The *in vitro* Effects of Nutraceutical-Treatment on Human Osteoarthritic Chondrocytes of Females of Different Age and Weight Groups

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Running head title: Nutraceuticals and osteoarthritis

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Abstract

The *in vitro* effects of four nutraceuticals, catechin hydrate, gallic acid, alpha tocopherol, and ascorbic acid, on the ability of human osteoarthritic chondrocytes of two female groups to form articular cartilage (AC) tissues and to reduce inflammation were investigated. Group 1 represented 13 females in the 50-69 years old range, average weight of 100 kg, and an average body mass index (BMI) of 34.06 kg/m^2 (obese). Group 2 constituted 3 females in the 70-80 years old range, average weight of 75 kg and an average BMI of 31.43 kg/m^2 (obese). The efficacy of nutraceuticals was assessed in monolayer cultures using histological, colorimetric and mRNA gene expression analyses. AC engineered-tissues of group 1 produced less total collagen, and COL2A1 (38-fold), and higher COL10A1 (2.7-fold), MMP13 (50-fold), and NOS2 (15-fold) mRNA levels than those of group 2. In comparison, engineered-tissues of group 1 had a significant decrease in NO levels from day 1 to day 21 (2.6-fold), as well as higher mRNA levels of FOXO1(2-fold) and TNFAIP6 (16-fold) compared to group 2. Catechin hydrate decreased NO levels significantly in Group 1 (1.5-fold), while increasing NO levels significantly in group 2 (3.8-fold). No differences from the negative control were observed in the presence of nutraceuticals for either group. In conclusion, engineered-tissues of the younger but heavier patients responded better to nutraceuticals than those from the older but leaner study participants. Finally, group 2 formed better AC tissues with less inflammation and better extracellular matrix than cells from group 1.

Chondrocytes of Females of Different Age and Weight Groups *in vitro*

1. Introduction

Articular cartilage (AC) is a tissue that surrounds moving joints, providing lubrication and serves as a load-bearing tissue in joints such as the knee. AC has a low cellular density of chondrocytes embedded in a highly organized extracellular matrix (ECM) composed mainly of collagen II and glycosaminoglycans (GAGs)[1]. The degradation of ECM due to injury[2], genetics[3], obesity[4], and aging[5] results in osteoarthritis (OA). OA affects more than 30 million people in the US, causing a huge financial burden of an estimated $137 billion annually[6]. OA worsens quality of life and often progresses to disability. AC has a very limited ability to self-heal due to its avascular, aneural, and alymphatic nature. The lack of pain sensation makes it difficult to detect OA in its early stages. OA has no approved disease-modifying drugs that can result in tissue healing. It is largely managed for symptomatic pain relief using pain killers, anti-inflammatory injections, and intra-articular injections of lubricating polymers such as hyaluronic acid. Eventually and as the disease progresses, total knee replacement (TKR) surgery is needed[7].

Many factors affect OA such as sex, aging, and obesity[4, 5]. In 2018, 49.6% of self-reported OA was in ages above 65 compared to 29.3% in ages 45-64 years old and 30.3% of the population aged 18 and above were females compared to 22.9% males[8]. With aging, changes in the joint can be systemic such as loss of activity and balance, as well as increased cell senescence, the exact pathway of which is unknown[9]. The first National Health and Nutrition Examination survey has shown that obese females were 4 times at higher risk of knee OA than none-obese women[10]. Studies relate the high levels of adipokines to an increase in metalloproteinases 13 (MMP13) levels in obese patients[11], while an increase in mechanical loading on the joints leads to further destruction of degraded joints[12].

The exact underlying molecular mechanisms behind the evolution of OA are not very well understood. However, a great deal of effort has been directed towards the study of inflammation of the joint marked by oxidative stress in OA[13]. In early stages of OA, chondrocytes express degradative enzymes like MMPs[14] that are exacerbated by the production of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α)[15] and the interleukins (IL)[16]. The increase in MMP levels leads to further degradation of the ECM denoted by a

reduction in collagen II and an increase in chondrocyte hypertrophy with higher levels of collagen I and collagen X expressed[17]. As such, controlling inflammation in OA is a step towards the control of ECM degradation.

Patients resort to natural and home remedies to alleviate the pain associated with OA and reduce inflammation[18]. Nutraceuticals are naturally-occurring anti-inflammatory and antioxidant chemicals available in everyday diets and sometimes are taken as supplements due to their general safety[19]. As antioxidants, nutraceuticals possess a reactive oxygen species (ROS) scavenging ability that restores the balance in ROS levels and alleviates the oxidative stress. Nitric oxide (NO) is a major ROS, produced by NO Synthase (NOS) in the event of inflammation, which acts as a mediator in pathophysiological processes in cells with an increase in NOS gene expression indicating more inflammation in OA joints[20]. The exact mechanism of oxygen scavenging is nutraceutical-dependent. Because nutraceuticals are not regulated by the food and drug administration (FDA), many options are available over-the-counter. The responsibility to test the efficacy of these supplements towards OA relies upon the scientific community.

The lack of controlled studies investigating the underlying interconnected mechanisms of aging and excessive weight in OA in response to nutraceutical treatments, motivated this study. We studied the effect of four nutraceutical treatment, catechin hydrate (C), an active ingredient in green tea, gallic acid (G), available in gallnut, alpha tocopherol (Alpha or Vitamin E), available in mixed nuts, and ascorbic acid (AA or Vitamin C), available in citrus fruits on inflammation reduction in chondrocytes obtained from female patients of two groups (group 1: 50-69 years old, an average weight of 100 kg and a average BMI of 34.06 kg/m^2) and (group 2: 70-80 years old, average weight of 75 kg and an average BMI of 31.43 kg/m^2). The effects of nutraceuticals on chondrogenic enhancements were also assessed. Implications for the use of nutraceuticals as complementary dietary components for patients with OA are discussed.

2. Materials and Methods

The following materials were acquired from Life Technologies Corp (Waltham, MA, USA): Alamar Blue, Collagenase Type I, Dulbecco′s Modified Eagle′s Medium (DMEM), Fetal Bovine Serum (FBS), Fungizone, MagMAX™-96 for Microarrays Total RNA Isolation kit, Penicillin/Streptomycin (Pen/Strep), Quant-iT PicoGreen Kit, and SuperScript® VILO™ Master

Mix. The following materials were acquired from Millipore Sigma (St. Louis, MO, USA): alpha-tocopherol, ascorbic acid, Bouin's Fixative, catechin hydrate, Chloroform, Dimethylsulfoxide (DMSO), Ethanol, Ethylenediaminetetraacetic acid disodium salt (EDTA), gallic acid, iso-Propanol, L-Cysteine HCl, Masson's Trichrome Kit, Phosphoric acid, Rat Collagen I, Sodium Acetate, Sodium Nitrite, Steriflip filters, Sulfanilamide, Toluidine Blue, TriZol, and Trypan Blue. Insoluble Collagen Kit from Biocolor LTd (UK). Phosphate Buffered Saline (PBS) from GE Healthcare Life Sciences (Marlborough, MA, USA). TaqMan® Gene Expression Master Mix from Applied Biosystems (Grand Island, NY).

2.1 Isolation of Human Articular Chondrocytes

Adult human articular chondrocytes (hAChs) were obtained from 16 consented (after the approval of the Institutional Review Board at Washington State University) osteoarthritic female, Caucasian patients with stage 4 OA (International Cartilage Repair Society Scale) who underwent TKR surgeries. AC tissues discarded during surgeries were provided by Dr. Edwin Tingstad. The study was performed on two groups. Group 1: 13 females in the age range of 50 – 69 years, average age, weight and BMI are 63 years, 100 kg and 34.06 kg/m^2, respectively. Group 2: 3 females in the age range of 70 – 80 years, average age, weight, and BMI are 76 years, 75 kg, and 31.43 kg/m^2, respectively. The mean weights between the two groups were statistically different (Figure S1, Supporting Information) while the mean BMIs were not statistically different. Since we can't control patients who undergo TKR, we selected our samples from those who did the surgery over a year. When it came to group 2, we were only able to collect samples from 3 female patients with criteria identified above. We can speculate that the limited number of patients in group 2 who underwent TKR during the year was in part due to elderly patients having additional medical issues that prevent them from doing TKR surgeries or possibly due to lack of elderly population in Pullman, WA which is a university small town.

AC tissues were dissected into pieces from seemingly less-inflamed regions. Tissues were washed three times with dissection medium (PBS containing 1% Pen/Strep and 1% Fungizone). AC tissues were digested overnight at 37 °C and 125 rpm using a digestion medium (DMEM/Ham's F-12 containing 0.1% Collagenase Type I, 2% FBS, 2% Pen/Strep, and 1% Fungizone), then filtered through 40 µm Steriflip filters. The enzyme was deactivated by diluting the digested tissue with an expansion medium (DMEM/Ham's F-12 containing 10% FBS, 1%

Pen/Strep, and 1% Fungizone), followed by three washes with DMEM and10-minute centrifugations at 1,500 rpm. Cells were counted using 0.4% Trypan Blue, then suspended in freezing medium (90% expansion medium and 10% DMSO) at a density of 1 million cells/mL in 2 mL freezing vials and frozen in a -84 °C freezer until use.

2.2 Cell Culture

Vials of frozen cells were thawed. Cells were washed three times with the expansion medium after thawing to get rid of residual freezing medium and counted with 0.4% Trypan Blue. Cells were cultured at a seeding density of 16 million cells/mL. Cultures were incubated in a humidified CO_2 incubator at 37 °C for 21 days with the medium changed every other day. To study the effect of the four nutraceuticals (C, G, Alpha, and AA) on OA hAChs, 4 expansion media were prepared with a final concentration of 50 μM of each nutraceutical as well as a negative control medium that had no nutraceuticals. To perform experiments designed, OA hAChs were pooled from different donors to yield a representative mixture of chondrocytes for the two age and weight groups to be investigated. This was done for several reasons. First, pooling of cells is a common practice to reduce variability amongst donors[21-24]. Second, OA hAChs dedifferentiate upon expansion; limiting the ability to increase cell numbers to cell densities desired *via* expansion[25]. Finally, we wanted to investigate responses of primary cells and not expanded cells. After pooling, OA hACh suspensions were seeded at random in wellplates and were investigated in technical triplicates.

2.3 Cell Viability

Alamar blue test was performed to assess the viability of the cultured hAChs at day 21, according to manufacturers' protocol. In short, Alamar blue reagent was added to make a 10% v/v of the medium volume and incubated at 37 °C and 5% CO_2 for 4 hours. The absorbance was measured against a blank of medium and Alamar blue at a wavelength of 570 nm using Cytation 5 Multiplate Reader (BioTek, Winooski, VT, USA). Samples were run in triplicates. The value of the measured absorbance is an indication of cell viability as only living cells can reduce the active ingredient.

2.4 Nitric Oxide Assay

To determine the NO levels in culture medium, Greiss reaction was employed as described previously with some modifications[26]. The reduction of NO^{3-} to NO^{2-} was detected spectrophotometrically by adding Griess reagents (0.1% N-1-napthylethylenediaminedihydrochloride (NED) in nanopure water (resistivity: 18.2 mΩ), and 1% Sulfanilamide in 5% phosphoric acid) to the medium. A standard was prepared using a stock solution of 0.1 M Sodium Nitrite in nanopure water. Sulfanilamide and NED solutions were equilibrated to room temperature for 30 minutes before use. Volumes of 50 µL from media were placed in a 96-well plate in triplicate and 50 µL of Sulfanilamide was added to each well. The mixture was incubated for 10 minutes at room temperature in the dark to which 50 µL of NED solution was added to each well. The new mixture was incubated for 10 more minutes at room temperature in the dark. The absorbance of the final mixture was then measured at 543 nm using a Cytation 5 Multiplate Reader.

2.5 Biochemical Analyses of Total Collagen and DNA

Total collagen produced was measured using an Insoluble Collagen Kit. A modified version of the manufacturer's protocol was used. Briefly, 400 µL/well of the dye was added and the plate was shaken for 30 minutes on an orbital shaker. The dye was then removed and 400 µL of acid/salt wash was added to remove the unbound dye. The mixture was incubated on the shaker for 10 minutes after which 400 µL of dye dissociation reagent was added to the wells to dissolve the bound dye. The dye was then taken to a 96-well plate and absorbance was measured at 550 nm using Cytation 5 Multiplate Reader. DNA's quantification was performed on the same samples after collagen quantification and dye removal and wash steps.

To determine the amount of DNA in the samples, Quant-iT PicoGreen Kit was used according to the manufacturer's protocol. Briefly, cells were digested overnight at 60 °C in 0.1 mg/mL Papain digestion medium (0.2 M Sodium Phosphate buffer containing 8 mg/mL Sodium Acetate, 4 mg/mL EDTA disodium salt, and 0.8 mg/mL L-Cysteine HCl). 12.5 µL of the samples were added to a 96-well plate. To that, 87.5 µL of 1X TE buffer was added. After that, a 100 µL of 1:1000 diluted PicoGreen reagent was added. The well plate was incubated in the Cytation 5 Multiplate Reader with gentle shaking for 3 minutes after which the fluorescence was measured at an excitation of 480 nm and an emission of 520 nm.

2.6 Histology

Qualitatively, total collagen and total GAG staining were done using Aniline Blue (Masson's Trichrome) and Toluidine Blue, respectively. For both tests, at day 21 cells were fixed using Bouin's Fixative for 15 mins at 56 °C. The fixative was washed with deionized water until its yellow color cleared. For total collagen, the staining was done according to the manufacturer's protocol. Briefly, Aniline Blue was added to the wells for 10 minutes. The dye was removed, and 1% acetic acid was added to the wells to differentiate the color. GAG's staining was carried out as previously described[27]. Briefly, the wells were covered by (0.1% Toluidine Blue in 5% acetic acid) for 5 minutes, the dye was washed using deionized water. Histological images of the stained cultures were captured using a Nikon inverted microscope (Nikon Corporation, Tokyo, Japan) at 10X magnification.

2.7 mRNA Isolation and Analysis

To quantify the amount of mRNA expressed in the cells, quantitative real time polymerase chain reaction (qRT-PCR) was used[28]. Briefly, total mRNA was isolated using TriZol™. Chloroform was used to achieve phase separation between aqueous phase and organic phase. The mRNA-containing aqueous phase was purified using MagMAX™-96 for Microarrays Total RNA Isolation kit as per manufacturer's protocol. Total mRNA (up to 2.5 µg) was reverse transcribed to core DNA (cDNA) using SuperScript® VILO™ Master Mix. cDNA was amplified with TaqMan® Gene Expression Master Mix on an ABI 7900HT Sequence Detection System (Applied Biosystems, Grand Island, NY) and probes that are specific for genes human beta actin (housekeeping gene), COL2A1, COL10A1, ACAN, SOX9, FOXO1, MMP13, BMP2, TNFAIP6, NOS2 were used. Relative gene expression was calculated using the $\Delta\Delta C_T$ method previously described[29], and fold differences were expressed as $2^{-\Delta\Delta C_T}$.

2.8 Statistical Analysis

To study the significance in differences between the two groups, Two-way ANOVA was performed with multiple comparison using Tukey's Test. Finally, the Grubbs' test was performed to remove outliers from the data (Grubbs 1950). GraphPad Prism (GraphPad Software, San Diego, CA) was used.

3. Results

3.1 Cell Viability

Chondrocytes derived from both groups were viable at day 21 with no significant differences in viability between treatment groups or between the two groups (Two-Way ANOVA) (Figure S2, Supporting Information).

3.2 Extracellular Matrix (ECM) Formation

Qualitatively, chondrocytes from both groups produced ECM proteins containing collagen and GAGs as indicated by 21-day cultures (representative images in Figure 1A). It has to be noted that the density of stained tissues in these images are not stoichiometrically correlated to collagen and GAG contents in tissues imaged. As such, darker staining of GAG or collagen in images does not necessarily indicate a higher content of the markers imaged in the tissues. To quantify collagen and GAG formation stoichiometrically from all sample content, colorimetric assays were used. Our results indicated that, while G showed the only statistically significant increase in collagen by 3.2-fold (Figure 1B, Two-Way ANOVA), the general trend of increasing values of 1.7, 2.1, 2.3 and 1.0-fold was observed for NC, C, Alpha, and AA treatments.

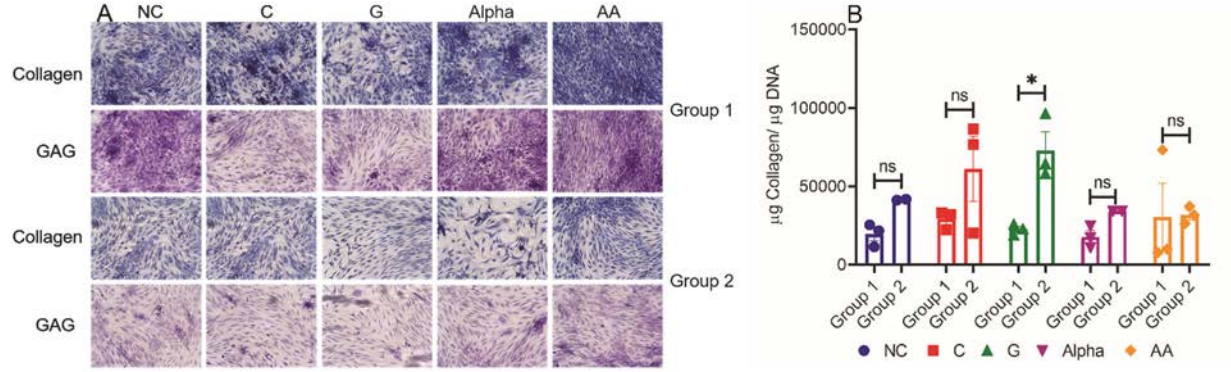

Figure 1

3.3 Nitric Oxide Levels

After culturing for 21 days, NO dropped for all studies, significantly for group 1 by an average 2.6-fold. Though not significant, the very similar collective average drop of 2.3-fold for all but C corroborated the group 1 data. While adding nutraceuticals showed average drops from NC of 1.3-fold and 1.4-fold for groups 1 and 2 (except for C), respectively, only one is significant that

for C in group 1 with a 1.5-fold drop form NC, while increasing NO levels by 3.8-fold for group 2.

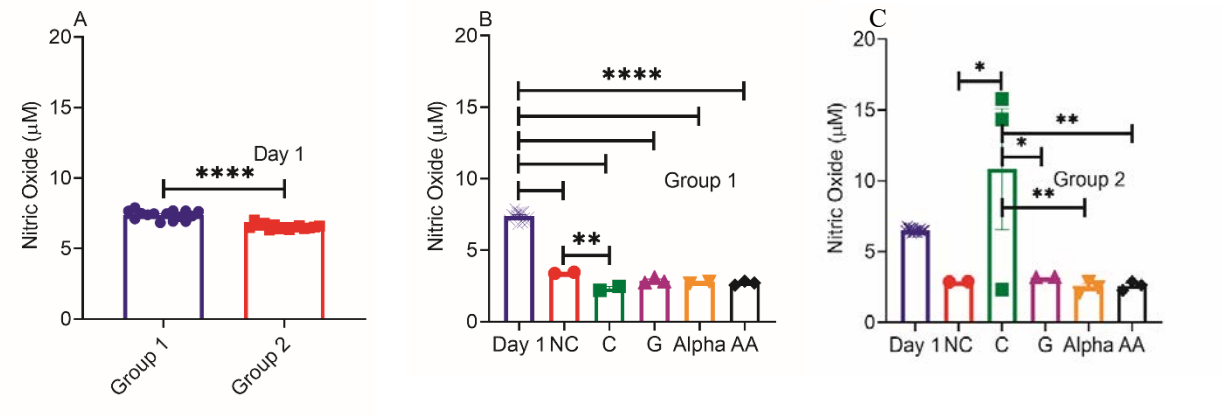

Figure 2

3.4 mRNA Relative Gene Expression of the ECM Proteins (COL2A1, COL10A1, and ACAN)

In characterizing the mRNA genes responsible for translating the main ECM components, a significant impact of participant weight was noted. We studied the chondrogenic genes COL2A1 and ACAN and the hypertrophic gene COL10A1. Our results indicated that group 2 expressed significantly more chondrogenic collagen II mRNA (Figure 3A) (38-fold) which coincides with enhanced collagen production inferred by the data in Figure 1B. Significantly less osteogenic collagen X mRNA (Figure 3B) (2.7-fold) was produced compared to group 1. Both groups (Figure 3C) expressed similar levels of ACAN with no significant differences observed among treatments nor compared to NC.

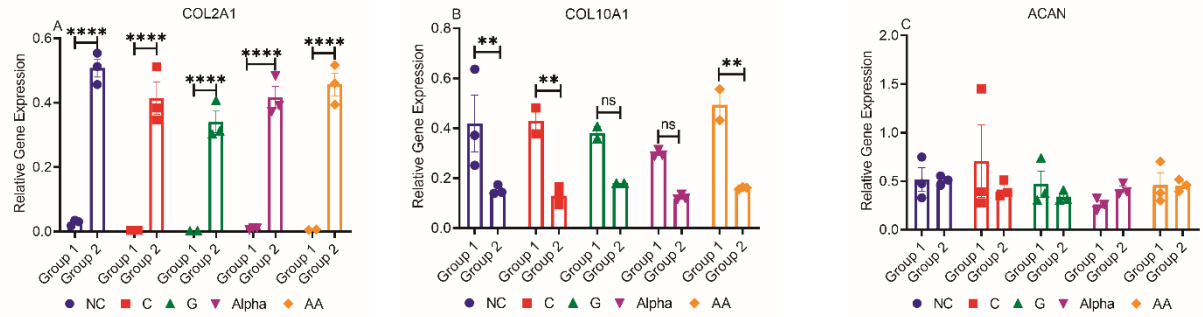

Figure 3

3.5 mRNA Relative Gene Expression of the Regulatory Proteins (BMP-2, FOXO1, and SOX9)

The relative gene expressions of three important regulatory proteins of AC homeostasis and function Bone Morphogenetic Protein-2, Forkhead-Box O1, sex-determining region Y (SRY) box-9 (BMP-2, FOXO1, and SOX9) were assessed. BMP-2 relative gene expression was higher in group 1 with significance from NC detected only in groups treated with C and G (5-fold). No significant differences were detected in group 2 between treatments and NC. Group 1 expressed significantly more FOXO1 than group 2 for all treatments (2-fold), with no significant differences between treatments and NC in both groups. The levels of SOX9 mRNA relative gene expression were not significantly different between the two groups except for NC. Group 1 NC were significantly higher than group 2 NC ($P<0.05$). No significant differences were detected between treatments and NC for both groups.

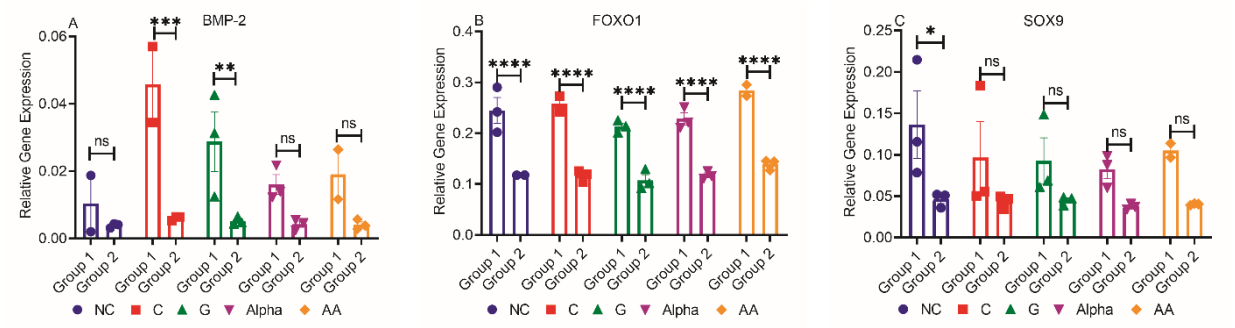

Figure 4

3.6 mRNA Relative Gene Expression of Inflammation Related markers (NOS2, MMP13, and TNFIAP6)

Group 1 expressed significantly more NOS genes than group 2 for all treatments (15-fold), with no significant differences between nutraceutical treatments for either group. These results confirmed our NOS assay results measured with Griess Reaction (Figure 2). Here, our results indicated that MMP13 levels for group 1 were higher than those of group 2 (50-fold), with significance only between NC and C-treated chondrocytes when comparing the two groups. Finally, group 1 expressed significantly more TNFIAP6 than group 2 (16-fold). This was true except for alpha-treated chondrocytes in which differences were insignificant between both groups. No significance was detected between treatments and NC.

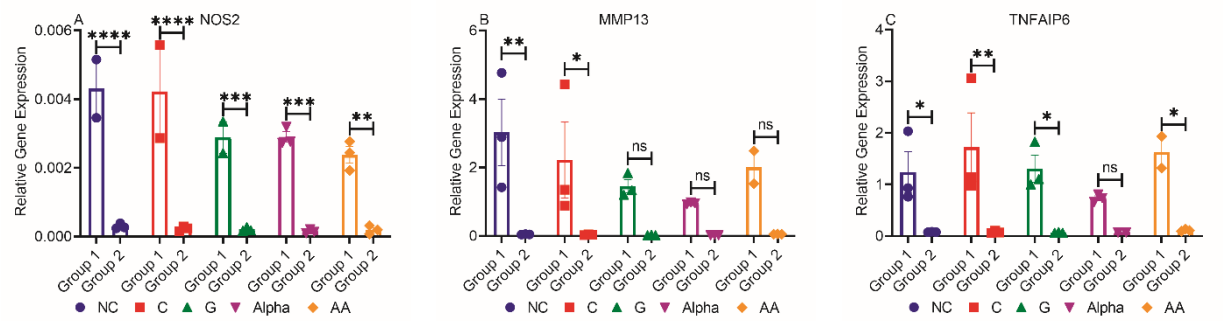

Figure 5

4. Discussion

The correlation between ECM markers, NO, and gene expression in this study provides insight towards understanding age and weight-dependent cellular responses to nutraceutical-treatments of OA chondrocytes. Our main findings are summarized in Figure 6A. Briefly, no significant differences were found in response to nutraceutical treatments when compared to NC in both groups. However, women patients in group 2 who were older yet leaner showed an inherently better phenotype and less inflammation than those of younger and more obese women patients of group 1 (Figure 6A).

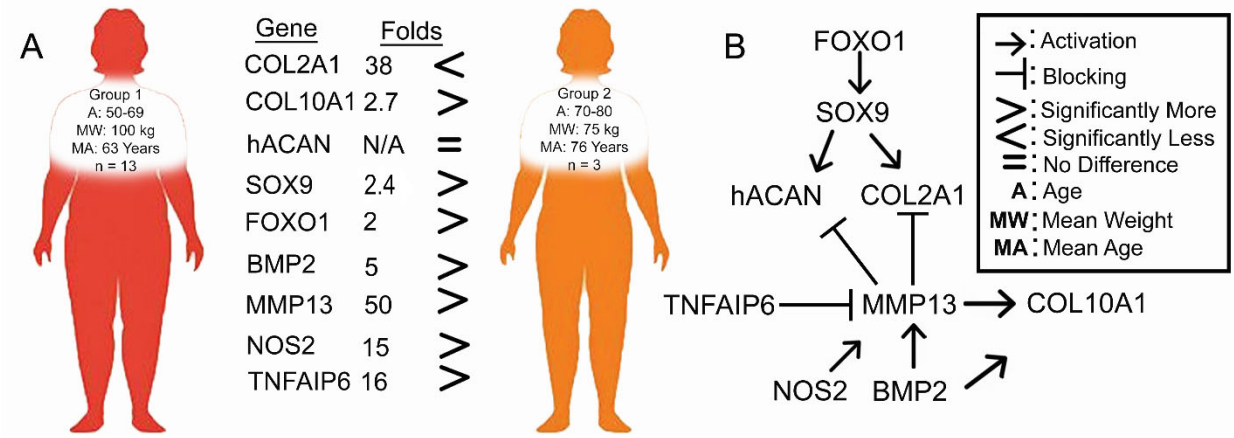

Figure 6

As we age, our body's natural ability to regenerate declines and we become prone to diseases. When it comes to OA, the changes can be at the whole joint or at the tissue level. At the joint level, changes such as weakened muscles or muscle loss, meniscus degeneration, and bone density decrease are observed[30]. At the tissue level, changes such as chondrocyte senescence

indicated by telomere shortening[31] and formation of advanced-glycation products (AGE). The creation of AGE's leads to a decrease in mechanical properties of AC[32]. Additionally, with increasing age, chondrocytes lose their ability to respond to growth factor stimulation such as Insulin-Like Growth Factor 1 (IGF-1) and Transforming Growth Factor Beta 1 (TGF-β1)[33]. Here, group 2 NO levels dropped from day 1 to day 21 but not significantly. However, group 1 and despite their NO levels being higher on day 1 than group 2, experienced a significant drop in NO by day 21. Note that no differences appeared between nutraceutical treatments and NC except for the C treatment. Similar results were observed previously by Bharrhan *et al*, where they found that C reduced the NO levels in alcohol induced liver injury[34]. It was also observed that C regulates the levels of NO production by downregulating the inducible (iNOS) *via* inhibition of nuclear factor kappa B (NF-kB)[35]. Additionally, Kaur *et al* showed *in vitro* that C-loaded nanoparticles reduced the NO levels by competing with oxygen to prevent the formation of nitrites tested by Griess reaction[36]. Interestingly, the opposite effect for C was observed in group 2, that requires further investigation. Obesity is regarded as a state of low-grade systemic inflammation, where high levels of tumor necrosis factor-α (TNF-α), interlukin-6 (IL-6), and leptin have been observed in obese compared to normal adipocytes. This increase in adipocyte inflammation can contribute to worsening of OA symptoms making it a comorbidity or a risk factor[37]. It has also been shown that weight loss leads to a decrease in inflammatory markers such as TNF-a and IL-6, and an increase in anti-inflammatory markers such as adiponectin[37]. Pallu S. *et. al* found that chondrocytes derived from obese patients treated with leptin showed an over expression of MMP13 suggesting a direct correlation between obesity and OA[38]. The worsening of OA in obese patients could also be attributed to excessive joint loading shown to increase levels of matrix degrading enzymes[12].

Among the undesired phenotypes found for group 1 in comparison to group 2 were the lower levels of COL2A1 as well as the higher levels of NOS2 and MMP13 mRNA. Van den berg *et al* reported that iNOS knockout in mice made them resistant to experimental OA[39]. Murrell *et al* found that the inhibition of NO produced by iNOS through TNF-α and IL-1β inhibited MMP activity[40]. In healthy mammalian cells, the MMP enzyme family plays a critical role in matrix remodeling like in wound healing. MMPs help in embryonic development of the cartilage-bone interface and they are involved in tissue turnover in adult cartilage[41]. However, the upregulation of MMPs, especially MMP13 have been linked to OA progression and cartilage

337 degradation[42]. MMP13 degrades the ECM matrix by degrading both collagen II and aggrecan
338 (Figure 5B)[7].

339 We further observed that for group 1, levels of BMP2 were higher than group 2,
340 accompanied by higher levels of the hypertrophic COL10A1 (Figure 5A). We also observed a
341 duality in SOX9 action in chondrocytes as both groups had similar levels of SOX9, yet group 1
342 had higher COL10A1 while group 2 had higher COL2A1 mRNA levels. BMP-2 and SOX9 are
343 two important proteins in controlling chondrocyte differentiation. BMP-2 is a regulatory protein
344 that is essential for chondrocyte functionality. It acts however as a two-edged sword promoting
345 chondrocytes synthesis of matrix ECM but also leading to terminal differentiation when
346 accompanied by other degradative enzymes from the MMP enzyme family such as MMP13[43].
347 The latter explains what we have observed with group1 (Figures 6A and 6B). SOX9 is a DNA-
348 binding protein that play a key role in early developmental stages of chondrocytes[44]. However,
349 the function of SOX9 is time-dependent; continued expression of SOX9 in differentiated
350 chondrocytes is essential for hypertrophy and survival, with latter inactivation in round
351 chondrocytes resulting in COL2A1 inhibition or under regulation, while flat chondrocytes
352 mature without hypertrophy that leads to apoptosis[45].

353 Furthermore, our results indicated that group 1 expressed more FOXO1 and TNFAIP6
354 than group 2 (Figure 6A). These results suggest that cells of group 1 are responding by reducing
355 inflammation and attempting repair of damaged tissue. FOXO transcription factors have gained a
356 lot of interest in recent years when it comes to OA[46]. FOXO family has been shown to be
357 upregulated in the inflammatory environments and have a chondroprotective role through
358 regulating stress-related, cell growth, and survival genes and by modulating autophagy[47].
359 FOXO1 is necessary for SOX9 gene expression as well as for cell cycle arrest in chondrogenic
360 differentiation *via* the TGF-β1 pathway (Figure 6B)[48]. It regulates the gene expression of
361 ACAN and COL2A1 (Figure 6B) [49]. TNFAIP6 is an anti-inflammatory protein that is induced
362 by TNF-α cytokine. TNFAIP6 has a hyaluronan-binding domain that serves a role in ECM
363 stability and cellular migration. Overexpression of TNFAIP6 has been found in patients suffering
364 from knee OA[50]. TNFAIP forms a complex with inter-α-inhibitor (I-α-I) which is a protease
365 inhibitor which in turn leads to inhibition of MMPs[51].

The differences observed in gene expression between NC and day 21 cultures for both groups as a function of nutraceutical treatment are summarized in Table S1 (Supporting Information) may provide insights that can be used in future studies since statistical significance was not detected. The nutraceuticals used did not protect against hypertrophy as the levels of COL2A1 and ACAN have been reduced compared to NC, while the levels of BMP-2 increased for all treatments (Figure 6B). However, all nutraceuticals for both groups reduced NOS and MMP13 levels compared to NC. This reduction suggests that nutraceutical treatment may help alleviate the inflammation and reduce matrix degradation. However, such positive effects are not sufficient to counteract hypertrophy which could have been manifested due to 2D culture dedifferentiation[52]. Cells treated with AA seemed to have a more hypertrophic phenotype compared to cells treated with other nutraceuticals as evident from the reduced levels of collagen II and aggrecan, and increased level of collagen X. This could be due to the dual functionality of AA as an osteogenic and a chondrogenic inducer[53]. All nutraceuticals led to an in increase in the chondroprotective TNFAIP6-gene expression. Chondrocytes of group 2 responded well to C and AA treatment as they have caused an increase in TNFAIP6 and a decrease in NOS2 and subsequently a decrease in MMP13. Akolkar *et al* noted that AA can act as an antioxidant by reducing NO levels via downregulating iNOS and endothelial NOS (eNOS) in doxorubicin induced inflammation in cardiac cells[54]. Similar effect for C has been observed where it downregulated iNOS levels in focal cerebral ischemia[35]. These results suggest a chondroprotective role for AA and C, with C significantly decreasing NO levels in cells of group 1.

In summary, our results showed that chondrocytes of elder and leaner females (group 2) showed inherently better phenotype and less inflammation than younger and more obese females (group 1) after 21-day culture *in vitro* by expressing higher COL2A1, and less MMP13, TNFAIP6, and NOS2, these findings stress the importance of weight management to help combat the progression and worsening of OA. However, chondrocytes of group 1 responded better to nutraceuticals compared to group 2 by expressing genes for anti-inflammatory proteins. C decreased NO levels in group 1 and increased NO levels in group 2 significantly, which suggests an age-dependent effect of C. Trends in nutraceutical treatment show that all nutraceuticals led to a reduction in gene expression of NOS2 and MMP13 and an increase in the chondroprotective protein TNFAIP6's expression in group 1. The nutraceuticals did not protect

against hypertrophy and dedifferentiation. Finally, our findings suggest the importance of 3D culture to ensure a proper chondrogenic phenotype while alleviating inflammation; that may in turn help improve AC regeneration. For future work, a full panel of inflammatory proteins could provide a better understanding of the mechanisms presented in this paper.

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Declaration of Interest

All authors declare no conflict of interest

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Figure Legends

Figure 1. A) Representative histological images of total collagen (Aniline Blue Staining) and GAG (Toluidine Blue Staining) for both groups (Objective: 10X), scale bar is shown in Figure S3 (Supporting Information) (n=3). B) Normalized total collagen per DNA measured at day 21 for chondrocytes of both groups. (Mean ± SEM, n=3), not significant (ns): $p > 0.05$ and * $p < 0.05$.

Figure 2. A) NO levels of both groups at day 1(Mean ± SEM, n=15). Prior to any treatment at day 0, the NO content was averaged for all 15 samples representing the technical replicate for the negative control and for each of the four nutraceuticals (4×3) investigated as they all come from the same pool prior to randomization of cells in wells. B and C) NO levels of both groups measured at day 21 *vs.* day 1, respectively (Mean ± SEM, n=3). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Figure 3. mRNA relative gene expressions of A) COL2A1, B) COL10A1, and C) ACAN for both groups. (Mean ± SEM, n=3). ** $p < 0.01$, **** $p < 0.0001$ and ns $p > 0.05$.

Figure 4. mRNA relative gene expressions of A) BMP-2, B) FOXO1, and C) SOX9 for both groups. (Mean ± SEM, n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and ns $p > 0.05$.

Figure 5. mRNA relative gene expression of A) NOS2, B) MMP13, and C) TNAIP6. (Mean ± SEM, n=3) for both groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and ns $p > 0.05$.

Figure 6. A) Summary of our gene expression differences found between the two groups investigated. B) The complex interplay between key markers of AC homeostasis in the literature.

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