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Variations in nanomechanical properties and tissue composition within trabeculae from an ovine model of osteoporosis and treatment

Jayme C. Burket^a, Daniel J. Brooks^a, Jennifer M. MacLeay^b, Shefford P. Baker^c, Adele L. Boskey^{d,e,f}, and Marjolein C.H. van der Meulen^{a,d}

Jayme C. Burket: jcb86@cornell.edu; Daniel J. Brooks: djb286@cornell.edu; Jennifer M. MacLeay: JMMacLeay@gmail.com; Shefford P. Baker: spb14@cornell.edu; Adele L. Boskey: boskeya@hss.edu

^aSibley School of Mechanical and Aerospace Engineering, Cornell University, Ithaca, NY, 14850, USA

^bDepartment of Clinical Sciences, Colorado State University, Fort Collins, CO, 80523, USA

^cDepartment of Materials Science and Engineering, Cornell University, Ithaca, NY, 14850, USA

^dMusculoskeletal Integrity Program, Hospital for Special Surgery, New York, NY, 10021, USA

^eDepartment of Biochemistry, Weill Medical College of Cornell University, New York, NY, 10021, USA

^fGraduate Program in Physiology, Biophysics, and Systems Biology, Weill Medical College of Cornell University, New York, NY, 10021, USA

Abstract

Osteoporosis and treatment may affect both composition and nanomechanical properties and their spatial distributions within the individual trabeculae of cancellous bone at length scales that cannot be captured by bulk measurements. This study utilized 25 mature adult ewes divided into 5 treatment groups. Four treatment groups were given a dietary model for human high-turnover osteoporosis, and two of these were treated with antiresorptive drugs, either zoledronate (ZOL) or raloxifene (RAL), to examine their effects on bulk tissue properties and nanoscale tissue composition and mechanical properties within trabeculae. Treatment effects were most pronounced at the nanoscale, where RAL increased indentation modulus and hardness throughout trabeculae by 10% relative to the osteoporosis model. In comparison, ZOL increased these properties exclusively at the surfaces of trabeculae (indentation modulus + 12%, hardness +16%). Nanomechanical alterations correlated with changes in tissue mineralization, carbonate substitution, crystallinity, and aligned collagen. Despite only minimal changes in bulk tissue tBMD, the nanomechanical improvements within trabeculae with both treatments greatly improved the predicted theoretical bending stiffness of individual trabeculae when idealized as cylindrical struts. Hence, small tissue-level alterations in critical locations for resisting trabecular failure could account for some of the discrepancy between the large reductions in fracture risk and the only modest changes in BMD with antiresorptive treatments.

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Corresponding Author: Marjolein C.H. van der Meulen, Address: 219 Upson Hall, Cornell University, Ithaca, NY 14853-7501, Telephone: (607) 255-1445, Fax: (607) 255-1222, mcv3@cornell.edu.

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Keywords

osteoporosis; bisphosphonate; SERM; composition; nanomechanical properties; cancellous bone

1. Introduction

One in three women and one in five men over the age of fifty will experience an osteoporotic fracture in their lifetime [1–3]. The risk for additional fractures increases markedly after experiencing the first fracture [4]. Antiresorptive agents and selective estrogen receptor modulators (SERMs) reduce fracture risk from 30–50%, but increase areal BMD only slightly by 0–8% [5–10], suggesting that BMD alone is unable to fully capture skeletal alterations with treatment. BMD reflects the total amount of bone mineral (and the overall level of mineralization) but cannot capture effects at smaller length scales where failure initiates. At these smaller length scales, skeletal fracture risk depends on tissue microarchitecture and properties of mineral and matrix constituents that can vary spatially within individual bone microstructures due to the remodeling process [11–18]. These spatial distributions are particularly important because small tissue property changes in critical locations for resisting trabecular failure can profoundly affect skeletal fracture resistance. Imbalances or alterations in the remodeling process with osteoporosis and treatment may alter levels and spatial distributions of mineral and matrix properties within bone microstructures and could play a crucial role in our understanding of fracture risk and our ability to develop and evaluate successful therapies.

The mineralization gradient within trabeculae of healthy human bone is positive from surface to center and produces a positive gradient in tissue stiffness [15,19–23]. The manner in which individual trabeculae bear load is influenced by these tissue-level properties and their arrangement within trabeculae. In postmenopausal women, osteoporosis reduces the overall heterogeneity of both tissue mineralization and mineral crystallinity [22,24,25], and bisphosphonate treatment further reduces heterogeneity in these properties [26–30]. However, the effects of osteoporosis and treatments on specific spatial distributions of compositional properties within trabeculae are not well-documented and may depend on treatment type. Mineral and matrix composition ultimately determine tissue mechanical function. Thus, any heterogeneity in tissue compositional properties results in nanomechanical heterogeneity that alters profiles of stress and strain within trabeculae and possibly affects the bone composite's ability to dissipate energy [14,17,18]. In healthy tissue, nanomechanical properties follow changes in mineralization and aligned collagen content [19,20,31]. Relationships between spatial compositional changes and the consequential nanomechanical alterations with osteoporosis and treatment have not yet been determined.

The goal of the present study was to compare spatial compositional and nanomechanical alterations in trabeculae with a large-animal model of osteoporosis and treatment. Two drugs were compared in this study. The first was a selective estrogen receptor modulator (SERM) that reduces turnover by acting through natural estrogen receptor pathways in bone and mimicking the effects of estrogen. The second treatment was from the widely-used bisphosphonate class of drugs that bind to bone matrix and directly inhibit osteoclastic activity upon resorption. SERMs and bisphosphonates reduce bone turnover to varying degrees yet produce similar reductions in fracture risk [32,33]. In a previous study, bisphosphonates altered collagen cross-linking in healthy female beagle vertebrae whereas SERMs did not [34]. SERMs improved vertebral mechanical performance independently of BMD in these same animals, suggesting that other changes in tissue composition might play an important role in fracture resistance [10,35].

Our hypotheses were that tissue stiffness and hardness would decrease in an ovine model of osteoporosis relative to healthy ewes due to alterations in tissue composition. Conversely treatment with either raloxifene (SERM) or zoledronate (bisphosphonate) would restore tissue nanomechanical properties through further alterations in tissue composition. Greatest effects were expected near surfaces of trabeculae in regions of active remodeling and bone formation. Additionally, nanoscale rather than bulk tissue measures were predicted to better capture these changes with treatment because of their better spatial resolution. As tissue age and the amount of turnover influence tissue properties [21, 22], Zoledronate was expected to alter these properties more markedly than raloxifene relative to the ovine model for osteoporosis because bisphosphonates reduce bone turnover to a greater degree than SERMs [33,36]. However, raloxifene treatment was expected to better restore cancellous tissue properties to those present in healthy, ovary intact female sheep, since SERMs act through natural estrogen receptor pathways [37].

2. Materials and Methods

The present study utilized a dietary-induced ovine model of osteoporosis and treatment to examine alterations in bone tissue compositional and nanomechanical properties, and changes in the spatial arrangements of these properties within trabeculae. The microstructure of sheep bone is similar to that of humans [38–42]. In a previous study, dietary-induced metabolic acidosis (MA) in sheep increased bone turnover and reduced bone mineral and whole bone strength similar to postmenopausal osteoporosis in humans [38,41,43]. In contrast, the ovine ovariectomy (OVX) model decreased bone turnover and caused osteomalacia [43], making MA the appropriate model choice for post-menopausal, high-turnover osteoporosis. Two different antiresorptive treatments were examined in this study: the SERM, raloxifene (RAL), and the bisphosphonate, zoledronate (ZOL).

2.1 Samples and Specimen Preparation

Femora were obtained from 25 mature adult Swiss-Ramboulet ewes (6 – 7 years old at the start of the experiment) from 2 experiments (approved by the Colorado State University Institutional Animal Care and Use Committee). Originally, the bisphosphonate and SERM treatments were planned as a single experiment; however, due to difficulty administering alendronate in the first study, the bisphosphonate study was repeated with zoledronate and the MA group for comparison. The first experiment had two groups. Both groups were fed a diet that induced compensated metabolic acidosis (MA) via the consumption anionic salts. Previously, this diet increased bone remodeling and decreased bone density as assessed by dual-energy x-ray absorptiometry [38,41,43] After an initial six months on the MA diet, the ewes continued this diet for another six months while receiving either vehicle (MA1, n = 5) or a clinically equivalent dose of raloxifene (RAL, 0.80 mg/kg daily, n = 4) administered daily through abomasal cannulae [41,43]. Both treatment groups started with n = 6 sheep, based on a power analysis for nanoscale measurements in a pilot study plus one additional sheep per treatment group in accordance with standard procedure for ovine experiments at Colorado State University. Two RAL sheep were euthanized early due to cannula pull-out and one MA sheep was euthanized due to injuries unrelated to the experiment. A group of healthy animals fed a normal diet (CONT, n = 5) were housed in the same facility and euthanized in the same season (summer) as Experiment 1. The second experiment had two treatment groups. Both groups received eight months of MA rather than six months due to a delay in receiving the medication for the study. Subsequently, one group continued on the MA diet for six months with no treatment (MA2, n = 6) and one group continued on the MA for six months with a clinically equivalent dose of zoledronate (ZOL, 5 mg/sheep, n = 6) at the beginning of the treatment period. Both groups were euthanized in the winter. The second experiment included a MA group to compare the effects of zoledronate treatment

with the ovine osteoporosis model, but did not include a normal diet control group. After euthanasia, femora were stored at -20°C prior to specimen preparation.

2.2 Bulk Cancellous Tissue Characterization

A single cylindrical core was taken from the medial-caudal quadrant of the distal femur from each sheep to characterize morphology and mechanical properties. Cores were excised with a diamond core drill (5 mm ID, Starlight Industries, Rosemont, PA) under constant irrigation with physiological saline, wrapped in saline-soaked gauze, and stored at -20°C prior to scanning.

2.2.1 Micro-Computed Tomography (MicroCT)—Tissue architecture within each cancellous core was quantified by microCT (55 kVp, 145 mA, 600 ms integration time, μCT 35, Scanco Medical, Bruttisellen, Switzerland). The axial central third of each core was scanned at $15\text{ }\mu\text{m}$ isotropic resolution. A calibrated HA standard was used to convert the linear attenuation for each voxel to g HA/ cm^3 . A 0.5 mm aluminum filter reduced the effects of beam hardening. A global threshold was chosen for all specimens. Outcomes included bone volume fraction (BV/TV), tissue mineral density (tBMD, mg/cm^3), and trabecular thickness and separation (Tb.Th and Tb.Sp, mm).

2.2.2 Compression Testing and Ashing—Bulk cancellous mechanical properties were assessed by compression testing of the bone cores. Prior to testing, press-fit brass end-caps were bonded to the ends of each core to minimize end artifacts [44]. The average gage length and diameter of the exposed core between endcaps was measured and used for subsequent stress and strain calculations. The load function consisted of 5 preconditioning cycles of 0 to 0.1% compressive strain before monotonically loading to 3% compressive strain at a rate of 0.5% strain/sec using a servohydraulic test system (Mini-Bionix 858, MTS Systems Corporation, Eden Prairie, MN). Displacement was measured with an extensometer attached across the endcaps (Model 634.11F-24, MTS Systems Corporation). The apparent compressive modulus (Young's modulus), yield strength, and ultimate compressive strength were determined for each core. The apparent compressive modulus was defined as the slope of the least-squares fit of the stress-strain data over 0.02–0.2% strain. The yield strength was determined using the 0.2% offset method, and the ultimate compressive strength was the maximum stress achieved [45]. Following compressive testing the bone cores were dried and heated to 600°C for 18 hours, and ash fraction was calculated as ash weight/dry weight [46].

2.3 Nanoscale Cancellous Tissue Characterization

For nanoscale characterization of compositional and nanomechanical properties, transverse cancellous sections were taken from the medial-cranial quadrants of the distal femora at the metaphysis, directly cranial to the cancellous cores excised for bulk tissue characterization. Sections were fixed, dehydrated, and embedded in polymethylmethacrylate [47]. A thin section ($3\text{ }\mu\text{m}$) was taken from the surface of each embedded bone block and mounted on a barium fluoride infrared spectroscopy window (Spectral Systems, Hopewell Jct., NY). The adjacent surface of the remaining block was polished anhydrously to a RMS surface roughness of less than 15 nm on a $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ AFM Scan (Dimension 3100, Veeco, Plainview, NY) [31,48,49].

Three trabeculae in each block and adjacent thin section were selected for characterization in the transverse plane. These trabeculae were chosen from a $1\text{ mm} \times 1\text{ mm}$ region in the caudal-most portion of the sample, nearest to the location of the cancellous cores excised for bulk tissue characterization. In both the blocks and adjacent thin sections, the mean width of selected trabeculae was greater than 70% of the mean Tb.Th computed for each animal

from the micro-CT measurements. This criterion ensured that selected trabeculae were sectioned near their midsection and not near their top or bottom edges, so tissue properties near trabecular surfaces and deeper within the trabeculae could be accurately probed. In the three trabeculae from the blocks, tissue nanomechanical properties and composition were probed at identical locations with nanoindentation, Raman spectroscopy, and second harmonic generation microscopy (SHG). In the blocks, the confocal mode of the SHG instrument was used to confirm that at least 60 μm of tissue was present below the surface of the sample. In the three trabeculae from the thin sections, tissue compositional parameters were assessed with Fourier transform infrared spectroscopy (FTIR). All nanoscale tissue property measurements were recorded in transverse lines across the trabeculae. For consistency, locations of remodeling at the time of euthanasia, visibly evident as regions of eroded trabeculae with scalloped surfaces, were avoided.

2.3.1 Nanoindentation—Tissue nanomechanical properties were assessed in a transverse line across the three trabeculae from the embedded bone block via nanoindentation. Indentations were made with a Berkovich diamond indenter at the center of each lamella (Hysitron, Inc., Minneapolis, MN) [50,51]. For accurate placement, surface topography scans (20 μm^2) were made with the scanning nanoindenter (TriboIndenter, Hysitron, Inc.) prior to each indentation [31,48–51]. The load function included a 10 second hold at 700 μN peak load with loading/unloading rates of 50 $\mu\text{N/s}$ [50]. The resulting indentation depth was approximately 150 nm. For nanoindentation, the depth of material below the indentation must be an order of magnitude greater than the indentation depth [52], i.e. 1.5 μm for our 150 nm-deep indentations. Even at the most superficial indentations, where less tissue was present due to the curvature of the trabecular surface, the tissue depth exceeded this requirement when measured by SHG.

Each trabecula contained a mean of 13 (± 3) lamellae, resulting in roughly 40 measurements per animal. Indentation modulus (E_i), a measure of tissue stiffness (i.e. elastic properties), and hardness, a measure of inelastic properties, were calculated from the unloading portion of the load-displacement curve and the mean pressure at maximum load, respectively [52].

2.3.2 Raman Spectroscopy—Tissue composition was characterized with Raman spectroscopy, as described previously [50], at the same lamellar locations characterized with nanoindentation (InVia microRaman, Renishaw, Gloucestershire, United Kingdom). The spot size was 2 μm^2 . For each measurement, Raman peak heights were identified from smoothed spectra after cubic polynomial background subtraction (Matlab, The Math Works, Inc., Natick, MA). Three bone parameters were calculated: mineral-to-matrix ratio (Raman mineral:matrix), B-type carbonate substitution, and crystallinity (Raman crystallinity) [50,53–58]. Mineral-to-matrix ratio, a measure of the degree of mineralization of the tissue, was calculated as the peak height ratio of the phosphate ν_1 ($\sim 965\text{ cm}^{-1}$) to the CH_2 wag ($\sim 1450\text{ cm}^{-1}$) peaks [50,54]. B-type carbonate substitution, a measure of the level of carbonate substitution for phosphate in the hydroxyapatite (HA) crystal lattice, was calculated as the peak height ratio of the carbonate ν_1 ($\sim 1070\text{ cm}^{-1}$) to the phosphate ν_1 peak [50,57]. Raman crystallinity, a measure of crystal size and perfection, was estimated as the reciprocal of the full width at half maximum (FWHM) of the phosphate ν_1 peak [50,56,58]. For this measure, a sharper phosphate ν_1 peak decreases FWHM and indicates greater mineral crystal size and perfection.

2.3.3 Second Harmonic Generation Microscopy (SHG)—Aligned collagen content within a longitudinal plane of the trabeculae was assessed via SHG microscopy as described previously [50] in the same trabecular regions studied with nanoindentation and Raman spectroscopy (Mai Tai Deep See, Spectra Physics, Santa Clara, CA; BX61W1, Olympus, Center Valley, PA). The pixel size was 0.318 μm^2 . Fiducial markers such as osteocyte

lacunae were compared with light microscopy images to co-locate SHG measurements with nanoindentation and Raman measurements.

The concentration of aligned collagen molecules (aligned collagen content) is equal to the square root of SHG intensity [59–63]. The aligned collagen content was calculated for a transverse line across each trabecula resulting in a profile of aligned collagen content with distance (Matlab, The Math Works, Inc.). The line was 6 pixels wide, corresponding to the Raman spot size of 2 μm . From this profile, maxima corresponding to lamellar aligned collagen and minima corresponding to interlamellar aligned collagen were recorded [31].

2.3.4 Fourier Transform Infrared Spectroscopy (FTIR)—Tissue composition was characterized in three trabeculae with FTIR spectroscopy, a complementary vibrational spectroscopy to Raman (Spectrum Spotlight 3000, Perkin Elmer Instruments, Waltham, MA) [51,64,65]. Due to the thin-section requirements for FTIR, these trabeculae were not the same as those characterized with the other techniques; however, FTIR sections were taken adjacent to the polished bone surfaces so anatomically similar areas were characterized. The pixel size was 6.25 μm^2 . Four outcome measures were calculated (ISYS 4.0, Spectral Dimensions, Olney, MD) [64,66]: Mineral-to-matrix ratio (FTIR mineral:matrix), a measure of tissue mineralization, was calculated as the integrated area of the phosphate peak from 916 – 1180 cm^{-1} over the area of the amide I mode from 1592 – 1712 cm^{-1} [67,68]; total carbonate substitution, a measure of all carbonate substituted in to the HA crystal lattice (substitution for phosphate and hydroxyl, and labile substitution) was calculated as the integrated area of the carbonate ν_2 peak from 840 – 892 cm^{-1} to the area of the phosphate peak [69]; FTIR crystallinity, a measure of stoichiometric to non-stoichiometric mineral content that correlates with c-axis line broadening as assessed by x-ray diffraction, was calculated as the ratio of the peak height sub-band at 1030 over the sub-band at 1020 cm^{-1} within the phosphate contour [70,71]; and finally, collagen cross-linking network maturity (XLR), a measure of the ratio of mature pyridinoline cross-links (PYR) to immature, reducible collagen cross-links (deH-DHLNL, deH-HLNL, and deH-HHMD) was calculated as the ratio of the peak height sub-band at 1660 to the sub-band at 1690 cm^{-1} [72]. From the FTIR images, the mean value of each parameter versus distance from the edge of the trabecula was calculated (Matlab).

2.4 Statistical Analysis

Statistical analyses were performed separately for Experiment 1 and 2, i.e. RAL was compared only with MA1 and CONT, while ZOL was compared only with MA2 (JMP Pro 9.0, Cary, NC). CONT animals were included with Experiment 1 for statistical analyses because they were housed in the same facility and euthanized shortly after the first experiment in the same season (summer). Experiment 1 and 2 analyses were performed separately due to the experiments being conducted separately and because animals were euthanized in the summer for Experiment 1 and winter for Experiment 2. In the sheep, BMD is constant during summer months; however seasonal variations can be present between summer and winter [73].

The effects of treatment on bulk tissue outcome measures (from microCT, compression testing, and ashing) were assessed with single-factor ANOVAs with Tukey post-hoc tests. All data satisfied the assumptions of the ANOVA models, the significance level was $p < 0.05$, and results stated are significant unless noted otherwise. Figures display mean values with error bars representing 95% confidence intervals.

2.4.1 Nanoscale Effects of Treatment and Region—To determine effects of treatment and distance from the edge of the trabeculae on nanoscale mechanical and

compositional properties from nanoindentation, Raman, SHG, and FTIR, each measurement was classified by region within the trabeculae: Superficial (Sup, < 20 μm from the trabecular edge), Intermediate (Int, 20 – 40 μm from the trabecular edge), and Central (Ctr, > 40 μm from the trabecular edge) [19,20,23,74,75]. Multi-factor, nested ANOVAs with Tukey post-hoc tests determined effects of treatment (CONT, MA, and RAL; MA2 and ZOL), region (Superficial, Intermediate, and Central), treatment*region, sheep (nested), and trabeculae (nested).

2.4.2 Correlations Between Compositional and Nanomechanical Parameters—

The contribution of compositional properties to alterations in tissue nanomechanical properties was determined by regression analysis. Raman and SHG but not FTIR compositional parameters were used for compositional correlations because Raman and SHG measurements were colocalized with nanoindentation measurements. The mean Superficial, Intermediate, and Central values for the compositional and mechanical parameters were calculated for each trabecula. Correlating these averaged values rather than each individual measurement location reduced variability from inherent heterogeneity of the bone tissue or slight misalignment between measurement locations.

Correlations were performed separately for Experiment 1 and Experiment 2. First, linear ANOVA models were performed for E_i and H vs. Raman mineral-to-matrix ratio, B-type carbonate substitution, Raman crystallinity, and lamellar aligned collagen. Subsequently, non-significant terms were removed from the model stepwise in order of decreasing p-value until only significant effects remained. Next, treatment and full interactions between compositional parameters and treatment were added to the ANOVA models. Non-significant terms were again removed in a stepwise fashion until only significant effects remained. For significant improvement in explanatory power, a minimum 5% increase in the (adjusted) R^2 value was considered practically meaningful.

3. Results

3.1 MA and Treatment

MA and treatment effects were more apparent at the nanoscale than at the bulk tissue level. MA did not differ significantly from controls for any of the bulk compression outcome measures (Figure 1), due at least in part to substantial variability between animals and small sample sizes. Bulk microCT demonstrated reduced tBMD with MA, with MA1 ewes having 3% lower tBMD than control animals (Figures 2). At the nanoscale, MA ewes had lower indentation modulus (–15%) and hardness (–13%) throughout trabeculae relative to control animals (Figure 3). Compositional measures at indentation locations showed reduced B-type carbonate substitution in Superficial (–11%) and Intermediate regions (–11%) in MA relative to control ewes (Figure 4). Complementary FTIR compositional measurements showed less total carbonate substitution throughout the trabeculae in MA sheep relative to controls, with the magnitude of the difference depending on region (Superficial –14%, Intermediate –8.3%, Central –12% vs. CONT; Figure 5). In addition, Superficial regions of MA trabeculae had lower FTIR mineral-to-matrix ratio (–8.1%), lower collagen cross-linking network maturity (–9.2%), and greater FTIR crystallinity (+3.4%) than that of controls (Figure 5).

Like MA, RAL treatment effects were greater at the nanoscale than at the bulk tissue level. The decreased bulk tBMD with MA was restored with RAL, with RAL-treated animals achieving tBMD levels equivalent to controls (Figure 2). At the nanoscale, indentation modulus (+9.6%) and hardness (+9.9%) increased with RAL compared to MA animals throughout trabeculae, but these properties were not fully restored to control levels (E_i –6.5%, H –4.6% vs. CONT; Figure 3). Measurements of tissue composition at indentation

locations showed that RAL did not alter the reduction in B-type carbonate substitution that occurred with MA (Figure 4); furthermore, total carbonate substitution was reduced even further with RAL over MA, with the magnitude of the decrease greater at Superficial regions than in deeper tissue (Superficial -36%, Intermediate -30%, Central -27% vs. CONT, Figure 5). RAL restored collagen cross-linking network maturity (XLR) to levels in controls, and increased FTIR mineral-to-matrix ratio (Superficial +9.2%, Intermediate +12%, Central +19% vs. CONT) and FTIR crystallinity (Superficial +6.6%, Intermediate +6.5%, Central +4.6% vs. CONT) throughout trabeculae, with the magnitudes of the increases varying by region. FTIR mineral-to-matrix ratio and crystallinity reached levels greater than those in control animals (Figure 5).

Treatment effects with ZOL relative to MA were different from those with Raloxifene relative to MA at both the bulk tissue level and the nanoscale. In bulk tissue ZOL decreased yield strain (-23%) and trended towards increased trabecular thickness (+ 30 μ m or 19%, $p < 0.06$) relative to MA animals (Figures 1 and 2). Unlike RAL, ZOL did not affect tBMD. Nanomechanical properties were altered exclusively in Superficial regions with ZOL, with these regions having greater indentation modulus (+12%) and hardness (+16%) than in MA animals (Figure 3). Nanoscale compositional measurements at indentation locations showed that Raman mineral-to-matrix ratio was also greater in Superficial regions with ZOL (+10%) than with MA, whereas B-type carbonate substitution increased (+4.7%) and aligned collagen content (lamellar -13%, interlamellar -12%) decreased throughout trabeculae with ZOL (Figure 4, 6). From the adjacent thin sections, FTIR mineral-to-matrix ratio, FTIR crystallinity, total carbonate substitution, and collagen cross-linking network maturity were altered with ZOL relative to MA (Figure 5), but percent changes were less than 3% and likely not physiologically meaningful although statistically significant.

3.2 Correlations between Compositional and Nanomechanical Parameters

For Experiment 1 (CONT, MA1, and RAL), indentation modulus and hardness correlated with B-type carbonate substitution, Raman crystallinity, and lamellar aligned collagen (Table 1, Figure 7). These three compositional measures explained 28% and 25% of the variation in indentation modulus and hardness, respectively. The predictive power for indentation modulus and hardness increased to ~35% when treatment and its effects on compositional relationships were included. When the contributions of each compositional parameter were examined individually, B-type carbonate substitution and Raman crystallinity were the strongest predictors of nanomechanical function and correlated positively with both indentation modulus and hardness. B-type carbonate substitution and Raman crystallinity, respectively, explained 20% and 10% of the variation in indentation modulus independent of treatment. In contrast, relationships between hardness and these two parameters varied between treatment groups. B-type carbonate substitution was only a significant predictor of hardness in MA and RAL ewes, both of which had lower levels of carbonate substitution than control animals. Raman crystallinity significantly predicted hardness only with RAL and accounted for 46% of the variation in hardness in these animals, the greatest explanatory effect measured.

The ANOVA models for Experiment 2 explained less of the variability in nanomechanical parameters than those for Experiment 1. Indentation modulus and hardness correlated positively with Raman mineral-to-matrix ratio and crystallinity. Hardness also correlated with lamellar aligned collagen. Composition accounted for 15% of the variability in indentation modulus and was not affected by treatment. Compositional parameters alone explained only 7% of the variability in hardness. Allowing the relationship between hardness and lamellar aligned collagen to vary with treatment increased the explanatory power to 12%. When the contributions of each compositional parameter to the ANOVA models were examined individually, Raman mineral-to-matrix ratio provided the most

explanatory power and predicted 11% of the variation in indentation modulus independent of treatment. Hardness also correlated with Raman mineral-to-matrix ratio, and though significant, this relationship explained only 2.4% of the variation in hardness and provided limited predictive utility.

Discussion

As hypothesized, raloxifene and zoledronate reversed changes in cancellous bone tissue properties in a large-animal model of human osteoporosis, and these improvements correlated with changes in composition. Bulk tissue properties such as tBMD were not sensitive enough to capture treatment effects, which became more apparent at the nanoscale and varied spatially within cancellous microstructures. Not surprisingly, nanoscale tissue property effects differed between the two treatments. Raloxifene reversed MA-induced losses in stiffness and hardness throughout the trabeculae but did not fully restore levels to those present in healthy ewes. Alterations in tissue mineralization, lamellar aligned collagen content, and Raman crystallinity accounted for one quarter to one third of the variation in nanomechanical parameters with MA and raloxifene. In comparison, zoledronate improved tissue stiffness and hardness over MA exclusively at the surfaces of trabeculae. Nanomechanical improvements with MA and zoledronate correlated with tissue mineralization, crystal size and perfection, and B-type carbonate substitution. In both experiments, the relationships between indentation modulus and compositional parameters were independent of treatment while relationships between hardness and compositional parameters differed between treatment groups.

The most pronounced treatment effects were expected at surfaces of trabeculae, regions where active remodeling and bone formation take place. Surface tissue is younger and less mineralized due to the remodeling process in cancellous bone and consequently has the greatest potential for gains in mineralization [21,76–78]. Tissue nanomechanical properties positively correlate with mineralization [50,76–79]. Additionally, bisphosphonates such as zoledronate target to bone matrix on trabecular surfaces where they directly inhibit osteoclast activity when resorbed by these cells [80–85]. Indeed, our results confirm zoledronate treatment effects were primarily on surfaces of trabeculae and locally increased mineral-to-matrix ratio followed by gains in tissue stiffness and hardness. These results confirm findings from 12 months of zoledronate treatment in an ovine OVX model [20].

In contrast, treatment effects with the SERM, raloxifene, were distributed throughout the thickness of the trabeculae and not limited to surfaces. Raloxifene acts as an estrogen agonist in bone and reduces turnover by binding to estrogen receptors and acting through estrogen response elements and coregulator proteins (IL-6, IL-7, TGF- β , etc.) [37,86]. Turnover is reduced less with raloxifene than with zoledronate and other bisphosphonates both in women with osteoporosis and animals with replete estrogen levels [10,33]. Bulk tissue measures in the current study showed no increase in trabecular thickness with RAL. Thus, gains in tissue stiffness and hardness throughout trabeculae were achieved by increased mineralization of existing trabecular tissue, as evidenced by the increase in FTIR mineral-to-matrix ratio throughout trabeculae.

Our results provide insight into a possible mechanism by which anti-resorptive treatments can reduce fracture risk despite only modest increases in BMD. If a single trabecula for each treatment were modeled as a perfect cylinder with average trabecular width and regional tissue stiffness as measured in these experiments, raloxifene and zoledronate would increase the theoretical trabecular bending stiffness over their corresponding MA groups by 60% and 110%, respectively. With raloxifene, the trabecular bending stiffness would still be 15% less than that of healthy trabeculae, but with zoledronate, trabecular bending stiffness would be

28% greater than that from a healthy sheep from Experiment 1. The greater increase in trabecular bending stiffness with zoledronate is attributable to both the trend towards greater trabecular thickness and the localized increase in tissue stiffness near surfaces of trabeculae. The trabeculae characterized in this experiment were all transverse to the primary (longitudinal) loading direction. During failure of cancellous bone, trabeculae transverse to the primary loading direction fail primarily due to bending while longitudinal trabeculae accumulate damage and fail predominantly by buckling due to compression [87]. Both bending and buckling are most influenced by the quantity and properties of tissue located farthest away from the center (or neutral axis) of the trabeculae. By increasing tissue stiffness near the surface, zoledronate targets the exact region most critical for resisting bending and buckling failure.

Although these effects within trabeculae did not translate to changes in the bulk tissue mechanical properties measured in this experiment, localized improvements with zoledronate treatment could account for some of the discrepancy between the small change in BMD and the much greater decrease in fracture risk observed clinically with bisphosphonate treatment. The bulk tissue samples of this study were tested in the primary loading direction, whereas many osteoporotic fractures occur due to falls transverse to the primary direction. Further study of transversely-loaded cancellous sections similar to a falling situation (and also longer-term treatment) may produce more pronounced effects at both the nanoscale and bulk-tissue levels.

The nature of mineral-to-matrix alterations differed between raloxifene and zoledronate, as evidenced by differences in Raman and FTIR compositional parameters. Zoledronate affected primarily the peak vibrations of phosphate ν_1 and CH_2 wag [50,54]. In contrast, raloxifene altered the entire phosphate ν_1 and Amide I vibrational modes [67,68] but had little effect on peak phosphate ν_1 and CH_2 wag vibrations. Zoledronate effects were most evident from Raman measurements made at peaks of lamellae, while raloxifene effects were most evident from SHG measurements that reflect both lamellar and interlamellar material due to the lower spatial resolution.

Previously, decreased carbonate substitution was associated with increased atomic order in synthetic apatites [88]. All types of carbonate substitution decreased with MA and raloxifene and indeed this corresponded with increased stoichiometric/non-stoichiometric crystallinity ratio measured by FTIR. Unlike raloxifene, zoledronate increased B-type carbonate substitution over MA but decreased the combination of A-type and labile carbonate substitution. As a result, the difference in total carbonate substitution with zoledronate was small and likely not physiologically significant.

The treatment-dependent relationships between hardness and compositional parameters suggest that the nature of mineral and matrix alterations differ between treatments and depend on additional factors not captured by the four compositional parameters correlated in this study. Furthermore, compositional correlations provided limited explanatory power for the nanomechanical parameters, particularly in Experiment 2, and also point towards contributions from aspects of mineral and matrix composition not measured here. Factors such as other non-enzymatic collagen cross-links, mineral crystal orientation, and non-collagenous proteins could also play a role in mechanical function. In addition, the nanoscale data from Experiment 2 fell in a narrow range, such that treatment and regional differences were on a scale not much larger than the biological variability.

The experimental design introduced several limitations that could affect the interpretation of the results. First, the sample size of the large animal model was adequately powered for nanoscale measurements that benefit from repeated measures but underpowered for bulk

tissue characterization. Additionally, large variations in bulk tissue measures between animals affected our ability to detect treatment effects; therefore, the lack of bulk-level effects reflects a combination of lower resolution and inadequate sample size. A posthoc power analysis showed that a three-fold increase in sample size was needed to measure significant changes in BV/TV and a two-fold increase for Tb.Th. Nonconcurrent performance of the two experiments was another limitation and prevented direct statistical comparison between all treatment groups. Ideally, controls would have been included as part of the experimental design; however, little effect was expected due to different treatment and euthanasia dates because control animals were housed in the same facility and euthanized in the same season. Additionally, performing statistical models without control animals did not significantly alter the results or predictive capability. The delay in administering zoledronate in Experiment 2 prolonged the MA period by two months. Also, animals in Experiment 2 were euthanized in a different season than Experiment 1 (winter vs. summer, respectively). Both factors could produce differences between the two MA groups at the start of the treatment period, which might influence drug effectiveness. Consequently, direct comparisons between Experiment 2 and both Experiment 1 and controls were avoided. The MA model presents another limitation because ewes were estrogen-replete, unlike post-menopausal osteoporotic patients. Notably, the presence of estrogen in the MA animals may have blunted the effects of raloxifene, since SERMs and estrogen act through the same estrogen receptor pathways [37,86].

The methods of analysis presented several additional limitations. First, newly formed tissue could not be identified. Previously, zoledronate increased mineralization and mineral crystallinity in newly formed tissue [89]. Future study of areas of visible resorption, identified by scalloped edges, and newly formed tissue, indicated by fluorochrome labeling, will allow full characterization of spatial nanoscale changes within trabeculae with treatment. Unfortunately, the nanoscale and bulk tissue measurements could not be performed in the same locations. However, the nanoscale characterization was performed directly adjacent (cranially) to the cancellous sample for the bulk tissue measures to minimize variability from anatomical location. Finally, FTIR measurements were not colocalized with other nanoscale measurements due to the thin-section requirement, limiting comparisons to trends rather than direct correlations among parameters.

In conclusion, both raloxifene and zoledronate improved cancellous tissue nanomechanical properties in the distal femur of a large-animal model for human high-turnover osteoporosis, and these improvements were attributable to changes in tissue composition. A companion study compares the FTIR changes in the distal femur to changes in other bones from the same animals [90]. In the present study, alterations were less evident at the bulk tissue level than at the nanoscale—the length scale at which failure initiates. Zoledronate improvements were localized to new tissue at surfaces of trabeculae, where the drug binds to bone matrix and directly reduces osteoclastic activity. In contrast, raloxifene acts through estrogen receptor pathways and improved tissue properties throughout the trabeculae. The nanoscale spatial alterations in composition and consequently nanomechanical properties resulted in improvements in estimated trabecular bending stiffness. As the fracture of cancellous bone ultimately arises due to the failure of individual trabecular elements, these findings may provide some explanation for the reductions in fracture risk with SERM and bisphosphonate treatment despite only modest gains in BMD.

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Abbreviations

BMD	bone mineral density
SERM	selective estrogen receptor modulator
MA	metabolic acidosis
CONT	control
RAL	raloxifene
ZOL	zoledronate
Sup	Superficial
Int	Intermediate
Ctr	Central
HA	hydroxyapatite
SHG	second harmonic generation
FTIR	Fourier transform infrared spectroscopy
Mineral	matrix, mineral-to-matrix ratio
XLR	collagen cross-linking network maturity
BV/TV	bone volume fraction
tBMD	tissue mineral density
Tb.Th	trabecular thickness
Tb.Sp	trabecular separation

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Highlights

- Raloxifene increases tissue stiffness and hardness throughout trabeculae.
- Zoledronate increases tissue stiffness and hardness only at surfaces of trabeculae.
- Nanomechanical gains correlate with increased mineral and carbonate substitution.
- Nano-scale, spatially-dependent changes were not measureable with bulk techniques.

Figure 1. Bulk tissue results from compression testing of cancellous cores. (A) Young's modulus, (B) ultimate stress, (C), yield stress, and (D) yield strain. Cores ashed after compression testing did not show any difference in ash content between treatment groups. * $p < 0.05$ Figures show mean $\pm$ 95% confidence interval.

Figure 2. Bulk tissue results from microCT of cancellous cores. (A) BV/TV, (B) tBMD, (C) Tb.Th., and (D) Tb.Sp. * $p < 0.05$ ** $p < 0.10$ Figures show mean $\pm$ 95% confidence interval.

Figure 3. Nanomechanical results. (A) Indentation modulus and (B) hardness for each region (superficial, intermediate, and central) by treatment. Raman measurements were made at nanoindentation locations. * = treatment effects for this experiment depended on region within the trabecula. C = different from Control for the region indicated. M = different from MA for the region indicated. S = different from Superficial within the treatment group indicated. I = Different from Intermediate within the treatment group indicated. $p < 0.05$. Figures show mean $\pm$ 95% confidence interval.

Figure 4.

Raman compositional results. (A) Raman mineral-to-matrix ratio, (B) Raman crystallinity, and (C) B-type carbonate substitution for each region (superficial, intermediate, and central) by treatment. Raman measurements were made at nanoindentation locations. * = treatment effects for this experiment depended on region within the trabecula. C = different from Control for the region indicated. M = different from MA for the region indicated. S = different from Superficial within the treatment group indicated. I = Different from Intermediate within the treatment group indicated. $p < 0.05$. Figures show mean $\pm$ 95% confidence interval.

Figure 5.

FTIR compositional results. (A) FTIR mineral-to-matrix ratio, (B) FTIR crystallinity, (C) total carbonate substitution, and (D) collagen cross-linking network for each region (superficial, intermediate, and central) by treatment. Raman measurements were made at nanoindentation locations. * = treatment effects for this experiment depended on region within the trabecula. C = different from Control for the region indicated. M = different from MA for the region indicated. S = different from Superficial within the treatment group indicated. I = Different from Intermediate within the treatment group indicated. $p < 0.05$. Figures show mean $\pm$ 95% confidence interval.

Figure 6. SHG results. (A) Lamellar aligned collagen and (B) interlamellar aligned for each region (superficial, intermediate, and central) by treatment. Raman measurements were made at nanoindentation locations. * = treatment effects for this experiment depended on region within the trabecula. C = different from Control for the region indicated. M = different from MA for the region indicated. S = different from Superficial within the treatment group indicated. I = Different from Intermediate within the treatment group indicated. $p < 0.05$. Figures show mean $\pm$ 95% confidence interval.

Figure 7. Measured values of indentation modulus and hardness from Experiment 1 (A and C) and Experiment 2 (B and D) plotted versus the predicted values from the ANOVA models including treatment and significant compositional parameters (Table 1). Relationships between nanomechanical and compositional parameters were allowed to vary between treatment groups.

Table 1

Coefficients of variation (R^2) for nanomechanical properties modeled as linear functions of Raman and SHG compositional parameters for each experiment.

Nanomechanical Parameter	Composition Only	Composition + Treatment	
Experiment 1	R^2	R^2	Significant Compositional Parameters
Indentation Modulus	0.28	0.34	B-type carbonate substitution Raman crystallinity Lamellar aligned collagen
Hardness	0.25	0.35	B-type carbonate substitution ⁺ Raman crystallinity ⁺ Lamellar aligned collagen
Experiment 2			
Indentation Modulus	0.15	0.15	Raman mineral:matrix Raman crystallinity
Hardness	0.07	0.12	Raman mineral matrix Raman crystallinity Lamellar aligned collagen ⁺

“Composition Only” indicates the explanatory power of the linear models with four compositional parameters: Raman mineral:matrix, B-type carbonate substitution, Raman crystallinity, and lamellar aligned collagen. “Composition + treatment” indicates the explanatory power of the models with the four compositional parameters, treatment, and the effects of treatment on the nanomechanical relationships with composition.

⁺ Indicates compositional parameters whose effects varied with treatment. An increase in R^2 0.05 is considered a meaningful improvement.