Changes of Elastic Constants and Anisotropy Patterns in Trabecular Bone During Disuse-Induced Bone Loss Assessed by Poroelastic Ultrasound

Luis Cardoso

Department of Biomedical Engineering,
The City College of New York,
New York, NY 10031

Mitchell B. Schaffler

Department of Biomedical Engineering,
The City College of New York,
New York, NY 10031

Currently, the approach most widely used to examine bone loss is the measurement of bone mineral density (BMD) using dual X-ray absorptiometry (DXA). However, bone loss due to immobilization creates changes in bone microarchitecture, which in turn are related to changes in bone mechanical function and competence to resist fracture. Unfortunately, the relationship between microarchitecture and mechanical function within the framework of immobilization and antiresorptive therapy has not been fully investigated. The goal of the present study was to investigate the structure–function relationship in trabecular bone in the real-world situations of a rapidly evolving osteoporosis (disuse), both with and without antiresorptive treatment. We evaluated the structure–function relationship in trabecular bone after bone loss (disuse-induced osteoporosis) and bisphosphonate treatment (antiresorptive therapy using risedronate) in canine trabecular bone using μ CT and ultrasound wave propagation. Microstructure values determined from μ CT images were used into the anisotropic poroelastic model of wave propagation in order to compute the apparent elastic constants (EC) and elastic anisotropy pattern of bone. Immobilization resulted in a significant reduction in trabecular thickness (Tb.Th) and bone volume fraction (BV/TV), while risedronate treatment combined with immobilization exhibited a lesser reduction in Tb.Th and BV/TV, suggesting that risedronate treatment decelerates bone loss, but it was unable to fully stop it. Risedronate treatment also increased the tissue mineral density (TMD), which when combined with the decrease in Tb.Th and BV/TV may explain the lack of significant differences in vBMD in both immobilization and risedronate treated groups. Interestingly, changes in apparent EC were much stronger in the superior–inferior (SI) direction than in the medial–lateral (ML) and anterior–posterior (AP) anatomical directions, producing changes in elastic anisotropy patterns. When data were pooled together, vBMD was able to explain 58% of ultrasound measurements variability, a poroelastic wave propagation analytical model (i.e., BMD modulated by fabric directionality) was able to predict 81% of experimental wave velocity variability, and also explained 91% of apparent EC and changes in elastic anisotropy patterns. Overall, measurements of vBMD were unable to distinguish changes in apparent EC due to immobilization or risedronate treatment. However, anisotropic poroelastic ultrasound (PEUS) wave propagation was able to distinguish functional changes in apparent EC and elastic anisotropy patterns due to immobilization and antiresorptive therapy, providing an enhanced discrimination of anisotropic bone loss and the structure–function relationship in immobilized and risedronate-treated bone, beyond vBMD. [DOI: 10.1115/1.4029179]

Keywords: bone loss, microarchitecture, fabric anisotropy, immobilization, antiresorptive therapy, poroelastic ultrasound

Introduction

Characterization of bone mass density loss and diagnosis of osteoporosis are generally based on measurements of the areal BMD (aBMD) estimated using a dual energy DXA device or by the volumetric BMD (vBMD) obtained using a peripheral quantitative computed tomography (pQCT) system [1–4]. In addition to a decrease in mass density, osteoporosis is characterized by

directional changes of microstructure [5], and thus BMD exhibit a limited ability to fully discriminate osteoporotic from nonosteoporotic tissues [6–9]. BMD-based approaches do not take into account the directional aspects of cancellous bone microarchitecture that are key to bone's mechanical integrity [10–16].

Trabecular bone adapts its porosity, microarchitecture, and tissue composition in response to its mechanical environment through a complex and well orchestrated bone remodeling process. Bone loss due to immobilization creates changes in bone microarchitecture, which in turn are related to changes in bone mechanical function and competence to resist fracture. Bone loss results in overall decreased apparent EC and changes in

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microstructure produce different elastic anisotropy patterns. Unfortunately, the relationship between microarchitecture and mechanical function [17–22] within the framework of immobilization and antiresorptive therapy has not been fully investigated.

The anisotropic EC and elastic anisotropy pattern of trabecular bone can be determined using ultrasound wave propagation in multiple directions. However, the structure–function relationship between microarchitecture, acoustic, and mechanical properties in cancellous bone is highly complex. To better understand the relationship between microarchitecture, acoustic, and mechanical properties in trabecular bone, we recently developed an architecture–density based approach for acoustic wave propagation in anisotropic poroelastic media [23–26]. The role of anisotropic architecture on the mechanical properties of trabecular bone was introduced by Cowin [17] using a fabric tensor $\mathbf{F}$. The fabric tensor $\mathbf{F}$ is a quantitative stereological measure of the degree of structural anisotropy and the mechanical principal orientations of cancellous bone. More recently, Cowin and Cardoso [23–26] introduced the fabric tensor in the wave propagation equations to calculate the velocity of longitudinal and shear waves in anisotropic porous media and along any direction within the porous media [23–27]. This approach provides the potential to use ultrasound measurements in bone to examine tissue architecture in addition to bone mass. Recent studies show that this approach significantly improves prediction of mechanical properties of bovine and human cancellous bone when compared to density-based approaches [28–30].

In the present study, we evaluated the structure–function relationship in trabecular bone after bone loss (disuse-induced osteoporosis) and bisphosphonate treatment (antiresorptive therapy using risedronate) in canine trabecular bone using ultrasound wave propagation and an anisotropic poroelastic model. vBMD, global, and directional changes in microarchitecture were determined using μ CT scanning. Ultrasound wave velocities were measured using a transit time technique along the three anatomical directions of bones. Microstructure values determined from μ CT images were used into the anisotropic poroelastic model of wave propagation in order to compute the anisotropic EC of bone along the main anatomical directions in samples from animals with and without disuse-induced osteoporosis, both treated with and without antiresorptive therapy. These computed results were compared to the EC derived from ultrasound measurements. It is expected that using the anisotropic poroelastic model, a better estimation of apparent EC and elastic anisotropy patterns of bone will result in an enhanced discrimination of anisotropic bone loss and the structure–function relationship in immobilized bone and risedronate treated bone.

Materials and Methods

Bone Samples. Fresh frozen humeri samples from animals that underwent immobilization and antiresorptive treatment with risedronate were obtained from a previous NSBRI study [31,32] and used for the experiments in this study. Briefly, disuse osteoporosis was induced in female retired breeder Beagle dogs (5–7 years old, $n = 28$, Marshall Farms) by immobilizing for 12 months the right forelimb of animals using a splint as described by Li et al. [32]. Age-matched controls, which consisted of animals in which the forelimb was not immobilized, were used to evaluate baseline and treatment effects. One half of animals in both control and immobilization groups were treated with risedronate sodium (RIS, Proctor & Gamble Pharmaceuticals, Cincinnati, OH) at a dose of 1 mg/kg orally daily (CN + RIS and IM + RIS). Antiresorptive treatment started 2 days after immobilization, and it continued for the 12 months duration of the experiment. The second half of animals in control and immobilization groups only received sterile water vehicle (CN + VEH and IM + VEH). The risedronate dose employed in the study was shown effective in inhibiting cortical and cancellous bone activation of bone

resorption in metacarpal dog bones [31,32]. Animals were killed by overdose of pentobarbital. All procedures were approved by Institutional Animal Care and Use Committees of Mount Sinai School of Medicine and VA Medical Center, Bronx, NY.

Humeri were harvested and cleaned of soft tissues, wrapped in saline-soaked gauze, and frozen at -40°C until testing. Cube-shaped trabecular bone samples, with an approximate dimension $1 \times 1 \times 1$ cm, were retrieved using a low speed diamond saw from humeral heads with faces aligned with the ML, AP, and SI anatomical planes. Either one or two samples were obtained from each humeral head, resulting in a total of 42 samples for this study. The marrow was conserved inside the samples and the samples were maintained humidified with a phosphate-buffered saline solution (PBS) with an antibiotic to minimize microbial proliferation and contamination during μ CT scanning and ultrasound testing.

MicroCT Image Acquisition. Trabecular bone samples were thawed until reaching room temperature prior to scanning with an 1172 SkyScan high resolution μ CT system (SkyScan, Belgium). X-ray projections were acquired at a nominal isotropic voxel size resolution of $10.0 \mu\text{m}$ using a 0.5 mm aluminum filter to eliminate beam hardening artifacts. X-ray projections were generated every 0.4 degrees of rotation, obtaining 512 consecutive projections. To produce high-contrast, low-noise images, the projections were averaged five times, and a median filter was used to prevent speckle noise formation. Hydroxyapatite rods (HA, 2 mm diameter, 0.25 and 0.75 gHA/cm^3) were scanned using the same protocol to calibrate images for TMD [30,33].

Image Processing. Approximately 1000 images per sample were reconstructed from X-ray projections using the back-projection reconstruction algorithm in NRECON software (Skyscan, V1.6.1.1, SkyScan). Hounsfield unit (HU) and TMD calibration procedures were performed in CTAN software (CT Analyzer, v.1.6.1, SkyScan, Kontich, Belgium). For calibration of mineral density, four volumes of interest were selected from regions that contained water, air, 0.25 or 0.75 gHA/cm^3 . The mean grayscale index value from water and air was used to calibrate images in HU, and the mean grayscale index values from the 0.25 and 0.75 gHA/cm^3 mineral rods were used to generate a calibration curve between the grayscale color in each pixel and the corresponding mineral density in gHA/cm^3 . After image density calibration, the separation (image segmentation) between mineralized and soft tissues in each scan was performed using a mean global thresholding procedure. The threshold value was determined by analyzing the images using an edge detection algorithm (IMAGEJ v 1.37, National Institutes of Health, Bethesda, MD). The TMD threshold obtained through this edge detection procedure was 0.45 gHA/cm^3 [30,33]. An anatomical Cartesian coordinate system with axes defined along the ML, AP, and SI directions was introduced to investigate the relationship between directional measurements of bone microarchitecture and ultrasound measurements.

Global Measurements of Microarchitecture. For each trabecular bone sample, global architectural parameters were measured, including the trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, $1/\text{mm}$), trabecular spacing (Tb.Sp, mm), bone volume to total volume ratio (BV/TV, nondimensional), and porosity (ϕ , nondimensional). Since images were calibrated for mineral density, vBMD (gHA/cm^3), and TMD (gHA/cm^3) were obtained using built-in algorithms in CTAN software employing the guidelines for assessment of bone microarchitecture using μ CT [34]. vBMD was quantified before segmentation of images which includes both mineralized tissue and marrow, and TMD was obtained after segmentation, comprising the mineralized tissue only.

Directional Measurement of Microarchitecture: Fabric. The fabric tensor serves as a measure of the degree of structural

anisotropy of the porous medium [17,26,35–40]. The eigenvectors of $\mathbf{F}$ are the principal axes of material symmetry of the porous solid medium and the eigenvalues of $\mathbf{F}$ provide a measure of the distribution of porous volume fraction in the direction of the principal axes of material symmetry. If the three eigenvalues of the fabric tensor, F_1 , F_2 , and F_3 , are distinct, $F_1 \neq F_2 \neq F_3$, the fabric is orthotropic; if two are equal, $F_1 = F_2 \neq F_3$, the fabric is transversely isotropic; and if all three are equal, $F_1 = F_2 = F_3$, the fabric is isotropic. As with any symmetric positive definite second order tensor in 3D, the fabric tensor may be represented as an ellipsoid. The ellipsoid is one with three unequal axes for an orthotropic fabric, one unique axis for a transversely isotropic fabric (forming an ellipsoid of revolution about the unique axis), and the ellipsoid becomes a sphere in the case of an isotropic fabric.

The directional variation of pore orientation was calculated via the measurement of MIL tensor $\mathbf{M}$ [41,42] using CTAN software. Then, values of the fabric tensor $\mathbf{F}$ eigenvalues F_1 , F_2 , and F_3 , were obtained by taking the square root of the eigenvalues of MIL tensor $\mathbf{M}$. Fabric components were normalized by dividing each one by the sum $F_1 + F_2 + F_3$. The three eigenvectors of $\mathbf{F}$ represent the principal axes of material symmetry, which also correspond to the principal orientations of trabeculae. The components of the fabric eigenvectors are expressed relative to the anatomical coordinate axis; their vector components are the directional cosines of eigenvectors in the anatomical coordinate system. The degree of anisotropy (DA) was measured as the ratio of the largest to the smallest directional component of MIL tensor.

Ultrasound Wave Propagation. Each cube-shaped sample was immersed in PBS solution at room temperature and tested along three anatomical directions. Two broadband ultrasound transducers (Panametrics V323, Waltham, MA) with a central frequency of 2.25 MHz (6.8 mm in diameter) were used in this test. A damped single pulse was generated by an ultrasonic source (Panametrics 5077PR, Waltham, MA) operated in a transmission mode. The ultrasound wave enters to the sample, and due to the porous nature of bone, it causes the genesis of two waves propagating with different velocities [28,29,43–49]. The received signal is amplified in 40 dB, digitized, and averaged ten times in order to increase the signal to noise ratio. These waves were identified as the fast and slow waves predicted by the Biot's theory [50–55]. As identification of these waves was sometimes compromised by superposition of both waves in the time domain, a time–frequency analysis was developed to identify each kind of wave in different frequency ranges of propagation [29]. Digital filtering was used to separate these waves; the quantification of the wave velocity was then possible using a conventional time transit technique method [45,46]. The signal-conditioning procedures were performed in MATLAB (version 2010, MathWorks, Natick, MA).

Anisotropic PEUS Wave Propagation Model. The role of porous media microarchitecture was recently introduced in the anisotropic poroelasticity theory of wave propagation [26]. Key to the development of such a theory was the incorporation of the

fabric tensor into the governing equations for wave motion in the linear theory of anisotropic poroelastic materials [17,56]. This new approach resulted in a poroelastic Christoffel equation for anisotropic poroelastic media with six roots. Four of those six roots are nonzero and correspond to the four wave modes of propagation in each direction in porous media, two of which are longitudinal and two are shear wave modes. Analytical expressions were given in Cowin and Cardoso [26] for the velocity and attenuation of each wave mode for the case in which the direction of wave propagation coincides with the normal to a plane of symmetry of the anisotropic medium. Later, that study was extended to the propagation of waves along an arbitrary direction in orthotropic porous media [23]. Global changes in velocity of wave propagation depend on the porosity and material properties of the solid and fluid constituents (i.e., density and EC of tissue matrix and marrow), while directional changes of ultrasound wave velocity are a function of the porous media microarchitecture as quantified by the fabric tensor. For the present study, the longitudinal fast wave velocity was computed using equations in Cardoso and Cowin [23] along three anatomical directions ML, SI, and AP, using the measurements of porosity and fabric from each trabecular bone sample. TMD from μ CT was used to calculate the tissue modulus for each sample [30,33] and the apparent density of the solid constituent. The material properties of the fluid representing the mixture of blood and marrow were defined as follows: mass density = 1055 kg/m³; Lamé constants $\lambda = 2634$ MPa and $\mu = 0$ MPa; and dynamic viscosity $\eta = 0.1$ Pa·s.

Statistical Analysis. Forty-two trabecular bone samples obtained from 28 canine humeri were analyzed using PRISM5 statistics software (GraphPad Software Inc., La Jolla, CA). The number of samples in the CN + VEH group was $n = 12$; samples in the IM + VEH was $n = 10$, in CN + RIS group was $n = 11$ and in the IM + RIS was $n = 9$. Repeated measures ANOVA with a significance level of $p < 0.05$ and Bonferroni's multiple comparison test was performed to identify differences in global and directional architectural parameters (Tb.Th, Tb.Sp, Tb.N, vBMD, TMD, BV/TV, F, and DA) among animals that were immobilized (CN + VEH versus IM + VEH) and treated with and without anti-resorptive therapy (CN + RIS versus IM + RIS), as well as per anatomical location (ML, SI, and AP). Raw data were reported and shown in figures as mean $\pm$ standard deviation. To analyze the correlation among microstructural parameters, Pearson's correlation coefficient was calculated and $p < 0.05$ was set as the significance level.

Results

Microarchitecture. Changes in microarchitecture of trabecular bone as a consequence of immobilization and antiresorptive treatment are illustrated in Fig. 1. A 3D reconstruction of a sample from the CN + VEH group acquired using μ CT scanning is shown in Fig. 1(a). Figure 1(b) displays a sample from the IM + VEH group, and a typical sample from the CN + RIS and IM + RIS groups is shown in Figs. 1(c) and 1(d), respectively. The different

Fig. 1 3D images from μ CT scanning. (a) shows the CN + VEH; (b) displays IM + VEH, in (c) an example from the CN + RIS group and a sample from IM + RIS is shown in (d). The different thickness in trabeculae can be observed in each group.

Fig. 2 Global changes in microarchitecture, TMD and vBMD as a consequence of immobilization and antiresorptive treatment. (a)–(c) show the Tb.Th, Tb.Sp, and Tb.N, respectively, and (d)–(f) correspond to BV/TV, TMD, and vBMD. There is a similar pattern for Tb.Th, Tb.N, BV/TV, TMD, and vBMD, and Tb.Sp exhibits the opposite trend. However, significant differences were only found in Tb.Th and BV/TV when comparing CN + VEH versus IM + VEH, indicating a significant effect of immobilization in Tb.Th and BV/TV, and when comparing CN + RIS versus IM + RIS, indicating that immobilization has an effect in Tb.Th and BV/TV, but not in Tb.N, Tb. Sp, TMD, or BMD. It was also found a difference in Tb.Th between IM + VEH and IM + RIS groups, but there is not a difference in Tb.Th or BV/TV when comparing CN + VEH versus IM + RIS, indicating that RIS treatment was effective in slowing the bone loss produced by immobilization.

trabecular thickness and plate/rodlike appearance for each group can be observed in the representative images in Fig. 1. Immobilization of a hind limb (IM + VEH) resulted in a statistically significant ($p < 0.05$) reduction of Tb.Th and BV/TV (Figs. 2(a) and 2(d), respectively) when compared to CN + VEH group. However, the difference between means was not statistically significant for Tb.N, Tb.Sp, TMD, and BMD (Figs. 2(b), 2(c), 2(e), and 2(f)).

The treatment of immobilized animals with Risedronate (IM + RIS) as an antiresorptive agent also exhibited a statistically significant reduction in Tb.Th and BV/TV when compared to its matching control group (CN + RIS). Tb.N, Tb.Sp, and BMD; however, exhibited no significant differences due to risedronate treatment in this skeletal site. Interestingly, the comparison between IM + VEH versus IM + RIS was statistically significant for differences in Tb.Th, showing a smaller decrease of Tb.Th in the IM + RIS group than in the IM + VEH group, thus indicating that the bone loss created by immobilization alone was slowed down by the antiresorptive therapy, but not fully stopped (Fig. 2(a)). Also, TMD was statistically different when comparing IM + VEH versus IM + RIS, indicating that risedronate had an effect in TMD [57], but there was not a significant effect of immobilization. vBMD was only different when comparing CN + VEH and CN + RIS, indicating that risedronate had an effect on the vBMD of controls, but there was not a significant effect due to

immobilization. All together, these results suggest that the lack of changes observed in vBMD is a consequence of a decrease in Tb.Th that is counterbalanced by an increase in TMD (Table 1).

Fabric Components. Values of F_1 , F_2 , and F_3 components of the fabric tensor for all groups of animals (Table 2) are illustrated in Figs. 3(a)–3(c), respectively. When comparing F_1 , F_2 , or F_3 among the four treated groups (e.g., F_1 in CN + VEH, IM + VEH, CN + RIS, and IM + RIS), it was observed that the mean value of each eigenvalue remained statistically similar in all four groups ($p > 0.05$), indicating that no changes in the value of F_1 , F_2 , and F_3 components of the fabric tensor occurred due to immobilization or risedronate. However, when comparing F_1 , F_2 , and F_3 within each treated group (e.g., F_1 versus F_2 and F_3 in CN + VEH), it is observed that all eigenvalues are statistically different (Table 2). These three unequal mean fabric values show that the trabecular bone architecture is orthotropic, and remained orthotropic during immobilization and risedronate treatment. The fabric tensor can be illustrated as an ellipsoid with three different fabric eigenvalues in the three different axes as is shown in Fig. 4. F_1 as the largest value of F_i ($i = 1, 2, 3$) corresponds to the main trabecular orientation. Similar variability on eigenvalue magnitude and eigenvector direction across the samples was found for the other two principal directions of fabric (i.e., F_2 and F_3).

Table 1 Descriptive statistics of global microarchitecture, BMD, and TMD as a consequence of immobilization and antiresorptive treatment

	CN + VEH	IM + VEH	CN + RIS	IM + RIS
Tb.Th (mm)	0.118 ± 0.009	0.091 ± 0.009	0.119 ± 0.008	0.107 ± 0.008
Tb.N (mm^{-1})	1.591 ± 0.205	1.505 ± 0.279	1.659 ± 0.210	1.479 ± 0.226
Tb.Sp (mm)	0.535 ± 0.050	0.550 ± 0.084	0.519 ± 0.058	0.547 ± 0.059
BV/TV (%)	18.850 ± 2.926	13.680 ± 2.465	19.670 ± 2.470	15.900 ± 2.621
TMD (gHA/cm^3)	0.975 ± 0.083	0.952 ± 0.062	1.039 ± 0.025	1.043 ± 0.0346
vBMD (g/cm^3)	0.320 ± 0.108	0.295 ± 0.081	0.426 ± 0.074	0.382 ± 0.075

Table 2 Descriptive statistics of fabric eigenvalues (F_1 , F_2 , and F_3 components) representing the magnitude of trabecular mass distribution along the primary, secondary and tertiary directions of bone samples. Statistical significance between F_1 , F_2 , and F_3 directions is reported.

	CN + VEH	IM + VEH	CN + RIS	IM + RIS
F_1	$0.41 \pm 0.02^{a,b}$	$0.40 \pm 0.02^{a,b}$	$0.40 \pm 0.02^{a,b}$	$0.41 \pm 0.01^{a,b}$
F_2	$0.33 \pm 0.01^{a,c}$	$0.33 \pm 0.02^{a,c}$	$0.33 \pm 0.02^{a,c}$	$0.33 \pm 0.01^{a,c}$
F_3	$0.27 \pm 0.01^{b,c}$	$0.27 \pm 0.01^{b,c}$	$0.27 \pm 0.02^{b,c}$	$0.27 \pm 0.02^{b,c}$

^a $p < 0.05$ between F_1 and F_2 .

^b $p < 0.05$ between F_1 and F_3 .

^c $p < 0.05$ between F_2 and F_3 .

Correlation Among Microarchitecture Parameters. The correlation between Tb.N, Tb.Th, Tb.Sp, BV/TV, TMD, vBMD, F_1 , F_2 , F_3 , and DA was analyzed for samples within each group (Tables 3–6). It was found that vBMD was strongly correlated to global descriptors of microarchitecture such as Tb.N, Tb.Th, and Tb.Sp. The directional parameters such as F_1 , F_2 , F_3 , and DA are however statistically not correlated to vBMD, confirming that fabric is a measurement of architecture independent of vBMD.

Effect of Fabric on Ultrasound Wave Propagation. Data from control and treatment groups were pooled together to investigate the correlation between experimental fast wave velocities, vBMD, theoretical poroelastic fast wave velocities, and apparent EC in trabecular bone. The fast wave velocity measured along the three anatomical directions (AP, ML, and SI) of samples varied within the 1500–2200 m/s range. Fast wave velocities exhibit a large variability when analyzed as a function of vBMD

(Fig. 5(a)). vBMD (and conversely, porosity) was found to be a moderate predictor of the waves velocities ($R^2 = 0.58$) in anisotropic cancellous bone after immobilization and risedronate treatment. Theoretical fast wave velocities were computed using the PEUS wave propagation theory [23,26] along each of the three tested directions with ultrasound. The measurements of fast wave velocities were well correlated to predicted velocities from the anisotropic poroelastic model of wave propagation ($R^2 = 0.81$) as shown in Fig. 5(b). The ability of the anisotropic poroelastic approach to predict measured wave velocities was greater than vBMD alone. This improvement in wave velocity predictability occurred because the poroelastic model includes both global (TMD and BV/TV) and directional (Fabric) measures of bone microarchitecture. The corresponding experimental apparent EC were determined from the ultrasound measurements as the product of the apparent density in the sample and the wave velocity squared. These experimental ECs were thus compared to theoretical EC obtained from the poroelastic model, in which the inputs of the model were the TMD, BV/TV, and the fabric measurements. Comparison between experimental and theoretical EC demonstrated a higher correlation coefficient ($R^2 = 0.91$) than BMD or ultrasound wave velocity alone.

Elastic Anisotropy Patterns. The measurements of apparent EC were also analyzed within the CN + VEH, IM + VEH, CN + RIS, and IM + RIS groups (Figs. 6(a)–6(d)). The results demonstrate a shift from an ellipsoid (CN + VEH) to a more spherical shape (IM + VEH). The antiresorptive therapy during immobilization (IM + RIS) exhibit an ellipsoid smaller than CN + RIS, consistent with bone loss, but it maintains the same anisotropy ratio as the control group. A similar trend was observed in the experimental EC obtained using measurements of apparent density and anisotropic wave velocities. Descriptive statistics of

Fig. 3 Magnitude of Fabric components F_1 , F_2 , and F_3 (eigenvalues) from trabecular bone samples in the CN + VEH, IM + VEH, CN + RIS, and IM + RIS groups. No significant differences were observed due to immobilization or risedronate treatment; however, the comparison of F_1 , F_2 , and F_3 within each treated group indicates that bone is orthotropic and remained orthotropic after immobilization and risedronate treatment.

Fig. 4 Fabric anisotropy in all three anatomical planes are shown as ellipsoidal plots for the CN + VEH, IM + VEH, CN + RIS, and IM + RIS groups. Each group shows mean-SD values (red solid inner ellipsoids), the intermediate ellipsoids (blue) represent the mean values and the open mesh ellipsoids show mean + SD. There is a clear fabric anisotropy ($p < 0.05$) given by directional dependent microarchitecture within each group. Such DA was found similar in all groups ($p > 0.05$), indicating that immobilization and risedronate treatment had no significant effect on anisotropy of microarchitecture.

Table 3 Correlation coefficient (R^2) and p value among architectural parameters, F_1 , F_2 , F_3 , DA, ϕ , vBMD, TMD, Tb.Th, Tb.Sp, and Tb.N measured on CN + VEH group. Correlation values are in the upper right side of the table and p values in the lower-left side of the table. Boldface values represent correlation coefficients with $p < 0.05$.

	Tb.Th	Tb.N	Tb.Sp	BV/TV	TMD	vBMD	F_1	F_2	F_3	DA
Tb.Th		0.08	-0.24	0.56	0.44	0.44	0.37	-0.06	-0.41	0.40
Tb.N	0.81		-0.91	0.87	-0.40	-0.22	0.22	0.05	-0.30	0.27
Tb.Sp	0.45	0.00		-0.87	0.28	0.05	-0.11	-0.17	0.25	-0.19
BV/TV	0.06	0.00	0.00		-0.10	0.05	0.36	0.01	-0.44	0.41
TMD	0.16	0.20	0.38	0.76		0.81	-0.30	0.26	0.19	0.31
vBMD	0.16	0.50	0.88	0.87	0.00		-0.22	0.00	-0.39	0.37
F_1	0.23	0.50	0.73	0.25	0.34	0.50		-0.56	0.26	-0.24
F_2	0.85	0.88	0.61	0.98	0.42	1.00	0.06		-0.02	-0.26
F_3	0.19	0.35	0.43	0.15	0.56	0.41	0.00	0.96		-0.97
DA	0.20	0.40	0.56	0.18	0.45	0.42	0.00	0.46	0.00	

Table 4 Correlation coefficient (R^2) and p value among architectural parameters, F_1 , F_2 , F_3 , DA, ϕ , vBMD, TMD, Tb.Th, Tb.Sp, and Tb.N measured on IM + VEH group. Correlation values are in the upper right side of the table and p values in the lower-left side of the table. Boldface values represent correlation coefficients with $p < 0.05$.

	Tb.Th	Tb.N	Tb.Sp	BV/TV	TMD	vBMD	F_1	F_2	F_3	DA
Tb.Th		-0.31	0.44	0.19	-0.18	-0.54	0.37	-0.41	0.06	0.15
Tb.N	0.41		-0.97	0.87	-0.30	0.59	0.53	-0.40	-0.14	0.39
Tb.Sp	0.23	0.00		-0.79	0.35	-0.65	-0.45	0.36	0.10	-0.31
BV/TV	0.63	0.00	0.01		-0.44	0.34	0.75	-0.63	-0.13	0.50
TMD	0.65	0.43	0.36	0.24		0.02	-0.34	0.69	-0.43	0.11
vBMD	0.13	0.09	0.06	0.37	0.95		0.41	-0.27	-0.16	0.33
F_1	0.33	0.15	0.23	0.02	0.37	0.28		-0.65	-0.41	0.80
F_2	0.27	0.29	0.34	0.07	0.04	0.48	0.06		-0.43	-0.07
F_3	0.88	0.71	0.80	0.73	0.25	0.69	0.28	0.25		-0.87
DA	0.69	0.30	0.41	0.17	0.79	0.38	0.01	0.86	0.00	

Table 5 Correlation coefficient (R^2) and p value among architectural parameters, F_1 , F_2 , F_3 , DA, ϕ , vBMD, TMD, Tb.Th, Tb.Sp, and Tb.N measured on CN + RIS group. Correlation values are in the upper right side of the table and p values in the lower-left side of the table. Boldface values represent correlation coefficients with $p < 0.05$.

	Tb.Th	Tb.N	Tb.Sp	BV/TV	TMD	vBMD	F_1	F_2	F_3	DA
Tb.Th		-0.26	0.32	0.30	0.47	0.18	0.29	0.38	-0.53	0.44
Tb.N	0.45		-0.86	0.84	-0.47	0.51	0.30	-0.46	0.06	0.05
Tb.Sp	0.35	0.00		-0.69	0.38	-0.68	-0.07	0.45	-0.26	0.18
BV/TV	0.36	0.00	0.02		-0.20	0.63	0.48	-0.26	-0.23	0.29
TMD	0.15	0.14	0.25	0.56		-0.12	0.26	0.32	-0.46	0.39
vBMD	0.60	0.11	0.02	0.04	0.73		-0.01	-0.30	0.23	-0.19
F_1	0.39	0.36	0.84	0.14	0.44	0.97		-0.23	-0.72	0.87
F_2	0.25	0.16	0.16	0.44	0.33	0.36	0.50		-0.51	0.27
F_3	0.10	0.86	0.44	0.49	0.16	0.50	0.01	0.11		-0.96
DA	0.18	0.89	0.61	0.38	0.23	0.59	0.00	0.42	0.00	

Table 6 Correlation coefficient (R^2) and p value among architectural parameters, F_1 , F_2 , F_3 , DA, ϕ , vBMD, TMD, Tb.Th, Tb.Sp, and Tb.N measured on IM + RIS group. Correlation values are in the upper right side of the table and p values in the lower-left side of the table. Boldface values represent correlation coefficients with $p < 0.05$.

	Tb.Th	Tb.N	Tb.Sp	BV/TV	TMD	vBMD	F_1	F_2	F_3	DA
Tb.Th		-0.17	0.18	0.31	0.13	-0.26	0.13	-0.28	0.05	-0.01
Tb.N	0.64		-0.96	0.88	-0.80	0.87	-0.04	0.37	-0.17	0.08
Tb.Sp	0.62	0.00		-0.85	0.80	-0.95	0.20	-0.16	-0.07	0.13
BV/TV	0.38	0.00	0.00		-0.73	0.74	0.00	0.21	-0.12	0.05
TMD	0.71	0.01	0.01	0.02		-0.72	0.34	-0.47	-0.02	0.15
vBMD	0.47	0.00	0.00	0.02	0.02		-0.35	0.10	0.21	-0.28
F_1	0.72	0.92	0.59	0.99	0.33	0.32		0.17	-0.85	0.93
F_2	0.44	0.30	0.66	0.56	0.18	0.79	0.63		-0.66	0.52
F_3	0.90	0.65	0.86	0.75	0.97	0.55	0.00	0.04		-0.98
DA	0.98	0.82	0.72	0.88	0.68	0.44	0.00	0.13	0.00

Fig. 5 Ultrasonic wave velocities and apparent EC as a function of vBMD (a), theoretical PEUS wave velocities (b), and theoretical EC (c). Coefficient of correlation between wave velocities and vBMD was $R^2 = 0.58$. Theoretical poroelastic wave propagation theory was able to predict 81% of experimental wave velocity variability ($R^2 = 0.81$), and the theoretical poroelastic constants were higher correlated to experimental constants ($R^2 = 0.91$) than vBMD or wave velocity.

Fig. 6 The shift from an ellipsoid (CN + VEH) to a more spherical shape (IM + VEH) shows that bone loss is not uniform in all directions. The antiresorptive therapy during immobilization (IM + RIS) exhibit an ellipsoid smaller than CN + RIS, consistent with bone loss, but it maintains the same anisotropy ratio as the control group, indicating a conservation of mechanical function.

apparent EC derived from ultrasound measurements in the SI, AP, and ML anatomical directions are summarized in Table 7. Two-way ANOVA confirmed that the apparent EC in the three anatomical directions (i.e., SI, ML, and AP) in the IM + VEH group were significantly different ($p < 0.05$) to the corresponding apparent EC in the CN + VEH group, demonstrating that the immobilization treatment affected the apparent EC of samples in all three directions. It was also found that the apparent EC in the IM + RIS group in the SI direction was significantly different to the EC in the CN + RIS group in the same direction, but no differences were found in the other two anatomical directions (ML and AP), demonstrating that risedronate treatment stopped the changes produced by immobilization in the ML and AP direction, but could not preserve unchanged the SI direction. Moreover, the apparent EC in the IM + RIS group in the SI direction was also significantly different to the EC in the IM + VEH group in the same direction, but no differences were found in the other two anatomical directions (ML and AP), demonstrating that risedronate treatment stopped the changes produced by immobilization in the ML

and AP direction, but could not preserve unchanged the SI direction. Moreover, the apparent EC in the SI direction in each of the CN + VEH, CN + RIS, and IM + RIS groups were significantly different to the EC in their respective ML and AP orthogonal directions, and only the apparent EC in the IM + VEH group became similar in all three anatomical directions. These observations indicate that immobilization changed the elastic anisotropy pattern of bone from anisotropic to isotropic; however, this change in elastic anisotropy pattern did not occur in all other three CN + VEH, CN + RIS, and IM + RIS groups. These results thus demonstrate that bone loss and the decrease in apparent mechanical properties of trabecular bone due to immobilization are not equal in all anatomical directions. Also, the fact that the elastic anisotropic pattern in the IM + RIS group was similar to the CN + VEH and groups, and it did not become isotropic as the IM + VEH group, indicates that risedronate treatment indeed helped to retain the anisotropy and mechanical function of trabecular bone.

Table 7 Descriptive statistics of apparent EC derived from ultrasound measurements in the SI, AP, and ML anatomical directions. Statistical significance between treatment groups and directions is reported.

	CN + VEH	IM + VEH	CN + RIS	IM + RIS
SI	4.038 ± 0.912 ^a	2.870 ± 0.418 ^b	4.491 ± 0.949 ^a	3.549 ± 0.796 ^{a,c,d}
AP	3.507 ± 0.575	2.770 ± 0.338 ^b	3.579 ± 0.488	3.067 ± 0.284
ML	3.554 ± 0.500	2.796 ± 0.240 ^b	3.751 ± 0.575	3.164 ± 0.358

^a $p < 0.05$ versus other two orthogonal directions.

^b $p < 0.05$ versus CN + VEH.

^c $p < 0.05$ versus CN + RIS.

^d $p < 0.05$ versus IM + VEH.

Discussion

In this study, we investigated the effect of immobilization and risedronate-based antiresorptive therapy on trabecular bone from canine humeri. Statistically significant changes in microarchitecture (i.e., Tb.Th, BV/TV) occurred as a consequence of immobilization. However, immobilization alone did not result in significant loss of trabecular number, TMD, BMD, or a significant increase in trabecular spacing. Moreover, immobilization combined with risedronate treatment resulted in reduced bone loss (less Tb.Th and BV/TV changes) when compared to the baseline control group, but it did not significantly change other descriptors of microarchitecture (Tb.Sp and Tb.N) or bone density (vBMD) at this skeletal site. Not surprisingly, risedronate treatment affected the TMD [57,58]. These results indicate that

risedronate treatment was effective in reducing the rate of bone loss (as assessed by changes in Tb.Th and BV/TV), but was unable to fully stop such process. The effect of immobilization and risedronate on bone microarchitecture was previously analyzed in metacarpals [31,32], but bone loss in metacarpals was more accentuated than in the humeral heads analyzed here, suggesting that bone loss is also nonuniform at different skeletal sites. It has been longtime known that bone loss due to immobilization is higher in distal sites when compared to proximal sites [59].

Trabecular bone was found orthotropic, and surprisingly, the fabric anisotropy due to immobilization was not changed in canine humeral heads. Even though trabeculae became thinner in our samples, and the volume fraction decreased due to immobilization, the observation that trabecular number, trabecular separation, and fabric anisotropy remained unchanged indicates that the anisotropic microarchitecture scaffold was not fully disrupted by immobilization. Indeed, risedronate treatment helped to reduce the thinning of trabeculae and the decrease of BV/TV. This is important because retaining the trabecular scaffold may allow anabolic treatments (e.g., PTH-based therapies) to help recover the trabecular thickness and volume fraction lost due to immobilization. If the trabecular scaffold may have being completely lost during immobilization, anabolic therapy will not have a trabecular surface in which osteoblasts may work to lay down new bone tissue.

Analysis of correlation between global and directional microarchitectural parameters demonstrate that vBMD is significantly correlated to other classical histomorphometry measurements (e.g., Tb.N, Tb.Th, Tb.Sp, and BV/TV), and it is independent of directional measurements (e.g., F_1 , F_2 , F_3 , and DA), suggesting that Tb.N, Tb.Th, Tb.Sp, and BV/TV are redundant measurements of vBMD, and that fabric is an additional independent measurement of microarchitecture. These observations are in agreement with previous studies of trabecular bone anisotropy in the human calcaneus [30]. These results suggest that vBMD and fabric could be considered as the main independent descriptors of trabecular bone microarchitecture.

The velocity and mechanical properties of anisotropic bone are best quantified when the global and directional microstructures are taken into account. Overall, vBMD was not different after immobilization or risedronate treatment, and it was capable of explaining 58% of data variability. However, when vBMD was modulated by the fabric anisotropy using the theoretical poroelastic model of wave propagation, the correlation between experimental and theoretical wave velocities was able to explain 81% of wave velocity variability, and significant differences due to immobilization and risedronate treatment were found. Importantly, the inputs in the poroelastic model to obtain each theoretical wave velocity are the TMD and BV/TV (i.e., the vBMD) and the fabric components. Furthermore, comparison of the EC derived from ultrasound measurements and from the theoretical poroelastic model showed that the model is able to explain up to 91% of data variability, demonstrating the importance of the anisotropy in addition to the vBMD alone to better describe anisotropic mechanical behavior of trabecular bone. This result suggests that vBMD and fabric are the main independent descriptors of not only microarchitecture but also the main independent microarchitecture determinants of wave propagation and apparent mechanical constants in trabecular bone [60,61]. Assessment of vBMD (or TMD + BV/TV) and fabric is capable of providing an improved estimate of apparent EC and distinguish changes in trabecular bone produced by immobilization and risedronate treatment, which are not distinguished by measurement of vBMD alone. These results strongly support the use of structure–function poroelastic analytical models to enhance the assessment of bone loss and bone quality beyond vBMD, which can be achieved using HR-pQCT or PEUS wave.

The structure–function relationship (i.e., microarchitecture–mechanical/acoustic) in immobilized and risedronate treated cancellous bone are well described by an anisotropic poroelastic

model of wave propagation. The apparent EC within each group were represented by a 3D shape that depicts not only the amount of mass (i.e., vBMD) but also its relative orientation along the anatomical directions of trabecular bone samples (fabric). The apparent EC reflect the mechanical function of each group of samples, and interestingly, during immobilization, the anisotropic EC pattern shows a shift from an ellipsoid (CN + VEH) to a more spherical shape (IM + VEH). This result indicate that changes in the mechanical function during bone loss is not uniform in all directions; however, such change is not simply reflected by changes in fabric, but a combination of fabric and vBMD, which is taken into account in the anisotropic poroelastic analytical model. The anti-resorptive therapy during immobilization (IM + RIS) exhibit an ellipsoid smaller than CN + RIS, consistent with bone loss, but it maintains the same anisotropy ratio as the control group, indicating a conservation of mechanical function. The results also show that ultrasound-derived EC can be used to identify both global and directional changes (change in ellipsoid size and shape) in bone loss due to immobilization-induced osteoporosis. Also important, the antiresorptive therapy was able to reduce both the global and directional bone losses during immobilization, thus preserving the possibility of recovering the original mass and mechanical function through remobilization/PTH treatment.

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