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Alterations to the gut microbiome impair bone tissue strength in aged mice

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ABSTRACT

Whole bone strength and resistance to fracture are determined by a combination of bone quantity and bone quality – key factors in determining risk for osteoporosis and age-related fractures. Recent preclinical studies have shown that alterations to the gut microbiome can influence bone quantity as well as bone tissue quality. Prior work on the gut microbiome and bone has been limited to young animals, and it is unknown if the gut microbiome can alter bone tissue strength in aged animals. Here we ask if alterations to the constituents of the gut microbiome influence bone strength in older mice (12–24 months of age). Male C57BL/6J mice raised on a standard chow diet until 12 months of age were assigned to one of three diets: high glycemic, low glycemic, or low glycemic diet containing antibiotics (ampicillin and neomycin) to modify the constituents of the gut microbiome. The group fed the low glycemic diet containing antibiotics showed reductions in whole bone strength that could not be explained by geometry, indicating reduced bone tissue strength (p < 0.007). The high glycemic diet group had larger bone cross-sectional area and moment of inertia and a corresponding greater bone strength as compared to the low glycemic groups, however tissue strength did not noticeably differ from that of the low glycemic group. These findings demonstrate that modifying the gut microbiome in aged mice can alter bone tissue quality.

1. Introduction

The ability of bone to resist fracture is determined by a combination of bone quantity and bone quality (Hernandez and Keaveny, 2006). Current interventions for osteoporosis focus primarily on improving bone quantity and do not directly address bone quality. Factors that influence bone quality therefore have the potential to influence bone fragility in ways that are not possible with current osteoporosis treatments. Tissue material properties are a major component of bone quality.

Recent studies have shown that the gut microbiome can regulate bone quantity, bone quality, and whole bone strength. The gut microbiome is the community of microbial organisms that inhabit the gastrointestinal tract. Preclinical studies have shown that the gut microbiome can influence bone loss induced by estrogen depletion (Li et al., 2020), continuous PTH treatment (Yu et al., 2020), and glucocorticoid treatment (Schepper et al., 2020), clearly demonstrating that the gut microbiome can influence bone quantity. Guss and colleagues found that disruption of the gut microbiome can lead to impaired tissue mechanical properties without noticeable changes in bone geometry (Guss et al., 2017), demonstrating that the gut microbiome can alter bone tissue quality. One limitation of prior studies of the microbiome and bone is that they included only young adult mice (less than 10 months of age (Li et al., 2020; Yu et al., 2020; Schepper et al., 2020)). For example, Guss and colleagues observed a reduction in bone tissue strength in mice following disruption of the gut microbiome during a period of rapid bone acquisition (1-4 months of age (Guss et al., 2017)). In most mouse strains, including C57BL/6J mice, bone growth continues after sexual maturity, increasing between 6 and 12 months of age (Ackert-Bicknell et al., 2016). It is unclear if modifications to the gut microbiome can influence bone tissue strength in older animals, which would more directly relate to fracture risk in older adults.

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Here we ask if alterations to the constituents of the gut microbiome via antibiotics or diet in older mice (12–24 months of age) influence bone tissue strength.

2. Materials and methods

2.1. Study design

Here we examine bones from an animal study designed to study the effects of diet and changes in the gut microbiome in mice. Specifically, the study was designed to determine the effects of a high-glycemic index diet (HG) and alterations to the gut microbiome on age-related retinopathy (Rowan et al., 2017). A Western-style diet is associated with changes in metabolism that are also correlated with increases in fracture risk (Tian and Yu, 2017). However, it is unknown if the same diet could influence age-related bone disease as well.

Male C57BL/6J retired breeder mice, obtained at 9-months of age (Jackson Laboratories, Bar Harbor, ME, USA), were individually housed in plastic micro-isolator cages and provided standard laboratory chow (Teklad 2916 irradiated diet, Envigo, USA) and water ad libitum. Starting at twelve months of age, mice were divided into three isocaloric dietary groups (n = 16/group, 48 total) with different dietary starch composition as follows: high glycemic (HG, 100% amylopectin (Amioca starch, Ingredion Inc., Bridgewater, NJ)), low glycemic (LG, 30% amylopectin/70% amylose (Hylon VII starch, Ingredion Inc., Bridgewater, NJ)), or low glycemic containing antibiotics shown to modify gut microbiome composition (LG Amp + Neo, 320 mg ampicillin /kg chow +640 mg neomycin /kg chow (Goldbio, St. Louis, MO, USA)). Diet composition was 542 g/kg starch, 200 g/kg casein, 85 g/kg sucrose, 56 g/kg soybean oil, 50 g/kg wheat bran, 2 g/kg DL methionine, 10 g/kg vitamin mix, and 35 g/kg mineral mix (Rowan et al., 2020). Macronutrient energy percentages were 65% carbohydrate, 21% protein, and 14% fat for both HG and LG diets. All diets were formulated by Bio-Serv (Frenchtown, NJ). Mice were group pair-fed to ensure equal consumption. The dietary treatment continued until age 24 months when animals were fasted for 6 h and subsequently killed. The final number of mice for analysis was n = 11 (HG), n = 13 (LG), n = 9 (LG Amp + Neo). Animal work was performed at the Tufts University HNRCA and approved by the Tufts University IACUC in adherence with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Femoral geometry of mid-diaphyseal cortical bone

The left femora were harvested, wrapped in saline soaked gauze, and stored in an airtight container at -20 °C. Images of the femoral diaphyseal cross-section were obtained by micro-computed X-ray tomography with a voxel size of $25\,\mu$ m (eXplore CT 120, GE, Fairfield, CT, USA; 80kVp, $32\,\mu$ A, 100ms integration time). A Gaussian filter (radius = 1) was used to remove noise, and a global threshold was used to segment mineralized tissue from surrounding nonmineralized tissue. Crosssectional geometry of the mid-diaphyseal cortical bone was determined using a volume of interest extending 2.5% of total bone length and centered midway between the greater trochanter and lateral condyle (BoneJ, version 1.3.3) (Doube et al., 2010; Bouxsein et al., 2010). Femur length was measured from the greater trochanter to the lateral condyle using digital calipers.

2.3. Mechanical testing

Femurs were thawed to room temperature and maintained hydrated during mechanical testing. Left femora were tested to failure in the anterior-posterior direction with three-point bending at a rate of 0.5 mm/s using a span length of 8.5 mm between outer loading pins (858 Mini Bionix; MTS, Eden Prairie, MN, USA). Force and displacement measurements were measured using a 100-pound load cell (SSM-100; Transducer Techniques, Temecula, CA, USA) and a linear variable differential transducer at a 100-Hz sampling rate (a new calibration curve spanning the expected force range was generated prior to testing). Bending stiffness was calculated as the slope of the linear portion of the force-displacement curve (Guss et al., 2017). Peak bending moment was calculated as one-half the peak load multiplied by one-half the span length. The relationship between whole bone strength and section modulus was examined as an indicator of modifications in tissue strength using the following equation:

$$\sigma = \frac{Mc}{I} \tag{1}$$

where M is peak bending moment, σ is bone tissue material strength, I is the moment of inertia, and c is the distance from the neutral axis to bone surface. The term I/c is the section modulus and incorporates the total geometric contributions to bending resistance for a given cross-section (Turner and Burr, 1993; Jepsen et al., 2015). Three specimens (2 LG, 1 LG Amp + Neo) were lost due to experimental error (motion artifacts in micro-CT or during mechanical testing).

2.4. Fecal microbiome analysis

After 22 months of age, fecal pellets were collected from empty sterile cages for microbiota composition analysis (n = 10/group). Microbial DNA was isolated using the QiaAMP PowerFecalPro DNA kit (QIAGEN, Hilden, Germany). 16S rRNA libraries were prepared using the Earth Microbiome Project protocol with primers as described previously (Caporaso et al., 2010; Caporaso et al., 2011). Demultiplexed paired end reads were imported into QIIME2 (version 2018.6) for quality control, feature table construction, and computation and significance testing of alpha- and beta-diversity. Taxanomic assignment was performed using QIIME's machine learning classifier trained on Greengenes sequences (version 13_8).

2.5. Blood plasma analysis

Fasting plasma was collected at euthanasia via cardiac puncture to measure concentrations of C-terminal telopeptide (CTX) for osteoclast activity (catalog #AC-06F1, Immunodiagnostic Systems, Tyne and Wear, United Kingdom) and Procollagen 1 Intact N-Terminal Propeptide (P1NP) for osteoblast activity (catalog #AC-33F1, Immunodiagnostic Systems, Tyne and Wear, United Kingdom). Plasma was analyzed at the Maine Medical Center Research Institute Physiology Core. One outlier was removed from the CTX measurement (Low Glycemic) after a Grubbs' test.

2.6. Statistical analyses

The effect of diet on measurements of bone geometry and alpha diversity was determined using a one-way ANOVA with group as the factor followed by Tukey post hoc for multiple comparisons (R v3.6).

Analysis of covariance (ANCOVA), implemented with a generalized linear model (GLM) with section modulus as a covariate was used to determine differences in whole bone strength among groups after adjusting for cross-sectional geometry (Eq. (1), R v3.6). This approach does not require the assumptions used when calculating tissue stress directly (such as constant cross-sectional area along the length of the diaphysis) (Caporaso et al., 2010). Additionally, the use of ANCOVA are can be more sensitive than ANOVA (Caporaso et al., 2011).

Beta-diversity significance testing was performed using PERMA-NOVA on unweighted UniFrac distances. Unweighted UniFrac distances were used to perform principal coordinate analysis (PCoA) in R (v3.6). Statistical tests were conducted using R (v.3.6). Statistical tests were performed with alpha = 0.05.

3. Results

The relationship between whole bone strength and section modulus was altered in the low glycemic Amp + Neo group (LG Amp + Neo), indicating a 22% reduction in whole bone strength compared to bones with similar cross-sectional geometry from other groups (Fig. 1A, B and Table 1). The relationship between whole bone strength and section modulus indicates alterations in tissue strength. Whole bone strength in the group fed a high glycemic diet (HG) was greater than that in other groups. However, the relationship between whole bone strength and section modulus in the HG and LG groups did not differ (Fig. 1A), indicating that differences in whole bone strength between those two groups could be explained primarily by cross-sectional geometry (Fig. 1D, C). Bones from the HG group showed increased cortical area and thickness compared to mice on a LG diet (Fig. 1C, Table 1) leading to a larger moment of inertia and a correspondingly greater whole bone stiffness (Table 1) than the LG group. No differences in cortical bone geometry were observed between the LG group and the LG Amp + Neo group.

No statistically significant differences in plasma markers of bone formation (P1NP) or resorption (CTX) were observed among groups (Table 1). Antibiotic treatment resulted in decreased alpha diversity of the gut microbiome, as estimated by the Shannon diversity index (Fig. 1D), and an altered abundance of many taxa including increased abundance of Bacteroidetes and Proteobacteria and reduced abundance of Firmicutes and Actinobacteria (Fig. 1F). Gut microbiome composition was significantly different in all pairwise comparisons of treatments (p= 0.001) although the LG Amp + Neo group showed greater separation from the other two treatments (Fig. 1E). Differences in gut microbiome composition, metabolism, and body weight between the HG group and LG group were comparable to our previously reported findings (Rowan et al., 2017).

4. Discussion

Here we show that modifying the gut microbiome of mice in late adulthood (12-24 months of age) via antibiotics causes a reduction in bone tissue strength without noticeable modifications in cortical bone geometry or bone turnover markers. The reductions in bone tissue strength observed here (22%) are similar to those reported by Guss et al. (15%) when altering the microbiome in young adult mice (1-4 months of age) using the same stimulus (ampicillin and neomycin). The findings in the present study demonstrate that a modification to the constituents of the gut microbiome late in life have the potential to lead to alterations in bone tissue mechanical properties. This is significant in the context of aging, where the prevalence of gut dysbiosis is increased, either due to age-related microbiome changes and/or lifelong exposure to an unhealthy diet or antibiotic use (Claesson et al., 2012; Jeffery et al., 2016). Antibiotic use has potential for long-term restructuring of the gut microbiome, as spontaneous recovery from antibiotic use does not restore baseline diversity, even after six months of use (Suez et al., 2018). Age-related bone loss, as seen in osteoporosis and osteopenia, may associate with dysbiosis, which our study shows causes a decrease in bone tissue mechanical properties (Wang et al., 2017). Not all changes to the gut microbiome led to impaired bone tissue mechanical properties, however. As expected from prior studies (Rowan et al., 2017), mice fed HG diets had increased body weight and alterations in gut microbiome composition compared to mice fed LG diets. Bones from HG-fed mice were larger and therefore stronger than bones from LG-fed mice, but the changes in bone strength were explained primarily by alterations in bone cross-sectional geometry (i.e., no change in bone tissue mechanical properties were observed).

While modifications to bone resorption, formation, and/or bone turnover can explain changes in bone *quantity*, there are relatively few known mechanisms for modulating bone tissue *quality* (Alliston, 2014; Castaneda et al., 2020). Prior studies have indicated that modifications

to the gut microbiome can regulate trabecular BV/TV and bone loss in mice by modulating bone resorption and/or bone formation (Li et al., 2020; Yu et al., 2020; Schepper et al., 2020; Yan et al., 2016). However, the modifications to the gut microbiome in the current study were not associated with noticeable changes in femoral bone geometry or plasma markers of bone turnover (similar to previous observations (Guss et al., 2019)). This study did not measure trabecular microarchitecture or other bone material properties outside of ultimate stress, which was significantly lower with antibiotic treatment (Table 1). However, in a previous study, we quantified tissue changes in the tibia of antibiotic treated mice and found a reduction in crystallinity and osteocalcin concentration (Guss et al., 2019), with no changes to trabecular microarchitecture except in cortical tissue mineral density (Guss et al., 2017). Our future work includes performing the same analysis on aged mice to verify the phenotype. Alterations in bone tissue quality in humans are often associated with genetic abnormalities (osteogenesis imperfecta) or increased tissue age (the length of time the tissue has been in the body, not to be confused with the age of the individual) that lead to altered tissue degree of mineralization, mineral composition, collagen quality/cross-linking, or microdamage accumulation. In our study, mice were of the same genotype, and we did not observe noticeable differences in bone turnover markers, suggesting that tissue age did not vary substantially among groups. Our prior work has associated ampicillin and neomycin-induced changes in the gut microbiome with the reduced capacity of the gut microbiome to synthesize vitamin K, decreased levels of vitamin K in the body, and reduced concentrations of the vitamin K dependent protein osteocalcin in bone matrix (Guss et al., 2017; Guss et al., 2019). The changes in the composition of the gut microbiota in the current study are consistent with those observed in our prior work, although we did not directly assess vitamin K. Although our findings indicate clear differences in the composition of the gut microbiome associated with antibiotic dosing, further study is required to understand the link between the observed changes in the gut microbiome and tissue mechanical properties.

The current study was not designed to evaluate bone as the primary endpoint, which created some limitations for our analysis and interpretation. The study was restricted to male retired breeder mice and lacked a baseline group to establish the bone phenotypes before alterations to the diet/gut microbiome. Furthermore, the current study uses special diets (LG and HG diets) rather than a more common mouse chow. The fact that the difference in bone tissue strength caused by the manipulation of the microbiome is similar to our prior findings using a standard mouse chow (Guss et al., 2017) makes it unlikely that the results are specific to the LG diet. These findings are consistent with the strong effect of antibiotic treatment on the metabolic environment of the gut, potentially modifying or overriding dietary effects (Cabral et al., 2019). Overall, these limitations do not influence the overall finding that changes in the gut microbiome late in life can influence bone tissue properties in a similar manner to what we have previously observed in younger animals.

Modifications in tissue strength can have profound effects on whole bone strength. Clinical studies using patient specific CT scans suggest that impairment of tissue strength can greatly increase bone fragility; a 20% reduction in whole bone strength can, in some cases, double the probability of experiencing hip fracture (Castaneda et al., 2020; Kopperdahl et al., 2014). Current pharmaceutical interventions for osteoporosis act primarily by altering bone *quantity*, and an intervention that directly improves bone tissue *quality* would be novel. Our findings show that bone tissue material properties can be impaired by an antibioticinduced alteration in the gut microbiome. It remains to be determined if modifications to the gut microbiome late in life can improve bone tissue material properties.

Transparency document

The Transparency document associated with this article can be found



Fig. 1. Alterations to the gut microbiota caused reductions in bone strength that could not be explained by geometry. (A) A multiple linear regression model of whole bone strength against section modulus with the R-squared value depicting the overall accuracy of the model using the two linear regressions (as opposed to one). (B) Whole bone strength measured as maximum moment from three-point bending. (C) Cross-sectional area of the femoral diaphysis. (D) Bacterial diversity decreased in Amp + Neo mice via Shannon diversity. (E) Beta diversity of the gut microbiota is shown using principal coordinate analysis of the unweighted UniFrac distances. (F) The gut microbial taxa at a phylum and family level of animals at 22 months is shown. Each column represents the gut microbiota composition of a single mouse. Groups: High Glycemic (HG), Low Glycemic (LG), and Low Glycemic with altered gut microbiota (LG + Amp + Neo). Sample size is n = 11 (HG), n = 11 (LG), n = 8 (LG Amp + Neo) for (A-B); n = 11 (HG), n = 13 (LG), n = 9 (LG Amp + Neo) for (C); n = 10 for (D–F).

Table 1

Markers from plasma analysis, body mass, diaphyseal geometry and whole bone properties are shown (mean ± SD with the range in parentheses).

		High glycemic (HG)	Low glycemic (LG)	Low glycemic Amp + Neo	One-way ANOVA <i>p</i> -value
Whole body	P1NP (ng/mL)	12.04 ± 2.54	10.98 ± 1.28	13.23 ± 2.31	0.08
	n	(7.52–17.37) 11	(8.20–12.98) 12	(9.22–17.76) 9	
	CTX (ng/mL)	11.46 ± 2.53	11.70 ± 6.00	11.76 ± 2.01	0.98
	n	(7.90-15.18)	(3.86–28.00)	(8.40-15.79)	
		11	13	9	
	Body mass (g)	43.5 ± 4.3	$36.8 \pm 3.5^*$	39.8 ± 5.3	0.005
	n	(35.2–49.0)	(30.6-44.0)	(35.5–50.5)	
		11	13	9	
Femoral diaphysis	Cortical thickness (mm)	0.24 ± 0.02	$0.19\pm0.01^{\ast}$	$0.20\pm0.03^{\ast}$	0.00005
	n	(0.20-0.27)	(0.16-0.22)	(0.14–0.24)	
		11	13	9	
	Cross-sectional area (mm ²)	1.02 ± 0.12	$0.81\pm0.09^{\ast}$	$0.82\pm0.11^{*}$	0.0002
	n	(0.89 - 1.20)	(0.61–0.99)	(0.60–1.00)	
		11	13	9	
	Moment of inertia (mm ⁴)	0.22 ± 0.04	$0.17\pm0.02^{*}$	$0.17 \pm 0.03^{*}$	0.0008
	n	(0.18 - 0.28)	(0.11–0.20)	(0.13–0.22)	
	2	11	13	9	
	Section modulus (mm ³)	0.30 ± 0.04	$0.21\pm0.03^*$	$0.24 \pm 0.04^{*}$	0.00005
	n	(0.23–0.37)	(0.15–0.31)	(0.18–0.30)	
		11	13	9	
Whole bone	Length (mm)	15.93 ± 0.37	16.20 ± 0.30	$16.47 \pm 0.21*$	0.004
	n	(15.33–16.65)	(15.56–16.70)	(16.17–16.93)	
		11	11	8	0.0007
	Peak Bending Moment (Nmm)	34.97 ± 5.62	27.57 ± 5.39*	$24.93 \pm 2.55^{*}$	0.0006
	n	(24.05–44.96)	(19.24–35.68)	(21.35–30.39)	
	Devidence of (General)	11	11	8	0.00
	Bending stimess (N/mm)	8/.00 ± 15.0/	75.71 ± 19.84	$63.46 \pm 14.56^{\circ}$	0.03
	n	(5/.5/-106.33)	(49.75–115.30)	(44.12-95.70)	
Tissue level	Illimate stress (whole have strength (section modulus)	110.00 10.70	11	8 102 70 ± 12 77 [#]	0.01
	(CDa)	(75,72,126,50)	(97.07.159.54)	103.79 ± 12.77	0.01
	(GPA)II	(/5./2-130.39)	(87.97-138.30)	(87.81-127.11)	
	Flastic modulus (GPa)	481 ± 0.82	5.46 ± 1.35	435 ± 0.82	0.48
	n	(3.92 - 6.04)	(3.69_8.18)	(3 25-5 87)	0.10
		11	11	8	
		**	**	0	

* p < 0.05 compared to high glycemic.

[#] p < 0.05 compared to low glycemic.

in online version.

CRediT authorship contribution statement

Macy Castaneda: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. Kelsey M. Smith: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. Jacob C. Nixon: Formal analysis. Christopher J. Hernandez: Conceptualization, Supervision, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. Sheldon Rowan: Conceptualization, Supervision, Funding acquisition, Resources, Writing – original draft, Writing – review & editing.

Declaration of competing interest

All authors state that they have no conflicts of interest.

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