Bone 105 (2017) 200–211

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

Obesity and type 2 diabetes, not a diet high in fat, sucrose, and cholesterol, negatively impacts bone outcomes in the hyperphagic Otsuka Long Evans Tokushima Fatty rat

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ARTICLE INFO

Article history:
Received 9 May 2017
Revised 14 July 2017
Accepted 8 September 2017
Available online 9 September 2017

Keywords:
Obesity
Type 2 diabetes
Western diet
Bone
Trabecular microarchitecture
Bone turnover markers

ABSTRACT

Background: Obesity and type 2 diabetes (T2D) increase fracture risk; however, the association between obesity/T2D may be confounded by consumption of a diet high in fat, sucrose, and cholesterol (HFSC).
Objective: The study objective was to determine the main and interactive effects of obesity/T2D and a HFSC diet on bone outcomes using hyperphagic Otuska Long Evans Tokushima Fatty (OLETF) rats and normophagic Long Evans Tokushima Otsuka (LETO) controls.
Methods: At 8 weeks of age, male OLETF and LETO rats were randomized to either a control (CON, 10 en% from fat as soybean oil) or HFSC (45 en% from fat as soybean oil/lard, 17 en% sucrose, and 1 wt%) diet, resulting in four treatment groups. At 32 weeks, total body bone mineral content (BMC) and density (BMD) and body composition were measured by dual-energy X-ray absorptiometry, followed by euthanasia and collection of blood and tibiae. Bone turnover markers and sclerostin were measured using ELISA. Trabecular microarchitecture of the proximal tibia and geometry of the tibia mid-diaphysis were measured using microcomputed tomography; whole-bone and tissue-level biomechanical properties were evaluated using torsional loading of the tibia. Two-factor ANOVA was used to determine main and interactive effects of diet (CON vs. HFSC) and obesity/T2D (OLETF vs. LETO) on bone outcomes.
Results: Hyperphagic OLEFT rats had greater final body mass, body fat, and fasting glucose than normophagic LETO, with no effect of diet. Total body BMC and serum markers of bone formation were decreased, and bone resorption and sclerostin were increased in obese/T2D OLETF rats. Trabecular bone volume and microarchitecture were adversely affected by obesity/T2D, but not diet. Whole-bone and tissue-level biomechanical properties of the tibia were not affected by obesity/T2D; the HFSC diet improved biomechanical properties only in LETO rats.
Conclusions: Obesity/T2D, regardless of diet, negatively impacted the balance between bone formation and resorption and trabecular bone volume and microarchitecture in OLETF rats.

1. Introduction

The prevalence of diabetes in adults has nearly doubled in the past decade, with type 2 diabetes (T2D) accounting for 90–95% of diagnosed cases [1,2]. Because excessive adiposity is the strongest driver of T2D, the increased incidence of T2D is tightly linked to the increase in overweight and obesity [3,4]. Data accumulated during the past decade show that fracture risk is increased in obesity/T2D [5–17], dispelling the long-standing assumption that obesity/T2D was beneficial to skeletal health because of increased bone mineral density (BMD) [18].

Because BMD is either normal or elevated in obesity/T2D [15], the increased fracture risk has been attributed to deterioration of bone quality, including changes in bone remodeling [19,20], and in bone's structural and material properties [19,21,22]. Bone remodeling rate is suppressed by obesity/T2D with a greater inhibition of bone formation

Abbreviations: AGE, advanced glycated endproducts; BMC, areal bone mineral content (g); BMD, areal bone mineral density (g/cm^2); BV/TV, bone volume; Conn.D, connectivity density; Ct.Ar, cortical area; Ct.Th., cortical thickness; CTx, C-terminal telopeptide of type I collagen; DA, degree of anisotropy; DXA, dual-energy X-ray absorptiometry; EF, ellipsoid factor; G, tissue-level stiffness; Imax, second moment of area around the major axis; Imin, second moment of area around the minor axis; J, polar moment of inertia; Ks, torsional stiffness; Le, length; LETO, Long-Evans Tokushima Otsuka; Ma.Ar, marrow area; OC, osteocalcin; OLETF, Otsuka Long-Evans Tokushima Fatty; P1NP, amino-terminal propeptide of type I collagen; PBS, phosphate-buffered saline; Su, tissue-level strength; T2D, type 2 diabetes; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; Tmax, maximum torque; TRAP5b, tartrate-resistant acid phosphatase isoform 5b; Tt.Ar, total cross-sectional area; U, energy absorbed to failure; μCT, microcomputed tomography.

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http://dx.doi.org/10.1016/j.bone.2017.09.003
8756-3282/

relative to resorption [23,24]. At the cellular level, bone formation is reduced because osteoblast differentiation and proliferation are inhibited [25,26], while adipogenesis is increased [27]. These alterations occur in both untreated T2D [31–33] and as result of treatment with thiazolidinediones (TZDs), which as PPARγ agonists, promote adipogenesis at the expense of osteoblastogenesis [28]. Inhibition of canonical Wnt signaling by the osteocyte-derived protein sclerostin promotes the shift from osteoblastogenesis to adipogenesis [29–32]. Obesity/T2D is associated with increased circulating sclerostin in humans and experimental animals [33–35]. Thus, decreased Wnt/β-catenin signaling appears to play a central role in the pathology underlying bone fragility in obesity/T2D. Obesity/T2D adversely affect bone's material properties by increasing the advanced glycated end-products (AGEs) content of bone [36–38], which increases propensity to fracture [36,38–40]. Data supporting this hypothesis are largely from experimental animals (reviewed in (38)); only one study has reported increased AGE content in bone from T2D patients and it was not clear if the bone was cortical, cancellous or a mixture [41].

Confounding the relationship between obesity/T2D and increased skeletal fragility is the coincident "westernization" of the diet [42,43]. The typical "Western Diet" is high in saturated fat, cholesterol and sucrose [42], each of which may impact bone independent of body weight or adiposity [44–48]. Although feeding a high-fat diet (i.e., 45–60 en% from fat) or Western Diet negatively impacts bone turnover [49–51], trabecular microstructure [49,52], and bone biomechanical properties [47,53–56] in rodents, the effects of obesity/T2D have not been dissociated from the effects of diet composition.

The OLETF rat is selectively bred for null expression of cholecystokinin-1 receptor in the hypothalamus, resulting in hyperphagia independent of diet composition [57]. Thus, an advantage of the OLETF rat is that a high-fat diet is not needed to induce obesity/T2D. The OLETF rat is an appropriate animal model in which to evaluate the skeletal effects of obesity/T2D due to a gradual progression from insulin resistance to T2D near the time of skeletal maturity, which recapitulates both the disease progression and skeletal alterations observed in humans [58,59].

The objective of the current study was to determine the effects of obesity/T2D and a diet high in fat, sucrose, and cholesterol (HFSC) on bone outcomes using hyperphagic OLETF rats and normophagic Long-Evans Tokushima Otsuka (LETO) controls. By use of a two-by-two factorial design (i.e., +/− obesity/T2D and +/− HFSC), we investigated the main and interactive effects of genotype (presence of obesity/T2D) and diet on bone outcomes. We hypothesized that hyperphagia resulting in obesity/T2D and WD would independently: decrease bone formation relative to resorption; reduce total body BMD and BMC relative to body mass; adversely affect trabecular bone volume and microarchitecture of the proximal tibia; decrease whole-bone and tissue-level biomechanical properties of the tibia mid-diaphysis; and increase AGE content. We also hypothesized that genotype and diet would have negative additive effects, such that OLETF rats fed the HFSC diet would have the worst bone outcomes.

2. Methods

2.1. Experimental design and tissue collection

The study used a 2-factor experimental design to determine the main and interactive effects of diet [a diet high in fat, sucrose, and cholesterol (HFSC) vs. control diet (CON)] and genotype [hyperphagic OLETF vs. normophagic LETO] in a 24-week longitudinal study. A portion of the animal characteristics have been published previously [60]. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Missouri. OLETF and LETO rats were obtained from the Tokushima Research Institute (Tokushima, Japan). At 8 weeks of age, the OLETF (O) and LETO (L) rats were randomized to either a control CON or HFSC diet (Tables 1 and 2), resulting in four treatment groups: L-CON ($n = 10$), L-HFSC ($n = 10$), O-CON (n

Table 1
Composition of the control (CON) and high-fat, high-sucrose, and high-cholesterol (HFSC) diets[a].

	CON		HFSC	
	g/kg	% kcal	g/kg	% kcal
Protein		20		20
Casein	189.6	(19.7)	230.8	(19.7)
L-Cystine	2.8	(0.3)	3.5	(0.3)
Carbohydrate		70		35
Corn starch	520.8	(54.2)	84.0	(7.2)
Maltodextrin 10	118.5	(12.3)	115.4	(9.9)
Sucrose	24.2	(2.5)	199.4	(17.0)
Cellulose, BW200	47.4		57.7	
Fat		10		45
Soybean oil	23.7	(5.6)	28.8	(5.6)
Lard	19.0	(4.4)	204.8	(39.4)
Cholesterol	0		9.8	
Minerals				
Mineral Mix S10026	9.5		11.5	
DiCalcium Phosphate	12.3		15.0	
Calcium Carbonate	5.2		6.3	
Potassium Citrate, w H2O	15.6		19.0	
Vitamins				
Vitamin Mix V10001	9.5	(1.0)	11.5	(1.0)
Choline bitartrate	1.9		2.3	
FD&C yellow dye #5	0.02		0	
FD&C blue dye #1	0.02		0	
Total	1000	100 (100)	1000	100 (100)

[a] Energy density of the CON diet was 3.85 kcal/g and for the HFSC diet was 4.68 kcal/g.

Table 2
Micronutrient content of the control (CON) and high-fat, high-sucrose, and high-cholesterol (HFSC) diets.

	CON		HFSC	
	Amount/100 kcal	g/kg	Amount/1000 kcal	g/kg
Vitamins				
Vitamin A, IU[a]	98.60	3792	98.60	4616
Vitamin D3, IU	24.65	948	24.65	1154
Vitamin E, IU	1.232	47.40	1.232	57.70
Vitamin K, mg[b]	0.012	0.47	0.012	0.58
Biotin, mg	0.005	0.19	0.005	0.23
Vitamin B12, ug[c]	0.246	9.48	0.246	11.54
Folic acid, mg	0.049	1.90	0.049	2.31
Niacin, mg[d]	0.739	28.44	0.739	34.62
Pantothenic acid, mg[e]	0.394	15.17	0.394	18.46
Vitamin B6, mg[f]	0.173	6.64	0.173	8.08
Vitamin B2, mg[g]	0.148	5.69	0.148	6.92
Vitamin B1, mg[h]	0.148	5.69	0.148	6.92
Minerals				
Calcium carbonate	3.204	5.21	3.204	6.35
Dicalcium Phosphate	1.356	12.32	1.356	15.00
Potassium citrate	4.067	15.64	4.067	19.04
Cl, g	0.025	0.95	0.025	1.15
Na, g	0.039	1.52	0.039	1.85
Mg, g	0.012	0.47	0.012	0.58
S, g	0.008	0.31	0.008	0.38
Mo, mg	0.039	1.52	0.039	1.85
Cr, mg	0.049	1.90	0.049	2.31
Cu, mg	0.148	5.69	0.148	6.92
Fe, mg	0.912	35.08	0.912	42.70
Mn, mg	1.454	55.93	1.454	68.09
I, mg	0.005	0.19	0.005	0.23
Fl, mg	0.022	0.85	0.022	1.04
Se, mg	0.004	0.15	0.004	0.18
Zn, mg	0.715	27.49	0.715	33.47

[a] Vitamin A palmitate.
[b] Menodione.
[c] Cyanocobalamin.
[d] Nicotinic acid.
[e] Calcium pantothenate.
[f] Pyridoxine-HCl.
[g] Riboflavin.
[h] Thiamin-HCl.

= 10), O-HFSC ($n = 16$). The sample size was estimated assuming 80% power and a per-comparison error rate (α) of 0.05 using differences in bone biomechanical properties between OLETF and LETO rats fed a standard chow diet [59]; we estimated that 10 animals were needed per group. Because the animals included in this study were part of a larger project, we had 16 O-HFSC animals; for some outcomes data from an animal were not usable and the sample size for each group is included in the table or figure legend. For the CON diet (D12110704; Research Diets Inc., New Brunswick, NJ), the fat, carbohydrate, and protein content as a percent of kilocalories was 10%, 70%, and 20%, respectively, with the fat provided as a mixture of soybean oil (5.6 en%) and lard (4.4% en%). The HFSC diet (D09071604; Research Diets Inc.) contained 1% cholesterol by weight and the fat, carbohydrate, and protein content as a percent of kilocalories was 45%, 35%, and 20%, respectively, with the fat provided as a mixture of soybean oil (5.6 en%) and lard (39.4 en%). The micronutrient content of the HFSC diet was increased ~20% by weight so that the micronutrients per kcal were the same between the CON and HFSC diets (Table 2) and both diets provided micronutrients in amounts consistent with recommendations for rodents [61]. All animals had ad libitum access to food and water and were individually housed in a temperature-controlled environment (21 °C) with a 0600–1800 h light-1800–0600 h dark cycle that was maintained throughout the duration of the study. Body weight was measured weekly, and food intake was assessed by weighing every three days.

At 32 weeks of age, animals were fasted for 12 h, and then between 8:00 and 10:00 AM, anesthetized with sodium pentobarbital (100 mg/kg) and a dual-energy X-ray absorptiometry (DXA) scan (Hologic Discovery A QDR series) performed to determine body composition, total body BMD, and BMC. Animals were then exsanguinated via cardiac puncture, and serum was separated by centrifugation and stored at −80 °C for future analysis. The right tibia was removed and placed in formalin for later assessment of trabecular microarchitecture. The left tibia and right radius were removed, cleaned of soft tissue, wrapped in PBS-soaked gauze, and stored at −80 °C. Biomechanical properties, AGE and collagen content were measured in the left tibia diaphysis; ash content was measured in the right radius.

2.2. Serum analyses

The concentrations of glucose (Sigma, St. Louis, MO) and insulin (Linco Research, St. Charles, MO) in serum were determined using commercially available methods as described previously [62]. Serum markers of bone formation, collagen type I propeptide (P1NP), and bone resorption, C-terminal telopeptide of type I collagen (CTX) and tartrate resistant acid phosphatase isoform 5b (TRAP5b), were measured using commercially available, rodent-specific ELISA kits (ImmunoDiagnostic Systems, Fountain Hills, AZ). Osteocalcin (OC) is an osteoblast-secreted peptide with three glutamic acid residues that can be carboxylated to form the mature peptide (i.e., carboxylated OC, cOC). cOC is secreted into the bone matrix [63], and is considered a marker of bone formation [64]. During bone resorption, OC is decarboxylated in the acidic environment of the resorption pit to form undercarboxylated osteocalcin (uOC) [65]; thus, uOC is sometimes viewed as an indicator of bone resorption. cOC and uOC were measured using commercially available, rat-specific ELISA kits (Takara Bio, Inc. USA, Mountain View, CA, USA). Total OC was calculated as the sum of cOC and uOC. The coefficient of variations were <4% for CTx, <3% for TRAP5b, <6% for P1NP, and <9% for cOC and uOC. Serum sclerostin was also measured using a commercially available rodent-specific ELISA kit (R&D Systems, Minneapolis, MN), with a CV of <4%.

2.3. Tibial trabecular microarchitecture

Trabecular microarchitecture was assessed using a SkyScan1174v2 microCT scanner (SkyScan Bruker-microCT, Kontich, Belguim) according to the guidelines described by Bouxsein et al. [66]. All scans were performed with an X-ray setting of 50 kV voltage and 800 μA current with a 3000-ms exposure using a bin of 1. In a single rotation, 460 projections were collected at 0.4-degree increments. Scans were performed with an effective voxel size of 11.6 μm. Images were reconstructed in NReconServer version 1.6.9 (SkyScan Bruker-microCT) with a post-alignment of −17.0, beam-hardening correction of 33%, and ring-artifacts reduction of 5. Trabecular bone structural properties were assessed in a 2.5-mm region of interest directly below the growth plate of the proximal tibia because it is a similar percentage of tibial length that is commonly quantified in mice [67]. A global upper threshold of 255 and lower threshold of 62 (μCT grey scale value) was used for all samples to separate the bone from the background and soft tissue. CTan version 1.14.1 (SkyScan, Bruker-microCT) was used to determine bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), connectivity density (Conn.D), ellipsoid factor (EF), and degree of anisotropy (DA). EF has been developed as a method for measuring rod- and plate-like geometry, independent of other geometric parameters, so it is not confounded by treatment differences in BV/TV [68]. Bony plates are approximated by discus-shaped ellipsoids, where EF approaches −1; rods are approximated by javelin-shaped ellipsoids where EF approaches 1, whereas transitional regions have less extreme geometry and are rugby ball-shaped where EF approaches 0 [68].

2.4. Tibial cortical morphometry

Cortical morphometry of the left tibia was assessed using micro-computed tomography (INVEON Micro-CT scanner, Siemens Medical, Malvern, PA, USA). Scans were performed with an X-ray setting of 80 kVp voltage and 500 μA current with a 180-ms exposure at a medium-high magnification using a bin of 4. In a single rotation, 360 projections were collected at one-degree increments and calibration images were collected prior to data acquisition. Scans were performed with an effective voxel size of 0.126 mm. Images were reconstructed in real-time using a Feldkamp cone beam filtered back projection algorithm (2D-FDP). Tibial cross-sectional morphometry was measured at the mid-slice of the diaphysis between the crest of the tibia and the distal edge of where the fibula joins the tibia. A 0.5-mm section of the tibia (0.25 mm proximal and distal to the mid-slice) was used to determine tibial cross-sectional morphometry. The ImageJ software (ver. 1.50d) plugin BoneJ (NIH public domain) [69] was used to determine length (Le), total cross-sectional area (Tt.Ar.), cortical area (Ct.Ar.), marrow area (Ma.Ar.), cortical thickness (Ct.Th.), second moment of area around the major (Imax) and minor (Imin) axis, and polar moment of inertia (J = Imax + Imin) [69]. Robustness was calculated by dividing Tt.Ar. by tibia length (Le).

2.5. Tibial biomechanical properties

Torsional loading to failure was used to assess whole-bone and tissue-level biomechanical properties of the left tibia mid-diaphysis. The distal and proximal ends of the left tibia were embedded in a steel cylindrical holder with a gauge length of 13 mm along the long axis of the tibia; the holder was then placed in a test fixture. A cross-bar was used to prevent the proximal end of the tibia from rotating about its long axis, while the distal end was rotated about its long axis at a speed of 10 mm/s with a load cell of 100 kg. The TA-HDi machine and control software (Stable Micro Systems, Surrey, UK) measured cable force (F) in grams and applied torque (T). The load displacement curve from this analysis is analogous to a torque-twist curve, and it was used along with geometrical properties determined from μCT (i.e. length of specimen and polar moment of inertia) to calculate: whole-bone strength [maximal torque (Tmax), the greatest torque a bone structure withstands before fracturing]; whole-bone stiffness [torsional stiffness (Ks), how much the entire bone deforms when loaded]; the

energy absorbed to failure (U, the work done by the applied load to deform and fail the bone); tissue-level strength [ultimate tensile strength or maximal shear stress (Su), highest load per unit area that the bone-tissue withstands before fracturing]; and, tissue-level stiffness [shear modulus of elasticity (G), the resistance to deformation of bone-tissue per unit area when loaded]. Tmax, Ks, Su, and G were calculated as previously described [59], and the energy absorbed to failure (U) was calculated as the area under the T versus θ graph from $T = 0$ to Tmax.

2.6. Bone collagen, AGE, and ash content

The right radius was defatted in hexane and then diethyl ether (12–24 h each). Radii were dried at 60 °C for 24 h to remove residual water. The entire radius was ashed in a muffle furnace (800 °C) for four days. Tibia diaphyses were acid-hydrolyzed, dried and reconstituted in 0.001 N HCL and the hydrolysate used to measure collagen and AGE content. Collagen content was estimated by determination of hydroxyproline using a colorimetric assay [70]. Total AGEs were quantified by fluorescence with excitation at 360 nm and an emission of 460 nm using quinine (Thermo Fisher Scientific, Watham, MA, USA) as a standard [71].

2.7. Statistics

Two-factor ANOVA or ANCOVA was used to test for significant main and interactive effects between diet and genotype. In the case of a significant diet-by-genotype interaction, a one-factor (group) ANOVA (or ANCOVA) with LSD pair-wise comparisons was used to locate the interaction. ANOVA was used for body-mass-independent outcomes. Because there were significant group differences in body weight gain, ANCOVA with was used to evaluate diet and genotype effects for body-mass-dependent outcomes (i.e., cortical geometry). Covariates examined for possible inclusion in the ANCOVA were: body mass at initiation of the dietary treatments (8 weeks of age), change in body weight from 8 to 32 weeks of age, peak body weight, and final body weight (32 weeks of age). Potential covariates were screened using Pearson's correlation (data not shown). Peak body mass was most strongly correlated with cortical geometry and was therefore included as a covariate in the ANCOVAs. In addition, Tt.Ar was included as a covariate in the ANCOVA for Ma.Ar and Ct.Ar, as recommended by Jepsen et al. [72], as these variables are dependent on bone size (i.e., cross-sectional area). Data are means ± standard error of the mean or adjusted means (estimated marginal means) ± standard error of the mean. Statistical analyses were performed using general linear model (GLM) in SPSS version 21 (Chicago, IL). Statistical significant was set at $P \leq 0.05$.

3. Results

3.1. Animal characteristics

As expected, the OLETF rats were hyperphagic and consumed more food (genotype: LETO 298.8 ± 4.3 vs OLETF 344.1 ± 3.9 g/kg/wk., main effect $P < 0.001$) and more dietary energy per body mass (genotype: LETO 1.21 ± 0.02 vs OLETF 1.36 ± 0.02 kcal/kg body weight/wk., main effect $P < 0.001$) compared to LETO controls (Table 3; Supplemental Fig. 1). Animals fed the HFSC diet consumed more dietary energy than those on the CON diet (diet: CON 633 ± 9 vs. HFSC 701 ± 8 kcal/wk, main effect $P < 0.001$); however, energy intake relative to body mass was not significantly increased by the HFSC diet (diet: CON 1.26 ± 0.02 vs HFSC 1.30 ± 0.02 kcal/kg/wk, main effect $P = 0.081$). Micronutrient consumption in all animals exceeded the recommendations for rodents [61]; data not shown. Consequently, there were significant main effects of diet and genotype on final body mass (Table 3; Supplemental Fig. 2A), such that the HFSC diet or hyperphagic OLETF genotype significantly increased final body mass (diet: CON 587.4 ± 15.4 g vs. HFSC 647.0 ± 13.8 g, main effect $P < 0.01$; genotype: LETO 547.3 ± 15.4 g vs. OLETF 687.3 ± 13.8 g, main effect $P < 0.001$). The hyperphagic OLETF rats had significantly greater absolute (genotype: LETO 162.4 ± 16.6 vs OLETF 201.4 ± 15.0 g, main effect $P < 0.001$) and relative fat mass compared with LETO rats (genotype: LETO 20.8 ± 1.7% vs. OLETF 34.6 ± 1.5%, main effect $P < 0.001$). There was no effect of diet on absolute or relative fat mass, although both tended to be greater in animals fed the HFSC diet ($P \sim 0.1$; Table 3). Absolute lean mass was not significantly affected by genotype or diet, but relative lean mass was significantly lower in hyperphagic OLETF rats compared with LETO controls (genotype: LETO 76.3 ± 1.7 vs OLETF 62.7 ± 1.5%, main effect $P < 0.001$).

Fasting glucose was greater in OLETF vs. LETO rats (genotype: LETO 11.2 ± 0.6 mmol vs. OLETF 18.9 ± 0.6 mmol, main effect $P < 0.001$). Moreover, fasting glucose in the OLETF rats was clearly above the threshold of 13.9 mmol for diabetes in rodents, while fasting glucose in LETO rats was at the upper limit of normal (11.0 mmol) [73]. Fasting insulin was not affected by genotype, which is expected in OLETF rats

Table 3
Body mass and composition, food intake, fasting glucose and insulin of LETO (L) and OLETF (O) rats fed a control (CON) or high-fat, high-sucrose, high-cholesterol (HFSC) diet from 8 to 32 weeks of age[a].

	L-CON	L-HFSC	O-CON	O-HFSC	*P*-value[b]		
					d	g	d × g
Body Mass, g	526.8 ± 21.7	567.7 ± 21.7	648.3 ± 21.7	746.9 ± 17.2	0.006	<0.001	0.375
Δ Body Mass[c], g	278.1 ± 21.2	311.9 ± 21.2	340.8 ± 21.2	425.4 ± 16.8	0.005	<0.001	0.210
Peak Body Mass, g	537.9 ± 15.8	575.5 ± 15.8	704.3 ± 15.8	770.9 ± 12.5	0.001	<0.001	0.342
Week of Peak Body Mass	30 ± 1	31 ± 1	26 ± 1	25 ± 1	0.954	<0.001	0.484
Δ from Peak Body Mass, g	−11.1 ± 8.6	−7.8 ± 8.6	−56.1 ± 8.6	−37.1 ± 6.8	0.183	<0.001	0.345
Δ from Peak Body Mass, %	−0.4 ± 1.1	−0.12 ± 1.1	−5.5 ± 1.1	−2.7 ± 1.1	0.177	0.002	0.284
Fat Mass, g	102.7 ± 23.6	130.0 ± 23.6	222.0 ± 23.6	272.9 ± 18.6	0.089	<0.001	0.602
Fat, %	19.1 ± 2.4	22.5 ± 2.4	32.9 ± 2.4	36.4 ± 1.9	0.143	<0.001	0.983
Lean Mass, g	413.2 ± 21.3	425.9 ± 21.3	411.4 ± 21.3	362.0 ± 16.9	0.372	0.113	0.134
Lean, %	77.9 ± 2.4	74.7 ± 2.4	64.3 ± 2.4	61.0 ± 1.9	0.157	<0.001	0.992
Food Intake[d], kcal/wk	514.9 ± 12.9[d]	554.7 ± 12.9[c]	751.0 ± 12.9[b]	846.4 ± 10.2[a]	<0.001	<0.001	0.029
Food Intake[d], kcal/kg/wk	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	0.081	<0.001	0.181
Food Intake[d], g/kg/wk	324.2 ± 6.1	273.4 ± 6.1	371.4 ± 6.1	316.9 ± 4.8	<0.001	<0.001	0.749
Glucose, mmol/L	11.9 ± 0.8	10.5 ± 0.4	18.8 ± 0.7	17.9 ± 0.5	0.551	<0.001	0.388
Insulin, pmol/L	650.1 ± 116.7	566.7 ± 116.7	850.0 ± 116.7	583.3 ± 100.0	0.159	0.384	0.488

[a] Data are means ± SEM; L-CON ($n = 10$), L-HFSC (n = 10), O-CON (n = 10), O-HFSC (n = 16) per group.
[b] Data were analyzed by 2-way ANOVA; *P*-values are for main effects of diet (d) and genotype (g), and diet-by-genotype (d × g) interaction. When a significant d × g interaction was found, group differences were evaluated by one-way ANOVA with least-significant difference post hoc pair-wise comparison; means with different letter superscripts are significantly different $P < 0.05$.
[c] Change (Δ) in body mass from the start of HFSC diet (8 weeks of age) to 32 weeks of age.
[d] Average weekly energy intake across the 24-week study.

with frank T2D. Consistent with disease progression in humans, OLETF rats develop insulin resistance that is associated with hyperinsulinemia followed by pancreatic β-cell insufficiency, atrophy and hypoinsulinemia [73]. At 20 weeks of age, OLETF rats fed the HFSC diet had fasting insulin concentrations of 1118.9 ± 150.3 pmol/L (unpublished data). The hyperphagic OLETF genotype had significantly greater total body BMC adjusted for peak body mass (genotype: LETO 16.0 ± 0.3 g vs. OLETF 18.5 ± 0.3 g, main effect $P < 0.001$; Supplemental Fig. 2B–C). There was a significant main effect of diet for total body BMC, such that animals fed the HFSC diet had significantly lower total body BMC (diet: CON 17.9 ± 0.1 g vs. HFSC 17.3 ± 0.1 g, main effect $P = 0.004$); BMD showed a similar, non-statistically significant, response (diet: CON 0.208 ± 0.003 g/cm^2 vs. HFSC 0.199 ± 0.003 g/cm^2, main effect $P = 0.056$).

3.2. Serum bone turnover markers

A significant interaction between the diet and the genotype was found for the serum bone formation markers P1NP and cOC (Fig. 1). The HFSC resulted in lower P1NP and cOC only in the LETO rats. Hyperphagic OLETF rats had lower P1NP than LETO rats, regardless of dietary treatment; and, OLETF rats fed the control diet had lower cOC than LETO rats fed the control diet. The circulating marker of osteoclast number and/or activity TRAP5b, was significantly reduced in hyperphagic OLETF rats compared to normophagic LETO rats regardless of diet (genotype: LETO 7.6 ± 0.3 U/L vs OLETF 5.1 ± 0.3 U/L, main effect $P < 0.001$). The HFSC diet tended to increase circulating TRAP5b compared to the CON diet (diet: CON 6.0 ± 0.3 U/L vs HFC 6.8 ± 0.2 U/L, main effect $P = 0.070$). The hyperphagic OLETF rats had significantly greater concentrations of the bone resorption marker CTx, compared to normophagic LETO rats (genotype: LETO 14.9 ± 1.5 ng/mL vs. OLETF 21.3 ± 1.3 ng/mL, main effect $P < 0.01$). The OLETF genotype was associated with a significantly greater resorptive index (CTx/TRAP5b) than the LETO genotype (genotype: LETO 2.01 ± 0.31 vs OLETF 4.52 ± 0.29, main effect $P < 0.001$). ucOC was significantly reduced in hyperphagic OLETF rats compared to normophagic LETO rats (genotype: LETO 102.1 ± 14.0 vs OLETF 49.1 ± 11.2 ng/mL, main effect $P = 0.006$. Serum sclerostin was significantly increased by the OLETF genotype compared to the LETO genotype, regardless of diet (genotype: LETO 135.5 ± 9.5 pg/mL vs. OLETF 183.4 ± 8.4 pg/mL, main effect $P < 0.001$). Serum sclerostin was positively correlated with CTx ($r = 0.703$, $P < 0.001$).

3.3. Trabecular microarchitecture of the proximal tibia

Representative three-dimensional reconstructions of the trabecular bone of the proximal tibia were generated by μCT image analysis (Fig. 2). The hyperphagic OLETF genotype had significantly reduced BV/TV (genotype: LETO 21.14 ± 0.76% vs, OLETF 12.59 ± 0.69%, main effect $P < 0.001$), Tb.Th (genotype: LETO 0.084 ± 0.002 mm vs. OLETF 0.077 ± 0.002 mm, main effect $P = 0.038$), Tb.N (genotype: LETO 2.50 ± 0.08 1/mm vs. OLETF 1.62 ± 0.06 1/mm, main effect $P < 0.001$), and Conn.D (genotype: LETO 161.41 ± 10.06 mm^3 vs. OLETF 118.97 ± 9.06 mm^3, main effect $P < 0.01$); and, Tb.Sp. was significantly increased in hyperphagic OLETF rats compared to normophagic LETO rats (genotype: LETO 0.34 ± 0.04 mm vs. OLETF 0.67 ± 0.03 mm, main effect $P < 0.001$) (Fig. 3A-G). Based on EF, there was a significant main effect for genotype, such that OLETF rats had a greater EF (i.e., more rod-like shape) than LETO rats that had more plate-like shaped trabeculae (genotype: LETO 0.047 ± 0.017 vs OLETF 0.148 ± 0.017, main effect $P < 0.001$; Fig. 3H). There was no effect of diet on these measures. DA was significantly increased in OLETF rats compared to LETO rats (genotype: OLETF 1.79 ± 0.02 vs. LETO 1.86 ± 0.02, main effect $P = 0.005$; Fig. 3I).

3.4. Cortical geometry and biomechanical properties of the tibia diaphysis

There were no effects of diet or genotype on unadjusted tibia length or Tt.Ar, (Table 4), indicating that the treatments did not affect tibia linear growth or periosteal expansion. Ct.Ar (genotype: LETO 6.90 ± 0.06 mm^2 vs. OLETF 7.03 ± 0.05 mm^2, main effect $P = 0.094$) and Ct.Ar/Tt.Ar (genotype: LETO 80.0 ± 0.7% vs. OLETF 81.5 ± 0.9%, main effect $P = 0.086$) tended to be increased in the OLETF rats. Compared to LETO rats, OLETF rats had greater Imax (genotype: LETO 6.53 ± 0.20 mm^4 vs. OLETF 7.06 ± 0.18 mm^4, main effect $P = 0.055$) and Imax/Imin ratios (genotype: LETO 6.53 ± 0.20 vs. OLETF 7.06 ± 0.06, main effect $P < 0.001$), suggesting that the cross-sectional geometry of the tibial diaphysis of the OLETF rats was less circular and more elliptical than the LETO rats. When peak body weight was included as a covariate in the 2-way ANCOVAs, the genotype effects on cortical geometry were no longer significant (Supplemental Table 1). Therefore, the differences in cortical geometry between OLETF and LETO rats can be attributed to differences in body mass. Adjusting for peak body mass, there was a significant diet effect for Imax (diet: CON 7.18 ± 0.2 mm^4 vs. HFSC 6.50 ± 0.2 mm^4) and robustness (diet: CON 1.95 ± 0.03 mm vs. HFSC 1.86 ± 0.03 mm^4, $P = 0.072$) and polar moment of inertia (diet: CON 12.2 ± 0.4 mm^4 vs. HFSC 11.2 ± 0.3 mm^4, $P = 0.082$) also tended to be affected by diet (Supplemental Table 1).

There were significant interactions between diet and genotype for whole-bone strength (Tmax: interaction $P < 0.01$), stiffness (Ks: $P = 0.047$), and energy absorbed to failure (U: $P = 0.021$) (Fig. 4), such that the HFSC diet increased Tmax in LETO rats only (L-CON vs. L-HFSC, $P = 0.032$). Ks and U tended to be increased in L-HFSC compared to L-CON ($P = 0.068$ and $P = 0.054$, respectively). Whole-bone biomechanical properties were similar in OLETF rats fed the CON or HFSC diets. Similar to the result observed for whole-bone strength, there was a significant interaction between diet and genotype for tissue-level strength (Su), such that Su was significantly greater in L-HFSC than L-CON ($P = 0.021$). There were no effects of diet or genotype on tissue-level stiffness (G).

3.5. Bone ash, collagen, and AGE content

Ash content of the radius normalized to dry tissue weight tended to be greater in the OLETF rats and in those fed the HFS diet (genotype: LETO 0.599 ± 0.005 vs. OLETF 0.610 ± 0.004 *w*/w, main effect $p = 0.082$; and diet: CON 0.599 ± 0.005 vs. HFSC 0.610 ± 0.004 w/w, main effect $p = 0.090$). A significant genotype-by-diet interaction was detected for the hydroxyproline content of the tibial diaphysis. L-HFSC (166.1 ± 5.2 μmol/g) had greater hydroxyproline content of the tibial diaphysis than L-CON (147.5 ± 4.9 μmol/g; $p < 0.05$), and no differences were observed between the O-CON (165.4 ± 4.6 μmol/g) and O-HFSC (161.4 ± 4.3 μmol/g). Neither diet nor genotype had an effect on AGE content of the tibial diaphysis normalized to bone (diet: CON 131.1 ± 5.4 vs. HFSC 128.4 ± 5.5 ng quinine/mg bone, main effect $p = 0.729$; and genotype: LETO 124.5 ± 5.7 vs. 134.9 ± 5.1 ng quinine/mg bone, main effect $p = 0.186$) or to hydroxyproline content (diet: CON 6.45 ± 0.31 vs HFSC 6.02 ± 0.31, main effect $p = 0.324$ ng quinine/μg OH-proline; and genotype: LETO 6.15 ± 0.33 vs. OLETF 6.32 ± 0.29, main effect $p = 0.688$ ng quinine/μg OH-proline).

4. Discussion

4.1. Summary of study results

Here, we were able to investigate the independent and interactive effects of obesity/T2D and diet composition on bone outcomes in the hyperphagic OLETF rat. We report that obesity/T2D, regardless of diet, has significant adverse effects on the balance between bone formation and resorption and cancellous bone volume and trabecular microarchitecture. That is, in the context of obesity/T2D, the HFSC diet

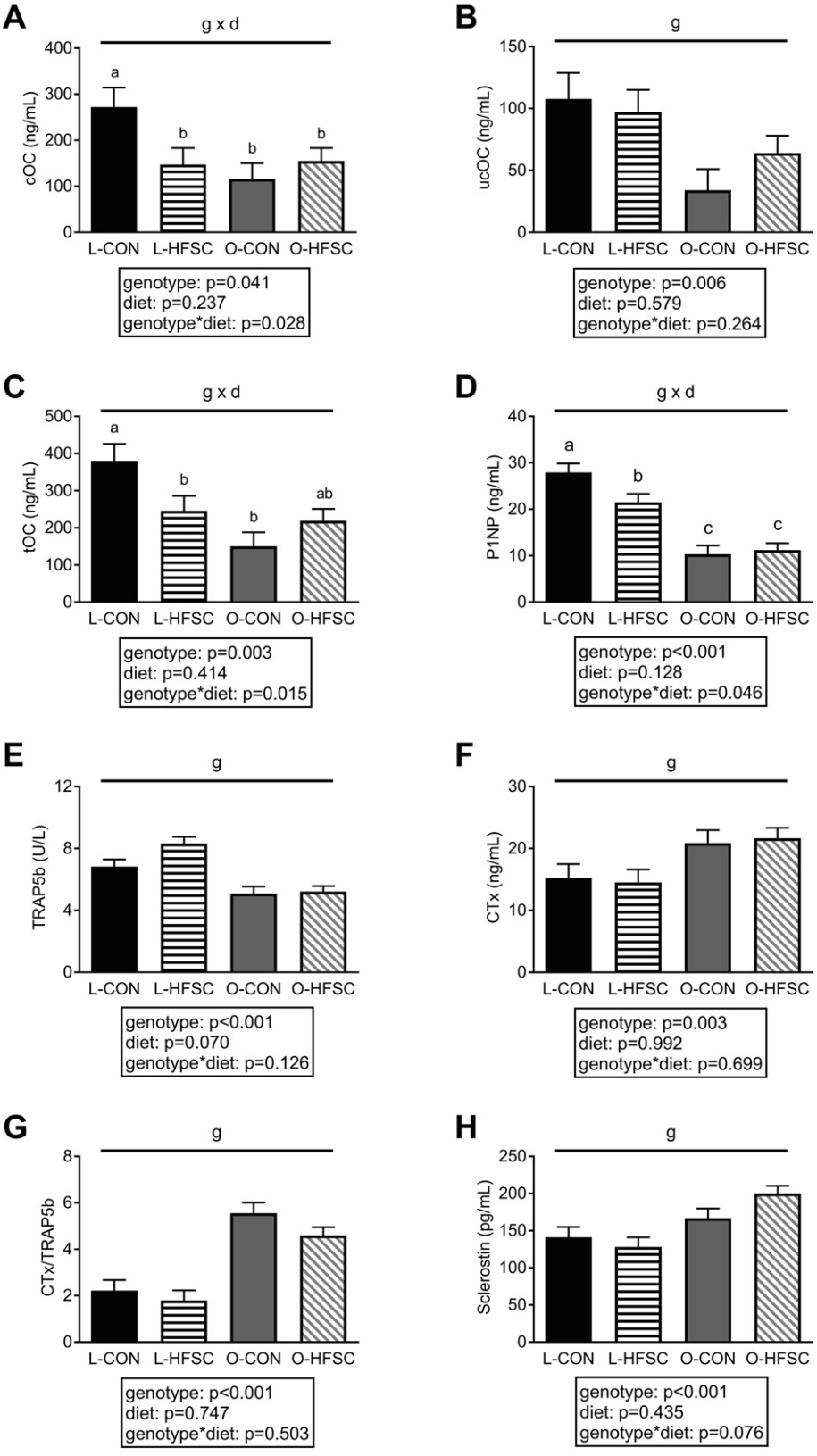

Fig. 1. Serum cOC (A), ucOC (B), tOC (C), P1NP (D), TRAP5b (E), CTx (F), CTx/TRAP5b (G), and sclerostin (H) in LETO or OLETF rats fed a CON or HFSC diet from 8 to 32 weeks of age. Data are means ± SEM; L-CON ($n = 9$), L-HFSC ($n = 10$), O-CON (n = 10), O-HFSC ($n = 16$) per group. Means with different letter superscripts are significantly different, $P \leq 0.05$. CON, control diet; CTx, C-terminal telopeptide of type I collagen; HFSC, diet high in fat, sucrose, and cholesterol; cOC, carboxylated osteocalcin; tOC, total osteocalcin; ucOC, undercarboxlyated osteocalcin; P1NP, N-terminal propeptide of type I collagen; TRAP5b, tartrate-resistant alkaline phosphatase.

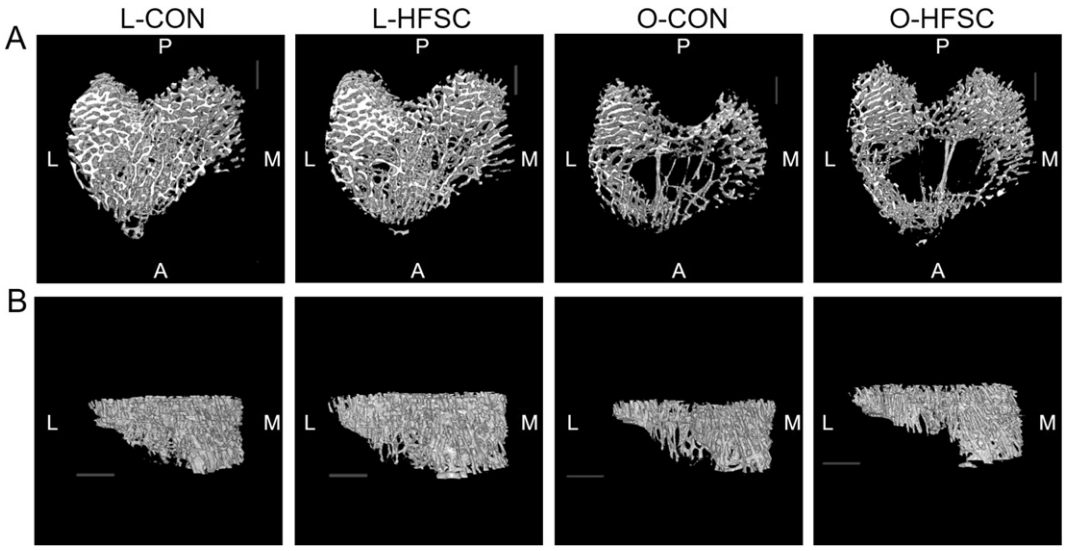

Fig. 2. Representative μCT images of the proximal tibia for LETO and OLETF rats fed a CON or HFSC diet from 8 to 32 weeks of age. Axial view (A) with the anterior (A) tibia down, posterior (P) tibia up, medial (M) tibia facing right, and lateral (L) tibia facing left. Coronal view of the anterior tibia (B) with the medial tibia on the right and the lateral tibia on the left of the image. Images were selected from the animals with a relative bone volume (BV/TV) most similar to the mean per group. CON, control diet; HFSC, diet high in fat, sucrose, and cholesterol.

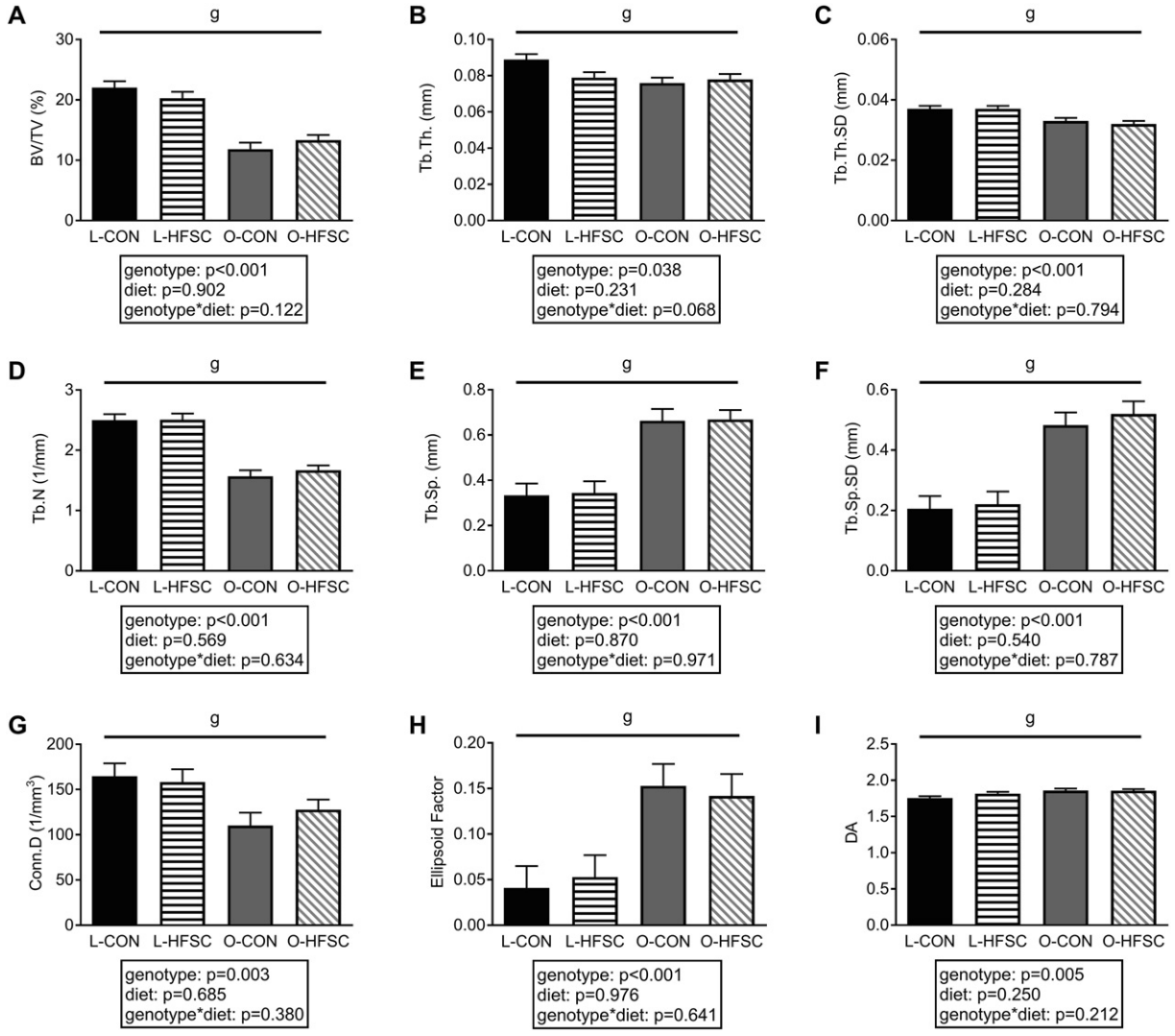

Fig. 3. Trabecular microarchitecture of the proximal tibia. BV/TV (A), Tb.Th (B), Tb.Th.SD (C), Tb.N (D), Th.Sp.(E), Tb.Sp.SD (F), Conn.D (G), EF (H), and DA (I) in LETO and OLETF rats fed a CON or HFSC diet from 8 to 32 weeks of age. Data are means ± SEM; L-CON (n = 9), L-HFSC (*n* = 10), O-CON (n = 10), O-HFSC (n = 16) per group. Means with different letter superscripts are significantly different, $P \leq 0.05$. BV/TV, relative bone volume; Conn.D, connectivity density; CON, control diet; DA, degree of anisotropy; EF, ellipsoid factor; Tb.N, trabecular number; HFSC, diet high in fat, sucrose, and cholesterol; Tb.Sp, trabecular separation; Tb.SpSD, trabecular number standard deviation; Tb.Th, trabecular thickness; Tb.ThSD, trabecular thickness standard deviation.

Table 4
Unadjusted tibia cortical morphometry of LETO (L) and OLETF (O) rats fed a control (CON) or a high-fat, high-sucrose, and high-cholesterol (HFSC) diet from 8 to 32 weeks of age[a,b,c,d].

	L-CON	L-HFSC	O-CON	O-HFSC	*P*-value		
					d	g	d × g
Length, cm	4.52 ± 0.02	4.57 ± 0.02	4.53 ± 0.02	4.54 ± 0.02	0.114	0.776	0.380
Tt.Ar., mm^2	8.70 ± 0.19	8.49 ± 0.19	8.80 ± 0.18	8.50 ± 0.17	0.174	0.740	0.802
Tt.Ar./Length	1.93 ± 0.04	1.86 ± 0.04	1.94 ± 0.04	1.87 ± 0.04	0.083	0.689	0.961
Ct.Th., mm	1.09 ± 0.02	1.06 ± 0.02	1.11 ± 0.02	1.07 ± 0.01	0.149	0.408	0.998
Ct.Ar.[d], mm^2	6.89 ± 0.08	6.90 ± 0.08	7.05 ± 0.08	7.01 ± 0.07	0.850	0.094	0.726
Ma.Ar.[d], mm^2	1.73 ± 0.08	1.72 ± 0.08	1.57 ± 0.08	1.61 ± 0.07	0.851	0.103	0.708
Ct.Ar./Tt.Ar., %	80.0 ± 0.9	80.0 ± 0.8	81.8 ± 0.9	81.3 ± 0.8	0.823	0.086	0.775
Imax, mm^4	6.66 ± 0.28	6.40 ± 0.28	7.28 ± 0.27	6.75 ± 0.25	0.111	0.055	0.506
Imin, mm^4	5.09 ± 0.25	4.76 ± 0.25	4.88 ± 0.24	4.54 ± 0.22	0.181	0.378	0.980
Imax/Imin	1.31 ± 0.05	1.35 ± 0.05	1.55 ± 0.05	1.49 ± 0.04	0.783	<0.001	0.260
J, mm^4	11.75 ± 0.50	11.16 ± 0.50	12.25 ± 0.48	11.29 ± 0.43	0.114	0.507	0.696

[a] Data are unadjusted means ± SEM; L-CON ($n = 9$), L-HFSC ($n = 10$), O-CON (n = 10), O-HFSC ($n = 16$) per group.
[b] Data were analyzed by 2-way ANOVA; *P*-values are for main effects of diet (d) and genotype (g), and diet-by-genotype (d × g) interaction. When a significant d × g interaction was found, group differences were evaluated by one-way ANOVA with least-significant difference post hoc pair-wise comparison; means with different letter superscripts are significantly different $P < 0.05$.
[c] Tt.Ar, total area; Ct.Th, cortical thickness; Ct.Ar, cortical area; Ma.Ar, marrow area; Imax, maximum moment of inertia; Imin, minimum moment of inertia; J, polar moment of inertia.
[d] Ct.Ar and Ma.Ar adjusted for Tt.Ar.

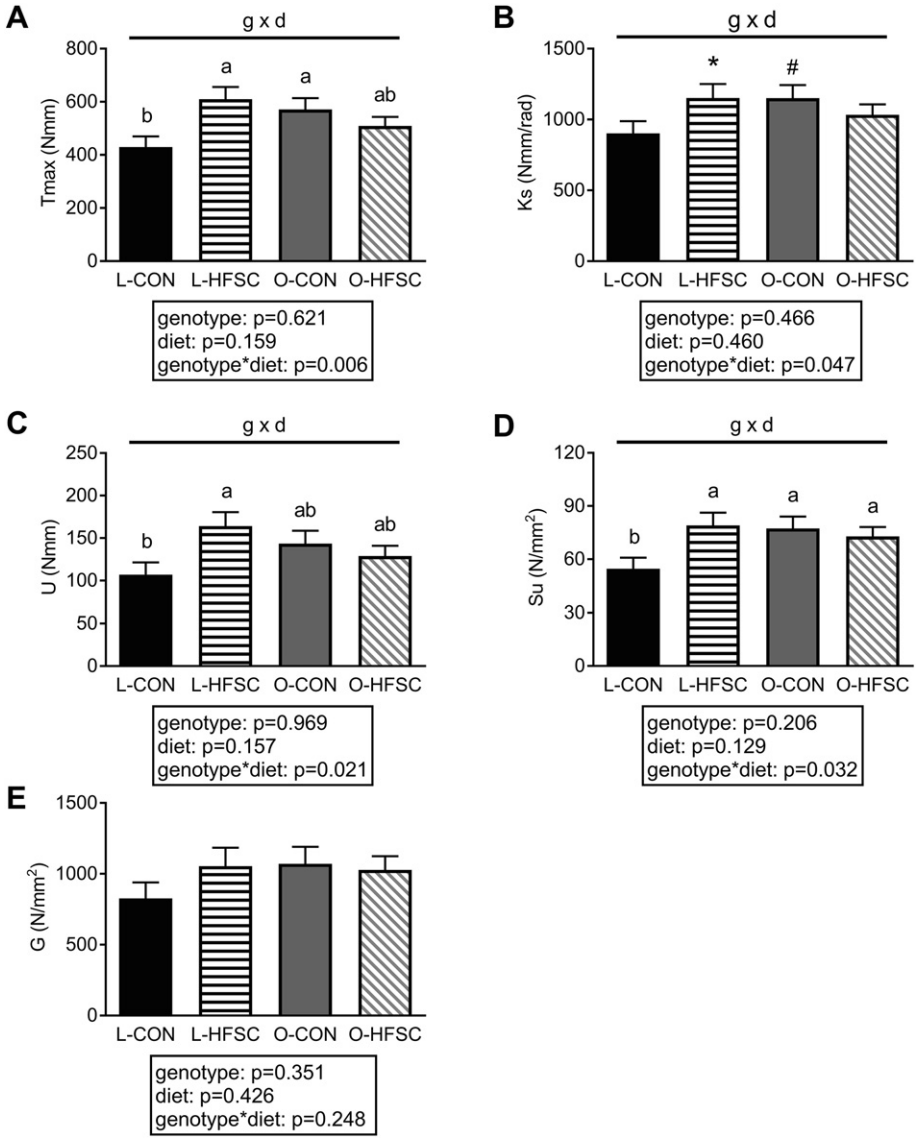

Fig. 4. Biomechanical properties of the tibia mid-diaphysis. Tmax (A), Ks (B), U (C), Su (D), and G (E) in LETO and OLETF rats fed a CON or HFSC diet from 8 to 32 weeks of age. Data are means ± SEM; L-CON ($n = 6$), L- HFSC (n = 10), O-CON (n = 10), O– HFSC ($n = 11$) per group. Means with different letter superscripts are significantly different, $P \leq 0.05$. CON, control diet; G, tissue-level stiffness; HFSC, diet high in fat, sucrose, and cholesterol diet; Ks, whole-bone stiffness; Su, tissue-level strength; Tmax, maximal torque; U, energy absorbed to failure.

did not result in worse skeletal outcomes than the CON diet. Somewhat surprisingly, the HFSC diet generally improved bone outcomes in normophagic, non-obese LETO controls.

Our observation that the HFSC diet did not aggravate the negative effects of obesity/T2D on bone is consistent with human data showing that the detrimental effects of a Western Diet on bone metabolism (greater CTx in people consuming a Western Diet vs. a Prudent diet) were no longer significant when adjusting for BMI [74]. However, in humans consuming a Western Diet, micronutrient intake may be below the recommendations, unlike the animals in our study. Similarly, a high-saturated-fat, high-sucrose diet exacerbated the detrimental effects of ovariectomy on trabecular microarchitecture, OPG:RANKL mRNA and PPARγ, due to greater weight gain in the ovariectomized rats fed the high-saturated fat, high-sucrose diet [75]. Thus, it appears that the negative effects of a diet high in fat and sucrose on bone are mediated by excessive adiposity rather than by the composition of the diet per se.

4.2. Biochemical markers of bone remodeling

In the present study, T2D OLETF rats had increased CTx but reduced TRAP5b, resulting in a significantly elevated resorptive index indicative of greater resorption per osteoclast [76]. The decrease in TRAP5b (osteoclast number) observed in OLETF rats is likely due to the significant reduction in cancellous bone volume, as the majority of osteoclasts reside in cancellous bone in the rat [76]. OLETF rats fed the control diet had lower cOC than LETO rats, and P1NP was reduced in the obese/T2D OLETF rat, regardless of diet. Taken together, obesity/T2D resulted in increased resorption relative to formation, consistent with decreased trabecular BV/TV. Circulating sclerostin, the Wnt/β-catenin antagonist, was also increased in OLETF rats. Sclerostin inhibits the activation of the canonical Wnt/β-catenin signaling pathway and prevents activation of β-catenin and the transcription of key osteogenic regulators such as Runx2 and osterix [77]. Individuals with T2D have elevated serum sclerostin, associated with a significant reduction in serum markers of bone remodeling [33,34]. Thus, the results of the present study are consistent with sclerostin inhibition of Wnt/β-catenin signaling playing a causal role in the bone deficits observed in obesity/T2D. These data support further investigation of how altered Wnt signaling leads to diabetic bone fragility by examination of dickkopf-1 and Wnt-ligand expression in bone.

The significantly lower ucOC in hyperphagic OLETF rats compared with normophagic LETO rats was somewhat surprising given the marked increase in CTx suggestive of increased bone resorption. A plausible explanation for these results is that reduced insulin signaling in osteoblasts of T2D OLETF rats lowers both osteocalcin production [78] and decarboxylation to form ucOC [79]. While we did not measure insulin signaling in osteoblasts in the present study, we have previously reported that hyperphagic OLETF rats exhibit whole body and skeletal muscle insulin resistance [80], suggesting that osteoblasts might also show reduced insulin signaling. We have also previously reported that T2D OLETF rats have significantly increased leptin concentrations [81], which might also contribute to reduced ucOC. Increased sympathetic nervous system activity secondary to elevated circulating leptin levels, reduces insulin signaling in osteoblasts resulting in lower ucOC [82].

4.3. Trabecular microarchitecture of the proximal tibia

Obesity/T2D caused a significant reduction in cancellous bone volume that was independent of diet. The reduction in BV/TV, Tb.N and Conn.D, as well as the shift from more plate-like to javelin-shaped rod-like trabeculae (i.e., greater EF) in OLETF rats, appeared due to a preferential loss of trabeculae and increased Tb.Sp in the central region of the proximal tibia (Fig. 2) where mechanical stress is the smallest [83]. This localized reduction in bone volume is likely because trabeculae exposed to the lowest stresses are the first locations of bone resorption [83]. Perforation of trabeculae and ultimately complete removal can occur as a result of trabecular thinning, increased frequency of remodeling activation, and/or increased resorption depth [84]. Tb.Th and variability in Tb.Th were not affected by T2D, suggesting that increased remodeling frequency or erosion pit depth were responsible for the loss of trabeculae in OLETF rats. Significant reductions in BV/TV, Conn.D and increased Tb.Sp, Tb.Sp.SD, and DA are associated with a reduction in trabecular bone elasticity and strength [85]. We previously reported that loss of trabecular bone volume and microarchitectural deterioration are not evident in insulin resistant OLETF rats (20 weeks of age), but only in animals with T2D [86]. Others have also reported that obesity with impaired glucose tolerance [87] or insulin resistance seems to preserve trabecular microarchitecture in animals [52,88–90] and humans [91], possibly to due to increased insulin, which plays an essential role in osteoblast differentiation and activity [78]. By contrast, obesity with T2D negatively impacts trabecular microarchitecture [52,56,88,92].

4.4. Cortical geometry and biomechanical properties of the tibia diaphysis

Obesity/T2D resulted in changes in cortical geometry at the mid-diaphysis of the tibia, regardless of diet. These changes were likely compensatory adaptations to increased body mass in the OLETF rats, as observed previously [59]. Bone is highly adaptive and changes in one trait can be compensated for by changes in other traits [93]. Thus, the changes in cortical geometry likely explained the preservation of whole-bone biomechanical properties (Tmax, Ks, U) in OLETF rats compared to LETO fats fed the CON diet, as Ct.Ar/Tt.Ar was positively correlated with Tmax ($r = 0.803$, $P < 0.001$), Ks ($r = 0.805$, $P < 0.001$), and U ($r = 0.691$, $P < 0.001$). Although the HFSC diet had no effect on cortical bone geometry in either LETO or OLETF rats, there was a significant positive effect of the HFSC diet on biomechanical properties in LETO rats only. The greater whole-bone strength, stiffness and energy absorbed to failure was largely due to tissue-level changes, as cortical geometry was not affected by the HFSC diet in LETO rats compared with those fed the CON diet.

4.5. Ash, collagen and AGE content and relationships with biomechanical properties

Thus, we investigated whether differences in ash (mineral), collagen or AGE content might explain differences in tissue-level biomechanical properties in L-HFSC. The ash content of the radius tended to be greater in the OLETF rats and rats fed the HFSC diet. The effects of diet and genotype were not due to differences in mineral intake. After adjusting for micronutrient intake in the two-factor ANCOVA, the diet main effect was statistically significant ($P = 0.032$, suggesting that differences in ash content were due to diet macronutrient composition. The greater hydroxyproline content (i.e. collagen) of the tibial diaphysis in the L-HFSC rats might also have explained the beneficial effect of the HFSC diet on tissue-level strength in LETO rats [94]. Collagen gives bone its tensile (elongation) strength, elasticity (the ability of a material to deform and return to its natural state), and toughness (the ability to absorb energy without fracturing) [85,95,96]. Hyperglycemia increases the availability of reducing sugars to form AGEs within and between collagen fibrils [38], and there is evidence of increased AGE bone content in T2D humans and animals [37,40]. Given the significant hyperglycemia observed in the OLETF rats, we hypothesized that T2D OLETF rats would have greater AGE content in the tibial diaphysis compared with LETO rats. We measured AGE in cortical bone to associate AGE content with biomechanical properties; however, AGE was not related to tissue-level biomechanical properties and did not differ among groups. It is worth noting, however, that cancellous bone has a much higher AGE content than cortical bone [97,98], and we might have detected effects of obesity/T2D and/or the HFSC diet on AGE content of cancellous bone.

4.6. Skeletal effects of obesity/T2D override diet composition

The results of this study provide evidence that the effects of obesity/T2D override the effects of diet composition, at least in the context of adequate micronutrient intake. In the absence of obesity/T2D, the HFSC diet generally had positive effects on bone outcomes, with the exception of P1NP, which was lower in LETO rats fed the HFSC diet compared with animals fed the control diet. However, in LETO rats, the HFSC diet increased cortical bone biomechanical properties apparently by altering the tissue-level (material) properties of the tibia diaphysis. This response could be attributed to, in part, by the high-fat content of the HFSC diet [99]. In rats, high dietary fat increases circulating HDL [100], which is emerging as an important determinant of bone health [101, 102]. Mice with severely reduced HDL concentrations have significantly reduced bone mass, deterioration of trabecular bone volume and microarchitecture, reduced bone formation and lower whole-bone strength and stiffness in association with reduced collagen crosslinks [103]. The effects of dietary sucrose/fructose on bone outcomes are confounded by differences in fat content. Consequently, the existing literature is equivocal, with some studies showing negative effects of sucrose/fructose on BMD or biomechanical properties [46] [104] and others reporting beneficial effects on bone quality and strength in growing male rats relative to glucose [48]. Although the HFSC diet did not result in obesity/T2D in normophagic LETO rats during the 24-week study period, over the long-term, the HFSC diet might result in increased adiposity and ultimately loss of glucose homeostasis. Based on the dominant negative effect of obesity/T2D on bone in the OLETF rats, we hypothesize that skeletal integrity would deteriorate with the onset of T2D in LETO rats.

4.7. Mechanisms by which obesity/T2D might adversely affect bone

In the present study, there was a significant main effect of obesity/T2D (i.e., genotype) on the majority of bone outcomes examined (total body BMC, bone remodeling markers, trabecular microarchitecture, cortical morphometry), while diet composition impacted cortical biomechanical properties, but only in the non-obese, non-diabetic LETO rats. Thus, the results of the present study provide convincing evidence that the adverse effects of obesity/T2D override any skeletal effects, beneficial or harmful, of a HFSC diet. The most likely explanation for this observation is that obesity/T2D is associated with a plethora of changes in metabolism, endocrine function, inflammation, and oxidative stress, which contribute to the comorbidities of obesity (reviewed in [105]). Research to identify how these diverse changes affect bone is still in its infancy. The effects of obesity endocrine function (e.g., sex steroids, PTH, vitamin D) and the skeletal consequences of these changes have been known for some time (reviewed in [106]). However, emerging data suggest that there are several additional pathways by which obesity/T2D might affect bone. In particular, cytokines, adipokines, pancreatic and gut hormones might affect bone both directly via receptors on osteoblasts, osteocytes, osteoclasts or bone marrow progenitors or indirectly via the sympathetic nervous system [107]. Likewise, bone marrow adipose tissue, which increases in obesity/T2D, acts locally to suppress osteoblastogenesis and enhance osteoclast activity (reviewed in (106)). The net result of these changes is increased adipogenesis at the expense of osteoblastogenesis, dysregulation of bone remodeling balance in favor of resorption, and deterioration of bone quality. Thus, we hypothesize, that because obesity/T2D affect bone through multiple pathways, many of which are distinct from the effect of diet composition (i.e., high-fat, cholesterol, sucrose), the effects of obesity/T2D supersede those of diet.

4.8. Study strengths and limitations

Use of the hyperphagic OLETF rat model of obesity/T2D is a strength of the present study. Many other genetic rodent models of obesity-associated T2D have limitations that might compromise the translational relevance of the model to human disease. In particular, these models are limited by: 1) the timing and rate of onset of hypoinsulinemia and hyperglycemia relative to skeletal maturity; 2) genetic alterations that affect leptin signaling (e.g., Zucker diabetic fatty), which is problematic because leptin has direct effects on bone [108]; and, 3) obesity and hyperinsulinemia, but normoglycemia [109]. By contrast, in the hyperphagic OLETF rat, the onset of hyperglycemia, insulin resistance and frank T2D occur after skeletal maturity, as typically occurs in humans. Also analogous to humans, the obesity and glucose dysregulation of the OLETF rat are entirely attributable to their hyperphagia [110]. We must acknowledge the possibility, however, that the absence of a functional hypothalamic CCK-1 receptor has yet unidentified effects on bone. Another potential study limitation is the difference in micronutrient intake between the animals fed the CON diet and those fed the HFSC diet. However, because all of the animals in our study consumed micronutrients in excess of the recommendations, and because the relationship between micronutrient intake and bone outcomes is not linear above the requirement, i.e., threshold effect [111], it is unlikely that these differences confounded the results. Finally, the study appears to have been slightly underpowered to detect differences in some bone outcomes. The required sample size was estimated using differences in bone biomechanical properties between OLETF and LETO rats fed a standard chow diet [59].

4.9. Conclusions

In summary, development of obesity/T2D, but not a diet high in fat, sucrose and cholesterol, negatively impacted the balance between bone formation and resorption and resulted in significant adverse changes in trabecular bone volume and microarchitecture in the proximal tibia. The diet high in fat, sucrose and cholesterol, independent of the development of obesity, did not have a detrimental impact on bone outcomes. From a clinical perspective, these data are highly relevant. Prevention and treatment of bone fragility associated with obesity/T2D should focus on achieving and maintaining health body mass and composition, as well as glucose homeostasis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2017.09.003.

Acknowledgments

MAL, RSR, LCO and PSH designed the research; MAL, LCO, RSR, RKD conducted the research; RSR provided the animals; LCO and PSH performed statistical analyses; PSH wrote the paper and has primary responsibility for the final content; all authors have read and approved the final manuscript. This work was supported by the Department of Nutrition & Exercise Physiology, University of Missouri; Margaret Mangel Research Catalyst Fund (DN530); Veteran Affairs (VA) Grants VA-CDA2 BX001299 (RSR) and VA-Merit Grant I01BX003271-01 (RSR) and by resources and the use of facilities at the Harry S. Truman Memorial VA Hospital in Columbia, MO.

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