Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 22 (2011) 665–672
Journal of Nutritional Biochemistry

# Effects of long-term supplementation with omega-3 fatty acids on longitudinal changes in bone mass and microstructure in mice☆

Nicolas Bonnet*, Serge L. Ferrari

*Division of Bone Diseases, Department of Rehabilitation and Geriatrics, WHO Collaborating Center for Osteoporosis Prevention, Geneva University Hospital, 1211 Geneva 14, Switzerland*

Received 20 January 2010; received in revised form 21 April 2010; accepted 19 May 2010

**Abstract**

A diet rich in omega-3s has previously been suggested to prevent bone loss. However, evidence for this has been limited by short exposure to omega-3 fatty acids (FAs). We investigated whether a diet enriched in eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) for the entire adult life of mice could improve bone microstructure and strength. Thirty female mice received a diet enriched in DHA or EPA or an isocaloric control diet from 3 to 17 months of age. Changes in bone microstructure were analyzed longitudinally and biomechanical properties were analysed by a three-point bending test. Bone remodelling was evaluated by markers of bone turnover and histomorphometry. Trabecular bone volume in caudal vertebrae was improved by EPA or DHA at 8 months (+26.6% and +17.2%, respectively, compared to +3.8% in controls, $P=.01$), but not thereafter. Trabecular bone loss in the tibia was not prevented by omega-3 FAs (BV/TV −94%, −93% and −97% in EPA, DHA and controls, respectively). EPA improved femur cortical bone volume (+8.1%, $P<.05$) and thickness (+4.4%, $P<.05$) compared to controls. EPA, but not DHA, reduced age-related decline of osteocalcin (−70% vs. −83% in controls, $P<.05$). EPA and DHA increased leptin levels (7.3±0.7 and 8.5±0.5 ng $ml^{-1}$, respectively, compared to 4.5±0.9 ng $ml^{-1}$ in controls, $P=.001$); however, only EPA further increased IGF-1 levels (739±108 ng $ml^{-1}$, compared to 417±58 ng $ml^{-1}$ in controls, $P=.04$). These data suggest that long-term intake of omega-3 FA, particularly EPA, may modestly improve the structural and mechanical properties of cortical bone by an increase in leptin and IGF-1 levels, without affecting trabecular bone loss.


*Keywords:* Omega-3; Long-term; Bone mineral density; Microarchitecture; Strength; Leptin

## 1. Introduction

Loss of bone mineral density (BMD) and increased fracture risk are common characteristics of the aging process. As average life expectancy continues to increase, the need to develop new strategies to prevent osteoporosis and fragility fractures is growing [1]. Dietary modifications may be considered a primary target to minimize bone loss and fragility [2].

Several epidemiological studies have shown a lower incidence of fractures in Mediterranean countries [3,4] and have suggested an association between the Mediterranean diet, which is rich in omega-3 FA and contains relatively low amounts of omega-6 fatty acid (FA), and a lower rate of bone loss in older adults [5–7]. However, this association may be confounded by a number of lifestyle-related factors, such as the level of physical activity, sun exposure and ensuing vitamin D levels and calcium intake. Nevertheless, long chain polyunsaturated FAs and their metabolites, best known for their cardioprotective role, can regulate bone metabolism [8,9] and may potentially play a role in the prevention of osteoporosis.

The Western diet contains predominantly saturated FAs and omega-6 FAs, whereas the content of omega-3 FAs is fairly low. There is increasing evidence that a high omega-6/omega-3 ratio favours bone loss in both rodents [10,11] and humans [12]. Compared to corn oil, which is rich in n-6 FAs, fish oil (FO) is rich in omega-3 FA and has been shown to have beneficial effects on bone [13] and to prevent bone loss in ovariectomized rodents [14,15].

There are several biological pathways whereby FO may regulate bone metabolism, including decreased expression of prostaglandins ($PGE_2$) and of the major osteoclast differentiation factor, receptor activator for nuclear factor κ B ligand (RANKL), which is produced by both osteoblasts [16] and T cells [14,17]. Moreover, FO may modulate a number of pro-inflammatory cytokines, increase production of insulin-like growth factor-I (IGF-1) and improve calcium accretion in bone [13]. Consequently, it has been proposed that omega-3 FAs could prevent age-related bone loss by inhibiting osteoclastogenesis while improving osteoblast differentiation and function [18]. The effect of omega-3 FAs on the skeleton seems to be further dependent on the ratio between the two main omega-3 FAs: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA is the major long-chain omega-3 FA present in FO [19] and has been most commonly used for *in vivo* studies. EPA has also been suggested to prevent fat mass gain with age and could thereby be more efficient than DHA in preventing

☆ These studies were supported by a Swiss National Science Foundation grant no. 310030-130550/1 (to S.L.F).
* Corresponding author. Tel.: +41 22 382 99 68; fax: +41 22 382 99 73.
*E-mail address:* nicolas.bonnet@unige.ch (N. Bonnet).

0955-2863/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2010.05.006

trabecular bone loss [20]. In contrast, *in vitro* studies demonstrated that DHA inhibits osteoclast differentiation, activation and function by inhibiting $PGE_2$ more potently than EPA [21]. Moreover, omega-3 FAs, particularly DHA, enhance calcium absorption by modifying the lipid composition of the intestinal cell membrane and decreasing intestinal calcium loss [22–24]. Finally, the only *in vivo* experiment that compared the effects of EPA compared to DHA on the skeleton in ovariectomized rats showed a greater prevention of femoral and lumbar spine BMD loss over 4 months with DHA [25].

These observations provide a strong rationale for the study of long-term supplementation with polyunsaturated FA on the age-related changes in bone mass, microstructure and strength. With age, mice lose BMD [26] and cancellous bone volume and show decreased cortical thickness in the appendicular skeleton [27] These changes are similar to the age-related changes in bone mass and microstructure observed in humans [28]. However, cortical bone size and cross-sectional moment of inertia (CSMI) progressively increase with age in mice, reflecting the continuous growth in this species, contrasting with the decrease of CSMI observed as humans age [29,30].

This suggests that the influence of omega-3 FAs on the skeleton could differ according to age depending on the level of bone turnover. The present study was designed to test the hypothesis that a diet supplemented with omega-3 FAs (EPA or DHA) would reduce trabecular bone loss and/or cortical thinning that occurs as a result of ageing in intact animals. A 14-month study of female mice was undertaken to examine the long-term effect of the two main omega-3 FAs (EPA and DHA) on bone metabolism, microstructure and strength of the appendicular and axial skeleton. We show here that lifelong supplementation with EPA and, to a lesser extent, DHA exerts modest and nonsustained effects on trabecular bone. However, EPA and DHA supplementation does have some beneficial effects on cortical bone structure and strength.

## 2. Methods and materials

### 2.1. Animals

Thirty-eight, 8-week-old female C57BL/6J mice were obtained from Harlan. Weight-matched mice were housed in a laboratory animal care facility in cages (five mice/cage) and fed a standard pellet diet for 4 weeks. At 12 weeks of age, mice were divided into three dietary groups ($n$=10 per group) and fed diets enriched with EPA, DHA or with bovine fat for the control group (KLIBA NAFAG mod 2222 AIN93G; Supplemental Table S1). The eight remaining mice were sacrificed at 12 weeks of age to characterize the baseline bone phenotype at baseline. Isocaloric intakes were ensured by the addition of bovine fat (55% saturated FA, 40% mono-unsaturated FA and 10% polyunsaturated FA, i.e., omega-6; Supplemental Table S2) in the control diet, thus providing the same energy intake.

The diet enriched in DHA contains 0.13 mg of DHA and 0.04 mg of EPA per gram of diet, the diet enriched in EPA contains 0.14 mg of EPA and 0.02 mg of DHA per gram of diet, and the control diet contained only traces of DHA or EPA (Supplemental Tables S2 and S3). The percentage of omega-3 FA added to the diet was calculated on the basis of a 3.5 g of food intake per day per mouse, so that the FA supplementations correspond to those recommended for humans. The estimated omega-3 intake per mouse per day is therefore 0.455 mg of DHA and/or 0.49 mg of EPA, respectively. The omega-3 FA preparation contained trace amounts of vitamin E to preserve the biological activity of the FAs and to limit peroxidation. The stability of the diets has been controlled by the provider ISONATURA (ref. notice, the diet is certified ISO 9001 and stable for 3 years). Supplements were freshly prepared every 2 weeks, stored in aliquots at −20°C and provided daily. Food intakes by the mice were measured weekly. Mice were maintained on a 12-h light/dark cycle at an ambient temperature of 22–25°C. All mice were sacrificed at the age of 17 months, following the observation of two spontaneous deaths in the control group.

Animal procedures were approved by the University of Geneva School of Medicine Ethical Committee and by the State of Geneva Veterinarian Office.

### 2.2. In vivo measurement of BMD and body composition

Lean body mass, body fat mass, total body mass, femoral and spinal BMD ($g\ cm^{-2}$) were measured *in vivo* at baseline and after 3, 5.5, 8, 12.5 and 17 months by dual-energy X-ray absorptiometry (PIXImus2, GE lunar, Madison, WI, USA) [31].

### 2.3. In vivo measurement of morphology and microarchitecture

A high-resolution *in vivo* microcomputed tomography system (microCT Skyscan 1076, Skyscan, Aartselaar, Belgium) was used to scan the left tibia and the caudal vertebrae at 3, 8 and 17 months of age. The *in vivo* microCT system consists of an X-ray source and detector rotating around the animal bed. The microCT machine is equipped with a 100-kV X-ray source with a spot size of 5 μm. A scan lasted approximately 20 min, resulting in shadow projections with a pixel size of 10 μm. A modified Feldkamp algorithm, using undersampling to reduce noise, was applied to the scan data, resulting in reconstructed 3D data sets with a voxel size of 20 μm [32]. The mice were scanned at baseline (3 months) and after 8 and 17 months of diets. A detailed description and validation of the algorithm is published elsewhere [33]. Cortical and trabecular bones were separated manually with the CT analyzer software (Skyscan, Aartselaar, Belgium). For the trabecular bone regions, we assessed the bone volume fraction (BV/TV, %), trabecular thickness (TbTh, μm), trabecular number (TbN, $mm^{-1}$), trabecular spacing (TbSp, mm) and structural model index (SMI). The structure model index was measured to determine the prevalence of plate-like or rod-like trabecular structures, where 0 represents "plates" and 3 "rods" [34]. For cortical bone at the tibial midshaft, cortical tissue volume (CtTV, $mm^3$), cortical bone volume (CtBV, $mm^3$), the marrow volume (BMaV, $mm^3$) and the average cortical thickness (CtTh, μm) were measured.

### 2.4. Histomorphometry

To measure dynamic indices of bone formation, mice received subcutaneous injections of calcein (10 mg $kg^{-1}$, Sigma, Buchs, Switzerland) 9 and 2 days before euthanasia. Tibias were embedded in methyl-methacrylate (Merck, Switzerland), and 8-μm-thick transversal sections of the midshaft were cut with a Leica Corp. Polycut E microtome (Leica Microsystems AG, Glattburg, Switzerland) and mounted unstained for fluorescence evaluation. Five-micrometer-thick cross-sectional sections were cut in the middle of the tibia and histomorphometric measurements were performed on the endocortical and periosteal bone surfaces, using a Leica Corp. Q image analyser at 40× magnification. All parameters were calculated and expressed according to standard formulas and nomenclatures [35]: mineral apposition rate (MAR, μm $day^{-1}$), single-labelled cortical perimeter (sLS/BPm, %) and double-labelled cortical perimeter (dLS/BPm, %). Mineralizing perimeter per bone perimeter (MPm/BPm, %) was calculated by adding dLS/BPm and one-half sLS/BPm. Bone formation rate at the cortical region (BFR/BPm, $\mu m^3\ \mu m^{-2}\ day^{-1}$) was calculated as the product of MPm/BPm and MAR.

### 2.5. Testing of mechanical resistance

The night before mechanical testing, the bones were thawed slowly at 7°C and then maintained at room temperature. The fibula was removed and the length of the tibia (distance from intermalleolar to intercondylar region) was measured using a calliper with electronic digital display, and the middle of the shaft was determined. The tibia was then placed in the material testing machine on two supports separated by a distance of 9.9 mm, and load was applied to the middle of the shaft, thus creating a three-point bending test. The mechanical resistance to failure was tested using a servo-controlled electromechanical system (Instron 1114, Instron Corp., High Wycombe, UK) with actuator displaced at 2 mm $min^{-1}$. Both displacement and load were recorded. Ultimate force (maximal load, N), stiffness (slope of the linear part of the curve, representing the elastic deformation, N $mm^{-1}$) and energy (surface under the curve, N mm) were calculated. Ultimate stress (N $mm^{-2}$) and Young's modulus (MPa) were determined by the equations previously described by Turner and Burr [36].

### 2.6. Collection of serum

Blood from all mice was obtained by retro-orbital collection at baseline and after 6 and 14 months of diets. After centrifugation, serum was removed and stored at −80°C until analysis. Serum TRACP5b (tartrate-resistant alkaline phosphatase form 5b) and osteocalcin were measured according to manufacturers' instructions (SBA Sciences, Turku, Finland, and Biomedical Technologies, Inc., Stoughton, MA, USA, respectively). IGF-I and leptin were measured in serum by immunoenzymometric assay (IEMA) with a kit from Immunodiagnostic Systems (Paris, France) following the manufacturer's instructions.

### 2.7. Data analysis

We first tested the effects of diet by a one-way ANOVA repeat measurements with the type of diet as a factor. As appropriate, *post hoc* testing was performed using Fisher's protected least squares difference (PLSD). Time comparisons between diets with the control group were performed by two-way ANOVA. The $P$ of interaction between the diet and the time of experiment was only mentioned when significant. Differences were considered significant at $P<.05$. Data are presented as mean±S.E.M.

## 3. Results

### 3.1. Influence of diet on body weight, body composition and BMD

Mice were fed *ad libitum* with EPA, DHA or a control (isocaloric, bovine-fat enriched) diet from 3 to 17 months of age. Overall, no significant differences in food intake were observed between groups, indicating that all the diets were well tolerated (Fig. 1). However, the reduced food intake in controls vs. mice fed the omega-3 FAs that appeared towards the end of the study (Fig. 1), as well as the death of two control mice at 17 months of age, suggests that omega-3s could improve the vitality and longevity of mice. By 5.5 months of age, the EPA and DHA groups had a lower body weight compared to controls, but these differences were not sustained. At 17 months of age, animals fed EPA and DHA had a higher body weight than controls (+11% and +8%, respectively, $P<.05$). Percentage fat mass increased in all groups with age and was higher in the DHA group at the end of the study (+25% compared to controls, $P<.05$) with a similar trend in the EPA group.

Femoral BMD increased until 6 months of age, then plateaued in all groups, with no significant difference among diets, although BMD was slightly higher in the EPA group vs. controls (+4.6%, nonsignificant). Lumbar spine BMD increased up to 8 months of age in control and DHA groups, then decreased sharply in both groups, resulting in a loss of −15.3% in DHA and −1.4% in controls between 8 and 17 months of age. In contrast, in the EPA group spine BMD remained constant between 3 and 17 months of age and did not differ from the control group at the end of the study (Fig. 2).

### 3.2. Influence of diet on age-related changes in trabecular bone microarchitecture

Age-related changes in trabecular microarchitecture followed a similar pattern in the proximal tibia and caudal vertebrae, although in the tibia the loss of BV/TV and TbN occurred earlier and was more dramatic (Fig. 3). Hence, the apparent increase of trabecular thickness between 8 and 17 months in all groups may be an indirect reflection of the absence of thin trabeculae. In the tibia, BV/TV, TbN and TbTh did not significantly differ between diet groups at any time point. In contrast, the age-related changes in trabecular microarchitecture in caudal vertebrae were influenced by diet. Vertebral BV/TV and TbN increased between 3 and 8 months of age with EPA and DHA but not in controls (interaction between age and diet, $P<.05$, by 2F-ANOVA). Trabecular thickness also slightly increased between 3 and 8 months with EPA or DHA vs. controls (Supplemental Table S4). However, at 17 months of age, no statistically significant interaction remained between age and diet, indicating that the early benefits of EPA and DHA on vertebral trabecular bone structure did not persist.

These age-related changes were confirmed by *ex vivo* microCT. Cancellous bone structure in distal femur and lumbar spine was significantly lower at 17 months vs. 3 months in all groups, without significant effects from omega-3 FA diets (Table 1).

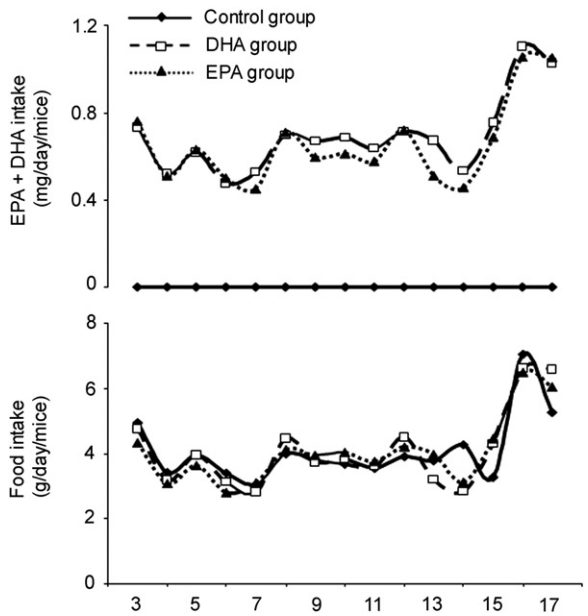


Fig. 1. Food and EPA+DHA intakes in controls, DHA and EPA FA groups in mice from 3 to 17 months of age.

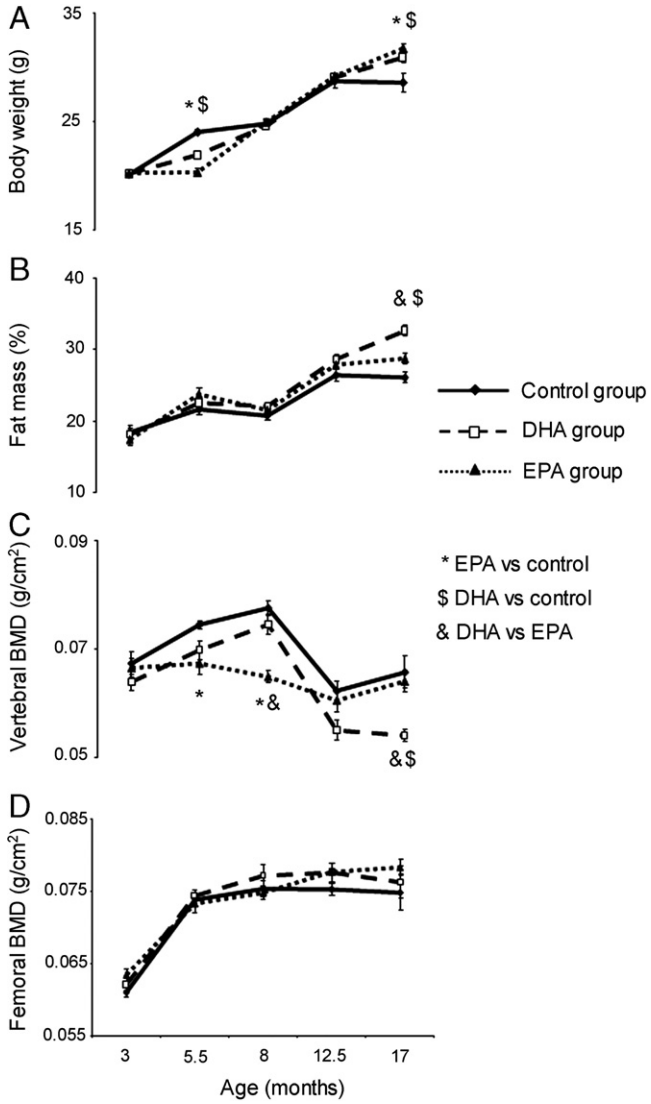


Fig. 2. Effects of DHA and EPA FAs on body composition and vertebral and femoral BMD. Bars show the mean (±S.E.M.) measured at 3, 5.5, 8, 12.5 and 17 months of age in each group (DHA, EPA and control, $n=10$ mice per group). (A) Body weight. (B) Percentage of fat mass. (C) BMD in vertebrae. (D) BMD in total femur. *P* value for differences between n-3 FA groups (1F-ANOVA, diet). *, EPA vs. control; $, DHA vs. control; &, EPA vs. DHA by *post hoc* Fisher's PLSD. Means±S.E.M.

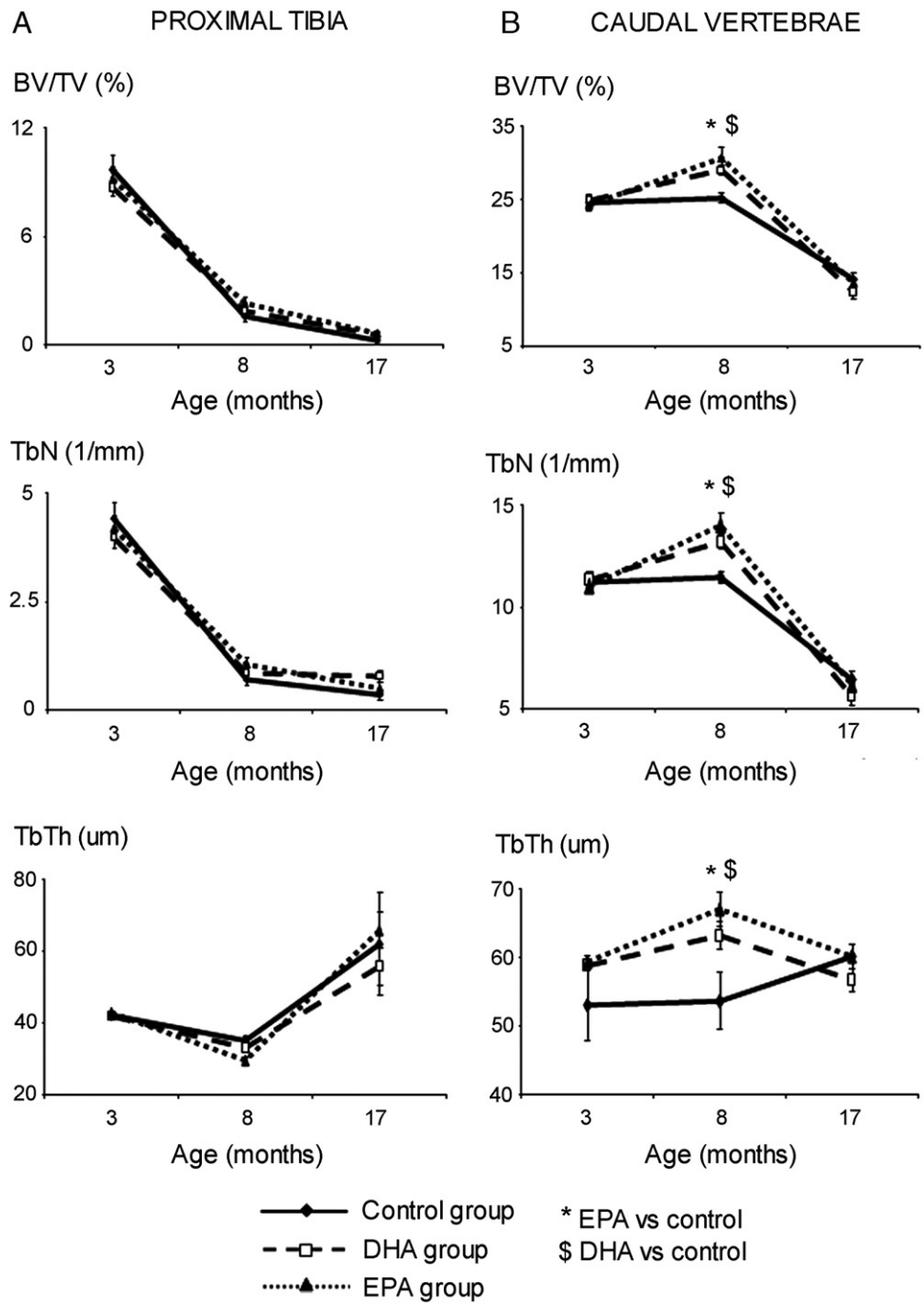


Fig. 3. Effects of DHA and EPA FAs on trabecular bone microarchitecture of the vertebrae and proximal tibia. Bars show the mean (±S.E.M.) measured at 3, 8 and 17 months of age in each group (DHA, EPA and control, $n$=10 mice per group). (A) Trabecular bone microarchitecture of the proximal tibia. (B) Trabecular bone microarchitecture of the vertebrae. Bone volume on tissue volume (BV/TV), trabecular number (TbN, 1/mm) and trabecular thickness (TbTh, μm). *P* value for differences between n-3 FA groups (1F-ANOVA, diet). *, EPA vs. control; $, DHA vs. control by *post hoc* Fisher's PLSD. Means±S.E.M.

### *3.3. Influence of diet on age-related changes in cortical bone microarchitecture*

Analysis of the midtibial diaphysis is shown in Fig. 4. Tissue volume (TV) and CSMI increased continuously from 3 to 17 months of age in all groups, consistent with continuous periosteal bone apposition (Fig. 4 and Table 1). Cortical bone volume significantly increased between 3 and 8 months of age independently of the diet and then remained constant to 17 months of age with DHA and control diets. However, CtBV increased further with EPA supplementation. Moreover, CtTh, which slightly increased in all groups between 3 and 8 months and then decreased from 8 to 17 months of age, decreased less in the EPA group. As a result, at 17 months of age, CtBV and CtTh were significantly higher in the EPA vs. the control group (Fig. 4).

These age-related changes were confirmed by *ex vivo* microCT of the femur where CtTV and CtBV were greater at 17 months than at 3 months (Table 1). Moreover, at 17 months of age, microCT confirmed a greater CtTV, CtBV and CtTh in the EPA compared to the control group (+6.3%, +8.1% and +4.4%, respectively, $P$<.05). The

Table 1
Influence of DHA and EPA on femoral and vertebrae trabecular and cortical microarchitecture

| | Baseline | Control | DHA | EPA |
|---|---|---|---|---|
| Distal femur | | | | |
| BV/TV (%) | 7.13±0.4 | 0.41±0.1 # | 0.56±0.1 # | 0.60±0.1 # |
| Conn D | 49.61±6.9 | 0.29±0.1 # | 0.21±0.1 # | 0.10±0.06 # |
| TbN (1/mm) | 4.06±0.1 | 1.55±0.1 # | 1.44±0.1 # | 2.23±0.65 # |
| TbTh (μm) | 41.2±0.7 | 59.6±7.4 # | 52.7±5.1 | 67.1±7.1 # |
| TbSp (mm) | 0.246±0.006 | 0.662±0.04 # | 0.709±0.036 # | 0.540±0.123 # |
| SMI | 2.96±0.05 | 3.67±0.2 # | 3.83±0.3 # | 3.32±0.2 |
| TV ($mm^3$) | 1.05±0.019 | 1.43±0.02 # | 1.52±0.02 # * | 1.52±0.02 # * |
| BV ($mm^3$) | 0.45±0.01 | 0.61±0.01 # | 0.63±0.01 # | 0.66±0.01 # * |
| CTh (μm) | 171±2.3 | 203±2.6 # | 203±3.3 # | 212±3.4 # * |
| BMaV ($mm^3$) | 0.60±0.009 | 0.825±0.02 # | 0.891±0.02 # * | 0.859±0.01 # |
| Vertebral body | | | | |
| BV/TV (%) | 19.4±0.7 | 14.2±0.8 # | 13.2±1.01 # | 13.5±0.5 # |
| Conn D | 159.85±5.95 | 32.56±3.0 # | 27.97±2.47 # | 29.35±3.63 # |
| TbN (1/mm) | 4.35±0.10 | 2.72±0.05 # | 2.65±0.10 # | 2.86±0.11 # |
| TbTh (μm) | 46.4±0.5 | 59.1±1.7 # | 56.7±1.7 # | 60.1±1.7 # |
| TbSp (mm) | 0.227±0.005 | 0.364±0.008 # | 0.385±0.01 # | 0.370±0.01 # |
| SMI | 0.99±0.07 | 1.13±0.07 | 1.39±0.12 # | 1.47±0.18 # |
| CTh (μm) | 73.3±1.1 | 84.4±3.2 # | 78.4±2.0 # | 85.8±2.2 # $ |

Values are means±S.E.M.
Cancellous bone microarchitecture was evaluated at the distal femur and vertebral body, and cortical microarchitecture at the midshaft femur by *ex vivo* microCT at 12 weeks of age (baseline, $n$=10 mice) and 17 months of age for control, DHA and EPA groups ($n$=10 per group). $P$ value for differences between n-3 FA groups (2F-ANOVA, diet and time). #, vs. baseline; *, vs. control; $, vs. DHA, by *post hoc* Fisher's PLSD.

DHA group presented an increase in CtTV (+6.3%, $P$<.05) without significant difference in CtBV and CtTh. This was partly due to greater bone marrow volume than in controls, indicating a higher endocortical bone resorption with the DHA diet (Table 1).

### 3.4. Influence of diet on biomechanical properties of the tibia

To evaluate whether differences in cortical architecture translated into differences in bone strength, three-point bending tests were performed on the tibias. In controls, the ultimate force, ultimate stress, elastic energy, stiffness and Young's modulus were higher at 17 months than at 3 months (+45.5%, +180%, +43.9%, +22.2% and +109.3%, respectively, all $P$<.01), consistent with the age-related increase of CtBV and CSMI.

Tibial bone strength was also significantly greater at 17 months than at 3 months of age in EPA and DHA groups. Moreover, at 17 months of age, EPA had significantly improved the ultimate force, ultimate stress and plastic energy compared to controls (+12.3%, +28.5% and +29.9%, respectively, all $P$<.05). Consistent with the structural data, DHA only significantly increased the ultimate stress compared to controls (+27.3%, $P$<.05) (Table 2).

### 3.5. Histomorphometrical and biochemical indices of bone turnover

Osteocalcin levels decreased steadily with age in controls, and long-term EPA and DHA supplementation did not affect this. Nevertheless, osteocalcin at 17 months of age was significantly higher in EPA and DHA compared to controls, although the absolute levels remained low (Fig. 5). In controls, TRAPc5b levels were stable from 3 to 8 months of age but then increased significantly from 8 to 17 months of age. EPA but not DHA intake resulted in significantly decreased TRAPc5b levels compared to controls between 3 and 8 months. However, at 17 months of age, both EPA and DHA displayed a higher TRAP level compared to controls (Fig. 5).

Bone-forming indices on cortical bone surfaces were evaluated by histomorphometry. At the periosteal (Ps) but not at the endocortical (Ec) surface, EPA significantly increased the MAR and bone forming rate (BFR/BPm) compared to controls (+315% and +472%, respectively, $P$<.05). The periosteal mineralizing perimeter (Ps MPm/BPm) in the EPA group also remained constant compared to baseline, whereas it tended to decrease in the control and DHA groups (Table 3). In contrast, DHA was associated with a significantly higher Ec MAR compared to controls (+112%, $P$<.05), without significant effects on Ec BFR/BPm and Ec MPm/BPm.

In summary, the analysis of biochemical and histomorphometry markers of bone turnover indicated that, in the long-term, omega-3 FAs, and particularly EPA, promoted cortical bone remodelling.

### 3.6. Influence of diet on age-related changes in leptin and IGF-1 levels

In order to further examine the potential mechanisms mediating the effects of omega-3 FAs on bone turnover, we measured two factors known to play an important role in bone remodelling, namely, leptin and IGF-1.

Leptin levels increased significantly between 3 and 8 months of age in the control group. In the EPA group, leptin levels also increased

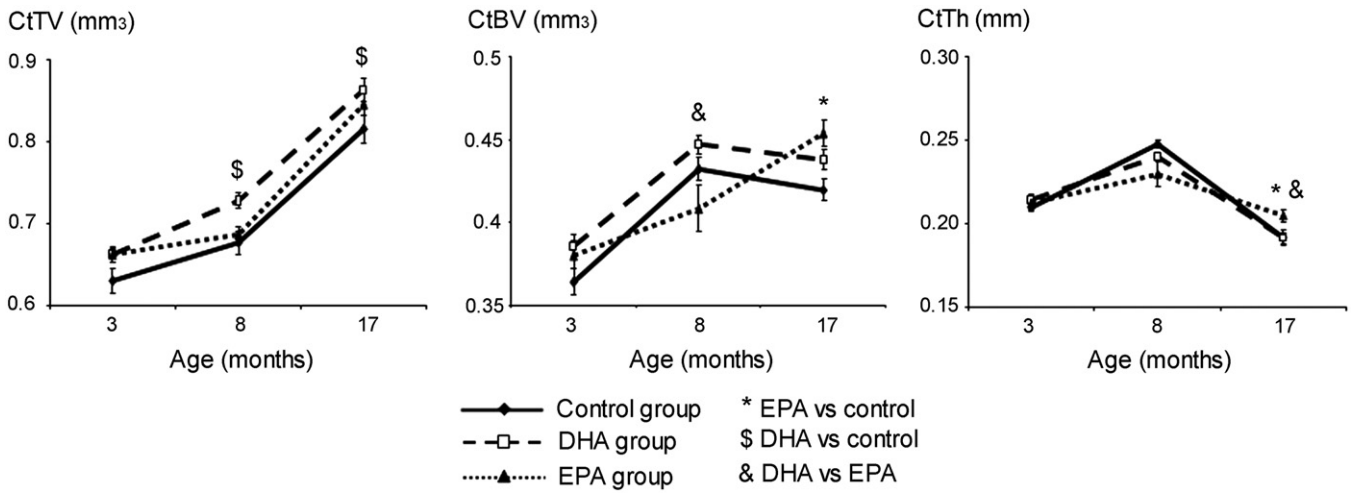


Fig. 4. Effects of DHA and EPA FAs on cortical bone microarchitecture of the midshaft tibia. Bars show the mean (±S.E.M.) measured at 3, 8 and 17 months of age in each group (DHA, EPA and control, $n$=10 mice per group). Cortical bone tissue volume (CtTV), cortical bone volume (CtBV), cortical thickness (CtTh). $P$ value for differences between n-3 FA groups (1F-ANOVA, diet). *, EPA vs. control; $, DHA vs. control; &, EPA vs. DHA by *post hoc* Fisher's PLSD. Means±S.E.M.

Table 2
Influence of DHA and EPA on tibiae biomechanical properties evaluated by the three-point bending test

| | Baseline | Control | DHA | EPA |
|---|---|---|---|---|
| Tibia | | | | |
| Ultimate force (N) | 12.76±0.7 | 18.83±0.6 # | 19.37±0.8 # | 21.15±0.7 * # |
| Ultimate stress (N mm$^{-2}$) | 740.0±156 | 2075.6±100 # | 2641.9±231 * # | 2667±134 * # |
| Elastic energy (N mm) | 2.23±0.1 | 3.21±0.2 # | 3.37±0.3 # | 2.92±0.2 |
| Plastic energy (N mm) | 5.37±0.2 | 5.62±0.4 | 6.49±0.6 | 7.3±0.8 * # |
| Stiffness (N mm$^{-1}$) | 43.83±3.1 | 56.36±2.1 # | 58.89±3.6 # | 59.19±2.8 # |
| CSMI (mm$^4$) | 0.092±0.028 | 0.156±0.002 # | 0.177±0.007 * # | 0.175±0.006 * # |
| Young's modulus (MPa) | 35,383.5±6603 | 74,062.3±2964 # | 87,484.2±6478 # | 86,496.7±6802 # |

Values are means±S.E.M.
Cortical bone mechanical properties of the tibiae were evaluated by three-point bending test at 12 weeks of age (baseline, $n$=10 mice) and at 17 months of age for control, DHA and EPA groups ($n$=10 per group). $P$ value for differences between n-3 FA groups (2F-ANOVA, diet and time). #, vs. baseline; *, vs. control by *post hoc* Fisher's PLSD.

with age, most dramatically between 8 and 17 months of age, whereas in the DHA group, leptin levels increased continuously. Hence, at the end of the experiment, leptin levels were significantly higher in the DHA and EPA groups compared to controls (Fig. 5). A statistically significant interaction occurred between diet and age ($P$=.02), indicating that patterns of age-related changes in leptin levels differ in mice receiving omega-3 FAs (EPA or DHA) compared to controls. As expected, age-related changes in leptin levels and fat mass were highly correlated ($r^2$=0.64, $P$<.0001).

IGF-1 levels were also significantly higher at 17 months with EPA supplementation compared to controls (739±108 vs. 417±58 ng ml$^{-1}$, $P$<.05) or DHA (396±84 ng ml$^{-1}$, $P$<.05) groups.

## 4. Discussion

Our study shows that omega-3 FAs exert complex effects on trabecular and cortical bone mass and structure, which vary with age and the type of omega-3 FAs. Most studies on the effects of omega-3 FAs on bone metabolism in growing and adult mice have used short-term supplementation and cross-sectional measurements of bone parameters. In contrast, this study was long-term and examined *in vivo* longitudinal changes in bone mass and microstructure. This allowed differentiation of the effects of omega-3 FAs on the skeleton in young vs. older adults.

Mice on a control diet increased body weight between 3 and 8 months of age. Meanwhile, trabecular bone microarchitecture was dramatically reduced in the long bones, while CtTV, bone volume and thickness increased continuously. Bone turnover was characterized by a marked decrease in osteocalcin levels, without significant changes in Trap5b levels. Our longitudinal data indicating that trabecular bone volume in the long bones declines more rapidly and more profoundly than in the vertebrae are consistent with previous cross-sectional studies [27,37]. This observation may be attributed to mechanical forces in the long bones being efficiently transmitted to the cortex; thus trabecular bone loss could be an adaptative, physiological mechanism to cortical bone expansion by eliminating trabeculae which participate little in maintaining bone strength [38]. However, in the vertebral body, load-transfer characteristics are different as a higher fraction of the load is carried by trabecular bone [38]. This may explain why cancellous bone is better preserved in the vertebrae at an early age. Additionally, increased adipogenesis from mesenchymal stem cells occurs at the expense of the osteoblast lineage; thus with age there is greater fat gain and a lower osteogenic potential in long bones than in vertebrae [39].

During early adulthood (up to 8 months of age), omega-3 FAs had no major influence on cancellous bone in the metaphyseal region of long bones nor on cortical bone mass. However, both EPA and DHA improved the cancellous bone volume in caudal vertebrae. EPA may exert these positive effects by decreasing leptin and TRAP levels. Indeed, leptin has been shown to induce trabecular bone remodelling in vertebrae by triggering β-adrenergic signalling in the CNS, which in turn increases RANKL expression in osteoblasts [40,41]. The effects of omega-3 FAs on bone resorption could also be mediated by a decrease in inflammatory cytokines such as IL-6, IL1-β or TNF-1 [42].

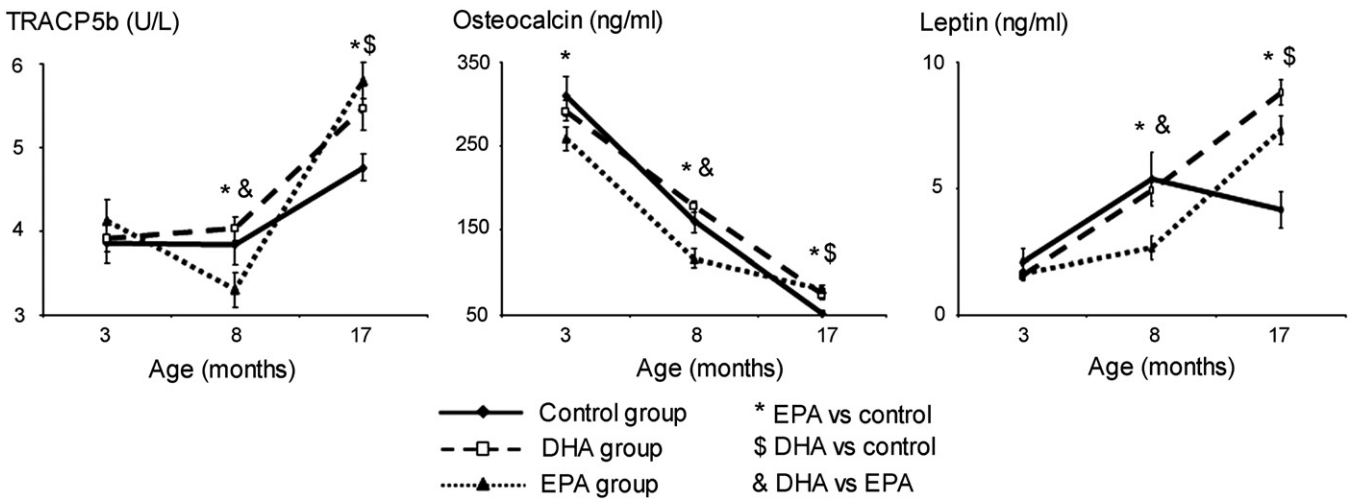


Fig. 5. Effects of DHA and EPA FAs on bone markers and leptin levels. Bars show the mean (±S.E.M.) measured at 3, 8 and 17 months of age in each group (DHA, EPA and control, $n$=10 mice per group). TRACP5b (tartrate-resistant alkaline phosphatase form 5b). $P$ value for differences between n-3 FA groups (1F-ANOVA, diet). *, EPA vs. control; $, DHA vs. control; &, EPA vs. DHA by *post hoc* Fisher's PLSD. Means±S.E.M.

Table 3
Influence of EPA and DHA on turnover indices at periosteal and endocortical bone surfaces

| | Parameters | Baseline | Control | DHA | EPA |
|---|---|---|---|---|---|
| Periosteal | Ps MAR (μm day$^{-1}$) | 0.22±0.11 | 0.13±0.09 | 0.15±0.09 | 0.57±0.10 *$#$ |
| | Ps BFR/BPm (μm$^2$ μm$^{-1}$ day$^{-1}$) | 0.004±0.004 | 0.018±0.018 | 0.025±0.018 | 0.103±0.032 *$#$ |
| | Ps MPm/BPm (%) | 0.18±0.09 | 0.09±0.04 | 0.05±0.04 | 0.17±0.05 |
| Endocortical | Ec MAR (μm day$^{-1}$) | 0.32±0.11 | 0.43±0.17 | 0.91±0.07 *$#$ | 0.57±0.21 |
| | Ec BFR/BPm (μm$^2$ μm$^{-1}$ day$^{-1}$) | 0.010±0.005 | 0.001±0.001 $#$ | 0.002±0.001 $#$ | 0.002±0.001 $#$ |
| | Ec MPm/BPm (%) | 0.26±0.06 | 0.16±0.06 | 0.21±0.04 | 0.22±0.05 |

Values are means±S.E.M.
Turnover indices of the tibiae were evaluated by histomorphometry at 12 weeks of age (baseline, $n$=10 mice) and at 17 months of age for control, DHA and EPA groups ($n$=10 per group). $P$ value for differences between n-3 FA groups (2F-ANOVA, diet and time). $#$, vs. baseline; *, vs. control; $, vs. DHA, by *post hoc* Fisher's PLSD.

From 8 to 17 months of age a remodelling imbalance, shown by a decrease in osteocalcin but an increase of TRAP activity, resulted in all groups losing trabecular bone microarchitecture both in the long bones and in the vertebrae. The favourable effects of omega-3 FAs observed earlier on vertebrae trabecular bone microarchitecture were not maintained. This suggests that additional mechanisms of trabecular bone remodelling become effective with aging on which omega-3 FAs have little or no effect. Cortical tissue volume continued to increase from 8 to 17 months, whereas the cortical bone volume and thickness remain constant as the bone marrow volume also expanded. These data are consistent with previous studies showing continued periosteal expansion with aging [27,29,37] but also a stabilisation of cortical bone volume and thickness through an increase of endocortical resorption [43]. Interestingly, EPA continuously stimulated the increase of CtBV and, to a lesser extent, CtTh, thereby improving the mechanical properties of the tibia. In rats, Reinwald et al. [44] also found a higher cross-sectional geometry and second moment of inertia of the femur with omega-3 intake. The current study indicates that long-term EPA/DHA supplementation increases bone formation (MAR) at periosteal surfaces which, together with modestly increased levels of osteocalcin at 17 months, suggests an anabolic effect of these FAs.

These effects on cortical bone paralleled a continuous increase of leptin levels that strongly correlated with fat mass gain in the DHA and EPA groups after 8 months of age. Leptin could be a mediator of the effects of omega-3 on cortical bone as it is known to improve cortical bone formation by a direct effect on osteoblasts [45,46]. Additionally, peripheral administration of leptin has been shown to increase cortical bone mass in obese (ob/ob) mice [47] and increase bone strength in adult male mice [48]. Other adipokines could also be involved, such as resistin which is usually decreased and adiponectin which is increased by omega-3 intake [49]. However, their precise role on bone remodelling remains to be elucidated [50]. IGF-1 levels were significantly increased at 17 months of age with EPA supplementation, paralleling higher indices of bone formation at periosteal surfaces. This data supports the study by Watkins et al. [51] showing higher IGF-1 levels in chicks fed diets rich in omega-3s [52]. IGF-1 administration is known to increase osteoblast recruitment and activity in the periosteum without any change in bone formation drifts at endocortical surfaces [53,54]. DHA but not EPA induced a higher MAR activity at the endocortical compartment. This supports the hypothesis that DHA could decrease bone loss mainly by increasing calcium accretion in bone, which is known to improve bone formation at the endocortical surface rather than at the periosteum [55]. These two last observations are further supported by increased bone formation at the cortical rather than at the trabecular compartment after a supplementation with omega-3 FAs [56]. However, DHA seemed to not only increase bone formation at the endocortical compartment but also to increase bone resorption, as suggested by an increase in bone marrow volume. Together these data indicate that omega-3 FAs increase bone remodelling at the endocortical compartment and promote periosteal bone formation. Some studies have reported a negative effect of omega-3 FAs on bone, depending on the intake. Hence *in vivo* a high dose of omega-3 FAs (1 g kg$^{-1}$ body weight per day during 9 weeks) decreased lumbar spine and femoral BMD and increased bone resorption (CTx) compared to ovariectomized and controls rats fed with nonsupplemented diet, whereas a low dose (0.1 g kg$^{-1}$ day$^{-1}$) did not have deleterious effects [57]. Although we did not have the ability to measure omega-3 FA levels in blood, the supplements provided in our study matched the recommended intake for cardiomuscular protection in humans (1500 mg DHA-EPA per day) and were therefore far below the high doses mentioned above.

Omega-3 FA supplementation lowered body weight compared to controls, as previously described [58], but this effect was not sustained. Hence, during the last months of life the two groups of omega-3 FAs had a higher body weight compared to controls. This result may be related to the death of two control mice, which could reflect the effect of long-term omega-3s on vitality and lifespan. Interestingly, the same observation has been made in a nationwide community-based cohort of Japanese men and women, in whom an inverse association between omega-3 FA dietary intakes and mortality was found [59].

In summary, we showed a two-phase positive effect of omega-3s, particularly EPA, on the skeleton, with an early impact on vertebrae trabecular BV/TV and a late improvement of cortical bone mass and strength. However, in the long-term, intake of omega-3 FAs did not prevent trabecular bone loss in the long bones and vertebrae. The cortical bone effects could be explained by an increase in leptin and IGF-1 levels, although this does not preclude modulation of expression of other adipokines and cytokines by omega-3 FAs [49]. These observations therefore indicate that long-term supplementation with omega-3 FAs could have a favourable impact on diaphyseal bone strength. However, omega-3 supplementation has a limited impact on trabecular bone microstructure and is therefore unlikely to effectively improve bone strength in vertebrae.

## Acknowledgments

We thank Dr. Martin Claire (Isonatura, Paris, France) for providing the omega-3 FAs and her assistance in designing these experiments. We thank Dr Sarah Frankland for editorial assistance and Ms. Fanny Cavat for her technical assistance.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.05.006.

## References

[1] Rossignol M, Moride Y, Perreault S, Boivin JF, Ste-Marie LG, Robitaille Y, et al. recommendations for the prevention of osteoporosis and fragility fractures. Int J Technol Assess Health Care 2002;18:597–610.

[2] Abellan van Kan G, Gambassi G, de Groot LC, Andrieu S, Cederholm T, André E, et al. Nutrition and aging, the Carla Workshop. J Nutr Health Aging 2008;12: 355–64.
[3] Ohnell O, Gullberg B, Allander E, Kanis JA. The apparent incidence of hip fracture in Europe: a study of national register sources MEDOS Study Group. Osteoporos Int 1992;2:298–302.
[4] Kannus P, Parkkari J, Sievänen H, Heinonen A, Vuori I, Järvinen M. Epidemiology of hip fractures. Bone 1996;18:57S–63S.
[5] Willett WC, Sacks F, Trichopoulou A, Drescher G, Ferro-Luzzi A, Helsing E, et al. Mediterranean diet pyramid: a cultural model for healthy eating. Am J Clin Nutr 1995;61:1402S–6S.
[6] Kontogianni MD, Melistas L, Yannakoulia M, Malagaris I, Panagiotakos DB, Yiannakouris N. Association between dietary patterns and indices of bone mass in a sample of Mediterranean women. Nutrition 2009;25:165–71.
[7] Puel C, Coxam V, Davicco MJ. Mediterranean diet and osteoporosis prevention. Med Sci 2007;23:756–60.
[8] Fernandes G, Bhattacharya A, Rahman M, Zaman K, Banu J. Effects of n-3 fatty acids on autoimmunity and osteoporosis. Front Biosci 2008;13:4015–20.
[9] Lavie CJ, Milani RV, Mehra MR, Ventura HO. Omega-3 polyunsaturated fatty acids and cardiovascular diseases. J Am Coll Cardiol 2009;54:585–94.
[10] Simopoulos AP. Omega-3 fatty acids in health and disease and in growth and development. Am J Clin Nutr 1991;54:438–63.
[11] Watkins BA, Li Y, Lippman HE, Seifert MF. Omega-3 polyunsaturated fatty acids and skeletal health. Exp Biol Med (Maywood) 2001;226:485–97.
[12] Weiss LA, Barrett-Connor E, von Mühlen D. Ratio of n-6 to n-3 fatty acids and bone mineral density in older adults: the Rancho Bernardo Study. Am J Clin Nutr 2005;81:934–8.
[13] Poulsen RC, Moughan PJ, Kruger MC. Long-chain polyunsaturated fatty acids and the regulation of bone metabolism. Exp Biol Med (Maywood) 2007;232:1275–88.
[14] Sun D, Krishnan A, Zaman K, Lawrence R, Bhattacharya A, Fernandes G. Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice. J Bone Miner Res 2003;18:1206–16.
[15] Fernandes G, Lawrence R, Sun D. Protective role of n-3 lipids and soy protein in osteoporosis. Prostaglandins Leukot Essent Fatty Acids 2003;68:361–72.
[16] Poulsen RC, Wolber FM, Moughan PJ, Kruger MC. Long chain polyunsaturated fatty acids alter membrane-bound RANK-L expression and osteoprotegerin secretion by MC3T3-E1 osteoblast-like cells. Prostaglandins Other Lipid Mediat 2008;85: 42–8.
[17] Bhattacharya A, Rahman M, Banu J, Lawrence RA, McGuff HS, Garrett IR, 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.
[18] Coetzee M, Haag M, Joubert AM, Kruger MC. Effects of arachidonic acid, docosahexaenoic acid and prostaglandin E(2) on cell proliferation and morphology of MG-63 and MC3T3-E1 osteoblast-like cells. Prostaglandins Leukot Essent Fatty Acids 2007;76:35–45.
[19] Frøyland L, Madsen L, Vaagenes H, Totland GK, Auwerx J, Kryvi H, et al. Mitochondrion is the principal target for nutritional and pharmacological control of triglyceride metabolism. J Lipid Res 1997;38:1851–8.
[20] Rosen CJ, Bouxsein ML. Mechanisms of disease: is osteoporosis the obesity of bone? Nat Clin Pract Rheumatol 2006;2:35–43.
[21] 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.
[22] Kruger MC, Horrobin DF. Calcium metabolism, osteoporosis and essential fatty acids: a review. Prog Lipid Res 1997;36:131–51.
[23] Claassen N, Coetzer H, Steinmann CM, Kruger MC. The effect of different n-6/n-3 essential fatty acid ratios on calcium balance and bone in rats. Prostaglandins Leukot Essent Fatty Acids 1995;53:13–9.
[24] Kruger MC, Schollum LM. Is docosahexaenoic acid more effective than eicosapentaenoic acid for increasing calcium bioavailability? Prostaglandins Leukot Essent Fatty Acids 2005;73:327–34.
[25] Poulsen RC, Firth EC, Rogers CW, Moughan PJ, Kruger MC. Specific effects of gamma-linolenic, eicosapentaenoic, and docosahexaenoic ethyl esters on bone post-ovariectomy in rats. Calcif Tissue Int 2007;81:459–71.
[26] Beamer WG, Donahue LR, Rosen CJ, Baylink DJ. Genetic variability in adult bone density among inbred strains of mice. Bone 1996;18:397–403.
[27] Glatt V, Canalis E, Stadmeyer L, Bouxsein ML. Age-related changes in trabecular architecture differ in female and male C57BL/6J mice. J Bone Miner Res 2007;22: 1197–207.
[28] Boutroy S, Bouxsein ML, Munoz F, Delmas PD. In vivo assessment of trabecular bone microarchitecture by high-resolution peripheral quantitative computed tomography. J Clin Endocrinol Metab 2005;90:6508–15.
[29] Ferguson VL, Ayers RA, Bateman TA, Simske SJ. Bone development and age-related bone loss in male C57BL/6J mice. Bone 2003;33:387–98.
[30] Somerville JM, Aspden RM, Armour KE, Armour KJ, Reid DM. Growth of C57BL/6 mice and the material and mechanical properties of cortical bone from the tibia. Calcif Tissue Int 2004;74:469–75.
[31] Iida-Klein A, Lu SS, Yokoyama K, Dempster DW, Nieves JW, Lindsay R. Precision, accuracy, and reproducibility of dual X-ray absorptiometry measurements in mice in vivo. J Clin Densitom 2003;6:25–33.
[32] Waarsing JH, Day JS, van der Linden JC, Ederveen AG, Spanjers C, De Clerck N, et al. Detecting and tracking local changes in the tibiae of individual rats: a novel method to analyse longitudinal in vivo micro-CT data. Bone 2004;34:163–9.
[33] Waarsing JH, Day JS, Weinans H. An automatic algorithm to segment 3D microCT scans of bone. The IXth Congress of the International Society of Bone Morphometry. Edinburgh, UK; 2002.
[34] Hildebrand T, Ruegsegger P. A new method for the model-independent assessment of thickness in the three-dimensional images. J Microsc 1997;185: 67–75.
[35] Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 1987;2:595–610.
[36] Turner CH, Burr DB. Basic biomechanical measurements of bone: a tutorial. Bone 1993;14:595–608.
[37] Halloran BP, Ferguson VL, Simske SJ, Burghardt A, Venton LL, Majumdar S. Changes in bone structure and mass with advancing age in the male C57BL/6J mouse. J Bone Miner Res 2002;17:1044–50.
[38] Tommasini SM, Morgan TG, van der Meulen MCh, Jepsen KJ. Genetic variation in structure–function relationships for the inbred mouse lumbar vertebral body. J Bone Miner Res 2005;20:817–27.
[39] Hamrick MW, Pennington C, Newton D, Xie D, Isales C. Leptin deficiency produces contrasting phenotypes in bones of the limb and spine. Bone 2004;34:376–83.
[40] Zwart SR, Pierson D, Mehta S, Gonda S, Smith SM. Capacity of omega-3 fatty acids or eicosapentaenoic acid to counteract weightlessness-induced bone loss by inhibiting NF-kappaB activation: from cells to bed rest to astronauts. J Bone Miner Res 2010;25:1049–57.
[41] Elefteriou F, Ahn JD, Takeda S, Starbuck M, Yang X, Liu X, Kondo H, Richards WG, Bannon TW, et al. Leptin regulation of bone resorption by the sympathetic nervous system and CART. Nature 2005;434:514–20.
[42] Kang JX, Weylandt KH. Modulation of inflammatory cytokines by omega-3 fatty acids. Subcell Biochem 2008;49:133–43.
[43] Seeman E. Reduced bone formation and increased bone resorption: rational targets for the treatment of osteoporosis. Osteoporos Int 2003;14:S2–8.
[44] Reinwald S, Li Y, Moriguchi T, Salem Jr N, Watkins BA. Repletion with (n-3) fatty acids reverses bone structural deficits in (n-3)-deficient rats. J Nutr 2004;134: 388–94.
[45] Thomas T, Gori F, Khosla S, Jensen MD, Burguera B, Riggs BL. Leptin acts on human marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes. Endocrinology 1999;140:1630–8.
[46] Thomas T. Leptin: a potential mediator for protective effects of fat mass on bone tissue. Joint Bone Spine 2003;70:18–21.
[47] Steppan CM, Crawford DT, Chidsey-Frink KL, Ke H, Swick AG. Leptin is a potent stimulator of bone growth in ob/ob mice. Regul Pept 2000;92:73–8.
[48] Cornish J, Callon KE, Bava U, Lin C, Naot D, Hill BL, Grey AB, Broom N, Myers DE, et al. Leptin directly regulates bone cell function in vitro and reduces bone fragility in vivo. J Endocrinol Invest 2002;175:405–15.
[49] Drevon CA. Fatty acids and expression of adipokines. Biochim Biophys Acta 2005;1740:287–92.
[50] Takeda S. Hormones and osteoporosis update. Regulation of bone remodeling by adipokines. Clin Calcium 2009;19:1009–14.
[51] Watkins BA, Shen CL, Allen KG, Seifert MF. Dietary (n-3) and (n-6) polyunsaturates and acetylsalicylic acid alter ex vivo $PGE_2$ biosynthesis, tissue IGF-I levels, and bone morphometry in chicks. J Bone Miner Res 1996;11:1321–32.
[52] Mazzuco H, McMurtry JP, Kuo AY, Hester PY. The effect of pre- and postmolt diets high in n-3 fatty acids and molt programs on skeletal integrity and insulin-like growth factor-I of White Leghorns. Poult Sci 2005;84:1735–49.
[53] Bourrin S, Ammann P, Bonjour JP, Rizzoli R. Dietary protein restriction lowers plasma insulin-like growth factor I (IGF-I), impairs cortical bone formation, and induces osteoblastic resistance to IGF-I in adult female rats. Endocrinology 2000;141:3149–55.
[54] Bateman TA, Zimmerman RJ, Ayers RA, Ferguson VL, Chapes SK, Simske SJ. Histomorphometric, physical, and mechanical effects of spaceflight and insulin-like growth factor-I on rat long bones. Bone 1998;23:527–35.
[55] Iwamoto J, Yeh JK, Takeda T, Ichimura S, Sato Y. Comparative effects of vitamin K and vitamin D supplementation on prevention of osteopenia in calcium-deficient young rats. Bone 2003;33:557–66.
[56] Matsushita H, Barrios JA, Shea JE, Miller SC. Dietary fish oil results in a greater bone mass and bone formation indices in aged ovariectomized rats. J Bone Miner Metab 2008;26:241–7.
[57] Poulsen RC, Kruger MC. Detrimental effect of eicosapentaenoic acid supplementation on bone following ovariectomy in rats. Prostaglandins Leukot Essent Fatty Acids 2006;75:419–27.
[58] Micallef M, Munro I, Phang M, Garg M. Plasma n-3 polyunsaturated fatty acids are negatively associated with obesity. Br J Nutr 2009;19:1–5.
[59] Yamagishi K, Iso H, Date C, Fukui M, Wakai K, Kikuchi S, et al. Fish, omega-3 polyunsaturated fatty acids, and mortality from cardiovascular diseases in a nationwide community-based cohort of Japanese men and women the JACC (Japan Collaborative Cohort Study for Evaluation of Cancer Risk) Study. J Am Coll Cardiol 2008;52:988–96.