Metabolic enrichment of omega-3 polyunsaturated fatty acids does not reduce the onset of idiopathic knee osteoarthritis in mice

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Abstract

Objective—We evaluated the effect of a reduction in the systemic ratio of n-6:n-3 polyunsaturated fatty acids (PUFAs) on changes in inflammation, glucose metabolism, and the idiopathic development of knee osteoarthritis (OA) in mice. We hypothesized that a lower ratio of n-6:n-3 PUFAs would protect against OA markers in cartilage and synovium, but not bone.

Design—Male and female *fat-1* transgenic mice (Fat-1), which convert dietary n-6 to n-3 PUFAs endogenously, and their wild-type (WT) littermates were fed an n-6 PUFA enriched diet for 9-14 months. The effect of gender and genotype on serum PUFAs, IL-6, TNF-α, and glucose tolerance was tested by 2-factor analysis of variance. Cortical and trabecular subchondral bone changes were documented by micro-focal computed tomography, and knee OA was assessed by semi-quantitative histomorphometry grading.

Results—The n-6:n-3 ratio was reduced 12-fold and 7-fold in male and female Fat-1 mice, respectively, compared to WT littermates. IL-6 and TNF-α levels were reduced modestly in Fat-1 mice. However, these systemic changes did not reduce osteophyte development, synovial hyperplasia, or cartilage degeneration. Also the *fat-1* transgene did not alter subchondral cortical or trabecular bone morphology or bone mineral density.

Conclusions—Reducing the systemic n-6:n-3 ratio does not slow idiopathic changes in cartilage, synovium, or bone associated with early-stage knee OA in mice. The anti-inflammatory and anti-catabolic effects of n-3 PUFAs previously reported for cartilage may be more evident at later stages of disease or in post-traumatic and other inflammatory models of OA.

Keywords

INTRODUCTION

Inflammation mediates osteoarthritis (OA) pathogenesis through a mosaic-like pattern of classical immune cell mediated cytokine signaling and activation of molecular inflammatory pathways in native cells of intra-articular joint tissues [1,2]. While these inflammatory responses are most evident in post-traumatic knee OA [3,4], they are also observed in primary knee OA, suggesting that age-dependent changes in inflammatory pathways contribute to an increase in OA risk [5,6].

Recent studies suggest that chronic dietary factors can exacerbate or inhibit joint inflammation and thus may be important mediators of aging-associated knee OA. Obesity is a well established risk factor for knee OA, and several recent studies indicate that altered joint biomechanics alone are insufficient to increase OA risk with obesity [7-10]. While most studies have focused on adipokines as systemic mediators of obesity-associated OA, lipids are also potent regulators of inflammation [11]. In particular, the ratio of omega-6 (n-6) to omega-3 (n-3) polyunsaturated fatty acids (PUFAs) is considered one of the most important dietary mediators of inflammation [12]. Arachidonic acid (AA), a major n-6 PUFA, promotes inflammation by being converted into pro-inflammatory eicosanoids, such as prostaglandins,

thromboxanes, and leukotrines. In contrast, n-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) inhibit inflammation and accelerate the resolution of inflammation. The anti-inflammatory effects of n-3 PUFAs occur through multiple mechanisms, including inhibition of the AA conversion into pro-inflammatory eicosanoids, synthesis of anti-inflammatory agents such as protectins and resolvins, and down-regulation of pro-inflammatory gene expression through n-3 receptor GPR120 [13,14]. Thus, variation in the dietary ratio of n-6:n-3 PUFAs, which is elevated in modern Western diets [15] and attributed to the increase in risk of numerous chronic diseases [16], may also contribute to differences in OA risk.

Previous studies support a role for n-6 and n-3 PUFAs in modifying OA severity. In middle-aged individuals without clinical knee OA, dietary intake of n-6 PUFAs was positively associated with the future prevalence, but not the incidence, of subchondral bone marrow lesions [17,18]. In individuals who have or are at high risk for knee OA, fasting plasma AA was positively associated with synovitis, whereas patella-femoral cartilage loss was negatively associated with DHA [19]. Animal and cell studies also indicate that n-3 PUFAs protect against OA. Feeding an n-3 enriched diet to OA-prone Dunkin-Hartley Guinea pigs reduced markers of OA without altering OA markers in a non-prone strain [20]. In addition, mice expressing the *fat-1* transgene were moderately protected from developing knee OA following transection of the medial meniscus, medial collateral ligament, and anterior crutiate ligament [21]. This transgene induces endogenous conversion of n-6 to n-3 PUFAs by encoding a desaturase enzyme absent in mammals that adds a double bond into the omega-3 position of an unsaturated fatty acid. The result is a systemic reduction in the n-6:n-3 ratio [22]. The protective effects of the *fat-1* transgene was attributed to a reduction in inflammation, decreased protein expression of matrix metalloproteinase-13 and ADAMTS-5, and enhanced autophagy [21]. In bovine cartilage explant and cell culture models, EPA and DHA inhibited the expression of pro-inflammatory and pro-catabolic genes and reduced glycosaminoglycan catabolism induced by exposure to interleukin-1 [23,24]. Yet not all aspects of n-3 PUFAs necessarily protect against OA. EPA and DHA levels are positively associated with bone strength and bone density [25,26], and a low ratio of n-6:n-3 fatty acids protected against ovariectomy-induced bone loss in mice [27]. These pro- anabolic effects of n-3 PUFAs on bone strength and mass may promote OA by stimulating osteophyte development or subchondral bone thickening.

Our goal was to determine how a life-long reduction in the ratio of n-6:n-3 PUFA levels affects the development of idiopathic knee OA in mice. We hypothesized that a low ratio of n-6:n-3 PUFAs protects against OA markers in cartilage and synovium, but not bone. We tested this hypothesis using a transgenic mouse model containing the *fat-1* transgene from *C. elegans* [22]. By feeding mice an n-6 PUFA-enriched diet, Fat-1 mice reduce the n-6:n-3 ratio in the serum and tissues approximately 20-fold [22]. Here we examined changes in serum lipids and inflammatory markers in 9-14 month-old male and female C57BL/6 mice with and without the *fat-1* transgene. We determined the effect of *fat-1* transgene expression on idiopathic knee OA changes in cartilage, bone, and synovium. We also conducted regression analyses to evaluate whether *fat-1* expression altered associations between cartilage and bone or synovium and bone OA outcome measures.

MATERIALS AND METHODS

Animals

We obtained *fat-1* transgenic breeder mice from Dr. J. X. Kang [22]. Animals were bred while housed in the University of Oklahoma Health Sciences Center (OUHSC) vivarium on a 12-hour light/dark cycle under temperature-controlled conditions (20–22°C). Animals were allowed *ad libitum* access to water and a modified AIN-76A purified rodent diet supplemented with 10% Safflower oil by weight (#180465; Dyets, Inc., Bethlehem, PA) as previously described [28]. The diet contains 59% kcal carbohydrate, 18% kcal protein, and 23% kcal fat, with an n-6:n-3 ratio of 274 [28]. Wild-type (C57BL/6) females were mated with male hemizygous *fat-1*$^{+/0}$ transgenic mice maintained on a C57BL/6 background. Male and female littermates carrying either one copy of the *fat-1* gene (hereafter referred to as Fat-1) or no copy of the *fat-1* gene (WT) were studied. Genotyping was conducted as previously described [28]. Our initial cohort of animals (cohort 1) included the following number and ages of animals: female WT mice (N=10; 59.6 ± 1.7 wks of age [58 – 62 wks], mean±SD [range]), female Fat-1 mice (N=10; 59.5 ± 1.6 wks of age [58 – 62 wks]), male WT mice (N=10; 55.4 ± 4.4 wks of age [52 – 61 wks]), and male Fat-1 mice (N=10; 57.7 ± 3.8 wks of age [51 – 61 wks]). Additional male mice were included (cohort 2) to increase the sample size for lipid, cytokine, and histologic analyses: male WT mice (N=8; 43.6 ± 4.3 wks of age [36 – 48 wks]) and male Fat-1 mice (N=7; 43.1 ± 3.9 wks of age [40 – 48 wks]). Although cohort 2 mice were younger than cohort 1, no significant age-dependent differences were observed so data were pooled for both cohorts. Following euthanasia, the hind limbs were dissected and stored in phosphate buffered saline at −80° C. All experimental procedures were conducted in accordance with a protocol approved by the OUHSC Animal Care and Use Committee.

Serum analyses

Blood was collected from anesthetized mice just before the mice were killed. The blood was allowed to clot for 60 minutes at room temperature and then centrifuged for 15 minutes at 3,500 revolutions per minute, and the serum was aliquoted for immediate storage at −80°C until analysis. Serum n-6 and n-3 PUFA analyses were performed as previously described [28]. Briefly, lipid classes of serum lipid extracts were resolved, derivatized to form fatty acid methyl esters, and analyzed using gas-liquid chromatography in an Agilent 6890N gas chromatograph with flame ionization detector (GC-FID) (Agilent Technologies, Wilmington, DE). Authentic standards (NU-CHEK PREP, Elysian, MN) were used to calculate relative mole percentages for the following fatty acids: 20:4n6, 18:3n3, 20:5n3, 22:5n3, and 22:6n3. Serum concentrations of IL-6 and TNFα were investigated as prognostic markers of the effect of fat-1 transgene expression on systemic inflammation. They were measured using quantitative enzyme linked immunosorbent assays (ELISA) designed specifically for mice (eBioscience, San Diego, CA). All samples were analyzed as recommended by the protocols provided by the manufacturer. Glucose tolerance tests were conducted as previously described [29].

Micro-CT analyses

Right knee joints of mice from Cohort 1 were thawed, fixed in 10% neutral buffered formalin, and scanned using a micro-computed tomography system (vivaCT; Scanco Medical, Basserdorf, Switzerland) as described previously [30]. We quantified the following parameters for the tibial epiphysis: subchondral bone thickness (mm), subchondral bone density (mg hydroxyapatite/cm^3), trabecular thickness (mm), trabecular bone spacing (mm), trabecular bone volume (BV, cm^3), total trabecular volume (TV, cm^3), and relative trabecular bone volume (BV/TV). Bone outcome measures were evaluated separately for the medial and lateral compartments.

Histomorphometric analyses

Following fixation, intact knee joints from all animals were processed and stained for histological grading as described previously [30]. OA severity scoring was conducted using a modified Mankin scoring system [31] for the following categories: changes in articular cartilage structure (score of 0-11), Safranin O staining (score of 0-8), tidemark duplication (score of 0-3) and hypertrophic chondrocytes (score of 0-2), for a maximum score of 24 per location. Scores were recorded by 3 experienced graders under blinded conditions at 4 locations within the joint—lateral femur, lateral tibia, medial femur, and medial tibia. We also quantified the severity of osteophyte formation along the anterior and posterior joint margins of the medial tibial plateau using a 0-3 histological grading scheme for each region as previously described based on a visual scale of progressive bone deposition at the peripheral osteochondral junction [32]. In addition, synovial changes were determined by measuring synovial thickness (distance between synovial lining and joint capsule at the midpoint of the anterior meniscus) and quantifying synovial extension into the joint space. We quantified synovial extension as the presence (1) or absence (0) of synovial tissue outgrowth parallel to the surface of the meniscus. Extensions were evaluated along the meniscus adjacent to the femoral and tibial surfaces in both the anterior and posterior regions for a maximum score of 4. Scores were averaged for two sections within the medial and lateral compartments per animal.

Statistical analyses

All statistical analyses were conducted using either JMP 8.02 (SAS Institute, Cary, NC) or Prism 6 (GraphPad Software, La Jolla, CA). The effect of the *fat-1* genotype and sex were determined by 2-factor analysis of variance, with Holm-Sidak's multiple comparison's post-hoc test to determine group differences. When cytokine concentrations were below the lowest levels of quantification (LLOQ), a value of one-half the LLOQ was used for statistical purposes. Statistical analyses were performed on log-transformed serum lipid and cytokine data to correct for non-normal distributions as determined by Shapiro-Wilk normality test. An F test was used to test for equality of variance between WT and Fat-1 male and female mice. Body weight and glucose AUC comparisons between genotypes within a given sex were conducted using two-tailed t-tests, and blood glucose levels during the glucose tolerance testing were analyzed by 2-way ANOVA with time as a repeated measure. For semi-quantitative histological scores, genotype and site-specific differences

were determined either by Mann-Whitney U tests (cartilage) or a Kruskal-Wallis test followed by Dunn's multiple comparisons test (osteophyte and synovial hyperplasia).

We constructed exploratory pair-wise and multivariable regression models to evaluate the effect of *fat-1* transgene expression on the association between cartilage and bone or synovium and bone outcome measures. Pair-wise comparisons were conducted using Spearman's Rho rank correlation analysis. No significant correlations were observed between any bone parameters (i.e., osteophyte score, subchondral bone thickness, or relative trabecular bone volume) and either modified Mankin OA scores or synovial thickness. Therefore, multivariable regression analyses were conducted that adjusted for *fat-1* expression and sex using Generalized Linear Modeling (GLM). Models contained either the modified Mankin OA score or synovial thickness as the outcome parameter. Osteophyte score, subchondral bone thickness, or relative trabecular bone volume were the independent predictors. For both pair-wise and GLM analyses, OA scores were site-matched to the corresponding bone parameter (e.g., medial tibial plateau OA score:osteophyte score). Statistical significance was reported at the 95% confidence level for all tests ($P < 0.05$). All data were reported as the mean $\pm$ 95% confidence intervals (CI).

RESULTS

Serum n-6:n-3 PUFAs and cytokines

Fat-1 transgene expression significantly reduced the n-6:n-3 PUFA ratio in male and female mice. In male mice, the n-6:n-3 ratio decreased more than 12-fold, from 38.8 to 3.2 in WT versus Fat-1 mice, respectively ($p < 0.001$; Fig. 1A). This change in PUFA ratio was due to a 14% reduction in the relative mole percentage of AA (17.6 to 15.2%) and a 9.7-fold increase in n-3 levels (0.50 to 4.87%). In female mice, the n-6:n-3 ratio decreased nearly 7-fold, from 35.1 to 5.1 in WT versus Fat-1 mice, respectively ($p < 0.001$; Fig. 1A). This reduction in n-6:n-3 ratio was attributed to a 14% reduction in AA (22.0 to 19.0%) and a 5.8-fold increase in n-3 PUFAs (0.67 to 3.86%). Thus, while *fat-1* expression dramatically reduced the n-6:n-3 ratio in serum in both sexes, the effect was greater in male mice due to a greater increase in n-3 PUFA concentrations. Along with the reduction in the n-6:n-3 PUFA ratio, *fat-1* expression also reduced serum IL-6 and TNF-α levels by approximately 50% and 33%, respectively, (Fig. 1B,C). For IL-6, *fat-1* expression reduced serum concentrations similarly in male and female mice (52% versus 46%; Fig. 1B). For TNF-α, *fat-1* expression reduced serum concentrations to a lesser extent in male mice (19%) compared to female mice (41%; Fig. 1C). Overall, however, serum cytokine concentrations were only significantly altered by the *fat-1* genotype and not gender.

Body mass and glucose tolerance

n-3 PUFAs have previously been shown to promote insulin signaling in diet-induced obese mice [13]. Therefore, to investigate potential links between n-3 mediated changes in metabolism and knee OA, we tested the effect of *fat-1* expression on body mass and glucose tolerance in aged mice. Male and female mice were compared separately because body weight is lower in female mice (Fig. 2A, D). In male mice, *fat-1* expression did not significantly alter body mass (Fig. 2A) or glucose tolerance (Fig. 2B,C). *Fat-1* expression

did not alter body mass or glucose tolerance in female mice either (Fig. 2D-F). Thus, unlike with obesity [13], increased n-3 PUFAs with *fat-1* expression did not improve glucose tolerance in aged mice.

Effect of fat-1 expression on knee OA in male mice

Cartilage OA changes generally ranged between 25-50% of the maximal OA scores in both WT and Fat-1 mice, although a small number of animals developed near maximal OA (Fig. 3A,B). Typical changes included cartilage loss and fibrillation in the surface zone and focal loss of Safranin-O staining in the central primary load-bearing regions (Fig. 3A). OA scores were similar between the femur and tibia in both the medial and lateral compartments of WT and Fat-1 animals (Fig. 3B). Furthermore, genotype did not alter any of the sub-component OA scores at these sites, including cartilage structural damage, tidemark duplication, Safranin-O staining loss, or hypertrophic chondrocytes. Osteophyte development was assessed in the anterior and posterior margins in the medial peripheral tibial plateau. Osteophyte development was greater in the anterior versus the posterior joint margin in WT animals (Fig. 3C). A similar trend was observed in Fat-1 mice. There was no effect of *fat-1* expression on osteophyte development at either location (Fig. 3C). Finally, we assessed changes in synovial morphology by quantifying anterior thickness and the outgrowth of synovium into the joint space, parallel to the meniscus surface. While there was a trend for greater synovial thickness in the lateral compartment, joint compartment and *fat-1* transgene expression did not alter synovial thickness (Fig. 3D). The occurrence of synovial extensions into the joint space was similar in both medial and lateral compartments and in both WT and Fat-1 mice (Fig. 3E).

Effect of *fat-1* expression on knee OA in female mice

In female animals, cartilage OA changes generally ranged between 15-30% of the maximal OA scores in both WT and Fat-1 mice, with a small number of animals with OA scores around 50% maximal (Fig. 4A,B). Similar to male mice, typical changes included cartilage loss and fibrillation in the surface zone and focal loss of Safranin-O staining in the central primary load-bearing regions (Fig. 4A). Although not statistically significant, tibial OA scores tended to be greater than femoral scores in both the medial and lateral compartments of WT and Fat-1 animals (Fig. 4B). Genotype did not significantly alter the overall OA score or any of the sub-component OA scores at any of the sites with the exception of hypertrophic chondrocytes. In the medial tibia, Fat-1 mice developed fewer hypertrophic chondrocytes than WT mice (1.5 ± 0.1 versus 1.0 ± 0.1; p=0.016). As in male mice, osteophyte development was greater in the anterior versus the posterior medial peripheral joint margin, although in female mice this was true for both WT and Fat-1 mice (Fig. 4C). Similarly, though, there was no effect of *fat-1* expression on osteophyte development at either location (Fig. 4C). In the synovium, the anterior thickness was 87% and 77% thicker in the lateral versus the medial compartment for WT and Fat-1 mice, respectively (Fig. 4D; p<0.001 for both genotypes). The number of synovial extensions into the joint space was also significantly greater in the lateral compartment for both genotypes (p<0.001; Fig. 4E). However, there was no effect of *fat-1* expression on synovial thickness or synovial extensions in any location

Subchondral cortical and trabecular bone

Both the morphology and density of the tibial subchondral cortical and trabecular bone were examined for male and female WT and Fat-1 mice (Fig. 5). Subchondral cortical bone thickness was significantly greater in the medial versus the lateral tibial plateau in both male (Fig. 5A) and female (Fig. 5B) animals (27% and 19%, respectively). The reduced subchondral bone thickness in the lateral compartment was associated with an 8% lower bone mineral density in male but not female mice (p<0.05; Fig. 5A). *Fat-1* transgene expression did not alter any subchondral cortical bone parameters in male or female mice. Medial versus lateral differences were also observed in the trabecular bone parameters, although these differences were primarily restricted to male mice (Fig. 5C,D). For both WT and Fat-1 male mice, the lateral trabecular bone compartment, compared to the medial compartment, had a lower relative bone volume (BV/TV), lower bone mineral density, reduced trabecular bone thickness, and increased trabecular spacing (Fig. 5C). No medial-lateral differences in trabecular bone parameters were detected in female mice, except for an 11% reduction in trabecular thickness in the lateral trabecular compartment of Fat-1 mice (p<0.05; Fig. 5D).

Effect of *fat-1* expression on the association of OA outcome markers in cartilage, synovium, and bone

To further investigate the potential effect of a reduction in the n-6:n-3 ratio on OA-related outcomes, we conducted multivariable analyses to determine if adjusting for the Fat-1 genotype or sex altered the associations between modified Mankin OA scores and each of the following bone parameters: osteophyte score, subchondral bone thickness, and trabecular bone volume (BV/TV). We repeated these models for synovial thickness as the outcome measure. Including sex and genotype in the regression analyses did not result in significant associations between any of the bone-related parameters and the cartilage OA score or synovial thickness. In each model, though, sex had a significant effect on the relationship between bone phenotypes and cartilage OA or synovium outcome measures. Genotype, however, was not a significant parameter for any of the models (data not shown). Thus, a reduction in the n-6:n-3 ratio by expression of the *fat-1* transgene did not alter the association between bone phenotypes and cartilage OA scores or synovium thickness.

DISCUSSION

We hypothesized that a low ratio of n-6:n-3 PUFAs caused by expression of the *fat-1* transgene would protect against idiopathic OA development in cartilage and synovium, but not bone. However, our results do not support this hypothesis. Despite a significant, albeit moderate, reduction in the systemic inflammatory cytokines TNF-α and IL-6 in Fat-1 mice, there were no significant differences in glucose tolerance and the development of spontaneous knee OA. In fact, in nearly every OA related outcome examined, mice expressing the *fat-1* transgene were nearly indistinguishable from WT mice. Moreover, when statistically controlling for differences between male and female mice, there was no effect of the *fat-1* transgene on associations between cartilage and bone or synovium and bone OA-related outcomes. Thus, a life-long reduction in the n-6:n-3 ratio does not alter the development of idiopathic early to moderate stage knee OA in middle-aged mice.

These findings suggest that a systemic shift in the relative levels of n-6 and n-3 PUFAs has a minimal effect on normal joint development and remodeling. This is surprising because n-3 PUFAs are ligands for the nuclear transcription factors peroxisome proliferator activated receptor-gamma and retinoid X receptor [33], which regulate cartilage development and homeostasis [34,35]. In addition, autophagy, which is implicated in aging-associated OA [36], is up-regulated in Fat-1 mice following joint injury [21]. The absence of a protective effect of the *fat-1* transgene on idiopathic OA is also surprising because n-3 PUFAs inhibit the activity of pro-inflammatory IKKβ/NFκB and JNK/AP1 signaling pathways [13]. In chondrocytes, IKKβ/NFκB and JNK/AP1 are critical inflammatory and mechanosensitive signaling pathways involved in regulating catabolic gene expression [5,37,38]. n-3 PUFAs inhibit these pathways directly through ligand-mediated activation of GPR120 and downstream inactivation of transforming growth factor-β activated kinase 1 (TAK1) [13]. Chondrocytes express GPR120 [39], but the physiological relevance of GPR120-mediated inactivation of TAK1 in articular chondrocytes is unknown.

With knee OA, the rate of subchondral bone remodeling increases [40] resulting in altered subchondral cortical and trabecular bone volume, morphology, and mineral content depending on the stage of disease [41]. Previous studies indicate that n-3 PUFAs reduce bone absorption [42] and increase bone mass and strength [26,43], including protection against ovariectomy-induced bone loss in mice supplemented with an n-3 PUFA enriched diet [44] or in *fat-1* transgene mice [27]. Therefore, the absence of any changes in subchondral bone parameters in Fat-1 mice was unexpected. A reduced ratio of n-6:n-3 PUFAs may alter skeletal disease pathology in post-traumatic OA models involving higher rates of bone remodeling.

There are several limitations of the current study. First, we do not know the extent to which n-6 and n-3 PUFAs were altered in the joint tissues of Fat-1 mice. Previous studies suggest less reduction in the n-6:n-3 ratio in cartilage (–57%) [21] compared to muscle (–96%) [22] and bone (–99%) [26] based on a ratio of AA/(EPA+DPA+DHA). The beneficial effects of n-3 PUFAs using dietary supplements may achieve lower n-6:n-3 ratios. Bhattacharya and colleagues [25] observed a beneficial effect of n-3 PUFAs on reducing inflammation and osteoclastogenesis in aging mice by manipulating the n-6:n-3 ratio using corn versus fish oil supplements. The fish oil supplement group obtained a serum n-6:n-3 ratio of 0.41, which is lower than we observed in Fat-1 mice. However, dietary supplements can also alter the macronutrient composition of diets, which may contribute to differences in study outcomes. An advantage of the *fat-1* transgene for mechanistic studies is that Fat-1 and WT littermate mice are fed the same diet and exposed to the same environmental conditions due to co-housing, factors which may contribute to differences in the development of idiopathic OA [45].

An additional limitation is that we did not examine older animals with more advanced OA. n-3 PUFAs inhibit inflammation and accelerate the resolution of inflammation [13,14]; however, the involvement of immune cell mediated cytokine signaling in mild to moderate idiopathic OA is not well-established in C57BL/6 mice. Analysis of older animals with more severe OA or in combination with post-traumatic models of OA [21] may be required to reveal the beneficial effects of n-3 PUFAs with aging. A recent study reported that DHA

supplementation reduces nociception and inflammation in an inflammatory mouse model of knee arthritis [46]. Thus, OA sub-types involving more active synovial inflammation may benefit from increased levels of n-3 PUFAs as suggested in individuals that have or are at high risk for developing knee OA [19].

In summary, a life-long reduction in the ratio of n-6:n-3 PUFAs causes a modest decrease in systemic TNF-α and IL-6 but does not alter the early-stage development of idiopathic knee OA in aging Fat-1 mice; 9-14 month-old male and female Fat-1 mice develop cartilage degeneration and osteophyte formation at levels comparable to WT mice. Furthermore, subchondral cortical and trabecular bone morphology and bone mineral density are similar in Fat-1 and age-matched WT mice. Overall, these findings suggest that high levels of n-6 relative to n-3 PUFAs do not exacerbate idiopathic changes in joint tissue homeostasis that initiate the development of knee OA. The anti-inflammatory and anti-catabolic effects of n-3 PUFAs previously reported for cartilage appear to be more evident in post-traumatic and other more inflammatory models of OA.

Acknowledgments

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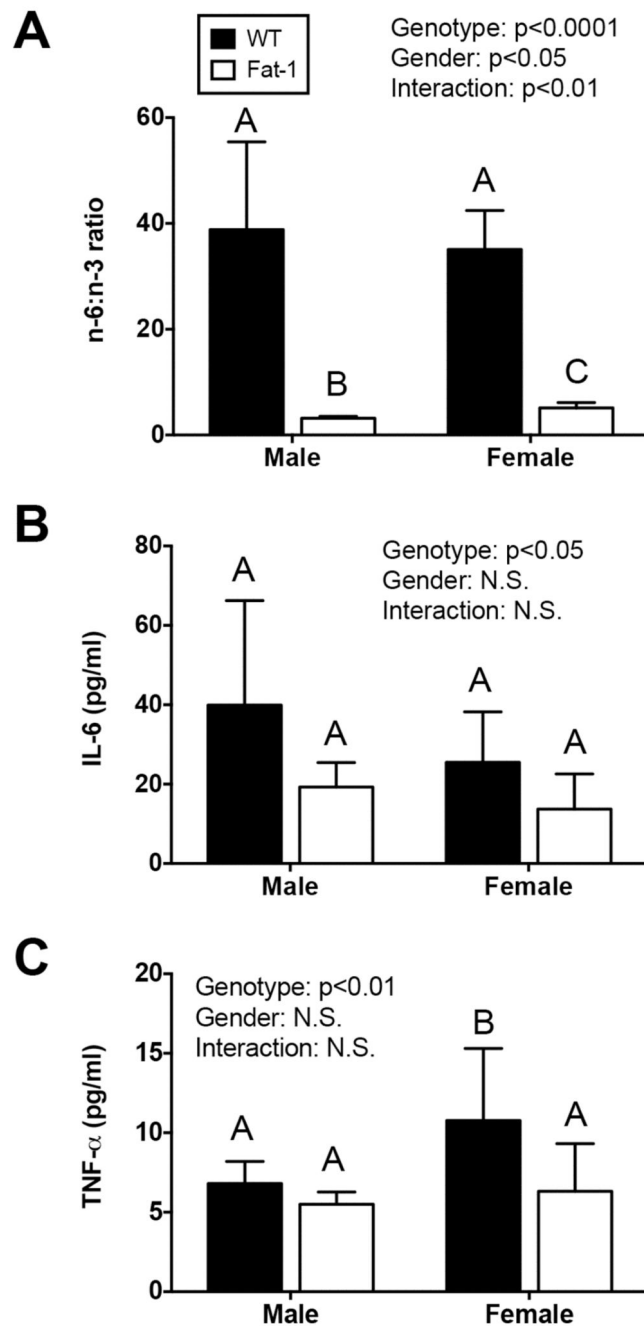
Figure 1. *Fat-1* transgene expression reduces n-6:n-3 serum fatty acids and systemic cytokines
A. *Fat-1* gene expression converts n-6 to n-3 fatty acids, resulting in a dramatically reduced n-6:n-3 lipid ratio in male and female mice. B. Serum IL-6 concentrations are marginally reduced in Fat-1 mice (p=0.049) independent of animal gender. C. Serum TNF-α is also reduced in Fat-1 mice (p=0.004) with the greatest differences in female mice. Samples sizes in (A) are N=5, 14, 10, and 8 for WT male, Fat-1 male, WT female, and Fat-1 female, respectively. Sample sizes in (B) and (C) are N=16, 16, 10, and 10, respectively. P-values are from a 2-Factor ANOVA; bars not sharing the same letter are statistically different from

each other as determined by post-hoc analysis (Holm-Sidak's multiple comparison test). Values are mean ± 95% CI.
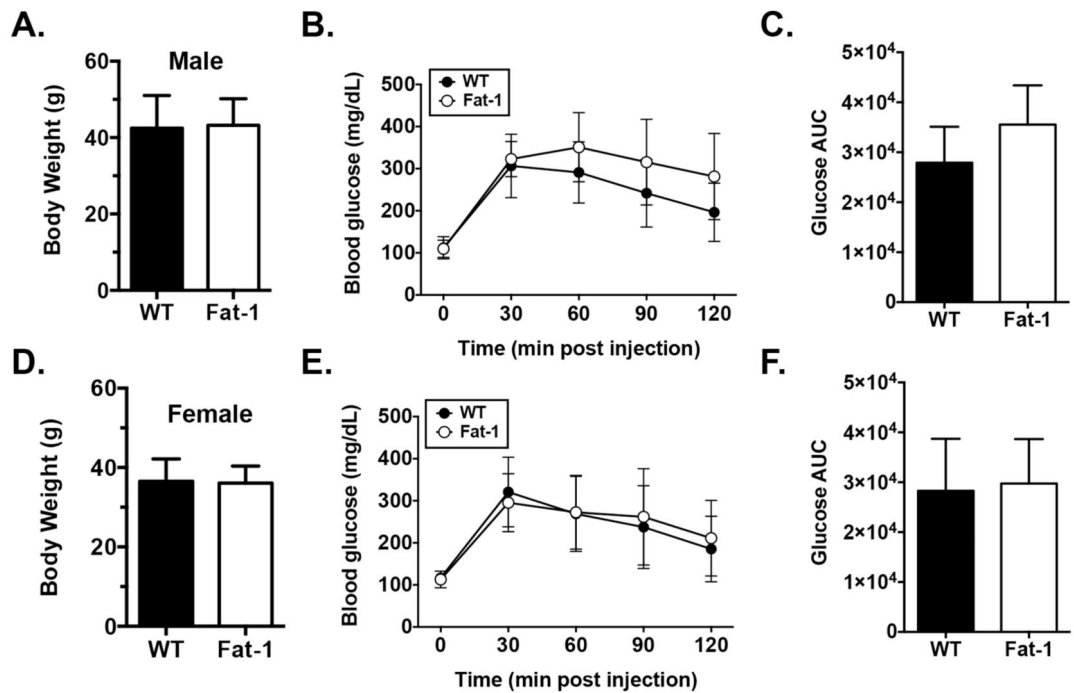
Figure 2. *Fat-1* transgene expression does not alter body weight or glucose tolerance in aged male (A-C) or female (D-F) mice

Body weight (A, D) and serum blood glucose clearance following a fasted glucose tolerance test (B, E) were not significantly different between either male or female WT and Fat-1 mice (p>0.05). Both male and female WT and Fat-1 mice produced similar glucose areas under the curve (AUC) values up to 120 min post glucose injection (C, F; mg/dL/min). N=10 per genotype and sex. Values are mean ± 95% CI.
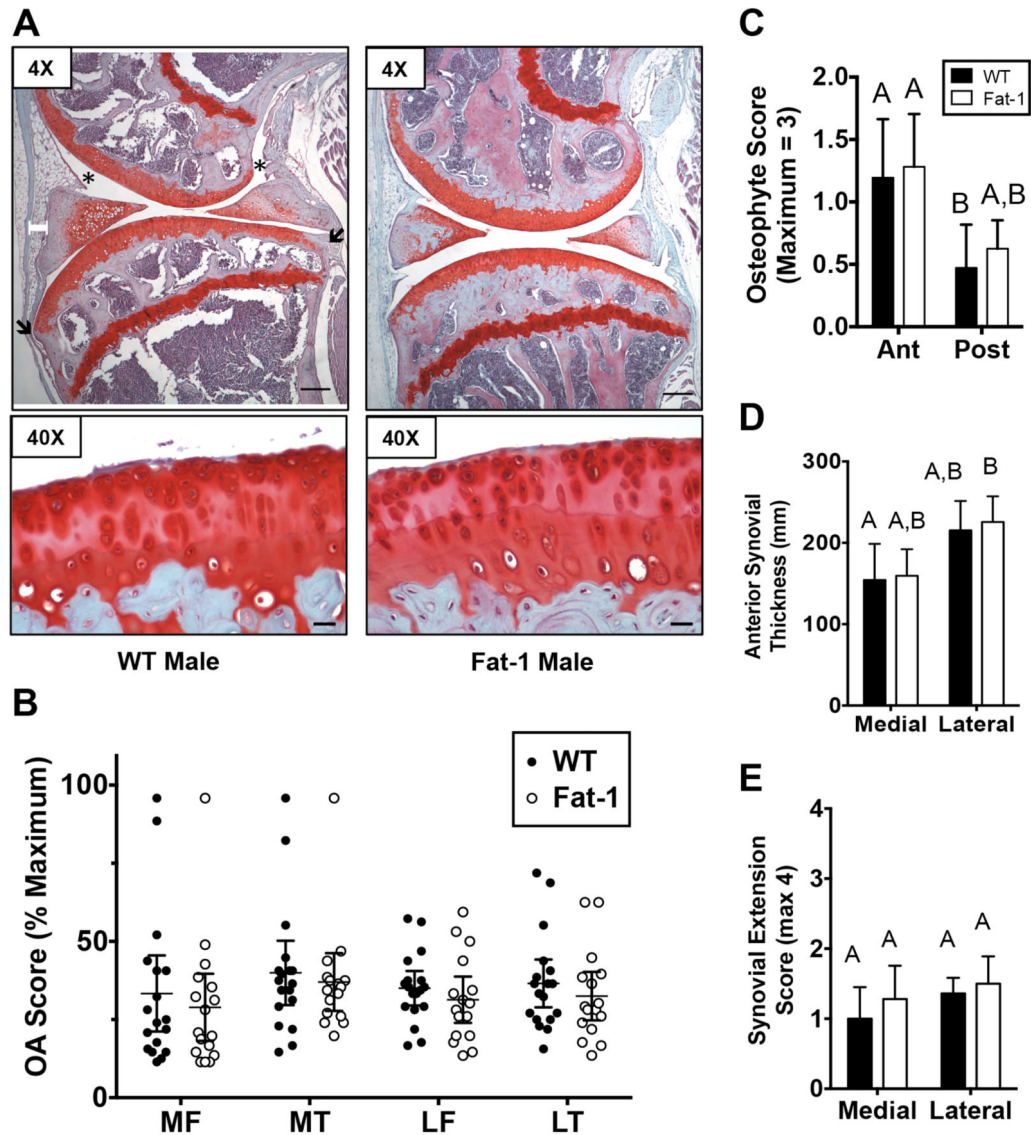
Figure 3. *Fat-1* **transgene expression does not alter knee joint OA outcomes in aged male mice**
A. Representative sagittal histology sections of the medial knee joint compartment in WT
and Fat-1 male mice. 4× images are from more peripheral medial sections used for
osteophyte grading and 40× images are from more central load-bearing regions. Capped
white bar superficial to the anterior horn of the meniscus indicates site of anterior synovial
thickness measurement. Asterisks indicate examples of synovial extensions into the joint
space. Black arrows indicate sites of osteophyte evaluation. Black bar = 200µm in 4× image
and 20 µm in 40× image. B. Modified Mankin OA score of the medial (M) and lateral (L)
tibia (T) and femur (F). Data points are mean values of multiple graded sections for
individual animals. Expression of the fat-1 transgene did not alter OA scores at any site. C.
Anterior (Ant) and posterior (Post) medial tibia osteophyte scores for WT and Fat-1 mice.
D. Medial and lateral anterior synovial thickness in WT and Fat-1 mice. E. Medial and
lateral synovial extension scores for WT and Fat-1 mice. N=18, WT; N=16, Fat-1 for all

scores. Bars that do not share the same letter are statistically different from each other ($p<0.05$) as determined by Dunn's multiple comparisons test. Values are mean ± 95% CI.
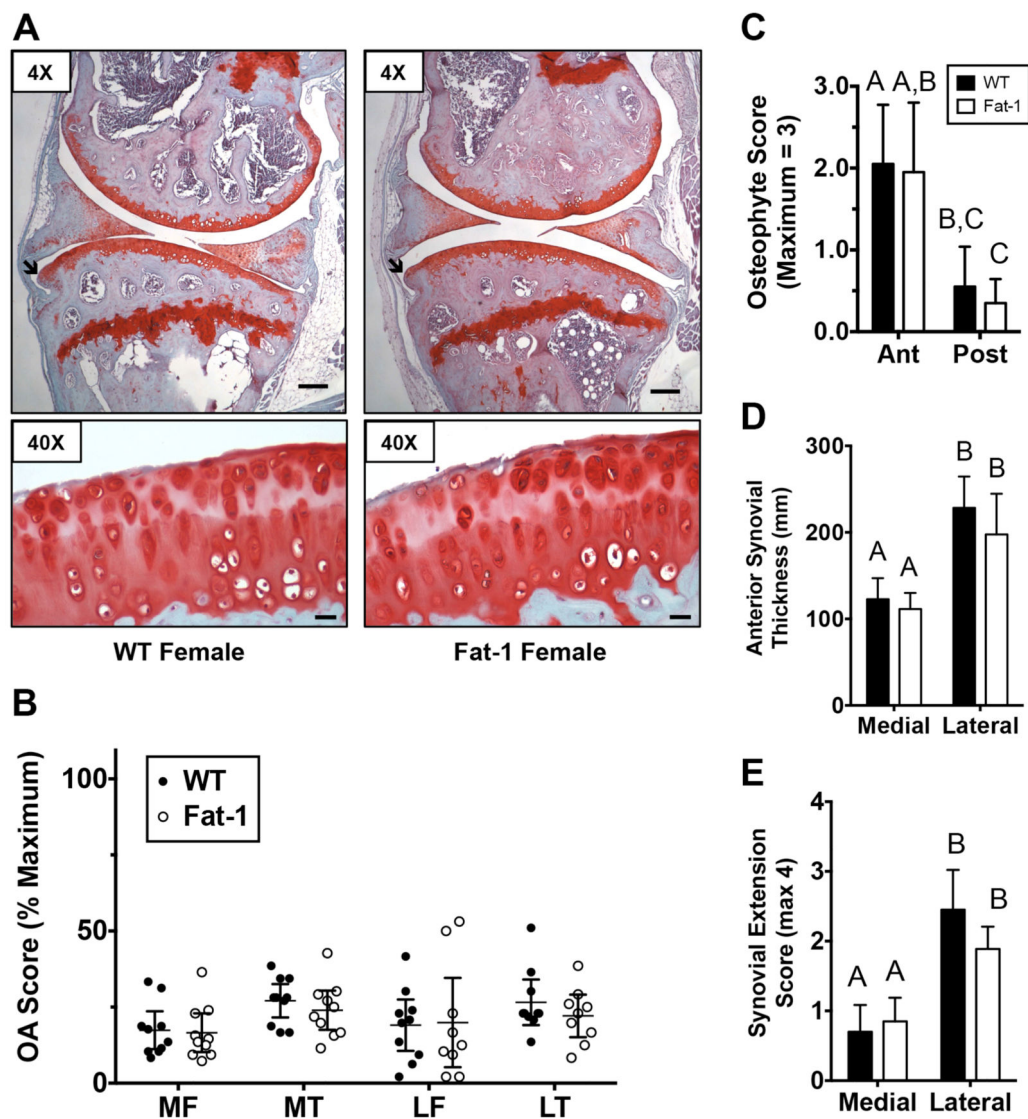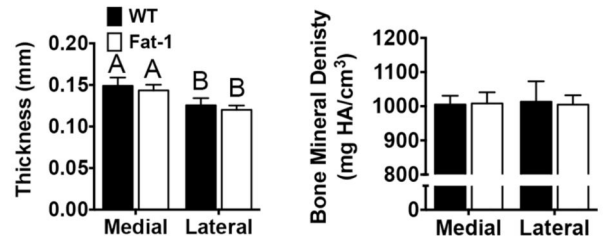
Figure 4. *Fat-1* transgene expression does not substantially alter knee joint OA outcomes in aged female mice

A. Representative sagittal histology sections of the medial knee joint compartment in WT and Fat-1 female mice. 4× images are from more peripheral medial sections used for osteophyte grading and 40× images are from more central load-bearing regions. Black arrows indicate anterior osteophytes. Black bar = 200μm in 4× image and 20 μm in 40X image. B. Modified Mankin OA score of the medial (M) and lateral (L) tibia (T) and femur (F). Data points are mean values of multiple graded sections for individual animals. Expression of the fat-1 transgene did not alter OA scores at any site. C. Anterior (Ant) and posterior (Post) medial tibia osteophyte scores for WT and Fat-1 mice. D. Medial and lateral anterior synovial thickness in WT and Fat-1 mice. E. Medial and lateral synovial extension scores for WT and Fat-1 mice. N=10, WT; N=10, Fat-1. Bars that do not share the same letter are statistically different from each other (p<0.05) as determined by Dunn's multiple comparisons test. Values are mean ± 95% CI.
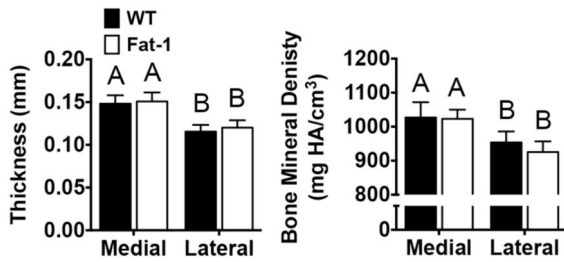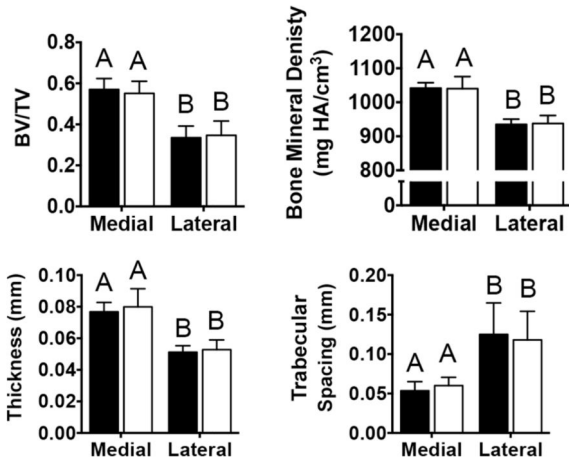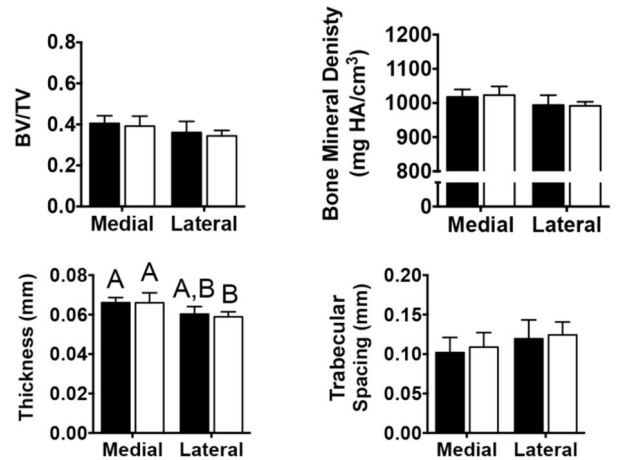
Figure 5. *Fat-1* **transgene expression does not alter proximal tibial subchondral or trabecular bone morphology or density in male and female mice**

Micro-CT evaluation of the proximal tibial plateau and epiphysis was used to examine effects of *fat-1* transgene expression on the bony changes related to knee OA. Medial and lateral subchondral bone thickness and bone mineral density were compared by genotype in male (A) and female (B) mice. Trabecular bone volume, density, thickness, and spacing were also compared in medial and lateral compartments in WT and Fat-1 male (C) and female (D) mice. Genotype did not alter any of the bone morphology or density outcome measures. Differences between the medial and lateral compartments were observed for several subchondral and trabecular bone measures in both male and female mice, as indicated by bars that do not share the same letter (p<0.05). Graphs without letters contain no significant group differences. Sample size: Males (N=10, WT; N=9, Fat-1); Females: (N=10 for WT and Fat-1). Values are mean ± 95% CI.