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# Diet and gene interactions influence the skeletal response to polyunsaturated fatty acids

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ABSTRACT

Diets rich in omega-3s have been thought to prevent both obesity and osteoporosis. However, conflicting findings are reported, probably as a result of gene by nutritional interactions. Peroxisome proliferator-activated receptor-gamma (PPARγ) is a nuclear receptor that improves insulin sensitivity but causes weight gain and bone loss. Fish oil is a natural agonist for PPARγ and thus may exert its actions through the PPARγ pathway. We examined the role of PPARγ in body composition changes induced by a fish or safflower oil diet using two strains of C57BL/6J (B6); i.e. B6.C3H-6T (6T) congenic mice created by backcrossing a small locus on Chr 6 from C3H carrying 'gain of function' polymorphisms in the *Pparγ* gene onto a B6 background, and C57BL/6J mice. After 9 months of feeding both diets to female mice, body weight, percent fat and leptin levels were less in mice fed the fish oil vs those fed safflower oil, independent of genotype. At the skeletal level, fish oil preserved vertebral bone mineral density (BMD) and microstructure in B6 but not in 6T mice. Moreover, fish oil consumption was associated with an increase in bone marrow adiposity and a decrease in BMD, cortical thickness, ultimate force and plastic energy in femur of the 6T but not the B6 mice. These effects paralleled an increase in adipogenic inflammatory and resorption markers in 6T but not B6. Thus, compared to safflower oil, fish oil (high ratio omega-3/-6) prevents weight gain, bone loss, and changes in trabecular microarchitecture in the spine with age. These beneficial effects are absent in mice with polymorphisms in the *Pparγ* gene (6T), supporting the tenet that the actions of n−3 fatty acids on bone microstructure are likely to be genotype dependent. Thus caution must be used in interpreting dietary intervention trials with skeletal endpoints in mice and in humans.



## Introduction

The loss of bone mineral density (BMD) and the increase in bone marrow adiposity are common hallmarks of the aging process [1]. As the average life expectancy continues to increase, the need to develop new strategies to prevent osteoporosis and fragility fractures is rising. Dietary factors have long been known to influence bone remodeling and fragility [2]. Several epidemiological studies have particularly focused on Mediterranean diets [3,4] and have suggested an association between fish oil consumption and lower rate of bone loss in older adults [5–7]. However, conflicting results are reported in clinical and preclinical studies. For example, in epidemiological studies, BMD was positively associated with n−3 polyunsaturated fatty acid (PUFA) [8] or fish [9,10] consumption. Cross sectional studies also reported a lower risk of osteoporosis fracture among women with a higher intake of sea food [11]. In contrast, others found no significant association between PUFA consumption, bone formation markers, BMD or risk fracture [12–14]. Moreover, other studies highlight opposite findings by showing a higher fracture risk in women taking higher polyunsaturated fat [15]. Although those discrepancies may arise due to various confounding factors (age, dose, time of treatment …), one of the main explanations might relate to the interaction between dietary intake and genome, since genetic factors are thought to contribute between 55% and 85% to the variance of bone loss with age [16].

B6.C3H-6T congenic (6T) mice were developed to gain insights into the genetics and biology underlying phenotypes of the C3H/HeJ (C3H, High BMD and IGF-1) and the C57BL/6J (B6, low BMD and IGF-1) mice. 6T mice were generated by intercrossing a region of chromosome 6 from C3H onto a B6 background and then backcrossing 10 generations onto B6. The chromosome 6 quantitative trait locus (QTL) encompasses close to 20 cM on mid-distal mouse chromosome 6; there are more than 500 genes in this region; however, finer mapping revealed a list of 21 candidate genes; of those there are hundreds of SNPs between C3H

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and B6; however there are only 3 coding non-synonymous SNPs, none of which differed between C3H and B6 [17]. Based on previous studies, 2 SNPs located in the distal UTR of PPARγ were identified to be the most important contributors to the 6T phenotype and both these were associated with a gain of function of PPARγ. In a previous work, low bone mass and reduced IGF-1 (systemic and locally) were the major phenotypic characteristics of the 6T mouse; both of these were worsened by feeding a high fat diet due to the increased activity of PPARγ [18]. This supported the possibility that PPARγ itself was one of the determinants of *Igf-1* gene expression and protein level [19]. Nocturnin, a circadian deadenylase, was found to be markedly enhanced when PPARγ was activated, and this in turn was thought to cause the low skeletal and circulating IGF-1 levels [20]. Association between bone and PPARγ was verified in a human cohort, showing different skeletal responses to dietary environment depending on the genetic variation of *Pparγ* [18,21].

Other genetic mouse models support the notion that enhanced PPARγ signaling worsens bone health with age. PPARγ regulates osteoclastogenesis directly through C-fos expression [22] and indirectly by decreasing the OPG/RANKL ratio in osteoblast/osteocytes [23,24]. As such PPARγ activation may have several effects on bone, including increased bone marrow adiposity, decreased osteoblastogenesis and increased osteoclastogenesis [20,22,23].

In vitro, fish oil has been shown to directly up-regulate PPARγ in different cell lines such as the C2C12 myocyte or HEK293T kidney cells [25–27]; however in vivo fish oil decreased the absolute amount of PPARγ by decreasing peripheral fat. In rodents, fish oil supplementation has been shown to reduce fat mass and to improve BMD in aging mice after 4 and 6 months of supplementation respectively in Balb/c and C57BL6J mice [28,29]. However, our group and others reported also in C57BL6J mice either a modest or no structural and mechanical effect on bone of the n − 3 fatty acids (FAs), respectively after 5 and 14 months of supplementation, mainly found in fish oil [30].

We therefore postulated that the conflicting results of fish oils on bone health with aging may result from a gene × diet interaction between fish oil intake and *Pparγ* genotype. In this study we used the 6T congenic mice to investigate independent and interactive effects of fish oil with *Pparγ* genotypes on bone mass, structure and strength in female mice aged from 3 to 12 months old.

## Materials and methods

### *Animals*

Forty 10 week-old female B6.C3H-6T (6T, n = 20) and C57BL/6J (B6J, n = 20) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The B6.C3H-6T congenic strain was made as previously described [17,18]. Weight-matched mice were housed in a laboratory animal care facility in cages (5 mice/cage) and fed a standard pellet diet for 2 weeks. At 12 weeks of age, B6.C3H-6T and C57BL/6J mice were divided into two dietary groups (n = 10 per group) and fed 22% safflower oil or 22% fish oil diets (Harlan Teklad TD.08324 and TD08323, Supplemental Tables S1–S2). Isocaloric diets were ensured by Harlan. The two diets were constituted by weight of pellet, 21.2% of protein, 42.3% of carbohydrate and 22.2% of fat. Animals were pair fed. Food consumption was recorded weekly in 6T safflower oil, 6T fish oil, B6 safflower oil or B6 fish oil groups and the smallest amount of food intake observed was then fed to all the 4 groups over the following week. Supplements were freshly prepared every three months, stored in aliquots at 4 °C and distributed every 2 or 3 days. 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 12 months which is considered old age in mice. Animal procedures were approved by the University of Geneva School Of Medicine Ethical Committee and the State of Geneva Veterinarian Office.

### *In vivo measurement of bone mineral density and body composition*

Total body mass, lean body mass, fat mass, and femoral and spinal bone mineral density (BMD, g/cm$^2$) were measured in vivo at baseline and just before euthanasia by dual-energy X-ray absorptiometry (PIXImus2, GE lunar, Madison WI) [31].

### *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 and 12 months of age. The in vivo microCT system consists of an X-ray source and a 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 12 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]. A detailed description and validation of the algorithm is published elsewhere [33]. Cortical and trabecular bones were separated manually with "CT analyzer" software. Outcome measure for the trabecular and cortical structures is described by Bouxsein et al. [34]. To evaluate bone marrow adiposity tibias, we processed ex-vivo protocol with osmium staining as described [35]. After labeling of lipids by osmium tetroxide, the bones were imaged using an energy of 45 keV (UCT40, Scanco Medical AG, Basserdorf Switzerland).

### *Histomorphometry*

Femur and lumbar spine L5 were embedded in methyl-methacrylate (Merck, Darmstadt, Germany) as previously described [36]. Five-8 μm thick sagittal sections were cut with a Leica Corp. Polycut E microtome (Leica Corp. Microsystems AG, Glattburg, Switzerland) and stained with modified Goldner's trichrome, and histomorphometric measurements were performed on the secondary spongiosa of the distal femur metaphysis and vertebral body of L5, using a Leica Corp. Q image analyzer at 40× magnification. Two sections for each stained sample were quantified per animal. TRAP was detected by using hexazotized pararosanilin (Sigma, St Louis, MO) and naphthol ASTR phosphate (Sigma, St Louis, MO) to reveal osteoclasts; non-osteoclastic acid phosphatase was inhibited by adding 100 mMol/L L(+)-tartaric acid (Sigma, St Louis, MO) to the substrate solution. The following parameters were recorded: the number of TRAP + osteoclasts in contact with trabeculae (N.Oc/TBPm; expressed in cells per millimeter of trabecular bone surface); the resorption surface (OcS/BS; expressed in %); and the average length of the zone of contact per osteoclast (Oc.Pm/N.Oc; given in microns).

### *Testing of mechanical resistance*

The night before mechanical testing, bones were thawed slowly at 7 °C and then maintained at room temperature. Femur was placed on the material testing machine on two supports separated by a distance of 9.9 mm and load was applied to the midpoint 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 an actuator displaced at 2 mm/min. Outcome measures were ultimate force, stiffness, and energy as described by Turner and Burr [37].

### *RNA extraction and quantitative PCR: bone*

The whole tibia was excised and immediately pulverized to a fine powder in peqGold Trifast (peQLab Biotechnologie GmbH) using a FastPrep System tube and apparatus (QBiogene, Illkirch, France) in