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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 PPARγ pathway. We examined the role of PPARγ in body composition changes induced by a fish or safflower oil diet using two strains of C57BL6J (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 C57BL6J 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 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

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genotype dependent. Thus caution must be used in interpreting dietary intervention trials with skeletal endpoints in mice and in humans.

Keywords

aging; Omega-3s; Bone-fat interactions; Genetic animal models; bone microCT

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 remodelling 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 pre-clinical studies. For example, in epidemiological studies, BMD was positively associated to n-3 polyunsaturated fatty acids (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 (12,13,14). 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 the genome, since genetic factors are thought to contribute between 55% to 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 20cM 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 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 contributor to the 6T phenotype and both these were associated with a gain of function of PPARγ. In previous work, low bone mass and reduced IGF-1 (systemic and locally) were the major phenotypic characteristics of the 6T mouse; both 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 PPARG 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 signalling 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 C2C12 myocyte or HEK293T kidney cells (25, 26, 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 groups and others reported also in C57BL6J mice either a modest or no structural and mechanical effect on bone of the n-3 fatty acids (FA), 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 weeks-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 table 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 feed. 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 consider to be old 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, 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 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. Outcomes measure for the trabecular and cortical structure are 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 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-8μm thick sagital 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 analyser at 40X magnification. Two sections for each stained sample were quantified per animal. TRAP was detected by using hexazotized pararosanilin (Sigma, St Louis, MO) and naphtol ASTR phosphate (Sigma, St Louis, MO) to reveal osteoclasts, ; non-osteoclastic acid phosphatase was inhibited by adding 100mMol/L L(+)-tartric 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 %) ; 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 actuator displaced at 2mm/minute. Outcomes 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 FastPrep System tube and appartus (QBiogene, Illkirch, France) in order to achieve quantitative RNA extraction. We used a lysis time and speed respectively of 14sec and 5 m/s, as recommended by the maker. Total RNA extraction and quantitative PCR were performed as previously described (36). The following pre-designed TaqMan® gene expression assays were used for the quantitative RT-PCR (References, *Gapdh*: Mm00437762_m1, *Igf-1*: Mm00439559_m1, *Il-6*: Mm00446190_m1, *Rankl*: Mm00441908_m1, *Opg*: Mm00435452_m1, *Fabp4*: Mm00445878_m1, *AdipoQ*: Mm01343606_m1, *Adipsin*: Mm00439559_m1, *Mcsf*: Mm00432689_m1, *Il1-ra*: Mm00446185, *Tnfα*: Mm99999068_m1, *Trap*: Mm00475698_m1, *Fsp27*: Mm00617672_m1, *Lpl*: Mm00434764_m1, *Pepck*: Mm00440635_m1, *Pparγ*: Mm01184322_m1 Applied Biosystems, Rotkreuz, Switzerland) consisting of two unlabeled primers and a FAM™ dye-labeled TaqMan® MGB probe, and the correspondent buffer TaqMan® Universal PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland). Relative quantities (RQ) were calculated with the formula RQ=E-Ct using an efficiency (E) of 2 by default. The mean quantity was calculated from triplicates for each sample and this quantity was normalized to the similarly measured mean quantity of the GAPDH normalization gene. Finally, normalized quantities were averaged for 3 to 4 animals and represented as mean ± SEM.

White adipose tissue (WAT)

Total RNA from peri-ovarian WAT was extracted using the Trizol reagent (Life technologies, Zug, Switzerland) to the manufacturer's protocol. Three micrograms total RNA were reverse-transcribed using 400 units of Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Invitrogen, Basel, Switzerland), in the presence of 1 unit/μl RNAsin (Promega Corp, Madison, WI, USA), 0.2 μg random primers (oligo(dN)6) (Promega Corp, Madison, WI, USA), 2 mM dNTP and 20 μM DTT (Invitrogen, Basel, Switzerland). The expression of the cDNAs was determined by quantitative real-time PCR using an ABI step one plus sequence detection System (Applera Europe, Rotkreuz, Switzerland) and were normalized using the housekeeping genes Ribosomal Protein S29. PCR products were quantified using the Power Master SYBR Green mix (Applera Europe, Rotkreuz, Switzerland) and results are expressed in arbitrary units (A.U) relative to the control group mean value. Primers sets were designed using the Primer Express software (Applera Europe, Rotkreuz, Switzerland) and were chosen when possible on both sides of an intron to avoid amplification of possible contaminating genomic DNA. The annealing temperature (60°C) and amplicon size (50–150 bp) were automatically determined by the software. Oligos were used at 217nM each (Microsynth, Switzerland). The sequence of the primers used is provided in supplemental table S3.

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 phosphatise form 5b) and osteocalcin were measured according to manufacturer's instructions (SB-TR103 SBA Sciences, Turku, Finland; and CN50-1300 Biomedical Technologies Inc., Stoughton, MA, USA, respectively). IGF-I (AC-18F1 Immunodiagnostic Systems, Paris, France), myostatin (9875-11 US biological life science, Salem, MA01970) and leptin (90030 Crystal Chem Inc, Downers Grove, IL 60515) were measured in serum by immunoenzymometric assay (IEMA) with a kit from IDS following the manufacturer's instructions.

Data analysis

We tested the significance of diet effects by a two way ANOVA repeat measurements with the type of diet and genotype as factors. As appropriate, *post hoc* testing was performed using Fisher's protected Least Squares Difference (PLSD). The p of interaction between the diet and genotype was only mentioned when significant. Differences were considered significant at $p < 0.05$. Data are presented as mean ± SEM.

Results

Effect of n-3/n-6 FAs ratio on body composition and gene expression of WAT in B6 and 6T

Overall, no significant differences in food intake were observed between groups (Fig. S1). At baseline, 6T tend to have a lower body weight, % fat mass and a higher % lean than B6. There are no significant differences in appendicular lean mass (Table S4). After 9 months of supplementation, the fish oil diet resulted in decreased body weight and % fat mass whereas it led to an increase in the %lean vs the safflower oil. These effects were similar in 6T and B6 (Fig. 1A–C). Interestingly, appendicular lean mass gain was lower in 6T than in B6 (+0.068±0.04% vs +0.162±0.05%, $p<0.05$) but was not influenced by fish oil. At sacrifice, WAT (peri-ovarian and peri-kidney) and BAT mass per kg of body weight significantly decreased with fish oil vs safflower oil diet in both 6T and B6 (Fig. 1D–E). Interestingly, the fish oil diet led to a more drastic reduction in WAT (around 54%) than BAT (around 20%) in both 6T and B6 (Fig. 1D–F).

In B6 mice, gene expression analysis of peri-ovarian WAT indicated that fish oil significantly decreased the markers of adipogenesis (*leptin*, *adiponectin*, *Fas*) and increased *Pparβ* (+158% vs safflower oil, $p<0.05$) (Table 1). In contrast, fish oil did not have any significant effect on *leptin*, *adiponectin* and *Pparβ* expression in 6T; rather it increased the expression of inflammatory markers *Fas* and *Tnfα* (respectively, +87% and +51% vs safflower, $p<0.05$).

Effect of n-3/n-6 FAs ratio on BMD and trabecular microarchitecture in B6 and 6T

At baseline, 6T mice exhibited lower total body BMD and trabecular bone volume at the lumbar spine compared to B6 mice (Table S4). In B6 mice, fish oil significantly increased lumbar spine BMD vs safflower oil whereas it did not have any impact on total body and femoral BMD. In contrast, in 6T mice, fish oil did not have significant effect on lumbar

spine BMD but significantly decreased the total body and femoral BMD vs safflower oil (Fig. 2 A–C). 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 was more dramatic (Fig. 2 D–I). At the vertebrae, fish oil significantly prevented the decrease of BV/TV and Tb.N with age in B6 mice. The same trend was observed in 6T mice but the difference was not significant (Fig. 2 D–F). At the proximal tibia, fish oil did not have any significant effect in B6, whereas in 6T mice, it significantly accentuated the loss of bone volume fraction and trabecular number with age (Fig. 2 G–I). In addition, 6T mice on a fish oil diet had increased bone marrow adiposity vs B6 mice on a long term fish oil diet (MicroCT AdV/TV, 0.12±0.05 vs 0.02±0.005, respectively, $p<0.05$).

Effect of n-3/n-6 FAs ratio on cortical architecture and mechanical properties in B6 and 6T

At baseline, 6T mice exhibited lower cortical tissue and bone volume at the mid tibia compared to B6 mice (Table S4). Cortical architecture evolution at the mid-tibia diaphysis is shown in figure 3. Gain in Cortical Bone volume (CtBV) between 3 and 12 months of age is lower in fish oil group's vs safflower oil either in B6 and 6T mice. However, this effect disappears after adjustment by body weight. Tissue volume (TV) increased equally from 3 to 12 months of age in all groups (around 18%), consistent with a continuous periosteal bone apposition. In B6 mice, bone marrow volume increased with age in a similar manner to fish and safflower oil (around 25%), therefore CtTh decreased equally in the two groups. In 6T, the bone marrow volume increase was higher in fish vs safflower oil; hence fish oil decreased CtTh more than safflower oil (Fig. 3A–E). As a result, in B6 mice fish oil did not significantly change ultimate force, stiffness, elastic, plastic and fracture energy. In contrast, in 6T mice, fish oil significantly decreased ultimate force, yield point, elastic, plastic and fracture energy (Fig. 4A–F). In sum when mice are fed with fish oil, 6T has lower ultimate force, plastic and fracture energy, −17.2%, −27.2% and −21.2% vs B6 respectively, all $p<0.05$.

Effect of n-3/n-6 FAs ratio on osteoclast number and bone marrow adiposity in B6 and 6T

In B6 mice, osteoclast surface and adipocyte volume in femur did not differ between fish and safflower oil (Fig. 5A&H). In lumbar spine, osteoclast surfaces and numbers were significantly decreased with the fish oil diet vs safflower oil and adipocytes were not detectable (Fig. 5D–F). In 6T, fish oil increased osteoclastic and adipocyte number, qualitatively by histmorphometry and quantitatively by microCT with osmium, vs safflower oil in femur whereas it had no significant effect in lumbar spine (Fig. 5G–I). Thus 6T mice fed with fish oil have higher osteoclast surface and numbers, as well as more marrow adiposity compared to B6.

Effect of n-3/n-6 FAs ratio on bone markers in B6 and 6T

At baseline, 6T mice tend to exhibit lower leptin and IGF-1 levels compared to B6 mice (table S4) similar to what has been reported previously (17). Levels of osteocalcin and TRAP5b were not significantly different between the congenic and B6 mice. In B6, osteocalcin levels decreased less with age in fish oil vs safflower oil diet and TRAP5b remained unchanged between groups. In contrast, in 6T mice, fish oil did not slow the osteocalcin decrease with age and tended to increased TRAP5b (Table S5). In accordance

with WAT mass changes, the increase of leptin with age was lower in mice fed the fish oil vs those fed the safflower oil in both 6T and B6 mice (Table S5).

Effect of n-3/n-6 FAs ratio on gene expression of the tibia in B6 and 6T

In order to clarify the molecular mechanisms by which fish oil mediates different skeletal responses to aging between B6 and 6T, we next examined gene expression in the tibia of both strains. In B6 mice, fish oil decreased markers of bone resorption (*Mcsf*, *Trap*) and elicited an anti-inflammatory effect as indicated by an increase in *Il1-ra* and a decrease in *Tnfa* vs safflower oil (+199% and −18% respectively, $p<0.001$). Fish oil also decreased *Pparγ* (−43% vs safflower, $p<0.01$), i.e. markers of adipogenesis (Table 2).

In 6T mice, fish oil decreased *Pparγ* expression (−21% vs safflower, $p<0.01$), however this decrease was lower than observed in B6. Interestingly, the fish oil diet in 6T increased others adipogenic markers such as adiponectin +33%, *Fsp27* +59% and *Pepck* +358% vs safflower oil (i.e an important indicator of gain of function in PPARγ), all $p<0.05$; as well as markers of inflammation (*Tnfα*: +28% vs safflower, $p<0.01$). Moreover, markers of bone resorption such as *Mcsf* and *Trap* remained unchanged in response to fish oil (Table 2).

Discussion

The mains goal of our study was to elucidate the role of genetic background on the skeletal response to n-3 PUFA. As omega-3 fatty acid is a known ligand for PPARγ (38, 39), we challenged mice with a fish oil diet, utilizing the very modest genotypic differences between the 6T congenic mouse and B6. The 30 cM QTL on mouse Chr 6 that originated from C3H/HeJ and was backcrossed onto B6 includes the *Pparγ* gene which exhibits a gain of function, due to polymorphisms in the 3' UTR. Previously these mice were characterized by their high marrow adiposity, low bone mass and reduced circulating IGF-I (40). In the current report, we found that fish oil intake, rich in omega n-3 FA, was associated with an increase in BMD and trabecular microarchitecture at the caudal vertebrae in B6 mice, mainly due to a decrease in osteoclast number through a decrease in inflammatory cytokines. However, fish oil did not significantly improve bone mechanical properties. In 6T, fish oil did not decrease the osteoclast number and surfaces and did not prevent trabecular bone loss with age. This suggests that, when PPARγ activity is initially increased, i.e. as in 6T bone, fish oil was unable to reduce its activity enough to prevent trabecular bone loss with age. Moreover, in 6T, fish oil reduces mechanical properties, i.e. ultimate force and plastic energy, associated with a decrease in cortical thickness and an increase in bone marrow volume. Therefore, these findings offer a potential explanation for the inconsistent effects of omega-3 fatty acid supplementation on the murine skeleton and may provide insight into the heterogeneity in the skeletal response to specific diets in humans.

In accordance with previous experiments, we confirmed that 6T mice had a low vertebral trabecular bone fraction, cortical bone microarchitecture with an increase in bone marrow adiposity and *Pparγ* expression in bone (41). We also confirmed lower bone marrow adipocytic density in the lumbar spine compared to the femur, similar to what has been reported in humans (42). Moreover we found that 6T did not exhibit more brown or brown

like fat, nor more leptin, but did have more osteoclasts, indicating that increased PPARγ activity in bone may directly increase osteoclast numbers (22).

In B6 mice, the fish oil diet significantly reduced the body weight and fat mass gain with age; at sacrifice the fish oil group had lower WAT and BAT mass, with a marked decrease in circulating leptin levels. Interestingly, the decrease of WAT was 3 fold higher than the decrease of BAT, suggesting that fish oil decreased fat mass but preserved a beneficial metabolic profile (43). The pattern of gene expression in white fat indicated a reduction of adipogenesis (*leptin*, *adiponectin*, *Fas*) and an increase of FA oxidation through *Pparβ* but without changes in *Pparγ* (44). At the bone tissue levels fish oil significantly decreased *Pparγ* and increased *Igf-1* gene expression while it preserved vertebral BMD and cancellous bone volume supporting the observations that PPARγ regulates IGF-I, probably through indirect mechanisms (19). Surprisingly, fish oil did not affect the other determinants of adipogenesis such as *Adiponectin*, *Adipsin*, *Fsp27* and *Pepck*, and as a result bone marrow adiposity was not significantly altered by fish oil in our model. In accordance with Sun et al (28), the fish oil bone effect could be mediated by inflammatory cytokines (TNFα, IL1-Ra). As we previously demonstrated in C57BL6J mice, long-term supplementation of omega-3 FA also may exert these positive effects by decreasing leptin (45). Indeed, intracerebroventricular leptin injection has been shown to induce trabecular bone remodelling in vertebrae by triggering β-adrenergic signalling in the CNS, which in turn increases osteoclast number and activity (46, 47). However, this decrease in osteoclasts had little influence on femoral BMD, cortical thickness or the mechanical properties.

In 6T mice, fish oil diet decreased body weight, fat gain, and circulating leptin in a similar manner to B6 mice. However, markers of adipogenesis that were decreased in B6 in response to fish oil were unchanged in 6T mice. Fish oil can modulate body fat metabolically by increasing lipolysis and fatty acid oxidation (48); or by reducing food intake through the endocannabinoid and mesocorticolimbic dopamine system (49). This second mechanism was not operative in our experiments due to the use of isocaloric diet and pair feeding. So these data suggest that the difference in adipogenesis markers at the gene expression levels were not translated into differences in fat mass gain. At the bone levels, 6T mice exhibited resistance to the positive effect of fish oil on cancellous bone. Moreover, fish oil consumption was associated with a further increase in bone marrow adiposity and a decrease in BMD, cortical thickness and finally femoral mechanical properties. These negative effects paralleled an increase in resorption, adipogenic and inflammatory markers including adiponectin, probably because 6T mice have a gain of function in the *Pparγ* gene compared to B6 mice. The interaction between omega-3 FA effect and level of PPARγ activation has also been found pharmacologically in skeletal muscle where inhibition of PPARγ, prevented the action of n-3 fatty acid on cell differentiation (50). *Pparγ* is likely not the only gene that contributes to the skeletal phenotype of the 6T congenic strain since *Cidea*, *Zfp422* and *Alox5* genes are within the QTL and could be potential candidates to explain both the bone marrow fat and skeletal response to fish oil diet, although these genes do not have functional polymorphisms between C3H and B6 (51).

There are several limitations to our study. First, the 6T congenic mouse has polymorphic differences in multiple genes that could impact the skeletal response to dietary interventions

relative to B6. In particular, this QTL contains other genes that may impact fat mass such as *Cidec*, a mediator of apoptosis in adipocytes. Notwithstanding, congenic rather than pure inbred mice, are more representative of the heterogeneity in the genetic background of most mammals. Hence our conclusions about diet×gene interactions are not inconsistent with larger cohort studies in humans. Second, *Ppar*γ gene expression does not equate with its nuclear receptor activity. Hence relative differences in *Ppar*γ gene expression by strain may not reflect in vivo activities. Indeed, marrow adiposity was increased in 6T in response to the fish oil diet despite a modest reduction in gene expression. Third, our studies only lasted nine months; hence the long term effects of diet on bone and body composition, particularly in older individuals may be affected by age related changes in several genes including *Ppar*γ. In conclusions, our observations confirm that long-term fish oil diet rich in omega-3 FAs could have a favourable impact on fat mass, skeletal integrity and bone loss with age, i.e bone mass and trabecular bone microarchitecture in vertebrae(52). However, at the skeletal level, the beneficial effects of a fish oil diet may be dependent on the presence of allelic variants in several genes, including *Ppar*γ. Therefore, our findings emphasize the need to consider nutrient by genotype interactions when considering the beneficial or harmful effects of a particular diet.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Fish oil reduced body weight and fat mass gain with age in B6 mice
- In B6, fish oil preserved vertebral BMD and cancellous bone by a decrease in osteoclast
- 6T mice exhibit resistance to fish oil effect on bone due to a gain of function of PPARγ
- In 6T mice, fish oil increase bone marrow adiposity, resorption and inflammatory markers
- Fish oil effects may dependent on allelic variants in several genes, including *Pparγ*

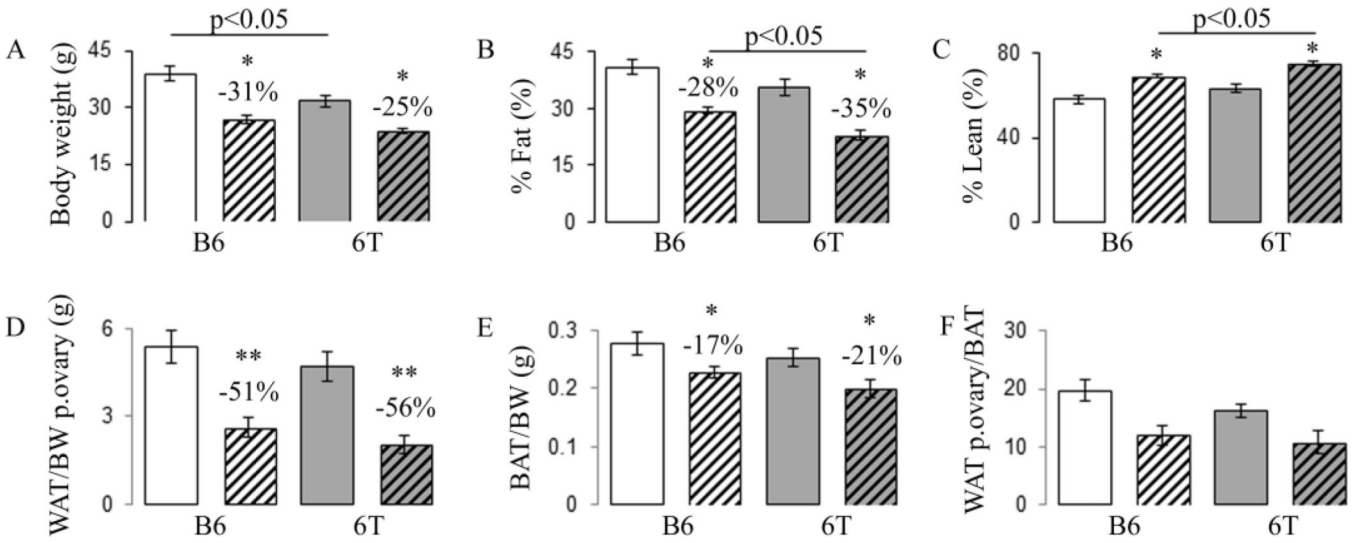

Fig 1.

Effects of 9 months of fish oil diet on body composition, WAT and BAT mass in 6T and B6. (A) Total body weight; (B) %fat and (C) %lean evaluated by piximus; (D) WAT ovary mass measured at sacrifice per body weight (BW); (E) BAT/BW; (F) WATovary/BAT. * p<0.05,** p<0.01 significant difference vs safflower mice. Grey bars,6T; open bars, B6; hatched bars, fish oil diet; non-hatched bars, safflower diet. Bars show means (± sem).

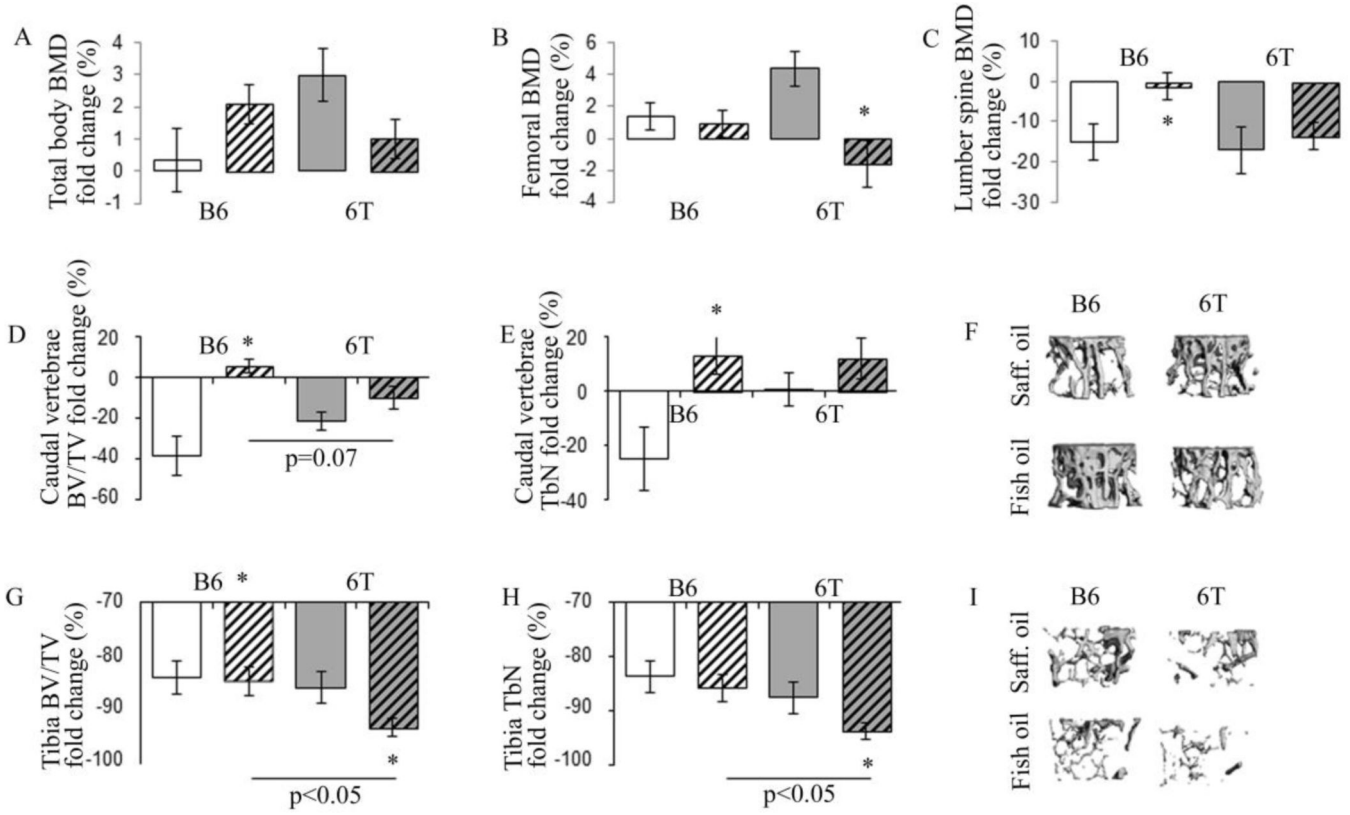

Fig 2.

Effect of fish oil on BMD, tibial and vertebrae trabecular microarchitecture changes with ages in 6T and B6. (A) Total Bone Mineral Density (BMD); (B) femoral BMD; (C) lumbar spine BMD; (D) Caudal vertebrae Trabecular bone volume on tissue volume (BV/TV); E) Caudal vertebrae trabecular number (TbN); (F) illustration of 3D trabecular structure of the vertebrae in mice aged of 12 months; (G) tibial BV/TV; (H) tibial TbN; (I) illustration of 3D trabecular structure of the tibia in mice aged of 12 months. * $p<0.05$ significant difference vs safflower mice. Grey bars,6T; open bars, B6; hatched bars, fish oil diet; non-hatched bars, safflower diet. Bars show means (± sem).

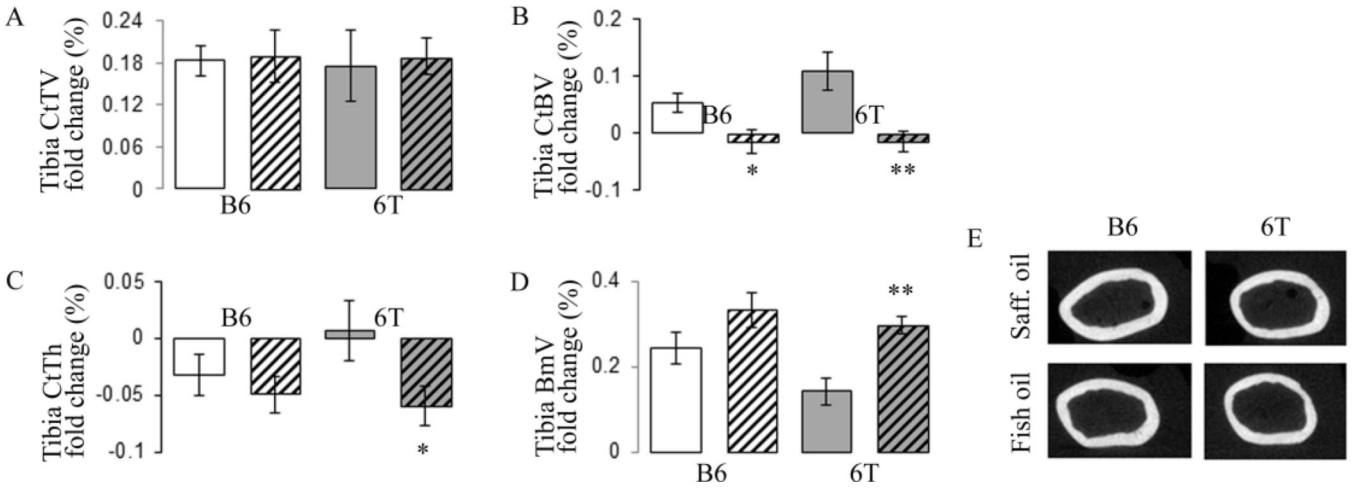

Fig 3.

Effect of fish oil diet on tibial cortical microarchitecture changes with ages in 6T and B6. (A) Cortical Tissue Volume (CtTV) at midshaft tibia; (B) Cortical Bone Volume (CtBV); (C) Cortical thickness (CtTh); (D) Bone marrow volume (BmV); (E) 2D reconstruction of cortical femur midshaft illustrating the lower CtTh in 6T mice under fish oil diet also found in femur. * $p<0.05$, ** $p<0.01$ significant difference vs safflower mice. Grey bars,6T; open bars, B6; hatched bars, fish oil diet; non-hatched bars, safflower diet. Bars show means (± sem).

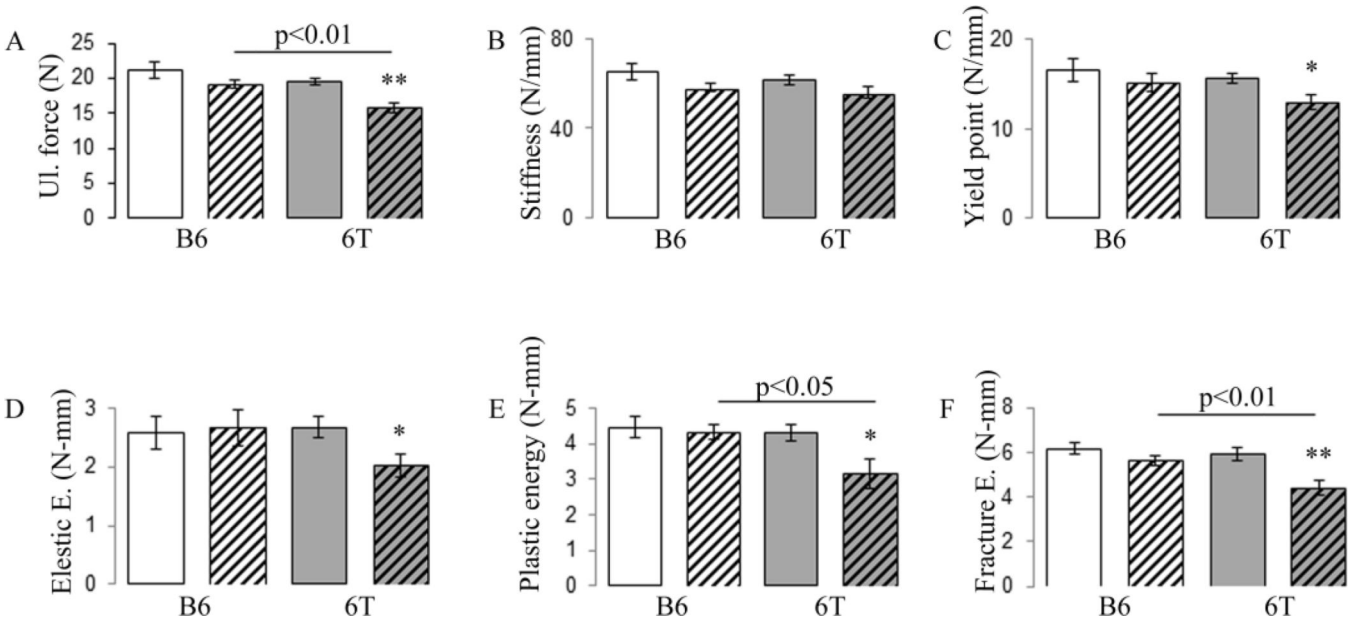

Fig 4.

Effect of fish oil diet on femur strength in 6T and B6. Mechanical properties have been evaluated by three point bending test. (A) Ultimate (Ul) force; (B) stiffness; (C) yield point; (D) elastic energy; € plastic energy; (F) fracture energy. * $p<0.05$, ** $p<0.01$ significant difference vs safflower mice. Grey bars,6T; open bars, B6; hatched bars, fish oil diet; nonhatched bars, safflower diet. Bars show means (± sem).

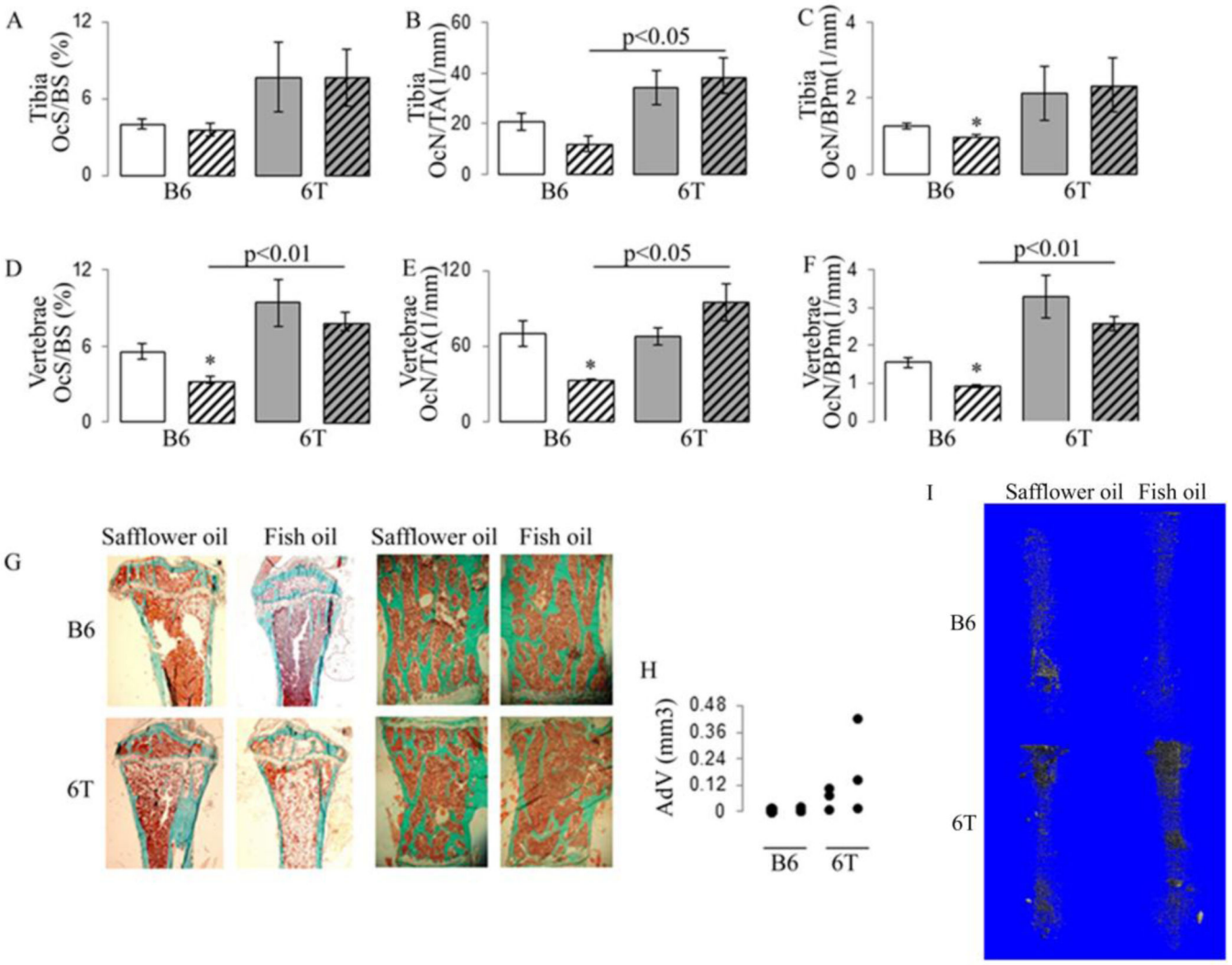

Fig 5.

Effect of fish oil diet on osteoclast and bone marrow adiposity in tibia and vertebrae in 6T and B6. (A) tibial Osteoclast Surface per Bone surface (OcS/BS); (B) tibial Osteoclast Numbers per Tissue Area (OcN/TA); (C) tibial Osteoclast Numbers per Bone Perimeters (OcN/BPm); (D) vertebrae OcS/BS; E) vertebrae OcN/TA; (F) vertebrae OcN/BPm; (G) histological illustration of increased adipocyte infiltration in tibial bone marrow of 6T under fish oil; and absence of adipocyte in bone marrow of vertebrae; (H) Adipocyte volume on tissue volume (Adv/TV) in distal femur quantified by microCT after osmium staining; (I) 3D reconstruction of adipocyte volume present in the marrow space of the all femur.

Table 1

Effects of fish oil on WAT gene expression after 9 months of supplementation in 6T and B6.

	B6		*6T*		*P Genotype*	*P Diet*	*P Int*
Gene	**Safflower**	**Fish**	**Safflower**	**Fish**			
Leptin	1.00±0.13	0.58±0.08**	0.69±0.13	0.59±0.12	NS	0.03	NS
AdipoQ	1.00±0.10	0.58±0.09**	0.54±0.10	0.51±0.19	0.05	0.08	NS
Pparβ	1.00±0.19	2.58±0.73*	1.91±0.67	1.92±0.45	NS	NS	NS
Tnfα	1.00±0.23	1.59±0.27	2.68±0.67	4.04±0.67$	0.006	0.01	NS
Pparγ	1.00±0.44	0.16±0.05	0.55±0.28	0.84±0.74	NS	NS	NS
Fas	1.00±0.10	0.63±0.17	0.90±0.17	1.68±0.39*	0.05	NS	0.02
Lpl	1.00±0.14	0.96±0.11	1.91±0.59	1.29±0.30	0.05	NS	NS
Srebp	1.00±0.14	1.94±0.75	1.63±0.65	2.58±1.07	NS	NS	NS

Gene expression was evaluated by qRTPCR (n=6–8 per group).

*p<0.05 significant difference vs safflower in each genotype;

$p<0.05 significant difference vs B6 in each diet by post-hoc fisher's PLSD (2F-ANOVA, genotype and diet). Interaction, Int. Means ± SEM.

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Table 2

Effects of fish oil on bone gene expression after 9 months of supplementation in 6T and B6.

	B6		*6T*		*P Genotype*	*P Oil*	*P Int*
Gene	**Safflower**	**Fish**	**Safflower**	**Fish**			
Igf-1	0.98±0.07	1.43±0.11**	1.50±0.10	1.74±0.13	0.0004	0.002	NS
Il-6	1.05±0.10	1.03±0.13	1.14±0.11	1.27±0.11	NS	NS	NS
Opg	1.18±0.22	0.61±0.13*	1.36±0.29	0.71±0.16*	NS	0.01	NS
Pparγ	1.08±0.14	0.62±0.03**	1.66±0.08	1.31±0.07**	0.0001	0.0001	NS
Rankl	1.05±0.10	0.84±0.10	1.05±0.06	0.94±0.11	NS	NS	NS
AdipoQ	1.03±0.08	1.19±0.08	1.52±0.15	2.03±0.18*	0.0001	0.01	NS
Adipsin	1.15±0.15	1.15±0.11	5.78±1.4	7.07±0.94	0.0001	NS	NS
Il-10	1.16±0.17	0.24±0.03***	0.43±0.04	0.31±0.04*	0.002	0.0001	0.0003
Il1-ra	1.04±0.14	3.11±0.43****	1.78±0.43	4.33±0.66**	0.05	0.0001	NS
Tnfα	1.00±0.02	0.82±0.04***	1.04±0.05	1.33±0.08**	0.0001	NS	0.0001
Mcsf	1.01±0.04	0.74±0.06**	1.00±0.04	1.11±0.07	0.001	NS	0.001
Trap	1.04±0.09	0.74±0.03**	1.17±0.06	1.23±0.02	0.0001	0.03	0.002
Ak2	1.11±0.13	1.16±0.10	1.34±0.24	1.30±0.17	NS	NS	NS
Fabp4	1.03±0.08	1.87±0.05***	1.43±0.18	2.19±0.19**	0.01	0.0001	NS
Fsp27	1.03±0.07	1.05±0.08	3.34±0.70	5.31±0.69*	0.0001	0.05	0.05
Lpl	1.08±0.13	1.49±0.15*	1.25±0.16	1.55±0.16	NS	0.02	NS
Pepck	1.15±0.24	1.64±0.20	2.54±0.61	11.64±1.61****	0.0001	0.0001	0.0001

Gene expression was evaluated by qRTPCR (n=6–8 per group).

*p<0.05 significant difference vs safflower in each genotype;

$p<0.05 significant difference vs B6 in each diet by post-hoc fisher's PLSD (2F-ANOVA, genotype and diet). Interaction, Int. Means ± SEM.