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The Kidney Sodium-Phosphate Co-Transporter Alters Bone Quality in an Age and Gender Specific Manner

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Abstract

Mutations in the kidney NaPiIIa co-transporter are clinically associated with hypophosphatemia, hyperphosphaturia (phosphate wasting), hypercalcemia, nephrolithiasis and bone demineralization. The mouse lacking this co-transporter system was reported to recover its skeletal defects with age, but the “quality” of the bones was not considered. To assess changes in bone quality we examined both male and female NaPiIIa knockout (KO) mice at 1 and 7 months of age using micro-computed tomography (micro-CT) and Fourier transform infrared imaging (FT-IRI). KO cancellous bones at both ages had greater bone volume fraction, trabecular thickness and lesser structure model index based on micro-CT values relative to age- and sex-matched wildtype animals. There was a sexual-dimorphism in the micro-CT parameters, with differences at 7 months seen principally in males. Cortical bone at 1 month showed an increase in bone volume fraction, but this was not seen at 7 months. Cortical thickness which was elevated in the male and female KO at 1 month was lower in the male KO at 7 months.. FTIRI showed a reduced mineral and acid phosphate content in the male and female KO's bones at 1 month with no change in acid phosphate content at 7 months. Collagen maturity was reduced in KO cancellous bone at 1 month. The observed sexual dimorphism in the micro-CT data may be related to altered phosphate homeostasis, differences in animal growth rates and other factors. These data indicate that the bone quality of the KO mice at both ages differs from the normal and suggests that these bone quality differences may contribute to skeletal phenotype in humans with mutations in this co-transporter.

Keywords

Bone quality; FTIR imaging; micro-computed tomography; NaPi IIa; knockout mice; sexual dimorphism

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Conflict of Interest

The authors have no conflicts of interest.

Introduction

Control of phosphate homeostasis is a complex process dependent on a multitude of interacting factors [1–8]. In general, ingested phosphate enters the intestine via the activities of two sodium-phosphate co-transporters, NaPi IIa¹ and NaPi IIc. The activities of these co-transporters are regulated by vitamin D, PTH, and several newer regulatory factors [3,5,9] including FGF23 and Klotho. Once in the circulation, inorganic phosphate (Pi) levels are controlled primarily by the kidney via the NaPi IIa transporter, its cofactor, Klotho, PTH, and vitamin D through the action of its 1- hydroxylase [6]. Other tissues important for Pi homeostasis are the intestines which control Pi absorption and excretion (action of co-transporter NaPi IIb) and the mineralized tissues (calcified cartilage, bones and teeth). These mineralized tissues express the intestinal co-transporter NaPi IIb, small amounts of NaPi IIa in osteoclasts and a type III co-transporter, Pit1 [10–12].

Heterozygous loss of function mutations of NaPi IIa in humans are associated with hypophosphatemia, hyperphosphaturia (phosphate wasting), hypercalcemia, nephrolithiasis (in males), and bone demineralization [13]. Mice, in which NaPi IIa is genetically ablated are smaller at birth than wild type animals, and are similarly hypophosphatemic and waste phosphate. The tibias of these KO mice, based on histomorphometric data, were shown to vary with age (21–184 days). This variance includes; (i) At 21 days reduced secondary ossification centers. (ii) At 45 days no appreciable differences. (iii) At 115 days increased metaphyseal trabeculae. (iv) Calcifications within the marrow cavity at older ages [14].

It is not clear if NaPi IIa has a direct effect on bone or if the observed global phenotype in the NaPi IIa KO and in patients with mutations (early hypercalcuria and osteomalacia) [15] is directly related to the observed phosphate wasting, to their hypercalcuria, or to their compensatory responses. The NaPi IIa KO animals are normophosphatemic but express elevated levels of NaPi IIb in the intestine. Because the process of bone formation is dependent on local Pi levels, we hypothesized that; were the bone phenotype in the NaPi IIa KO due to a decreased local Pi concentration (in the bone microenvironment) there would be a corresponding decrease in the mineral content in all areas of new bone formation, and newly formed crystals would grow to a lesser extent than those in the WT. However, if the bone phenotype occurred due to a general decrease in circulating Pi, then the ability of the osteoblasts to maintain a normal local Pi and Ca level in their microenvironment, would result in minimal changes in mineral properties. These changes would be commensurate with a smaller boney structure in the wild type. Since NaPi IIa deficient animals were reported to lack a bone phenotype when they were older [14], we predicted that according to these hypotheses, bone in older animals would appear normal in quality (composition, collagen cross links, micro-architecture). This quality would still be less in older mice compared with changes in younger mice.

As noted within, earliest radiographic and morphologic reports described a bone phenotype in young animals that disappeared as the animals aged [14]. To gain more insight into new bone formation and remodeling processes in the presence and absence of NaPi IIa, the specific aims of this study are to compare bone properties of 1 and 7 month old NaPi IIa KO mice to their age- and sex matched WT controls.

¹Abbreviations: BS/BV- bone surface/bone volume; BV/TV- bone volume fraction; Ca-calcium; FGF23 – fibroblast growth factor-23; HYP-hypophosphatemic; KO – knockout; micro-CT- micro computed tomography; NaPi II – sodium phosphate cotransporter type II; Pi – inorganic phosphate; PMMA- poly-methyl methacrylate; PTH-parathyroid hormone; SMI- structure model index; TMD-tissue mineral density; WT-wild type;

Material and Methods

Study Design

To characterize how bone mineral properties varied with age and genotype, male and female wild type (WT) and NaPi IIa knockout (KO) mice at 1 and 7 months of age were compared. The variables studied were the geometry, architecture, and density based on micro-CT. Bones of a 1 month old mouse would demonstrate mainly new bone formation. The 7 month old skeletally mature mouse would, similarly, exhibit changes in mature bone. Mineral compositional changes were based on Fourier transform infrared imaging microscopy. The covariates were age and sex. Based on a power-study, for an $\alpha = 0.05$, $\beta = 0.20$ (i.e. 80% power), four mice per group were needed for FTIRI and five per group for the micro-CT.

Animals

Four to 6-month-old male and female B6 WT and Npt2a KO mice (acquired from Jackson laboratories) weighing 18 to 30 gm were used as breeders at U. Fla Gainesville under IACUC approval and following NIH guidelines. The breeders and their pups were given regular powdered sterile rodent chow moistened with water and formed into small balls. Urinary and serum calcium and phosphate levels were measured as detailed previously [16,17]. NaPi IIa KO and WT pups (5 per genotype per sex) were sacrificed at 1 month and at 7 months, and their long bones stored in 90% ethanol were used for the analyses detailed below. Bones (5/group) from the following groups of mice were examined: 1 month old male WT, 1 month old female WT, 1 month old male KO, 1 month old female KO, 7 month old male WT, 7 month old female WT, 7 month old male KO, 7 month old female KO. Total of 40 animals.

MicroCT

Femora, cleaned of soft-tissue and placed in 90% ethanol were used to determine the morphometry and density of cortical and cancellous bone by micro-Computed Tomography (microCT) using a Scanco μ CT35 system operating at 55KVp, 6 μ m resolution (Scanco Medical, Basselsdorf, Switzerland). Three D-reconstructions were performed automatically with a user-defined beam hardening correction factor. Mineral density calibrations were done based on preset by Scanco algorithms for the 55KVp during the reconstructions. Evaluation of the 3D reconstructed volumes was performed using the Scanco morphometry and densitometry software for open VMS on a Hewlett-Packard RAID server. The parameters evaluated [18] were bone volume fraction (BV/TV), Porosity(%), cortical thickness (Ct.Th(mm)), tissue mineral density (TMD(mg/cc)), and polar moment of inertia (pMOI[mm⁴]) for cortical bone. The parameters for cancellous bone were BV/TV, TMD, trabecular number (Tb.N (1/mm)), trabecular thickness (Tb.Th. (mm)) and trabecular separation (Tb.Sp.(mm)), the ratio of bone surface to bone volume (BS/BV), connectivity density (Conn-Dens.), and structure model index (SMI).

FTIR Imaging

Following micro-CT femurs were embedded in polymethyl methacrylate (PMMA). Multiple 1–2 μ m semi-thin sections were cut from each PMMA block using a heavy duty sledge microtome (Polycut 3500, Leica, Germany). These sections were examined by a Fourier Transform Infrared Spectroscopic Imaging System (Perkin Elmer model 100 imaging system) as detailed elsewhere [19]. For the one month old bones, cortical, cancellous and proximal growth plates were examined separately. In the 7 mo bones only cortical and cancellous bone areas were examined. For each bone sample three areas ($\sim 100\mu\text{m} \times 200\mu\text{m}$) per section of trabecular or cortical bone were imaged with a 6.25 μm spatial and a 4 cm^{-1}

spectral resolution. ISYS software (Spectral Dimensions, Olney, MD) was used to process the data, including a subtraction of the PMMA spectral contribution and base-lining the data. Five image parameters were calculated: mineral to matrix ratio, carbonate to phosphate ratio, crystallinity, acid phosphate substitution, and collagen maturity. Mineral to matrix which is linearly related to the tissue's ash content [20] was calculated as the relative integrated intensities of the ν_1, ν_3 phosphate band, $\sim 900\text{--}1200\text{cm}^{-1}$ to that of the protein amide I band ($1585\text{cm}^{-1} - 1720\text{cm}^{-1}$). Carbonate ($855\text{cm}^{-1} - 890\text{cm}^{-1}$) to phosphate band area ratio indicates the extent of carbonate substitution in the apatite lattice [21]. Crystallinity, related to crystallite size and perfection [20], was calculated based on the peak height ratios of stoichiometric (subband at 1030cm^{-1}) and non-stoichiometric (subband at 1020cm^{-1}) apatite [19]. Acid phosphate substitution was calculated from the intensity ratios of subbands at ($1128\text{cm}^{-1}/1096\text{cm}^{-1}$) [22]. Collagen maturity was calculated from the peak height ratios of subbands in the collagen amide I peak, related to reducible (1660cm^{-1}) and non-reducible (1690cm^{-1}) fibrillar collagen components [23], it does not reflect non-enzymatic cross links, but based on studies in osteonal bone is related to tissue age [24].

Statistics

Mean and standard deviations were calculated for each bone type, each gender, genotype and age. Linear regression was used to assess the effect of genotype while controlling for age and gender. Interaction effects between genotype, age, and gender were also considered in the regression. When an interaction effect was significant, we compared the two genotypes specifically for each age group or gender group. We conducted regression diagnostics including tests of collinearity, independence of residual errors, normality of residuals, and homogeneity of variance [25]. Specifically, variance inflation factor, Durbin-Watson test, Shapiro-Wilks test, and scatterplots of residuals versus predicted values were used to assess collinearity, independence, normality, and homogeneity, respectively. The criteria except for homogeneity were met in all regression analyses. Non-normality was found in regression analysis of three micro-CT parameters (porosity, Ct.Th, and TMD). However, linear regression has been proven to be very robust to the assumption of normality in statistical literature [26–28]. In addition, the test of normality is likely to be unreliable when the sample size is small. A $p < 0.05$ was accepted as significant. These calculations were performed using SAS 9.2 (SAS Institute, Cary, NC). Significant results are shown in the Table and Figures.

Results

Micro-CT

In the cortical bone, independent of gender and genotype, between one and seven months, bone volume fraction (BV/TV), cortical thickness (Ct.Th), tissue mineral density (TMD) and polar moment of inertia (pMOI) increased. The only aspect which was reduced with time was bone porosity (Table 1). There were age*genotype interaction effects in BV/TV, Ct.Th. and TMD. BV/TV, Ct.Th. and TMD for the KO differed significantly from the WT at 1 Mo, but BV/TV was not different for the KO vs. WT at 7 Mo. Ct.Th. and TMD were significantly different for KO and WT at 7 Mo.

In the cancellous bone comparing 1 and 7 month old animals (Table 1), BV/TV was lower in the wild type, but was greater in the KO in juxtaposition. There were gender*genotype interaction effects in trabecular thickness (Tb.Th.) and trabecular bone surface/bone volume (BS/BV). There were significant age*genotype interactions in BV/TV, trabecular number (Tb.N.), trabecular separation (Tb.Sp.), TMD, and structure model index (SMI). BV/TV also had significant gender differences, and the KO differed significantly from the WT at both 1 and 7 months of age. Trabecular thickness differed significantly when male WT and KO

were compared. Trabecular BS/BV ratio also showed significant age effects and female WT differed significantly from the female KO mice. TMD only varied with the genotype at 7 months, Connectivity density had significant age and genotype effects. Structure model index (SMI) differed significantly between WT and KO only at 7 months.

Micro-CT of the femurs showed an increase in BV/TV of the male and female KO proximal growth plates as compared to the WT at 1 month, and a decrease in the proximal growth plate TMD. Figure 1 illustrates typical 3D images of the cancellous bones of 1 and 7 month old male and female animals.

FT-IRI

FT-IR images demonstrated visible differences in the distribution of mineral and matrix parameters in the one month animals (Figure 2). In the cortical bone (Figure 3) mineral to matrix ratio showed a significant age*genotype interaction, with lower mineral content in both gender KOs at 7 months but not at 1 month of age (Figure 3A). The acid phosphate content showed a significant age*genotype interaction, the KO with reduced content at 1 month but not at 7 months (Figure 3B). Collagen maturity showed an age specific variation, but no effect of genotype or gender (Figure 3C). Crystallinity was not significantly effected (Figure 3D), while the carbonate:phosphate ratio showed an age dependent effect, increasing with age in both WT and KO mice (Figure 3E).

Comparing the WT and KO cancellous bone at 1 month of age, (Figure 4), a significant age*genotype interaction was noted, and the KO mineral to matrix ratio was reduced (Figure 4A). Acid phosphate content showed a significant gender*genotype interaction, with the male KO significantly different from the male WT at 1 and 7 months of age (Figure 4B). Collagen maturity also showed a significant age*genotype interaction, with the KO significantly reduced in maturity relative to the WT at one month (Figure 4C). Significant age and genotype effects were seen for crystallinity (Figure 4D). Carbonate:phosphate ratio showed a significant age effect (Figure 4E) but KO and WT did not differ in this parameter. In the one month growth plate there were no significant differences between the KO and WT FTIRI parameters (Figure 5). In the 7 month animals the growth plate was not detectable.

Discussion

This investigation demonstrates that bone quality, known to be altered in younger mice relative to WT mice [14], remains altered in older mice which lack the NaPi IIa co-transporter. Hydroxyapatite present in the KO bones differed in its composition from that of the WT. The mineral to matrix ratio was reduced in NaPi IIa deficient bones. The crystallinity was invariant in the cortex, but decreased with age in the cancellous bones, possibly due to retention or incorporation of carbonate. As expected [21], the carbonate to phosphate ratio increased with age, and was independent of genotype and gender. The acid phosphate substitution was less in the KO and showed a sexual dimorphism. Acid phosphate substitution, in general, is associated with new hydroxyapatite deposition [22], suggesting less new mineral was deposited.

Decreases in mineral to matrix ratio are directly associated with decreased mechanical strength (elastic modulus or stiffness) as are decreases in carbonate substitution and collagen maturity at both the nano- [29–31] and macro- [32] levels. The changes in the bones of the NaPi IIa knockout resemble alterations that have been noted in patients with high turnover chronic kidney disease [33].

The data are consistent with our second proposed scenario – i.e., that a generalized lower Pi level, results in decreased mineral formation (hence the reduced mineral/matrix ratio) and

that the local maintenance of normocalcemia and normophosphatemia enables the mineral crystals that do form, to grow and attain age-appropriate mineral properties. The invariant BV/TV at an early age and the lack of genotype-specific differences in crystallinity and carbonate substitution, agree with this concept. The relatively small, but significant changes in SMI [34] also support this view. The decreased proportion of acid phosphate in younger bones may indicate a greater mineral turnover, or may suggest, that the absence of the phosphate cotransporter which is specific [12] for $\text{H}_2\text{PO}_4^{-2}$, may result in altered phosphate equilibrium. A greater bone turnover is most likely due to the fact that hyperphosphaturic animals must maintain their normal serum Pi levels at the expense of increased bone turnover. It is important to compare these bone compositional parameters to that which was observed in the hypophosphatemic (HYP) male mouse at young and mature ages. At one and nine months the HYP males had reduced serum phosphate and showed increased phosphate clearance. FGF23 levels were also elevated [19]. At 9 months, histomorphometry showed increased BV/TV and an increased in osteoid volume in the male HYP animals. Bone mineral in male HYP mice contained larger crystals at 35 days of age than those in the male controls [35]. The crystal size in the male HYP mice at nine months remained significantly greater than that in the wild type [19]. Therefore, while both the NaPi IIa KO and the HYP mice have hypophosphatemic rickets, their bone quality is quite distinct. The observation that the NaPi IIa KO animals are normophosphatemic may explain the difference in the crystal sizes between these two animal models. Unfortunately micro-CT measurements were only done on the teeth of the HYP animals [19].

Bones of the NaPi IIa KO mouse have not previously been characterized by micro-CT and their composition was not been evaluated. FGF2 transgenic mice, however, which have decreased NaPi IIa levels, increased PTH, normal 1,25 di-hydroxyvitamin D, decreased serum Pi and Pi wasting. These mice had their L3 vertebrae examined by microCT at 3 months [36]. Similar to our findings at 2 months, these animals had decreased SMI, reduced BV/TV (72%), a 15% reduction in trabecular thickness, decreased trabecular number and increased trabecular spacing. They was also a widened disorganized growth plate in the L3 vertebrae which was not detected in the long bones of the NaPi IIa KO mice.

The reason for the sexual dimorphism that was observed in the 7 month old KO mice may be related to the larger size of the male animals. Increase in mineral content could reflect the body's attempt to maintain normocalcemia in the face of Pi wasting, or the compensatory effect of NaPi IIb in other tissues, since its expression is associated with bone and cartilage calcification [10,11]. However, the direct effect of NaPi IIb on mineralization is not yet understood. NaPi IIa KO animals weigh more at all ages as reported in a study of animals ranging in age from 4–20 weeks [37]. Histomorphometry of the bones of these mice at 12 wks showed that the bone volume fraction increased with no change in mineral apposition rate [38]. Earlier studies [39] showed increases in the Vitamin D 1- and 24,25- hydroxylases which could contribute to changes in vitamin D activity. It is also possible that PHEX, which is an x-linked regulator of phosphate handling, is affected in the male mice leading to the observed dimorphism.

The role of PHEX cannot be ascertained from the present data, despite the fact that mice treated with cadmium showed an increased in FGF23 and phosphate wasting without altered PHEX gene expression in their long bones [40]. Future studies will be needed to explain the sexual dimorphism in the NaPi IIa knockout animals. This information will be important because of observed dimorphism in patients with mutations in NaPi IIa [13].

It is important to note the studies main limitations. No mechanical tests could be performed on the bones studied here since they were preserved in alcohol. Only two ages of animals were evaluated preventing time dependent changes from being measured. However, the

micro-CT and FTIRI data, based on studies in other systems, showed changes between the KO and the WT that should impact mechanical strength. Future studies will need to verify that suggestion. Our findings, even without mechanical testing, are clinically significant due to the parallels to chronic kidney disease and the high incidence of hereditary hypophosphatemic rickets [41] in which this co-transporter's activity is altered.

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Highlights

- The kidney sodium-phosphate co-transporter (NaPi IIa) is a major regulator of Pi homeostasis.
- NaPiIIa knockout and wild type mice were analyzed by micro-CT and FTIRI to assess bone quality.
- Alterations in geometric and density properties were age (1mo vs. 7mo) and sex-dependent.
- Increased mineral and acid phosphate content was seen in NaPiIIa knockout mice at both ages.
- Bone quality in this hypophosphatemic model is distinct from that in the HYP mouse.

Figure 1.

Typical micro-CT images of cancellous bone in 1 and 7 month old Female and Male wild type (WT) and NaPi IIa knockout (KO) mice. (A) Male WT, 1 mo (B) Male KO, 1mo (C) Female WT, 1mo (D) Female KO, 1mo (E) Male WT, 7mo (F) Male KO, 7mo (G) Female WT, 7 mo (H) Female KO, 7 mo. Bar = 100 um.

Figure 2.

Typical FTIRI images of 1 month old female NaPi IIa knockout (KO) and wild type (WT) cancellous bones. The scale bar indicates the dimensions of each image in both directions. The color bars are specific for each parameter shown.

Figure 3.

Cortical FTIR parameters. Box plots showing the 25th to 75th percentile of the FTIR data, with whiskers from the edge of the box indicates the 5th and 95th percentile. The solid line in the box is the median value. Data is shown for 1 and 7 month male (m) and female (f) femurs. Parameters are A) Mineral:Matrix, B) Acid Phosphate Substitution, C) Collagen Maturity, D) Crystallinity and E) Carbonate:Phosphate. Statistical comparisons are indicated as: a) significant interaction age*genotype; b) significant interaction gender*genotype. c) WT significantly different from KO, 1 mo d) WT significantly different from KO, 7mo, e) significant age effect; f) significant gender effect; g) male WT significantly different from male KO, h) female WT significantly different from female KO, i) significant genotype effect.

Figure 4.

Cancellous bone FTIR parameters. Box plots showing the 25th to 75th percentile of the FTIR data, with whiskers from the edge of the box indicates the 5th and 95th percentile. The solid line in the box is the median value. Data is shown for 1 and 7 month male (m) and female (f) wild type (WT) and NaPi IIa KO (KO, darker lines) femurs. Parameters shown A) Mineral:Matrix, B) Acid Phosphate Substitution, C) Collagen Maturity, D) Crystallinity and E) Carbonate:Phosphate. Statistical comparisons are indicated as: a) significant interaction age*genotype; b) significant interaction gender*genotype. c) WT significantly different from KO, 1 mo d) WT significantly different from KO, 7mo, e) significant age effect; f) significant gender effect; g) male WT significantly different from male KO, h) female WT significantly different from female KO i) Significant genotype effect..

Figure 5.

Growth plate parameters: Box plots showing the 25th to 75th percentile of the FTIRI data, with whiskers from the edge of the box indicates the 5th and 95th percentile. The solid line in the box is the mean value. Data is shown for 1 month femurs. Parameters are A) Mineral:Matrix, B) Acid Phosphate Substitution, C) Collagen Maturity, D) Crystallinity and E) Carbonate:Phosphate. Statistical comparisons are indicated as: a) WT significantly different from KO, b) significant gender effect.

Table 1
Micro-CT Parameters in Female (F) and Male (M) WT and NaPi IIa KO Mice Tibias as a Function of Age (mean±SD)

Parameter and statistics	One Month				Seven Months			
	WT-F	KO-F	WT-M	KO-M	WT-F	KO-F	WT-M	KO-M
Cortical Bone								
BV/TV (<i>a,c</i>)	0.249±0.0071	0.300 ± 0.0147	0.250±0.0093	0.312±0.0145	0.378±0.047	0.388 ± 0.053	0.410±0.0187	0.3958±0.0212
Porosity (%) (<i>e</i>)	12.5±2.40	11.79±0.60	11.75±1.01	11.95±0.775	7.586±0.335	7.87±0.57	7.586±0.33	7.87±0.57
Ct.Th. (mm) (<i>a,c,d</i>)	0.0718±0.0032	0.099±0.0057	0.071±0.0031	0.100 ± 0.0035	0.173±0.038	0.1512±0.026	0.190 ± 0.0087	0.167±0.011
TMD (mg/cc) (<i>a,c,d</i>)	881.8±12.3	917.92±9.83	885.8±12.6	942.45±15.2	1205.3±33.7	1100.2±86.9	1195.6±15.8	1147.9±20.9
pMOI (mm ⁴) (<i>e</i>)	0.0686 ± 0.0068	0.137±0.018	0.064±0.005	0.145±0.012	0.459±0.154	0.310±0.095	0.457±0.091	0.539±0.205
Cancellous Bone								
BV/TV (<i>a,c,d,f</i>)	0.06014±0.0053	0.088±0.006	0.0611±0.0074	0.094±0.010	0.0378±0.017	0.0784±0.028	0.0485±0.016	0.137 ± 0.054
Tb.N (1/mm) (<i>a,d,f</i>)	3.958 ± 0.291	4.13 ± 0.20	4.0138±0.32	4.18±0.22	2.27±0.537	3.259±1.144	2.915±0.210	3.839±0.483
Tb.Th. (mm) (<i>b,g</i>)	0.0267±0.0010	0.0304±0.0013	0.026±0.0008	0.0309±0.0016	0.0407±0.0052	0.0403 ± 0.0061	0.036±0.0017	0.046 ± 0.006
Tb.Sp. (mm) (<i>a,d,f</i>)	0.255 ± 0.019	0.245±0.0129	0.252±0.023	0.241±0.0137	0.454 ± 0.089	0.339 ± 0.12	0.337 ± 0.025	0.255±0.040
BS/BV (mm ⁻¹) (<i>b,e,g,h</i>)	99.007±3.77	87.45±3.790	100.34 ± 3.50	84.66 ± 5.284	70.28 ± 8.71	69.09±8.05	76.64±4.90	55.43±8.75
TMD (mg/cc) (<i>a,d</i>)	809.85±4.579	812.09±14.224	811.14±11.47	806.13 ± 7.27	909.80±22.33	870.78±22.47	899.8±23.09	893.0±10.7
Conn. Dens. (<i>e,i</i>)	242.16 ± 28.456	339.2±37.07	248.02 ± 49.044	332.0±26.2	47.04 ± 54.95	214.2±156.6	76.64 ± 4.90	55.42 ± 8.75
SMI (<i>a,d</i>)	2.243±0.033	2.035±0.099	2.225 ± 0.060	1.946 ± 0.133	2.437±0.302	1.933±0.020	2.33±0.58	1.37±0.64
Growth Plate								
BV/TV (<i>e</i>)	0.527±0.0253	0.587±0.0038	0.5960 ± 0.0104	0.621±0.0785				
TMD (gm/cc) (<i>e</i>)	312.55±10.265	283.06±13.15	319.58±13.35	296.13±5.70				

a) Significant interaction effect: age*genotype, p<0.0001

b) Significant interaction effect: gender*genotype, p<0.0001

c) WT significantly different from KO at 1 mo, p<0.001

d) WT significantly different from KO at 7 mo, p<0.001

e) Significant age effect, p<0.001

- f*) Significant gender effect, $p < 0.04$
- g*) male WT significantly different from male KO, $p < 0.001$
- h*) female WT significantly different from female KO, $P < 0.020$
- i*) Significant genotype effect, $P < 0.0001$