day 21 for F, M and M vs. F, respectively. (d–f) TNFAIP6 gene expression on day 21 for F, M and M vs. F respectively. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001.

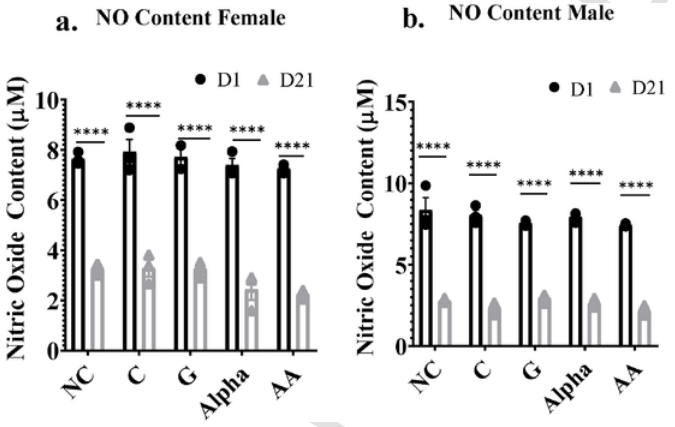

Fig. 2. (a–b) NO content at day 1 (D1) *vs.* day 21 (D21) for female (F) and male (M) groups of cells, respectively. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001.

3.4. Total collagen and GAG content

On day 21, AC engineered tissues were stained for total collagen and GAG representation and adequate ECM formation (Fig. 4). Male AC tissues had an average 2.35-fold higher total collagen relative to the female group for all treatments, and a significant 3.27-fold increase with G (P = 0.0494, Fig. 5a). Though insignificant, Alpha-treatment resulted in the highest collagen content within female and male groups by an average 1.38-fold and 1.13-fold, respectively (Fig. 5a). The male group had higher GAG's content compared to the females in all treatments except for AA, which had significantly higher GAG compared to NC in the female group (P = 0.0442, Fig. 5b). In comparison, Alpha-treatment of male cells had, insignificantly, the highest overall GAG's content (Fig. 5b).

3.5. Gene expression for degradation, osteogenic and hypertrophic markers

A downregulation in MMP13, BMP2 and COL10 expression was observed. MMP13's expression in male cells was significantly less than female cells by an average 1.78-fold (P ≤ 0.001, Fig. 6a). Interestingly, while female cells expressed higher COL2A1 than male cells, they expressed higher MMP13 (Figs. 3a and 6a). G-treated female cells had the lowest MMP13 level amongst nutraceuticals, significantly less compared to Alpha and AA (P = 0.0008, P = 0.0006) and significantly 1.67-fold less than NC (P ≤ 0.0001, Supporting information S.5, Fig. S3a). In comparison, Alpha-treated male cells showed a significant 2-fold and 1.91-fold decrease of MMP13 expression compared to NC and AA respectively (P ≤ 0.0001, P = 0.0001, Supporting information S.5, Fig. S3b).

For all treatments, female cells expressed higher COL10 by an average 1.93-fold and significantly so in C and Alpha-treatments relative to males (P = 0.0048, P = 0.0005, Fig. 6b). Though insignificant, G and Alpha-treated female and male cells, respectively, resulted in the lowest COL10 values (Fig. 6b). Though insignificant, the lowest BMP2's expression was observed when male cells were treated with Alpha by an

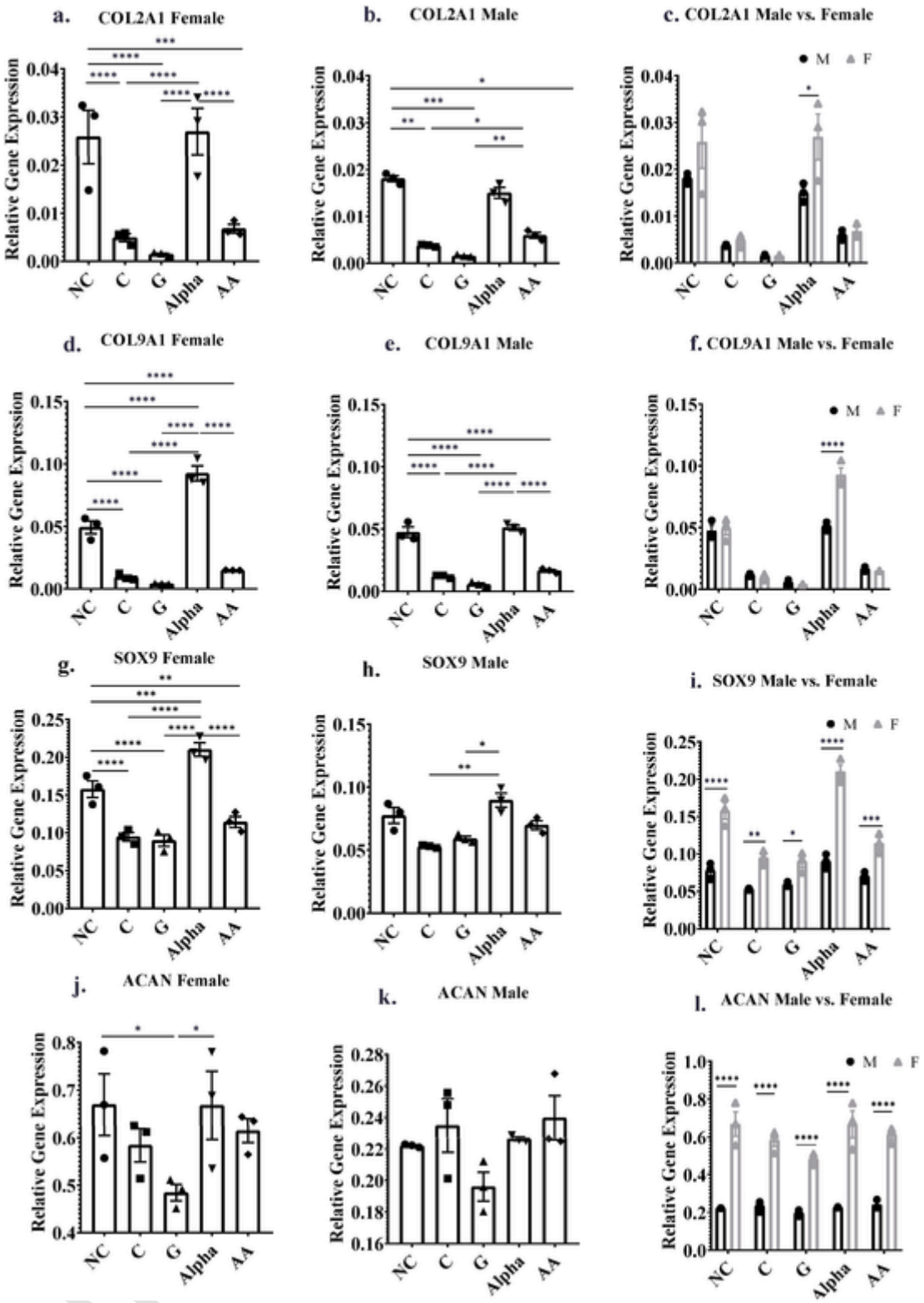

Fig. 3. Relative gene expression of chondrogenic markers as a function of sex and nutraceutical treatments. (a, d, g, and j) represent the female (F) cells, (b, e, h, and k) represent the male (M) cells and (c, f, i, and l) represent M vs. F for the gene expressions of COL2A1, COL9A1, SOX9, and ACAN's respectively. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001.

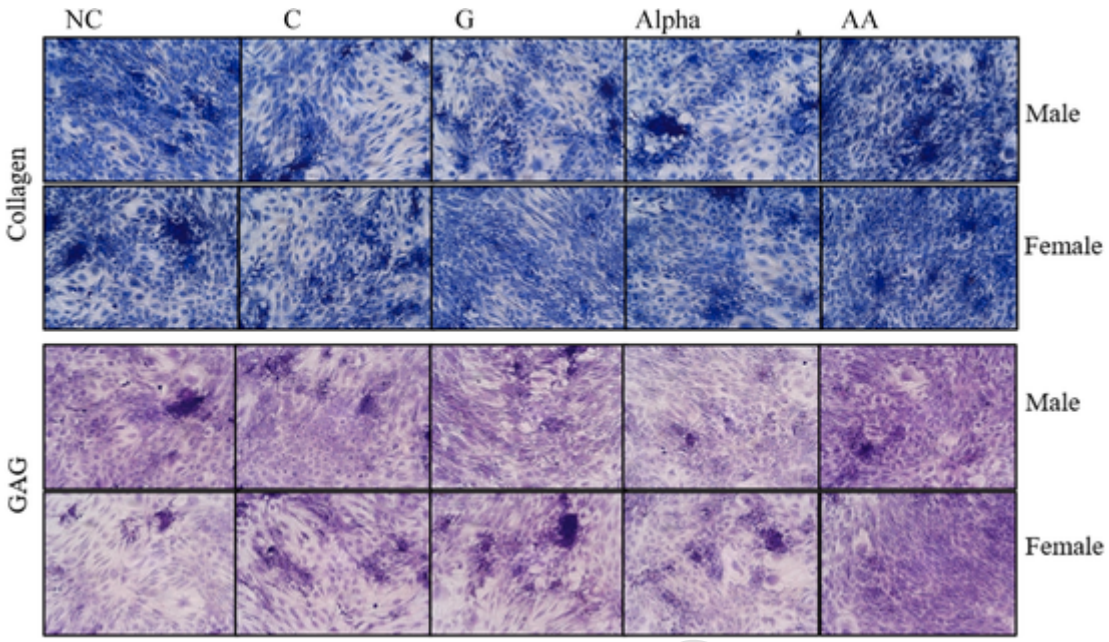

Fig. 4. Representative images of histological staining for total collagen and total GAG content of male vs. female OA human chondrocyte cultures after 21 days of treatment (chosen from n = 3 replicates). Objective lens 10×, scale bar on representative figure in supporting information S.4 (Fig. S2).

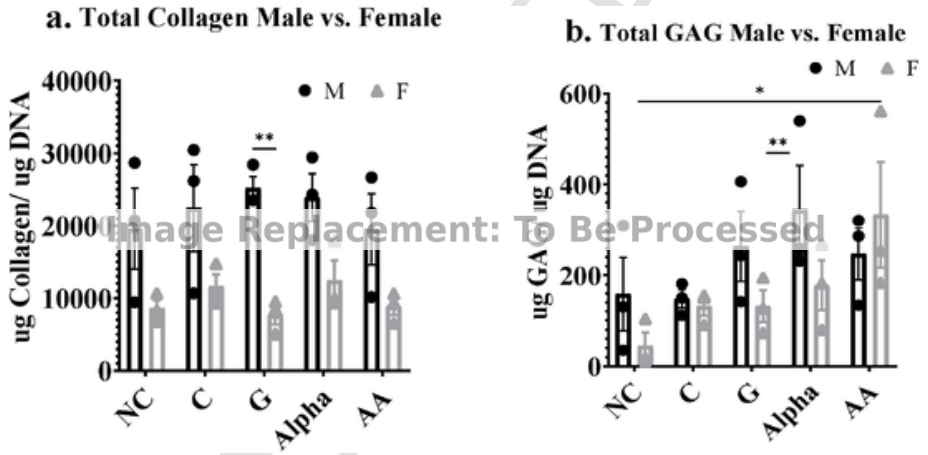

Fig. 5. (a–b) Total collagen and total GAG content normalized by the DNA contents at day 21 for the male (M) and female (F) groups, respectively. *P ≤ 0.05, **P ≤ 0.01..

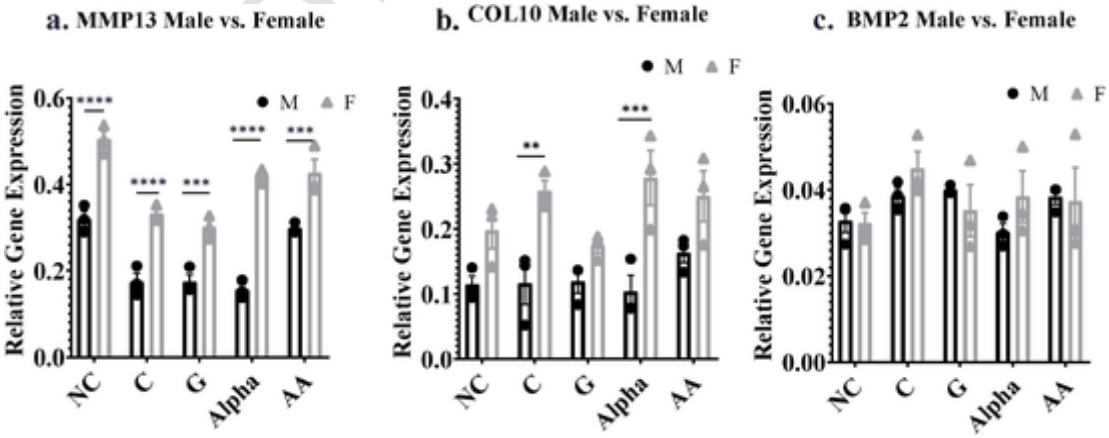

Fig. 6. Relative gene expression of matrix degradation and osteogenic markers: (a) MMP13 male (M) vs. female (F), (b) COL10 M vs. F, and (c) BMP2 M vs. F. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001.

average 1.24-fold and with NC for female cells by an average 1.21-fold (Fig. 6c).

4. Discussion

Currently used long-term OA treatments, including the use of nonsteroidal anti-inflammatory drugs for pain relief, contribute to cardiovascular and gastro-intestinal risks.[30,37] Moreover, there is a void in the market for actual disease modifying treatments rather than the temporary relief of OA-symptoms.[30,37] Motivated by this need, we examined the effects of antioxidant nutraceuticals on the inflammatory environment of OA as well as on the ability of male and female OA HACs to produce ECM. Our main findings indicated that cells of both sexes respond to treatment with a decrease in NO content. Furthermore, male OA chondrocytes had an enhanced response to treatment in reduction of inflammatory markers and increasing certain chondrogenic markers relative to female OA chondrocytes. These findings will be discussed below.

Inflammatory cytokines released due to damaged cartilage induce catabolic genes through mechanisms involving the activation of Nuclear factor-kappaB (NF-κB), an inducible transcription factor that plays a major role in immune and inflammatory responses.[38,39] Inflammatory stresses activate signal transduction pathways in cartilage leading to phenotypic changes, an imbalance of homeostasis, increase in MMPs' and ECM' remodeling, release of chondrocytes and hypertrophy. Following OA, chondrocytes are unable to maintain tissue homeostasis and replace key ECM components such as COL2.[39]

As an indicator of inflammation, nitrites, stable metabolites of NO, are known to cause chondrocytes' damage and lead to degradation of AC by inhibiting collagen and proteoglycan synthesis and upregulating MMPs.[40,41] Irrespective of sex, an average 2.82-fold reduction in NO content from day 1 to day 21 in all treatments was quantified. Although not significant compared to NC, AA-treatment had the least NO content in both sexes. NO is synthesized by a family of NOSs, which also plays a role in inhibiting collagen and proteoglycan synthesis.[40] The decrease in NO content was supported by the downregulation of NOS2's expression in nutraceutical treated cultures compared to NC for both sexes. Furthermore, when investigating nutraceutical effects on downregulation of NOS2, we showed female HACs responded to G by a 2.3-fold reduction while male HACs responded better to Alpha by a 1.9-fold reduction compared to NC. Alpha was shown to prevent induced inflammation and to act as a free radical scavenger.[12,42] Similarly, G's potential in scavenging ROS has been linked to its structure having strong antioxidant ability due to three hydroxyl groups bonded to the aromatic ring in an ortho-position.[14,43]

To further assess the inflammatory state of the HACs, expression of TNFAIP6 which is produced in response to proinflammatory cytokine stimuli and is elevated in OA cartilage was investigated.[44–46] While TNFAIP6 was shown to play a role in regulating inflammatory-response,[47,48] a recent study highlighted that it is not relevant to the context of inflammation in established OA-cases.[49] Indeed, in our study, TNFAIP6's expression was significantly lower in Alpha-treatment compared to nutraceuticals for both sexes by an average 1.66-fold. Overall, male cells expressed lower levels of inflammatory markers at the end of the study suggesting that reduction of inflammation has a greater effect for males compared to females with similar OA grades. With no cytotoxicity of the nutraceuticals and evident suppression of NOS2, our findings point to potential benefits for the particular use of G and Alpha, to mitigate OA's inflammation. Chondrocytes are embedded in an ECM consisting primarily of collagens and proteoglycans, which are critical for maintaining healthy load-bearing AC tissue and to chondrocytes' ability to withstand stresses.[31,50] Interestingly, there was a strong trend in treatment response between COL2A1 and COL9A1 expressions in all treatments for both sexes offering supporting evidence that their production is controlled by similar signaling pathways.[51,52] Additionally, COL9A1 is involved in COL2A1 formation.[53] COL2A1's expression was significantly higher in female cells than in male cells, while total collagen content quantified in the ECM was lower in the female group than in the male group. This lower collagen content may contribute to factors influencing expression of COL2A1 mRNA, i.e. to increase collagen production in ECM. Translation of gene expression to collagen in the matrix requires 14 days.[54] Our findings suggest that male chondrocytes produced collagen at faster rates, presumably leading to developing a mature ECM earlier. SOX9, an important transcription factor, required for ECM formation and cartilage function, also regulates COL2A1 production.[55,56] In this study, Alpha-treated cells had the highest SOX9 expression for both sexes. Remarkably, the highest COL2A1 expressions were also observed for Alpha-treated cells for both sexes. Accordingly, a strong trend between COL2A1 and SOX9 in response to nutraceutical treatment was observed in the female and male groups. At that time, male cultures were characterized by a more mature ECM compared to female cultures, signaling less need to produce collagen and GAG as reflected by lower COL2A1 and ACAN expression. Previously, we showed that lower Young's modulus of AC tissues corresponded to higher mRNA levels of β1-integrins to signal upregulation of these cell-ECM proteins.[33]

SOX9 regulates chondrocytes' differentiation and has been found to aid in the downregulation of hypertrophic marker COL10.[57] High levels of COL10 signals for bone tissue formation, chondrocytes' dedifferentiation, and is found at increased levels in arthritis.[58,59] Our findings showed a downregulation in COL10's expression for all treatments. Moreover, Alpha-treated male cells had the highest SOX9 expression, which corresponded to the lowest COL10 expression, whereas G-treated female cells corresponded to the lowest COL10. Chondrocyte hypertrophy is regulated by the NF-κB pathway through SOX9, BMP2 and hypoxia-inducible factor-2 (HIF-2α). HIF-2α acts as a promoter of MMP13, and COL10 activity.[38] Our results suggest that NF-κB activity should be further explored in other studies to confirm its suppression due to Alpha-treatment which would align with the decreased expression of COL10, BMP2, and higher SOX9 observed here for the male cells.

As a significant component of AC ECM, the highest GAG content varied based on sex. Treatment of female and male cells with AA (vitamin-C) and Alpha (vitamin-E), respectively, resulted in the highest GAG content. Interestingly, a vitamin-E deficient diet was shown to decrease GAG in male rat aortas.[60] Vitamin-C was shown to increase ACAN's expression for HACs in the presence of induced oxidative stress.[61] Our results suggest that the increase in GAG was influenced by AA's or Alpha's ability to diffuse and remain, respectively, in female and male cultures. AA and Alpha are naturally present in cells as small molecule antioxidants. However, AA, a polar acid, resides in the cytosol while Alpha is hydrophobic and is located mainly in the cell membrane.[62,63] Our findings support the premise that GAG's deposition in female cultures is due to the ECM's hydrophilic nature allowing higher diffusion of AA. In comparison, we hypothesize the presence of hydrophobic domains in the male cultures may have enhanced the accessibility of Alpha. Similarly, a hydrophilic synthetic derivative of G, was shown to enhance GAG's deposition due to its diffusibility in the polar membrane phase.[64] To test our hypotheses, contact angle measurements as well as atomic force microscopy maps of hydrophobicity of AC tissues will be performed in future studies.

As a GAG marker, the highest ACAN expression in the female group was observed in NC and Alpha-treatments which remarkably had the lowest TNFAIP6 expression. Interestingly, TNFAIP6, a hyaluronan-binding protein, can also bind to other GAGs including ACAN.[44] TNFAIP6 is associated with ECM remodeling during inflammation and competes with ACAN on binding hyaluronan, thus inhibiting robust ECM formation.[47] Presence of TNFAIP6 alone was correlated with a dose-dependent inhibition of ACAN binding to hyaluronan.[49] As such, the higher ACAN levels observed with Alpha-treatment in this study could be the result of suppressed TNFAIP6 levels.[47,48]

In addition to vital markers in ECM formation, we also explored MMP13's expression, which contributes to COL2A1 degradation and ECM damage.[9] Our findings showed reduced levels of MMP13 in all nutraceuticals compared to NC, suggesting that the use of nutraceuticals may lead to less AC degradation. Compared to male cells, MMP13 levels were higher in female cells, corresponding to decreased collagen content for these cultures. MMP13's higher expression indicates that female cells are susceptible to higher matrix degradation effects than male cells. Overall, females are at greater risk of developing OA, which rises in severity with menopause. Sex-hormones play a role in knee tissue development and maintenance and recent evidence shows estrogen's role in cartilage maintenance and menopause is associated with a decrease in estrogen.[7,65] Our age group, 60-70 years-old women were all postmenopausal. Further, another study revealed that while estrogen receptors are present in both male and female chondrocytes, female chondrocytes exhibit a higher sensitivity and rapid response to 17-β Estradiol as mediated by protein kinase C (PKC).[66] Another study revealed sex-differences in response to the active form of vitamin D, with reduced sunburn inflammation in the female group that was not observed in estrogen receptor-β knockout male mice.[67] Our findings call for treatments that suppress MMP13 expression, especially for females. In our study, G-treated female cells and Alpha-treated male cells had the lowest MMP13 expression by an average 1.36-fold and 1.43-fold, respectively.

As described earlier, G-treatment also resulted in the lowest NOS2 expression in female cells while Alpha-treatment in the male group showed the lowest NOS2 expression. Elevated NOS2 levels contribute to further degradation of AC tissue in OA.[40] Our findings suggest an interplay between MMP13 and NOS2. The reduced MMP13 along with reduced NOS2 expression suggest use of G and Alpha for females and males, respectively, may have a chondroprotective role in OA through suppressed inflammation and matrix degradation markers.

Summarizing the effects of nutraceuticals compared to NC on gene expression (Table 1), our main findings show that Alpha-treatment in the male group resulted in less MMP13, NOS2, COL10, BMP2 and TNFAIP6 expressions while promoting COL9A1, SOX9 and ACAN expressions. Contrarily, G-treatment in female cells resulted in less expression of MMP13, NOS2 and COL10, whereas Alpha-treatment in female cells promoted COL2A1, COL9A1, and SOX9 expression. Intrinsically, it seems that Alpha has more positive effects towards possible enhanced ECM maintenance for both sexes, while G and Alpha seem to be associated with positive effects for mitigating OA's inflammatory environment for females and males, respectively. This suggests the need for a follow-up temporal study of the effects of these nutraceuticals in male and female chondrocytes on downregulation of inflammatory markers and on ECM composition with the addition of a full protein panel to complement the results we observed.

Table 1
Gene expression for female (Left) and male (Right) chondrocytes as a function of nutraceutical treatment at day 21. Values were normalized to NC quantified at day 21 as well. Down-pointing arrows represent normalized values less than 1 compared to NC and up-pointing arrows represent values greater than 1 compared to NC. In the top half of the Table, a decrease in the expression of these markers is desired and as such, down-pointing arrows are preferred. In the bottom half, an increase in the expression is desired and as such, upward pointing arrows are favored. (* represents at least $P \leq 0.05$ compared to NC).

Female	C	G	Alpha	AA	Male	C	G	Alpha	AA
NOS2	↓*	↓*	↓*	↓*	NOS2	↓	↓	↓	↓
TNFAIP6	↑*	↑*	↑	↑*	TNFAIP6	↑	↑*	↓	↑*
MMP13	↓*	↓*	↓*	↓*	MMP13	↓*	↓*	↓*	↓
BMP2	↑	↑	↑	↑	BMP2	↑	↑	↓	↑
COL10	↑	↓	↑	↑	COL10	↑	↑	↓	↑
COL2	↓*	↓*	↑	↓*	COL2	↓*	↓*	↓	↓*
COL9	↓*	↓*	↑*	↓*	COL9	↓*	↓*	↑	↓*
SOX9	↓*	↓*	↑*	↓*	SOX9	↓	↓	↑	↓
ACAN	↓	↓*	↓	↓	ACAN	↑	↓	↑	↑

Interestingly, there was a notable difference in how male and female cells responded to nutraceutical treatments. In general, female cells expressed higher levels of inflammatory, matrix degradation and hypertrophic markers after similar treatments. This suggests females have higher levels of inflammation *in-vivo* than males as is evident from the higher prevalence of knee and wrist OA in the female population.[8] With such a disadvantage from the outset, this may be the reason females are prone to a more severe inflammatory response, inciting further matrix degradation. Furthermore, females generally expressed higher chondrogenic markers to signal for more protein production of necessary ECM components.

To further explore these results, future studies should address some of the limitations of this study. For example, using cocultures of chondrocytes with cells such as synoviocytes that are highly involved in the inflammatory response, would represent OA as a whole joint disease better. Furthermore, the use of pro-inflammatory cytokines to induce further inflammation and as such enhance our abilities to observe the efficacy of nutraceuticals at mitigating the inflammatory response would be recommended.

In summary, our research indicates that nutraceuticals and media play important roles in reducing the inflammatory environment. Furthermore, the variable nutraceuticals investigated assisted in maintaining ECM or in arresting undesired phenotypes in different ways relative to a nutraceutical-free media. Finally, our findings show that chondrocytes' inflammatory and chondrogenic responses to nutraceuticals' treatment are sex-dependent.

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Author contributions

All authors made contributions to the conceptualization of the study, drafting or editing of the manuscript to provide intellectual input and approved the final manuscript. In addition, **Dr. Van Wie**, **Dr. Idone**, **Dr. Gozen**, **Dr. Mendenhall** and **Dr. Abu-Lail** obtained funding to support the study. **Dr. Tingstad** performed all TKA surgeries, provided tissues and de-identified data on patients' osteoarthritis history. **Alia Mallah** performed experiments, analyses and wrote the original draft of the manuscript. **Mahmoud Amr** performed experiments and contributed to review and editing. **Haneen Abusharkh** performed experiments and contributed to review and editing. **Vincent Idone** represented Regeneron who performed the mRNA experiments. **Dr. Abu-Lail** is the advisor of **Alia Mallah**, the first author of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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