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Research Article

Sex-specific effects of *Fat-1* transgene on bone material properties, size, and shape in mice

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Abstract

Western diets are becoming increasingly common around the world. Western diets have high omega 6 (ω-6) and omega 3 (ω-3) fatty acids and are linked to bone loss in humans and animals. Dietary fats are not created equal; therefore, it is vital to understand the effects of specific dietary fats on bone. We aimed to determine how altering the endogenous ratios of ω-6:ω-3 fatty acids impacts bone accrual, strength, and fracture toughness. To accomplish this, we used the *Fat-1* transgenic mice, which carry a gene responsible for encoding a ω-3 fatty acid desaturase that converts ω-6 to ω-3 fatty acids. Male and female *Fat-1* positive mice (*Fat-1*) and *Fat-1* negative littermates (WT) were given either a high-fat diet (HFD) or low-fat diet (LFD) at 4 wk of age for 16 wk. The *Fat-1* transgene reduced fracture toughness in males. Additionally, male BMD, measured from DXA, decreased over the diet duration for HFD mice. In males, neither HFD feeding nor the presence of the *Fat-1* transgene impacted cortical geometry, trabecular architecture, or whole-bone flexural properties, as detected by main group effects. In females, *Fat-1*-LFD mice experienced increases in BMD compared to WT-LFD mice; however, cortical area, distal femur trabecular thickness, and cortical stiffness were reduced in *Fat-1* mice compared to pooled WT controls. However, reductions in stiffness were caused by a decrease in bone size and were not driven by changes in material properties. Together, these results demonstrate that the endogenous ω-6:ω-3 fatty acid ratio influences bone material properties in a sex-dependent manner. In addition, *Fat-1* mediated fatty acid conversion was not able to mitigate the adverse effects of HFD on bone strength and accrual.

Keywords: fatty acids, omega-6, omega-3, fracture toughness, bone strength, bone accrual

Lay Summary

As Western diets increase in popularity, it is becoming imperative to understand the impact of fatty acids in these diets on bone health. Western diets, known to increase the risk of bone fractures, contain large amounts of omega-6 (ω-6) fatty acids. Although evidence suggests that ω-6 fatty acids may be detrimental to bone health, it is important to consider the interaction between both ω-6 and omega-3 (ω-3) fatty acids, as fats are not consumed in isolation. To control the ratio of ω-6:ω-3 fatty acids, we used a transgenic mouse model that converts ω-6 fatty acids to ω-3 fatty acids within every cell. However, male and female *Fat-1* positive (*Fat-1*) and *Fat-1* negative (WT) mice were given either a high-fat diet (HFD) or low-fat diet (LFD) for 4 mo. In males, bone strength remained unchanged, but resistance to fracture decreased when the ω-6:ω-3 fatty acid ratio was reduced. In females, bone strength was reduced, but resistance to fracture remained unchanged. The evidence provided reveals that sex significantly influences how dietary fat impacts bone strength and fracture resistance, thereby enhancing our understanding of the relationship between bone health and fat.

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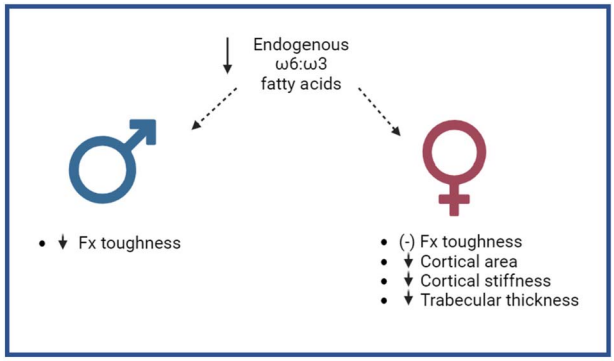
Graphical Abstract



We tested whether reducing the endogenous ω-6:ω-3 fatty acid ratio in mice under high-fat diet conditions leads to improved skeletal outcomes using the *Fat-1* transgenic mouse model. We found sex-specific effects of the *Fat-1* transgene where male mice experienced reductions in fracture toughness and females experienced reductions in cortical area, cortical strength, and trabecular thickness.

Introduction

The relationship between bone health and fat consumption is a topic that has been of great interest in society and research. However, many unresolved questions remain surrounding the effects of specific fats on bone outcomes. Western diets, which are high in animal-based nutrients, fat, and sugar content, are becoming more common across the world and have been linked to bone loss in both animals and humans.[1,2] With the increased pervasiveness of western diets, it is becoming imperative to tease out the intricacies of the effects of specific dietary fats on bone. Dietary fats are not created equal and can be subcategorized into two groups: saturated and unsaturated fats. Saturated fatty acids induce lipotoxicity in osteoblasts and osteocytes, which could lead to reductions in bone formation.[3,4] In contrast, unsaturated fatty acids have more convoluted effects. Monounsaturated fatty acids show positive associations with BMD in men and women.[5] Polyunsaturated fats such as omega-6 (*ω*-6) and omega-3 (*ω*-3) fatty acids have mixed impacts on bone outcomes. *ω*-6 fatty acids are generally associated with an increase in inflammatory markers,[6] inhibition of osteoblastogenesis, and induction of adipogenic differentiation of human mesenchymal stem cells.[7] In contrast, *ω*-3 fatty acids have anti-inflammatory effects, are associated with increased peak bone mass at the hip in young women, positively associated with BMD in women with osteopenia, reduced risk of hip fracture in men and women, and reduced bone resorption markers in men and women.[8-11] The ratio of *ω*-6:*ω*-3 fatty acids may be as important, if not more important, for bone outcomes than the absolute levels of each fat class. The *ω*-6:*ω*-3 ratio is high in western diets, meaning w-6 fatty acids are abundant compared to w-3 fatty acids. Recent evidence reveals that increasing this ratio (eg, increasing *ω*-6 and reducing *ω*-3 fatty acids) lowers hip BMD in both men and women and reduces whole body and spine BMC in young male rats.[11,12]

Diets rich in saturated, monounsaturated, and polyunsaturated fatty acids cause rapid absorption of fatty acids into the gut and possibly increase fatty acid clearance in other tissues compared to a high carbohydrate diet.[13,14] Femoral and tibial cortical and marrow compartments have a diverse fatty acid profile, including a large portion of unsaturated fatty acids.[15] Osteoblasts utilize these fatty acids during mineralization[16] and therefore changes in fatty acid composition could alter osteoblast function.[4,16,17] These alterations could potentially reduce bone mineralization, bone accrual, bone quality, and, thus, fracture resistance.[18,19] Nevertheless, it remains unclear whether different types of dietary fat have different impacts on bone outcomes. Understanding the consequences of alterations in bone accrual, bone strength, and fracture resistance with diet composition is paramount to minimizing fracture rates in humans.

A significant limitation in understanding the effects of different fats on bone is that most studies only consider fat intake from diet. The ratio of *ω*-6: *ω*-3 fatty acids available to and accessed by bone cells is likely substantially altered from that ingested as food. First, dietary fat must travel through the digestive system, eventually packaged in chylomicrons that will either transit to the bloodstream or the liver. Once in the bloodstream, the fatty acids in the chylomicrons can either be used for oxidation in tissues such as muscle, heart, and bone or be stored in adipose tissue. In the liver, the chylomicrons can be further broken down to generate endogenous fatty acids to be sent to the bloodstream for oxidation or storage.[20] Second, ingested fats in diet or supplements are known to alter and be altered by the gut microbiome, influencing BMD via the factors produced by the microbiome itself.[21,22] Therefore, any alterations in bone outcomes may not directly relate to the ingestion dietary composition.

In this study, we aimed to determine how altering the endogenous ratio of *ω*-6:*ω*-3 fatty acids impacts bone accrual, bone strength, and fracture toughness. To accomplish this, we utilized the *Fat-1* transgenic mouse model.[23,24] These mice carry the *Fat-1* gene from *Caenorhabditis elegans* that encodes for an *ω*-3 fatty acid desaturase, which allows for the conversion of *ω*-6 to *ω*-3 fatty acids. The *Fat-1* gene is not found in mammals, and the ubiquitous expression of this transgene in all tissues alters the *ω*-6:*ω*-3 ratio in tissues, such

as bone, relative to WT controls. Previous studies indicate that *Fat-1* mice, when fed a diet containing 10% safflower oil, which is high in the ω-6 polyunsaturated fatty acid linoleic acid, had reduced ω-6:ω-3 fatty acids in the femoral cortical bone compared to WT mice.[25] Herein, we hypothesized that a reduction in the endogenous ω-6:ω-3 fatty acid ratio would increase bone accrual, bone strength, and fracture toughness.

Materials and Methods

Animal studies

Animal procedures were approved by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee (IACUC, #00406). All original stocks of mice were purchased from The Jackson Laboratory, and experimental mice were bred in-house. WT male C57BL/6 J mice (#000664) were mated with female *Fat-1* (Tg[CAG-fat-1]1Jxk) heterozygous mice (#020097). Male and female weanlings were identified by PCR genotyping at 21 d, and *Fat-1* positive mice (*Fat-1*) and *Fat-1* negative littermates (WT) were used for the study design (Figure 1A). At 4 wk of age, mice were switched from rodent chow to either a high-fat diet (HFD) with 46% corn oil (Research Diets, D12344, HFD) or AIN-76A Rodent Diet with 12% corn oil (Research Diets, D11724, low-fat diet [LFD]) for 16 wk. The end groups were WT LFD mice (males: $n = 12$, females: $n = 12$), *Fat-1* LFD mice (males: $n = 10$, females: $n = 12$), WT HFD mice (males: $n = 9$, females: $n = 10$), and *Fat-1* HFD mice (males: $n = 9$, females: $n = 9$).

Gas chromatography–mass spectrometry

Fatty acid analysis was performed on tibia and humeri from *Fat-1* and WT controls. Bones were harvested, cleaned of muscle, placed in liquid nitrogen, and pulverized using pestle and mortar. Bone marrow samples were then placed in 6875 Freezer/Mill High Capacity Cryogenic Grinder to obtain small bone particulates. Samples were placed in a 1:2 water to methanol (MeOH) solution. Samples were taken to the Nutrition Obesity Research Center Lipidomics Core Laboratory for total fatty acid analysis. Bone homogenate (1 mg) was saponified with 0.5 N NH4OH in 50% MeOH for 2 h at 37°C, mixed with a mixture of stable isotope-labeled fatty acid standards [d2] myristic, [13C4] palmitic, [d4] stearic, [d8] arachidonic and [d5] docosahexaenoic acids, after saponification samples were acidified and extracted using isooctane. Samples were then dried and derivatized with pentafluorobenzyl bromine in N,N-diisopropylethylamine as described previously.[26] Analysis of the samples was performed by negative ion chemical ionization gas chromatography–mass spectrometry (GC/MS) on a Finnigan DSQ GC/MS system (Thermo Finnigan). The mass spectrometer was operated in the negative ion chemical ionization mode using methane as reagent gas. Data were acquired by selected ion monitoring of the following fatty acids: lauric (m/z 199), myristic (m/z 227), palmitic (m/z 255), stearic (m/z 283), linolenic (m/z 277), linoleic (m/z 279), oleic (m/z 281), eicosapentaenoic (m/z 301), arachidonic (m/z 303), and docosahexaenoic acid (m/z 327). The ions at m/z 229, 259, 287, 311, and 332 were monitored for [d2] myristic, [13C4] palmitic, [d4] stearic, [d8] arachidonic and [d5] docosahexaenoic acids, respectively. Concentration was determined using stable isotope dilution with standard curves generated for each free fatty acid. Data are expressed in nanogram of fatty acid per milligram of bone homogenate. The total ω-6:ω-3 ratio is the sum of ω-6 divided by the sum of ω-3 fatty acids, in other words (linoleic acid + arachidonic acid)/ (gamma-linolenic acid + alpha-linolenic acid + eicosapentaenoic acid + docosahexaenoic acid).

Dual-energy X-ray absorptiometry

The Faxitron DXA system by Hologic was used to measure body composition, areal BMD (g/cm^2), and areal BMC (g) after 4, 8, 12, and 16 wk of diet. The region of interest included the entire body minus the skull. The coefficient of variation for BMC is 0.93% and 0.75% for BMD.

Micro-computed tomography

The trabecular bone architecture of the distal metaphysis and cortical bone morphology of the mid-diaphysis were assessed for the left femurs using a μCT50 instrument (SCANCO Medical). The bones were scanned in 70% ethanol and scans were acquired using a 10 μm^3 voxel size, 70 kVP, 200 μA, 500 ms integration time, and were subjected to Gaussian filtration and segmentation. Image acquisition and analysis were done as previously described.[27] Trabecular bone microarchitecture was evaluated at the region between 7% and 12% of the femoral length proximal to the distal growth plate. A threshold of 550 mgHA/cm^3 was used to segment bone from soft tissue. Trabecular microarchitecture was analyzed using the standard trabecular bone morphology script in the Scanco Evaluation Program. The following architectural parameters were measured: trabecular bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, mm), and trabecular separation (Tb.Sp, mm). Cortical bone was assessed in transverse slices in a region between the mid-diaphysis and 12% of the length of the femoral bone above the growth plate. Cortical bone was segmented using a fixed threshold of 700 mgHA/cm^3. The following variables were computed: total cross-sectional area (bone + medullary area) (Tt.Ar, mm^2), cortical bone area (Ct.Ar, mm^2), medullary area (Ma.Ar, mm^2), bone area fraction (Ct.Ar/Tt.Ar, %), as well as maximum, minimum, and polar moments of inertia (I_{max}, I_{min}, and PMOI, mm^4), which describe the shape/distribution of cortical bone (larger values indicate a higher bending strength).

Whole bone flexural mechanical testing

Following micro-computed tomography (μCT), left femurs were thawed and tested by three-point bending to measure whole bone flexural properties. Each femur was placed anterior side down on 2 mm diameter supports, spaced evenly from mid-diaphysis at ~50% of femur length. Loading was applied at the midspan via a 2 mm diameter cylindrical nose and was performed to failure at 0.1 mm/s, with force and displacement data acquired at 10 Hz (TA Instruments, ElectroForce). Structural strength and maximum deflection were defined at the maximum applied bending moment to account for differences in femoral length. All discrete values of measured deflection were scaled to a standard reference length in the ratio $(L_{ref}/L)^3$ per standard bending formula.[28] Bending stiffness was calculated as the linear region of the force–deflection curve. Femur length was measured before μCT scanning using calipers. Elastic modulus was calculated using the bending stiffness, femur length, and moment of inertia, as previously described.[28,29] Tissue bone strength was calculated using the maximum applied bending moment, moment of inertia, and the distance from the neutral axis to the bone surface, as previously described.[28,29]

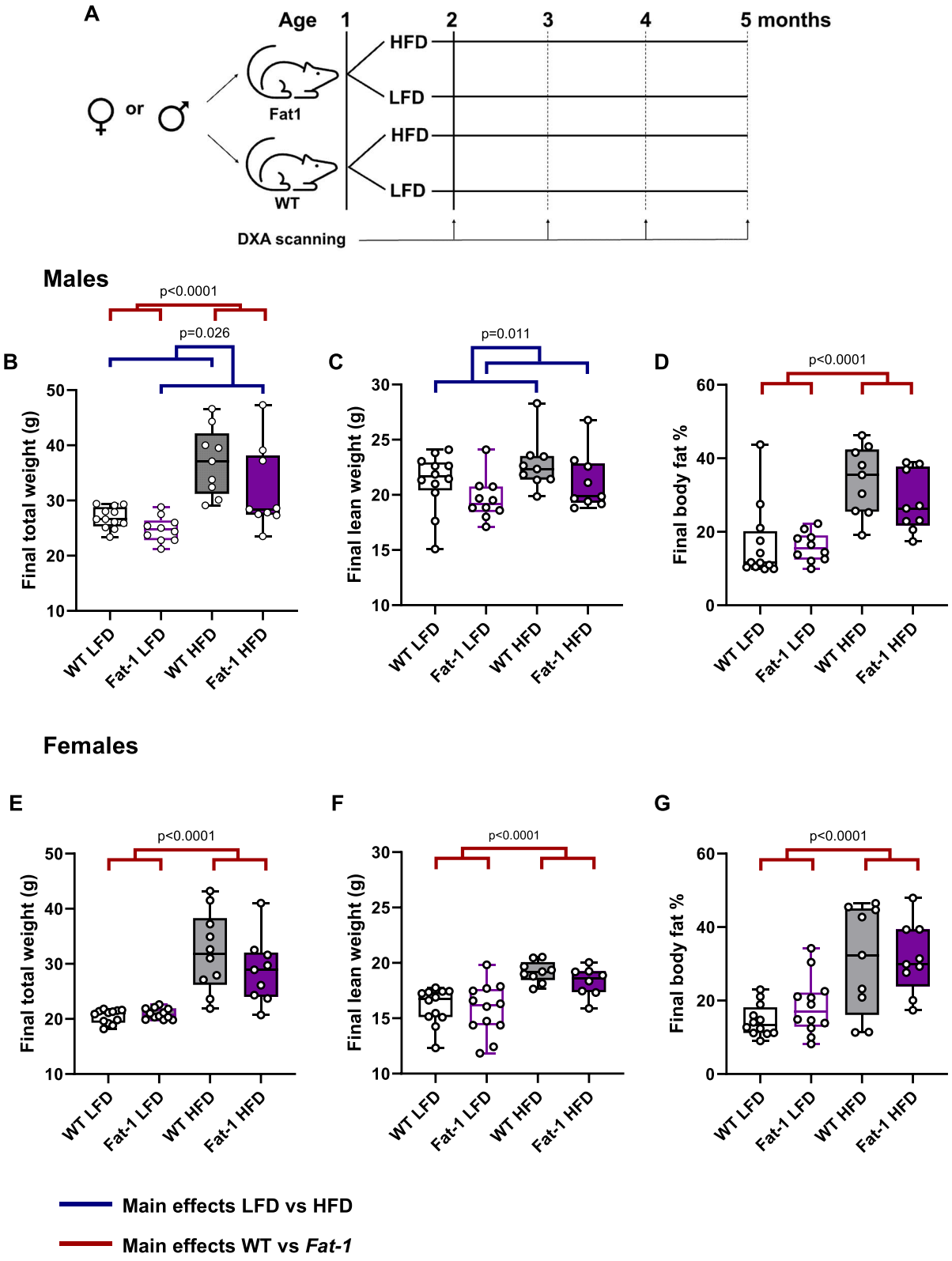


Figure 1. Study design and body composition. Animal study design (A). Four-week-old male and female mice were given either a HFD or LFD for 16 wk. Total weight was measured using a scale and body composition was measured using DXA. Total weight of each group at the end of the study for males (B). Lean weight of each group at the end of the study for males (C). Body fat percentage of each group at the end of the study for males (D). Total weight of each group at the end of the study for females (E). Lean weight of each group at the end of the study for females (F). Body fat percentage at the end of the study for females (G).

Table 1. Total fatty acid analysis in male mice. Total lipids were extracted, methylated, and subjected to analysis using gas chromatography-mass spectrometry. Lipids were extracted from tibial and humeri cortical bone and bone marrow. Units are in nanogram of fatty acid per milligram of bone homogenate. The total ω-6:ω-3 ratio is the sum of ω-6 divided by the sum of ω-3 fatty acids, in other words (linoleic acid + arachidonic acid)/ (gamma-linolenic acid + alpha-linolenic acid + eicosapentaenoic acid + docosahexaenoic acid).

		HFD			LFD		
Common name	Notion	WT Mean ± SEM	*Fat-1* Mean ± SEM	*P*-value	WT Mean ± SEM	*Fat-1* Mean ± SEM	*P*-value
Gamma-linolenic acid	18:3 g (ω-3)	0.228 ± 0.064	0.205 ± 0.027	.753	0.233 ± 0.020	0.245 ± 0.012	.613
Alpha-linolenic acid	18:3 a (ω-3)	0.768 ± 0.201	0.864 ± 0.080	.673	0.383 ± 0.066	1.622 ± 0.570	.095
Eicosapentaenoic acid	20:5 (ω-3)	5.330 ± 2.754	10.037 ± 1.235	.136	1.308 ± 0.456	22.277 ± 4.460	.012
Docosahexaenoic acid	22:6 (ω-3)	59.418 ± 16.376	110.963 ± 23.285	.108	52.827 ± 8.760	172.226 ± 38.232	.034
Linoleic acid	18:2 (ω-6)	92.532 ± 22.792	93.668 ± 9.343	.835	54.349 ± 5.270	95.573 ± 26.665	.199
Arachidonic acid	20:4 (ω-6)	134.192 ± 47.832	89.694 ± 15.784	.403	236.199 ± 35.220	88.181 ± 15.336	.022
Total Ω-6		226.724 ± 64.687	183.363 ± 22.404	.544	290.548 ± 40.357	183.754 ± 41.096	.06
Total Ω-3		64.679 ± 17.402	122.068 ± 24.549	.093	54.751 ± 9.175	196.370 ± 42.831	.028
ω-6:ω-3		4.203 ± 0.796	1.612 ± 0.165	.03	5.438 ± 0.430	0.959 ± 0.101	< .001

HFD, high-fat diet; LFD, low-fat diet

Table 2. Total fatty acid analysis in female mice. Total lipids were extracted, methylated, and subjected to analysis using gas chromatography-mass spectrometry. Lipids were extracted from tibial and humeri cortical bone and bone marrow. Units are in nanogram of fatty acid per milligram of bone homogenate. The total ω-6:ω-3 ratio is the sum of ω-6 divided by the sum of ω-3 fatty acids, in other words (linoleic acid + arachidonic acid)/ (gamma-linolenic acid + alpha-linolenic acid + eicosapentaenoic acid + docosahexaenoic acid).

		HFD			LFD		
Common name	Notion	WT Mean ± SEM	*Fat-1* Mean ± SEM		WT Mean ± SEM	*Fat-1* Mean ± SEM	
Gamma-linolenic acid	18:3 g (ω-3)	0.230 ± 0.013	0.258 ± 0.03	*P* = .787	0.178 ± 0.018	0.230 ± 0.032	*P* = .195
Alpha-linolenic acid	18:3a (ω-3)	0.658 ± 0.135	0.959 ± 0.090	*P* = .101	0.423 ± 0.087	0.796 ± 0.118	*P* = .035
Eicosapentaenoic acid	20:5 (ω-3)	0.754 ± 0.076	10.169 ± 2.353	*P* = .016	2.979 ± 2.368	20.649 ± 3.019	*P* = .012
Docosahexaenoic acid	22:6 (ω-3)	38.165 ± 15.33	98.247 ± 20.376	*P* = .06	49.440 ± 11.182	168.123 ± 15.333	*P* = .012
Linoleic acid	18:2 (ω-6)	98.841 ± 16.853	108.340 ± 9.966	*P* = .641	47.609 ± 4.067	62.771 ± 6.642	*P* = .087
Arachidonic acid	20:4 (ω-6)	113.823 ± 15.781	124.494 ± 21.692	*P* = .701	135.897 ± 21.906	95.737 ± 5.610	*P* = .144
Total Ω-6		212.671 ± 22.581	232.834 ± 26.135	*P* = .576	183.506 ± 21.414	158.508 ± 10.212	*P* = .323
Total Ω-3		39.808 ± 6.187	109.633 ± 22.673	*P* = .035	53.019 ± 13.618	189.798 ± 17.085	*P* < .001
ω-6:ω-3		5.954 ± 1.256	2.452 ± 0.501	*P* = .037	4.327 ± 0.856	0.851 ± 0.064	*P* = .022

HFD, high-fat diet; LFD, low-fat diet

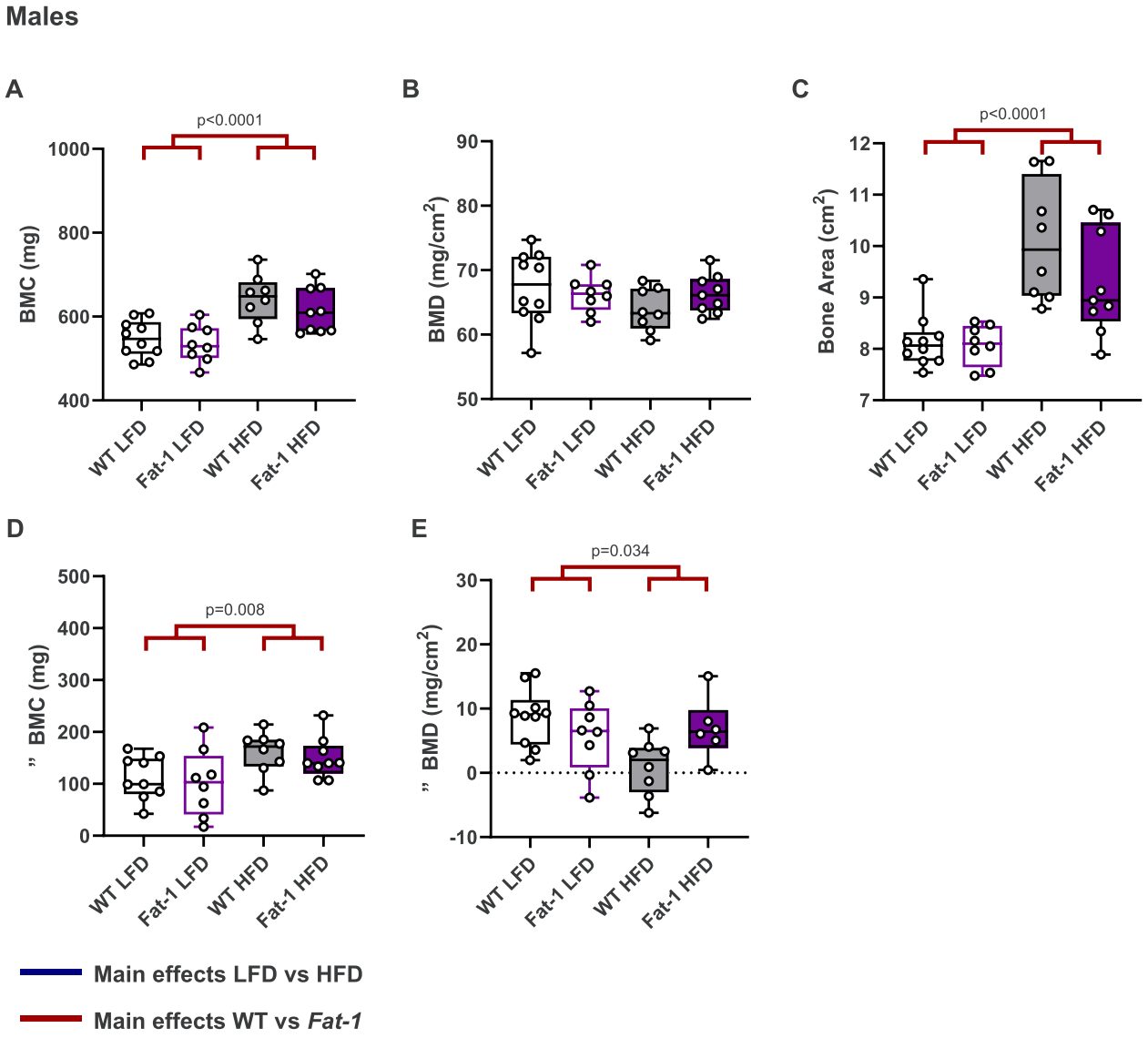


Figure 2. Whole body BMC and density accrual in male mice, assessed using DXA. Bone mass accrual was measured with DXA. BMC at the end of the study (A). BMD at the end of the study (B). Bone area at the end of the study (C). BMC accrual over the course of the study (D). BMD accrual over the course of the study (E).

Fracture toughness testing

Fracture toughness was quantified with notched three-point bending following methods described previously.[30] The notch was created on the posterior aspect of the midshaft femur to a target depth of 0.3× anterior–posterior width using a custom notching apparatus. Femurs were hydrated using PBS and were loaded on the anterior aspect until fracture at a rate of 0.001 mm/s (1 kN load cell Instron 5543). The distal half of the femur was air-dried and then imaged with variable pressure SEM (20 Pa, 15 kV; Zeiss SUPRA 55VP) to obtain the initial notch angle and cross-sectional geometry. Notches that were >30 degrees off the lateral-medial axis were discarded from the analysis. The critical stress intensity factor (Kc) was calculated for two different criteria, first at crack initiation (Kc, yield) and then at the maximum load (Kc, max) using the initial notch angle and the yield and maximum loads, respectively.[30] The yield load was calculated using the secant method where the secant line was considered to be 90% of the measured stiffness from the linear elastic portion of the load–displacement curve.

Statistical analysis

Data were analyzed with SAS version 9.4. To compare the individual (main effects) and combined (interaction) effects of genotype and diet on bone accrual, bone morphometry, and bone mechanical and material properties, a two-way ANOVA was used. Main effects (genotype and diet) were explored when the interaction effects were not significant. Significant interactions between genotype and diet were followed up with a Tukey–Kramer post hoc test. Model residuals were checked for normality and homoscedasticity to ensure the ANOVA assumptions were valid. Outliers were removed if the value was outside the range of the mean ± three SD.

Results

This study aimed to test how altering the endogenous ratios of ω-6:ω-3 fatty acids impacts bone accrual, strength, and fracture toughness. The effects of ω-6:ω-3 fatty acids, independent of diet were evaluated by comparing outcomes in *Fat-1* mice and pooled WT controls. We assessed the effect of diet,

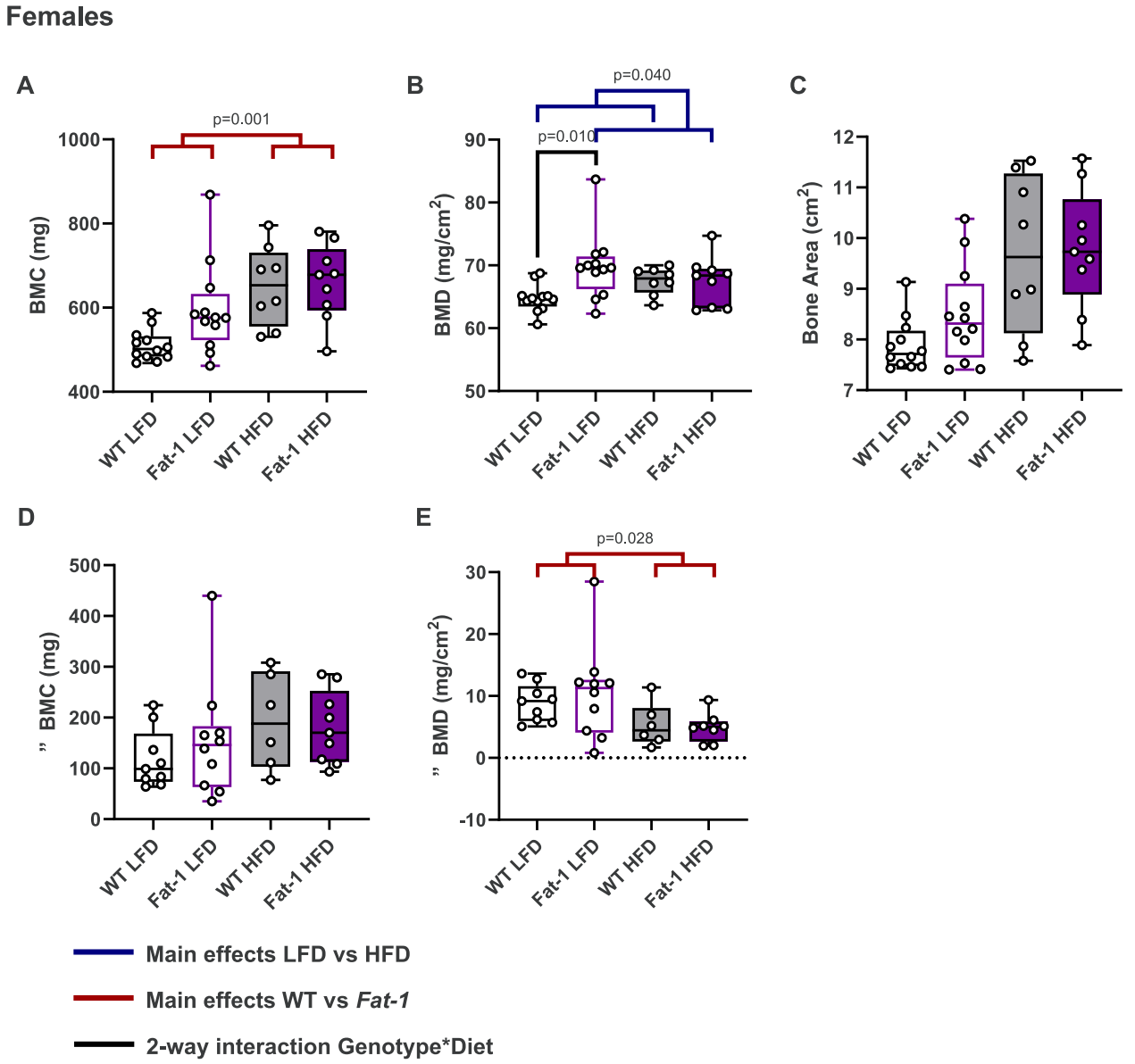


Figure 3. Whole body BMC and density accrual in female mice, assessed using DXA. Bone mass accrual was measured with DXA. BMC at the end of the study (A). BMD at the end of the study (B). Bone area at the end of the study (C). BMC accrual over the course of the study (D). BMD accrual over the course of the study (E).

independent of genotype, by comparing outcomes in HFD mice and pooled LFD mice. The main effects comparisons are not biologically relevant, and therefore direct comparisons between groups were made as well, only statistically significant outcomes are outlined in the results.

Total fatty acid analysis in bone

To verify the functionality of the *Fat-1* transgene, total fatty acids of tibiae and humeri were analyzed after the 16 wk of diet. Four separate analyses were run to compare WT and *Fat-1* mice in each sex and for each diet. In males, there was a significant increase in ω-3 fatty acids in mice fed both a HFD and LFD when compared to WT controls (Table 1). The total ω-6:ω-3 fatty acid ratio was therefore reduced in *Fat-1* mice. The same was observed in females (Table 2).

Body composition and BMD changes

After 16 wk of diet (Figure 1A), the final body weight was lower in male *Fat-1* mice when compared to WT mice, as detected by main group effects (Figure 1B). Male mice on a HFD had increased body weights compared to those on a LFD, as detected by main group effects (Figure 1B). Lean mass at the end of the study was lower in *Fat-1* male mice when compared to WT controls, as detected by main group effects (Figure 1C). The final body fat percentage was increased in male mice on a HFD when compared to mice on a LFD, as detected by main group effects (Figure 1D). In females, at the end of the 16 wk on a diet, there was an increase in final body weight in mice on a HFD compared to mice on a LFD, as detected by main group effects (Figure 1E). Lean mass was increased in HFD fed mice compared to mice on a LFD (main group effects) (Figure 1F). The final body fat percentage was highest in mice on a HFD

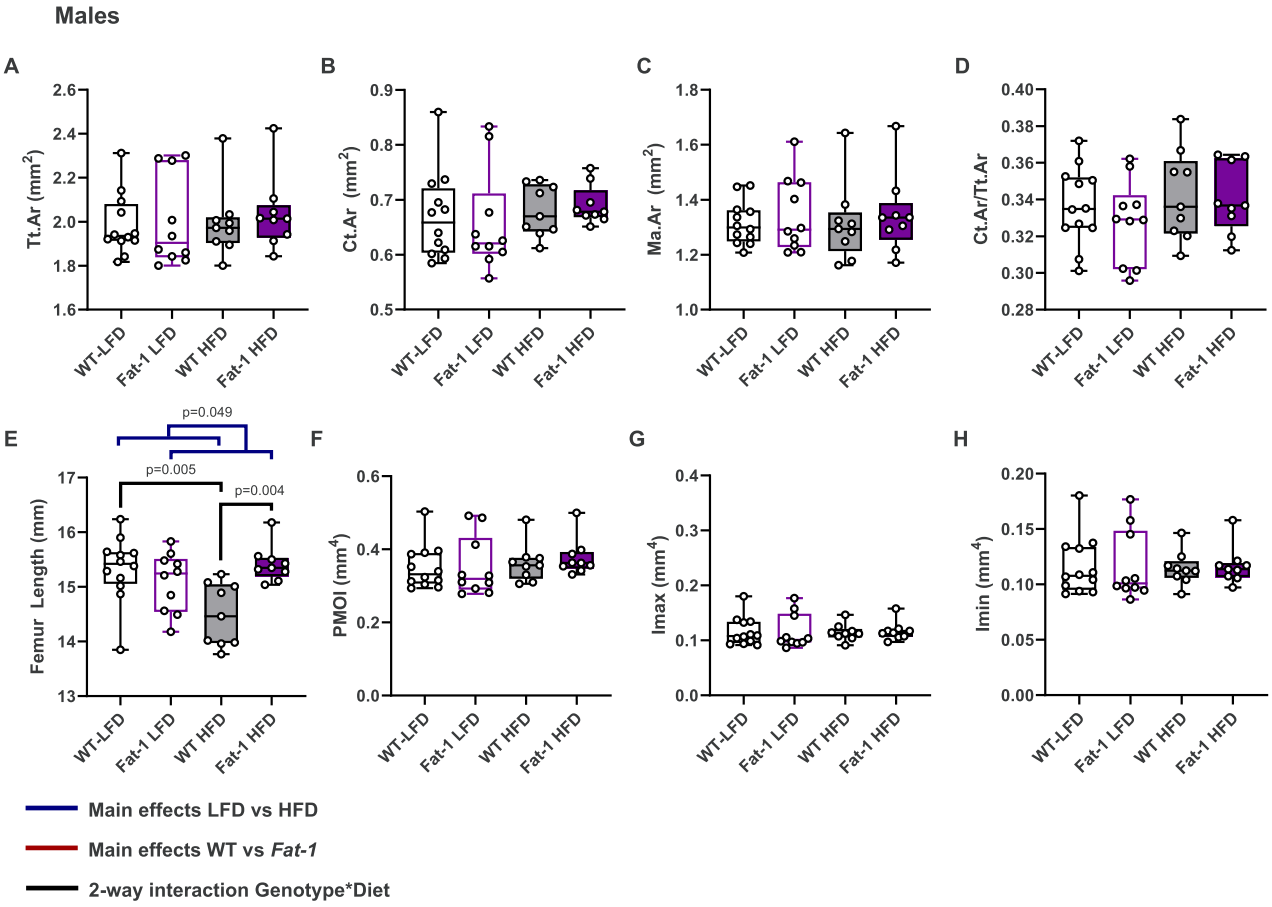


Figure 4. Cortical geometry at the femoral midshaft in male mice, assessed using μCT. All outcomes were measured at the end of the study. Tt.Ar of the femoral midshaft (A). Ct.Ar of the femoral midshaft (B). Ma.Ar of the femoral midshaft (C). Cortical area fraction (Ct.Ar/Tt.Ar) of the femoral midshaft (D). Femoral length (E). PMOI of the femoral midshaft (F). Maximum moment of inertia (I_{max}) of the femoral midshaft (G) and minimum moment of inertia (I_{min}) of the femoral midshaft (H).

in females when compared to mice on a LFD, as detected by main group effects (Figure 1G) (Supplementary Figure 2).

The final BMC in males was highest in mice fed a HFD compared to mice on a LFD, as detected by main group effects (Figure 2A). However, this was not accompanied by increased BMD (Figure 2B). The bone area was highest in male mice on a HFD when compared to mice on a LFD, as detected by main group effects (Figure 2C). Over the course of the study, HFD increased BMC accrual when compared to mice on a LFD (main group effects) (Figure 2D). However, a HFD decreased BMD accrual when compared to LFD fed mice (main group effects) (Figure 2E). In females, BMC at the end of the 16 wk was highest in mice fed a HFD when compared to LFD fed mice (main group effects) (Figure 3A). BMD was increased in *Fat*-1-LFD mice when compared to WT-LFD mice (Figure 3B). Bone area was not altered in females (Figure 3C). It is important to note that HFD feeding did not alter BMC accrual (Figure 3D) but did reduce BMD accrual in females when compared to LFD fed mice (main group effects) (Figure 3E). BMC and BMD for each timepoint and different regions of interest can be found in Supplementary Figures 3–7.

Cortical geometry

Cortical geometry in males was not altered between groups, except for femur length (Figure 4A–H). The femoral length was suppressed in WT-HFD mice, but this was rescued by the introduction of the *Fat-1* transgene (Figure 4E). In females, HFD feeding increased the Tt.Ar of the femur at the mid-diaphysis when compared to mice fed LFD (main group effects) (Figure 5A). Cortical bone cross-sectional area (Ct.Ar) was reduced in *Fat-1*-LFD and WT-LFD mice when compared to WT-HFD (Figure 5B). Marrow area (Ma.Ar) was increased in mice on a HFD when compared to LFD fed mice (main group effects) (Figure 5C). The ratio of Ct.Ar/Tt.Ar was lower in *Fat-1* female mice when compared to WT controls (main group effects) (Figure 5D). Mice on a HFD had reductions in Ct.Ar/Tt.Ar when compared to LFD mice, as detected by main group effects (Figure 5D). The femoral length was highest in mice fed a HFD when compared to LFD fed mice (main group effects) (Figure 5E). The PMOI was reduced in the female *Fat-1*-LFD and WT-LFD mice when compared to WT-HFD mice (Figure 5F). Mice on a HFD had increased maximum (I_{max}) (Figure 5G) and minimum (I_{min}) (Figure 5H) when compared to LFD fed mice (main group effects).

Trabecular architecture of the distal femur

At the end of the 16 wk on diet, male mice did not experience any changes in trabecular architecture of the distal femur (Figure 6A–D). However, females on a HFD experienced increases in total bone volume ratio (BV/TV) when compared to mice on a LFD (main group effects) (Figure 6E). Trabecular number (Tb.N) was not altered between groups

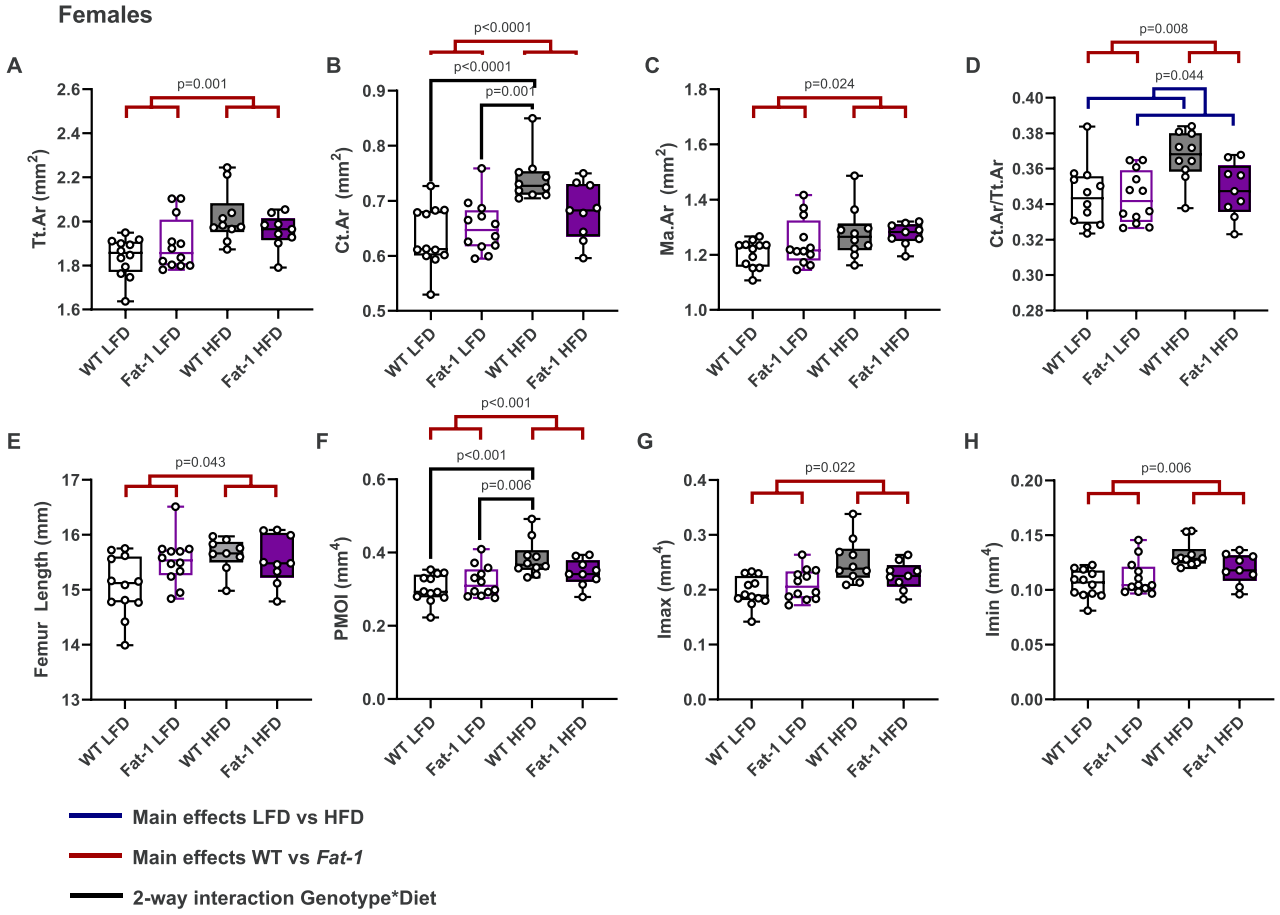


Figure 5. Cortical geometry at the femoral midshaft in female mice, assessed using μCT. All outcomes were measured at the end of the study. Tt.Ar of the femoral midshaft (A). Ct.Ar of the femoral midshaft (B). Ma.Ar of the femoral midshaft (C). Cortical area fraction (Ct.Ar/Tt.Ar) of the femoral midshaft (D). Femoral length (E). PMOI of the femoral midshaft (F). Maximum moment of inertia (I_{max}) of the femoral midshaft (G) and minimum moment of inertia (I_{min}) of the femoral midshaft (H).

(Figure 6F). Tb.Th was reduced in female *Fat-1* mice when compared to WT controls (main group effects) (Figure 6G). Tb.Th was increased in mice on a HFD compared to mice on a LFD, as detected by main group effects (Figure 6F). Trabecular spacing (Tb.Sp) was reduced in female *Fat-1* mice when compared to the WT controls (main group effects) (Figure 6H).

Mechanical and material properties

Three-point bending assessed the mechanical properties of the femurs after 16 wk on diet. In males, stiffness, maximum force, and fracture force were not different between groups (Figure 7A–C). In females, stiffness was reduced in *Fat-1* compared to WT controls (main group effects) and increased HFD fed mice compared to LFD fed mice (main group effects) (Figure 7D). Maximum force was higher in female mice fed a HFD when compared to LFD fed mice (main group effects) (Figure 7E). Fracture force was not different among groups in female mice (Figure 7F).

Material properties were estimated using traditional and notched three-point bending after 16 wk on diet. In males, modulus and ultimate strength were not different among groups (Figure 8A and B). Fracture toughness estimated at crack initiation (Kc_{yield}) was reduced in *Fat-1* male mice compared to WT controls and reduced in mice on a HFD compared to LFD fed mice (main group effects) (Figure 8C). Fracture toughness measured at the maximum load before failure (Kc_{max}) was not different among groups in males (Figure 8D). In females, the modulus was not altered among groups (Figure 8E). However, ultimate strength (ie, apparent strength) was increased in female mice on a HFD when compared to LFD fed mice, as detected by main group effects (Figure 8F). Kc_{yield} (Figure 8G) and Kc_{max} (Figure 8H) were not different among groups in females.

Discussion

Countries around the world continue to adopt a western diet that is known to have a high ω-6:ω-3 ratio and high levels of these fatty acids.[6] Consumption of a western diet has been linked to an increased risk of obesity[31,32] and inflammatory diseases.[6] Additionally, increases in dietary ω-6 and ω-3 fatty acids are associated with reduced hip BMD in women.[33] Work in this area has strongly suggested that the overconsumption of a western diet harms the body and, more specifically, bone.[1,2] However, it remains unknown whether the available ω-6 and ω-3 fatty acids and their relative amounts influence bone accrual and bone material properties. In this study, we aimed to test whether changes in the absolute amounts of fatty acid availability to bone cells altered bone accrual, strength, and fracture toughness. This was accomplished by feeding mice either a HFD or a LFD. In conjunction, we altered the endogenous ratio of ω-6:ω-3 fatty acids available to the bone

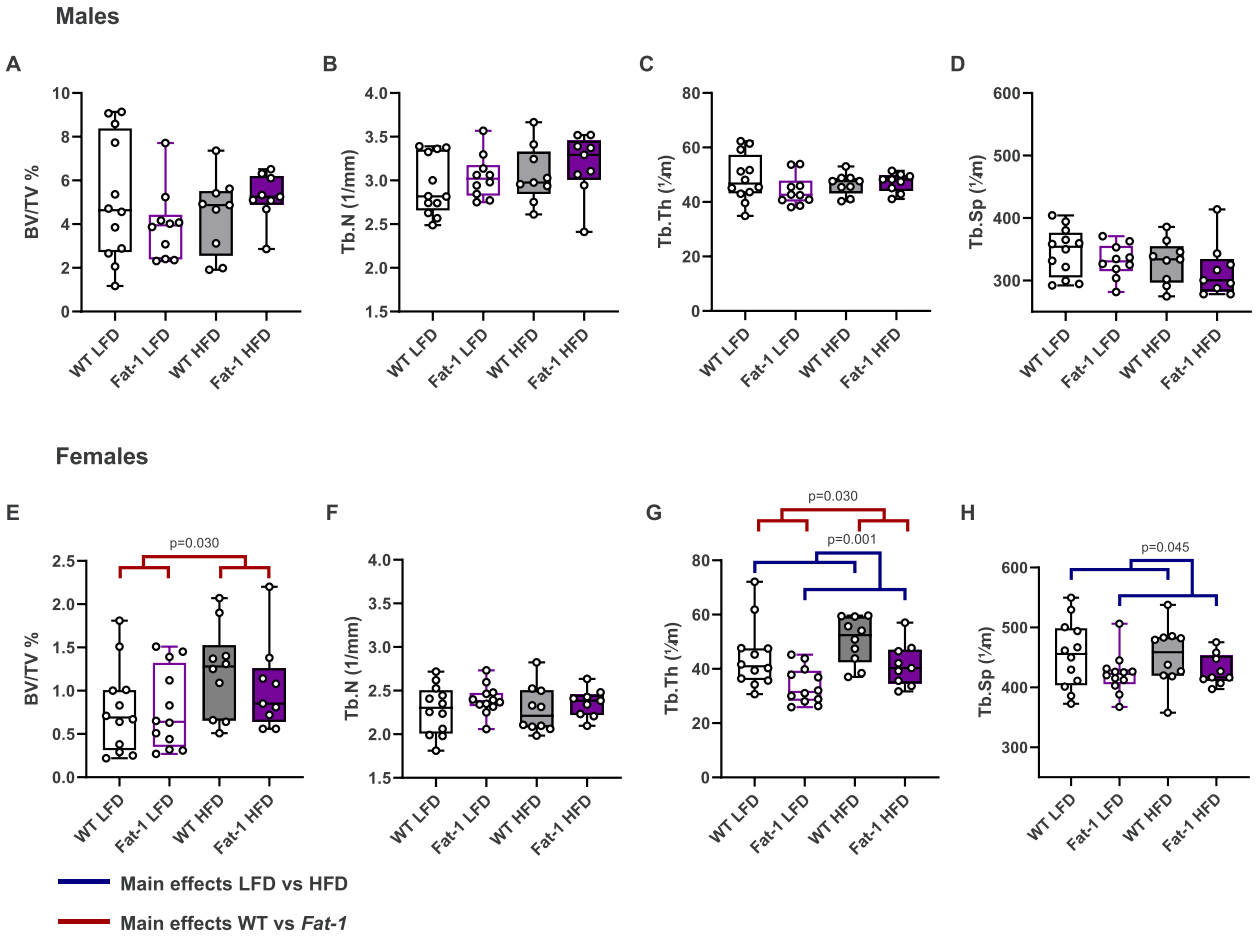


Figure 6. Trabecular architecture at the distal femur, assessed using μCT. All outcomes were measured at the end of the study. BV/TV of the distal femur for males (A). Tb.N of the distal femur for males (B). Tb.Th of the distal femur males (C). Tb.Sp of the distal femur for males (D). BV/TV of the distal femur for females (E). Tb.N the distal femur for females (F). Tb.Th of the distal femur for females (G). Tb.Sp of the distal femur for females (H).

by leveraging the *Fat-1* transgenic mouse model, which has previously been shown to have altered fatty acid profiles in bone.[25]

Our primary finding was that fracture toughness was reduced in *Fat-1* male mice compared to WT controls, and in the male mice fed a HFD compared to LFD fed mice (main group effects). However, this was not accompanied by reductions in other components of fracture resistance, including whole bone strength (ie, maximum load) or apparent strength. These findings suggest that the changes in endogenous ω-6:ω-3 fatty acid ratio are not able to override the negative effects of a HFD with regards to overall bone fracture resistance.

Optimizing peak bone mass during growth is critical to reducing the risk of osteoporosis in older age. However, genetics, environment, physical activity, and diet can all influence peak bone mass.[34,35] Studies have found mixed results on whether a HFD increases peak bone mass and strength in rodents,[36-38] and peak bone mass in mice is generally considered to be achieved at approximately 16 wk of age.[39] This study looked at the effects of dietary fat intake and the endogenous ratio of ω-6:ω-3 fatty acids on bone accrual in growing female and male mice. This was done in a period before they reached peak bone mass and extended into the early adulthood phase. We found that BMD was not altered in males. In females, the *Fat-1* transgene increased BMD at the end of the study compared to WT controls, as detected by main group effects. However, at this time point, the *Fat-1* transgene did not have a significant impact on bone accrual in males and females (Figures 2 and 3) that was sustained into early adulthood. Evidence shows that osteoblasts actively differentiating from mesenchymal stem cells heavily rely on fatty acid uptake during the early stages of differentiation.[16] Whether the transgene has an impact on osteoblast differentiation that would lead to a change in bone accrual is unknown, and further studies are required to investigate this.

At 16 wk, the weight separation between *Fat-1* HFD and WT HFD males became significant (Supplementary Figure 1). If the study had been carried out further, this separation could have become more pronounced,[40] and there may have been a clear alteration in mesenchymal stem cell differentiation to adipocytes vs osteoblast due to the change in endogenous fatty acids.[7] We hypothesize that over time, this would have deleterious impacts on bone accrual, which may or may not be accompanied by changes in bone resorption. As BMD is a function of both formation and resorption, understanding the impact of changing this fatty acid ratio on both osteoblast and osteoclast function should be explored.

Our findings stand in contrast to others that have found that an increase in endogenous ω-3 fatty acids protects *Fat-1* mice against ovariectomy-induced bone loss when compared to WT mice.[41,42] However, this is primarily an

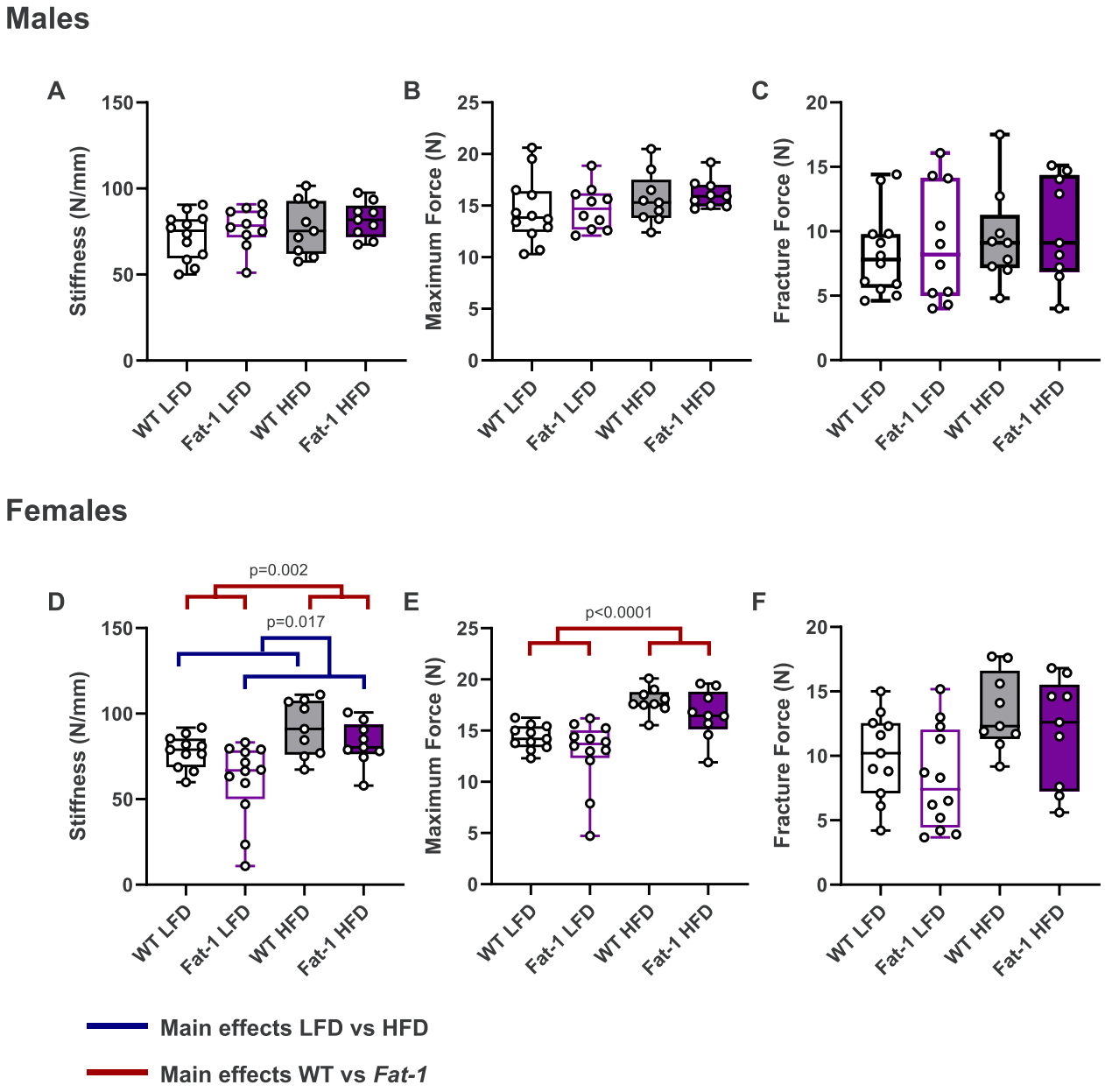


Figure 7. Mechanical properties of the femur, assessed using traditional three-point bending. All outcomes were measured at the end of the study. Stiffness for males (A). Maximum force required for post-yield displacement in males (B). Fracture force for males (C). Stiffness for females (D). Maximum force required for post-yield displacement for females (E). Fracture force for females (F).

osteoclast-mediated phenotype.[42] Furthermore, these studies utilized diets containing 10% corn oil-based fats, making it difficult to compare these results to our study directly since our diets have 46% (HFD) and 12% (LFD) corn oil composition. Zhan et al. found that a dietary supplement of Antarctic krill oil (lower ω-6:ω-3) improved BMD, mineral apposition rate, and trabecular structure in ovariectomized mice when compared to mice fed a diet supplemented with arachidonic acid.[43] The reduced ω-6:ω-3 fatty acids in Antarctic krill oil was from an increase in ω-3 fatty acids, which regulated osteoclastogenesis via the NF-κB signaling pathway.[43] The timing of diet introduction must also be considered, as alterations in the ω-6:ω-3 fatty acid ratio may be primarily beneficial only after skeletal maturity, providing protection against age-related decline. Furthermore, this study was not designed to explore the possibility that the ω-6:ω-3 ratio may have a role during fetal growth and lactation, as embryonic stem cell programming could be influenced by the altered availability of fatty acids. Further studies are required to understand if age influences the effects of fatty acids on bone accrual.

As with bone accrual, longitudinal growth may be impacted by genetics, diet, and environment. Existing studies with a HFD show no effect or an increase in femur length with a HFD in mice.[44,45] In male mice, we observed that a HFD suppressed femoral growth in the WT animals, but this effect (main group effect) was rescued by the introduction of the *Fat-1* transgene. The reduction in ω-6:ω-3 fatty acids may be playing a role, as previous studies found that a decrease in the availability of lipids in the bone microenvironment promotes skeletal

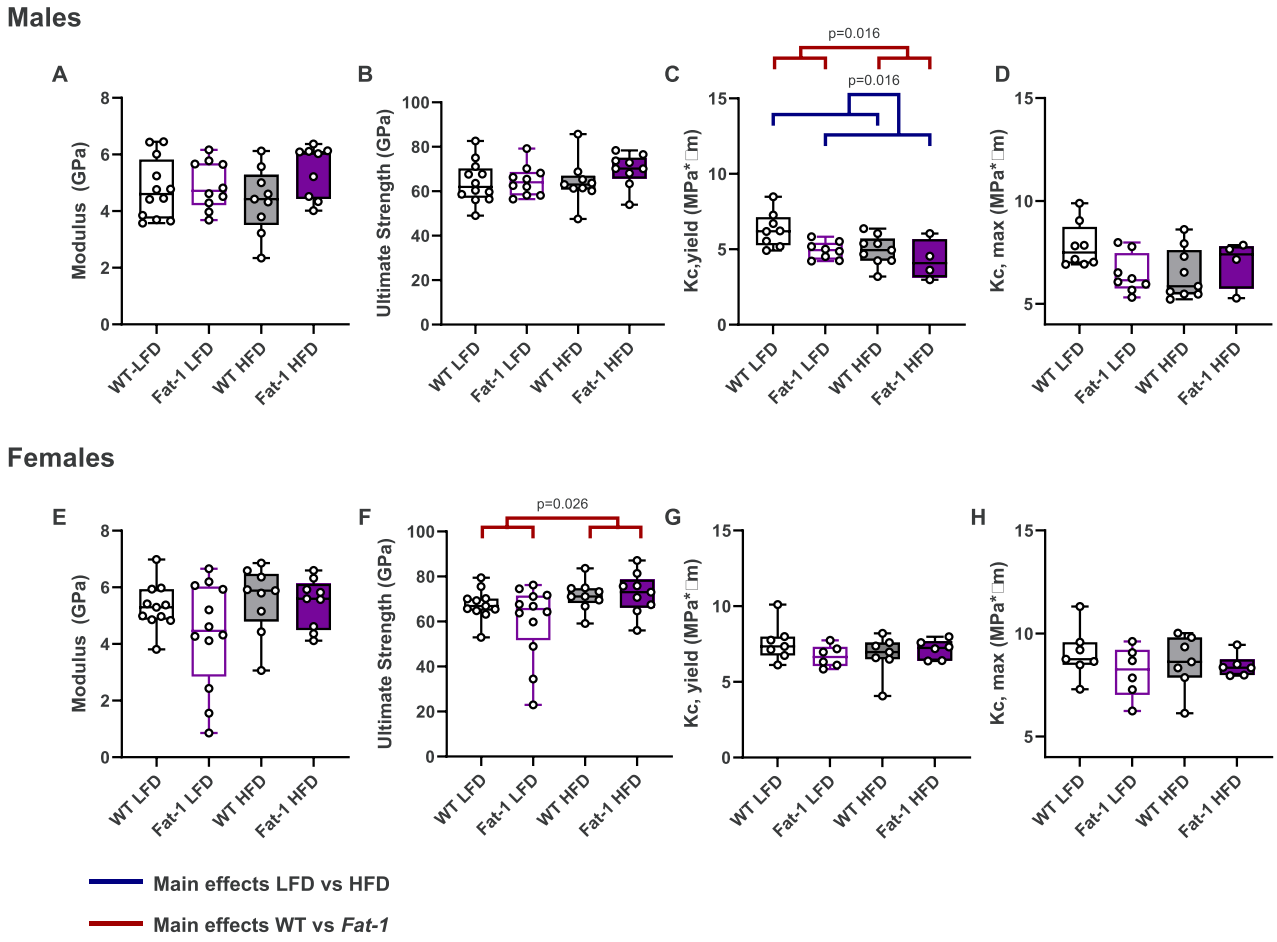


Figure 8. Material properties of the femur, assessed using traditional and notched three-point bending. All outcomes were measured at the end of the study. Bone tissue modulus for males (A). Ultimate strength for males (B). Fracture toughness at crack initiation for males (C). Fracture toughness at stable-crack propagation for males (D). Bone tissue modulus for females (E). Ultimate strength for females (F). Fracture toughness at crack initiation for females (G). Fracture toughness at stable-crack propagation for females (H).

progenitor cell differentiation to chondrocytes vs osteoblasts.[46] The *Fat-1* mice may have an advantageous effect, but more studies are required to better understand how types of fatty acids alter skeletal progenitor cell differentiation and how this effect manifests in the growth plate. It is also curious that this effect on long bone growth was not observed in the females.

This study demonstrates that although bone accrual is not affected by the *Fat-1* transgene, several other aspects related to bone quality are impacted, albeit in a sex-dependent manner. For males, cortical and trabecular architecture outcomes were not independently altered by genotype or diet throughout the study. For females, we found that WT-HFD mice had an increased Ct.Ar, but this cortical increase was suppressed by the introduction of the *Fat-1* transgene. As expected, this translated to changes in predicted bone strength in torsion (PMOI), as this is a calculated value derived from geometry and size. However, the alterations in Ct.Ar and PMOI in phenotypes could be partially attributed to changes in final body weight, as the WT mice were heavier than *Fat-1* mice (main group effect). Additionally, *Fat-1* females exhibited a reduction in Tb.Th and increases in Tb.Sp. These results disagree in some aspects with prior work with the *Fat-1* mouse model, including increases in cortical stiffness in *Fat-1* mice on a normal chow diet.[38] However, the study design and the diet used were dissimilar to our study and may explain the differences in results. Also, osteoclast activity is reduced in *Fat-1* ovariectomized and sham mice.[42] However, it is unknown if reduced osteoclast activity is occurring in the *Fat-1* mice in this study since it was not measured. Reduced osteoclast activity may play a role in the quantity and quality of bone turnover occurring in trabecular bone. This may explain why there is a reduction in Tb.Th, but further studies are required.

Bone strength is often used as an adequate predictor of fracture risk and therefore helps inform existing data on how the mechanical behavior of bone is altered in varying conditions. Studies have reported that supplementation of polyunsaturated fatty acids has led to increases in bone strength.[47,48] In males, mechanical properties were not altered among groups. In females, the *Fat-1* transgene reduced cortical stiffness, and a HFD increased both cortical stiffness and maximum force (main group effects). However, the reductions in mechanical properties associated with the *Fat-1* transgene in females are attributed to increases in Ct.Ar and PMOI. The relationship between whole bone strength and section modulus in females is similar between genotypes, but diet affects the linear relationship between strength and geometry (Supplementary Figure 8). This indicates that alterations in bone strength are not explained by geometry differences. Section modulus is a geometric property and therefore the unaltered whole bone strength in the *Fat-1* transgene further

supports that changes in bone strength are explained partly by geometry.

Material properties were estimated to better understand tissue level changes throughout the study. In females, ultimate strength (ie, tissue apparent strength) was increased in mice on a HFD (main group effect), but we did not observe a genotype effect. In males, fracture toughness in *Fat-1* mice and mice on a HFD is reduced when compared to WT controls and LFD fed mice (main group effects), respectively, and suggests that tissue-scale bone properties important to resist crack propagation are degraded. This is in agreement with previous data that has found that a HFD reduced fracture toughness in older (15-wk-old) mice compared to mice fed a LFD.[36] However, our results disagree with studies that observed no changes in fracture toughness in Wistar rats on a HFD.[49] It is unclear why the *Fat-1* transgene reduces fracture toughness, but it is possible that collagen or mineral properties were altered. Sex-specific differences are not uncommon for bone phenotypes in mice,[50] and this represents an interesting direction for future work.

There were several limitations in this study, some of which have been noted above. To begin, no randomization occurred when mice were switched to a HFD or LFD. This was due to diet shortages at the beginning of the coronavirus disease (COVID-19) pandemic, and therefore, the first cohorts were fed a HFD and LFD with 3–6 mo periods in between. After this time period, there was a more even switch off in diet randomization between later cohorts. Sample processing was still done in large batches to reduce the batch effect for tissue analysis. We did not record food intake, although previous studies have found that there is a decrease in energy intake in *Fat-1* mice compared to WT controls after 5 d of exposure to a high-fat, high-sucrose diet.[40] We did not quantify blood glucose or circulating lipids, as mice were not fasted during harvest. Lastly, we did not perform histological analysis to determine whether there were any changes in osteoblast and osteoclast activity that could better inform our results.

Conclusion

Our results suggest that a reduction in ω-6:ω-3 fatty acids influences bone strength and material properties in a unique way that is dependent on sex. Our results indicate that reducing ω-6:ω-3 fatty acids may not be beneficial in males, but further studies are required to understand the cellular mechanisms.

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Author contributions

Beatriz Bermudez (Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Writing—original draft, Writing—review and editing), Kenna Brown (Data curation, Formal analysis, Writing—review and editing), Ghazal Vahidi (Formal analysis, Writing—review and editing), Ana C.F. Ruble (Conceptualization, Data curation, Formal analysis, Methodology, Writing— review and editing), Chelsea Heveran (Funding acquisition, Methodology, Resources, Supervision, Validation, Writing—review and editing), Cheryl L. Ackert-Bicknell (Funding acquisition, Supervision, Writing— review and editing), and Vanessa Sherk (Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing—review and editing)

Supplementary material

Supplementary material is available at *JBMR PLUS* online.

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Conflicts of interest

None declared.

Data availability

Investigators interested in collaboration with data or with questions, can email VSD and CLAB at vanessa.sherk@nih.gov and cheryl.ackert-bicknell@cuanschutz.edu.

Human and animal rights

Animal procedures were approved by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee.

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