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Metabolic, Endocrine, and Genitourinary Pathobiology

Hypercholesterolemia Promotes an Osteoporotic Phenotype

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A role for hypercholesterolemia in the development of osteoporosis has been suggested in published reports. However, few studies contain direct evidence of a role for maintenance of cholesterol homeostasis in bone health. Using isocaloric high-fat/high-cholesterol and low-fat/no-cholesterol diets in a 4-month feeding study combined with micro computed tomography analysis, we demonstrated in two different mouse strains that mice with hypercholesterolemia lose cortical and trabecular bone in the femurs and vertebrae (bone mineral density was decreased on average by ≈90 mg/mL in the cortical vertebrae in one strain) and cortical bone in the calvariae (bone mineral density was decreased on average by ≈60 mg/mL in one strain). Mechanical testing of the femurs demonstrated that loss of bone in the mice with hypercholesterolemia caused changes in the mechanical properties of the bone including loss of failure load (failure load was decreased by ≈10 N in one strain) and energy to failure. Serologic and histomorphologic analyses suggested that hypercholesterolemia promotes osteoclastogenesis. These studies support a role for hypercholesterolemia in the development of osteoporosis and provide a model with which to test intervention strategies to reduce the effects of hypercholesterolemia on bone health. *(Am J Pathol 2012, 181:928–936; http://dx.doi.org/10.1016/j.ajpath.2012.05.034)*

Osteoporosis is the most common bone disorder, and is characterized by systemic reduction in bone mass and changes in the bone microarchitecture that result in fragility fractures.[1] The increased risk of spontaneous and traumatic bone fractures can lead to disability, often severe and permanent; placement in institutional care; and death. Osteoporotic fractures of the hip and spine result in mortality of up to 20% for 1 year after the fracture event.[2]

Recent evidence links the risk of developing osteoporosis to both cardiovascular disease and metabolic syndrome[3–10] (defined as central obesity with insulin resistance, and/or dyslipidemia and high blood pressure), which suggests that a common element between these conditions may also be operating to increase the risk of osteoporosis. Although not always present in individuals with cardiovascular disease or metabolic syndrome, hypercholesterolemia is a common factor in those with either disease.

Osteoblasts (cells that make bone) and osteoclasts (cells that resorb bone) synthesize cholesterol endogenously via the mevalonate pathway and absorb cholesterol-containing lipoproteins from the circulation. Consequently, control of cellular cholesterol content is a balance between metabolic processes intrinsic to the cell and the regulation of cholesterol distribution by the organism. Maintenance of the overall cholesterol content of cell membranes is determined by a complex set of processes including synthetic pathways in the endoplasmic reticulum, regulation of lipogenic genes by cholesterol-regulated sterol-response element binding proteins, receptor-mediated internalization, intracellular transport mechanisms, and cholesterol efflux via lipoprotein complexes.[11–13] Despite the elaborate mechanisms for main-

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taining correct cholesterol balance, osteoblasts and osteoclasts, like other cells, are subject to disruptions in cholesterol homeostasis as a consequence of dysregulation in endogenous synthetic pathways, poor cholesterol efflux, and/or systemic changes in serum cholesterol concentrations. These changes may result in altered cellular responses to specific growth factors and/or cytokines, as well as changes in the ability of bone cells to mature, differentiate, and stabilize bone.

Epidemiologic evidence indicates elevated serum cholesterol concentration as a risk factor in development of osteoporosis.[5,6,14–21] Other studies have demonstrated that statin drugs, which inhibit the rate-limiting step in cholesterol synthesis,[22–24] increase bone mineral density (BMD) in humans, lower fracture risk, and cause changes in bone cell (osteoblast) biochemistry *in vivo* and in tissue culture.[25–39] When provided in typical dosages in humans, statins work almost exclusively in the liver, where they suppress cholesterol synthesis, leading to reduced serum cholesterol concentrations. Simply stated, the level of extrahepatic statin is too small and for too short a time for the purported direct extrahepatic tissue effects of statins to occur.[22–24,40–50] Consequently, if statins alter risk of developing osteoporosis, this phenomenon likely stems from their effects on circulating cholesterol concentrations, and not from direct effects on osteoblasts and osteoclasts.

Because of the suggested links between cardiovascular disease, metabolic syndrome, and osteoporosis,[3–10] previous studies that have suggested an effect of elevated cholesterol concentration and statins on BMD and fracture risk,[5,6,14–21,25–39] and our understanding of the roles of cholesterol in bone cell biology, we hypothesized that altering the level of cholesterol in membranes of osteoblasts and/or osteoclasts, as a consequence of elevated serum cholesterol concentrations, will contribute to development of osteoporosis.

In a previous study, an atherogenic diet (high-fat/high-cholesterol [HFHC] with sodium cholate) fed to mice led to significantly reduced femoral mineral content (43% lower) and mineral density (15% lower) when compared with a low-fat/no-cholesterol (LFNC) diet fed to control animals.[51] A more recent study showed that rats fed a HFHC diet also demonstrated loss of femoral BMD when compared with rats fed normal chow.[52] Neither of these studies used isocaloric diets, and, therefore, did not rule out energy effects, resulting in inconclusive interpretation of a specific role for cholesterol in reducing BMD.

The objective of the present study was to test the effect of hypercholesterolemia on bone integrity. Using a novel diet scheme that included isocaloric diets lacking sodium cholate, we demonstrated that hypercholesterolemia is associated with alterations in BMD, bone volume fraction (BVF; bone volume/total volume), number of trabeculae, trabecular spacing, and other physical measures, accompanied by changes in the mechanical properties of the bone and an increase in bone resident osteoclasts.

Materials and Methods

Mice and Diets

All animal experiments were approved by the Institutional Animal Care and Use Committee of Children's Hospital Boston (protocol A07-06-084). Five-week-old male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) and C.B-17/Icr-SCID/Sed-Prkdcscid mice (Massachusetts General Hospital, Boston, MA) were fed an LFNC diet (catalog No. D12102; Research Diets, Inc., New Brunswick, NJ) for 2 weeks, after which blood was drawn from the saphenous tail vein, and the serum cholesterol concentration was determined using the Infinity Cholesterol Liquid Stable Reagent (Thermo Electron Corp., Waltham, MA). The mice were then divided into groups that received either the HFHC diet (catalog No. D12108; Research Diets) or the LFNC diet for 4 months. Although these diets differ in composition, they are used isocalorically, ie, by feeding mice the same number of calories in each diet. Terminal blood samples were obtained via cardiac puncture for serologic assay (pyridinoline cross-links assay; Quidel Corp., San Diego, CA) and osteocalcin assay (Biomedical Technologies, Inc., Stoughton, MA). Assays were performed according to the manufacturers' instructions. Triglyceride, insulin, and bilirubin concentrations, and other liver function tests were performed at the Yale University Mouse Phenotyping Center (New Haven, CT), and androgen level tests were performed at the Hormone Assay Core, Vanderbilt University (Nashville, TN).

Histomorphologic Analyses

Femurs were fixed in 10% neutral buffered formalin and decalcified for 2 weeks at 4°C using 10% EDTA (pH 7.4). The bones were dehydrated in graded ethanol (50%, 70%, 90%, and 95%) and twice in 100% ethanol for 1 hour each, cleared twice in xylene for 1 hour each, and embedded in paraffin. Five-micrometer sections were cut, deparaffinized, and stained using the TRAP kit (Acid Phosphatase, Leukocyte kit; Sigma Corp., St. Louis, MO) according to the manufacturer's instructions, and were counterstained with hematoxylin. TRAP-stained cells with three nuclei or more were counted as osteoclasts for quantification.

Bones, Micro Computed Tomography, and Mechanical Testing

The calvaria, right femurs, and L4 vertebrae were removed immediately after the animals were euthanized, and were washed in lactated Ringer's solution. All specimens were scanned and then reconstructed onto 18-μm voxels using a cone beam micro-computed tomography (microCT) system (GE Healthcare BioSciences Corp., Piscataway, NJ) to assess geometric and morphologic properties. A single threshold was applied to the three-dimensional dataset, as determined from evaluation of the calibrated density histograms. Cortical mineralization and geometric analyses were performed over a 3-mm mid-diaphyseal segment in the femurs, and trabecular

morphologic analysis over a standardized volume of bone segmented using a spline algorithm, starting at the distal growth plate and moving proximally to 20% of the total femur length. The elements of the spline were interpolated to generate a three-dimensional region of interest. Data from each femur were averaged over the entire region of interest to provide representative values for the entire three-dimensional mid-diaphysis. Outcome measures included cross-sectional area, cortical thickness, and bending moment of inertia. In addition to the femoral properties, two standardized volumes of trabecular bone were segmented from the proximal and distal ends of vertebral bodies. Volumes were determined by identifying the largest volume of trabecular bone possible by digitizing each section to define a series of splines that interpolated the volume of interest. The trabecular bone in both the femoral metaphyses and the vertebrae were evaluated to determine BVF, trabecular thickness, degree of anisotropy, number of trabeculae, and trabecular spacing. The analyses were performed using the advanced analysis programs and MicroView software suites in the GE MicroCT systems (GE Healthcare Biosciences). Morphologic analyses of the calvariae were performed on segments created using a region of interest growing tool. All of the algorithms have been validated in the Orthopaedic Research Laboratories at the University of Michigan (Ann Arbor).

Biomechanical Properties

Before biomechanical testing, all specimens were maintained frozen at −20°C. Testing was performed at room temperature, and the specimens were maintained moist using Ringer's solution. Whole-bone mechanical properties of the femurs to failure were performed in four-point bending using a servohydraulic testing machine (858 Mini Bionix II; MTS Systems Corp., Eden Prairie, MN) at a constant displacement rate of 0.5 mm/second. Femors were loaded in the anteroposterior direction so that the posterior side of the bone was in tension and the anterior side was in compression. All four loading points were placed in contact with the bone by adjusting the height of the upper two points independently. Regions loaded in four-point bending corresponded to those measured using microCT. Displacement was monitored using a linear variable differential transducer (Lucas Schaevitz 050 MHR; Measurement Specialties, Inc., Hampton, VA), and load data were collected using a load cell (Sensotec, Inc., Columbus, OH) in series with the actuator. Load and displacement data were sampled at 2000 Hz using the TestStar IIs system (version 2.4; MTS Systems). Load-displacement curves were then analyzed for whole-bone yield load, ultimate load, stiffness, failure energy, and displacement ratio (ultimate displacement:yield displacement) using MATLAB software (version 6.5; The MathWorks, Inc., Natick, MA). The predicted tissue modules and yield strength were calculated using standard mechanics of materials equations and geometric properties calculated during microCT analysis. These equations predict inherent material properties of the extracellular matrix, assuming a bending mode of failure.

Statistical Analysis

Statistical significance was determined using Student's *t*-test. $P \leq .05$ was considered significant.

Results

To understand how hypercholesterolemia affects various measures related to bone quality, we used two cohorts of mice fed isocaloric HFHC and LFNC diets, respectively, for 4 months. Previous testing of these diets indicated that to raise cholesterol concentrations in mice, a diet required both additional fat and cholesterol, ie, HFHC diet,[53] whereas the LFNC diet is well-defined and is composed essentially of normal mouse chow. Cohorts of mice fed these diets did not differ by weight even after 4 months (mean ± SD, 37.66 ± 3.68 *g* LFNC versus 38.61 ± 5.30 *g* HFHC for C57BL/6 mice, and 31.5 ± 2.93 *g* LFNC versus 31.31 ± 2.95 *g* HFHC for SCID mice) (Figure 1A; see also Supplemental Table S1 at *http://ajp.amjpathol.org*), but demonstrated significant differences in serum cholesterol concentrations (122.23 ± 22.53

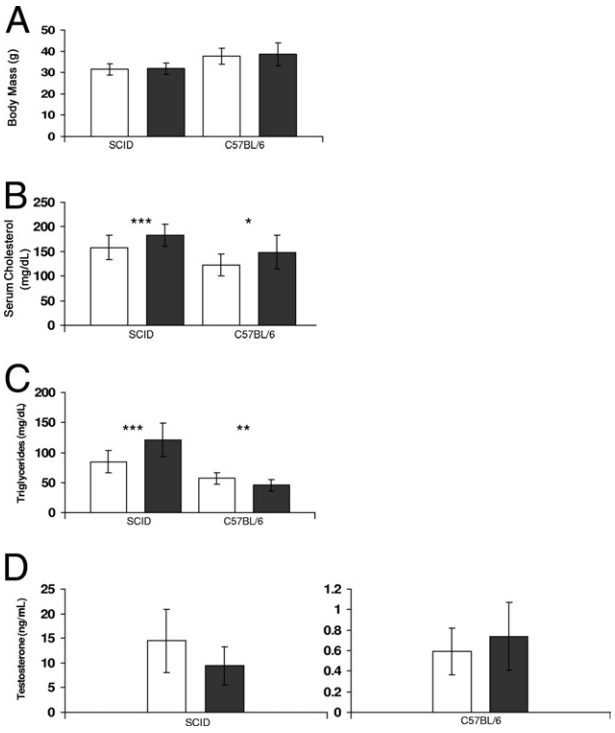

Figure 1. Effects of diet on weight and blood chemistry. Mice (14 to 16 per group) fed an isocaloric high-fat/high-cholesterol diet (black bars) or a low-fat/no-cholesterol diet (white bars) for 4 months were weighed (**A**), and serum cholesterol (**B**), triglyceride (**C**), and testosterone (**D**) concentrations were determined. **A:** Body mass. Data are plotted as mean ± SD versus diet group. There is no statistically significant difference between the 2 groups in either strain. **B–D:** Serologic measures. Mice were bled via cardiac puncture, and the serum was subjected to various tests. **B:** Serum cholesterol concentration was measured in the collected serum via Infinity colorimetric assay. Data are plotted as mean ± SD cholesterol concentration versus group. **C:** Triglyceride concentration was measured as described in *Materials and Methods*. Data are plotted as triglyceride concentration versus group. **D:** Testosterone concentration was measured as described in *Materials and Methods*. Data are plotted as mean ± SE testosterone concentration versus diet group. In all cases, the Student's *t*-test was used to calculate significance. Data were considered significant at $P < 0.05$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.005$.

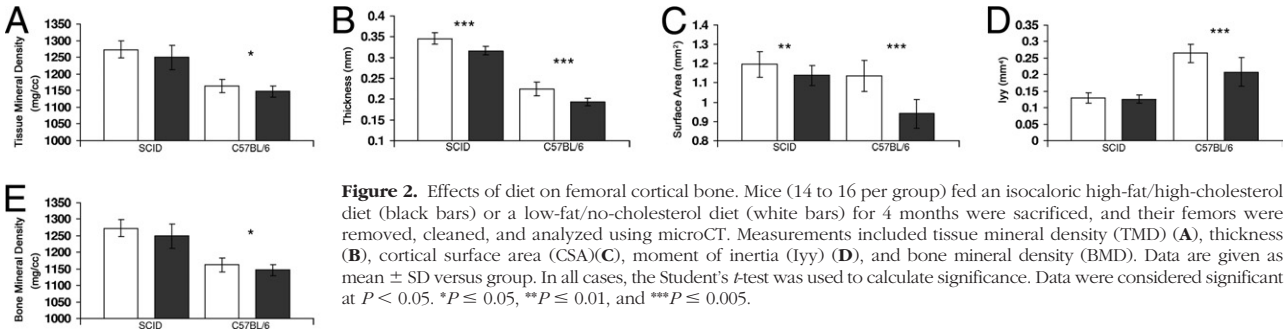

Figure 2. Effects of diet on femoral cortical bone. Mice (14 to 16 per group) fed an isocaloric high-fat/high-cholesterol diet (black bars) or a low-fat/no-cholesterol diet (white bars) for 4 months were sacrificed, and their femors were removed, cleaned, and analyzed using microCT. Measurements included tissue mineral density (TMD) (**A**), thickness (**B**), cortical surface area (CSA)(**C**), moment of inertia (Iyy) (**D**), and bone mineral density (BMD). Data are given as mean ± SD versus group. In all cases, the Student's *t*-test was used to calculate significance. Data were considered significant at $P < 0.05$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.005$.

mg/dL LFNC versus 152.37 ± 36.10 mg/dL HFHC for C57BL/6 mice, and 158.11 ± 22.76 mg/dL LFNC versus 181.53 ± 24.82 mg/dL HFHC for SCID mice) (Figure 1B; see also Supplemental Table S1 at *http://ajp.amjpathol.org*). Mice fed the HFHC diet had hypercholesterolemia, whereas the other group had normal cholesterol concentrations. The HFHC diet did raise triglyceride concentrations significantly in SCID mice, but not in C57BL/6 mice (Figure 1C). Liver toxicity, as determined by liver function test results, was never detected, and insulin levels were not significantly different between the diet cohorts, although they trended higher in the C57BL/6 HFHC diet cohort (data not shown). There were no significant differences in androgen levels (Figure 1D).

To determine whether elevated serum cholesterol concentrations affected bone health, the calvariae, femors, and vertebral bodies of the mice were removed, cleaned, and subjected to microCT.

The hypercholesterolemic mice demonstrated significant changes in their femors when compared with mice fed a normocholesterolemic diet, ie, significantly reduced tissue mineral density (TMD) in the cortical bone regions (1163.5 ± 20.4 mg/mL LFNC versus 1147.5 ± 16.1 mg/mL HFHC) in the C57BL/6 mice, which also trended similarly but did not reach statistical significance in the SCID mice (1273.0 ± 25.7 mg/mL LFNC versus 1249.8 ± 36.9 mg/mL HFHC), and significantly thinner diaphyseal cortical bone with a smaller cortical surface area in both strains (Figure 2, A–C; see also Supplemental Table S1 at *http://ajp.amjpathol.org*). We also measured significant differences in the moment of inertia (Iyy) and BMD in LFNC versus HFHC diet cohorts of C57BL/6 mice (0.27 ± 0.03 mm^4 LFNC versus 0.21 ± 0.04 mm^4 HFHC) (Figure 2, D and E; see also Supplemental Table S1 at *http://ajp.amjpathol.org*). These values trended similarly between the diets in the SCID mice, but did not reach statistical significance. The trabecular bone in the femors of the hypercholesterolemic cohorts also demonstrated statistically significant changes as compared with normocholesterolemic mice, with a smaller BVF (0.43 ± 0.06 LFNC versus 0.27 ± 0.05 HFHC for the C57BL/6 mice, and 0.46 ± 0.10 LFNC versus 0.37 ± 0.08 HFHC for the SCID mice), larger trabecular spacing (0.10 ± 0.02 mm LFNC versus 0.16 ± 0.03 mm HFHC for the C57BL/6 mice, and 0.09 ± 0.02 mm LFNC versus 0.12 ± 0.07 mm HFHC for the SCID mice), fewer trabeculae in both strains (5.59 ± 0.42 LFNC versus 4.51 ± 0.49 HFHC for the C57BL/6 mice, and 6.21 ± 0.53 LFNC versus 5.53 ± 1.0 HFHC for the SCID mice) (Figure 3, A–C; see also Supplemental Table S1 at *http://ajp.amjpathol.org*), and significantly less trabecular thickness and TMD in the LFNC diet cohort (691.05 ± 25.43 mg/mL) versus the HFHC diet cohort (717.37 ± 15.16 mg/mL) of the C57BL/6 strain. These values trended similarly in the SCID mice (Figure 3, D and E; see also Supplemental Table S1 at *http://ajp.amjpathol.org*).

Calvariae from mice fed the HFHC diet versus those fed the LFNC diet demonstrated significant reductions in BMD (763.5 ± 55.12 mg/mL LFNC versus 700.98 ± 45.53 mg/mL HFHC in the C57BL/6 mice, and 713.16 ± 69.13 mg/mL LFNC versus 664.5 ± 42.6 mg/mL HFHC in the SCID mice) and mean thickness in both strains (0.21 ± 0.02 mm LFNC versus 0.20 ± 0.02 mm HFHC in the C57BL/6 mice, and 0.33 ± 0.05 mm LFNC versus 0.29 ± 0.04 mm HFHC in the SCID mice) (Figure 4,A and B; see also Supplemental Table S1 at *http://ajp.*

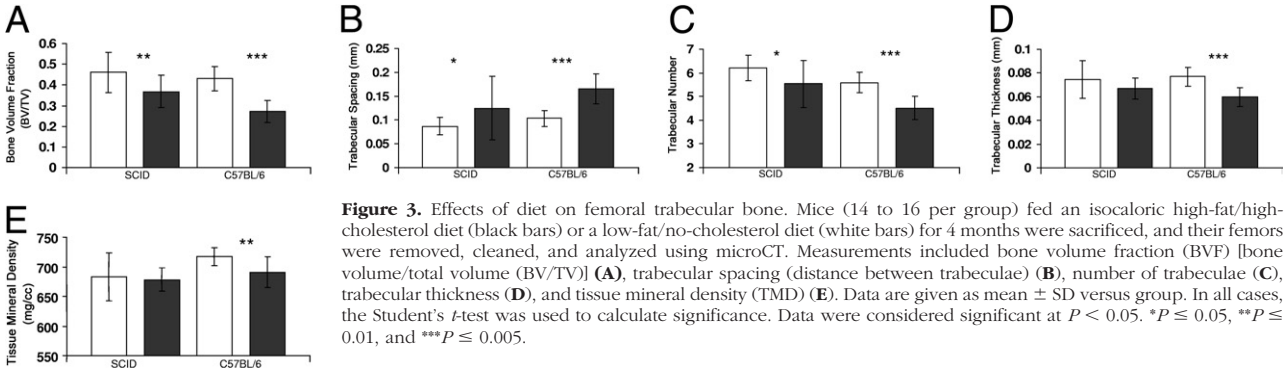

Figure 3. Effects of diet on femoral trabecular bone. Mice (14 to 16 per group) fed an isocaloric high-fat/high-cholesterol diet (black bars) or a low-fat/no-cholesterol diet (white bars) for 4 months were sacrificed, and their femors were removed, cleaned, and analyzed using microCT. Measurements included bone volume fraction (BVF) [bone volume/total volume (BV/TV)] **(A)**, trabecular spacing (distance between trabeculae) (**B**), number of trabeculae (**C**), trabecular thickness (**D**), and tissue mineral density (TMD) (**E**). Data are given as mean ± SD versus group. In all cases, the Student's *t*-test was used to calculate significance. Data were considered significant at $P < 0.05$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.005$.

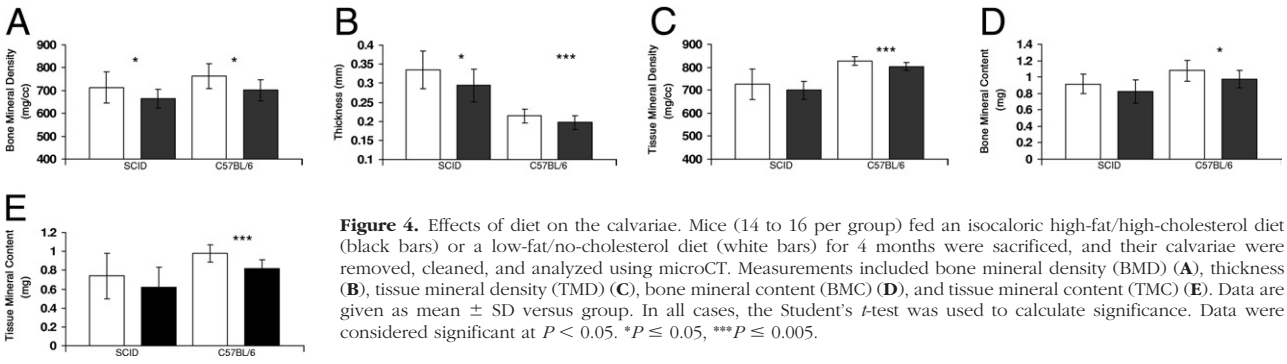

Figure 4. Effects of diet on the calvariae. Mice (14 to 16 per group) fed an isocaloric high-fat/high-cholesterol diet (black bars) or a low-fat/no-cholesterol diet (white bars) for 4 months were sacrificed, and their calvariae were removed, cleaned, and analyzed using microCT. Measurements included bone mineral density (BMD) (**A**), thickness (**B**), tissue mineral density (TMD) (**C**), bone mineral content (BMC) (**D**), and tissue mineral content (TMC) (**E**). Data are given as mean ± SD versus group. In all cases, the Student's *t*-test was used to calculate significance. Data were considered significant at $P < 0.05$. $^{*}P \leq 0.05$, $^{***}P \leq 0.005$.

amjpathol.org). The calvariae from the C57BL/6 mice fed the LFNC diet also had significantly higher TMD (826.6 ± 18.21 mg/mL versus 802.44 ± 17.65 mg/mL), bone mineral content (1.08 ± 0.13 mg versus 0.97 ± 0.11 mg), and tissue mineral content (0.98 ± 0.09 mg versus 0.82 ± 0.09 mg) when compared with mice fed the HFHC diet, and these measures also trended similarly in the SCID mouse cohort (Figure 4, C–E; see also Supplemental Table S1 at *http://ajp.amjpathol.org*).

The trabecular bone of the vertebral bodies from the C57BL/6 and SCID HFHC cohorts demonstrated significantly fewer trabeculae (7.71 ± 0.19 LFNC versus 7.21 ± 0.46 HFHC for the C57BL/6 mice, and 6.90 ± 0.78 LFNC versus 5.96 ± 0.79 HFHC for the SCID mice) and larger trabecular spacing (0.05 ± 0.01 mm LFNC versus 0.08 ± 0.01 mm HFHC for the C57BL/6 mice, and 0.09 ± 0.02 mm LFNC versus 0.11 ± 0.03 mm HFHC for the SCID mice) than in the LFNC cohorts (Figure 5,A and B; see also Supplemental Table S1 at *http://ajp.amjpathol.org*). In addition, there were also significant differences between the C57BL/6 HFHC diet mice and LFNC diet mice in both trabecular thickness (0.08 ± 0.01 mm LFNC versus 0.06 ± 0.01 mm HFHC) and BVF (0.61 ± 0.05 LFNC versus 0.45 ± 0.06 HFHC) (Figure 5, C and D; see also Supplemental Table S1 at *http://ajp.amjpathol.org*).

In contrast to the overall similarity of the effects of diet on the C57BL/6 and SCID mice, the effects of diet on cortical vertebral bone varied between the two strains. In the C57BL/6 strain, bone mineral content (0.52 ± 0.08 mg LFNC versus 0.45 ± 0.07 mg HFHC), BMD (479.62 ± 25.59 mg/mL LFNC versus 389.53 ± 41.74 mg/mL HFHC), TMC (0.42 ± 0.07 mg LFNC versus 0.31 ± 0.06 mg HFHC), and TMD (621.43 ± 17.22 mg/mL LFNC versus 597.03 ± 19.46 mg/mL HFHC) were all significantly reduced, whereas in the SCID mice, there were no significant differences in any of these measurements between the LFNC and HFHC groups, although bone mineral content, BMD, and TMC measurements all trended similarly to those of the C57BL/6 strain (Figure 6, A–D; see also Supplemental Table S1 at *http://ajp.amjpathol.org*).

Because the HFHC versus LFNC diet was associated with significant changes in both mineral content and microarchitecture of the femors, we next determined whether these changes resulted in alterations in the mechanical properties of the bone (Figure 7; see also Supplemental Table S1 at *http://ajp.amjpathol.org*). The femors from the C57BL/6 HFHC cohort were less stiff (323.08 ± 35.36 N/mm LFNC versus 244.0 ± 42.43 N/mm HFHC), had significantly smaller ultimate and failure loads (38.41 ± 5.71 N LFNC versus 27.94 ± 4.24 N HFHC), and less energy to failure (4.28 ± 1.96 N/mm LFNC versus 2.76 ± 1.29 N/mm HFHC) than did the femors from the C57BL/6 LFNC group. The mechanical properties of the femors did not differ in the SCID HFHC group when compared with the SCID LFNC group (Figure 7, A–D; see also Supplemental Table S1 at *http://ajp.amjpathol.org*).

Serologic testing indicated that there were no differences in osteocalcin levels between the hypercholesterolemic HFHC cohort and the normocholesterolemic LFNC cohort, which suggested that altered osteoblast activity was unlikely to explain the observed changes in the bone (data not shown). However, we did observe differences in the level of pyridinoline cross-links found in collagen I fragments, a measure of osteoclast activity (Figure 8A). This suggested that increased osteoclast activity contributes to the osteopenic phenotype of the hypercholesterolemic mice. Consistent with an effect of the HFHC diet on osteoclastogenesis, histomorphometric

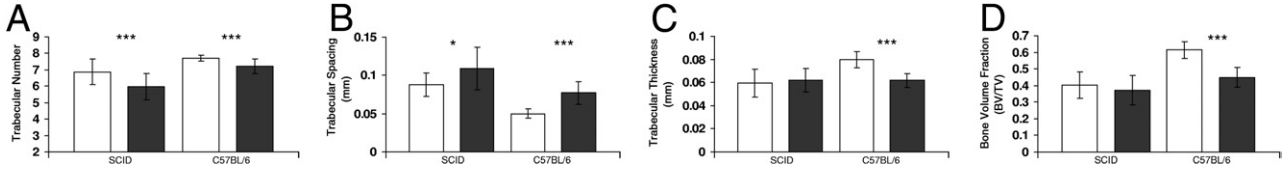

Figure 5. Effects of diet on the trabecular bone of vertebrae. Mice (14 to 16 per group) fed an isocaloric high-fat/high-cholesterol diet (black bars) or a low-fat/no-cholesterol diet (white bars) for 4 months were sacrificed, and L4 was removed, cleaned, and analyzed using microCT. Measurements included number of trabeculae (**A**), trabecular spacing distance between trabeculae (**B**), trabecular thickness (**C**), and bone volume fraction (BVF) [bone volume/total volume (BV/TV)] (**D**). Data are given as mean ± SD versus group. In all cases, the Student's *t*-test was used to calculate significance. Data were considered significant at $P < 0.05$. $^{*}P \leq 0.05$, $^{***}P \leq 0.005$.

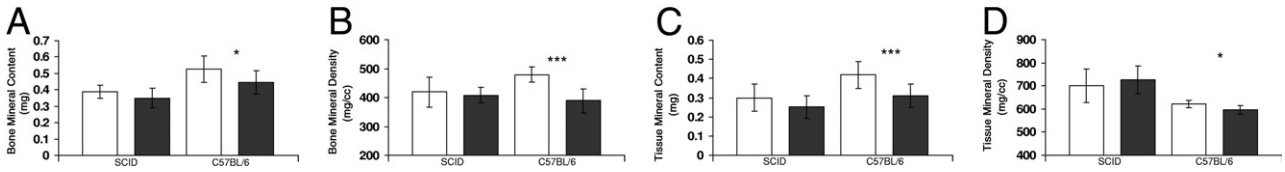

Figure 6. Effects of diet on the cortical bone of the vertebrae. Mice (14 to 16 per group) fed an isocaloric high-fat/high-cholesterol diet (black bars) or a low-fat/no-cholesterol diet (white bars) for 4 months were sacrificed, and L4 was removed, cleaned, and analyzed using MicroCT. Measurements included bone mineral content (BMC) (**A**), bone mineral density (BMD) (**B**), tissue mineral content (TMC) (**C**), and tissue mineral density (TMD) (**D**). Data are given as mean ± SD versus group. In all cases, the Student's *t*-test was used to calculate significance. Data were considered significant at $P < 0.05$. *$P \leq 0.05$, ***$P \leq 0.005$.

analysis revealed a significant increase in the number of osteoclasts present in the bones of the hypercholesterolemic versus the normocholesterolemic groups (Figure 8, B and C).

Discussion

In the present study, we demonstrated that diet-induced hypercholesterolemia in mice is associated with a reduction in bone quality measures that resemble the bone quality in humans with osteoporosis.

We concluded that there is likely a direct influence of hypercholesterolemia on bone health due to the following: i) mice were fed isocaloric diets, and, thus, there is no effect of additional calories in this model, even though the diets differed in their levels of fat and cholesterol; ii) the mice did not gain weight with the HFHC diet versus the LFNC diet, and, thus, any changes that occurred in bone are not reflective of differences in animal weight (Figure 1A); iii) multiple parameters of bone quality were altered by diet, and, thus, the changes detected are likely to originate in a systematic loss of bone homeostasis and are not due to an isolated event (Figures 2 to 6); iv) femors, calvariae, and vertebrae were all affected, indicating that the source of altered bone quality is systemic (Figures 2 to 6); v) the C57BL/6 HFHC cohort exhibited reduced mechanical properties (Figure 7); and vi) diet did not influence androgen levels (Figure 1D).

Mice received the HFHC or LFNC diets for 4 months, which is approximately 16% to 25% of their lifespan. This should be sufficient time to affect cortical and trabecular bone, should either diet have any effect.

Testing of the femors of the C57BL/6 mice demonstrated significant loss of their mechanical properties, including substantial decreases in stiffness, failure load, ultimate load, and energy to failure in the HFHC cohort, which suggests that the loss of bone mineral and thinning of the trabeculae were substantial enough to significantly weaken the bone. The loss of both physical and mechanical properties implies a resemblance to osteoporosis in humans. The similarities to osteoporosis in humans include thinning of the diaphyseal cortical bone, reduced BVF, thinning trabeculae, loss of trabeculae, increased osteoclast numbers, and reductions in failure load and energy to failure. Given the loss of femoral stiffness, which is likely a reflection of the thinner bone, it is expected that the bones would be somewhat less brittle; rather, we found that the bones were more brittle. Thus, the loss of energy to failure induced by the HFHC diet likely reflects a change in the material properties of the bone.

Although in many respects the physical properties noted in the C57BL/6 mice fed the HFHC versus the LFNC diet were also present in the SCID mice, for the most part, they were less pronounced, and most mechanical measures were not affected by diet in the SCID strain.

Although not all of the factors that contribute to native BMD are known, a combination of environmental and genetic factors is most probably involved. The effect of serum cholesterol concentrations on development of an osteoporotic phenotype is likely, to some degree, also dependent on native BMD (ie, the BMD before cholesterol manipulation). On the basis of previous analyses,[54–56] and our own data (Figure 2E) for the native femoral BMD of these strains, C57BL/6 mice have a lower BMD than does the SCID strain, which shares a background with Balb/C mice. This result suggests that the native BMD may influence the degree to which hypercholesterolemia affects bone. A study by Ishimori et al[57] suggested that the differential effect of atherogenic diets in C57BL/6 and 129 strains, which displayed different

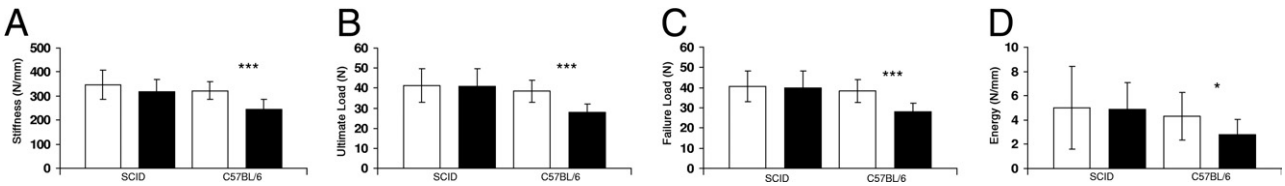

Figure 7. Mechanical properties of femors. Mice (14 to 16 per group) fed an isocaloric high-fat/high-cholesterol diet (black bars) or a low-fat/no-cholesterol diet (white bars) for 4 months were sacrificed, and their femors were removed, cleaned, and analyzed via mechanical testing. Whole-bone mechanical properties of the femors to failure were performed in 4-point bending using a servohydraulic testing machine. Load-displacement curves were then analyzed for whole-bone yield load, ultimate load, stiffness, failure energy, and displacement ratio (ultimate displacement:yield displacement), and other measures using MATLAB software. The difference between "start" (centroid coordinate position) and "end" positions was used to calculate stiffness (**A**), ultimate load (**B**), failure load (**C**), and energy (**D**). Data are given as mean ± SD. In all cases, the Student's *t*-test was used to calculate significance. Data were considered significant at $P < 0.05$. *$P \leq 0.05$, ***$P \leq 0.005$.

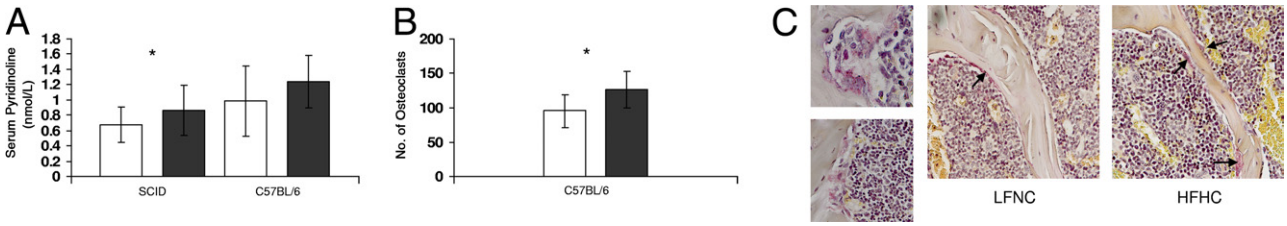

Figure 8. Bone loss in mice with hypercholesterolemia is linked to increased osteoclastic activity and osteoclasts. Mice fed an isocaloric high-fat/high-cholesterol diet (black bars) or a low-fat/no-cholesterol diet (white bars) for 4 months were sacrificed, and serum pyridinoline (PYD) concentration in the murine blood was determined via enzyme-linked immunosorbent assay (**A**), the femors were analyzed at histology, and the osteoclasts were counted (**B**). **A:** Data are plotted as mean ± SD PYD concentration versus group (n = 8 or 9 mice per group). **B:** Five-micrometer sections of femors were cut and stained using the Acid Phosphatase, Leukocyte (TRAP) kit, and the total number of osteoclasts in 5 ×20 fields were counted. Data are given as mean ± SD number of osteoclasts versus group (n = 7 bones per group). In all cases, the Student's t-test was used to calculate significance. Data were considered significant at $P < 0.05$. **C:** Histomorphometric images demonstrate osteoclasts (**arrows**) in trabecular regions of the femors in C57BL/6 mice. These are representative images of the ×20 fields used in the osteoclast quantification at ×40 magnification. **Left:** Most panels are enlargements of original ×60 images demonstrating osteoclasts used for quantification with characteristic TRAP staining (light pink) and multiple nuclei (purple). *$P \leq 0.05$.

total and vertebral BMD after being fed an atherogenic diet, are linked to four quantitative trait loci: Bmd9, Bmd20, Bmd21, and Bmd22, which collectively accounted for 21.6% of total BMD and 17.3% of vertebral BMD of the F_2 population variances. It is likely that genetic variations, which influence BMD, may have a role in the effect of diet on the development of osteopenia in mice.

Another potential reason for the disparate response between C57BL/6 and SCID mice is that SCID mice are profoundly immunodeficient owing to the inability of these mice to perform VDJ recombination, rendering them without functional T and B cells. Although certain inflammatory responses from natural killer cells and macrophages remain, most inflammation is impaired in these animals. In a separate study (accepted for publication, PLOS One), we measured particular inflammatory responses in the animals as related to diet, and found that the HFHC diet did contribute to inflammation in the C57BL/6 mice, which was absent in the SCID mice, consistent with the known effects of hypercholesterolemia on inflammation.

Proinflammatory cytokines have a role in osteoclastogenesis and bone resorption, and are increased during menopause, which suggests that inflammation and osteoporosis are linked.[58] Our results are consistent with a role for inflammation in response to hypercholesterolemia as a mediator of bone loss, but also suggest that noninflammatory pathways likely mediate some of the effect of diet on development of osteoporosis because the SCID mice did lose bone.

Our data (Figure 8) indicate that hypercholesterolemia increases the number and activity of osteoclasts within bone. Although increased osteoclast activity could explain the reduced BMD in both strains, it is notable that statistically significant effects of hypercholesterolemia were observed only for serum pyridinoline concentration (a measure of osteoclast activity) in SCID mice, whereas more extensive effects of diet on bone were evident in C57BL/6 mice. This is likely a reflection of the timing of the assay, which was necessarily performed as an experimental end point (ie, after 4 months on the diet) at which the loss of bone in C57BL/6 mice had already peaked.

Our data suggest that hypercholesterolemia promotes osteoclastogenesis, consistent with unpublished observations from our laboratory that increasing the exogenous cholesterol available to macrophages increases the number and size of the polykaryon (data not shown). It is possible that hypercholesterolemia affects the membrane composition of both osteoblasts and osteoclasts, which we hypothesized would in turn affect their ability to respond to environmental cues. We have published[59,60] and unpublished evidence that altering membrane cholesterol levels in osteoblasts affects their responses to growth factors, but we have no evidence that this is occurring *in vivo*.

A number of studies have suggested that high serum cholesterol concentrations, per se, are associated with low BMD and osteoporosis in humans.[5,14–21] For example, Broulik and Kapitola[14] measured higher cholesterol concentrations in patients with osteoporosis with and without vertebral fractures, respectively, compared with age-matched patients without osteoporosis. Poli et al[21] reported that women with plasma low-density lipoprotein (LDL) cholesterol concentrations ≥160 mg/dL had a more than twofold probability of having osteopenia than did women with lower LDL cholesterol concentrations (47.9% versus 21.2%, respectively). Of note, some studies have not found an association between high cholesterol concentration (or LDL concentration) and osteoporosis.[61,62] For example, Samelson et al,[61] using data from the Framingham Study, examined the relationship between BMD (femoral head) and total cholesterol concentration, but found no correlation between high cholesterol concentrations and osteoporosis. Of importance, this study did not use vertebral measures, LDL cholesterol concentrations were unknown, and, perhaps most critical, the subjects were enrolled in 1948, yet their BMD was not measured until 1988–1989,[61] thereby effectively skewing the data in favor of the null hypothesis because many subjects with high cholesterol concentrations and low BMD were likely deceased by 1989. Assessing the complex relationships between diet, cholesterol concentrations, and osteoporosis in a human population is problematic because all important variables cannot be controlled and data are difficult to obtain.

In the present study, we created a hypercholesterolemic animal model with which we can determine how hypercholesterolemia affects bone health. By controlling calories, animal weight, and other variables, we were

able to accurately measure the effect of hypercholesterolemia in mice. It is noteworthy that a previous analysis of diet and bone in mice determined that an atherogenic diet that raised cholesterol concentrations was correlated with loss of BMD. The diet we used in our study also raised serum cholesterol concentrations significantly, but was not atherogenic. Mice do not naturally develop atherosclerotic lesions in the aorta similar to those found in humans.[63] An atherogenic diet in mice requires the addition of sodium cholate, a bile acid.[63] Sodium cholate is not required to raise cholesterol concentrations, but does cause liver toxicity. Thus, hypercholesterolemic non-atherogenic diets, such as those used in the present study, are preferred when determining the relationship between hypercholesterolemia and disease.

Although the type of analysis performed in the present study will be helpful in hypothesis testing and in discovery, this model is not without caveats; measuring osteoporosis in mice will not accurately predict which bones and bone regions will be most affected by hypercholesterolemia in humans because weight distribution in mice is not the same.

In conclusion, a hypercholesterolemic non-atherogenic diet contributes to development of an osteoporotic bone phenotype in mice, including an increase in osteoclasts, loss of trabeculae, thinning of trabeculae and cortex, and reductions in failure load and energy to failure. This model of osteoporosis may provide an opportunity to critically evaluate various interventional strategies to counteract the changes induced by a hypercholesterolemic diet. Our data suggest that the correlation between hypercholesterolemia and osteoporosis may be a manifestation of failure to maintain cholesterol homeostasis and an increase in osteoclast activity. Given the increase in hyperlipidemia, metabolic syndrome, and obesity in the United States and around the world, we anticipate an increase in diet-induced osteoporosis, making our novel model particularly timely.

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