

Vertebrae of Developing Fat-1 Mice Have Greater Strength and Lower N-6/N-3 Fatty Acid Ratio

BEATRICE Y. Y. LAU,* WENDY E. WARD,* JING X. KANG,† AND DAVID W. L. MA*,‡,¹

*Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, Toronto, Canada M5S 3E2; †Department of Medicine, Harvard Medical School, Boston, Massachusetts 02129; and ‡Department of Human Health and Nutritional Sciences, College of Biological Sciences, University of Guelph, Guelph, Canada N1G 2W1

Incorporation of dietary n-3 polyunsaturated fatty acids (PUFA) into bone may optimize bone development. The study objective was to use the *fat-1* mouse, a transgenic model that synthesizes n-3 PUFA from n-6 PUFA, to determine if bone mineral density (BMD) and biomechanical bone strength were favourably modulated by lowering the n-6/n-3 PUFA ratio in vertebrae. Male and female wild-type and *fat-1* mice were fed an AIN93-G diet containing 10% safflower oil from weaning through 12 weeks of age. Vertebrae BMD was determined by dual energy x-ray absorptiometry and peak load, a surrogate measure of fracture risk, was measured by a materials testing system. Vertebrae fatty acid composition was measured by gas liquid chromatography. At 12 weeks of age, vertebrae peak load was higher in *fat-1* mice compared to wild-type ($P = 0.026$). *Fat-1* mice also had lower n-6/n-3 PUFA ratio in vertebrae than wild-type ($P < 0.001$) and this ratio was negatively correlated with BMD and peak load ($P = 0.005$). Moreover, n-3 PUFA including α -linolenic acid, eicosapentaenoic acid and docosahexaenoic acid were positively correlated ($P < 0.05$) with BMD and peak load. Therefore, a lower vertebrae n-6/n-3 PUFA ratio is associated with stronger vertebrae and suggests a positive role for n-3 PUFA in bone development. *Exp Biol Med* 234:632–638, 2009

Key words: n-3 polyunsaturated fatty acids; bone mineral density; bone biomechanical strength; lumbar vertebrae; *fat-1* mice

Introduction

Vertebral compression fractures resulting from osteoporosis present a significant clinical challenge in 25–40% of postmenopausal women (1, 2). Associated with high rates of morbidity (3) and even mortality (4–6), prevention strategies are needed to reduce the occurrence of vertebral compression fractures. Provision of healthful foods and/or food components during development to encourage the acquisition of strong, healthy bones may provide an effective strategy (7). Emerging research suggests that this could be done through the balanced intake of dietary bioactive substances such as n-3 polyunsaturated fatty acids (PUFA), e.g., α -linolenic acid (ALA; 18:3 n-3), eicosapentaenoic acid (EPA; 20:5 n-3), and docosahexaenoic acid (DHA; 22:6 n-3), and n-6 PUFA, e.g., linoleic acid (LA; 18:2 n-6) and arachidonic acid (AA; 20:4 n-6). The degree of balance between the two types of fatty acids can be expressed by the n-6/n-3 ratio, and lowering the n-6/n-3 ratio has been implicated in the prevention and/or treatment of cardiovascular disease, arthritis, asthma, cancer, and mental illness (8).

Most research investigating fatty acid status and bone development has been conducted using rodent models. Their overall results suggest a beneficial effect of lowering the n-6/n-3 ratio on bone health. Feeding three-week-old male rats a diet of 1.2:1 n-6/n-3 ratio resulted in greater serum alkaline phosphatase activity, an indication of higher bone formation, and a greater bone formation rate in the tibia at 9 weeks of age compared to rats with a ratio of 23.8:1 (9). Femur bone mineral density (BMD) of male rats fed a corn and fish oil diet with a 1.4:1 n-6/n-3 ratio from three to eight weeks of age was higher than rats fed a soybean diet with a ratio of 7.1:1 (10).

Few studies have investigated the effects of PUFA on bone health in both males and females. Three-week-old rats that were fed a low dietary n-6/n-3 ratio containing 7% flaxseed oil for 12 weeks had greater whole body bone

The authors would like to thank Bristol-Myers Squibb/Mead Johnson for support from a Freedom to Discover Grant to the Department of Nutritional Sciences at University of Toronto and the Natural Sciences and Engineering Research Council of Canada (NSERC) for Discovery Grants to D.W.L. Ma and W.E. Ward.

¹ To whom correspondence should be addressed at Department of Human Health and Nutritional Sciences, Animal Science/Nutrition Building, Rm 243, College of Biological Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1. E-mail: davidma@uoguelph.ca

Received August 18, 2008.
Accepted February 5, 2009.

DOI: 10.3181/0808-RM-247
1535-3702/09/2346-0632\$15.00
Copyright © 2009 by the Society for Experimental Biology and Medicine

mineral content (BMC) and BMD compared to their counterparts fed 7% corn oil (11). The authors also noted that the effect of flaxseed oil on bone may be sex specific, as they did not observe greater whole body BMD in females (11). Feeding growing male and female mice flaxseed oil from weaning to 13 weeks of age, thus lowering the dietary n-6/n-3 ratio to 1:4, resulted in no change in BMD or bone strength in the femur and lumbar vertebrae (LV) compared to mice that were fed corn oil with an n-6/n-3 ratio of 57:1 (12). It is possible that a dietary n-6/n-3 ratio of 1:4 was simply too small to have a favourable effect on bone. Nevertheless, the authors did not observe any adverse outcomes as a result of the ratio.

It is possible that a lower n-6/n-3 ratio has site specific effects on the developing skeleton. The lumbar spine has a higher proportion of trabecular bone, which has a higher turnover rate and is more metabolically active, than cortical bone (13). Since trabecular bone may be more sensitive to dietary factors (13), subsequent changes in functional measures of bone may be more pronounced in the lumbar vertebrae than sites with a lower trabecular bone and higher cortical bone content, such as long bones (14). Despite the fact that trabecular bone may be more responsive to PUFA changes, studies have predominately focused on long bones rather than both long bones and vertebrae. In one study, male rats that were fed tuna oil, which has a low n-6/n-3 ratio and a high DHA content, had a higher LV BMD than rats that were fed corn oil (15). However, functional measures, including biomechanics of the lumbar vertebrae, were not determined.

Some studies have assessed the fatty acid status of the trabecular portion of long bones to provide some insight into potential mechanisms of action (16, 17), but these studies have not measured the fatty acid composition of lumbar vertebrae. Because of the insufficient data in the available literature on the fatty acid profile of lumbar vertebrae and gender effects of PUFA on bone health, the goal of the present study was to link PUFA status in the vertebrae with the BMC, BMD, and biomechanical strength in both genders using the *fat-1* mouse model. *Fat-1* mice can convert n-6 to n-3 PUFA through an n-3 desaturase encoded by the *fat-1* transgene (18). The specific study objectives were to determine if the *fat-1* gene could lower the n-6/n-3 PUFA ratio in the vertebrae of developing *fat-1* male and female mice, and if this translates to greater vertebrae mineral density and biomechanical strength. Because the *fat-1* gene can convert both carbon 18 and 20 PUFA (19), another goal of the study was to establish any potential relationships between specific n-6 and n-3 PUFA levels and measures of bone health.

Materials and Methods

Animals and Diet. C57BL/6 $\times$ C3H *fat-1* breeders were obtained from Dr. Jing X. Kang (Harvard Medical School). All mice were housed in temperature- and

humidity-controlled standard clean environmental conditions with a 12-hour light/dark cycle throughout the study. F1 progeny ($n = 12$ male wild-type mice, $n = 14$ male *fat-1* mice, $n = 11$ female wild-type mice, $n = 12$ female *fat-1* mice) were obtained by breeding male C57BL/6 $\times$ C3H *fat-1* males with female C57BL/6 mice from Charles River Laboratories (Saint-Constant, Quebec, Canada). Mice were weaned at three weeks of age and housed two to four per cage with littermates of the same gender. All mice including dams and progeny had *ad libitum* access to water and a modified AIN-93G diet with 10% safflower oil, rich in LA (Product #D04092701; Research Diets, New Brunswick, NJ). The fatty acid composition of the diet was reported previously (20).

Mice were euthanized with CO₂ at 12 weeks of age and their final body weights were measured. LV1–3, LV4 and LV5–6 were excised, cleaned of soft tissue, and stored at -80°C until analyses were performed. All experimental procedures respected the policies set out by the Canadian Council on Animal Care (21) and were approved by the Animal Ethics Committee at the University of Toronto, Toronto, Canada.

Fatty Acid Analysis of LV5–6. Vertebrae fatty acid composition was determined from total lipids of LV5–6. LV5–6 from each gender and genotype were excised at termination ($n = 4$ –5 to obtain statistical significance). The spinal cord was gently removed by forceps. Bone tissue was placed in liquid nitrogen and pulverized using a pestle and mortar. Lipids were extracted from the bone tissue with chloroform/methanol (2:1, v/v) as described by Folch *et al.* (22). The upper aqueous layer was removed and the lower chloroform fraction was evaporated under a gentle stream of nitrogen. 0.2 mg of lipids was transferred to a clean screw cap tube with Teflon cap and saponified using 2 ml of 0.5 M KOH in methanol for 1 h at 100°C . After cooling, 2 ml of hexane and 2 ml of 14% boron trifluoride in methanol (Cat. No. 1252; Sigma-Aldrich, Oakville, Ontario, Canada) were added to transesterify the fatty acids for 1 h at 100°C . 2 ml of water was then added to stop the methylation process, and the mixture was centrifuged at $\sim 100 \times g$ for 10 min to separate phases. The upper hexane layer containing the fatty acid methyl esters (FAMES) was extracted and then quantified on an Agilent 6890 gas chromatograph equipped with a flame ionization detector and separated on an Agilent J&W fused silica capillary column (DB-23; 30 m, 0.25 μm film thickness, 0.25 mm i.d.; Agilent, Palo Alto, CA, USA). Samples were injected in splitless mode. The injector and detector ports were set at 250°C . FAMES were eluted using a temperature program set initially at 50°C and held for 2 min, increased at $20^{\circ}\text{C}/\text{min}$ and held at 170°C for 1 min, increased at $3^{\circ}\text{C}/\text{min}$ and held at 212°C for 10 min to complete the run. The carrier gas was helium set to a 0.7 ml/min constant flow rate. Fatty acids were identified by comparing the relative retention times with those of a known standard mixture (GLC 463; Nu-Chek-Prep, Elysian, MN). The area under each peak was determined using

ChemStation (version B.01.01; Agilent). Values are expressed as the percent of total fatty acids.

BMC and BMD of LV1–3. Intact spines (LV1–3) were scanned in air at room temperature for determination of BMC and BMD using dual energy x-ray absorptiometry (pDEXA Sabre, Model 932937; Stratec Medizintechnik GmbH, Germany) and a specialized software program (Host software version 3.9.4, Scanner software version 1.2.0) (20). The percent coefficient of variance for LV1–3 area, BMC, and BMD was 1.6, 2.2, and 1.8, respectively.

Biomechanical Strength Testing of LV4. The biomechanical strength of LV4 was determined by conducting compression testing using a materials testing system (Model 4442 Universal Testing System; Instron Corp., Canton, MA) and a specialized software program (Instron Series IX Automated Materials Tester, Version 8.15.00; Instron Corp.) as previously described (23–25). LV4 were soaked in physiological saline (9 g NaCl/L) for 4 h at room temperature prior to testing. Spinal processes were removed. Immediately prior to testing, LV4 were weighed and the height, depth, and width of each vertebra were measured using electronic precision calipers. Each LV4 was positioned in anatomical position on the centre of a stainless-steel plate. A compressive force was applied by lowering a second stainless-steel plate at a constant rate of 2 mm/min until the vertebra was compressed. Each vertebra was visually inspected during each testing to ensure that it did not shift from its original position during the process of force application. Peak load, the maximum force that the bone withstands before fracture, was determined to be at the first peak on the load-deformation curve.

Statistical Analyses. Statistical analyses were performed using SigmaStat version 2.0 (Jandel Corp., San Rafael, CA). All data are expressed as mean $\pm$ standard deviation (SD). Data were analyzed by two-way ANOVA with gender and genotype as main effects, and the interaction of gender $\times$ genotype. The Tukey post-hoc test was used to determine the differences among the four groups when a statistical interaction of gender $\times$ genotype was observed. Pearson's correlation was performed to determine the relationships between LV5–6 fatty acid concentrations and LV1–3 BMD and LV4 peak load. Differences were considered significant at $P < 0.05$.

Results

Final Body Weight. At 12 weeks of age, male mice were significantly heavier ($P < 0.001$) than female mice, but the final body weight of wild-type mice was not significantly different from *fat-1* mice (data not shown).

Fatty Acid Composition of LV5–6. Gender, genotype, and their interaction did not result in any significant effects on the overall percent compositions of saturated, unsaturated, and monounsaturated fatty acids (Table 1). Male mice had a significantly greater percent composition of

total PUFA ($P = 0.003$) in their LV5–6 than female mice (Table 1).

However, both gender and genotype were modifiers of total n-6 PUFA percent composition: males and wild-type mice had higher total n-6 PUFA percent compositions than females and *fat-1* mice, respectively ($P < 0.001$ for both gender and genotype) (Table 1). Male mice also had higher percent compositions of LA ($P = 0.005$), eicosadienoic acid (20:2 n-6) ($P = 0.005$), and eicosatrienoic acid (20:3 n-6) ($P = 0.004$) than female mice (Table 1). No significant effects of gender, genotype, and their interaction were seen in the percent compositions of γ -linolenic acid (18:3 n-6) and docosadienoic acid (22:2 n-6) (Table 1). *Fat-1* mice had lower percent compositions ($P < 0.001$) of AA, adrenic acid (22:4 n-6), and docosapentaenoic acid (DPA, 22:5 n-6) than wild-type mice (Table 1). Furthermore, the percent composition of DPA was higher in males than females ($P = 0.004$) and an interaction between gender and genotype was also observed ($P = 0.021$) (Table 1).

Fat-1 mice had higher total n-3 PUFA in LV5–6 than wild-type mice ($P < 0.001$) (Table 1), and no gender or interaction by genotype was observed. No significant effects of gender and genotype were seen in the percent compositions of stearidonic acid (18:4 n-3) and eicosatrienoic acid (20:3 n-3) (Table 1). *Fat-1* mice had higher percent compositions ($P < 0.001$) of ALA, EPA, DPA (22:5 n-3), and DHA than wild-type mice (Table 1). The n-6/n-3 PUFA ratio of LV5–6 was lower in *fat-1* than wild-type mice ($P < 0.001$) (Table 1).

LV1–3 BMC and BMD. Male mice had significantly greater BMC ($P = 0.007$) and BMD ($P = 0.014$) than female mice (Table 2). Genotype and its interaction with gender did not have a significant effect on both BMC and BMD (Table 2).

LV4 Weight, Dimensions and Peak Load. There was no significant effect of gender, genotype and their interaction on the weight and dimensions of LV4 (Table 2). A significantly greater peak load ($P = 0.026$) was observed in *fat-1* mice compared to wild-type mice, although it was independent of gender (Table 2).

Correlations Between Fatty Acid Concentrations and Vertebrae Outcomes. BMD and peak load were significantly and positively correlated with the three n-3 PUFA that are potential modulators of bone metabolism: ALA ($r = 0.700$, $P < 0.001$ for BMD; $r = 0.469$, $P = 0.043$ for peak load), EPA ($r = 0.753$, $P < 0.001$ for BMD; $r = 0.708$, $P < 0.001$ for peak load), and DHA ($r = 0.756$, $P < 0.001$ for BMD; $r = 0.654$, $P = 0.002$ for peak load) (Table 3 and Fig. 1). Although LA was not correlated with LV1–3 BMD and LV4 peak load, significant negative correlations were observed with AA ($r = -0.773$, $P < 0.001$ for BMD, $r = -0.672$, $P = 0.002$ for peak load) (Table 3 and Fig. 2). Negative correlations were seen between the n-6/n-3 PUFA ratio and LV1–3 BMD ($r = -0.611$, $P = 0.005$) and LV4 peak load ($r = -0.611$, $P = 0.005$) (Table 3).

Table 1. Selected Fatty Acid Composition of LV5–6^a

Fatty acid	Percentage of total fatty acids				Two-way ANOVA		
	Males		Females		(P value)		
	Wild-type	<i>Fat-1</i>	Wild-type	<i>Fat-1</i>	Gender	Genotype	Interaction
n-6 PUFA							
18:2 n-6 (linoleic)	24.0 ± 1.9	23.7 ± 3.0	20.6 ± 1.9	20.7 ± 1.9	0.005	NS	NS
18:3 n-6 (γ-linolenic)	0.4 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	0.6 ± 0.4	NS	NS	NS
20:2 n-6 (eicosadienoic)	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.005	NS	NS
20:3 n-6 (eicosatrienoic)	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.04	0.4 ± 0.1	0.004	NS	NS
20:4 n-6 (arachidonic)	5.2 ± 0.3	2.7 ± 0.2	5.1 ± 0.4	2.9 ± 0.5	NS	<0.001	NS
22:2 n-6 (docosadienoic)	ND	ND	ND	ND	NS	NS	NS
22:4 n-6 (adrenic)	1.4 ± 0.2	0.7 ± 0.3	1.4 ± 0.1	0.6 ± 0.4	NS	<0.001	NS
22:5 n-6 (docosapentaenoic)	3.8 ± 0.4 ^a	0.6 ± 0.2 ^c	2.8 ± 0.5 ^b	0.4 ± 0.2 ^c	0.004	<0.001	0.021
n-3 PUFA							
18:3 n-3 (α-linolenic)	0.1 ± 0.1	0.2 ± 0.03	0.1 ± 0.1	0.2 ± 0.01	NS	<0.001	NS
18:4 n-3 (stearidonic)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	NS	NS	NS
20:3 n-3 (eicosatrienoic)	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.004	0.1 ± 0.1	NS	NS	NS
20:5 n-3 (eicosapentaenoic)	ND	0.3 ± 0.04	ND	0.3 ± 0.04	NS	<0.001	NS
22:5 n-3 (docosapentaenoic)	ND	0.7 ± 0.2	ND	0.6 ± 0.1	NS	<0.001	NS
22:6 n-3 (docosahexaenoic)	0.3 ± 0.3	3.2 ± 0.8	0.3 ± 0.4	2.8 ± 0.3	NS	<0.001	NS
Total saturates	41.8 ± 1.8	42.8 ± 6.3	44.9 ± 2.6	45.9 ± 2.3	NS	NS	NS
Total unsaturates	58.2 ± 1.8	57.2 ± 6.3	55.1 ± 2.6	54.1 ± 2.3	NS	NS	NS
Total monounsaturates	21.8 ± 1.2	23.3 ± 3.4	23.2 ± 2.6	23.8 ± 1.5	NS	NS	NS
Total polyunsaturates	36.4 ± 2.1	33.9 ± 3.8	31.9 ± 1.6	29.7 ± 2.0	0.003	NS	NS
Total n-6 PUFA	35.8 ± 1.9	29.2 ± 3.0	31.1 ± 1.6	26.0 ± 1.9	<0.001	<0.001	NS
Total n-3 PUFA	0.6 ± 0.2	4.7 ± 0.8	0.7 ± 0.4	4.3 ± 0.5	NS	<0.001	NS
n-6/n-3	68.4 ± 30.8	6.3 ± 0.9	57.3 ± 29.0	6.2 ± 0.8	NS	<0.001	NS

^a All data are expressed as mean ± SD; *n* = 5 per gender per genotype, except for female *fat-1*, where *n* = 4. Within a row, statistically different values are marked with different superscripts when a significant interaction was observed. NS, not significant (*P* ≥ 0.05); ND, not detected.

Discussion

LV of *fat-1* mice had a lower n-6/n-3 ratio, with a corresponding higher total n-3 PUFA percent composition and lower total n-6 PUFA percent composition, than wild-type mice (Table 1). Although no differences were seen in BMD between genotypes, *fat-1* mice had stronger LV than wild-type mice (Table 2). Vertebrae BMC and BMD of

male mice were higher than those of female mice, but this effect was modest as a gender-specific effect was not reflected by differences in vertebra dimensions or peak load.

Given the significant difference in LV peak load, LV BMD and peak load were correlated with the n-6/n-3 ratio, ALA, EPA, DHA, and AA to determine if changes in fatty acid composition were associated with vertebrae strength.

Table 2. Bone Mineral of LV1–3 and Dimensions and Peak Load of LV4^a

	Two-way ANOVA				(P value)		
	Males		Females		(P value)		
	Wild-type	<i>Fat-1</i>	Wild-type	<i>Fat-1</i>	Gender	Genotype	Interaction
LV1–3							
BMC (mg)	13.8 ± 2.3	15.3 ± 1.4	13.1 ± 2.1	12.6 ± 2.6	0.007	NS	NS
BMD (mg/cm ²)	47.2 ± 5.2	49.8 ± 4.1	44.9 ± 5.4	44.4 ± 6.5	0.014	NS	NS
LV4							
Weight (mg)	28.3 ± 4.4	29.3 ± 4.3	27.4 ± 4.7	26.3 ± 3.2	NS	NS	NS
Height (mm)	3.1 ± 0.2	3.2 ± 0.2	3.1 ± 0.4	3.0 ± 0.2	NS	NS	NS
Depth ^b (mm)	2.7 ± 0.1	2.8 ± 0.1	2.7 ± 0.1	2.7 ± 0.1	NS	NS	NS
Width ^c (mm)	2.9 ± 0.2	2.9 ± 0.2	2.9 ± 0.2	2.8 ± 0.2	NS	NS	NS
Peak load (N)	41.7 ± 6.6	47.0 ± 5.8	41.1 ± 6.3	44.1 ± 6.6	NS	0.026	NS

^a All data are expressed as mean ± SD; *n* = 12 male wild-type, *n* = 14 male *fat-1*, *n* = 11 female wild-type, *n* = 12 female *fat-1*. NS, not significant (*P* ≥ 0.05).

^b Depth refers to the anteroposterior width.

^c Width refers to the mediolateral width.

Table 3. Correlations Between Fatty Acid Concentrations and LV Outcomes^a

Variables	LV1–3 BMD		LV4 peak load	
	<i>r</i>	<i>P</i>	<i>R</i>	<i>P</i>
Fatty acid				
18:2 n-6 (linoleic)	0.0892	NS	0.00379	NS
18:3 n-3 (α-linolenic)	0.700	<0.001	0.469	0.043
20:5 n-3 (eicosapentaenoic)	0.753	<0.001	0.708	<0.001
n-6/n-3	−0.611	0.005	−0.611	0.005

^a *n* = 19, with 5 per gender per genotype, except 4 in female *fat-1*. NS, not significant (*P* ≥ 0.05).

Significant correlations were observed suggesting that a balance of fatty acids favoring n-3 over n-6 PUFA has a positive effect on bone development (Table 3; Figs. 1 and 2). These correlations are strong and therefore may give the impression that the effects of n-3 PUFA are very persuasive. It should be noted that the experiment was designed to specifically evaluate whether n-3 PUFA can impact on bone fatty acid composition and strength and to reduce experimental variation. Figures 1 and 2 show that the data is clustered within wild-type and transgenic genotypes. This

is not surprising given the use of wild-type and heterozygous transgenic littermates, which would reduce variability between animals. Whether the beneficial effects of n-3 PUFA on vertebrae can be extrapolated to humans requires further study.

We hypothesized that the *fat-1* gene would result in a lower n-6/n-3 ratio within the vertebrae. Indeed, the n-6/n-3 ratios of *fat-1* mice were 6.3 ± 0.4 for males and 6.2 ± 0.4 for females, and the ratios of wild-type mice were 68.4 ± 13.8 for males and 57.3 ± 13.0 for females. Comparing

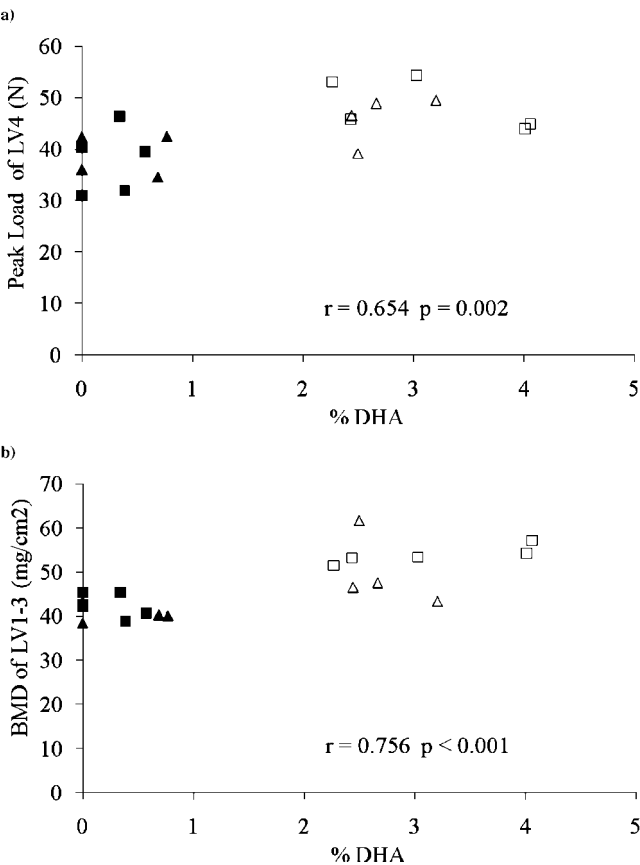


Figure 1. Correlations between DHA content and (a) peak load of LV4, (b) BMD of LV1–3. *n* = 19, with 5 per gender per genotype, except 4 for female *fat-1*. Symbols used: ■, male wild-type; □, male *fat-1*; ▲, female wild-type; △, female *fat-1*. DHA, an n-3 PUFA is observed to be positively correlated with both peak load of LV4 and BMD of LV1–3.

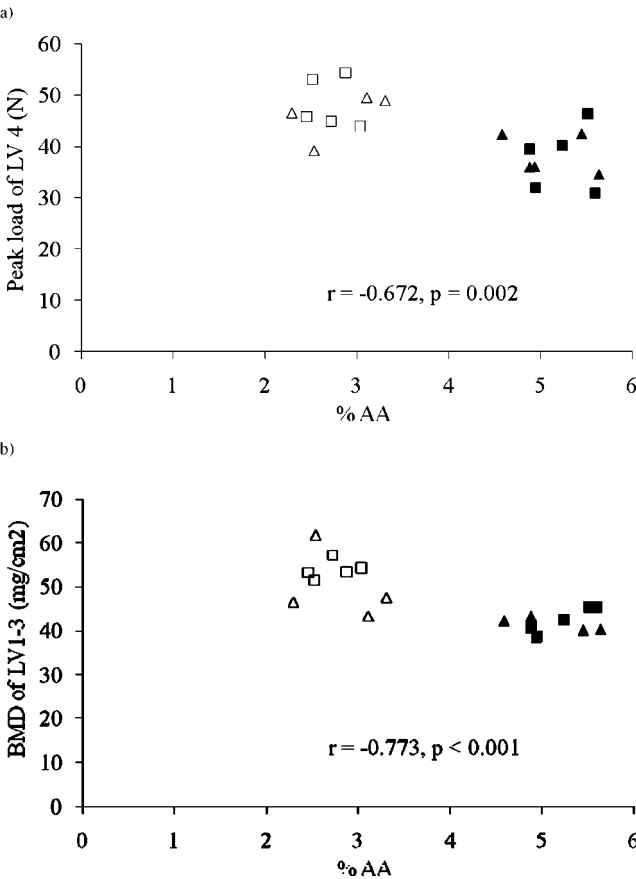


Figure 2. Correlations between AA content and (a) peak load of LV4, (b) BMD of LV1–3. *n* = 19, with 5 per gender per genotype, except 4 for female *fat-1*. Symbols used: ■, male wild-type; □, male *fat-1*; ▲, female wild-type; △, female *fat-1*. AA, an n-6 PUFA is observed to be negatively correlated with both peak load of LV4 and BMD of LV1–3.

these results with what we have previously reported in the femur (20), it seems that the n-6/n-3 ratio is site specific in bone, as the ratio was higher in the femur than the vertebrae within each genotype. Our data showed that the percent composition of total n-3 PUFA was similar between vertebrae and femur within each genotype; however, total n-6 PUFA was higher in the femur (20). These results suggest that different ratios of trabecular to cortical bone, as exemplified by vertebrae and femurs, may lead to changes in n-3 and n-6 PUFA incorporation and turnover within bone tissue. Further study is warranted to determine if this is indeed true.

Interestingly, results from middle-aged male rats showed no such pattern: the n-6/n-3 PUFA ratio in cortical bone was similar to that in trabecular bone within each dietary treatment (16, 26). However, it should be noted that this group analyzed trabecular bone of the tibia and not the vertebrae. Determination of the fatty acid composition of trabecular bone in the long bones of *fat-1* mice is technically challenging due to the small size of mouse bones. Rat bones are comparatively larger and thus allow for the isolation of trabecular bone.

To our knowledge, this was the first study conducted to examine changes in PUFA levels in vertebrae; hence we were unable to compare literature values at the present time. However, it is our hypothesis that the PUFA composition of *fat-1* and wild-type vertebrae is similar to that of animals fed a diet higher in n-3 PUFA and a diet devoid of n-3 PUFA, respectively, as we have previously shown in femurs of *fat-1* and wild-type mice (20). Femurs of wild-type mice had 45.0%, 0.7% and 96.8 in n-6 PUFA, n-3 PUFA, and the n-6/n-3 ratio, respectively. A dietary study where the control group was fed a safflower oil diet reported similar values (48.9%, 0.8% and 77.8) (17). Likewise, the fatty acid composition in *fat-1* femurs was comparable to that of the rats fed a high n-3 PUFA diet in the same study (17). The similarity of the two sets of results allows us to infer that the fatty acid levels achieved in the *fat-1* and wild-type vertebrae are attainable through diet manipulation. Thus, the *fat-1* mouse is a physiological and viable model for studying the effects of n-3 PUFA on bone metabolism. The relevance of the model is further strengthened by observations made by our group about the phenotype of the mouse. One concern of all transgenic mice is the unknown compensatory effects due to the insertion of a transgene, which may lead to the misinterpretation of study results. In our experience, outwardly, the *fat-1* mice do not have physical defects, produce viable young of expected litter size, life expectancy upwards of two years and have successfully transmitted the gene through more than eight generations.

As expected, the *fat-1* genotype altered the percent composition of ALA, AA, EPA, and all of the carbon 22 PUFA except for docosadienoic acid (22:2 n-6) in the vertebrae. Gender had a lesser effect than genotype, and only affected some of the n-6 PUFA, with males having a

greater percent composition than females. This is a departure from the results obtained in the femur where males had a greater percent composition of n-3 PUFA, including the long chain EPA and DHA, than females (20). It is possible that the ability to incorporate n-3 PUFA in cortical bone is gender specific.

Genotype affected lipid status and was associated with changes in the vertebrae peak load. Bone strength is influenced by both bone mineral and matrix proteins. A higher peak load can result from favourable changes in bone matrix structure, which is influenced by collagen crosslinks formation. As our results demonstrated differences in bone strength but not mineral content at the vertebrae, future research should investigate bone matrix composition in the skeleton of *fat-1* mice.

Compression analysis of mouse vertebrae is challenging given the small size and the irregular surface of the vertebral body endplate. This can introduce error into the measurement but given the sample size used in the present study, based on our previous studies, vertebral shape did not markedly affect the analyses (23–25). The study findings suggest that peak load is favourably enhanced by n-3 PUFA using one approach, and warrants further investigation using alternate methods to corroborate these results.

Because vertebrae n-6/n-3 ratio was affected by genotype alone, we postulated that PUFA status is associated with biomechanical strength in the vertebra. Indeed, we determined that the n-6/n-3 ratio was negatively correlated with peak load. Because various types of n-6 fatty acids are substrates for the desaturase encoded by the *fat-1* gene (19), correlations were performed between vertebrae outcomes and specific PUFA that have been identified as potential modulators of bone health. Thus, we selected ALA and LA, and the bioactive long chain PUFA: AA, EPA, and DHA. Not only peak load had the aforementioned negative correlation with n-6/n-3 ratio, its increase was also associated with a higher percent composition of the n-3 PUFA, ALA, EPA, and DHA; and a lower percent composition of the long chain n-6 PUFA, AA, while the essential n-6 PUFA, LA, was not correlated. These correlations followed a similar trend previously reported for the femur (20), where EPA composition was correlated with peak load of the femur neck, and both EPA and DHA composition were correlated with BMC. The correlations for vertebra were stronger and included more PUFA types, specifically ALA and AA. This finding supports our hypothesis that vertebrae may be more responsive to fatty acid changes than long bones. These fatty acids were also correlated with BMD. However, because correlations do not demonstrate causal effects, it is important that more research is done to assess the individual effects of ALA, EPA, DHA and AA on vertebrae.

In conclusion, the study findings have shown that the *fat-1* mouse model is useful to study the effect of PUFA on vertebrae status, and that a higher level of n-3 PUFA and a lower level of n-6 PUFA are associated with an improve-

ment in LV strength of growing animals. Whether this beneficial effect persists into old age, a time when there is a higher risk of vertebral fractures, warrants further investigation.

1. Melton LJ III, Kan SH, Frye MA, Wahner HW, O'Fallon WM, Riggs BL. Epidemiology of vertebral fractures in women. *Am J Epidemiol* 129:1000–1011, 1989.
2. Melton LJ III. Epidemiology of spinal osteoporosis. *Spine* 22:2S–11S, 1997.
3. Nevitt MC, Thompson DE, Black DM, Rubin SR, Ensrud K, Yates AJ, Cummings SR. Effect of alendronate on limited-activity days and bed-disability days caused by back pain in postmenopausal women with existing vertebral fractures. Fracture Intervention Trial Research Group. *Arch Intern Med* 160:77–85, 2000.
4. Cauley JA, Thompson DE, Ensrud KC, Scott JC, Black D. Risk of mortality following clinical fractures. *Osteoporos Int* 11:556–561, 2000.
5. Center JR, Nguyen TV, Schneider D, Sambrook PN, Eisman JA. Mortality after all major types of osteoporotic fracture in men and women: an observational study. *Lancet* 353:878–882, 1999.
6. Kado DM, Browner WS, Palermo L, Nevitt MC, Genant HK, Cummings SR. Vertebral fractures and mortality in older women: a prospective study. Study of Osteoporotic Fractures Research Group. *Arch Intern Med* 159:1215–1220, 1999.
7. Heaney RP, Abrams S, Wason-Hughes B, Looker A, Marcus R, Matkovic V, Weaver C. Peak bone mass. *Osteoporos Int* 11:985–1009, 2000.
8. Simopoulos AP. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother* 56:365–379, 2002.
9. Watkins BA, Li Y, Allen KG, Hoffmann WE, Seifert MF. Dietary ratio of (n-6)/(n-3) polyunsaturated fatty acids alters the fatty acid composition of bone compartments and biomarkers of bone formation in rats. *J Nutr* 130:2274–2284, 2000.
10. Green KH, Wong SC, Weiler HA. The effect of dietary n-3 long-chain polyunsaturated fatty acids on femur mineral density and biomarkers of bone metabolism in healthy, diabetic and dietary-restricted growing rats. *Prostaglandins Leukot Essent Fatty Acids* 71:121–130, 2004.
11. Weiler HA, Kovacs H, Nitschmann E, Bankovic-Calic N, Aukema H, Ogborn M. Feeding flaxseed oil but not secoisolariciresinol diglucoside results in higher bone mass in healthy rats and rats with kidney disease. *Prostaglandins Leukot Essent Fatty Acids* 76:269–275, 2007.
12. Cohen SL, Ward WE. Flaxseed oil and bone development in growing male and female mice. *J Toxicol Environ Health A* 68:1861–1870, 2005.
13. Malluche HH, Fraugere MC. Atlas of Mineralized Bone Histology. New York: Karger Publishing, pp136, 1986.
14. Baeksgaard L, Andersen KP, Hyldstrup L. Calcium and vitamin D supplementation increases spinal BMD in healthy, postmenopausal women. *Osteoporos Int* 8:255–260, 1998.
15. Kruger MC, Schollum LM. Is docosahexaenoic acid more effective than eicosapentaenoic acid for increasing calcium bioavailability? *Prostaglandins Leukot Essent Fatty Acids* 73:327–334, 2005.
16. Shen CL, Yeh JK, Rasty J, Chyu MC, Dunn DM, Li Y, Watkins BA. Improvement of bone quality in gonad-intact middle-aged male rats by long-chain n-3 polyunsaturated fatty acid. *Calcif Tissue Int* 80:286–293, 2007.
17. Watkins BA, Li Y, Seifert MF. Dietary ratio of n-6/n-3 PUFAs and docosahexaenoic acid: actions on bone mineral and serum biomarkers in ovariectomized rats. *J Nutr Biochem* 17:282–289, 2006.
18. Kang JX, Wang J, Wu L, Kang ZB. Transgenic mice: fat-1 mice convert n-6 to n-3 fatty acids. *Nature* 427:504, 2004.
19. Watts JL, Browse J. Genetic dissection of polyunsaturated fatty acid synthesis in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* 99:5854–5859, 2002.
20. Lau BY, Ward WE, Kang JX, Ma DW. Femur EPA and DHA are correlated with femur biomechanical strength in young fat-1 mice. *J Nutr Biochem* (in press), 2009.
21. Canadian Council on Animal Care. Guide to the care and use of experimental animals. Ottawa, 1984.
22. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226:497–509, 1957.
23. Ealey KN, Kaludjerovic J, Archer MC, Ward WE. Adiponectin is a negative regulator of bone mineral and bone strength in growing mice. *Exp Biol Med* 233:1546–1553, 2008.
24. Fonseca D, Ward WE. Daidzein together with high calcium preserve bone mass and biomechanical strength at multiple sites in ovariectomized mice. *Bone* 35:489–497, 2004.
25. Ward WE, Fonseca D. Soy isoflavones and fatty acids: effects on bone tissue postovariectomy in mice. *Mol Nutr Food Res* 51:824–831, 2007.
26. Shen CL, Yeh JK, Rasty J, Li Y, Watkins BA. Protective effect of dietary long-chain n-3 polyunsaturated fatty acids on bone loss in gonad-intact middle-aged male rats. *Br J Nutr* 95:462–468, 2006.