Monounsaturated and Saturated, but Not n-6 Polyunsaturated Fatty Acids Decrease Cartilage Destruction under Inflammatory Conditions: A Preliminary Study

Y.M. Bastiaansen-Jenniskens¹, M. Siawash¹, C.H.A. van de Lest², J.A.N. Verhaar, MD¹, M. Kloppenburg³, A.-M. Zuurmond⁴, V. Stojanovic-Susulic⁵, G.J.V.M. Van Osch¹,⁶, and S. Clockaerts¹,⁷

Abstract

Purpose: Osteoarthritis (OA) is associated with obesity in which altered fatty acid levels have been observed. We investigated whether the most common fatty acids in synovial fluid influence cartilage deterioration in OA. Design: Cartilage was obtained from OA patients undergoing total knee arthroplasty. Chondrocytes or cartilage explants were cultured with linoleic (n-6 polyunsaturated), oleic (monounsaturated), or palmitic (saturated) acid. After preculture, media were renewed and inflammation was simulated in half of the samples by addition of 10 ng/mL tumor necrosis factor-α (TNFα) with or without the fatty acids. Effects on lipid uptake (Oil-Red-O), cell toxicity (lactate dehydrogenase), prostaglandin-E2 (PGE2) release and gene expression for prostaglandin-endoperoxide synthase-2 (PTGS2), matrix metalloproteinase-1 (MMP1), and MMP13, and a disintegrin and metalloproteinase with thrombospondin motifs 4 were determined on chondrocytes in monolayer. Effects on glycosaminoglycan (GAG) release were evaluated on cartilage explants. Results: None of the fatty acids were cytotoxic and all were taken up by the cells, resulting in a higher amount of intracellular lipid in chondrocytes. Linoleic acid increased PGE2 production in the presence of TNFα. Oleic acid and palmitic acid inhibited MMP1 gene expression in chondrocytes stimulated with TNFα. In cartilage explants, GAG release was also inhibited by oleic acid and palmitic acid, and oleic acid decreased PTGS2 gene expression in stimulated chondrocytes. Conclusions: Linoleic acid has a pro-inflammatory effect on cartilage whereas oleic acid and palmitic acid seem to inhibit cartilage destruction. These results indicate that altered fatty acid levels may influence loss of cartilage structure in OA.

Keywords

chondrocyte, cartilage, fatty acids

Introduction

Osteoarthritis (OA) is the most common form of arthritis, in which biomechanical, genetic, and inflammatory factors are involved. Major risk factors for OA are age, gender (female), and obesity.¹,² The association between OA and obesity has mainly been attributed to increased loading forces on the joint. However, OA of the non-weightbearing joints of the hands is also associated with obesity, which indicates that other factors may also explain the role of obesity in OA.¹,² Emerging evidence exists that adipose tissue may contribute to the OA disease process by producing cytokines or adipokines that alter the OA disease process.³ An additional hypothesis, however, is that altered serum and synovial fluid levels of fatty acids (FAs) may influence cartilage metabolism and thereby contribute to the OA disease process.

Fatty acids are covalently bound to cholesterol esters, triglycerides, or phospholipids, but can also be present as

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¹Department of Orthopaedics, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands
²University of Utrecht, Utrecht, The Netherlands
³Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands
⁴TNO, Leiden, the Netherlands
⁵Janssen, The Pharmaceutical Companies of Johnson&Johnson, Fort Washington, PA, USA
⁶Department of Otorhinolaryngology, Erasmus MC University Medical Center Rotterdam, Rotterdam, the Netherlands
⁷Department of Orthopaedic Surgery and Traumatology, University Hospital of Antwerp, Antwerp, Belgium

Corresponding Author:
S. Clockaerts, Department of Orthopaedics, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands.
Email: s.clockaerts@erasmusmc.nl
“free” FAs. In the free form, they are transported in the blood stream bound to albumin. In general, saturated FAs have been associated with cardiovascular disorders, not only through their incorporation into atherosclerotic plaques but also by enhancing inflammation in various tissues by activating protein kinase C and nuclear factor-κB (NFκB) leading to the production of pro-inflammatory cytokines. In general, n-3 polyunsaturated FAs (PUFAs) inhibit inflammation and n-6 PUFAs enhance inflammation. For instance, n-6 PUFA increase cyclooxygenase-2 (COX2) protein levels in cartilage. n-3 PUFAs might compete with n-6 PUFA in eicosanoid metabolism leading to more anti-inflammatory resolvins and prostaglandins than with n-6 PUFA. In addition, COX2 gene expression and interleukin-1 production decreases in response to n-3 PUFA.

The role of monounsaturated FA (MUFA) remains unclear.

Previous publications already indicated that cartilage metabolism can be influenced by FAs. n-3 PUFAs lower glycosaminoglycan (GAG) release under inflammatory conditions whereas n-6 PUFAs increase GAG release under inflammatory conditions. In addition, n-3 PUFA-rich diet reduced the signs of OA in guinea pigs and an inverse relation was found between n-3 PUFA levels in serum and patellofemoral cartilage loss in humans.

However, other FAs than those studied are mainly present in the synovial fluid. The most prominent FAs in synovial fluid are linoleic acid (n-6 PUFA with 18 carbon atoms and 2 double bonds), oleic acid (n-9 monounsaturated FA with 16 carbon atoms), and palmitic acid (saturated FA with 16 carbon atoms). These 3 FAs constitute approximately 80% of the total FAs in the synovial fluid. Fatty acid levels in synovial fluid are correlated with fatty acid levels in serum, but the ratio between synovial fluid and serum levels depends on the type of joint disease involved. Absolute levels also depend on joint disease.

Taken together, FAs such as n-3 PUFAs might influence cartilage, but the most prominent FAs present in synovial fluid have not been investigated for their effect on cartilage. In this study, we aimed to investigate the effect of linoleic acid (n-6 PUFA), oleic acid (n-9 monounsaturated FA), and palmitic acid (saturated FA) on inflammatory and destructive processes in chondrocytes and cartilage explants. To examine this hypothesis, we cultured chondrocytes with tumor necrosis factor-α (TNFα) to mimic an arthritic environment, and co-cultured chondrocytes and cartilage explants with the FA. Then, we analyzed inflammatory (prostaglandin-endoperoxide synthase [PTGS2; gene encoding for COX2], prostaglandin-E2 [PGE2]) and destructive markers (matrix metalloproteinases [MMPs] -1, -3, -13, a disintegrin, and metalloproteinase with thrombospondin motifs-4 [ADAMTS4], GAG release).

Methods

Dissolving Fatty Acids

Fatty acids (Sigma-Aldrich, St. Louis, MO) were prepared by dissolving 300 µg FA/mL in a solution containing 3.5 mg/mL FA-free human serum albumin (Sigma-Aldrich), Dulbecco’s modified eagle medium low glucose, 1% insulin-transferring–selenium A (ITS), 0.1% gentamycine, 0.6% fungizone and incubating for 16 hours at 37 °C under roller agitation followed by filtering (0.2 µm pore size, Sartorius Ltd, Göttingen, Germany) to sterilize the solution and remove the unbound excess of FA. Measurement of actual FA concentration after dissolving and filtration was done by mass spectrometry. For this, the samples were first spiked with a known amount of deuterated palmitic acid, to determine recovery. After acidification of the samples by adding 0.5% of 6 M HCl, the FAs were extracted by 3 washes with hexane, and dried under nitrogen. After this, the samples were analyzed as described previously.

Measurement revealed that the concentration in stock solutions of linoleic, oleic, and palmitic acids were 20.4, 17.1, and 11.1 µg/mL, respectively.

Cartilage Isolation

Cartilage was obtained anonymously as redundant material from total knee replacement surgery procedures at Erasmus MC, University Medical Centre (MEC2004-322). The average age was 72 years (range = 62-88 years), average body mass index was 31.3 kg/m² (range = 24.9-38.0 kg/m²), and 2 out of 3 were female. Cartilage was removed from underlying bone under sterile conditions. For explants culture, cartilage punches were prepared with a 6 mm diameter biopsy punch. For cell culture, cartilage was first digested with 0.1% pronase (Sigma, St. Louis, MO) in saline for 1.5 hours at 37 °C with roller agitation and then further digested overnight with 0.15% collagenase B (Roche Diagnostics, Mannheim, Germany) in Dulbecco’s modified Eagle medium, 4.5 g/L glucose with 10% fetal calf serum, 0.1% gentamycin, 0.6% fungizone (all Invitrogen, Paisley, Scotland).

Experiment Setup and Culture

Passage 3 chondrocytes were seeded as monolayers in 12-well plates (50,000 cells/cm²) with 0.5 mL Dulbecco’s modified Eagle medium low glucose containing 10% fetal calf serum, 0.1% gentamycin, and 0.6% fungizone (all Invitrogen) and cultured overnight. After 16 hours, culture medium was replaced with Dulbecco’s modified Eagle medium low glucose containing 10% fetal calf serum, 0.1% gentamycin, and 0.6% fungizone (all Invitrogen) and cultured overnight. After 16 hours, culture medium was replaced with Dulbecco’s modified Eagle medium low glucose containing 10% fetal calf serum, 0.1% gentamycin, and 0.6% fungizone (all Invitrogen) and cultured overnight. After 24 hours, the culture medium was removed and fresh medium with or without FA and with or without 1 ng/mL...
TNFα was prepared. We used TNFα instead of interleukin-1β because we wanted to have a mild inflammatory reaction, preventing having a large inflammatory effect that cannot be modified by FAs. Culture medium containing 3.5 mg/mL albumin was used as control medium. Previously, it was shown that when FAs are bound to albumin, it takes approximately 8 hours to reach an equilibrium between culture medium and inside of the cell.17 We wanted to have reached this equilibrium first before adding the TNFα.

Then, culture medium was harvested and stored in aliquots at −80 °C and monolayers were suspended in 350 µL RNeasy lysis buffer (Qiagen, Hilden, Germany) with 1% β-mercaptoethanol and stored also at −80 °C. Each condition was cultured in triplicate (3 wells).

To analyze the effects of FAs on GAG release, explants (diameter 6 mm) of 3 donors were cultured for 24 hours. For each donor, 3 samples per condition were cultured. Each sample consisted of 3 explants cultured in 1 mL culture medium containing Dulbecco’s modified Eagle medium low glucose, 1% ITS, 0.1% gentamycin, 0.6% fungizone, and with or without FA. Then, the medium was removed and fresh medium with or without FA and with or without 1 ng/ml TNFα was added and cultured for another 48 hours. Hereafter, culture medium and explants were separately snap frozen in liquid nitrogen and stored at −80 °C.

**Oil-Red-O Staining and Quantification**

To verify the FA uptake by chondrocytes, Oil-Red-O staining was used. After completion of the experiment, culture medium was removed and wells were washed 3 times with saline. Hereafter, cells were fixed for 10 minutes in 500 µL of 10% formalin solution in phosphate-buffered saline. After 10 minutes, the formalin solution was removed and cells were stained for 15 minutes using solution of isopropanol with Oil-Red-O 0.5% diluted 3:2 with distilled water. After staining, culture plates were washed 3 times using saline and 600 µL of 100% isopropanol was added to each well for 15 minutes of incubation at 37 °C. The content of the wells was transferred into 2-mL tubes and centrifuged during 5 minutes at 10,000 rpm. Supernatant was moved to a 96-well plate and extinction was measured with a spectrophotometer at 510 nm.

**Lactate Dehydrogenase Assay**

Viability of the chondrocytes was determined using the spectrophotometric LDH Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s protocol. Medium sample (100 µL) was mixed with 100 µL dye solution and incubated for 15 minutes at room temperature. After 15 minutes, a stop solution was added, and absorbance was read at 490 nm, with reference filter at 655 nm.

**RNA Extraction and RT-PCR**

After culture, chondrocytes were lysed in RLT buffer containing 1% of β-mercaptoethanol followed by RNA isolation using RNeasy Micro Kit (Qiagen, Hilden, Germany). Quantification of the RNA concentration was performed using Nanodrop ND-1000 spectrophotometer (NanoDrop ND1000, Isogen Life Science, De Meern, the Netherlands). From each sample 500 ng total RNA was reverse transcribed into cDNA using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Schwerte, Germany). TaqMan Universal PCR Master Mix (Applied Biosystems, Capelle a/d Ijssel, Netherlands) was used to perform real-time gene expression analysis for GAPDH, MMP-1, MMP-3, MMP-13, and ADAMTS4. For PTGS2, qPCR Mastermix Plus SYBR Green I (Eurogentec, Maastricht, the Netherlands) was used followed by melting curve analysis and using an ABI PRISM 7000 (Applied Biosystems, Foster City, CA) with SDS software, version 1.2.3. GAPDH (when compared with β-actin [ACTB] and hypoxanthine phosphoribosyltransferase 1 [HPRT1]) was found to have good stability for all samples. Relative gene expression was calculated by means of the 2−dCT formula.

Primer sequences for the genes were as follows: glycer-aldehyde-3-phosphate dehydrogenase (GAPDH, reference gene) (forward GTCAACGATTTGCTGTATGGG, reverse GCCATGGTGGAATCATATGGG, and probe Fam-TGGCGCCCCAACCAGGC; MMP1 (forward CTC AATTCTACTTGTATTCTGTGG, reverse CATACTCTGTCCGG CAAATTCGT), probe CACAACCTGCAAATGGGCTT GAAGC); MMP13 (forward AAGGAGCATGCGCACT TCT, reverse TGGCCCAAGGAAAGAC, probe CCC TCTGCCTGCGGCTCA); ADAMTS4 (forward TCGAG GACAAGGAGGCC, reverse TCGAGGTTGACGTGCTG TAGAGA, probe ATGGAACACGATGCCTTTCACCAC GA); PTGS2 (assay-on-demand Hs01573474.g1, Applied Biosystems, Capelle a/d Ijssel, the Netherlands). In pilot experiments (on 2 donors), we found no effects of FA on ADAMTS4, and MMP13 gene expression (data in Suppl. Figs. S1 and S2). Therefore, we only continued with Mmp1 and Ptgs2 gene expression.

**Dimethylmethylene Blue Assay**

The release of GAG from cartilage explants into the culture medium was analyzed with dimethylmethylene blue. The metachromatic reaction with dimethylmethylene blue was monitored with a spectrophotometer at 530 and 590 nm and the ratio between the extinctions at 530 and 590 (A530:A590) was used to determine the GAG amount with chondroitin sulfate C (Shark; Sigma, St. Louis, MO) as standard. Analyses were performed in duplicate and the averages of these results were used for statistical analysis.
**Prostaglandin-E2 Quantification**

PGE2 immunoassay (R&D Systems, Minneapolis, MN) was used to measure PGE2 concentration in chondrocyte culture supernatants. After completion of the assay following the manufacturer’s protocol, plate was read within 30 minutes at 450 nm absorbance (and 540 nm for correction).

**Statistical Analysis**

Experiments with cartilage explants and chondrocytes in monolayer were performed with 3 different donors performed with triplicate samples per donor per condition. Data were analyzed with mixed linear model to account for the biological variability and post hoc test least significant difference, using SAW statistics 17.0 (SPSS, Inc, Chicago, IL). Normal distribution of the residuals was confirmed using a Wilks–Shapiro test. A subject variable was included in the mixed linear model to indicate that 3 × 3 explants or cell culture samples were performed with tissues harvested from 3 donors.

**Results**

After making the solution, the concentration in stock solutions of linoleic acid, oleic acid, and palmitic acid appeared to be 20.4, 17.1, and 11.1 µg/mL, respectively. Therefore, the concentration used in culture (1:3 diluted) was 6.8 µg/mL for linoleic acid, 5.7 µg/mL for oleic acid, and 3.7 µg/mL for palmitic acid. These concentrations are in the physiological range and comparable to what is used previously.

**Intracellular Lipids Quantity and Cytotoxicity**

Uptake of FAs by chondrocytes was examined through quantification of Oil-Red-O staining. In all conditions, intracellular lipid levels increased with the addition of FA to the culture medium and were independent of TNFα stimulation (Fig. 1). Lactate dehydrogenase assay on culture media of all conditions showed that neither TNFα nor the FA increased release of cellular lactate dehydrogenase (Suppl. Fig. S3).

**MMP1 and PTGS2 Gene Expression by Chondrocytes**

Without TNFα, FAs did not change gene expression of MMP1. With TNFα, oleic acid (P = 0.03) and palmitic acid (P = 0.02) decreased MMP1 gene expression whereas linoleic acid did not exert an effect (Fig. 2a). Since COX2 action is often a target for drug therapies in OA process and an important enzyme in inflammatory pathways, we investigated the effects of FA on the gene expression of this enzyme, encoded by PTGS2. In non-inflammatory conditions,
**PTGs2 expression** was not significantly affected by FA. However, in the presence of TNFα, oleic acid decreased PTGs2 expression \((P = 0.01)\) whereas linoleic acid and palmitic acid did not affect gene expression (Fig. 3).

**PGE2 Release by Chondrocytes**

We examined PGE2 release into culture medium of cell cultures. Under non-inflammatory conditions, FA did not affect PGE2 release. With TNFα, linoleic acid increased PGE2 release in all 3 patients \((P = 0.03)\) whereas no effects were observed for oleic acid and palmitic acid (Fig. 4).

**Glycosaminoglycan Release by Cartilage Explants**

We examined the effect of FAs on proteoglycan degradation in cartilage explant cultures. Under non-inflammatory conditions, GAG release was decreased by oleic acid \((P = 0.004)\) and palmitic acid \((P = 0.03)\) whereas linoleic acid did not induce an effect. Under inflammatory conditions, oleic acid \((P = 0.03)\) and palmitic acid \((P = 0.04)\) lowered GAG release compared with control with TNFα alone whereas linoleic acid did not affect GAG release (Fig. 5).

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**Figure 3.** 
PTgs2 gene expression by chondrocytes in response to fatty acids (FAs) and/or tumor necrosis factor-α (TNFα). Data are shown as mean with the 25th and 75th percentile with the minimum and maximum as whiskers. \(N = 3\) donors with triplicate samples per donor. Values are corrected for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Indicates \(P < 0.05\) compared with control without TNFα. #Indicates \(P < 0.05\) compared with control with TNFα.

**Figure 4.** 
Prostaglandin-E2 (PGE2) release by chondrocytes in response to fatty acids (FA) and/or tumor necrosis factor-α (TNFα). Data are shown as mean with the 25th and 75th percentile with the minimum and maximum as whiskers. \(N = 3\) donors with triplicate samples. Values are corrected for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Indicates \(P < 0.05\) compared with control without TNFα. #Indicates \(P < 0.05\) compared with control with TNFα.

**Figure 5.** 
Glycosaminoglycan (GAG) release from cartilage explants shown as micrograms (µg) GAG per explant in response to fatty acids (FAs) with and without tumor necrosis factor-α (TNFα). Data are shown as mean with the 25th and 75th percentile with the minimum and maximum as whiskers. \(N = 3\) donors with triplicate samples per donor. *Indicates \(P < 0.05\) compared with control without TNFα. #Indicates \(P < 0.05\) compared with control with TNFα.
Discussion

The association between OA and obesity has mainly been attributed to increased loading forces on the joint. However, other pathogenetic factors are believed to be involved, since hand OA is also associated with obesity.2

An alternative hypothesis for the link between obesity and OA might be the dysregulated lipid metabolism in obesity, resulting in increased serum levels and altered distribution of triglycerides and cholesterol.20,21 We examined the in vitro effects of linoleic acid (n-6 PUFA), oleic acid (monounsaturated FA), and palmitic acid (saturated FA) on inflammatory and destructive processes in osteoarthritic cartilage.

We found that oleic acid and palmitic acid exert anti-destructive effects on chondrocytes and cartilage in vitro. It should be noted that passage 3 chondrocytes have other characteristics than primary chondrocytes as shown previously22 but by confirming anti-destructive effects of oleic acid and palmitic acid in the cartilage explants, we show that chondrocyte monolayers can be used as a model culture system. MMP-1 gene expression by chondrocytes in the presence of TNFα was lowered in response to oleic acid and palmitic acid. Although MMP13 and ADAMTS4 were not altered by these FAs in pilot experiments, we confirmed the anti-destructive effect of oleic acid and palmitic acid on cartilage explants, where similar effects were observed on GAG release. Earlier, we observed that addition of inflammatory cytokines lowers expression of cartilage matrix components such as aggrecan combined with an increase in release of GAG. This indicates that GAG release is mainly influenced by the breakdown of cartilage rather than the production of new cartilage matrix. The lack of association with aggrecan gene expression also confirmed that GAG release is mainly an indicator of cartilage breakdown.23 GAGs are one of the essential compounds of cartilage matrix and their loss can occur because of elevated circulating inflammatory mediators such as TNFα.24 Anti-destructive effects of n-3 PUFA on cartilage have been reported before,12,17 but to our knowledge, these effects on cartilage have not been observed before for oleic and palmitic acid, FAs that are most abundant in the synovial fluid. Future studies about the levels of oleic acid and palmitic acid in different disease stages and accompanying in vitro experiments are needed to verify the anti-destructive effects of these FAs on cartilage. Care must be taken, however, because oleic and palmitic acid might induce inflammatory responses to other cells resident in the joint such as synovial lining macrophages or fibroblasts, which in turn can induce cartilage degradation.

Oleic acid decreased PTGS2 by chondrocytes in the presence of TNFα. Linoleic acid, on the other hand, induced PGE2 production in the presence of TNFα. These results, combined with the differences between cartilage explants with and without TNFα, and the lack of effect on MMP13 and ADAMTS4, indicate that specific cellular processes are targeted by the different FA. It has been shown that FA influence inflammation in various cell types through numerous mechanisms. For example, n-6 PUFAs such as linoleic acid are precursors of arachidonic acid, which is metabolized by lipoxygenase and COX, resulting in the production of PGE2. In this study, we confirmed that similar mechanisms exist on chondrocytes, since we also observed an increase in PGE2 production in stimulated chondrocytes. Direct inhibition of PTGS2 expression in cartilage has previously been reported for n-3 PUFA.11 This study shows that oleic acid also acts as a potential PTGS2 gene expression inhibitor under pro-inflammatory conditions.

Other potential cellular mechanisms have been described that may explain the results we observed. FAs act on intracellular signaling pathways that are also activated in OA chondrocytes, such as signaling via NFκB,25 signaling via protein kinase C,26 and toll like receptor 4 (TLR4).27 TLR4 and NFκB activation is often seen after stimulation of cells with palmitic acid.28,29 FAs are also known to target nuclear receptors such as peroxisome proliferator-activated receptor (PPAR) α/β/γ, which have been mentioned as potential therapeutic targets in OA.30,31 More specifically, oleic acid downregulated TLR signaling, and upregulated proteins involved in PPAR signaling in a recent study.32 Linoleic acid is hypothesized to signal via phospholipase C, phospholipase A, and PPARs.33 Also, FAs are metabolized by COX and lipoxygenase but result in less inflammatory eicosanoids as tromboxane-3 and tromboxane-5 series, lipoxins, and eicosanoids. This way, there might be a competitive inhibition of inflammatory eicosanoid production.35 Future research could be performed to elucidate these underlying mechanisms.

Fatty acids are not only a source of energy but also form a hydrophobic barrier in the human body, and they play an essential role in transducing signals between cells and within cells.34 Bonucci and Silvestrini35 showed that lipids are present in cytoplasmic dense bodies of chondrocytes in vivo. Other studies demonstrate that FA levels in cartilage specimens are increased in OA and that relative values change: In young healthy cartilage, there are more n-9 PUFA and less n-6 PUFA, whereas in older OA cartilage, this ratio is inversed.36 Because of technical limitations, we were not able to equalize the FA concentrations in the culture media. We performed Oil-Red-O staining to confirm that FAs were indeed transported into the cell in vitro and that FAs were present in the chondrocytes. In addition, we did not find any indication that the differences in concentration between FAs may explain the observed results.

These results give an additional explanation for the link between obesity and OA. These insights provide new therapeutic targets for treatment of OA. Dietary advises could be designed and investigated. Lipid-lowering drugs may also be investigated from this point of view, as changes in
serum lipid profiles might also change synovial fluid lipid composition and consequently intracellular lipid composition in the cartilage. However, the pathogenesis of OA in obese patients is very complex involving adipokines, fatty acids, inflammatory cytokines, growth factors, and lipid mediators. It is not feasible to test all these variables and their combined effects on the OA development in an in vitro model. Although we found that FAs may alter catabolism in chondrocytes, it is possible that these FAs and their interactions may have different effects on the cartilage when applied to the whole joint.

In summary, this study indicates that FAs are capable of altering destructive and inflammatory processes in cartilage. Each type of FA seems to influence these mechanisms differently. Linoleic acid has a pro-inflammatory effect on cartilage whereas oleic and palmitic acids seem to inhibit cartilage destruction and inflammation. These results suggest new therapeutic targets for the treatment of OA.

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Ethical Approval
This study was approved by our institutional review board.

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