Published in final edited form as:

Bone. 2012 March ; 50(3): 596–604. doi:10.1016/j.bone.2011.12.012.

Changes in Intracortical Microporosities Induced by Pharmaceutical Treatment of Osteoporosis as Detected by High Resolution Micro-CT

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Abstract

Bone's microporosities play important biologic and mechanical roles. Here, we quantified 3D changes in cortical osteocyte-lacunae and other small porosities induced by estrogen withdrawal and two different osteoporosis treatments. Unlike 2D measurements, these data collected via synchrotron radiation-based μ CT describe the size and 3D spatial distribution of a large number of porous structures. Six-month old female Sprague-Dawley rats were separated into four groups of age-matched controls, untreated OVX, OVX treated with PTH, and OVX treated with Alendronate (ALN). Intracortical microporosity of the medial quadrant of the femoral diaphysis was quantified at endosteal, intracortical, and periosteal regions of the samples, allowing the quantification of osteocyte lacunae that were formed primarily before versus after the start of treatment. Across the overall thickness of the medial cortex, lacunar volume fraction (Lc.V/TV) was significantly lower in ALN treated rats compared to PTH. In the endosteal region, average osteocyte lacunar volume ($\langle \text{Lc.V} \rangle$) of untreated OVX rats was significantly lower than in age-matched controls, indicating a decrease in osteocyte lacunar size in bone formed on the endosteal surface after estrogen withdrawal. The effect of treatment (OVX, ALN, PTH) on the number of lacunae per tissue volume (Lc.N/TV) was dependent on the specific location within the cortex (endosteal, intracortical, periosteal). In both the endosteal and intracortical regions, Lc.N/TV was significantly lower in ALN than in untreated OVX, suggesting a site-specific effect in osteocyte lacuna density with ALN treatment. There also were a significantly greater number of small pores (5–100 μm^3 in volume) in the endosteal region for PTH compared to ALN. The mechanical impact of this altered microporosity structure is unknown, but might serve to enhance, rather than deteriorate bone strength with PTH treatment, as smaller osteocyte lacunae may be better able to absorb shear

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Disclosures

All authors have no conflict of interest.

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forces than larger lacunae. Together, these data demonstrate that current treatments of osteoporosis can alter the number, size, and distribution of microporosities in cortical rat lamellar bone.

Keywords

PTH; Alendronate; OVX; osteocyte lacunae; synchrotron micro-CT; cortical bone porosity

1. Introduction

In addition to decreases in the quantity of tissue present in the skeleton, inherent defects in the material can also result in an increased susceptibility to fracture. Current FDA approved pharmaceutical treatments of osteoporosis focus on retaining or improving bone mass by targeting the activity of osteoclasts (resorption) or osteoblasts (formation). Both anti-resorptive (e.g., bisphosphonates) [1–2] and anabolic (e.g., intermittent parathyroid hormone, PTH) [3–4] treatments for musculoskeletal diseases may influence not only bone's quantity but also its quality, ultimately improving (or compromising) bone's ability to resist load [5–6]. However, little is known about how osteoporosis treatments affect the immediate environment of the most abundant cell type in bone – osteocytes.

Osteocytes are the longest living cells in the skeleton and perform multiple functions associated with the regulation of bone remodeling. Osteocytes generally are thought to contribute to the regulation of bone remodeling in response to mechanical and micro-environmental changes by signaling to osteoblasts and osteoclasts [7–12]. In addition, mechanical and biochemical stimuli may cause osteocytes to directly contribute to the modulation of bone quality and quantity by directly remodeling its surrounding environment [13–14]. Therefore, these multi-purpose cells have potential as therapeutic targets for osteoporosis [15–17].

While the density, distribution, and geometry of osteocyte lacunae provide a time history of the 3D development of its surrounding mineral matrix, quantification of these variables is not entirely straightforward. Previous studies have reported changes in osteocyte lacunae and the cells that they contain. Evidence of osteolytic osteolysis in mice includes enlarged lacunae [18] and reduced radiodensity [19], suggestive of the ability of osteocytes to remodel its surroundings. However, current evidence is largely histological and radiographical, and thus limited by two dimensional methods. Irregular morphology, variability in spacing, and lack of consistent orientation of osteocyte lacunae are just a few of the technical challenges when using 2D measurements [20–21]. Most techniques to describe lacunar networks are either technically challenging or do not meet the required spatial resolution [22]. Therefore, advanced methods are necessary to understand the effect of 3D lacunar structures on bone quality.

The current standard evaluation of bone mass and architecture in small animal models is based on micro-computed tomography (μ CT). Although desktop μ CT scanners can provide critical information on the geometry of cortical and trabecular bone, at least in bones from small animals, small ($<10\ \mu\text{m}$) porosities within the tissue typically cannot be detected. Switching from a local x-ray source to a synchrotron source provides the brightness, collimation, and micro-focused beam needed to increase both the signal-to-noise ratio and spatial resolution and to allow for determining the size and geometry of osteocyte lacunae and vascular channels at about $1\ \mu\text{m}$ resolution [21, 23–24] in bones from rodents [25] to humans [22].

Here we combined conventional desktop μ CT with high-resolution synchrotron radiation-based μ CT to quantify changes in macro- and microscopic cortical bone structural properties induced by two different osteoporosis treatments in the ovariectomized (OVX) rat. Results from pharmaceutical interventions in the OVX rat model, such as bisphosphonates or PTH, generally match the effects of these agents in clinical trials in women with post-menopausal osteoporosis. In rats, bisphosphonates including the FDA-approved Alendronate [26], have been used successfully to stem the erosion of bone and restore bone strength by inhibiting bone cell activity and thus bone turnover [2]. In contrast, PTH injections, an anabolic treatment of osteoporosis, result in an increase in new bone formation by stimulating bone remodeling [27]. Little is known on whether these pharmaceutical treatments may modulate bone's internal structure. Here, we hypothesized that estrogen withdrawal will result in an increase in microporosities and that PTH treatment will induce subtle increases to the size and distribution of intracortical microporosities that previously may have gone undetected. Further, it was expected that Alendronate treatment will reduce the size and distribution of intracortical microporosities. Results from this study can offer critical information on how hormonal changes and current drug treatments alter a measure of matrix quality and, ultimately, may provide insight into why drug treatments have shown variable clinical effectiveness across different studies [3, 26, 28–30].

2. Materials and Methods

2.1. Experimental design

Six-month old female Sprague-Dawley rats were separated into either age-matched control (Ctrl), untreated OVX, OVX treated with subcutaneous injections of parathyroid hormone 1–34 (PTH, 15 μ g/kg/d), or OVX treated with subcutaneous injections of Alendronate (ALN, 100 μ g/kg/2x/wk). Rats in the OVX groups were ovariectomized at 5 months of age (4 weeks prior to starting the experimental protocol). To standardize environmental conditions, all rats were allowed access to a standard rodent chow (Purina Rodent Chow 5001) and water *ad libitum*, subjected to a 12-h light:dark cycle, and raised in individual cages in the same room. All rats were sacrificed at 12 months of age (n=6/group). The doses used in this study were derived from recent studies in rats [31–33]. They represent the therapeutic equivalent for the clinical treatment of osteoporosis extrapolated to the rat model [34–35] and previously were shown to have differential effects on bone quantity and quality [2]. All procedures were approved by the Stony Brook Institutional Animal Care and Use Committee.

The ovariectomized rat is currently the most studied animal model of post-menopausal osteoporosis. The focus of this study was to examine changes to specific aspects of bone quality during estrogen withdrawal and pharmaceutical treatment. The effects of OVX on rat long bones are well documented. The withdrawal of estrogen causes a rapid increase in bone turnover associated with a substantial decrease in bone mass [36–39]. Further, these previous studies did not find any effect of sham OVX surgery on bone mass. Therefore, no sham-surgery rats were included here. Treatment was administered for six months, a duration which ostensibly produces relatively large variation in bone quality parameters between groups [40–41].

Previous studies indicated that changes in bone morphology of OVX Sprague-Dawley rats treated with Alendronate and PTH for 2 months focus on trabecular rather than cortical bone [39, 42–44]. Nevertheless, the cortical mid-diaphysis was chosen as the focus of this study because in OVX rats, the contribution of cortical bone to whole-bone mechanical properties is greater than that of trabecular bone [45], allowing subtle changes in mineral density [46–49] or porosity [25] to have a large impact on bone's mechanical properties. Such bone

quality consequences of osteoporosis treatments may have previously been ignored because of insufficient μ CT resolution.

2.2 Macroscopic morphology via conventional desktop μ CT

After sacrifice, the left femoral diaphyses were preserved in 70% EtOH and scanned at a resolution of 36 μ m (55kV energy, 145 μ A intensity, 300ms integration time) using a desktop μ CT scanner (μ CT40; Scanco Medical AG, SUI) as described previously [50]. Mineral density was calibrated in units of mg HA/cc using a standard hydroxyapatite phantom (Scanco Medical AG). Cortical bone morphology and mineral composition including cortical thickness (Ct.Th), area (Ct.Ar), polar moment of inertia (J), and tissue mineral density (TMD) were determined for a 1.8-mm long volume of interest (VOI) at the mid-diaphysis.

2.3 Intracortical micro-porosity via synchrotron radiation μ CT

After scanning the femurs by conventional desktop μ CT, longitudinal bone strips were cut from the quartered diaphysis (Fig. 1) using a low-speed diamond wafer blade (South Bay Technology Inc.). Strips were 1mm $\times$ ~600 μ m $\times$ 5mm. To assess treatment-induced changes in intracortical microporosity, medial strips were scanned via high resolution, synchrotron radiation-based μ CT at beamline 2-BM at the Advanced Photon Source (APS) at Argonne National Laboratory.

Stacks of images were acquired at a photon energy of 20.98keV along the length of each cortical strip (image plane perpendicular to long axis of femur). The image stacks were generated by filtered back-projection from sets of 1500 projection images. Each projection image was exposed for 450ms and the angular rotation between each image was 0.12°. The reconstructed datasets consisted of approximately 2048 slices with a 2048 $\times$ 2048 in-plane data matrix, yielding a 750nm isotropic voxel size. Typical scan time was 25 minutes per specimen.

At the selected resolution, both large pores such as vascular canals and small pores such as osteocyte lacunae were readily visible (Fig. 2). The high signal-to-noise ratio and high resolution of the synchrotron μ CT provided clear boundaries between anatomical features for the algorithm, facilitating the segmentation of the images even in the presence of differences in tissue density. A sinogram-based algorithm removed ring artifacts which arose from defects on the scintillator of the optical system. Minimal noise within the reconstructed images was removed by a Gaussian filter. To avoid potential bias associated with differences in the degree of mineralization between groups, images were individually thresholded to segment bone tissue from porosity and soft tissue via a standard thresholding algorithm [51], rather than using a global threshold. This algorithm assumes that an image is composed of two classes of pixels – foreground and background. It then computes an optimal threshold value for each image that minimizes the intra-class variance. The resulting binarized images were inverted for the quantification of porosities.

All objects were evaluated in 3D using the Particle Analyser tool as part of the BoneJ plugin for ImageJ (v.1.43, NIH). We examined a stack of 896 reconstructed slices corresponding to a height of 675 μ m. Objects were classified as cannular or osteocyte lacunae based on size (Fig. 2). To decrease errors associated with the limits of spatial resolution, pores with a volume less than 5 μ m³ (maximum cross-sectional area of 2–3 pixels) were excluded. Cannular structures representing vasculature and/or bone remodeling units within cortical bone were classified as objects greater than 1,000 μ m³ in volume. The overall canal network was quantified by cannular indices as defined previously [25]. Parameters included canal volume (Ca.V) and canal volume density (canal volume divided by total cortical volume;

Ca.V/TV). Porosity comprising the osteocyte lacunar system was classified as objects 100–1,000 μm^3 in volume. For all specimens, approximately 99% of the osteocyte lacunae were 100–600 μm^3 . Osteocyte lacunar indices included number of lacunae (Lc.N), lacuna number density (Lc.N/TV), total lacuna volume (Lc.V), and lacuna volume density (Lc.V/TV), and average lacuna volume ($\langle\text{Lc.V}\rangle = \text{Lc.V}/\text{Lc.N}$). Percent porosity was defined as the total pore volume per total cortical volume. The number density of small pores ranging between 5–100 μm^3 in volume (Sm) were also quantified.

The spatial distribution of microporosity across the cortex of the transverse diaphysis was examined at its medial aspect to obtain differences in osteocyte lacunar structure in intracortical older bone (present prior to treatment) and younger bone at the periosteal and endosteal surfaces (formed after pharmaceutical treatment commenced). Three regions of interest (ROI) across the medial aspect of the diaphysis included the endosteal and periosteal surface as well as the central cortex (Fig. 1). The width of each ROI was defined as 20% of cortical thickness.

Comparisons in cross-sectional geometry between baseline (6-month old) and endpoint (12-month old) revealed that the intracortical bone present at the start of the study remained throughout the protocol. Differences in periosteal and endosteal diameter revealed that bone was formed on both surfaces during treatment (Fig. 3). On average, periosteal diameter was 3.31mm at 6-months of age and 3.65mm at 12-months. Bone marrow diameter measured 2.06mm at 6-months of age and 1.88mm at 12-months. Thus, the midshaft of the femur expanded in both directions, facilitating the separation of intracortical old bone from relatively new bone at the surfaces. The individual ROIs were approximately similar in width to the amount of bone added to both the periosteal and endosteal surfaces (Fig. 3).

2.4 Statistical Analysis

Non-parametric statistics were used to provide for more robust analyses given the relatively small sample sizes. To preserve statistical power, the number of comparisons between groups was limited. The primary analysis that directly tested for treatment effects and for differences between them compared ALN, PTH, and untreated OVX groups to each other via a non-parametric one-way ANOVA (Kruskal-Wallis) (GraphPad Prism 3.0). For instances where the Kruskal-Wallis found a significant difference between groups ($p < 0.05$), a Mann-Whitney test examined differences between specific groups. The secondary analysis testing for the effects of estrogen withdrawal compared untreated OVX to age-matched controls via a Mann-Whitney non-parametric test. Percent differences between groups were based on medians. Spearman's rho correlation coefficients were calculated to test for associations between cortical morphology, composition, and porosity. P-values for the interaction between location within the cortex (endosteal, intracortical, or periosteal) and treatment (OVX, ALN, PTH) were determined by two-way ANOVA (SPSS Statistics 17.0) because Q-Q plots confirmed data normality when including all 54 observations (6 rats * 3 treatment groups * 3 locations) for each trait. The significance level was set at 0.05.

3. Results

3.1 Animals and macroscopic morphology via desktop μCT

All rats tolerated treatment without apparent side-effects. There were no differences in body mass at baseline as well as at 12mo of age between any of the groups (Table 1). Conventional desktop μCT was used to test for differences in cortical morphology and tissue mineral density for effects of estrogen withdrawal and drug treatment. Compared to age-matched controls, untreated OVX had 1.9% lower ($p < 0.01$) tissue mineral density (TMD), but similar cortical thickness (Ct.Th), cortical area (Ct.Ar), and polar moment of inertia (J)

(Table 1). Comparing ALN and PTH treatments to untreated OVX revealed that ALN and PTH rats had 7% greater Ct.Th ($p < 0.01$; $p < 0.05$ respectively) compared to untreated OVX. ALN rats also had 1.5% higher ($p < 0.05$) TMD than untreated OVX. There were no significant differences in Ct.Ar or J between untreated OVX and treatment groups. Additionally, no significant differences in macroscopic porosity were detected between groups with conventional desktop μ CT (data not shown).

3.2 Overall intracortical microporosity via synchrotron μ CT

Improvement in spatial resolution and signal-to-noise ratio with synchrotron-based x-ray μ CT allowed the detection of bone microporosities. For the ROI that included the overall cortical thickness (Fig. 1), there were no significant differences between age-matched controls and untreated OVX in any porosity parameter measured (Table 2).

When comparing untreated OVX to PTH and ALN groups, lacunar volume fraction (Lc.V/TV), was 16% lower ($p = 0.02$) in ALN treated rats compared to PTH (Fig. 4). There were no significant differences in the number density (N/TV) of all pores, the number density of osteocyte lacunae (Lc.N/TV), cannular volume fraction (Ca.V/TV), or % porosity between groups (Table 2, Fig. 4). Across all groups, correlations showed that lacuna number density (Lc.N/TV) was inversely related to TMD (Spearman $\rho = -0.42$, $p < 0.01$) and positively related to Ct.Ar (Spearman $\rho = 0.41$, $p < 0.01$).

3.3 Region-specific micro-porosity via synchrotron μ CT

In the endosteal and intracortical ROI, there were no significant differences in any microporosity variable between age-matched controls and untreated OVX with the exceptions of the average lacuna volume ($\langle \text{Lc.V} \rangle$) in the endosteal ROI, which was 16% lower ($p < 0.01$) in untreated OVX than in age-matched controls and lacuna number density (Lc.N/TV) in the endosteal ROI, which was 29% higher ($p < 0.01$) in untreated OVX (Table 3).

Comparing untreated OVX to ALN and PTH groups demonstrated that Lc.N/TV was 28% ($p < 0.01$) and 14% ($p < 0.01$) greater in the endosteal and intracortical ROI of untreated OVX when compared to ALN (Fig. 5). Lc.V/TV was 43% greater ($p < 0.05$) in PTH than in ALN rats in the endosteal ROI and 22% lower ($p < 0.01$) in ALN compared to untreated OVX in the intracortical ROI (Table 3).

For the average osteocyte lacunar volume ($\langle \text{Lc.V} \rangle$), PTH was 20% greater ($p < 0.01$) than untreated OVX at the endosteal ROI. In the periosteal region, $\langle \text{Lc.V} \rangle$ was 18% lower ($p < 0.01$) in ALN compared to untreated OVX. In the endosteal ROI, % porosity was 45% higher ($p < 0.01$) in PTH compared to untreated OVX and 53% higher ($p < 0.01$) compared to ALN. In the periosteal ROI, % porosity was 38% higher ($p < 0.05$) in PTH compared to untreated OVX. Additionally, there was a 70% greater ($p < 0.01$) number of small pores in PTH than in ALN at the endosteal region.

To test whether the effect of treatment (OVX, ALN, PTH) on micro-porosities depended on the anatomical location (endosteal, intracortical, periosteal), a two-way ANOVA was performed. The p-value for the interaction between location and treatment was significant for Lc.N/TV, Lc.V/TV, $\langle \text{Lc.V} \rangle$, and % porosity (Table 4). Thus, the effect of treatment on the number of lacunae per tissue volume, lacunar volume fraction, average osteocyte lacunar volume, and % porosity was location dependent.

4. Discussion

Desktop and synchrotron radiation μ CT was used to volumetrically quantify differences in bone morphology, density and intracortical microporosity associated with estrogen withdrawal and different osteoporosis treatments in the OVX rat. OVX resulted in lower TMD compared to age-matched controls while ALN rats had higher TMD than untreated OVX. Both treatments resulted in greater Ct.Th compared to untreated OVX. Increasing scan resolution by an order of magnitude allowed the detection of site-specific differences in bone's microstructure between groups. Estrogen withdrawal resulted in smaller average osteocyte lacunar size in bone formed on the endosteal surface. Compared to untreated OVX, ALN treated rats had fewer osteocyte lacunae at endosteal and intracortical areas while PTH treated rats had larger average osteocyte lacunar size at the endosteal surface. PTH treated rats also had a higher lacunar volume fraction in bone formed during treatment compared to ALN treated rats. These data demonstrate the relatively subtle but significant changes that occur in the 3D internal structure of rat cortical bone with estrogen withdrawal and drug treatment. Together, they may serve towards a better understanding of the relationship between post-menopausal osteoporosis treatments, osteocyte biology, and bone quality.

The recent improvements in spatial resolution and throughput of 3D imaging provided the ability to identify and measure osteocyte lacunae and vascular canals and to relate their size and distribution to their development during estrogen withdrawal and pharmaceutical treatments of osteoporosis. The inverse relationship between TMD and porosity highlights the limitations of conventional μ CT, as TMD includes these smaller, undetected porosities. Still, there are limitations to the current study. Although aged rodents can have Haversian systems in cortical bone, the use of this model may be limited because of the predominant lamellar cortical structure and the lack of Haversian remodeling in young adult rats. Thus, it is unknown whether data from this study can be extrapolated to Haversian bone. Also, differences in cross-sectional geometry between baseline and 12-month old rats were used to estimate the thickness of bone formed after the start of treatment on periosteal and endosteal surfaces. As the width of the ROIs used to evaluate micro-porosities in the endosteal, periosteal, and intracortical regions was similar to the width of bone added to surfaces after treatment, each ROI should have contained predominantly either bone formed prior or post treatment. Nevertheless, future studies should use histological techniques to verify this assumption. Lastly, access to the beamline at the synchrotron is in high demand, restricting time and limiting the number of samples that can be scanned. Increasing the sample sizes and raising statistical power may enable the identification of additional group differences.

The size and number density of osteocyte lacunae reported in this study are generally consistent with findings from synchrotron light μ CT performed in mice [21, 25]. Across the cortex of the medial femoral diaphysis, PTH treatment resulted in a larger percent volume of lacunae compared to Alendronate, a change that could be attributed to osteocytic osteolysis [18–19]. Generally thought to contribute to the regulation of bone remodeling in response to mechanical and micro-environmental changes by signaling osteoblasts and osteoclasts [7–12], recent advances in osteocyte technology have provided evidence that osteocytes directly contribute to the modulation of bone quality by directly remodeling its surrounding matrix [13]. The osteocyte's role in mineral ion homeostasis through osteocytic osteolysis would result in changes to bone quality by subtly increasing lacunae and canaliculi size and altering the bone mineral matrix [52–53]. This especially may be true in a rodent model. With no (or very limited) osteonal remodeling, osteocyte remodeling could be a means of maintaining intracortical bone quality. Previous measurements of lacunar mineral uptake showed that osteocytic osteolysis is not sufficient for calcium regulation [54]. The surface area of the complete lacunar-canalicular network, however, makes a role in mineral

homeostasis ideal. Although changes to lacunar morphology may reflect changes to the mineral matrix surrounding osteocyte lacunae, synchrotron μ CT was not used to measure mineral density in this paper. Future studies, using a number of high-resolution imaging techniques (e.g., nano-CT, Fourier Transform Infrared, or electron microscopy) together with histologic techniques to assess the metabolic state of the osteocyte are needed to confirm osteocytic osteolysis as a way of altering the bone mineral matrix and to identify the interdependent links between treatments for osteoporosis, osteocyte biology, and bone quality.

The osteocyte lacunar network provides a history of the development of the surrounding mineral tissue. As osteoblasts secrete matrix, they become entombed by the mineral and transform into osteocytes. Thus, the location and number of osteocyte lacunae is an indicator of the development of the mineralized tissue. With the high spatial resolution provided by synchrotron light, intracortical microporosity was evaluated in areas of bone that were formed before and after treatment. The appearance of primary lamellar bone in the intracortical region was distinct compared to the lamellae visible near the bone surfaces. Thus, lacunae in the intracortical ROI were expected to be formed prior to the start of the treatment protocol. There were no significant differences between the different treatment groups in average lacunar volume in the intracortical ROI. Thus, the different pharmaceutical treatments for osteoporosis did not appear to affect *existing* lacunar size. In contrast, ALN treated rats had a significantly lower intracortical lacunar number density than untreated OVX rats suggesting a change in the number of osteocyte lacuna-size pores.

Inherently, any change to the internal micro-morphology of the matrix has the potential to modulate bone's mechanical properties and not surprisingly, intracortical pore size has been associated with bone's ability to absorb shear force [41]. Smaller pores may be better able to absorb shear forces than larger pores [41] and inability of the matrix to absorb shear force can result in microcracks, apoptosis, or initiate signals that elevate bone remodeling [55–56]. Here, we found a greater number of small pores in the endosteal region of PTH treated rats than in ALN rats. Thus, the increase in the number of smaller pores may be related to improved bone strength with PTH treatment.

Geometrical lacunar changes may not only directly influence bone's mechanical properties, but may also alter bone's sensitivity to mechanical signals as changes to the lacunar-canalicular geometry could affect fluid shear forces sensed by the cells [57–59]. Even with the increased spatial resolution provided by synchrotron based μ CT, canaliculi of individual osteocytes were not detectable. However, small porosities ($5\text{--}100\text{ }\mu\text{m}^3$ in volume) could be an indicator of changes to canalicular morphology, either in size or connectivity. Nano-CT at much higher resolutions ($\sim 50\text{nm}$) can determine the size and distribution of the canalicular processes of intracortical osteocytes [60], facilitating the quantification of canalicular morphology and perhaps clarify the origin of the differences in small porosities found between ALN and PTH treated rats.

Differences between the endosteal, intracortical, and periosteal regions of interest are consistent with previous studies on bone remodeling and drug treatment. The higher percent volume of lacunae in PTH-treated rats compared to ALN was a result of higher percent lacunar volume in the mineral matrix closest to the endosteal surface. Increased remodeling activity with PTH treatment can lead to larger and more active osteoblasts that remain larger when entombed by the mineral matrix [22]. Alternatively, the difference in percent lacunar volume between treatments could be the differential treatment effects on the formation or maturation of the perilacunar bone matrix, as previously observed in rats [22, 61–62]. Further, risedronate, another bisphosphonate, has been reported to reduce intracortical porosity in women with osteoporosis [63]. A reduction in remodeling with bisphosphonate

treatment would result in fewer osteocyte lacunae porosity as less active osteoblasts are recruited to remodeling sites. For the untreated OVX rats, the average osteocyte lacunar volume ($\langle \text{Lc.V} \rangle$) was significantly higher in the periosteal region compared to endosteal and intracortical, suggesting an increase in osteocyte lacunar space with estrogen withdrawal. Together, these data emphasize that altered levels of cellular activity can result in structural changes to the mineralized matrix.

Subtle changes to cortical bone material properties can have significant effects on whole bone mechanical properties [64]. Particularly, intracortical porosity has been linked to the stiffness and strength of cortical bone specimens from humans and other vertebrates. Furthermore, intracortical porosity increases the risk of femoral neck fractures, and can account for a 76% of the reduction in bone strength [25, 65]. Currently, intracortical microporosity is an often overlooked aspect of bone quality. It is possible that differences in the size and distribution of porosities may explain at least in part the variability in clinical effectiveness of pharmaceutical interventions of osteoporosis. Building upon the data presented here, future studies will need to investigate the contributions of microporosity to material level mechanical properties. Understanding how osteocytes contribute to the modulation of bone quality, both in the size and distribution of porosity and modulation of perilacunar matrix may represent an important step towards improving current osteoporosis treatments.

Three dimensional quantification of the size and distribution of intracortical microporosities provides insight into the influence of osteoporosis treatments on bone quality. Although previous studies have demonstrated changes to lacunar morphology with PTH treatment, 3D quantification of the size and distribution of lacunae during treatment had not been presented. Further, differences between anabolic and anti-resorptive treatments of osteoporosis were not assessed. Our data indicate that relatively subtle changes occur in the 3D internal structure of rat cortical bone with estrogen withdrawal and drug treatment. While estrogen withdrawal resulted in locally smaller average osteocyte lacunar size, ALN treated rats had fewer osteocyte lacunae and PTH treated rats had larger average osteocyte lacunar size at specific bone surfaces. These changes to intracortical microporosity due to estrogen loss or pharmaceutical intervention could contribute to the efficacy of both pharmaceutical and mechanical therapies for osteoporosis. By combining state of the art imaging modalities including high resolution μCT and nano-CT with techniques such as electron microscopy, immunohistological staining, and mechanical testing, osteocyte properties can be correlated with specific aspects of bone quality. Therefore, studies investigating how hormonal regulation and osteoporosis intervention affect osteocyte lacunae will further our understanding of how different osteoporosis treatments alter bone quality and bone strength.

Acknowledgments

Funding: National Institutes of Health (AR052778)

The authors thank the National Institutes of Health (AR052778) for their support of this research. Statistical advice from Dr. Jie Yang was greatly appreciated.

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Highlights

- We quantified the influence of two distinct pharmaceutical osteoporosis treatments on 3D osteocyte-lacunar porosity in the estrogen deprived rat.
- These data were collected via synchrotron radiation-based micro-CT and describe the size and 3D spatial distribution of porous structures.
- Estrogen withdrawal resulted in smaller average osteocyte lacunar size in bone formed on the endosteal surface.
- ALN treated rats had fewer osteocyte lacunae and PTH rats had larger average osteocyte lacunar size at specific bone surfaces.
- PTH treated rats also had a higher lacunar volume fraction in bone formed during treatment compared to ALN treated rats.

FIG. 1.

A. The femoral diaphysis was cut into longitudinal strips for each anatomical quadrant. Medial strips were scanned using synchrotron μ CT. **B.** Regions of interest near the endosteal and periosteal surface, and within intracortical bone were defined as 20% of total cortical thickness (white lines). The overall ROI encompassed the entire medial cortex (dashed line)

FIG. 2.

Images were thresholded and binarized to evaluate porosity. Pores were classified as small ($5\text{--}100\ \mu\text{m}^3$), lacunae ($100\text{--}1000\ \mu\text{m}^3$), and canals ($>1000\ \mu\text{m}^3$). The histogram of pores sizes of a representative sampled showed that 99% of lacunae had a volume of $100\text{--}600\ \mu\text{m}^3$.

FIG. 3.

Differences in cross-sectional geometry between baseline (6-months) and the 12-month endpoint. Periosteal, intracortical, and endosteal ROIs (dashed lines) evaluated bone that existed before (gray) and after (black) the start of treatment. Average dimensions of all samples: periosteal diameter of 12-month bone = 3.65mm; marrow cavity diameter of 12-month bone = 1.88mm; periosteal diameter of 6-month bone = 3.31mm; marrow cavity diameter of 6-month bone = 2.06mm; width of the ROIs = 20% of 12-month Ct.Th = 0.18mm.

FIG. 4.

Box-plots of **A)** the total number of pores per tissue volume (N/TV), **B)** the total number of lacunae per tissue volume (Lc.N/TV), and **C)** lacunar volume fraction (Lc.V/TV) across the cortical thickness of the medial femoral quadrant. Dashed line represents the median of age-matched controls. Lacunar volume fraction was 16% lower ($p < 0.05$) in ALN treated rats compared to PTH.

FIG. 5.

Box-plots of Lc.N/TV in the **A)** endosteal, **B)** intracortical, and **C)** periosteal ROIs. * Lc.N/TV was 28% ($p < 0.01$) and 14% ($p < 0.01$) greater in the endosteal and intracortical ROI of untreated OVX when compared to ALN. + Lc.N/TV in the endosteal ROI of the untreated OVX was significantly greater than age-matched controls ($p < 0.01$).

Table 1

Cross-sectional geometry and density of the femoral mid-diaphysis cortical bone measured using desktop μ CT.

	Ctrl	OVX	ALN	PTH
Body Mass [g]	427.0 (86.5)	456.5 (91.5)	460.5 (148.5)	449.5 (97.0)
Ct.Th [mm]	0.73 (0.06)	0.71 (0.09) ^{A,p}	0.76 (0.05) ^O	0.76 (0.07) ^O
Ct.Ar [mm ²]	6.58 (0.61)	6.73 (0.89)	6.64 (0.75)	6.81 (0.69)
J [mm ⁴]	14.0 (3.61)	15.5 (4.84)	14.4 (3.49)	15.2 (3.45)
TMD [mg HA/cc]	1026 (19) ^O	1007 (16) ^{C,a}	1022 (20) ^O	1010 (23)

Data are represented as median (interquartile range). A Mann-Whitney test was performed between Ctrl and OVX. A Kruskal-Wallis test was performed between OVX, ALN, and PTH. For each parameter that showed a significant difference with Kruskal-Wallis ($p < 0.05$), a Mann-Whitney test was performed to test for differences between specific groups.

^C different from Ctrl ($p < 0.01$).

^O different from OVX ($p < 0.01$).

^O different from OVX ($p < 0.05$).

^A different from ALN ($p < 0.01$).

^a different from ALN ($p < 0.05$).

^p different from PTH ($p < 0.05$).

Table 2

Porosity measured across the entire thickness of the medial quadrant of the femoral diaphysis (overall ROI).

	Ctrl	OVX	ALN	PTH
Lc.N/TV [mm ⁻³]	56,470 (13,710)	63,670 (14,110)	59,510 (9,370)	63,810 (9,420)
Lc.V/TV [%]	1.50 (0.25)	1.62 (0.53)	1.33 (0.29) ^p	1.58 (0.29) ^a
<Lc.V> [mm ³]	266.0 (43.3)	248.4 (44.9)	237.0 (47.2)	268.1 (18.3)
Ca.V/TV [%]	0.94 (0.16)	0.92 (0.16)	1.07 (0.17)	1.12 (0.44)
% porosity	2.40 (0.35)	2.50 (0.25)	2.45 (0.45)	2.70 (0.60)
Sm.N/TV [mm ⁻³]	11,660 (9,268)	11,410 (7,535)	10,180 (3,491)	15,160 (21,250)

Data are represented as median (interquartile range). A Mann-Whitney test was performed between Ctrl and OVX. A Kruskal-Wallis test was performed between OVX, ALN, and PTH. For each parameter that showed a significant difference with Kruskal-Wallis ($p < 0.05$), a Mann-Whitney test was performed to test for differences between specific groups.

^a different from ALN ($p < 0.05$)

^p different from PTH ($p < 0.05$)

Table 3

Porosity measured with synchrotron high-resolution CT in ROIs near the endosteal surface (Endo), intracortically (Intra), and near the periosteal surface (Peri).

Endo	Ctrl	OVX	ALN	PTH
Lc.N/TV [mm ⁻³]	43,370 (15,480) ^O	61,500 (7,410) ^{C,A}	48,030 (17,330) ^O	62,480 (16,840)
Lc.V/TV [%]	1.40 (0.44)	1.40 (0.22)	1.05 (0.49) ^P	1.50 (0.40) ^A
<Lc.V> [mm ³]	284.9 (98.9) ^O	238.0 (14.1) ^{C,P}	256.0 (60.4)	285.6 (37.9) ^O
Ca.V/TV [%]	0.77 (0.45)	0.90 (0.10)	0.92 (0.81)	1.53 (1.25)
% porosity	2.36 (0.53)	2.29 (0.18) ^P	2.17 (1.02) ^P	3.33 (1.52) ^{O,A}
Sm.N/TV [mm ⁻³]	13,450 (5,830)	10,950 (2,369)	8,608 (6,062) ^P	14,650 (31,100) ^A
Intra				
Lc.N/TV [mm ⁻³]	65,250 (15,020)	73,000 (7,840) ^A	63,990 (10,550) ^O	66,580 (16,520)
Lc.V/TV [%]	1.51 (0.64)	1.79 (0.34) ^A	1.39 (0.39) ^O	1.59 (0.50)
<Lc.V> [mm ³]	272.1 (32.2)	258.0 (26.3)	230.7 (49.2)	258.5 (23.0)
Ca.V/TV [%]	0.94 (0.31)	0.88 (0.21)	0.97 (0.63)	0.98 (0.64)
% porosity	2.74 (0.66)	2.77 (0.33)	2.38 (0.63)	2.36 (0.98)
Sm.N/TV [mm ⁻³]	16,410 (19,730)	16,000 (8,340)	12,870 (4,720)	20,610 (27,430)
Peri				
Lc.N/TV [mm ⁻³]	47,000 (8,430)	49,500 (12,830)	64,020 (10,290)	57,400 (27,870)
Lc.V/TV [%]	1.45 (0.15)	1.51 (0.44)	1.63 (0.25)	1.74 (0.52)
<Lc.V> [mm ³]	302.6 (71.3)	317.5 (51.7) ^A	261.5 (46) ^O	273.2 (72.3)
Ca.V/TV [%]	0.41 (0.29)	0.32 (0.23) ^A	0.80 (0.32) ^O	0.77 (0.88)
% porosity	1.89 (0.19)	1.84 (0.64) ^P	2.45 (0.51)	2.53 (0.99) ^O
Sm.N/TV [mm ⁻³]	19,210 (17,990)	17,050 (25,480)	19,110 (14,120)	24,540 (16,430)

Data are represented as median (interquartile range). A Mann-Whitney test was performed between Ctrl and OVX. A Kruskal-Wallis test was performed between OVX, ALN, and PTH. For each parameter that showed a significant difference with Kruskal-Wallis ($p < 0.05$), a Mann-Whitney test was performed to test for differences between specific groups.

^C different from Ctrl ($p < 0.01$).

^O different from OVX ($p < 0.01$).

^O different from OVX ($p < 0.05$).

^A different from ALN ($p < 0.01$).

^P different from PTH ($p < 0.01$).

^P different from PTH ($p < 0.05$).

Table 4

P-values resulting from the two-way ANOVA testing the effects of location (Endo, Intra, Peri), treatment (OVX, ALN, PTH), and the interaction between location and treatment on micro-porosities.

	Location	Treatment	Location $\times$ Treatment
Lc.N/TV	0.001	0.389	<0.001
Lc.V/TV	0.002	0.006	0.002
<Lc.V>	0.002	<0.001	0.035
Ca.V/TV	0.001	<0.001	0.074
% porosity	0.005	0.025	0.002
Sm.N/TV	0.025	0.053	0.817