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## Endogenous n-3 fatty acids protect ovariectomy induced bone loss by attenuating osteoclastogenesis

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### Abstract

Beneficial effects of n-3 fatty acids (FA) on bone mineral density (BMD) have been reported in mice, rats and human beings, but the precise mechanisms involved have not been described. This study used the Fat-1 mouse, a transgenic model that synthesizes n-3 FA from n-6 FA to directly determine if outcome of bone health were correlated with n-3 FA. Ovariectomized (Ovx) and sham operated wild-type (WT) and Fat-1 mice were fed an AIN-93M diet containing 10% corn oil for 24 weeks. BMD was analysed by dual energy x-ray absorptiometry. Fat-1 Ovx mice exhibited significantly lower level of osteotropic factors like receptor activator of NF- $\kappa$ B ligand and tartrate-resistant acid phosphatase (TRAP)5b in serum and higher BMD in distal femoral metaphysis, proximal tibial metaphysis, femoral diaphysis and lumbar vertebra as compared to WT Ovx mice. LPS-stimulated bone marrow (BM) cells from Fat-1 Ovx mice produced significantly lower level of pro-inflammatory cytokines like tumour necrosis factor- $\alpha$ , interleukin (IL)-1- $\beta$ , IL-6 and higher level of anti-inflammatory cytokines like IL-10, IFN- $\gamma$  and higher level of nitric oxide as compared to BM cells from WT Ovx mice. LPS-stimulated COX-II activity as well as NF- $\kappa$ B activation in BM cells from Fat-1 Ovx mice was significantly less as compared to BM cells from WT Ovx mice. Furthermore, Fat-1 BM cells generated significantly less number of TRAP osteoclast-like cells as compared to WT BM cells. In conclusion, we offer further insight into the mechanisms involved in preventing the BMD loss in Ovx mice by n-3 FA using a Fat-1 transgenic mouse model.

**Keywords:** n-3 fatty acids • bone mineral density • inflammation • osteoporosis • osteoclasts

### Introduction

Post-menopausal osteoporosis due to oestrogen deficiency is a major health problem, primarily because of the severe morbidity and mortality associated with osteoporotic fractures. Oestrogen and/or hormone replacement therapies (ERT and/or HRT) are able to prevent osteoporotic bone loss, however, accompanied by adverse side-effects, such as uterine, ovarian and breast cancer and increased risk of cardiovascular diseases [1, 2]. Therefore, diet therapies that minimize bone loss would be an ideal alternative.

Recently, there has been increasing evidence that deficiency of certain fatty acids (FA) in the diet may contribute to bone loss [3–5]. A body of scientific evidence based on results in cell cultures [6, 7], animals [6–11] and human beings [4] indicates that long-chain n-3 polyunsaturated FA may protect skeletal health and potentially improve conditions associated with osteoporosis. In animal models, it has been shown that n-3 FA deficiency caused severe osteoporosis [12]. When deficient animals were replenished with n-3 FA, the ratio of n-3 to n-6 FA in bone compartments was restored and the process of bone degradation was reversed [13]. Different dietary ratios of n-6 to n-3 FA were tested in piglets and shown that higher n-3 FA levels in blood were associated with lower bone resorption [14]. In a clinical trial of 65 elderly women whose diet was low in calcium, supplementation with a lower ratio of n-6 and n-3 FA plus calcium resulted in decreased bone degradation and increased BMD [4]. In another randomized trial in 40 patients with osteoporosis, individuals taking a supplement rich in n-3 FA showed better calcium absorption and increased markers of bone formation as compared to placebo group [12].

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We speculate that by modulating the dietary ratio of n-6/n-3 FA, bone growth can be optimized.

Mammalian cells can neither synthesize n-3 FA nor convert n-6 to n-3 FA as they lack the converting enzyme, n-3 desaturase. The consumption of fish rich in n-3 FA is recommended for its health benefits to protect against heart disease, diabetes and potentially cancer [15]. High-fat diets are pervasive in Western cultures. American people consume very minimal n-3 FA in relation to the amount of n-6 FA. n-3 FA stimulate production of anti-inflammatory eicosanoids that attenuate the production of cytokines and associated bone resorption, whereas its cousin in n-6 FA stimulate production of pro-inflammatory eicosanoids that stimulate bone resorption by releasing cytokines to activate NF- $\kappa$ B [12, 16].

In 2004, Kang *et al.* generated transgenic Fat-1 mouse (Fat-1) on C57BL6 background carrying the *fat-1* gene from *Caenorhabditis elegans*, which encodes for an n-3 desaturase enzyme that can synthesize n-3 FA from n-6 FA [17]. Different tissues of Fat-1 mice show increase in n-3 FA and decrease in n-6 FA leading to a significant decrease in n-6/n-3 FA ratio. Thus, Fat-1 transgenic mice have an n-6/n-3 FA ratio of ~1: 1 compared to wild-type (WT) mice with ratio of 20–30: 1. Preliminary studies with Fat-1 mouse have already yielded interesting results. We and others have shown that Fat-1 mice attenuate inflammatory response following bacterial lipopolysaccharide (LPS) challenge [18, 19]. To examine the effect of FA, dietary lipid feeding studies using an intact animal model system are useful; however, these are confounded by the need to formulate isocaloric diets with respect to fat content. In addition, to formulate diets with different n-6 to n-3 ratios requires the blending of several oil sources; thus, the fat composition between control and experimental diets is difficult to control. The Fat-1 mouse model is not subject to these potential confounders, given that Fat-1 mice can endogenously synthesize n-3 FA; thus, only one diet needs to be provided to both WT and Fat-1 mice. Thus, the Fat-1 mouse represents a significant advance in the development of a more sophisticated research model to investigate the effect of n-3 FA and n-6/n-3 FA ratio on physiological parameters, inflammation and molecular mechanisms without providing exogenous n-3 FA in form of fish oil. Although the research on n-3 FA and bone health is promising, researchers have yet to establish a clear mechanism of action. In this study, we used this Fat-1 transgenic ovariectomized (Ovx) mouse model to establish n-3 FA as a preventive drug to postmenopausal osteoporosis, and to dissect the molecular mechanisms underlying this effect.

## Materials and methods

### Animals and diet

Male transgenic Fat-1 C57BL6 mice were obtained from Dr. Jing Kang at the Harvard Medical School. They were mated with WT C57BL6 female

mice to obtain female *fat-1* positive C57BL6 mice (Fat-1) and *fat-1* negative C57BL6 mice (WT) identified by genotyping using REExtract-N-Amp Tissue PCR Kit from Sigma (St Louis, MO, USA) and analyzing the FA composition of tails by using gas chromatography as described previously [19]. Weight-matched mice were housed in a laboratory animal care facility in cages (three to four mice/cage) and fed semi-purified AIN-93M diets containing 10% corn oil (CO) (MP Biomedicals, Irvine, CA). CO is high in linoleic acid (18: 2n-6) and Fat-1 mice convert n-6 FA to n-3 FA. The composition of the semi-purified diet per kilogram of diet was: 140 g of casein, 424.3 g of corn starch, 145 g of dextrinized corn starch, 90 g of sucrose, 50 g of fibre, 35 g of AIN-93 mineral mix, 10 g of AIN-93 vitamin mix, 1.8 g of L-cystine and 2.5 g of choline bitartrate. Diets were prepared weekly and stored in aliquots at  $-20^{\circ}\text{C}$ . Fresh diet was provided daily, and leftover food was removed to prevent rancidity. At 2 months age, 40 weight-matched WT mice and Fat-1 mice were sham operated (10 mice per group) or Ovx (10 mice per group). Forty mice with four groups of 10 were maintained on 10% CO diet for 24 weeks until killing. The National Institutes of Health guidelines provided in 'The Guide for the Care and Use of Laboratory Animals' 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.

### Serum RANKL and TRAP5b measurement

Four weeks before termination of the study, blood was collected retro-orbitally and serum was separated. Serum receptor activator of NF- $\kappa$ B ligand (RANKL) and tartrate resistant acid phosphatase (TRAP) were measured using mouse free soluble (s)RANKL and mouse TRAP5b ELISA assay kits from Immunodiagnostic System (IDS) Inc. (Fountain Hills, AZ, USA) according to the manufacturer's instructions [20].

### Measurement of bone mineral density (BMD)

BMD was measured by dual energy x-ray absorptiometry (DEXA) at baseline (8 weeks) and after 24 weeks on 10% CO diet using a Lunar PIXImus mouse bone densitometer (General Electric, Madison, WI, USA) and data analysis was carried out manually with PIXImus software as described previously [21, 22].

### Isolation of whole bone marrow cells and culture

Whole bone marrow (BM) cells were aseptically isolated as described elsewhere [23]. Cells were counted and viability was determined by trypan blue exclusion method. Cells ( $10 \times 10^6$ /well) were plated in 12-well plates and bacterial LPS was added at the concentration of 5.0  $\mu\text{g}/\text{ml}$  for 24 hrs at  $37^{\circ}\text{C}$  in a humidified atmosphere of air/CO<sub>2</sub> 95: 5 (mol%). After 24 hrs, cells and culture medium were collected together and centrifuged at 2000 rpm for 5 min. The pellets were stored at  $-80^{\circ}\text{C}$  for transcription factor assays and supernatants were analysed for tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6, IL-10, interferon (IFN)- $\gamma$  and nitric oxide.

### Analysis for fatty acids in bone marrow cells

Whole BM cells ( $\sim 1 \times 10^6$  cells) were used for the extraction of total lipids by the method of Folch *et al.* using chloroform: methanol (2: 1) as

described previously [24, 25]. FA methyl esters were separated and quantified by gas-liquid chromatography using a Hewlett-Packard 5890A series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA), equipped with a DB225MS capillary column (J&W Scientific, Folsom, CA, USA). FA methyl esters were identified by comparison of retention times with FA methyl ester standard (FIM-FAME-7) from Matreya, Inc. (Pleasant Gap, PA, USA). Quantification was performed by an integrator (Hewlett-Packard 3396 series II) attached to a gas liquid chromatography (GLC) machine, and results were expressed as area percentages.

### Cytokine measurement in bone marrow culture supernatants

TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10 and IFN- $\gamma$  were measured by ELISA using BD OptEIA™ ELISA kits from BD Biosciences Pharmingen (San Diego, CA, USA) according to the manufacturer's instruction.

### Nitric oxide measurement in bone marrow culture supernatants

Nitric oxide was measured in LPS-treated BM culture supernatant using quantichrome nitric oxide assay kit (DINO-250) from Bioassay Systems (Hayward, CA, USA).

### Protein preparation

After 24 hrs of BM culture in the presence of LPS, cells were collected. Cytosolic and nuclear proteins were prepared as described previously [23]. Protein concentrations of the nuclear extracts, and cytosolic extracts were determined using a bicinchoninic acid (BCA) protein assay kit.

### Cyclo-oxygenase-II (COX-II) activation assay

One hundred micrograms of cytosolic protein of 24-hr LPS-treated BM cells from WT and Fat-1 mice were analysed for COX-II activity using CAYMAN COX Activity Assay Kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instruction.

### Western blot analysis

Thirty micrograms of cytosolic extracts were subjected to SDS-PAGE. Proteins were transferred to immunoblot polyvinylidene difluoride membranes (BioRad, Hercules, CA, USA) and subjected to Western blot analysis. Rabbit polyclonal antibody against I $\kappa$ B- $\alpha$  and mouse monoclonal antibody against phosphorylated I $\kappa$ B- $\alpha$  were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA).

### NF- $\kappa$ B activation assay

LPS-treated BM cells pellets obtained after collecting supernatants were analysed for LPS-stimulated activation of NF- $\kappa$ B using NF- $\kappa$ B transcription factor assay kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. NF- $\kappa$ B-DNA binding was analysed for

NF- $\kappa$ B p65 and NF- $\kappa$ B p50 subunits. Briefly, a total of 10  $\mu$ g of nuclear extracts were incubated with mild agitation for 1 hr at room temperature with binding buffer in microwells coated with probes containing the NF- $\kappa$ B consensus binding sequence. The microwells were then washed three times. Anti-NF- $\kappa$ B antibody was added to each well and incubated for 1 hr at room temperature. The microwells were then washed three times before being incubated with HRP-conjugated antibody for 1 hr at room temperature. The microwells were then washed four times and then exposed to tetramethylbenzidine for 10 min. at room temperature before the stop solution was added. The optical density was read at 450 nm using a microplate reader (Dynex Technologies, Worthing, UK).

### Osteoclast differentiation in BM cultures

BM cells from the tibias and femurs of WT and Fat-1 mice were collected and cultured as described previously by Rahman *et al.* [23]. Briefly, cells were suspended in  $\alpha$ -MEM containing 15% foetal calf serum and cultured in 48-well plates ( $1 \times 10^6$  cells/ml). Osteoclast differentiation was induced in the presence of macrophage colony-stimulating factor (M-CSF) (20 ng/ml) and sRANKL (30 ng/ml) for 4 days. 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 osteoclast. TRAP<sup>+</sup> cells with more than three nuclei were counted as osteoclast (multinucleated cells).

### Statistics

Data are expressed as means  $\pm$  S.E.M. To test the significance either Student's t-test or Newman-Keuls' one-way ANOVA was used. The significance of differences in BMD from baseline to end of study between WT and transgenic groups were analysed by unpaired t-test. The GraphPad Prism 4.0 was employed for the statistical analyses. Differences were considered significant when  $P < 0.05$ .

## Results

### Fatty acid profiles of bone marrow cells

The *fat-1* gene of *C. elegans* encodes an n-3 fatty-acid desaturase enzyme that converts n-6 to n-3 FA and which is absent in most animals, including mammals [26]. Both WT and Fat-1-transgenic littermates born to the same mother and were maintained on an identical diet that was high in n-6 but deficient in n-3 FA. However, the fatty-acid profiles of the two groups turned out to be quite different in BM cells (Table 1). During this dietary regime, Fat-1 mice had significantly higher amounts of n-3 FA, such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), in BM cells compared with WT mice (Table 1). The ratio of the long-chain n-6 FA (18: 2n-6 + 20: 4n-6 + 22: 4n-6 + 22: 5n-6) to the long-chain n-3 FA (18: 3n-3 + 20: 5n-3 + 22: 5n-3 + 22: 6n-3) was 5.9 in Fat-1 mice and 20.6 in WT mice and an arachidonic acid (AA)/(EPA+DPA+DHA) ratio

**Table 1** Profiles of polyunsaturated n-6 and n-3 fatty acids in bone marrow cells from WT or *fat-1* transgenic (Fat-1) mice

PUFAs	WT	Fat-1
18: 2n-6 (LA)	25.34 ± 0.41	24.27 ± 0.83
18: 3n-3	0.24 ± 0.09	0.38 ± 0.01*
20: 4n-6 (AA)	6.48 ± 0.10	4.16 ± 0.30*
20: 5n-3 (EPA)	0.20 ± 0.02	1.05 ± 0.10*
22: 4n-6	0.25 ± 0.01*	ND
22: 5n-6	0.37 ± 0.04	0.12 ± 0.01*
22: 5n-3 (DPA)	ND	0.32 ± 0.01*
22: 6n-3 (DHA)	1.14 ± 0.07	3.17 ± 0.40*
n-6/n-3	20.58 ± 1.10	5.89 ± 0.90*
AA/EPA + DPA + DHA	4.84 ± 1.11	0.92 ± 0.59*

Total lipids of serum were extracted, methylated and subjected to analysis by gas chromatography. The values (% of total fatty acids) are means of three independent measurements ± S.E.M.  $n = 7$ . ND, not detected.

\*Significant difference ( $P < 0.05$ ) between WT and Fat-1 transgenic mice.

Ratio of n-6/n-3 fatty acids is expressed as (18: 2n-6 + 20: 4n-6 + 22: 4n-6 + 22: 5n-6)/(18: 3n-3 + 20: 5n-3 + 22: 5n-3 + 22: 6n-3).

of 0.92 *versus* 4.84. This means transgene is functionally active *in vivo* and transmittable.

### Effect of endogenous n-3 fatty acids on serum sRANKL and TRAP5b

We measured the sRANKL and TRAP5b levels in serum collected retro-orbitally 4 weeks before termination of the study to determine the bone resorbing status in sham and Ovx mice. RANKL is one of the most pivotal osteoclastogenic factors [27, 28] and serum TRAP5b level indicates the current status of osteoclasts function, *i.e.* TRAP activity. Interestingly, we found both serum RANKL and TRAP5b levels were significantly less in Fat-1 Ovx mice than in WT Ovx mice (Fig. 1A and B). The results indicate that the key bone resorbing osteoclastogenic factors are reduced due to the presence of endogenous n-3 FA, which supports the earlier findings that n-3 FA down-regulate osteoclastogenic factors [10, 29].

### Effect of endogenous n-3 fatty acids on BMD

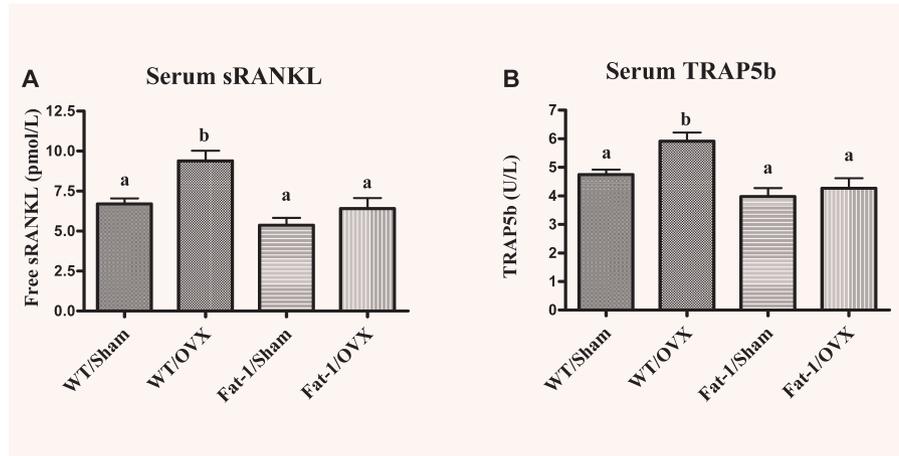
We have examined the baseline BMD of different bone regions prior to sham and Ovx surgery using DEXA. There were no differences in baseline BMD values among the groups (data not shown). To examine the effect of endogenous n-3 FA on Ovx-

induced bone loss, we measured the BMD of femur, tibia and lumbar regions 24 weeks after sham and Ovx surgery using DEXA. The results are shown in Table 2. The BMD in the distal end of the femur, the proximal end of the tibia and the lumbar regions of the spine of WT Ovx mice were significantly lower than that in WT sham mice. The BMD of different regions of Fat-1 Ovx mice was also lower than in Fat-1 sham mice. However, the reduction in BMD was not significant. Comparing between WT Ovx mice (without endogenous n-3 FA production) and Fat-1 Ovx mice (with endogenous n-3 FA production), the BMD loss was significantly higher in femoral, tibial and third lumbar regions of the WT mice group. Thus, lower ratio of n-6/n-3 FA due to endogenous production of n-3 FA maintains higher BMD in Fat-1 Ovx mice compared to WT Ovx mice. These findings indicate that Fat-1 mice, rich in n-3 FA, are at least better protected in oestrogen deficient BMD loss. To confirm the oestrogen status of the Ovx or sham mice, the uterine wet weight was measured at the time of killing. Ovariectomy performed in 8-week-old mice significantly decreased the uterus weight of both WT and Fat-1 mice (data not shown). The *fat-1* transgene had no effect on the uterus weight of sham or Ovx mice.

### Effect of endogenous n-3 fatty acids on LPS stimulated cytokine production by bone marrow cells

We next examined whether the Ovx-induced BMD loss protection observed in Fat-1 mice had an impact on bone resorbing inflammation-related cytokines expression. Pro-inflammatory cytokines like IL-1 $\beta$ , IL-6 and TNF- $\alpha$  are key regulators of osteoclastogenic activity and have been shown to increase bone resorption [30–32]. Interestingly, we found significant increase in IL-1 $\beta$  and TNF- $\alpha$  production by BM cells of WT Ovx mice than that of WT sham mice, whereas no increase of these cytokines was observed in Fat-1 Ovx mice when compared to Fat-1 sham mice (Fig. 2). However, there was no significant difference in IL-6 production between the sham and Ovx mice in both WT and Fat-1 groups. Significantly higher level of IL-1 $\beta$ , TNF- $\alpha$  and IL-6 was observed in WT Ovx mice when compared to Fat-1 Ovx mice. Surprisingly, we found significantly lower level of TNF- $\alpha$  in Fat-1 Ovx group than in Fat-1 sham group. We then examined if reduced n-6/n-3 FA ratio due to endogenous conversion of n-6 to n-3 FA can stimulate the production of anti-inflammatory cytokines like IL-10 and IFN- $\gamma$ . IL-10 is reported to inhibit bone resorption in inflammatory disorders [33, 34] and IFN- $\gamma$  is a strong suppressor of osteoclastogenesis [35]. Interestingly, we observed significantly higher level of both IL-10 and IFN- $\gamma$  production by BM cells of Fat-1 Ovx mice when compared to WT Ovx mice (Fig. 2). The results indicate that the reduction of n-6/n-3 FA ratio may prevent Ovx-induced BMD loss indirectly by inhibiting the production of osteoclastogenic pro-inflammatory cytokines and by enhancing the production of anti-osteoclastogenic anti-inflammatory cytokines.

**Fig. 1** Endogenous n-3 fatty acids reduce serum RANKL and TRAP5b. After 20 weeks on experimental diet serum was separated from blood collected retro-orbitally from WT and Fat-1 transgenic sham and Ovx mice (seven mice/groups). Serum was analysed for (A) free serum RANKL and (B) TRAP5b levels using standard ELISA kits. Each bar represents the mean  $\pm$  S.E.M. of seven duplicate samples. Value with different superscripts are significantly different at  $P < 0.05$  by Newman-Keuls' one way ANOVA with multiple comparison test.



**Table 2** Effect of endogenous n-3 fatty acids on bone mineral density (BMD) ( $\text{mg}/\text{cm}^2$ ) of ovariectomized (Ovx) mice<sup>a</sup>

Bone regions	WT				Fat-1				P-value <sup>2</sup>	P-value <sup>3</sup>
	Sham	Ovx	P-value <sup>1</sup>	% change	Sham	Ovx	P-value <sup>1</sup>	% change		
DFM	87.02 $\pm$ 2.91	72.87 $\pm$ 1.43	0.001*	-16.26	89.90 $\pm$ 1.87	87.23 $\pm$ 1.90	0.340	-2.97	0.423	0.0001*
PTM	72.17 $\pm$ 1.25	62.73 $\pm$ 1.99	0.003*	-13.08	72.62 $\pm$ 1.46	72.83 $\pm$ 1.78	0.927	0.29	0.820	0.004*
FD	73.97 $\pm$ 1.62	69.27 $\pm$ 1.90	0.089	-6.35	80.87 $\pm$ 2.52	78.63 $\pm$ 2.23	0.522	-2.77	0.044*	0.01*
TD	44.93 $\pm$ 0.86	45.40 $\pm$ 0.42	0.596	1.05	48.63 $\pm$ 0.84	49.52 $\pm$ 1.52	0.622	1.83	0.018*	0.026*
L2	62.92 $\pm$ 3.36	46.70 $\pm$ 2.47	0.003*	-25.78	58.47 $\pm$ 4.46	47.88 $\pm$ 6.63	0.215	-18.11	0.444	0.870
L3	60.98 $\pm$ 3.49	39.27 $\pm$ 2.96	0.001*	-35.60	57.35 $\pm$ 4.78	51.80 $\pm$ 4.82	0.433	-9.68	0.553	0.05*
L4	56.23 $\pm$ 1.72	43.03 $\pm$ 2.64	0.002*	-23.48	50.43 $\pm$ 2.73	48.82 $\pm$ 2.75	0.685	-3.19	0.102	0.160

<sup>a</sup> Values are means  $\pm$  S.E.M.,  $n = 8$ .

<sup>1</sup> Student's t-test comparing Ovx to sham.

<sup>2</sup> Student's t-test comparing wild-type (WT) sham to Fat-1 transgenic (Fat-1) sham.

<sup>3</sup> Student's t-test comparing WT Ovx to Fat-1 Ovx.

DFM: distal femoral metaphysis; PTM: proximal tibial metaphysis; FD: femoral diaphysis; TD: tibial diaphysis; L2: lumbar vertebra 2; L3: lumbar vertebra 3; L4: lumbar vertebra 4; \* $P < 0.05$  was considered significant.

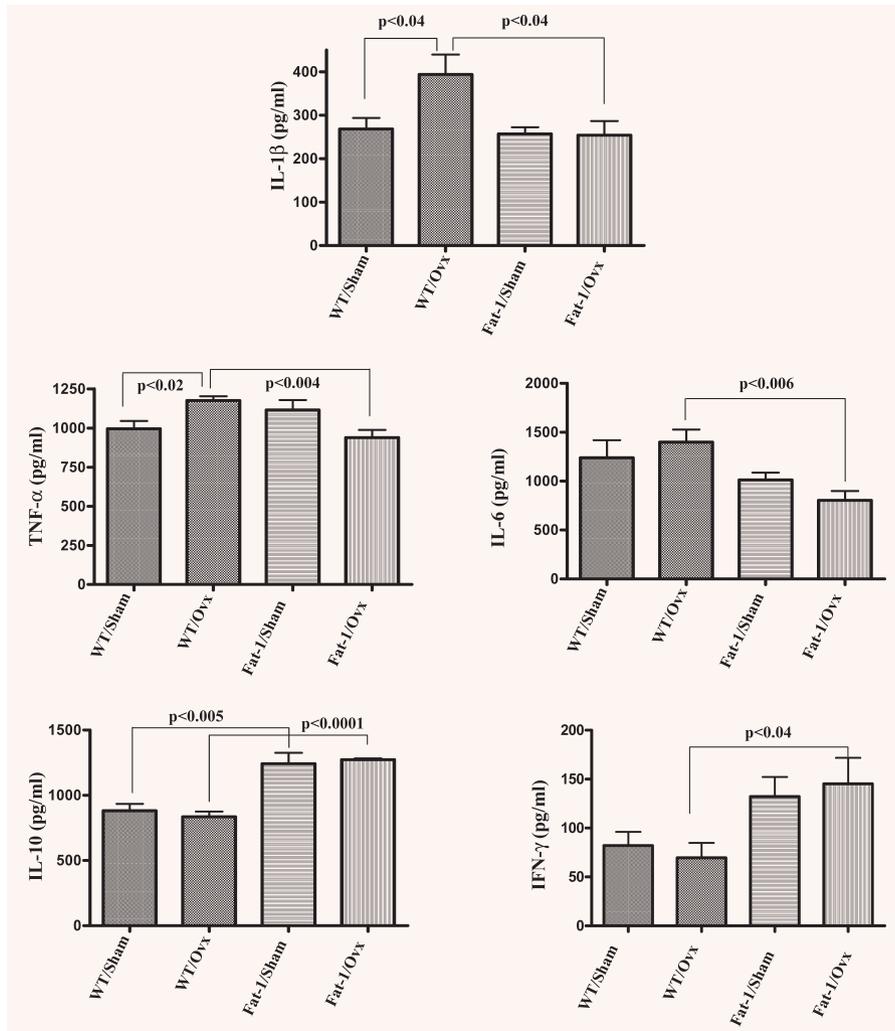
### Effect of endogenous n-3 fatty acids on LPS stimulated nitric oxide production by bone marrow cells

Nitric oxide has been reported to be a potent anti-osteoclastogenic and anti-osteoporotic in severe inflammatory and oestrogen deficient animals. Therefore, we next determined if there is any effect of endogenous n-3 FA on LPS-stimulated nitric oxide production by BM cells. Interestingly, we observed higher levels of nitric oxide production in BM cells from Fat-1 mice both sham and Ovx than from WT mice (Fig. 3). That might be another anti-

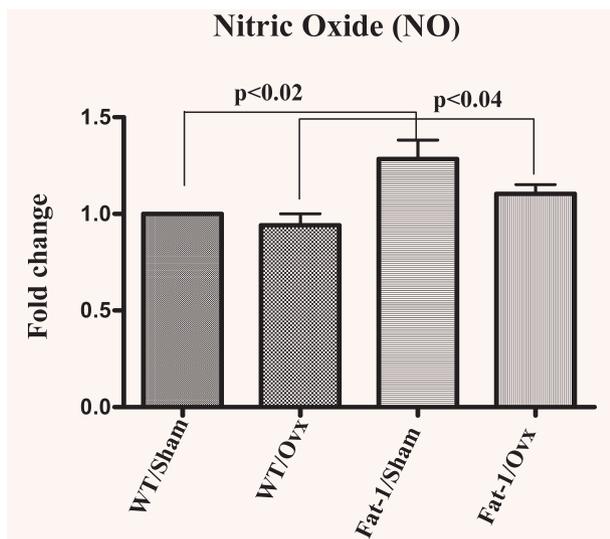
osteoclastogenic mechanism exerted by endogenous n-3 FA to protect Ovx-induced BMD loss.

### Effect of endogenous n-3 fatty acids on LPS stimulated COX-II activity

Over expression of COX-II stimulates osteoclastogenesis and bone resorption [36] and n-3 FA are reported to down-regulate COX-II expression. Therefore, we examined whether lower ratio of n-6/n-3 FA due to endogenous production of n-3 FA also have the COX-II reducing ability which might be one of the mechanisms by which it



**Fig. 2** Endogenous n-3 fatty acids modulate LPS-stimulated cytokines production. Bone marrow cells from WT and Fat-1 transgenic mice were cultured in the presence of LPS (5 μg/ml). After 24 hrs, culture media were collected and analysed for TNF-α, IL-1β, IL-6, IL-10 and IFN-γ by standard ELISA techniques. Each value represents the mean ± S.E.M. of two independent triplicate cultures. *P*-value < 0.05 was considered significant by Student's *t*-test.



**Fig. 3** Endogenous n-3 fatty acids increase LPS-stimulated nitric oxide production. Bone marrow cells from WT and Fat-1 transgenic mice were cultured in the presence of LPS (5 μg/ml). After 24 hrs, culture media were collected and analysed for nitric oxide using quantichrome nitric oxide assay kit. Each value represents the mean ± S.E.M. of two independent triplicate cultures. *P*-value < 0.05 was considered significant by Student's *t*-test.

is attenuating osteoclastogenesis and bone resorption. Interestingly, we found significant reduction of COX-II activity in LPS-treated BM cells from Fat-1 mice as compared to WT mice (Fig. 4).

### Effect of endogenous n-3 fatty acids on LPS stimulated activation of NF- $\kappa$ B

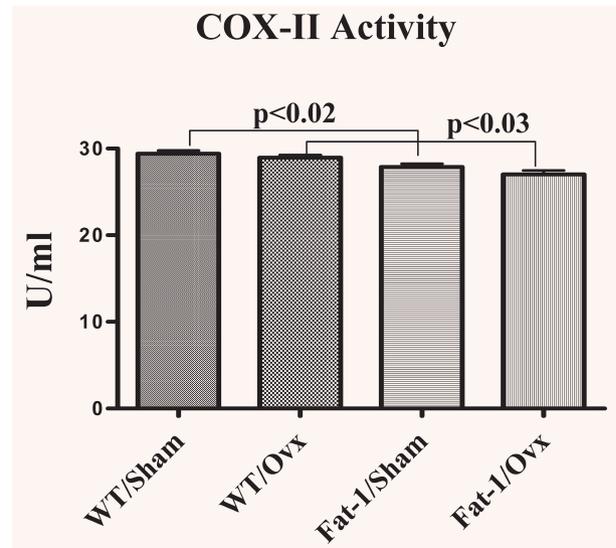
We further examined whether endogenous n-3 FA had any impact on the activation of NF- $\kappa$ B signalling. NF- $\kappa$ B is one of the most vital transcription factor associated with inflammatory bone destruction [37]. NF- $\kappa$ B activation is regulated by three major steps, phosphorylation of I- $\kappa$ B $\alpha$ , I- $\kappa$ B $\alpha$  degradation and nuclear translocation of p50/p65 subunits. LPS-stimulated I- $\kappa$ B $\alpha$  phosphorylation and I- $\kappa$ B $\alpha$  degradation in BM cells were analysed by western blot. Both I- $\kappa$ B $\alpha$  phosphorylation and I- $\kappa$ B $\alpha$  degradation were significantly lower in Fat-1 Ovx group when compared to WT Ovx group (Fig. 5A). Phosphorylation of I- $\kappa$ B $\alpha$  was significantly higher in WT Ovx as compared to WT sham (Fig. 5A). However, there was no significant difference in I- $\kappa$ B $\alpha$  phosphorylation and I- $\kappa$ B $\alpha$  degradation between Fat-1 Ovx and sham groups (Fig. 5A). LPS-stimulated activation of p65 and p50 NF- $\kappa$ B subunits were also analysed using transcription factor assay kit. Interestingly, LPS-stimulated activation of both p65 and p50 NF- $\kappa$ B were significantly lower in Fat-1 Ovx group than in WT Ovx group (Fig. 5B). Surprisingly, significantly lower level of activated p65 NF- $\kappa$ B was observed in Fat-1 Ovx group as compared to Fat-1 sham group (Fig. 5B).

### Effect of endogenous n-3 fatty acids on RANKL stimulated osteoclast like cells formation

Mouse BM cells can differentiate into TRAP<sup>+</sup> osteoclasts-like cells in the presence of RANKL and M-CSF [23]. We examined if endogenous n-3 FA have any effect on RANKL-stimulated osteoclast formation. Interestingly, significant reduction of osteoclasts formation was observed in BM cultures from Fat-1 mice as compared to WT mice (Fig. 6A and 6B).

## Discussion

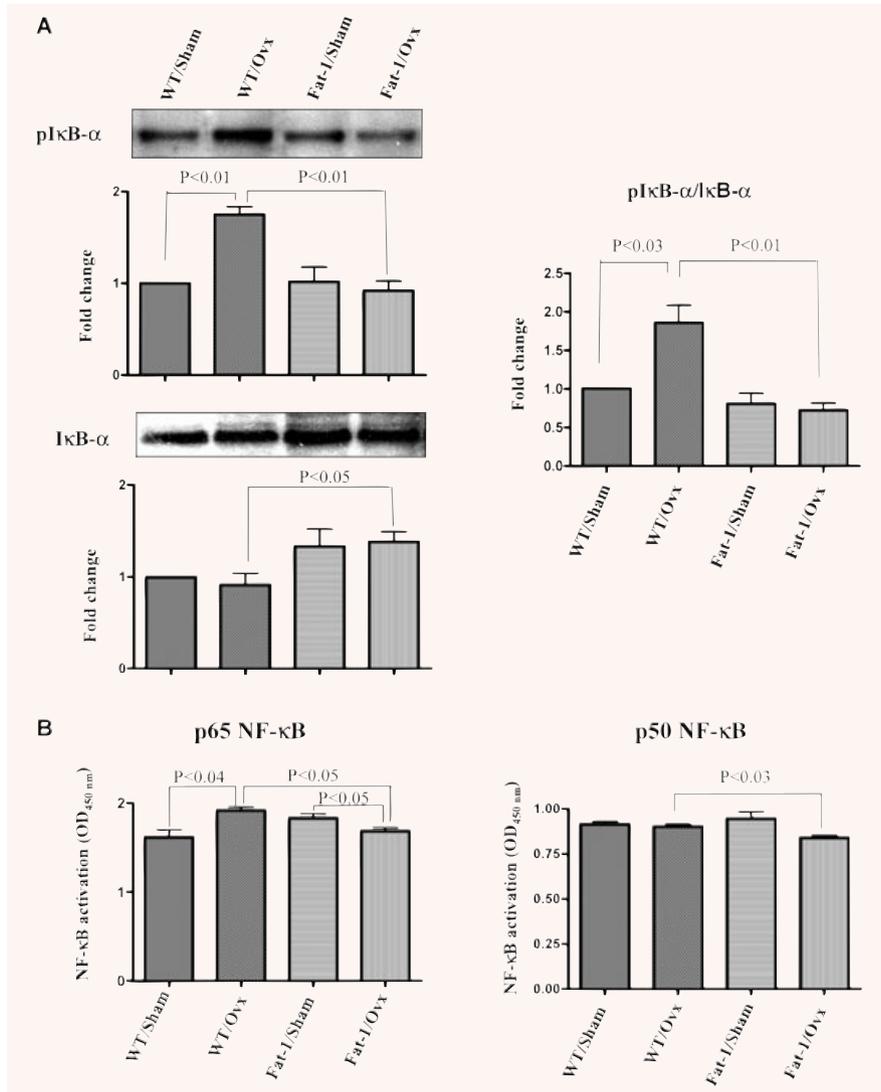
The present study was designed to examine the effect of an endogenously decreased n-6/n-3 FA status due to endogenous conversion of n-6 to n-3 FA on Ovx-induced BMD loss in Fat-1 mice versus WT control littermates. This study demonstrated that higher n-3 FA and lower n-6/n-3 FA level in BM phospholipids in Fat-1 mice could maintain higher BMD in oestrogen deficient condition when compared to that of WT mice. The decreased BMD loss in femur, tibia and lumbar regions of Fat-1 mice was accompanied by a lower incidence of osteoclastogenesis. These changes were, on a molecular level, accompanied by a lower activation NF- $\kappa$ B together with lower activity of COX-II, reduced production of pro-inflammatory cytokines TNF- $\alpha$ , IL-6 and IL1- $\beta$ , increased



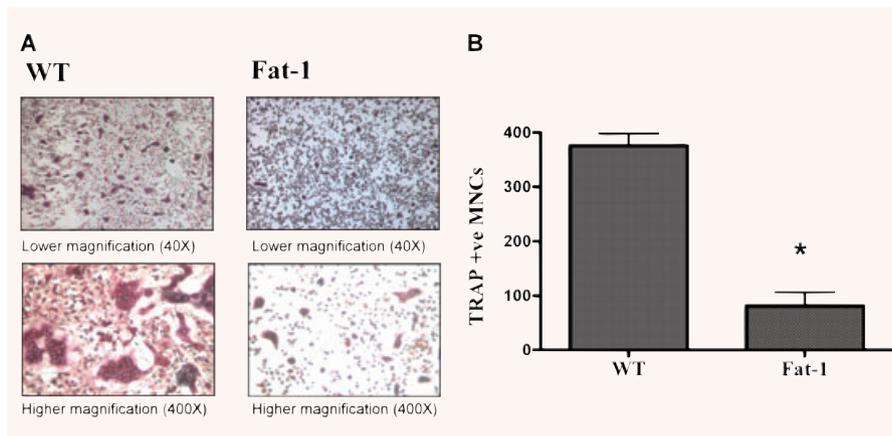
**Fig. 4** Endogenous n-3 fatty acids decrease LPS-stimulated COX-II production. Bone marrow cells from WT and Fat-1 transgenic mice were cultured in the presence of LPS (5  $\mu$ g/ml). After 24 hrs, cells were collected and cytosolic proteins were prepared. 100  $\mu$ g of cytosolic proteins were analysed for COX-II activity using COX Activity Assay Kit. Each value represents the mean  $\pm$  S.E.M. of two independent triplicate cultures. *P*-value < 0.05 was considered significant by Student's *t*-test.

production of anti-inflammatory cytokines IL-10, IFN- $\gamma$  and increased production of nitric oxide in BM cells of Fat-1 mice. We previously showed that dietary n-3 FA fed mice exhibit less Ovx-induced BMD loss accompanied by decreased osteoclastogenesis [10]. The molecular mechanisms underlying this protective effect are not clear yet. As Fat-1 transgenic mouse model eliminates confounding factors between control and experimental diets, we have chosen the Fat-1 transgenic mice to elucidate the molecular mechanisms underlying the protective effect of increased n3 and decreased n-6/n-3 FA tissue status.

The relative ratio between n-6 and n-3 FA is an important determinant in the overall health benefits of consuming n-3 FA [38, 39]. The n-6 to n-3 ratio consumed in the present Western diet is between 10: 1 and 20: 1; however, our ancestors had a diet closer to 1: 1 [40]. A lower n-6 to n-3 FA ratio may be optimal for one's health. Efforts have been made to incorporate n-3 FA into the food supply because of their health benefits and concern over the high n-6: n-3 FA in Western diets [17]. We observed a marked reduction in the n-6 to n-3 ratio in Fat-1 mice as compared to WT mice. The n-6 FA, especially AA, is a precursor of prostaglandins (PGs), leukotrienes and related compounds that influence the synthesis of eicosanoids which may enhance inflammation and bone loss [10, 18]. There was a trend towards higher levels of EPA, DPA and DHA and reduced levels of AA in Fat-1 mice, suggesting that elongation and desaturation were inhibited by the presence of n-3 FA. Consuming increased amounts of n-3 FA results in a partial replacement of the AA in cell membranes by EPA and DHA [18, 26]. This leads to decreased production of AA-derived



**Fig. 5** Endogenous n-3 fatty acids decrease LPS-stimulated NF-κB activation. Bone marrow cells from WT and Fat-1 transgenic mice were cultured in the presence of LPS (5 μg/ml). After 24 hrs, cells were collected and cytosolic nuclear proteins were prepared. **(A)** 30 μg of cytosolic proteins were analysed for phosphorylated I-κB-α and total I-κB-α level by western blot. Relative expression of I-κB-α, pI-κB-α and pI-κB-α/total I-κB-α is shown. The intensity of the bands was determined by densitometry. **(B)** 10 μg of nuclear proteins were analysed for p65 NF-κB and p50 NF-κB-DNA binding activity using TransAM Transcription Factor Assay kit. Each value represents the mean ± S.E.M. of two independent triplicate cultures. *P*-value <0.05 was considered significant by Student's *t*-test.



**Fig. 6** Endogenous n-3 fatty acids suppress osteoclast differentiation in bone marrow (BM) cell culture. BM cells ( $1 \times 10^6$ ) from WT and Fat-1 mice were cultured in the presence of sRANKL and macrophage colony-stimulating factor (M-CSF). **(A)** Formation of TRAP+ multinucleated cells in cultures of BM cells isolated from WT and Fat-1 transgenic mice in the presence of sRANKL and M-CSF. **(B)** TRAP+ multinucleated cells count in cultures of BM cells isolated from WT and Fat-1 mice.. \*Significantly different from WT control at *P* < 0.001 by Student's *t*-test.

pro-inflammatory mediators, *i.e.* PGE2. Dr. Kang group has already established that transgenic overexpression of *fat-1* gene lowers PGE2 expression in both cells and tissues by reducing the availability of AA [41–43]. COX-II is the key enzyme responsible for the conversion of AA to PGE2 and selective inhibition of COX-II can attenuate osteoclastogenesis as well as bone loss in inflammatory bone diseases [44–47]. It has been further reported that n-3 FA can down-regulate COX-II activity and also lower the production of PGE2 in local tissues [41, 48]. We have also found significantly reduced COX-II activity in Fat-1 mice. Thus, modification of membrane FA composition is one of the mechanisms by which n-3 FA may protect osteoporotic BMD loss possibly by reducing the pro-inflammatory mediators.

We and others earlier have described the inhibition of pro-inflammatory cytokines production by n-3 FA in cells and tissues [10, 48–50]. Our present data also show significant reduction of inflammatory cytokines production in LPS-treated BM cells from Fat-1 mice. This correlates well with previous findings of inflammatory cytokine suppression by n-3 FA [18, 41, 51, 52]. Our present data also show a significant increase in the LPS-stimulated production of IL-10 and IFN- $\gamma$  in BM cells. IL-10 has a critical role in the *in vivo* regulation of pro-inflammatory cytokine levels and has been reported to suppress osteoclastogenesis [53]. Further, IFN- $\gamma$  is also known to suppress osteoclastogenesis [35]. In addition, nitric oxide is postulated to play an important role in bone metabolism, and it is also known that both EPA and DHA enhance nitric oxide formation [3, 54, 55]. We have also detected higher level of nitric oxide production in Fat-1 mice. It was reported that osteoclast formation and bone resorption were inhibited by elevated levels of nitric oxide *in vivo* and *in vitro* [56–60]. Moreover, high nitric oxide levels and nitric oxide generating compounds inhibit osteoclast formation and bone resorption and prevent bone loss in severe inflammation or oestrogen-deficient animals [57, 61–64]. Further, iNOS deficiency or pharmacological inhibition of nitric oxide can accelerate osteoclast formation and bone resorption *in vivo* and *in vitro*, decrease normal bone mass, exacerbate bone destruction in arthritis or osteoporosis models, interfere with normal fracture healing and also iNOS knockout mice are known to exhibit more alveolar bone loss [65–67]. Thus, this might be another mechanism of the anti-osteoporotic action of n-3 FA.

It is now clearly emerging that n-3 FA might exert their effects on inflammatory gene expression through direct actions on the intracellular signalling pathways. Previous studies have shown that n-3 FA can down-regulate the activity of NF- $\kappa$ B. It has been reported that EPA prevents TNF- $\alpha$ -induced activation of NF- $\kappa$ B in cultured pancreatic cells [68]. In another study, EPA was reported to decrease endotoxin-induced activation of NF- $\kappa$ B and mitogen activated protein kinases (MAPK) in human monocytes [69–71]. Previously, we have also reported that EPA and DHA alone or in combination inhibits RANKL-induced NF- $\kappa$ B activation in BM cells [10]. Others have also showed that fish oil can inhibit LPS-induced NF- $\kappa$ B activation in a macrophage cell line [72]. These observations suggest direct effects of n-3 FA on inflammatory gene expression through the inhibition of NF- $\kappa$ B activation. In this

study, we also observed reduced NF- $\kappa$ B activation in BM cells of Fat-1 mice compared to that of WT mice. The role of NF- $\kappa$ B in the pathogenesis of osteoporosis is well documented. Mice null for NF- $\kappa$ B developed osteopetrosis and contain very few osteoclasts compared with normal controls [73]. This indicates the essential role of the NF- $\kappa$ B signalling pathway in osteoclast generation and activation [74, 75]. Activation of p38 MAPK and cJun N terminal kinase (JNK) is required for osteoclastogenesis [23]. The p38 MAPK pathway is also known to be involved in the regulation of bone resorption induced by oestrogen deficiency and selective inhibitors of this pathway have potential for prevention of bone loss in post-menopausal osteoporosis [76]. In a very recent study, we have found decreased activation of p38 MAPK and JNK in n-3 FA-treated BM cells (data not shown). Therefore, the observed protection of BMD loss due to oestrogen deficiency by increased n-3 FA and reduced n-6/n-3 FA tissue status is probably due to the reduced activation of NF- $\kappa$ B, and MAPK signalling pathways.

We previously reported that n-3 FA inhibited TRAP activity and osteoclast formation in primary BM cells [10]. In our present study, we found that higher endogenous n-3 FA and lower endogenous n-6/n-3 FA status in BM cells commensurate with lower RANKL-stimulated BM osteoclastogenesis. Stimulation of osteoclast differentiation is one of the mechanisms by which oestrogen deficiency causes bone loss [27, 28, 32, 77]. Thus, reduction of osteoclastogenesis might be one of the mechanisms by which n-3 FA exert its protection against osteoporotic BMD loss.

Our studies on Fat-1 transgenic mice provide compelling evidence for the effectiveness of n-3 FA to be a novel dietary FA to prevent post-menopausal osteoporosis. Endogenous conversion of n-6 FA to n-3 FA and maintaining lower ratio of n-6/n-3 FA not only prevents BMD loss in Ovx mice but also inhibits the inflammatory response that underlies the disease. As human beings cannot synthesize n-3 FA, they can however lower the n-6/n-3 FA ratio by consuming more n-3 FA, either as supplement or *via* foods enriched with n-3 FA, to prevent osteoporotic BMD loss. However, extensive pharmacological evaluation of this approach is required to fully determine the effect of long-term use of n-3 FA to prevent osteoporosis and related inflammatory bone loss. Very recently, fish oil rich in n-3 FA has been approved by the Food and Drug Administration (FDA) to use as a prescription drug to treat high triglyceride level as well as for cardiovascular diseases [78]. Because n-3 FA has many other beneficial effects, such as cardio-protective effect [79], anti-carcinogenic effect [41], triglyceride lowering effect [78, 80], as well as protective effect against inflammatory diseases [19, 81], supplementation with n-3 FA to prevent osteoporotic bone loss is a new strategy worth pursuing soon.

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## References

- Rosen CJ. Clinical practice. Postmenopausal osteoporosis. *N Engl J Med*. 2005; 353: 595–603.
- Lacey JV Jr, Mink PJ, Lubin JH, *et al*. Menopausal hormone replacement therapy and risk of ovarian cancer. *Jama*. 2002; 288: 334–41.
- Das UN. Essential fatty acids and osteoporosis. *Nutrition*. 2000; 16: 386–90.
- Kruger MC, Coetzer H, de Winter R, *et al*. Calcium, gamma-linolenic acid and eicosapentaenoic acid supplementation in senile osteoporosis. *Aging*. 1998; 10: 385–94.
- Watkins BA, Lippman HE, Le Bouteiller L, *et al*. Bioactive fatty acids: role in bone biology and bone cell function. *Prog Lipid Res*. 2001; 40: 125–48.
- Sakaguchi K, Morita I, Murota S. Eicosapentaenoic acid inhibits bone loss due to ovariectomy in rats. *Prostaglandins Leukot Essent Fatty Acids*. 1994; 50: 81–4.
- Watkins BA, Li Y, Lippman HE, *et al*. Modulatory effect of omega-3 polyunsaturated fatty acids on osteoblast function and bone metabolism. *Prostaglandins Leukot Essent Fatty Acids*. 2003; 68: 387–98.
- Kruger MC, Horrobin DF. Calcium metabolism, osteoporosis and essential fatty acids: a review. *Prog Lipid Res*. 1997; 36: 131–51.
- Schlemmer CK, Coetzer H, Claassen N, *et al*. Oestrogen and essential fatty acid supplementation corrects bone loss due to ovariectomy in the female Sprague Dawley rat. *Prostaglandins Leukot Essent Fatty Acids*. 1999; 61: 381–90.
- Sun D, Krishnan A, Zaman K, *et al*. Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice. *J Bone Miner Res*. 2003; 18: 1206–16.
- Watkins BA, Reinwald S, Li Y, *et al*. Protective actions of soy isoflavones and n-3 PUFAs on bone mass in ovariectomized rats. *J Nutr Biochem*. 2005; 16: 479–88.
- Heaney RP, Carey R, Harkness L. Roles of vitamin D, n-3 polyunsaturated fatty acid, and soy isoflavones in bone health. *J Am Diet Assoc*. 2005; 105: 1700–2.
- Reinwald S, Li Y, Moriguchi T, *et al*. Repletion with (n-3) fatty acids reverses bone structural deficits in (n-3)-deficient rats. *J Nutr*. 2004; 134: 388–94.
- Weiler HA, Fitzpatrick-Wong SC. Modulation of essential (n-6): (n-3) fatty acid ratios alters fatty acid status but not bone mass in piglets. *J Nutr*. 2002; 132: 2667–72.
- Connor WE. Importance of n-3 fatty acids in health and disease. *Am J Clin Nutr*. 2000; 71: 171S–5S.
- Fernandes G. Attenuation of osteoporosis by n-3 lipids and soy protein. In: Holick MF (editor). *Nutrition and bone health*. New Jersey: Humana Press Inc.; 2004. pp. 575–92.
- Kang JX, Wang J, Wu L, *et al*. Transgenic mice: fat-1 mice convert n-6 to n-3 fatty acids. *Nature*. 2004; 427: 504.
- Hudert CA, Weylandt KH, Lu Y, *et al*. Transgenic mice rich in endogenous omega-3 fatty acids are protected from colitis. *Proc Natl Acad Sci USA*. 2006; 103: 11276–81.
- Bhattacharya A, Chandrasekar B, Rahman MM, *et al*. Inhibition of inflammatory response in transgenic fat-1 mice on a calorie-restricted diet. *Biochem Biophys Res Commun*. 2006; 349: 925–30.
- Rahman MM, Bhattacharya A, Banu J, *et al*. Conjugated linoleic acid protects against age-associated bone loss in C57BL/6 female mice. *J Nutr Biochem*. 2007; 18: 467–74.
- Bhattacharya A, Rahman M, Banu J, *et al*. Inhibition of osteoporosis in autoimmune disease prone MRL/MpJ-Fas(lpr) mice by N-3 fatty acids. *J Am Coll Nutr*. 2005; 24: 200–9.
- Nagy TR, Clair AL. Precision and accuracy of dual-energy X-ray absorptiometry for determining in vivo body composition of mice. *Obes Res*. 2000; 8: 392–8.
- Rahman MM, Kukita A, Kukita T, *et al*. Two histone deacetylase inhibitors, trichostatin A and sodium butyrate, suppress differentiation into osteoclasts but not into macrophages. *Blood*. 2003; 101: 3451–9.
- Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*. 1957; 226: 497–509.
- Bhattacharya A, Sun D, Rahman M, *et al*. Different ratios of eicosapentaenoic and docosahexaenoic omega-3 fatty acids in commercial fish oils differentially alter pro-inflammatory cytokines in peritoneal macrophages from C57BL/6 female mice. *J Nutr Biochem*. 2007; 18: 23–30.
- Kang JX. Fat-1 transgenic mice: a new model for omega-3 research. *Prostaglandins Leukot Essent Fatty Acids*. 2007; 77: 263–7.
- Lacey DL, Timms E, Tan HL, *et al*. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*. 1998; 93: 165–76.
- Kong YY, Yoshida H, Sarosi I, *et al*. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature*. 1999; 397: 315–23.
- Rahman MM, Bhattacharya A, Fernandes G. Docosahexaenoic acid is more potent inhibitor of osteoclast differentiation in RAW 264.7 cells than eicosapentaenoic acid. *J Cell Physiol*. 2008; 214: 201–9.
- Jilka RL, Hangoc G, Girasole G, *et al*. Increased osteoclast development after estrogen loss: mediation by interleukin-6. *Science*. 1992; 257: 88–91.
- Manolagas SC. Role of cytokines in bone resorption. *Bone*. 1995; 17: 63S–7S.
- Manolagas SC, Jilka RL. Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. *N Engl J Med*. 1995; 332: 305–11.
- Liu D, Yao S, Wise GE. Effect of interleukin-10 on gene expression of osteoclastogenic regulatory molecules in the rat dental follicle. *Eur J Oral Sci*. 2006; 114: 42–9.
- Owens JM, Gallagher AC, Chambers TJ. IL-10 modulates formation of osteoclasts in murine hemopoietic cultures. *J Immunol*. 1996; 157: 936–40.
- Takayanagi H, Ogasawara K, Hida S, *et al*. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. *Nature*. 2000; 408: 600–5.
- Kasukawa Y, Miyakoshi N, Srivastava AK, *et al*. The selective cyclooxygenase-2 inhibitor celecoxib reduces bone resorption, but not bone formation, in ovariectomized mice *in vivo*. *Tohoku J Exp Med*. 2007; 211: 275–83.
- Jimi E, Aoki K, Saito H, *et al*. Selective inhibition of NF-kappa B blocks osteoclastogenesis and prevents inflammatory bone destruction *in vivo*. *Nat Med*. 2004; 10: 617–24.
- Gago-Dominguez M, Yuan JM, Sun CL, *et al*. Opposing effects of dietary n-3 and n-6 fatty acids on mammary carcinogenesis: The Singapore Chinese Health Study. *Br J Cancer*. 2003; 89: 1686–92.

39. **Ma DW, Ngo V, Huot PS, et al.** n-3 polyunsaturated fatty acids endogenously synthesized in fat-1 mice are enriched in the mammary gland. *Lipids*. 2006; 41: 35–9.
40. **Leaf A, Weber PC.** A new era for science in nutrition. *Am J Clin Nutr*. 1987; 45: S1048–S53.
41. **Jia Q, Lupton JR, Smith R, et al.** Reduced colitis-associated colon cancer in Fat-1 (n-3 fatty acid desaturase) transgenic mice. *Cancer Res*. 2008; 68: 3985–91.
42. **Kang JX.** From fat to fat-1: a tale of omega-3 fatty acids. *J Membr Biol*. 2005; 206: 165–72.
43. **Kang ZB, Ge Y, Chen Z, et al.** Adenoviral gene transfer of Caenorhabditis elegans n-3 fatty acid desaturase optimizes fatty acid composition in mammalian cells. *Proc Natl Acad Sci USA*. 2001; 98: 4050–4.
44. **Kaneko H, Mehrotra M, Alander C, et al.** Effects of prostaglandin E2 and lipopolysaccharide on osteoclastogenesis in RAW 264.7 cells. *Prostaglandins Leukot Essent Fatty Acids*. 2007; 77: 181–6.
45. **Coon D, Gulati A, Cowan C, et al.** The role of cyclooxygenase-2 (COX-2) in inflammatory bone resorption. *J Endod*. 2007; 33: 432–6.
46. **Richards JB, Joseph L, Schwartzman K, et al.** The effect of cyclooxygenase-2 inhibitors on bone mineral density: results from the Canadian Multicentre Osteoporosis Study. *Osteoporos Int*. 2006; 17: 1410–9.
47. **Kesavalu L, Bakthavatchalu V, Rahman MM, et al.** Omega-3 fatty acid regulates inflammatory cytokine/mediator messenger RNA expression in Porphyromonas gingivalis-induced experimental periodontal disease. *Oral Microbiol Immunol*. 2007; 22: 232–9.
48. **Kelley VE, Ferretti A, Izui S, et al.** A fish oil diet rich in eicosapentaenoic acid reduces cyclooxygenase metabolites, and suppresses lupus in MRL-lpr mice. *J Immunol*. 1985; 134: 1914–9.
49. **Fernandes G, Bysani C, Venkatraman JT, et al.** Increased TGF-beta and decreased oncogene expression by omega-3 fatty acids in the spleen delays onset of autoimmune disease in B/W mice. *J Immunol*. 1994; 152: 5979–87.
50. **Fernandes G.** Effects of calorie restriction and omega-3 fatty acids on autoimmunity and aging. *Nutr Rev*. 1995; 53: S72–7; discussion S77–9.
51. **Endres S, Ghorbani R, Kelley VE, et al.** The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *N Engl J Med*. 1989; 320: 265–71.
52. **Schmocker C, Weylandt KH, Kahlke L, et al.** Omega-3 fatty acids alleviate chemically induced acute hepatitis by suppression of cytokines. *Hepatology*. 2007; 45: 864–9.
53. **Evans KE, Fox SW.** Interleukin-10 inhibits osteoclastogenesis by reducing NFATc1 expression and preventing its translocation to the nucleus. *BMC Cell Biol*. 2007; 8: 4.
54. **Das UN.** Nitric oxide as the mediator of the antiosteoporotic actions of estrogen, statins, and essential fatty acids. *Exp Biol Med*. 2002; 227: 88–93.
55. **Hirafuji M, Machida T, Tsunoda M, et al.** Docosahexaenoic acid potentiates interleukin-1beta induction of nitric oxide synthase through mechanism involving p44/42 MAPK activation in rat vascular smooth muscle cells. *Br J Pharmacol*. 2002; 136: 613–9.
56. **MacIntyre I, Zaidi M, Alam AS, et al.** Osteoclastic inhibition: an action of nitric oxide not mediated by cyclic GMP. *Proc Natl Acad Sci USA*. 1991; 88: 2936–40.
57. **Kasten TP, Collin-Osdoby P, Patel N, et al.** Potentiation of osteoclast bone-resorption activity by inhibition of nitric oxide synthase. *Proc Natl Acad Sci USA*. 1994; 91: 3569–73.
58. **Brandt ML, Hukkanen M, Umeda T, et al.** Bidirectional regulation of osteoclast function by nitric oxide synthase isoforms. *Proc Natl Acad Sci USA*. 1995; 92: 2954–8.
59. **Collin-Osdoby P, Rothe L, Bekker S, et al.** Decreased nitric oxide levels stimulate osteoclastogenesis and bone resorption both *in vitro* and *in vivo* on the chick chorioallantoic membrane in association with neoangiogenesis. *J Bone Miner Res*. 2000; 15: 474–88.
60. **Zheng H, Yu X, Collin-Osdoby P, et al.** RANKL stimulates inducible nitric-oxide synthase expression and nitric oxide production in developing osteoclasts. An autocrine negative feedback mechanism triggered by RANKL-induced interferon-beta via NF-kappaB that restrains osteoclastogenesis and bone resorption. *J Biol Chem*. 2006; 281: 15809–20.
61. **van't Hof RJ, Ralston SH.** Cytokine-induced nitric oxide inhibits bone resorption by inducing apoptosis of osteoclast progenitors and suppressing osteoclast activity. *J Bone Miner Res*. 1997; 12: 1797–804.
62. **van't Hof RJ, Ralston SH.** Nitric oxide and bone. *Immunology*. 2001; 103: 255–61.
63. **van't Hof RJ, Armour KJ, Smith LM, et al.** Requirement of the inducible nitric oxide synthase pathway for IL-1-induced osteoclastic bone resorption. *Proc Natl Acad Sci USA*. 2000; 97: 7993–8.
64. **Jamal SA, Browner WS, Bauer DC, et al.** Intermittent use of nitrates increases bone mineral density: the study of osteoporotic fractures. *J Bone Miner Res*. 1998; 13: 1755–9.
65. **Diwan AD, Wang MX, Jang D, et al.** Nitric oxide modulates fracture healing. *J Bone Miner Res*. 2000; 15: 342–51.
66. **Veihelmann A, Landes J, Hofbauer A, et al.** Exacerbation of antigen-induced arthritis in inducible nitric oxide synthase-deficient mice. *Arthritis Rheum*. 2001; 44: 1420–7.
67. **McCartney-Francis NL, Song X, Mizel DE, et al.** Selective inhibition of inducible nitric oxide synthase exacerbates erosive joint disease. *J Immunol*. 2001; 166: 2734–40.
68. **Cuzzocrea S, Mazzon E, Dugo L, et al.** Inducible nitric oxide synthase mediates bone loss in ovariectomized mice. *Endocrinology*. 2003; 144: 1098–107.
69. **Lo CJ, Chiu KC, Fu M, et al.** Fish oil decreases macrophage tumor necrosis factor gene transcription by altering the NF kappa B activity. *J Surg Res*. 1999; 82: 216–21.
70. **Novak TE, Babcock TA, Jho DH, et al.** NF-kappa B inhibition by omega-3 fatty acids modulates LPS-stimulated macrophage TNF-alpha transcription. *Am J Physiol Lung Cell Mol Physiol*. 2003; 284: L84–9.
71. **Zhao Y, Joshi-Barve S, Barve S, et al.** Eicosapentaenoic acid prevents LPS-induced TNF-alpha expression by preventing NF-kappaB activation. *J Am Coll Nutr*. 2004; 23: 71–8.
72. **Camandola S, Leonarduzzi G, Musso T, et al.** Nuclear factor kB is activated by arachidonic acid but not by eicosapentaenoic acid. *Biochem Biophys Res Commun*. 1996; 229: 643–7.
73. **Iotsova V, Caamano J, Loy J, et al.** Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. *Nat Med*. 1997; 3: 1285–9.
74. **Wong BR, Josien R, Lee SY, et al.** The TRAF family of signal transducers mediates NF-kappaB activation by the TRANCE receptor. *J Biol Chem*. 1998; 273: 28355–9.
75. **Darnay BG, Haridas V, Ni J, et al.** Characterization of the intracellular domain of receptor activator of NF-kappaB

- (RANK). Interaction with tumor necrosis factor receptor-associated factors and activation of NF-kappaB and c-Jun N-terminal kinase. *J Biol Chem*. 1998; 273: 20551–5.
76. **Caverzasio J, Higgins L, Ammann P.** Prevention of Trabecular Bone Loss Induced by Estrogen Deficiency by a Selective p38alpha Inhibitor. *J Bone Miner Res*. 2008; 23: 1389–97.
77. **Pacifici R.** Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. *J Bone Miner Res*. 1996; 11: 1043–51.
78. **Bays HE, Tighe AP, Sadovsky R, et al.** Prescription omega-3 fatty acids and their lipid effects: physiologic mechanisms of action and clinical implications. *Expert Rev Cardiovasc Ther*. 2008; 6: 391–409.
79. **Wang C, Harris WS, Chung M, et al.** n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. *Am J Clin Nutr*. 2006; 84: 5–17.
80. **Qi K, Fan C, Jiang J, et al.** Omega-3 fatty acid containing diets decrease plasma triglyceride concentrations in mice by reducing endogenous triglyceride synthesis and enhancing the blood clearance of triglyceride-rich particles. *Clin Nutr*. 2008; 27: 424–30.
81. **Calder PC.** Immunomodulation by omega-3 fatty acids. *Prostaglandins Leukot Essent Fatty Acids*. 2007; 77: 327–35.