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# Effect of fish oil on bone mineral density in aging C57BL/6 female mice☆,☆☆

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**Abstract**

Life expectancy has increased considerably over the last century in the United States. It is expected that this longevity will be accompanied by an increase in the prevalence of osteoporosis and accompanying complications in the elderly population. Age-related loss of bone mass and bone fragility are major risk factors for osteoporosis, leading to an increased risk of fractures. Therefore, nutritional strategies and lifestyle changes that prevent age-related osteoporosis and improve the quality of life for the elderly population are urgently needed. Hence, the present study compared the effects of corn oil (CO; $n-6$ fatty acids; commonly present in Western diets) and fish oil (FO; $n-3$ fatty acids) on bone mineral density (BMD) in aging C57BL/6 female mice. After 6 months of dietary treatment, we found that 18-month-old FO-fed mice maintained higher BMD in different bone regions compared to CO-fed mice. These findings were accompanied by a decreased activity of pro-inflammatory cytokines, tumor necrosis factor-α and interleukin-6 in stimulated splenocytes; a nonsignificant but greater increase in bone formation markers alkaline phosphatase and osteocalcin in the serum; and lower osteoclast generation in bone marrow cell cultures in FO-fed mice. In conclusion, these findings suggest that providing $n-3$ fatty acids may have a beneficial effect on bone mass during aging by modulating bone formation and bone resorption factors.


*Keywords:* Fish oil; Cytokines; Osteoclast; Aging; Bone; $n-3$ fatty acids

## 1. Introduction

Bone-related disorders are one of the major causes of mortality and morbidity in the United States. As life expectancy increases in our fast-growing elderly population, the need to improve their quality of life requires attention. As opposed to rapid bone loss associated with menopause in women, termed Type 1 osteoporosis, there is a slow but gradual loss of bone in both men and women, termed Type 2 osteoporosis [1]. After attaining peak bone mass between the ages of 20 and 30 years, both men and women start losing bone at a rate of 0.5–1% per year. Osteoporosis-related costs are a major economic concern, with the cost of osteoporosis-related treatments in the United States expected to increase to US$131 billion by year 2050, putting a tremendous financial burden on society [2]. The type of dietary fat intake and lifestyle choices (sedentary vs. exercise) are important determinants of age-related osteoporosis. The fats present in Western diets consist predominantly of saturated fatty acids and $n-6$ polyunsaturated fatty acids (PUFAs) derived from sources such as corn, safflower, sunflower and soybean oils [3]. The predominant $n-6$ fatty acid in these oils is linoleic acid (LA; 18:2$n-6$), which is found to act as a pro-inflammatory fatty acid. In contrast, there is very low intake of $n-3$ fatty acids such as α-linolenic acid (ALA; 18:3$n-3$) from sources such as flaxseed and canola oils, or eicosapentaenoic acid (EPA; 20:5$n-3$) and docosahexaenoic acid (DHA; 22:6$n-3$) from sources such as marine fishes or fish oils (FOs), thereby increasing the $n-6/n-3$ fatty acid ratio in the diet to 20–25:1 [3]. Increasingly available literature mainly from animal studies suggests that an increase in the intake of $n-6$ fatty acids with a decrease in the intake of $n-3$ fatty acids and, consequently, high levels of $n-6/n-3$ ratio may be positively associated with risks for cardiovascular diseases, cancer, rheumatoid arthritis and bone loss [4–6].

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Some recent studies in humans also correlate an increase in $n-6/n-3$ fatty acid ratio with lower hip bone mineral density (BMD) and increased risk for periodontal disease and cancer [7–9].

There is considerable evidence to indicate that FO has beneficial effects against inflammatory diseases such as systemic lupus erythematosus, rheumatoid arthritis, cardiovascular diseases and osteoporosis [5,6,10,11]. The beneficial effects of FO or $n-3$ fatty acids have been ascribed in their ability to alter membrane phospholipid fatty acid composition, which in turn decreases the production of pro-inflammatory prostaglandins (PGs) and thromboxanes (TXs) ($PGE_2$ and $TXA_2$) derived from the $n-6$ fatty acid arachidonic acid (AA; 20:4$n-6$; downstream product of LA) through the cyclooxygenase (COX) pathway. EPA competes with AA for the synthesis of $PGE_3$ through the COX pathway [12], thereby suppressing the production of inflammatory mediators derived from $n-6$ fatty acids. Although $PGE_3$ has inflammatory properties, it is synthesized with either low efficiency or no efficiency [12]. It is well established that $PGE_2$ in high concentrations is a potent inducer of bone resorption [13]. FO and low $n-6/n-3$ fatty acids decrease $PGE_2$ levels in bone organ cultures [14–16]. Apart from pro-inflammatory eicosanoids, pro-inflammatory cytokines such as tumor necrosis factor (TNF) α, interleukin (IL) 1β and IL-6 also induce bone resorption through osteoclast activation and osteoclastogenesis [17–19]. $n-3$ fatty acids exhibit anti-inflammatory properties through the inhibition of these cytokines and the up-regulation of anti-inflammatory cytokines such as IL-10 [5,6,20].

Studies have shown that FO prevents bone loss in ovariectomized mice and rats models of postmenopausal bone loss, compared to diets enriched in $n-6$ fatty acids, such as corn oil (CO) [11,21,22]. FO and low $n-6/n-3$ fatty acids ratios have also been shown to promote bone formation in growing animals [14,15,23–26]. However, very few studies have focused on the effects of FO on bone metabolism during aging in middle-aged or older animals [27–29]. Thus, the present study was designed to determine the effect of FO on BMD in aging C57BL/6 female mice. We measured TNF-α and IL-6, and alkaline phosphatase (ALP) and osteocalcin (OC) in FO-fed mice to determine if the beneficial effects are associated with the modulation of pro-inflammatory cytokines and bone formation biomarkers. Since TNF-α and IL-6 are potent stimulators of osteoclast activity, we also performed tartarate-resistant alkaline phosphatase (TRAP) staining in bone marrow cells derived from CO-fed and FO-fed mice as a marker for osteoclast formation.

## 2. Materials and methods

### 2.1. Animals and experimental diets

Ten-month-old female C57BL/6 mice were obtained from Harlan (Indianapolis, IN). Weight-matched mice were housed in a laboratory animal care facility in cages (5 mice/cage) and fed a standard pellet diet (Harlan Teklad LM-485) for 2 months. At 12 months of age, mice were divided into two dietary groups and fed AIN-93M semipurified diets with AIN-93M vitamin and mineral mixes [30] containing either 10% CO or 10% FO (MP Biomedicals, Irvine, CA). One percent CO was added to the FO diet to prevent essential fatty acid (EFA) deficiency. Both diets were supplemented with equal levels of antioxidants. The composition of diet and oils is shown in Tables 1 and 2, respectively. Fresh diet was prepared weekly, stored in aliquots at −20°C and provided daily. Mice were maintained on a 12-h light/dark cycle in an ambient temperature of 22–25°C at 45% humidity. National Institutes of Health guidelines were strictly followed, and all studies were approved by the Institutional Laboratory Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

### 2.2. Measurement of body composition and BMD

BMD, lean body mass and body fat mass were measured by dual-energy X-ray absorptiometry (DEXA) at baseline (12 months) and after 6 months of FO treatment (18 months) using a Lunar PIXImus mouse bone densitometer (General Electric). Data analysis was carried out manually with PIXImus software [10]. Calibration of the instrument was conducted, as suggested by the manufacturer. An aluminum/lucite phantom (total bone mineral density [TBMD] = 0.0700 g/cm$^2$; % fat=14.0%) was placed on the specimen tray and measured 25 times without repositioning. Thereafter, the phantom was analyzed daily before animal testing for quality control purposes. Before bone scanning was performed, mice were anesthetized with intramuscular injections of ketamine/Rompun/NaCl (3:2:5). The densitometer was calibrated daily with a phantom supplied by the instrument's manufacturer. During measurements, the animals lay in prone position, with posterior legs maintained in external rotation with tape. The hip, knee and ankle articulations were in 90° flexion. Upon completion of scanning, BMD was determined in the following bone areas using the PIXImus software (version 1.46): distal femoral

Table 1
Composition of experimental diets[a]

| Diet ingredients | Percentage |
|---|---|
| Casein | 14.00 |
| Corn starch | 42.43 |
| Dextronized corn starch | 14.50 |
| Sucrose | 9.00 |
| Cellulose | 5.00 |
| AIN-93 mineral mix | 3.50 |
| AIN-93 vitamin mix | 1.00 |
| L-cystine | 0.18 |
| Choline bitartrate | 0.25 |
| TBHQ | 0.10 |
| Vitamin E | 0.04 |
| CO and/or FO[b] | 10.00 |

TBHQ, tert-butylhydroquinone.
[a] All diet ingredients were purchased from MP Biomedicals.
[b] FO diet supplemented with 1% CO (FO=9%, CO=1%).

Table 2
Selected fatty acid composition of oils[a]

| Fatty acids | CO | FO |
|---|---|---|
| 14:0 | 0.14 | 9.10 |
| 16:0 | 10.50 | 17.02 |
| 16:1 | ND | 12.87 |
| 18:0 | 2.06 | 2.78 |
| 18:1*n*−9 | 27.76 | 8.08 |
| 18:2*n*−6 | 56.06 | 1.27 |
| 18:3*n*−3 | 1.27 | 1.67 |
| 18:4*n*−6 | ND | 3.64 |
| 20:4*n*−6 | ND | 0.81 |
| 20:5*n*−3 | ND | 14.27 |
| 22:5*n*−3 | ND | 2.05 |
| 22:6*n*−3 | ND | 8.70 |

ND, not detected.
[a] Expressed as percentage of total fatty acids.

metaphysis (DFM) including cancellous (trabecular) bone, proximal tibial metaphysis (PTM), femoral diaphysis (FD), tibial diaphysis (TD) and lumbar vertebra 2 ($L_2$). The intrascan coefficient of variation ($CV_i$) was 0.79%, 3.30%, 1.35%, 3.48% and 1.19% for DLF, PLT, CLF, CLT and $L_3$, respectively. The interscan coefficient of variation ($CV_r$) was 5.47%, 3.86%, 5.12%, 1.36% and 2.37% for DFM, PTM, FD, TD and $L_3$, respectively. The CV are in agreement with studies examining the precision and accuracy of the PIXImus densitometer [31,32].

### 2.3. Collection of serum, bone marrow and tissues

At 18 months, the mice were sacrificed, and blood was collected and centrifuged at 10,000×*g* for 10 min at 4°C. Serum was removed and stored at −70°C until analysis. Spleens were collected aseptically in complete RPMI 1640 medium for splenocyte preparation [11]. Bone marrow was collected in DMEM medium [33].

### 2.4. Serum biochemistry

The serum activity of total ALP was measured with a Quantichrom Alkaline Phosphatase Assay Kit (Bioassay Systems, Hayward, CA). OC was measured with a Mouse Osteocalcin EIA Kit (Biomedical Technologies, Inc., Stoughton, MA).

### 2.5. Splenocyte preparation and culture

Spleens were aseptically removed and placed in 5 ml of RPMI 1640 media (Gibco, Grand Island, NY) supplemented with 25 mmol/L HEPES, 2 mmol/L glutamine, 100,000 U/L penicillin and 100 mg/L streptomycin (Gibco). Single-cell suspensions were made by teasing spleens between frosted ends of two sterile glass slides. After a 5-min centrifugation at 100×*g* to separate cells from debris, the cells were washed twice in RPMI medium. Splenic lymphocytes were isolated by layering over Histopaque (Sigma, St. Louis, MO), centrifuging at 1000 rpm for 15 min at 22°C and then washing twice in RPMI 1640 complete medium. Cells were counted, and viability was determined by trypan blue exclusion method. Cells ($1\times10^6$ cells/well) were plated in 96-well plates, and concanavalin A (conA) was added at a concentration of 1.0 μg/ml for 48 h at 37°C in a humidified atmosphere of air/$CO_2$ 95:5 (mol%). After 48 h, the culture medium was collected and analyzed for TNF-α? and IL-6 by standard enzyme-linked immunosorbent assay (ELISA) techniques [11].

### 2.6. Cytokine measurement in cultured splenocytes

TNF-α and IL-6 were measured by ELISA with BD OptEIA ELISA kit (BD Biosciences Pharmingen, San Diego, CA) [34]. Assay sensitivity was approximately 1 pg/ml. In brief, each well of flat-bottom 96-well microtiter plates was coated with 100 μl of purified anti-TNF-α and anti-IL-6 antibodies (4 μg/ml in binding solution) overnight at 4°C. The plates were rinsed four times with washing buffer, and the culture medium was added, followed by incubation for 2 h at room temperature. The plates were washed four times with washing buffer, followed by the addition of biotinylated anticytokine antibodies. The plates were incubated in room temperature for 1 h and then washed four times with washing buffer. Streptavidin–ALP conjugate was added, and the plates were incubated for 30 min at room temperature. The plates were again washed four times with washing buffer, and chromogen substrate was added. The plates were then incubated at room temperature to achieve the desired maximum absorbance and were read at 410 nM in an ELISA reader (Dynex Technologies, UK).

### 2.7. Bone marrow cell culture

Bone marrow cells from the tibias and femurs of CO-fed and FO-fed mice were cultured as described previously by Rahman et al. [33]. Briefly, cells were suspended in α-MEM

Table 3
Effect of CO and FO on body composition and BMD in aging C57BL/6 female mice[a]

| Parameters | | Diet | | *P* |
|---|---|---|---|---|
| | | CO | FO | |
| Body weight (g) | Baseline | 25.06±0.40 | 25.63±0.37 | NS |
| | Final | 38.16±0.41 | 39.60±0.38 | |
| Total body fat mass (g) | Baseline | 4.04±0.34 | 3.94±0.31 | NS |
| | Final | 14.78±0.35 | 15.26±0.32 | |
| Total body lean mass (g) | Baseline | 16.92±0.31 | 16.96±0.29 | NS |
| | Final | 15.13±0.32 | 15.06±0.30 | |
| DFM ($g/cm^2$) | Baseline | 0.090±0.002 | 0.092±0.001 | <.001 |
| | Final | 0.091±0.002 | 0.111±0.002[b] | |
| PTM ($g/cm^2$) | Baseline | 0.074±0.002 | 0.074±0.002 | <.01 |
| | Final | 0.075±0.002 | 0.092±0.002[b] | |
| FD ($g/cm^2$) | Baseline | 0.063±0.002 | 0.064±0.001 | <.05 |
| | Final | 0.079±0.002 | 0.087±0.001[b] | |
| TD ($g/cm^2$) | Baseline | 0.046±0.001 | 0.047±0.001 | <.05 |
| | Final | 0.048±0.001 | 0.054±0.001[b] | |
| $L_2$ ($g/cm^2$) | Baseline | 0.066±0.002 | 0.066±0.002 | NS |
| | Final | 0.055±0.002 | 0.047±0.002 | |

Baseline, 12 months; final, 18 months; NS, not significant.
[a] Values are expressed as mean±S.E.M. ($n=5$).
[b] Significantly different from CO control at $P<.05$ (unpaired *t* test; 18-month-old mice).

Table 4
Effect of CO and FO on serum bone formation biomarkers in 18-month-old C57BL/6 female mice[a]

| Serum parameters | CO | FO | P |
|---|---|---|---|
| OC (ng/ml) | 7.75±1.46 | 12.84±4.85 | NS |
| ALP (U/L) | 14.61±1.30 | 18.06±1.95 | NS |

[a] Values are expressed as mean±S.E.M. ($n=5$).

containing 15% fetal calf serum and cultured in 48-well plates ($1\times10^6$ cells/ml). Osteoclast differentiation was induced in the presence of macrophage colony-stimulating factor (M-CSF) (20 ng/ml) and soluble receptor activator of nuclear factor (NF) κB ligand (sRANKL) (30 ng/ml) for a 4-day culture. At the end of the culture, the cells were fixed and then stained with a commercial kit for TRAP (no. 387A; Sigma), a marker enzyme for osteoclasts. $TRAP^+$ cells with more than three nuclei were counted as osteoclasts [multinucleated cells (MNCs)].

### 2.8. Fatty acid analysis

Serum total lipids were extracted by the method of Folch et al. [35] using chloroform–methanol (2:1). The organic phase, containing total lipid extracts, was dried under a stream of nitrogen, and the residue was methylated according to the method of Kates [36]. Fatty acid methyl esters were separated and quantified by gas–liquid chromatography using a Hewlett-Packard 5890A series II gas chromatograph, equipped with a DB225MS capillary column (J&W Scientific, Folsom, CA) and a flame ionization detector (FID). The injection and detector port temperatures were 225°C and 250°C, respectively. The oven temperature was maintained at 170°C for 1 min and then increased to 215°C at a rate of 5°C/min. Helium was used as the carrier gas. The running time of each sample was approximately 36 min. Fatty acid methyl esters were identified by the comparison of their retention times with the fatty acid methyl ester standard from Matreya LLC (Pleasant Gap, PA). Quantification was done by an integrator (Hewlett-Packard 3396 series II) attached to a GLC machine, and results were expressed as area percentages. Oil samples were treated similarly beginning at the methylation step [37].

### 2.9. Statistics

Data are expressed as mean±S.E.M. Results were analyzed by unpaired $t$ test using Graph Prism software, and $P<.05$ was considered significant.

## 3. Results

### 3.1. Effect of dietary fat on body composition and BMD

Body weight and fat mass increased, and lean mass decreased, with time in both CO-fed and FO-fed mice. However, there was no difference in any of these parameters between CO-fed and FO-fed mice after 6 months of dietary treatment. However, BMD significantly increased in DFM (+20.6% vs. baseline), PTM (+24.3% vs. baseline) and TD (+14.9% vs. baseline) bone regions in FO-fed mice, whereas BMD increased minimally in CO-fed mice (DFM: +1.1% vs. baseline; PTM: +1.4% vs. baseline; TD: +4.4% vs. baseline). BMD increased significantly in the FD bone region in both CO-fed and FO-fed mice, but the increase was higher in FO-fed mice (CO: +25.4% vs. baseline; FO: +35.9% vs. baseline). Although BMD was decreased in the $L_2$ bone region in both CO-fed and FO-fed mice compared to baseline (CO: −16.7% vs. baseline; FO: −28.8% vs. baseline), no difference in BMD values was observed between the dietary groups at the final time point (Table 3). Thus, our results suggest that FO-fed aging mice maintain higher cancellous and cortical BMD compared to CO-fed mice in major bone compartments.

### 3.2. Serum biomarkers

Higher ALP and OC activities were observed in FO-fed mice compared to CO-fed mice, but the results were not statistically significant (Table 4).

### 3.3. Pro-inflammatory cytokine production in activated splenocytes

TNF-α activity in conA-stimulated splenocytes was significantly decreased in FO-fed mice compared to CO-fed mice. IL-6 was also decreased in FO-fed mice; however, the effect was not statistically significant (Fig. 1).

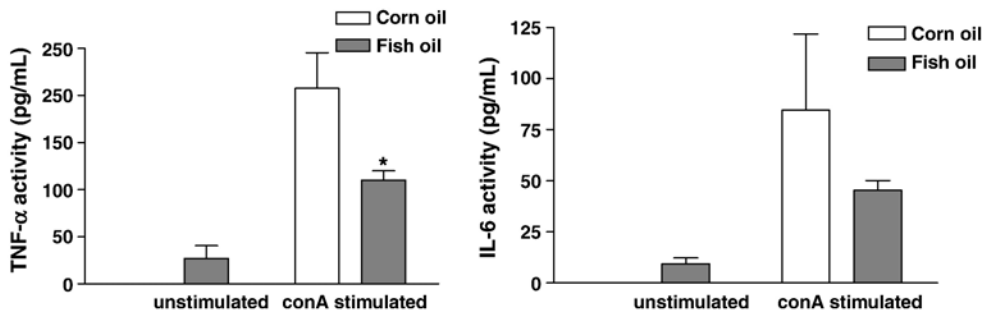


Fig. 1. TNF-α and IL-6 production in conA-stimulated splenocytes isolated from 18-month-old C57BL/6 female mice fed CO or FO for 6 months. *Significantly different from conA-stimulated CO control at $P<.05$ (unpaired $t$ test).

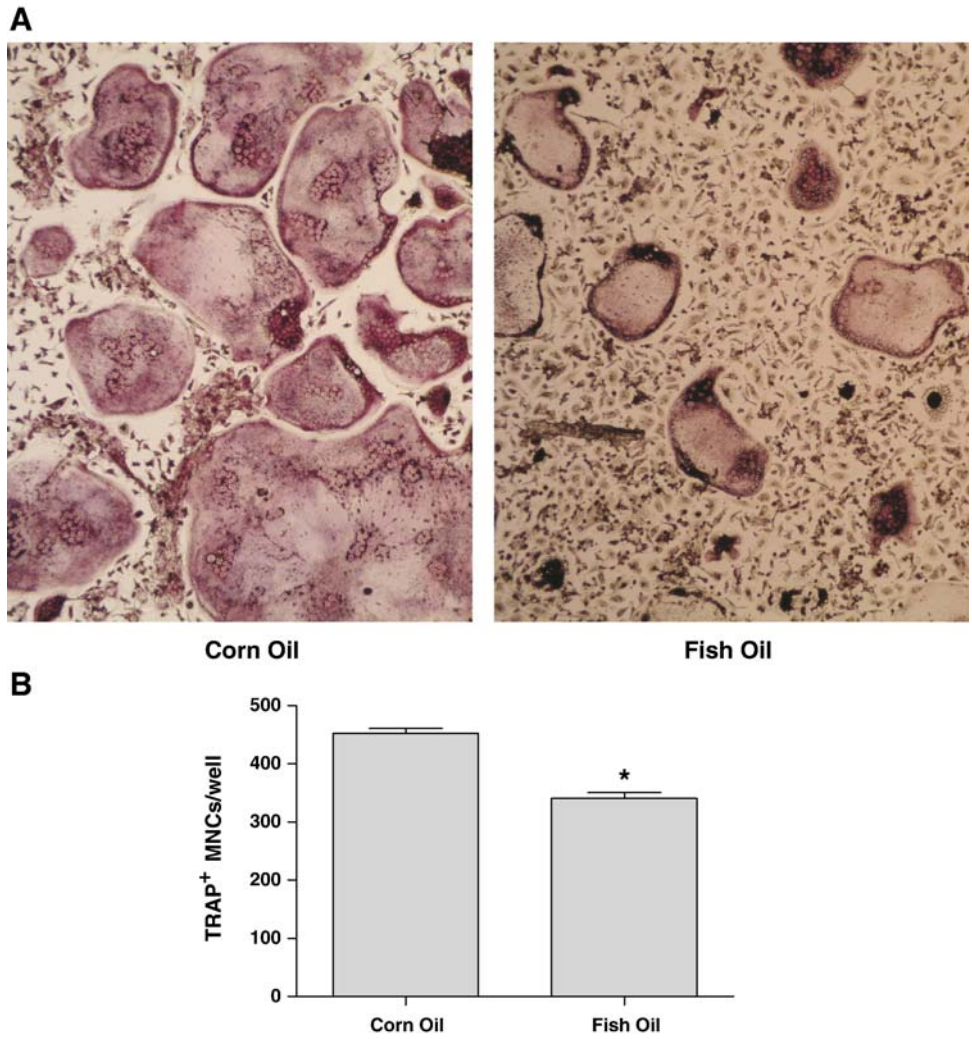


Fig. 2. (A) Formation of TRAP$^+$ MNCs in mouse bone marrow cultures isolated from CO-fed and FO-fed C57BL/6 female mice and treated with sRANKL and M-CSF. (B) MNC count in CO-fed and FO-fed mice. *Significantly different from CO control at $P<.01$ (unpaired $t$ test).

### 3.4. Bone marrow osteoclast formation

In cultures of mouse bone marrow cells, TRAP$^+$ osteoclast-like cells are formed in the presence of M-CSF and sRANKL [33]. We examined the formation of osteoclast-like cells in CO-fed and FO-fed mice. Our results clearly indicate significantly decreased TRAP$^+$ MNCs in FO-fed mice compared to CO-fed mice (Fig. 2A and B).

### 3.5. Serum fatty acids

The effect of dietary $n-3$ and $n-6$ fatty acids on the fatty acid composition of serum total lipids is presented in Table 5. CO-fed mice had higher levels of $n-6$ fatty acids (18:2$n-6$, 20:3$n-6$ and 20:4$n-6$) and 18:0 compared to FO-fed mice, which maintained significantly higher levels of 16:1$n-9$ and 22:6$n-3$. While 20:5$n-3$ and 22:5$n-3$ were present only in FO-fed mice, 22:4$n-6$ and 22:5$n-6$ were detectable only in CO-fed mice.

Table 5
Selected fatty acid composition of serum total lipids in 18-month-old C57BL/6 female mice[a]

| Fatty acids | CO | FO |
|---|---|---|
| 14:0 | 0.31±0.00 | 1.15±0.07[b] |
| 16:0 | 17.02±0.51 | 19.08±0.55 |
| 16:1$n-9$ | 2.21±0.04 | 5.59±0.67[b] |
| 18:0 | 11.63±0.02 | 7.31±0.24[b] |
| 18:1$n-9$ | 12.98±0.21 | 12.82±1.79 |
| 18:2$n-6$ | 22.35±0.61 | 7.77±0.72[b] |
| 20:3$n-6$ | 0.82±0.08 | 0.24±0.01[b] |
| 20:4$n-6$ | 25.71±1.74 | 6.79±0.11[b] |
| 20:5$n-3$ | ND | 24.96±1.73[b] |
| 22:4$n-6$ | 0.15±0.00 | ND |
| 22:5$n-6$ | 0.45±0.01 | ND |
| 22:5$n-3$ | ND | 0.81±0.08[b] |
| 22:6$n-3$ | 4.59±0.04 | 10.09±0.86[b] |

[a] Values are expressed as percentage of total fatty acids and as mean±S.E.M. of 5 mice/group.
[b] Significantly different from CO control at $P<.05$ (unpaired $t$ test).

## 4. Discussion

Most $n-3$ fatty acid studies on bone metabolism have been performed in growing mice, rats, chicks and piglets [14–16,23–26,38–42]. However, higher bone mineral content and cortical+subcortical BMD were recently reported in middle-aged male rats fed 20% FO compared to rats fed 20% safflower oil (SFO) [27], which correlated to higher bone $PGE_2$ and serum pyridinoline production in SFO-fed rats. In the present study, we measured BMD in vivo using DEXA in cancellous and cortical bones of the femur, tibia and lumbar spine in aging C57BL/6 female mice. When 12-month-old mice were fed either 10% CO or 10% FO for 6 months, FO-fed mice were found to maintain higher BMD in both pure cortical bone and cancellous bone. These findings correlated with a decreased activity of pro-inflammatory cytokines (TNF-α and IL-6 in activated splenocytes), a nonsignificant but greater increase in bone formation markers (OC and ALP in serum) and lower osteoclast generation in bone marrow cell cultures.

The importance of PUFAs in bone metabolism was exhibited when Kruger and Horrobin [43] found that EFA-deficient animals develop osteoporosis together with increased renal and arterial calcification. They further found that γ-linolenic acid (GLA)+EPA inhibit bone resorption in EFA-deficient young growing male rats by decreasing bone turnover parameters compared to mice fed LA+ALA [24]. Moreover, in calcium (Ca)-deficient elderly women, 18 months of treatment with Ca, along with EPA+GLA, showed improved lumbar spine and femoral BMD [44]. More recently, rats deficient in $n-3$ lipids were shown to have a decreased structural integrity of the tibia compared to rats adequately supplemented with $n-3$ lipids [45]. When deficient rats were repleted with $n-3$ lipids, accelerated bone modeling and improved second moment were observed. These studies suggest that PUFAs, in particular $n-3$ fatty acids, could be key modulators of bone metabolism. Claassen et al. [23] have indicated the importance of PUFAs in the regulation of Ca metabolism. Rats fed GLA+EPA showed increased Ca absorption from the intestine, decreased loss of urinary Ca and increased bone Ca content. Moreover, rats fed EPA-enriched FO also showed decreased Ca deposition in the kidneys and aortas [46]. This may be one of the mechanisms by which FO or $n-3$ fatty acids improve BMD.

It is well established that aging is associated with an increase in pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α [47–51]. These cytokines are key regulators of osteoclastogenic activity and have been shown to increase bone resorption [52–54]. Furthermore, these cytokines induce the expression of COX-2 in osteoblastic and stromal cells, resulting in an increased production of $PGE_2$, which is also an essential factor in osteoclastogenesis [55,56]. Our present study found a lower activity of TNF-α and IL-6 in FO-fed mice, which, in part, could explain the maintenance of higher BMD in these mice. Previous studies have shown that FO and $n-3$ fatty acids decrease the expression and activity of these cytokines, both in vivo and in vitro [11,57,58]. We have also shown a decreased mRNA expression of these cytokines in immune cells and kidney tissues from autoimmune disease animal models treated with $n-3$ fatty acids or FO [59–62]. $n-3$ fatty acids or FO has also been shown to decrease $PGE_2$ production in bone organ cultures and gingival tissues, which may be associated with improved BMD [14–16,28,63]. Although we did not measure $PGE_2$ in bone organ cultures in the present study, it is likely that a decreased activity of pro-inflammatory cytokines may have lowered $PGE_2$ levels in these mice, which could play an additional role in the beneficial effect of FO on BMD. Our recent data showed decreased COX-2 expression and $PGE_2$ activity in kidneys of FO-fed and calorie-restricted autoimmune-disease-prone NZB/W female mice [64].

A decreased activity of IL-6 and TNF-α may also explain the lower bone marrow osteoclast generation in FO-fed mice in the present study. Previous studies have shown that TNF-α and IL-6 promote bone resorption by increasing osteoclast differentiation [17–19]. We earlier showed that EPA and DHA, alone or in combination, decrease $1,25(OH)_2D_3$-stimulated osteoclast formation in cultured bone marrow cells, compared to treatment with LA and AA [11]. Moreover, Iwami-Morimoto et al. [25] showed decreased osteoclast formation and alveolar bone resorption in rats fed 10% FO compared to rats fed 10% CO. NF-κB is recognized as one of the key factors involved in the pathogenesis of osteoporosis, which is involved in a signaling pathway that leads to increased osteoclast generation and activation [11]. Our previous in vitro study found significant inhibition of RANKL-mediated NF-κB activation in bone marrow macrophages by EPA and DHA, alone or in combination, in contrast to $n-6$ fatty acids, which had no effect [11]. Although we did not measure NF-κB activation in the present study, its inhibition may be one of the potential mechanisms in the beneficial effect of FO on BMD.

We measured ALP and OC in the serum as biomarkers of bone formation. Although FO-fed mice showed a higher activity of these markers, the results, however, were not statistically significant. A lower ratio of $n-6/n-3$ fatty acids previously increased bone-specific ALP activity, but not OC activity, in growing rats, indicating higher bone formation [14]. Based on our results, it may be reasonable to conclude that higher bone formation may not alone account for the beneficial effect of FO in the present study. Inhibition of pro-inflammatory cytokines and lower osteoclast generation suggest that higher BMD in FO-fed mice may be associated, in part, with both decreased bone resorption and higher bone formation. More studies with $n-3$ fatty acids and different ratios of $n-6/n-3$ fatty acids are warranted soon in middle-aged and older animals to establish their mechanism of action in modulating bone mass during aging.

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