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Gender-dependence of bone structure and properties in adult osteogenesis imperfecta murine model

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Abstract

Osteogenesis imperfecta (OI) is a dominant skeletal disorder characterized by bone fragility and deformities. Though the *oim* mouse model has been the most widely studied of the OI models, it has only recently been suggested to exhibit gender-dependent differences in bone mineralization. To characterize the impact of gender on the morphometry/ultra-structure, mechanical properties, and biochemical composition of *oim* bone on the congenic C57BL/J6 background, 4-month-old *oim/oim*, *+oim*, and wild-type (*wt*) female and male tibiae were evaluated using micro-computed tomography, three-point bending, and Raman spectroscopy. Dramatic gender differences were evident in both cortical and trabecular bone morphological and geometric parameters. Male mice had inherently more bone and increased moment of inertia than genotype-matched female counterparts with corresponding increases in bone biomechanical strength. The primary influence of gender was structure/geometry in bone growth and mechanical properties, whereas the mineral/matrix composition, hydroxyproline content of bone were influenced primarily by the *oim* collagen mutation. This study provides evidence of the importance of gender in the evaluation and interpretation of potential therapeutic strategies when using mouse models of OI.

Keywords

Bone; Osteogenesis Imperfecta; *oim/oim*; Mouse; Gender

Introduction

Osteogenesis imperfecta (OI) is a hereditary connective tissue disorder primarily characterized by bone fragility and deformities. It affects 1 in 10,000–60,000 individuals in the United States³⁴. The clinical severity varies widely, ranging from extreme skeletal fragility and prenatal lethality to milder forms with minimal fractures^{11, 14}. Approximately 85% of patients with a clinical diagnoses of osteogenesis imperfecta possess gene defects in one of the two type I collagen genes (COL1A1 and COL1A2), which result in abnormal bone formation and high bone turnover^{11, 14, 22}. Currently, there is no cure for OI and

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Conflict of Interest Disclosure

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treatment strategies target improvement of patients' symptoms primarily through pharmacological approaches to increase bone mineral density with variable outcomes⁴⁵. Recent work with animal models has brought into question the validity of increasing bone density as a measure of bone quality⁴⁴.

Investigations using mouse models of OI have been performed since the 1980s^{14, 18}. The osteogenesis imperfecta murine (*oim*) mouse model, the most extensively studied, carries a functional null mutation in its COL1A2 gene⁹. Homozygous *oim/oim* mice synthesize only homotrimeric type I collagen, $\alpha 1(I)_3$, and phenotypically model human moderately severe OI type III [Sillence classification⁴²] with frequent fractures, small size, osteopenia, and bone deformities^{4, 9, 24, 39}. Heterozygote (*+oim*) mice represent a milder form of OI^{4, 9, 24, 39}. Studies of *oim/oim* mice demonstrate altered mineral/matrix composition^{5, 33} and reduced bone mechanical properties^{4, 24, 25, 28}, including increased brittleness, reduced stiffness, and decreased load to failure compared with *wt* bone. The deficient material properties of demineralized bone matrix of the *oim/oim* mice are unable to account for the severe fragility²⁷.

It is well established that genetic and non-genetic (diet, exercise, and gender) parameters impact bone quality and strength, yet, very few studies of the *oim* mouse or other OI mouse models address any non-genetic factors⁸. Gender differences in bone have been associated with specific sex steroids and growth hormone levels in both animal and human studies^{19, 23}. Some studies with the *oim* mouse suggest the bone phenotype is gender independent^{16, 25, 28, 31}, and many have used bones from mixed genders to maximize sample size^{4, 6, 24, 45}. Although a few studies have indicated use of bones from approximately equal numbers of males and females of each genotype²⁷, the majority of studies appear not controlling for the gender of the mouse at all. Yet there is growing evidence that *oim* male and female mouse bones are not equivalent and may not respond equally to specific treatments. *Oim* mice have been used in a number of studies to assess the effect of bisphosphonates^{3, 6, 13, 25, 28, 35}, RANKL inhibition^{1, 2, 12}, and stem cell therapy^{16, 26, 45} to decrease bone pain, increase bone strength, and reduce fracture incidence. Vanleene et al.⁴⁵ reported that human fetal bone stem/stromal cell transplantation affected male and female *oim/oim* mice differently. Evans et al.¹³ found that higher dose alendronate treatments reduced bone length in males, but not in females. In a bisphosphonate *oim* mouse treatment study, Rao et al.³⁵ analyzed males and females separately due to gender differences in dose-response. These findings bring to question many studies in which the gender of the *oim/oim*, *+oim* and wildtype mice was not considered in the analyses and interpretation of the results.

In the following study we systematically evaluate male and female *oim/oim*, *+oim* and wildtype mouse tibiae and demonstrate at multi-scale levels the differential impact of gender on the phenotypic severity of the type I collagen defect and provide further insight into the pathogenesis of OI and baseline values for potential therapeutic targets. In addition, we demonstrate by using a single tibia from each mouse that we can quantitatively investigate the ultra-structural geometry, biochemical composition, and the biomechanical and material properties by μ CT, Raman spectroscopy and three-point bending, thus allowing for direct correlation of multi-scale parameters of bone structure and function.

Materials and Methods

Animals

The *oim* mutation is maintained on a C57BL/6J congenic background and heterozygous (*+oim*) mice were bred to obtain the necessary wildtype (*wt*), *+oim* and *oim/oim* mice in an

AAALAC accredited facility at the University of Missouri-Columbia⁷. All experimental protocols were approved by Animal Care and Use Committee at University of Missouri^{7,33}.

The mice of *wt* (8 females, 8 males), *+oim* (8 females, 8 males), and *oim/oim* (8 females, 8 males) were sacrificed at four months of age, the age of peak bone mass. Left and right tibiae were harvested, and cleaned of soft tissue. The fibula was carefully removed from each bone using a scalpel. The tibia samples were wrapped in phosphate-buffered saline (PBS)-soaked gauze and stored in a -20°C freezer until use. The desired number of animals for each group ($N=8$) was compromised due to bones with evident fracture callus formation which were excluded from the study. In addition, due to the fragility of the bones, some were accidentally fractured during handling.

Experimental Design and Procedures

The whole bone properties including structural morphology, biomechanical, and chemical composition were evaluated using left tibial bones. The cortical and trabecular bone morphological parameters were determined using micro-computed tomography (μCT). Following the μCT analysis, the same bones were loaded to failure using three-point bending to acquire the mechanical properties, which was then followed by analysis of the chemical and matrix composition of the cortical bone using Raman micro-spectroscopy. Right tibiae were used to evaluate the collagen content indirectly by hydroxyproline quantitation.

μCT Evaluation

Left tibiae were thawed and brought to room temperature prior to testing. A viva CT 40 microCT (Scanco Medical AG, Bassersdorf, Switzerland) was used to assess cortical bone and trabecular bone properties. The vivaCT 40 has a rotating gantry, and a microfocus X-ray source (45–70kVp) where the detector rotates around the sample. All scans were performed with a source voltage of 55keV, current of 145 μA , and 10 μm voxel resolution. A hydroxyapatite (HA) calibration was performed to convert μCT values to a mineral-equivalent value, normally in milligrams per cubic centimeter (mg/ccm) for each voxel.

The tibiae were placed in 70% ethanol in Falcon 15 mL polypropylene conical tubes (Thermo Fisher Scientific Inc, Hudson, NH)) with low density foam to position the specimens firmly. The specimens were aligned with the vertical axis of the scanner. Three-dimensional images were reconstructed and each data set was arranged as a series of 10 μm -thick slices oriented along the long axis of the tibia. Cortical bone was analyzed at midshaft starting 1 mm above fibula-tibia junction. Then a 0.5 mm region was selected to get the average cortical thickness and density. Proximal metaphysic trabecular bone was analyzed 1 mm below the growth plate. The morphometric variables of trabecular bone include bone volume fraction (BV/TV), mean/density (BV), trabecular thickness (Tb. Th), trabecular bone number (Tb.N), trabecular separation (Tb.Sp), connectivity density (Conn.D) which was developed to characterize the redundancy of the trabecular connections, and the structure model index (SMI) which was designed to be 0 for perfect plate, 3 for perfect rod, and 4 for perfect spheres¹⁷. A global threshold 253 (μCT gray value) was used for all samples to extract the bone phase.

Mechanical Testing

Following the μCT measurements, the tibia bone structural and material mechanical behavior was measured via three-point bending to failure. All the tests were performed using an ElectroForce 3220 test instrument (Bose Corporation, Eden Prairie, MN, USA) with a 50 lb (225N) load cell. The span between the outer loading points was fixed at 11.2 mm⁴¹. For *oim/oim* mice, the tibia chosen for testing was confirmed to contain no fractures or

deformations²⁷. The tibia was positioned horizontally with the anterior surface upwards and the load force was directed vertically to the midshaft of the tibia. Each tibia was loaded at a constant speed of 0.2 mm/s until failure. The force-displacement data was collected every 0.005 s.

The measured data was converted to a load-displacement curve in Microsoft Excel 2007 software. The structural (whole bone) properties and material (tissue) property of bone were calculated. The ultimate force was defined as the maximum load the bone sustained during testing; the stiffness (k) was calculated as the slope of the linear region of the load – displacement curve.

The moment of inertia, I , is a measurement of an object's resistance to bending based on its geometry, which was calculated using a customized Matlab script. The script first identified the neutral axis of a cross-sectional microCT image of the tibia midpoint. The moment of inertia was then calculated as the sum of the squared distances of each pixel from the neutral axis. The Young's modulus E (GPa) was calculated as:

$$E = \frac{kL^3}{48I}$$

Where k is the stiffness (N/mm), L is the span of the lower supports (mm), I is the moment of inertia⁴¹.

Raman Spectroscopy

Raman spectra were acquired from cortical bone cross-section for each group using a LabRam HR 800 Raman spectrometer (Horiba JobinYvon, Edison, NJ). The Raman spectra were excited by a helium-neon laser (633 nm) with a 20 X water immersion objective in order to get the general or averaged bone biochemical composition. Raman spectra were recorded from 700 to 1800 cm^{-1} range, allowing acquisition of peaks attributed to the bone minerals and proteins. Each spectrum was performed with 60 s exposure time and 2 accumulations. Each tibia was examined at 4 points from anterior, posterior, medial and lateral cross section and data were averaged for each sample. Spectra analyses were performed to measure the area of hydroxyapatite $\nu_1 \text{PO}_4^{3-}$ peak (960 cm^{-1}), type B $\nu_1 \text{CO}_3^{2-}$ peak (1070 cm^{-1}), and matrix CH band (1450 cm^{-1}). The ratios phosphate/CH and carbonate/CH, which increase with bone mineral content, were assessed. The ratio of phosphate/carbonate, which decreases with maturation of mineralized matrix, was calculated.

Hydroxyproline Content

Hydroxyproline, an amino acid unique to collagen, was quantified as a relative measure of collagen content⁴³. Right tibiae were lyophilized for three hours and then cut in half. Each half was weighed (Model AG245, Mettler Toledo, Columbus, OH) and measured. The proximal half was used for the determination of hydroxyproline content/mg bone as described by Stegemann and Stalder⁴³.

Statistical Analysis

Results are presented as means $\pm$ SD. The interactions between gender and genotype were analyzed using two-way ANOVA. If there is no interaction, the differences between the female and the male were evaluated by two-tailed Student's t -test. The differences among genotypes of wt controls, +/oim, and oim/oim mice were evaluated with Tukey's post-hoc

test. Statistical analysis was performed using SAS v9.3 (SAS Institute Inc., Cary, NC, USA). Differences with $P < 0.05$ were considered to be statistically significant.

Results

μ CT Evaluation

Representative two-dimensional cortical bone and three-dimensional reconstructions of the trabecular bone of the proximal tibiae of *wt*, *+oim*, and *oim/oim* female and male mouse tibiae were generated by μ CT image analyses (Fig. 1). In male proximal tibiae the distinct plate-like structure of the trabecular bone could easily be seen and the connecting rods were well maintained in comparison to female trabecular bone. However, within each gender from *wt* to *+oim* and to *oim/oim* tibiae, the plate-like structure of the trabeculi was progressively disrupted, and many of the connecting rods appeared absent.

By μ CT analyses, the cortical thickness of male *wt* tibiae was significantly greater by 8.5% compared to female *wt* tibiae. Female *oim/oim* mice showed markedly decreased cortical thickness, by 9.4% and 9.1%, compared to *wt* mice and *+oim* mice, respectively (Fig. 2). The cortical thickness in male *oim/oim* mice was also decreased by 12.4% compared to male *wt* mice. Although there were no significant differences in cortical bone density between genders of each genotype, a trend towards higher cortical densities in females was noted when compared to genotype-matched males. The cortical bone density of female *oim/oim* mice was increased by 6.3% and 3.2% compared to *wt* and *+oim* mice, respectively. Similar genotype effects were seen in male mice; the cortical bone density of male *oim/oim* mice increased 6.6% compared to *wt* male mice.

The trabecular bone volume/total bone volume fraction (BV/TV) in female *wt*, *+oim*, and *oim/oim* mice was only 42.6%, 43.1% and 56.7% of genotype-matched male mice, respectively. The BV/TV of female *oim/oim* was decreased by 18.5% as compared to female *wt* mice. Whereas the BV/TV in male *oim/oim* mice exhibited more dramatic declines compared to male *wt* mice; male *oim/oim* BV/TV decreased by 38.7% compared to male *wt* mice.

Interestingly, there was no significant difference in trabecular bone density between genotype-matched male and female tibiae. Whereas, trabecular bone density in female *oim/oim* mice was increased by 12.5% and 6.7% relative to female *wt* and *+oim* trabecular bone density; the trabecular bone density of female *+oim* mice was also increased by 5.4% relative to *wt* mice. The trabecular bone density in male *oim/oim* mice was increased by 8.3% compared to *wt* mice.

The trabecular thickness (Tb.Th) of the tibiae in male *wt* mice was also 10.5% greater than female *wt* mouse trabecular thickness. The trabecular thickness of female *oim/oim* tibiae was increased by 31.3% and 15.7% compared to female *wt* and *+oim* trabecular thickness, respectively, with similar trends in males.

Trabecular number (Tb.N) in the female *wt*, *+oim*, and *oim/oim* mice was significantly less than genotype-matched male trabecular number, with female *wt*, *+oim*, and *oim/oim* mice having only 58.2%, 53.4% and 72.5% of male *wt*, *+oim*, and *oim/oim* trabecular number. Though there was no difference in trabecular number among female mice of the different genotypes, there was a trend toward reduced trabecular number with increasing number of mutant alleles (*wt* > *+oim* > *oim/oim*). The trabecular number in male *oim/oim* mice was only 64.4% and 69.0% of male *wt* and *+oim* trabecular number, respectively.

The trabecular separation (Tb.Sp) in female *wt*, *+oim*, and *oim/oim* mice was increased by 89.5%, 97.1%, and 35.6% compared to genotype-matched male mice. There were no differences in trabecular separation in female mice between the different genotypes, however the trabecular separation trended toward increasing values with increasing number of mutant alleles ($wt < +oim < oim/oim$). Within the male mice, the trabecular separation in *oim/oim* mice increased by 66.0% and 52.4% compared to *wt* and *+oim* trabecular separation.

The trabecular connectivity (Conn.D) in female *wt*, *+oim*, and *oim/oim* mice was only 35.7%, 23.3%, and 20.1% of genotype-matched male trabecular connectivity values, respectively. Within the female mice, the trabecular connectivity in *oim/oim* mice was significantly reduced to only 19.0% and 36.3% of female *wt* and *+oim* trabecular connectivity, respectively. Male mice exhibited similar results, with the trabecular connectivity in *oim/oim* mice only 33.6% and 42.0% of *wt* and *+oim* trabecular connectivity.

The trabecular structure model index (SMI), a relative indicator of the prevalence of rods and plates, was found to be elevated in female *wt* and *+oim* mice by 36.8% and 26.5% compared to male *wt* and *+oim* mice, which suggests that in the female mice the trabecular bone is more rod-like than that in male mice. There was no significant difference in trabecular SMI in the female mice among the different genotypes. Within the male mice, the trabecular SMI of *oim/oim* mice was 67.9% greater than that of *wt* mice, which suggested that the normal plate-like trabeculae observed in *wt* mice had been converted to more rod-like structures in *oim/oim* mice.

Tibial Bone Mechanical Testing (Three-point Bending)

Across all genotypes, female tibiae exhibited moments of inertia that were significantly less than genotype-matched males (Fig. 3). Female *wt*, *+oim*, and *oim/oim* mouse moments of inertia were only 50.6%, 66.4%, and 60.0% of male mice. For both males and females the impact of the *oim* gene defect resulted in decreased moment of inertia in *oim/oim* and *+oim* tibiae relative to their *wt* counterparts. The moment of inertia of female *oim/oim* mice was decreased by 31.7% and 28.7% compared to *wt* and *+oim* mice. While the moments of inertia of male *oim/oim* and *+oim* tibiae were decreased by 42.4% and 26.9% relative to *wt* tibiae.

There were significant gender-dependent differences and genotype dependent differences in bone biomechanical integrity. The ultimate force of female *wt* and *+oim* tibiae was only 70.9% and 79.0% of genotype-matched male tibiae. The ultimate force in female *oim/oim* mice was only 62.0% and 65.7% of female *wt* and *+oim* tibiae, respectively. In male mice, the ultimate force of *oim/oim* tibiae was also decreased compared to male *wt* and *+oim* tibiae, with male *oim/oim* tibiae having only 50.3% and 59.4% of *wt* and *+oim* tibial ultimate force, respectively.

The stiffness of male *wt* tibiae was 27.4% greater than female *wt* tibiae. The stiffness of male *oim/oim* tibiae was decreased by 35.4% compared to male *wt* tibiae, whereas that of female *oim/oim* tibiae was decreased by 22.6% compared to female *wt* tibiae. *Oim/oim* tibiae showed decreased ultimate force and stiffness compared to their gender-matched *wt* counterparts, corresponding to increased fragility. However, interestingly, Young's modulus values of male *wt*, *+oim*, and *oim/oim* were decreased by 37.79%, 30.45%, and 32.08% compared to female counterparts, respectively. In addition, the Young's modulus values of both male and female mice trended toward increasing values with increasing number of *oim* mutant alleles ($wt < +oim < oim/oim$), though they did not reach significance.

Raman Spectroscopy

Following mechanical testing we used Raman spectroscopy to evaluate the cortical bone phosphate/CH ratio (relative amount of mineral phosphate to matrix), carbonate/CH ratio (relative amount of mineral carbonate to matrix), and phosphate/carbonate ratio (indication of carbonate substituting for phosphate in the crystal lattice) in female and male *wt*, *+oim*, and *oim/oim* mice (Figure 4).

No gender-dependent differences were found in the phosphate/CH and carbonate/CH ratios between genotype-matched female and male mouse tibiae; however, there were genotype-dependent differences. Female and male *oim/oim* tibial phosphate/CH ratios were 24.0% and 15.7% greater than gender-matched *wt* tibial phosphate/CH ratios, respectively. Though there were no significant gender-dependent differences in the carbonate/CH ratio, there was a trend toward elevated carbonate/CH ratios with increasing number of mutant alleles in female mice. Male *+oim* mice had significantly increased carbonate/CH ratio relative to male *wt* tibial carbonate/CH ratio, increased by 14.3%. Female and male *oim/oim* tibiae had 9.5% and 8.2% greater phosphate/carbonate ratios than their *wt* counterparts, respectively. On the other hand, there were no gender-dependent differences in the tibial phosphate/carbonate ratio between the female and male mice; however, the *oim/oim* tibiae exhibited elevated phosphate/carbonate ratios relative to *wt* tibiae independent of gender.

Hydroxyproline Content

Hydroxyproline is an indirect measure of collagen. Although there were no gender-dependent differences in hydroxyproline content /mg of bone between genotyped-matched male and female mice, significant differences were present between genotypes (Figure 5). There was a strong inverse correlation in hydroxyproline content /mg of bone and number of mutant alleles (*wt* > *+oim* > *oim/oim*). Female and male *oim/oim* tibiae had only 62.0% and 63.2% of the hydroxyproline content per mg of bone as female and male *wt* tibiae respectively, with *+oim* having intermediate levels.

Discussion

Recent studies in our laboratory with the *oim* mouse on the congenic C57BL/6 background and the use of high resolution μ CT and mechanical analyses of torsional loading to failure suggested the *oim* mouse might exhibit gender-dependent differences in muscle strength and function, and in bone morphometry and mechanical strength^{8, 15}. The paucity of studies in OI mouse models addressing the potential role of gender in bone quality and strength in light of new studies demonstrating gender-dependence of the *oim* mouse's response to specific treatments^{13, 35, 45} demands careful characterization of the role of gender in the pathogenesis of OI and in the *oim* mouse as well as other mouse models of OI. The majority of studies using the *oim* mouse address age and genotype¹³, with several studies claiming no gender-dependence^{2, 4, 6, 28}. The absence of gender dependence in these studies may in part reflect low sample numbers of *oim/oim* bones used (due to lower pup survival and the need to exclude bones with fracture calluses or damage during the harvest), as well as the non-congenic background of the *oim* mouse from the Jackson Laboratory^{7, 9}. The *oim* mouse COL1A2 gene defect originally arose spontaneously at the Jackson Laboratory in an outbred mouse strain, B6C3Fe, on which the *oim* mutation is still currently maintained⁹. The B6C3Fe strain is an F₂ outbred of two inbred congenic mouse strains, C57BL/6J and C3H/HeJ. These two congenic mouse strains independently have very different bone mineral densities and biomechanical strengths³⁷, and when used to maintain the *oim*-B6C3Fe mouse model they also result in greater bone phenotypic variation, than that which is found in the *oim*-C57BL/6J mouse model⁷.

The objective of this investigation was to systematically characterize the impact of gender on the bone structural, biochemical and biomechanical properties at a multi-scale level of adult age-matched *oim/oim*, *+oim* and *wt* tibiae on the congenic C57BL/J6 background. As often the case, the number of *oim/oim* mice and intact bones are limiting, thus, we devised methodology that allows use of a single tibia to quantitatively evaluate the structural geometry by μ CT, the biomechanical properties by three-point bending, and the biochemical composition by Raman spectroscopy, thus allowing for direct correlation of multi-scale parameters of bone structure and properties in the same bone.

In the current study, the dramatic gender differences were evident in both cortical and trabecular bone morphological and geometric parameters. Male mice had inherently more bone than genotype-matched female mice, with significantly greater cortical bone thickness, trabecular bone volume fraction (BV/TV), trabecular thickness, number, and connectivity, and correspondingly less trabecular separation. These findings were further reflected by the decreased moment of inertia in female mice relative to their genotype-matched male counterparts and in the decreased biomechanical properties exhibited in female mice of all genotypes.

There were also incremental decreases in bone mass/volume as well as incremental increases in bone fragility with increasing number of mutant $\alpha 2(I)$ collagen alleles (or presence of homotrimeric type I collagen), with the resultant outcome of female *oim/oim* mice exhibiting the least amount of bone and highest fragility in contrast to male *wt* mice with the greatest amount of bone and biomechanical integrity. The *oim/oim* mouse bone has lower trabecular BV/TV, trabecular number, and trabecular connectivity, accompanied by an increase in SMI as compared to the *wt* mice. The SMI, a measure of the relative prevalence of rods and plates in a 3D structure, suggested a more rodlike architecture in *oim* mice. A large number of rods and a small number of plates result in a lot of free-ending trabeculae and disconnected 3D spongy lattice¹⁷. This is consistent with previous studies in human patients with OI who also are characterized by decreased trabecular bone mass, which is largely due to decreased trabecular number³⁶.

The potential impact of not controlling for the variable of gender when investigating the *oim* mice and potentially other mouse models of OI can be seen from the following. For example, when comparing geometric parameters such as the cortical bone thickness (0.2075 ± 0.0059 mm) and trabecular bone volume fraction (BV/TV; 0.0942 ± 0.0093 %) of female *wt* mice with those of male *oim/oim* mice such as cortical bone thickness (0.1972 ± 0.0226 mm, $p=0.2369$) and trabecular bone volume fraction (BV/TV; 0.1354 ± 0.0619 %, $p=0.0848$), no significant difference would be observed. Yet, when compared to gender-matched female *oim/oim* mouse cortical bone thickness (0.1879 ± 0.0207 mm) and trabecular bone volume fraction (BV/TV; 0.0768 ± 0.0099 %), the female *wt* mice have significantly greater cortical thickness ($p=0.0228$) and BV/TV ($p=0.0039$). The impact of mixed genders not only contributes to significant variance, but it also can significantly influence interpretation of subsequent therapeutic treatment strategy results. Bargman et al.² found that RANKL inhibition and alendronate treatment both increased trabecular number for *oim/oim*. In such a study, one can envision if the trabecular number of *oim/oim* male mice were compared to that of *wt* female mice, the outcome would result in the *oim/oim* mice having higher trabecular number independent of the treatment.

μ CT analyses suggest that cortical and trabecular bone density values are gender independent, which is consistent with Raman spectroscopy results indicating that the mineral/matrix composition (i.e., phosphate/CH ratio) of bone is also gender independent (Figure. 4). The differences in density values exhibited between genotypes. Female and male *oim/oim* mouse tibiae have thinner cortices, higher density as compared to the *wt*

counterparts. These μ CT results were supported by mechanical testing of the tibiae that examined whole-bone strength and mechanical properties. Tibiae of *oim/oim* mice showed decreased ultimate force and stiffness compared to *wt* controls, which corresponds to increased fragility. The collagen phase is generally associated with strength (or ductility) and the mineral phase is associated with stiffness⁴⁶. Besides bone geometry (thinner cortices), the decrease in ultimate force and stiffness of the *oim/oim* bone may also be attributed to changes in both the collagen and mineral phase. Interestingly, although not significant, there was a trend that Young's modulus was greater in the *oim/oim* mice compared to the *wt* mice (Figure 3). Since Young's modulus is calculated by dividing the stress by the strain, higher modulus values of the *oim/oim* bone may be caused by the decreased strain (a reflective of the presence of homotrimeric type I collagen (or absence of α 2(I) chains), as compared to those of the *wt* control²⁸.

The mechanical property including strength and stiffness of any structure is a function of its geometry as well as its material³⁵. Analyses of the hydroxyproline content demonstrated no gender dependence in genotype-matched mice, but there was a significant reduction in collagen content per bone mass with increasing number of mutant α 2(I) collagen alleles as previously reported¹⁰. In the present study, both the cortical and trabecular bone density values were increased in *oim/oim* tibiae, with the *oim/oim* tibiae having the lowest collagen content, which is consistent with previous reports^{4,7}. Raman data revealed significantly higher phosphate/CH and lower carbonate/matrix ratios in *oim/oim* mice compared to *wt* mice. Camacho⁵ suggested that increased mineral/matrix (phosphate/CH ratio) in the *oim/oim* was likely attributable to a reduced collagen content rather than increased mineral based on decreased ash weight in bones from a transgenic mouse model of OI³². However, analyses of mineral content by neutron activation analyses demonstrated significant reduction in the ppm of calcium and phosphorous in *oim/oim* C57BL/J6 tibiae as compared to age-matched *wt* C57BL/J6 tibiae, suggesting the mineral content of *oim/oim* mice is also reduced⁷. However, the increase in the mineral to matrix ratio would suggest that the *oim/oim* bone has a greater reduction in the organic type I collagen component than in the mineral component. Although the cause of the reduced carbonate content in the *oim/oim* tibiae is unknown, it is possible that the *oim/oim* mice have an increased rate of bone turnover, and thus insufficient time is allowed for carbonate to be incorporated into the mineral⁵. Earlier studies have demonstrated changes in crystal morphology and solubility associated with altered mineral carbonate content which may in turn influence bone remodeling⁵. Vanleene et al demonstrated by transmission electron microscopy that the apatite mineral crystals in the *oim/oim* bone matrix were smaller and more disorganized than the *wt*, and by quantitative backscattered electron microscopy (qBEM) that the amount of bone mineral was higher in females versus males across genotypes⁴⁴. In this study, although the bone density was found to be gender-independent by μ CT, a trend towards higher cortical densities in females versus males was noted. This result is consistent with the above observations by qBEM.

Gender differences in bone morphology, mechanical properties and response to loading have been described in many congenic and transgenic mouse models^{21,29,30,47}. In 2003, Evans provided the first evidence of gender specific differences in growth plate maturation and in alendronate uptake into *oim/oim* bone, with a differential response to higher dose alendronate treatment regimens¹³. Only a few studies using the *oim* mouse model have addressed gender, yet each demonstrated that tissue parameters were specifically impacted by gender^{15,35,44,45}. Sex steroids and growth hormone are well established regulators of bone growth and development^{20,38}, and gender differences in long bone structure are proposed to arise from the different hormonal environments that male and female bone cells reside. Sex steroid receptor knock-out mice clearly demonstrate the impact of sex steroids on the ability of bone cells to respond to mechanical loading and disuse^{23,40}. The present

study demonstrated the importance of controlling sex in the *oim* mouse model and provided baseline values for future or potential therapeutic treatments.

One of the limitations of the current study is that only one age (4-month) group was used. Future studies would be needed to determine the gender dependence using different age groups. Another limitation may be associated with low sample size, especially for mechanical testing. However, within limitations of this study, our findings confirmed the genotype effect of the *oim* mutation resulting in compromised mineral/matrix composition, structural geometry, hydroxyproline content and biomechanical integrity, as well as demonstrating the significant impact of sexual dimorphism in the bone biomechanical properties. The primary influence of gender in the *oim* model and in the *wt* appears to be structural in bone growth and mechanical properties, whereas the mineral/matrix composition and hydroxyproline content of bone appear less gender-dependent with the mutant collagen being the primary factor. We also demonstrate using a single tibia multi-scale analysis approach that quantitatively investigates the ultra-structural geometry, biochemical composition, and biomechanical properties. In addition we provide evidence of the importance of gender in the evaluation and interpretation of potential therapeutic strategies when using mouse models of OI.

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Fig. 1. Representative μ CT images of 2D cortical bone cross-sections in the midshaft region (top) and 3D trabecular architecture in longitudinal sections (bottom) of the proximal tibiae from *wt*, *+/oim*, and *oim/oim* female and male mice

Fig. 2.

μ CT cortical and trabecular morphological parameters of the proximal tibiae from *wt* (8 females, 8 males), *+/-oim* (8 females, 8 males), and *oim/oim* (7 females, 6 males) mice. Values are expressed as mean $\pm$ SD; ^a $p < 0.05$ as compared to *wt* male; ^b $p < 0.05$ as compared to *+/-oim* male; ^c $p < 0.05$ as compared to *oim/oim* male; ^d $p < 0.05$ as compared to *wt* female; ^e $p < 0.05$ as compared to *+/-oim* female; ^f $p < 0.05$ as compared to *wt* female; ^g $p < 0.05$ as compared to *wt* male; ^h $p < 0.05$ as compared to *+/-oim* male; ⁱ $p < 0.05$ as compared to *wt* male

Fig. 3. Mechanical testing results (three-point bending) of the tibiae from *wt* (8 females, 8 males), *+/oim* (8 females, 7 males), and *oim/oim* (7 females, 6 males) mice. Values are expressed as mean $\pm$ SD; ^a*p* < 0.05 as compared to *wt* male; ^b*p* < 0.05 as compared to *+/oim* male; ^c*p* < 0.05 as compared to *oim/oim* male; ^d*p* < 0.05 as compared to *wt* female; ^e*p* < 0.05 as compared to *+/oim* female; ^f*p* < 0.05 as compared to *wt* female; ^g*p* < 0.05 as compared to *wt* male; ^h*p* < 0.05 as compared to *+/oim* male; ⁱ*p* < 0.05 as compared to *wt* male

Fig. 4.

The cortical bone phosphate/CH ratio (relative amount of mineral phosphate to matrix), carbonate/CH ratio (relative amount of mineral carbonate to matrix), and phosphate/carbonate ratio (indication of carbonate substituting for phosphate in the crystal lattice) in *wt* (8 females, 8 males), *+oim* (8 females, 7 males), and *oim/oim* (7 females, 6 males) mice. Values are expressed as mean $\pm$ SD; ^a*p* < 0.05 as compared to *wt* male; ^b*p* < 0.05 as compared to *+oim* male; ^c*p* < 0.05 as compared to *oim/oim* male; ^d*p* < 0.05 as compared to *wt* female; ^e*p* < 0.05 as compared to *+oim* female; ^f*p* < 0.05 as compared to *wt* female; ^g*p* < 0.05 as compared to *wt* male; ^h*p* < 0.05 as compared to *+oim* male; ⁱ*p* < 0.05 as compared to *wt* male

Fig. 5.

Hydroxyproline content of the right tibiae from *wt* (8 females, 8 males), *+/oim* (8 females, 8 males), and *oim/oim* (8 females, 8 males) mice. Values are expressed as mean $\pm$ SD; ^a*p* < 0.05 as compared to *wt* male; ^b*p* < 0.05 as compared to *+/oim* male; ^c*p* < 0.05 as compared to *oim/oim* male; ^d*p* < 0.05 as compared to *wt* female; ^e*p* < 0.05 as compared to *+/oim* female; ^f*p* < 0.05 as compared to *wt* female; ^g*p* < 0.05 as compared to *wt* male; ^h*p* < 0.05 as compared to *+/oim* male; ⁱ*p* < 0.05 as compared to *wt* male