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HBM MICE HAVE ALTERED BONE MATRIX COMPOSITION AND IMPROVED MATERIAL TOUGHNESS

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Abstract

The G171V mutation in the low density lipoprotein receptor-related protein 5 (LRP5) leads to a high bone mass (HBM) phenotype. Studies using HBM transgenic mouse models have consistently found increased bone mass and whole-bone strength, but little attention has been paid to the composition of the bone matrix. The current study sought to determine if the cortical bone matrix composition differs in HBM and wild-type mice and to determine how much of the variance in bone material properties is explained by variance in matrix composition. Consistent with previous studies, HBM mice had greater cortical area, moment of inertia, ultimate force, bending stiffness, and energy to failure than wild-type animals. The increased energy to failure was primarily caused by a large increase in post-yield behavior, with no difference in pre-yield behavior. The HBM mice had increased mineral-to-matrix and collagen cross-link ratios, and decreased crystallinity, carbonate, and acid phosphate substitution as measured by Fourier transform infrared microspectroscopy, but no differences in crystal length, intra-fibular strains, and mineral spacing compared to wild-type controls, as measured by X-ray scattering. The largest between genotype difference in material properties was a 2-fold increase in the modulus of toughness in HBM mice. Step-wise regression analyses showed that the specific matrix compositional parameters most closely associated with material properties varied between the wild-type and HBM genotypes. Although the mechanisms controlling the paradoxical combination

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of more mineralized yet tougher bone in HBM mice remain to be fully explained, the findings suggest that LRP5 represents a target to not only build bone mass, but also to improve bone quality.

Keywords

HBM; LRP5; Matrix Composition; Mineralization; Collagen Cross-linking; Toughness

INTRODUCTION

Wnt signaling is a critical mediator of bone mass [1]. The link between Wnt signaling and bone was established when mutations in the Wnt co-receptor low-density lipoprotein receptor-related protein 5 (LRP5) were identified as the cause for the low bone mass disease osteoporosis pseudoglioma syndrome (OPPG) [2]. Later, gain of function mutations in LRP5 were identified as the cause for high bone mass (HBM) phenotypes [3, 4]. HBM mice, recapitulating a specific glycine-to-valine amino acid substitution at position 171 (G171V) in LRP5 [5] verified the importance of the LRP5 receptor for the maintenance of bone mass [5, 6]. Ultimately, these studies have led to promising clinical trials for osteoporosis with antibodies to sclerostin, a soluble antagonist to Wnt signaling that interacts directly with LRP5 [7–10].

Although it is clear that targeting the Wnt pathway represents a means to increase bone mass, it is unclear what role Wnt has on bone quality. Bone quality describes the factors that contribute to bone strength apart from bone quantity and is measured at length-scales varying from macroscopic to molecular [11]. The composition of the bone matrix is now recognized as an important contributor to bone quality and whole-bone mechanical behavior [12]. Alterations to bone matrix composition due to genetic mutations, such as osteogenesis imperfecta [13–17], pharmacological treatments for osteoporosis, such as sodium fluoride [18], or osteoporosis itself [19, 20] provide examples of the detrimental effects that aberrant matrix composition can have on bone strength. Direct measurements of HBM bone tissue have shown increased hardness [21] and assessment of material properties based on whole-bone mechanical testing combined with bone geometry suggests the formation of stronger bones at both the structural and material levels [6, 21]. Although one study referred to unpublished data on altered matrix composition in HBM mice [21], there has not been a published study investigating the composition of bone matrix in these mice.

Bone derives its fracture resistance from an ability to dissipate energy during loading, a parameter referred to as toughness. Bone loses toughness during aging [22, 23], which can lead to an increased risk of fracture at low stresses. Although previous studies have evaluated the strength of HBM bones [5, 6, 24], none have reported the mechanical behavior in the post-yield or plastic region, where most of bone's toughness is derived. Therefore, the goals of the current study were three-fold: (1) to compare the matrix composition of HBM bones to wild-type controls, (2) to evaluate the mechanical properties at both the whole-bone and derived material levels, and (3) to determine whether the variance in the material properties could be explained statistically by the variance in the compositional properties.

METHODS AND MATERIALS

Animals

The current study utilizes HBM transgenic mice carrying the human LRP cDNA with a single glycine-to-valine point mutation at amino acid 171 in the LRP5 protein (G171V) [3]. The transgene is under the control of the 3.6kb Col I promoter. Female and male HBM mice on a C57Bl/6J background [5] were bred and aged to 25 weeks of age, an age corresponding with peak bone mass in this strain [25]. Non-transgenic age- and gender-matched wild-type C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Right and left femurs were dissected free of soft tissues following euthanasia by CO₂ and cervical dislocation. Right femurs were fixed in 70% ethanol, while the left femurs were immediately wrapped in phosphate buffered saline soaked gauze and frozen at -20°C.

Micro-Computed Tomography (μCT)

Intact right femurs were scanned longitudinally using 55 kVp and 145 μA with an integration time of 300 ms and 10 μm isotropic voxel size (Scanco μCT 40, Brüttisellen, Switzerland). Cortical bone cross-sectional geometry was assessed using a 1 mm segment of the femoral midshaft to determine cortical area (mm²), total area (mm²), medullary area (mm²), cortical thickness (mm), the polar moment of inertia (mm⁴), and the moment of inertia in the medial-lateral plane (mm⁴). All μCT parameters are reported using conventional nomenclature [26]. A total of 9 female and 9 male wild-type and 16 female and 9 male HBM mice were used to obtain cortical geometry parameters.

Fourier Transform Infrared Imaging (FTIRI)

After microCT scanning, the right femurs were dehydrated in a graded series of ethanol solutions and then embedded in a non-infiltrating epoxy resin (EpoThin, Buehler, Lake Bluff, IL), which was allowed to harden overnight. Transverse sections were cut (Isomet 5000, Buehler) from the mid-diaphysis. Cut sections were fixed to plastic slides and ground to a final thickness of ~200 μm using fine grit sand paper (BuehlerMet II, 600 grit, Buehler) under anhydrous conditions to prevent demineralization of the bone matrix [27]. Specimen surfaces were then polished to a mirror finish using a graded series of diamond suspensions to a final finish of 3 μm (Metadi, Buehler).

Matrix composition was assessed by Fourier-transform infrared imaging (FTIRI) at the National Synchrotron Light Source at Brookhaven National Laboratory. Bone spectra were collected in a specular reflectance configuration with a spectral range of 650 to 4000 cm⁻¹ and a spectral resolution of 8 cm⁻¹. Cortical spectra was collected at the U10B beamline using an infrared microscope (Hyperion 3000, Bruker, Billerica, MA) attached to the FITR spectrometer (Vertex 80v, Bruker) using the global source and 128×128 pixel focal plane array (FPA) detector. The pixel size was 21.6 × 21.6 μm and a total of 64 scans were averaged per region of interest. Cortical spectra were collected in the anterior and posterior quadrants spanning the transverse thickness of the cortical shell. In total an average area of 4*10⁶ μm² was averaged to determine the matrix composition.

Primary measurements included the mineral-to-matrix ratio, the collagen cross-link ratio, mineral crystallinity and the carbonate and acid phosphate substitutions. Each parameter was derived using previously published spectral analysis methods [28]. The mineral-to-matrix ratio was calculated using the phosphate peak (integrated at 900–1200 cm^{-1} , baseline correction at 900–1200 cm^{-1}) and the amide I peak (integrated at 1600–1700 cm^{-1} , baseline at 1300–1800 cm^{-1}). The collagen cross-link ratio was calculated as the ratio between 1661–1659 cm^{-1} / 1691–1689 cm^{-1} (baseline: 1300–1800 cm^{-1}). Crystallinity was measured using the stoichiometric / non-stoichiometric phosphate ratio (1037–1033 cm^{-1} / 1027–1023 cm^{-1} , baseline: 900–1200 cm^{-1}). The carbonate substitution was assessed by normalizing the carbonate peak (1414–1424 cm^{-1} , baseline: 1300–1800 cm^{-1}) by the phosphate peak and the acid phosphate substitution was assessed with the ratio of the peaks at 1127 cm^{-1} / 1096 cm^{-1} (baseline: 900–1200 cm^{-1}) [29]. A total of 9 female and 8 male wild-type and 13 female and 11 male HBM animals were averaged to determine bone matrix compositional parameters.

Three-Point Bending

Left femurs were thawed in phosphate buffered saline prior to mechanical testing. Femurs were loaded to failure in the anterior-posterior direction with a span length of 8 mm, a strain rate of 0.1 mm/s, and a data acquisition rate of 100 Hz (MTS Criterion™, MTS Systems Corporation, Eden Prairie, MN). A preload of ~1.5 N was applied to the bones to prevent shifting during testing. The resulting load-displacement curves were used to determine structural mechanical properties including, the bending stiffness (N/mm), yield force (N), yield displacement (mm), ultimate force (N), and ultimate displacement (mm). The energy to failure (mJ) and two subsets of this energy, namely, the energy to yield (mJ) and the post-yield energy (mJ), were determined by calculating the appropriate areas under the load displacement curve. A total of 9 female and 8 male wild-type and 15 female and 8 male HBM mice were tested in three-point bending.

Material properties were calculated based on the whole-bone 3-point testing data and the geometric properties from μCT scans, using previously derived equations [30] and assuming bilateral femoral symmetry. Specifically, Young's Modulus (MPa), yield stress (MPa), yield strain, post-yield strain, ultimate stress (MPa), ultimate strain, and modulus of toughness (MPa) were calculated and compared between groups. The sample size for the derived material properties was 9 female and 8 male wild-type and 15 female and 8 male HBM mice.

Wide and Small Angle X-ray Scattering (WAXS and SAXS)

WAXS and SAXS measurements were performed at the 1-ID beamline of the Advanced Photon Source (APS) at the Argonne National Laboratory (Argonne, IL). Left tibia were dehydrated in a graded series of ethanol solutions and left to air dry overnight. 48 total samples were mounted on a wheel which was rotated stepwise to bring each sample into the x-ray beam. This wheel included WAXS and SAXS standards described below.

Transmission-based measurements were made using a 71.676 keV monochromatic X-ray beam which passed through the mid-diaphysis of each tibia. The hydroxyapatite crystal size and average lattice spacing were derived from WAXS patterns, which were captured from

four GE-41RT area detectors arranged in a flower pattern around the transmitted beam. These WAXS detectors were set 2.759 m downstream of the specimen wheel. The inter-mineral spacing was derived from the SAXS patterns, which were captured by a Pixirad CdTe area detector set 6.6 m downstream of the specimen wheel.

WAXS and SAXS spectra were processed using a previously published protocol [31]. Briefly, the patterns were converted from radial to Cartesian coordinates and integrated over the azimuthal ranges of $0 \pm 20^\circ$ and $180 \pm 20^\circ$, representing the longitudinal and transverse sample directions, respectively. Standards for WAXS (LaB6 and CeO2) and SAXS (Si nano-grating) were first evaluated to accurately determine instrumental parameters including detector distances, beam positions and instrumental broadening contributions. For WAXS, hydroxyapatite diffraction peaks 002, 222 and 004 from these two directions were fit using a pseudo-Voigt function and peak centers were converted to d-spacings using Bragg's law. Mineral crystal sizes and microstrains were determined using the instrumentally-corrected 002 and 004 peak widths and the Williamson-Hall approximation [32]. The average mineral spacing between collagen fibrils (~67 nm) was determined from the position of the 3rd order peak in the SAXS patterns.

Statistical Analysis

As the current study represents the first study to characterize the matrix composition and many of the material properties in HBM mice, it was not possible to perform an a priori power analysis. Using previously published data on whole-bone mechanical properties, such as the ultimate load [5, 33] and the bending stiffness [33], it was determined that at least 6 animals per genotype were required to detect differences in these parameters (assuming a power of 0.8 and significance level of $p < 0.05$). Additional animals were added to increase the power in matrix compositional parameters. The effects of genotype and gender were assessed using a two-way analysis of variance (ANOVA). Significance was defined as a p-value less than 0.05. If the ANOVA was significant, post-hoc comparisons were performed using independent t-tests with Bonferroni correction ($p < 0.025$). The contribution of each cortical matrix composition parameter (mineral-to-matrix ratio, collagen crosslink ratio, crystallinity, carbonate and acid phosphate substitution, crystal length, and intra-fibular strain) to the derived material properties was determined with a step-wise regression analysis using data from 36 animals (16 wild-type and 20 HBM animals). Commercial software was used (SPSS version 19, Chicago, IL).

RESULTS

Consistent with previous reports, the HBM genotype was found to increase the total and cortical areas (Fig. 1, $p < 0.001$ for each, ANOVA). The increase in total area was smaller in females (6%) than in males (13%, $p < 0.001$ for each, t-test), accounting for the significant interaction term ($p = 0.047$, ANOVA). Cortical area increased by similar amounts in females (17%) and males (20%, $p < 0.001$ for each, t-test), and the interaction term was not significant ($p = 0.239$). There was no genotype effect for the medullary area (Fig. 1c), suggesting the cortical expansion in HBM mice primarily occurs in the periosteal compartment, leading to an increased cortical thickness (Fig. 1d, $p < 0.001$, ANOVA). This increase in cortical

thickness was consistent in both female and male HBM mice compared to wild-type controls (14% increase in both genders, $p < 0.001$, t-test). The polar moment of inertia (Fig. 1e) had a significant genotype effect ($p < 0.001$, ANOVA) and a significant interaction term ($p = 0.014$) due to a more dramatic increase in males (31%, $p < 0.001$, t-test) than in females (18%, $p = 0.002$). The moment of inertia about the medial-lateral axis (I_{M-L} , Fig 1f) was also found to be affected by genotype ($p = 0.003$, ANOVA), yet, the post-hoc analysis failed to reveal specific differences between gender-matched animals.

HBM mice had significantly altered bone matrix composition (Fig. 2). The mineral-to-matrix ratio was significantly elevated in HBM mice (Fig. 2a, $p < 0.001$, ANOVA). Specifically, there was a significantly increased ratio in females (13%, $p = 0.002$, t-test), but the increase in males was not significant (5%, $p = 0.045$, t-test). Male mice of both genotypes were found to have a reduced ratio compared to females, but this difference was only significant in the HBM animals ($p < 0.001$, t-test). Collagen cross-link ratio was elevated in HBM mice (Fig. 2b, $p = 0.018$, ANOVA), but gender-specific post-hoc comparisons between genotypes failed to reach significance. Mineral crystallinity was reduced in HBM animals (Fig. 2c, $p < 0.001$, ANOVA), but post-hoc analysis only found a significant genotype difference in males ($p = 0.008$, t-test). Carbonate substitution was decreased in HBM mice (Fig 2d, $p = 0.003$, ANOVA), but this difference was only significant in males ($p = 0.015$, t-test). Despite a significant gender effect in the two-way ANOVA, neither genotype-specific post-hoc comparison was significant for carbonate substitution. The acid phosphate substitution was significantly reduced in the HBM animals (Fig 2e, $p = 0.001$, ANOVA), however, the post-hoc comparisons failed to reach significance.

None of the WAXS and SAXS derived matrix measurements, the average mineral crystal size, longitudinal intra-fibular strain, and collagen d-spacing, were impacted by either genotype or gender. The average mineral size was measured at 556 ± 101 and 458 ± 64 Å for female and male wild-type mice, respectively, and 529 ± 110 and 501 ± 93 Å for female and male HBM mice. The longitudinal intra-fibular strain was 0.006 ± 0.0005 and 0.005 ± 0.0005 for female and male wild-type mice and 0.006 ± 0.0008 and 0.006 ± 0.007 for female and male HBM mice, respectively. Finally, the collagen d-spacing parameter was 6.25 ± 0.15 and 6.23 ± 0.19 for female and male wild-type mice and 6.24 ± 0.12 and 6.24 ± 0.08 for female and male HBM mice, respectively.

The HBM mutation increased the whole-bone mechanical properties of the femoral diaphysis (Fig. 3). HBM mice had elevated bending stiffness (Table 1, $p = 0.001$, ANOVA), ultimate force ($p < 0.001$), and ultimate displacement ($p = 0.005$). Post-hoc analyses revealed that, despite a 10% increase in both females and males, the gender-specific differences in bending stiffness failed to reach significance. Ultimate force was significantly elevated in both females (16%, $p = 0.002$, t-test) and males (22%, $p = 0.013$). Only female mice were found to have increased ultimate displacement (31%, $p = 0.009$), while a similar 31% increase in males failed to reach significance. The energy to failure was increased by the HBM mutation ($p < 0.001$, ANOVA), which was significant in both females (59%, $p = 0.003$, t-test) and males (75%, $p = 0.020$). When separated between the pre- and post-yield energy, the primary differences were seen in the post-yield region, where HBM animals had dramatically increased post-yield energy ($p < 0.001$, ANOVA) in both females (120%,

p=0.007, t-test) and males (143%, p=0.012). None of the whole-bone mechanical properties were impacted by gender.

Derived material properties were calculated using μ CT structural information for the contralateral femoral diaphysis and the whole-bone mechanical properties. The HBM genotype was associated with lower yield stress (Fig. 4b, p=0.022, ANOVA), higher post-yield strain (Fig. 4d, p<0.001), and increased modulus of toughness (Fig. 4f, p=0.007). Despite these significant genotype effects, only the post-yield strain in female mice reached significance in the gender-specific post-hoc tests (103%, p=0.013, t-test). The post-yield strain was also increased in male HBM mice (130%), but this difference failed to reach significance (p=0.028, t-test). Ultimate stress, yield strain, and the Young's modulus were not different between genotypes. Gender effects existed in the ultimate stress (Fig. 4a, p<0.001, ANOVA), yield stress (Fig. 4b, p<0.001), and the Young's Modulus (Fig. 4e, p<0.001), all of which were associated with lower values in the male mice of both genotypes.

Step-wise regression analyses were performed to determine the contribution of various cortical matrix composition parameters to the derived material properties (Table 2). The correlation was performed using specimens of both genotypes (Combined Specimens) and separately for animals of each genotype to determine whether the matrix contribution to the derived material properties differed due to the HBM mutation. The correlations found in the combined specimen population were relatively weak, with no more than 30% of the total variance in each derived material property explained by the matrix composition. When split by genotype, the correlations were much stronger and the data revealed differences in the contribution of bone matrix composition to material properties between wild-type and HBM mice. In wild-type animals, the correlation analysis found that collagen cross-link ratio and collagen d-spacing parameter contributed to both the yield stress and the Young's modulus. In both cases the collagen cross-link ratio was negatively correlated, while the d-spacing was positively correlated with the material properties. In the HBM animals, the mineral crystallinity was positively correlated with the yield stress. Further, the pre-yield strain was found to be positively correlated with the carbonate substitution and the crystal size, while the post-yield strain was negatively associated with the collagen d-spacing. Correlation matrices are reported in the supplemental information and include all correlations between dependent and independent variables (Supplemental Table 1) and the association between all independent variables (Supplemental Table 2).

DISCUSSION

The current study suggests that in addition to increasing bone mass, the G171V mutation in LRP5 is associated with mechanically favorable alterations to the composition and plastic behavior of bone tissue. HBM mice were found to have significantly altered matrix composition in each of the five parameters measured. At the structural level, HBM mice had significantly stronger bones. At the material level, the differences between wild-type and HBM were primarily limited to the post-yield region, where HBM mice were found to have significantly greater toughness.

Previous studies of the HBM mouse model have consistently reported increased bone mass in both trabecular and cortical compartments. This increased bone mass has presented as increased trabecular bone volume fraction (BV/TV) [5, 33, 34] and increased cortical area [5, 33]. Similarly, previous studies of the femoral diaphysis of cortical bone have found increased whole-bone strength [5, 33]. Our current study confirms these findings, further demonstrating the anabolic capacity of the targeted disruption of LRP5.

Previous research in which certain material properties of HBM femurs were derived using whole bone mechanical and cross-sectional geometric data found increased ultimate load at the whole-bone level, but no difference in ultimate stress at the material level (i.e., bone material strength) between HBM mice and wild-type controls [5, 33], consistent with our findings. Cui et al. [24] previously reported increased energy to failure in HBM mice, a finding that is confirmed in the current study. Our results show for the first time that this increased energy to failure is primarily due to an increase in the post-yield region, indicating that HBM bone matrix has a greater ability to deform plastically. This increase in post-yield energy was due to an increase in post-yield strain in the absence of a difference in the ultimate stress. Additionally, the HBM mice were found to have an increased modulus of toughness, a finding that to our knowledge has not previously been reported in these animals. The A214V missense mutation in LRP5 has also been reported to increase the energy to failure in mice carrying a type I collagen mutation causing osteogenesis imperfecta [35].

The results presented in the current study represent the first evaluation of bone matrix composition in HBM mice, and are consistent with previous characterization of HBM tissues. For instance, the increased mineral-to-matrix ratio is consistent with the previously reported increase in ash density of HBM bones [33]. The increase in mineral-to matrix ratio and the increased collagen cross-link ratio are likely the cause for the increased tissue-level hardness previously reported [21]. Altered crystallinity, carbonate and acid phosphate substitution represent novel findings and further point to a role for LRP5 in the control of bone matrix development.

Toughness relates to bone's ability to resist fracture and is derived from several mechanisms at various hierarchical levels [36], including matrix composition [37]. Our data raise some new questions that will require further study. For instance, previous studies have associated reduced toughness with increasing mineral-to-matrix ratios [21, 38, 39], while we found increased modulus of toughness and increased mineral-to-matrix ratio in animals with HBM mutations. Similarly, an increase in the enzymatic collagen cross-link ratio is thought to reduce the toughness of bone [40–43], while in the current study HBM mice presented with increased toughness despite the increased collagen cross-link ratio. The reduced crystallinity we observed in the HBM femurs is consistent with an increase in toughness [38, 44]. Crystallinity is a composite measure of both crystalline size and perfection [45], therefore, we combined FTIRM with WAXS measurements to determine whether crystal size changes were driving the crystallinity differences noted between genotypes. There were no detectable differences in the crystal size, implying that additional current unknown matrix-level mechanisms must be contributing to the unique material behavior measured in HBM mice. Further, results from the step-wise regression analysis point to distinct differences between

the contribution of matrix composition to material properties in wild-type and HBM mice. Potential mechanisms contributing to the increased toughness observed in our study could include micro-level porosity, the arrangement or orientation of either the organic or mineral matrices, the interaction between the organic and mineral matrices, or the amount of bound water within the matrix [36].

Our findings are consistent with previous studies demonstrating that tissue mineral density is a means by which more slender bones are able to maintain bone strength [46, 47]. Specifically, female mice of both genotypes had smaller cortices, and yet the structural strength was unaffected by gender. Thus, it is likely the increased matrix-to mineral ratio in the female mice leads to stronger and stiffer material properties, evident in the increased Young's Modulus, which compensates for their more slender bones.

The current study is limited by the use of non-littermates as wild-type controls. The HBM animals studied were from a colony homozygous for the HBM genotype and, therefore, it was not possible to obtain wild type littermates. However, the use of animals of the same genetic background and the similarity of several of our findings on the primary endpoints with previously published reports comparing the same HBM mice with littermate wild type controls suggests that the reported results are attributed to the G171V mutation in LRP5. Specific similarities to previously published studies using littermate controls are described above but include, HBM-induced increases in cortical thickness [5, 33], polar moment of inertia [33], ultimate load [5, 33], stiffness [33], and energy to failure [24], with no genotypic effects in the ultimate stress [5, 33] and Young's Modulus [33]. In addition to the similarities mentioned above, Lopez Franco et al. [21] reported an increased collagen cross-link ratio in HBM animals in unpublished preliminary data, further corroborating our findings.

The current results suggest that in addition to its known role in controlling bone quantity, the Wnt signaling pathway may contribute to the quality of the matrix. This is a relatively unexplored area as most attention has been paid to bone mass [4, 48–52] and those few studies which have looked at matrix composition following modulation of this pathway have found somewhat disparate results. In the present study, the HBM animals (G171V LRP5 mutation) were found to have increased mineral-to-matrix ratio, which is distinctly different from a previously reported reduction in this ratio in sclerostin deficient mice [53]. While in our previous work we have found no effect of sclerostin antibody on tissue mineral density and mineral-to-matrix ratio in rats and nonhuman primates, except that the antibody seemed to be associated with accelerated mineralization in Haversian remodeling [28]. Although these studies used different techniques to measure matrix composition (FTIR vs. Raman spectroscopy), this data may suggest that the effect on the matrix composition depends upon where in the Wnt signaling pathway the perturbation is introduced and warrants further study within the same species and strain.

The current study represents the first direct evaluation of the bone matrix composition of HBM mice. The findings show that the HBM genotype is associated with significantly altered bone matrix, and significantly increased ability of the whole bone and matrix to deform plastically prior to failure. Despite matrix changes generally associated with tissue

embrittlement, the HBM mice were found to have a dramatic increase in the modulus of toughness. More research is needed to determine how HBM bones can have increased mineral-to-matrix and collagen cross-link ratios coincident with increased tissue toughness. However, the findings suggest that the Wnt signaling pathway represents a target to not only build greater bone quantity, but to also improve bone quality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

Cortical geometry at the femoral midshaft. a) Total area (mm²), b) cortical area (mm²), c) medullary area (mm²), d) cortical thickness (mm), e) the polar moment of inertia (mm⁴), and f) the area moment of inertia in the medial-lateral plane (mm⁴). The data are presented as the mean with error bars showing the standard deviation. Results from the two-way ANOVAs are presented within the legends. Significant post-hoc differences ($p < 0.025$) are reported above the data, with bars highlighting significant genotype differences between animals of the same gender and brackets highlighting significant gender differences between animals of the same genotype. * indicates a $p < 0.025$, ** $p < 0.01$, and *** $p < 0.001$.

Figure 2:

Cortical matrix composition at the femoral midshaft. a) mineral-to-matrix ratio (mineralization), b) collagen cross-link ratio, c) crystallinity, d) carbonate substitution, and e) acid phosphate substitution. The data are presented as the mean with error bars showing the standard deviation. Results from the two-way ANOVAs are presented within the legends. Significant post-hoc differences ($p < 0.025$) are reported above the data, bars representing significant genotype differences between animals of the same gender and brackets representing significant gender differences between animals of the same genotype. * indicates a $p < 0.025$, ** $p < 0.01$, and *** $p < 0.001$.

Figure 3:
Representative load-displacement curves for wild-type (gray) and HBM (black) mice, demonstrating the increased structural properties in HBM mice.

Figure 4:

Derived material properties determined from whole bone mechanical properties and cortical geometry. a) Young's Modulus (MPa), b) yield stress (MPa), c) yield strain, d) post-yield strain, e) ultimate stress (MPa), and f) modulus of toughness (MPa). The data are presented as the mean with error bars showing the standard deviation. Results from the two-way ANOVAs are presented within the legends. Significant post-hoc differences ($p < 0.025$) are reported above the data, bars representing significant genotype differences between animals of the same gender and brackets representing significant gender differences between animals of the same genotype. * indicates a $p < 0.025$, ** $p < 0.01$, and *** $p < 0.001$.

TABLE 1:**WHOLE-BONE MECHANICAL PROPERTIES**

Structural Property	Female WT	Female HBM	Male WT	Male HBM	Genotype Effect	Gender Effect	Interaction Term
Bending Stiffness (N/mm)	110.47 (11.23)	127.38 (17.18)	102.82 (11.11)	121.15 (18.52)	0.001	0.162	0.162
Yield Force (N)	14.21 (1.91)	14.22 (1.58)	13.71 (0.98)	14.18 (3.40)	0.722	0.692	0.732
Yield Displacement (mm)	0.11 (0.02)	0.11 (0.03)	0.12 (0.01)	0.10 (0.02)	0.073	0.927	0.363
Ultimate Force (N)	16.63 (1.10)	19.26 (2.61) ^a	16.18 (1.96)	19.72 (3.03) ^a	<0.001	0.997	0.541
Ultimate Displacement (mm)	0.18 (0.02)	0.23 (0.07) ^a	0.20 (0.04)	0.26 (0.09)	0.005	0.329	0.874
Energy to Failure (mJ)	1.92 (0.42)	3.05 (1.18) ^a	2.08 (0.67)	3.63 (1.55) ^a	<0.001	0.290	0.535
Energy to Yield (mJ)	0.93 (0.24)	0.86 (0.29)	0.94 (0.12)	0.87 (0.32)	0.411	0.875	0.985
Post-Yield Energy to Failure (mJ)	1.00 (0.56)	2.19 (1.20) ^a	1.14 (0.62)	2.76 (1.49) ^a	<0.001	0.308	0.538

Data reported as the means (standard deviation).

^a. different from WT mice of the same sex (p<0.025).

TABLE 2:
STEP-WISE REGRESSION ANALYSIS SHOWING THE CONTRIBUTIONS OF MATRIX COMPOSITION TO THE DERIVED MATERIAL PROPERTIES

Dependent Variable	Independent Variable Added	Adjusted R ² Value	Significance of Change (p-value)	Model Significance (p-value)
Combined Specimens				
Yield Stress	Collagen Cross-Link Ratio	0.308	0.002	0.002
Pre-Yield Strain	Carbonate Substitution	0.241	0.007	0.007
Post-Yield Strain	Acid Phosphate Substitution	0.280	0.004	0.013
Modulus of Toughness	Mineral-to-Matrix Ratio	0.233	0.014	0.014
Wild-Type Animals				
Yield Stress	Collagen Cross-Link Ratio	0.526	0.011	0.011
	Collagen d-spacing	0.744	0.031	0.004
Young's Modulus	Collagen Cross-Link Ratio	0.373	0.027	0.027
	Collagen d-spacing	0.723	0.021	0.006
HBM Animals				
Yield Stress	Crystallinity	0.370	0.012	0.012
Pre-Yield Strain	Carbonate Substitution	0.355	0.015	0.015
	Crystal Size	0.645	0.007	0.001
Post-Yield Strain	Collagen d-spacing	0.334	0.018	0.018

Note: only matrix compositional parameters that significantly improved the step-wise regression fit were included in the current table.