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Intraskelatal variation in human cortical osteocyte lacunar density: Implications for bone quality assessment

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ABSTRACT

Osteocytes and their lacunocanalicular network have been identified as the regulator of bone quality and function by exerting extensive influence over metabolic processes, mechanical adaptation, and mineral homeostasis. Recent research has shown that osteocyte apoptosis leads to a decrease in bone quality and increase in bone fragility mediated through its effects on remodeling. The purpose of this study is to investigate variation in cortical bone osteocyte lacunar density with respect to major factors including sex, age, and intracortical porosity to establish both regional and systemic trends. Samples from the midshaft femur, midshaft rib and distal one-third diaphysis of the radius were recovered from 30 modern cadaveric individuals (15 males and 15 females) ranging from 49 to 100 years old. Thick ground undecalcified histological (80 μm) cross-sections were made and imaged under bright field microscopy. Osteocyte lacunar density (Ot.Lc.N/B.Ar) and intracortical porosity (%Po.Ar) were quantified. No significant sex differences in Ot.Lc.N/B.Ar or %Po.Ar were found in any element. Linear regressions demonstrated a significant decrease in osteocyte lacunar density (Ot.Lc.N/B.Ar) and increase in intracortical porosity (%Po.Ar) with age for the sex-pooled sample in the femur ($R^2 = 0.208, 0.297$ respectively) and radius ($R^2 = 0.108, 0.545$ respectively). Age was unable to significantly predict osteocyte lacunar density or intracortical porosity in the rib ($R^2 = 0.058, 0.114$ respectively). Comparisons of regression coefficients demonstrated a systemic trend in the decrease in osteocyte lacunar density (Ot.Lc.N/B.Ar) and increase in intracortical porosity (%Po.Ar) with age. In each element, intracortical porosity was significantly negatively correlated with lacunar density for which the radius demonstrated the strongest relationship ($r = -0.746$). Using pore number (Po.N) as a proxy for available vascularity to support the osteocyte population, Po.N was able to predict 61.8% of variation in osteocyte lacunar number (Ot.Lc.N) in the rib. The femur and radius also demonstrated significant relationships between these variables ($R^2 = 0.560$ and 0.397 respectively). The results from this study indicate that although the femur, radius and rib may be experiencing systemically influenced declines in osteocyte lacunar density, there may be differential effects at each anatomical site potentially due to age related changes in mechanical loading. With decreasing osteocyte lacunar density in each element, intracortical porosity increased with likely direct impacts on gross bone strength. This study provides a foundation upon which to build interpretations of osteocyte lacunar density values and their effect on differential fracture risk for aging individuals.

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1. Introduction

Bone strength, or the ability to resist fractures and successfully incur regular insults while functioning properly, is determined by its composition and structure (Brandi, 2009; Currey, 2003; Seeman and Delmas, 2006) and better conceptualized as “bone quality.” Moving beyond solely considering measures of bone mass, research into the multi-faceted and hierarchical components of bone quality have increasingly included factors affecting the cellular machinery of bone. In fact, increasing age has been shown to increase fracture risk independently from measured

bone mass or mineral density (Nicks et al., 2012; Seeman, 2007) supporting a paradigm shift in the way in which bone quality is quantified. Determining factors of bone quality have been expanded to include not only mass, but also microarchitecture, material properties of the extracellular matrix, microdamage accumulation, osteocyte density, and remodeling rate (Burr, 2014; Burr and Akkus, 2014). Recently, another paradigmatic shift towards the importance of cortical microstructure in fracture resistance has been introduced (Agnew and Bolte, 2011; Nicks et al., 2012; Seeman, 2015). In order to better elucidate bone quality and quantify fracture risk for individuals at any age, the field must move beyond a “trabeculo-centric” view (Seeman, 2015). This manuscript investigates variation in one of the multi-factorial and hierarchical aspects of cortical bone maintenance and fracture resistance: the osteocyte.

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The mechanism by which the physiological (both mechanical and systemic influences) environment affects change (i.e., functional adaptation) in cortical bone is orchestrated by the osteocyte lacunocanalicular network conducting a cellular team towards maintaining bone quality and preventing gross failure. Osteocytes are ideally situated to integrate both metabolic (systemic) stimuli (Bellido and Hill, 2014; Bellido et al., 2013) and mechanical stimuli as the mechanotransducers of the skeletal system (Han et al., 2004; Klein-Nulend et al., 2013; Nicoletta et al., 2006; Schaffler et al., 2014) maintaining their local microenvironment and in turn the integrity of the bone as a whole (Bonewald, 2007, 2011; Seeman, 2006). Recently, Jilka and O'Brien (2016) provided an extensive review concerning the pivotal role of osteocyte control on age related bone loss. In order to govern bone functional adaptation, there must be adequate osteocyte cell numbers in any given bone (Ma et al., 2008; Vashishth et al., 2002). Osteocyte apoptosis, classified as a form of tissue damage itself (Seeman, 2006), can result from a multitude of endogenous and exogenous factors and can lead to increased fracture risk by an overall decrease in bone quality (Ma et al., 2008; Noble and Reeve, 2000; Noble et al., 1997). Systemic factors including increasing endogenous oxidative stress and glucocorticoids, and decreasing levels of sex hormones with increasing age have all been shown to have a pro-apoptotic effect on osteocytes (Almeida, 2010; Bellido and Hill, 2014; Bonewald, 2011; Jilka et al., 2013; Piemontese et al., 2015; Tomkinson et al., 1997). In addition to the systemic factors presumably affecting the global osteocyte population, maintaining site specific physiological or optimal mechanical loading, is essential to osteocyte viability (Aguirre et al., 2006; Hughes and Petit, 2010). Optimal levels of mechanical strains are necessary for movement of interstitial fluid throughout the lacunocanalicular network responsible for delivery of nutrients to the entombed cell population (Hughes and Petit, 2010; Jilka et al., 2013) and has demonstrated a protective mechanism to mitigate adverse systemic factors (Bonewald and Johnson, 2008). Disuse and/or linear microcracks interrupting fluid flow can result in hypoxia and apoptosis (Burr, 2014; Frost, 1960a; Jilka et al., 2013; Kennedy et al., 2014; Verborgt et al., 2000). The lacunar space occupied by viable osteocytes is maintained until apoptosis, upon which time either targeted removal and repair by a basic multicellular unit (BMU) is initiated (Burr, 2014; Cardoso et al., 2009) or the empty lacuna is filled with mineralized tissue (Frost, 1960b). Thus, the existence of the lacunae is dependent on the viability of its occupant (Knothe Tate et al., 2004). Apoptotic osteocytes signal surviving neighboring osteocytes to release RANKL to signal initiation and direct a targeted intracortical remodeling event (Kennedy et al., 2012). These remodeling events result in new intracortical porosity as well as supplying a new complement of osteocytes embedded in the recently laid down matrix. Thus, as the bones' mechanosensing cell, osteocytes conduct the adaptive response to its loading environment by translating mechanical signals into chemical signals affecting bone metabolism, but are themselves dependent on a customary strain level of mechanical loading to properly function.

Changes in bone strength and resulting differential fracture risk are likely due to site-specific changes in the cortex of susceptible bones rather than global changes affecting all skeletal envelopes equally (Thomas et al., 2006). To investigate the effects of such influences as chronological age and sex on changes in osteocyte lacunar density and ultimately how this contributes to differential fracture risk across the skeleton, it is imperative to establish intra-individual and inter-individual variation from all skeletal envelopes of comparable anatomical locations. This study is the first to attempt to establish a baseline understanding of intra-skeletal variation in osteocyte lacunar density in human cortical bone. Osteocyte lacunar density has often been used as a proxy for osteocyte cell density in both cortical and trabecular bone (Bach-Gansmo et al., 2015, 2016; Miskiewicz, 2016; Qing et al., 2012; Teti and Zallone, 2009). Before reported patterns of decreasing density with age or variation between sexes can be translated to tangible effects on fracture risk, a systematic investigation into basic factors

affecting variation in osteocyte density must be conducted. Thus, the goals of this study were two-fold: First, we investigated systemic variation in cortical osteocyte lacunar density between multiple anatomical sites as it relates to influential factors, specifically chronological age and sex. This study is the first to analyze systemic human cortical bone variation in osteocyte lacunar density represented by three anatomical sites experiencing varying mechanical stimuli to establish age and sex related trends. Second, by initiating and controlling the components of the basic multicellular unit (BMU), osteocyte density has a direct impact on intracortical porosity; a relationship we are the first to explore across the entirety of the cortex at multiple skeletal sites with implications for bone quality and fracture resistance.

2. Materials and methods

2.1. Samples

Skeletal samples were obtained from modern embalmed post-mortem human subjects (PMHS) received through The Ohio State University's Whole Body Donor program. A total of 30 individuals of known demographics were chosen as representative of the population for which fracture risk assessments are routinely performed. The total sample ranged in age from 49 to 100 years old including 15 males (mean age of 77.8 ± 13.45 years) and 15 females (mean age of 75.87 ± 11.89 years). Cause of death was not a determining factor as these individuals were experiencing a myriad of conditions which may influence osteocyte viability systemically and/or mechanically as a consequence of lifestyle variations. The only exclusion criterion was evidence of macroscopic changes to the skeletal elements of interest which could have included healed or active infections, prosthetics, or gross evidence of bony metastases.

Each PMHS was represented by three anatomical locations: midshaft femur, distal one-third of the diaphysis of the radius, and midshaft of the 6th rib; samples were obtained from each location in 2 cm blocks. These sites were chosen due to their varying loading environments and clinical significance. The femur represents a weight bearing bone which may experience age related changes associated with activity decline (Robling and Stout, 2003). The rib experiences a consistent loading environment from pulmonary ventilation independent of age and is often used as an indicator of systemic effects on skeletal metabolism within an individual (Agnew and Stout, 2012; Eleazer and Jankauskas, 2016). Lastly, the cortex of the distal one third of the diaphysis of the radius undergoes an intermediate amount of variation in mechanical loading with advancing age and is an important clinical site for age-associated fragility fractures (Court-Brown and Caesar, 2006). The total sample consisted of 30 femoral and radius sections and 29 rib sections (ribs were unavailable for one PMHS).

Each skeletal block was macerated, cleaned of remaining soft tissue and marrow, and de-greased. Ribs were embedded in epoxy resin to prevent damage to the fragile cortex during sectioning, neither femoral nor radius sections were embedded. Undecalcified histological sections were prepared using standard techniques (Maat et al., 2001). Thick sections were cut using an Isomet saw (Buehler, IL) and ground to a uniform thickness of $80 \mu\text{m}$ to reduce any lacunar density quantification errors attributable to variability in section thickness. The resulting 89 sections were mounted to glass slides using Permount. Imaging of slides was performed with CellSens dimension software on an Olympus VS120 slide scanner at $40\times$ magnification under bright field light for visualization of osteocyte lacunae.

2.2. Data collection

Composite images were used to quantify basic histomorphometric parameters as well as osteocyte lacunar data across each cross-section (Table 1). Due to their size, femoral total subperiosteal area (Tt.Ar) and endosteal area (Es.Ar) were measured using ArcGIS version 10.1

(ESRI© 2012) which has been introduced as a valuable analytical tool for bone histomorphometry (Rose et al., 2012). All other variables for each anatomical element were measured or calculated using ImageJ software (NIH; Rasband, 2013). In order to maximize the ability of ImageJ to automatically detect osteocytic lacunae and measure intracortical porosity, the variation in bone color within and between samples was addressed through image manipulation within ImageJ. Contrast between the extracellular matrix and the lacunae, Haversian canals, resorption spaces, and Volkmann's canals was increased prior to thresholding to produce a binary image. Each cross-section was manipulated and thresholded consistently resulting in a binary image demonstrating “white” extracellular matrix and “black” pores (see Fig. 1).

To quantify cortical osteocyte lacunar density for the entire cross-section an automated procedure in ImageJ was utilized. A pilot study was performed to assess the capacity of ImageJ to automatically identify and count osteocytic lacunae (Ot.Lc.N) accurately. Using a subsample of ten radii, the previously described image manipulation and subsequent automated procedures were performed for each entire cross-section. To automatically quantify Ot.Lc.N, the “Analyze Particles” function in ImageJ was used. The command searches the image for the edges of an “object” with specified size and shape parameters, outlines the encountered object, measures it, adds it to the ROI manager, and then proceeds to scan the rest of the image repeating the process to identify each “object”. Size and shape parameters were held constant so Ot.Lc.N output for each cross-section included all lacunae and excluded other pores to be later included in intracortical porosity (Po.Ar) measures. Additionally, Ot.Lc.N in each total cross-section was manually point-counted in ImageJ for direct comparison with automated counts. No significant differences ($p = 0.725$; %error < 3.45%) in Ot.Lc.N were found between counting methods. Therefore, to reduce potential observer error and standardize the procedure, Ot.Lc.N was quantified using the automated method described here for all 89 cross-sections.

Intracortical porosity was quantified for each cross-section using a semi-automated method in ImageJ, reported to produce the same results as manual calculation of total porosity area (Cole and Stout, 2015). For this study, binary images used for Ot.Lc.N quantification were used to count pores (Po.N) comprised of Haversian canals, resorption spaces, and Volkmann's canals and to measure porosity area (Po.Ar) within the cortex. Using the “Colony Blob Count Tool” and appropriate size and shape parameters, ImageJ counted and measured the area of all identified pores. The entire cross-section was manually checked by a histomorphometrist (RLH) to verify the program's ability to accurately identify pore edges, and alterations were made as appropriate. The total Po.Ar for the section was subsequently subtracted from cortical area (Ct.Ar) to calculate B.Ar (see Table 2) for a true measure of 2D bone quantity within the cross-section. Comparisons of intracortical porosity required normalization, thus percent porosity area (%Po.Ar) was calculated to investigate its relationship with osteocyte lacunar density for each anatomical element.

Bone areas (Ct.Ar or B.Ar) were used to control for size when investigating the effect of age and sex on osteocyte lacunae at each

anatomical site. Bone area (B.Ar) was incorporated into calculation of osteocyte lacunar density (Ot.Lc.N/B.Ar) for all regional and systemic analyses. However, to quantify the relationships between osteocyte density and intracortical porosity at each respective skeletal site, cortical area (Ct.Ar), which does not take into account intracortical porosity, was used to calculate osteocyte lacunar density (Ot.Lc.N/Ct.Ar).

2.3. Statistical analysis

Power was assessed a priori using G*power statistical software (Faul et al., 2009) and achieved using a sample size of 30 PMHS. All other data analyses were performed using SPSS (v.22) statistical software (IBM, 2013). Normalized variables (%Po.Ar, Ot.Lc.N/Ct.Ar and Ot.Lc.N/B.Ar) were tested for sex differences using independent samples *t*-tests for each of the three anatomical elements. ANOVA with Bonferroni post-hoc test was performed to assess intra-individual variation in Ot.Lc.N/B.Ar. For the rib, femur and radius, linear regressions were performed to investigate the impact of age on Ot.Lc.N/B.Ar and %Po.Ar. The interrelatedness between intracortical porosity (%Po.Ar) and osteocyte lacunar density (Ot.Lc.N/Ct.Ar) for each element were investigated using Pearson correlation coefficients for each element. Furthermore, normalized counts of pore number, as a proxy for available vascularity, was tested to predict osteocyte lacunar density using linear regressions. Significance was determined for all tests using an a priori alpha level of 0.05.

3. Results

For the femur, independent sample *t*-tests indicate no significant differences between males and females in Ot.Lc.N/B.Ar ($t(28) = 1.05$; $p = 0.30$) or intracortical porosity (%Po.Ar) ($t(28) = -0.33$; $p = 0.75$). Additionally, there were no significant sex differences in rib lacunar density ($t(28) = -0.79$; $p = 0.44$) or intracortical porosity ($t(27) = 0.06$; $p = 0.95$). Similarly, in the radius, male and female lacunar density were not statistically different ($t(28) = 0.40$; $p = 0.69$) nor was intracortical porosity ($t(28) = -0.43$; $p = 0.67$). Consequently, all further analyses were performed on the total sample including males and females for femora ($n = 30$), ribs ($n = 29$), and radii ($n = 30$).

Shapiro-Wilk tests revealed a normal distribution for all variables at all three anatomical sites. Descriptive statistics are provided in Table 2 for osteocyte lacunar density and intracortical porosity in the total sample. The largest amount of variation in Ot.Lc.N/B.Ar is found in the midshaft femur followed by the radius. Lastly, the least amount of variation in Ot.Lc.N/B.Ar for these three elements was found in the midshaft rib (Fig. 2).

ANOVA revealed significant intra-individual (i.e., systemic) variation in osteocyte lacunar density ($F(1,28) = 7.97$; $p < 0.001$). Bonferroni post-hoc examination indicated the radius had a significantly higher density (1074.50 mm^{-2}) than the rib or femur ($p = 0.003$), which were not significantly different ($p = 1.00$) from each other at 939.23 mm^{-2} and 939.80 mm^{-2} , respectively. An ANOVA comparing the amount of intracortical porosity (%Po.Ar) between the rib, radius

Table 1
Variables of interest.

Variable	Abbreviation	Description	Equation
Total subperiosteal area	Tt.Ar	Total area within periosteal border (mm^2)	Measured
Endosteal area	Es.Ar	Area of the marrow cavity (mm^2)	Measured
Cortical area	Ct.Ar	Area of cortical bone between periosteal and endosteal surfaces (mm^2)	$Tt.Ar - Es.Ar$
Pore number	Po.N	Total number of pores within cortex	Measured
Porosity area	Po.Ar	Total area of intracortical porosity (mm^2)	Measured
Percent porosity area	%Po.Ar	Relative area of intracortical porosity (%)	$Po.Ar / Ct.Ar * 100$
Bone area	B.Ar	Total area of cortical bone present (mm^2)	$Ct.Ar - Po.Ar$
Osteocyte lacunar number	Ot.Lc.N	Total number of osteocyte lacunae in cortex	Measured
Osteocyte lacunar density	Ot.Lc.N/B.Ar	Relative to amount bone present in cortex ($\#/ \text{mm}^2$)	$Ot.Lc.N / B.Ar$
Osteocyte lacunar density	Ot.Lc.N/Ct.Ar	Relative to cortical area ($\#/ \text{mm}^2$)	$Ot.Lc.N / Ct.Ar$

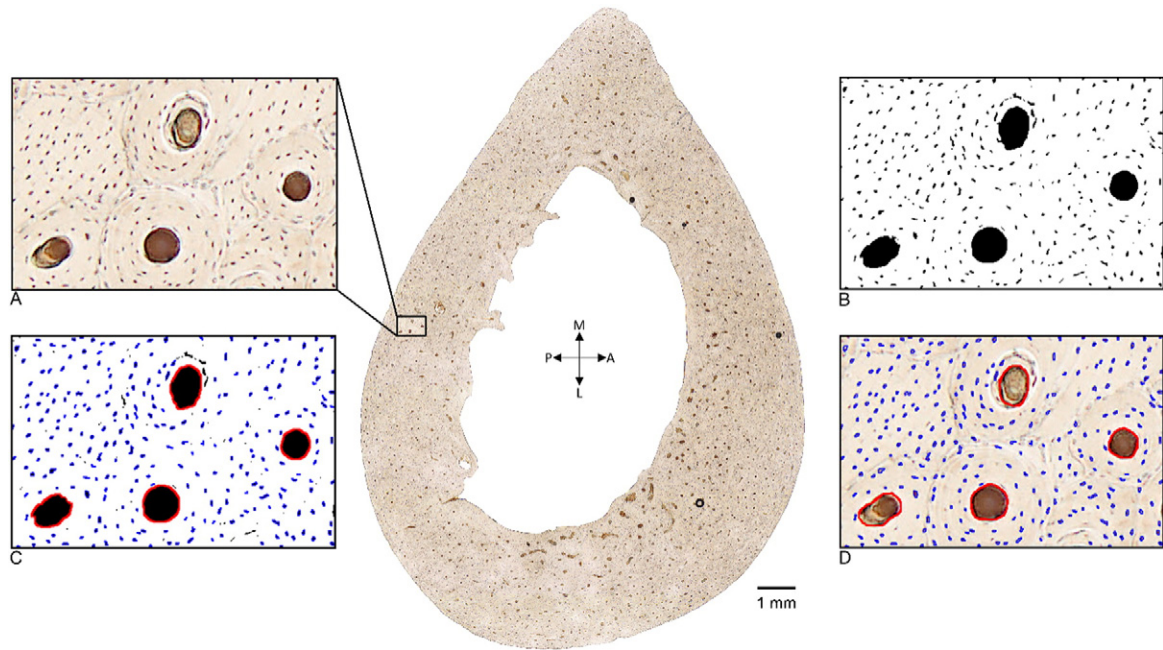


Fig. 1. Cross-section distal one-third radius the entirety of which was analyzed for osteocyte lacunar density and intracortical porosity; A–D are magnified areas representative of the methodology for automated Ot.Lc.N quantification and semi-automated Po.Ar quantification. A) Original input to ImageJ prior to any manipulation. B) Resulting binary image on which Ot.Lc.N and Po.Ar are quantified. C) Following the use of “Analyze Particles,” detected lacunae are counted (blue) and all pores, excluding lacunae, are measured for area (red). D) ImageJ results in reference to the original demonstrating the accuracy of the process (<3.45% error when compared to manual counting methods).

and femur indicated no significant differences between elements ($F(2,84) = 0.52$; $p = 0.60$).

3.1. Age related trends

Osteocyte cell population and therefore, lacunar density was expected to decrease with age due to an increase of pro-apoptotic systemic and mechanical factors. An observed trend consistent with this expectation was supported here for all skeletal elements. Table 3 and Fig. 3 demonstrate the relationship between age and Ot.Lc.N/B.Ar. At the midshaft femoral site, Ot.Lc.N/B.Ar significantly decreased with age ($F(1,28) = 7.33$; $p = 0.01$). Age was able to account for the highest amount of variation, 20.8%, in osteocyte lacunar density in the femur compared to the radius or rib. For the radius, linear regressions also demonstrated a decline in osteocyte lacunar density with age although this relationship was found to be insignificant ($F(1,28) = 3.41$; $p = 0.08$) explaining only 10.8% of variation in Ot.Lc.N/B.Ar. The relationship between Ot.Lc.N/B.Ar and age was weakest and not significant in the rib ($F(1,27) = 1.67$; $p = 0.21$) capable of predicting only 5.8% of the variation in lacunar density.

As expected, intracortical porosity (%Po.Ar) in all three elements increased with increasing age, although with varying predictive capabilities. Linear regression indicated significant increase in %Po.Ar with age in the radius ($F(1,28) = 33.75$; $p < 0.0001$) and femur ($F(1,28) = 11.85$; $p = 0.002$), although the relationship was insignificant in the

rib ($F(1,27) = 3.50$; $p = 0.07$). Age demonstrated stronger predictive capabilities of intracortical porosity than with the aforementioned osteocyte lacunar density explaining 54.5% of variation in the radius, 29.7% of variation in the femur, but only 11.4% of variation in the rib.

3.2. Systemic trends in Ot.Lc.N/B.Ar decline with age

To elucidate systemic patterns in the rates of osteocyte lacunar density decline with age between the three anatomical sites, regression coefficients (B) for the femur, radius and rib were compared. Overlap in their 95% confidence intervals indicated a statistically similar rate of decline in Ot.Lc.N/B.Ar with age and therefore demonstrated a systemic relationship (Table 3). Although statistically similar, osteocyte lacunar density in the femur demonstrated a steeper slope (-6.490) and appeared to change more substantially than in the radius (-3.694) and rib (-2.542) (Fig. 3). Similarly, the rate of increase in %Po.Ar between the elements demonstrated a systemic trend with statistically significant overlap in 95% confidence intervals of the regression coefficients (B) (Table 3).

3.3. Osteocyte lacunar density (Ot.Lc.N/Ct.Ar) and intracortical porosity (%Po.Ar)

As osteocyte apoptosis is linked to the initiation of intracortical remodeling events, the relationship between osteocyte lacunar density and intracortical porosity (i.e., bone loss) was explored. To further elucidate the direction of this relationship, Pearson correlations, rather than linear regressions, are reported here. To account for inter-individual size differences, Ct.Ar was used to normalize both pore area (Po.Ar) and osteocyte lacunar number (Ot.Lc.N). %Po.Ar demonstrated a significant negative correlation with Ot.Lc.N/Ct.Ar for all elements (Fig. 4). The strongest relationship was found within the radius ($r = -0.746$; $p < 0.0001$), while the weakest relationship between intracortical porosity and osteocyte lacunar density was observed in the rib ($r = -0.486$; $p = 0.004$). In the weight bearing femur, there was a strong and significant correlation between %Po.Ar and Ot.Lc.N/Ct.Ar ($r = -0.626$; $p < 0.0001$) intermediate to the radius and the rib. Pore

Table 2
Descriptive statistics per skeletal site for intracortical porosity (%Po.Ar) and osteocyte lacunar density (Ot.Lc.N/B.Ar).

Anatomical location	%Po.Ar (%)			Ot.Lc.N/B.Ar (#/mm ²)		
	Mean (SD)	Min	Max	Mean (SD)	Min	Max
Femur	13.21 (0.96)	4.01	24.27	939.80 (178.22)	515.17	1222.95
Radius	12.18 (1.08)	2.99	25.41	1074.49 (137.09)	738.26	1301.56
Rib	13.58 (0.99)	4.69	27.50	939.23 (132.33)	620.91	1141.51

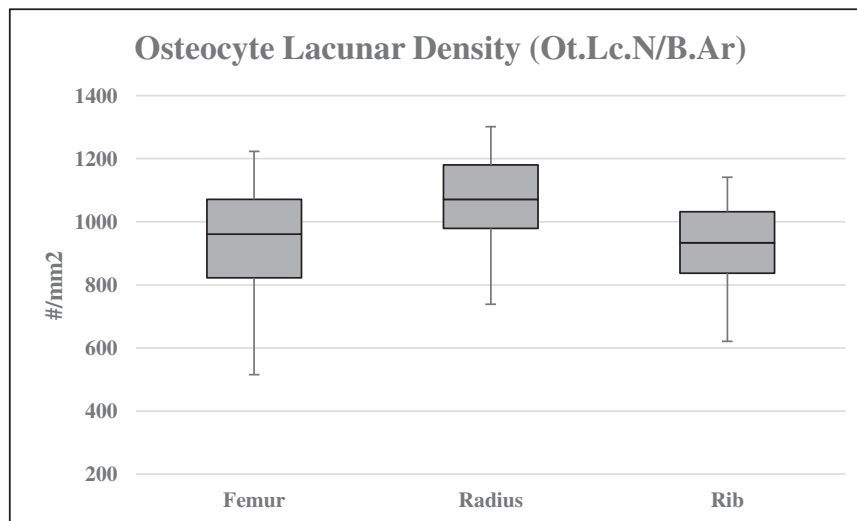


Fig. 2. Boxplot for the total sample ($n = 30$ PMHS) demonstrating no significant differences between femur and rib Ot.Lc.N/B.Ar, but significantly higher lacunar density in the radius than both femur and rib. Also notable is a larger amount of variation in the midshaft femur relative to the other elements.

number (Po.N) was used as a proxy for the available vasculature to support the viability of neighboring osteocyte populations. Thus, linear regressions were used to determine the ability of Po.N to predict Ot.Lc.N. Log transformations normalized Po.N distributions prior to analysis and all relationships were shown to be highly statistically significant. The rib demonstrated the strongest relationship with pore number (Po.N) capable of explaining 61.8% ($F(1,27) = 43.73$; $p < 0.0001$) of variation in osteocyte lacunar number (Ot.Lc.N) (Fig. 5). The femur maintained its intermediate status ($F(1,27) = 34.43$; $p < 0.0001$) with an R^2 of 0.560. The radius displayed the weakest relationship between pore number and osteocyte lacunar number ($F(1,27) = 17.76$; $p < 0.0001$) with an R^2 of 0.397.

4. Discussion

The purpose of this study was to establish an objective foundation for understanding regional and systemic variation in cortical bone osteocyte lacunar density with respect to major factors considered in the assessment of bone quality: sex, age, and cortical bone loss.

4.1. Effect of chronological age on osteocyte lacunar density

Lacunar density reduction with increasing age is a culmination of the effects of both systemic and mechanical influences on the viability of osteocytes themselves but has demonstrated conflicting results in the literature. For this sample, in the cortical bone of the femur and radius, the increasing chronological age of these individuals was significantly related to a decline in osteocyte lacunar density (Ot.Lc.N/B.Ar). Conversely, we found that rib Ot.Lc.N/B.Ar did not demonstrate a significant

relationship with age. Multiple studies have demonstrated a decrease in human osteocyte viability as well as lacunar density with age (Bach-Gansmo et al., 2016; Busse et al., 2010; Frost, 1960a; Mullender et al., 1996; Qiu et al., 2002b, 2006; Vashishth et al., 2002). Yet, within some of these same studies exist conflicting data on the importance of age on osteocyte density. Using transiliac biopsies, Mullender et al. (1996) and Bach-Gansmo et al. (2016) found significant declines in osteocyte lacunar density with increasing age but these relationships were dependent on subsamples and did not hold across all groups. Conversely, in trabecular bone of the vertebral bodies, Vashishth et al. (2005) reported a significant increase in lacunar density in females and a nonsignificant decrease in males with increasing age. Limited data are directly comparable to the anatomical locations sampled here; however, Carter et al. (2013) found no significant decrease in osteocyte lacunar density with age in the midshaft femur. Clearly, the predictive ability of an individual's chronological age on osteocyte lacunar density is limited and may be due to comparisons being made across varying anatomical locations and skeletal envelopes encompassing both different sensitivities to systemic and mechanical influences as well as localized tissue ages compared to chronological age.

Our study seeks to ameliorate the aforementioned conflicting patterns of osteocyte lacunar density changes with age by systematically investigating multiple anatomical sites from which the entire cross-section of bone is analyzed. Recently, Skedros et al. (2016) have proposed a theoretical framework within which to conceptualize the contributions of multiple mechanobiological influences on osteocyte viability, including availability of nutrition, microstructural constraints, microdamage, communication and skeletal metabolism. The relative contribution of these influences may vary within individuals and across anatomical sites depending on the histologic composition or skeletal envelopes present. Carter et al. (2014) had similarly cautioned against comparisons of osteocyte density between skeletal sites of varying developmental and loading history even within the cortex of a single cross section of bone. Frost (1960a), in his foundational work on osteocytes, once argued this methodological approach as a necessity. To our knowledge, the current study is the first investigation of the effects of age and sex on osteocyte lacunar density in the cortical bone of the human radius; however, comparative data are available for the midshaft femur and rib. Our findings are consistent with Vashishth et al. (2002) who reported significant decreases in osteocyte lacunar density with age for males and females in the cortical bone of the midshaft femur. In our sample, age was only able to explain 20.8% of variation in Ot.Lc.N/B.Ar, whereas, Vashishth et al. (2002) reported much stronger relationships: 91% and

Table 3

Osteocyte lacunar density (Ot.Lc.N/B.Ar) declines significantly with age only in the midshaft femur. Comparisons of the slopes (B) of the regression lines for the femur, radius and rib indicate significant overlap in their 95% confidence intervals suggesting a systemic trend in the rate of decline in Ot.Lc.N/B.Ar with age.

Variable	Age			95% confidence interval	
	R^2	p	B	Lower bound	Upper bound
Femur Ot.Lc.N/B.Ar	0.208	0.011	-6.490	-11.398	-1.581
Radius Ot.Lc.N/B.Ar	0.114	0.068	-3.694	-7.687	0.299
Rib Ot.Lc.N/B.Ar	0.058	0.207	-2.542	-6.579	1.495
Femur %Po.Ar	0.297	0.002	0.223	0.090	0.356
Radius %Po.Ar	0.545	<0.001	0.340	0.220	0.461
Rib %Po.Ar	0.115	0.072	0.145	-0.014	0.304

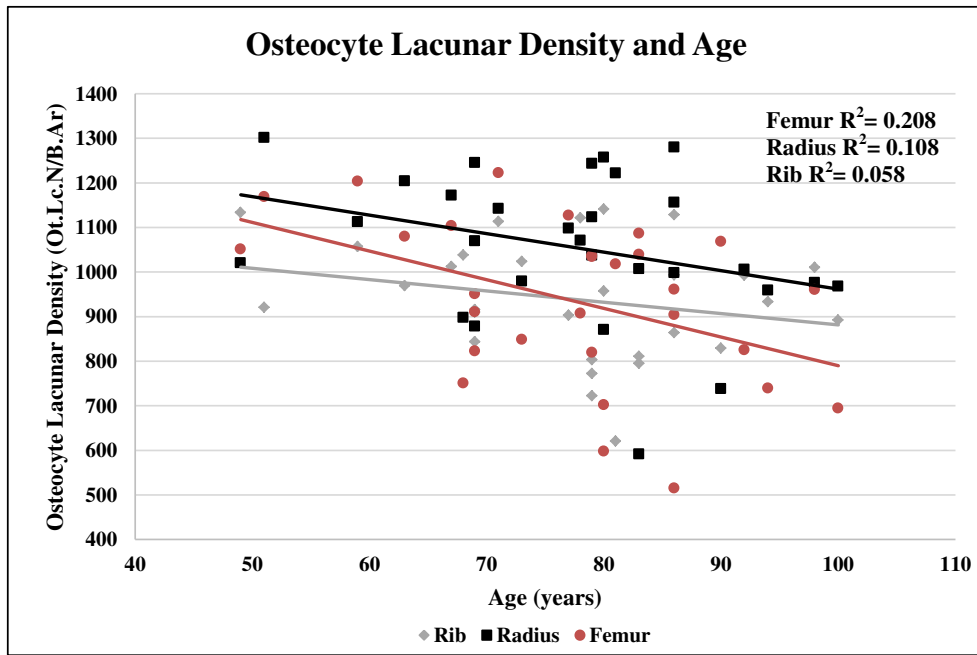


Fig. 3. Osteocyte lacunar density (Ot.Lc.N/B.Ar) decreases with increasing age in all elements. Although density demonstrates a significant decline with age in the femur only, low R^2 values for all three elements indicate chronological age is a weak predictor of osteocyte lacunar density.

52% for males and females respectively. Busse et al. (2010) reported significant decreases in lacunar density within the femoral cortices of both males and females with a greater decline in the endosteal envelope compared to the periosteum. Little work has been done in human ribs to investigate the relationship between osteocyte lacunar density and age. However, Bloch et al. (2012), whose sample encompassed individuals from fetal development through end of life stages, did find an exponential decline ($R^2 = 0.83$) in viable osteocytes with age in the rib. The PMHS included in our study were limited in age range (49 to 100 years old) which may explain the stronger decline in osteocyte lacunar density found in studies that included younger individuals. Significantly higher lacunar densities have been demonstrated in transiliac cortical

bone in younger individuals compared to older individuals (Bach-Gansmo et al., 2016). Thus, the effect of increasing age on cortical osteocyte density in the midshaft femur, midshaft rib and distal radius may be amplified with the inclusion of individuals in earlier stages of life.

It is estimated that 2.5% of osteocytes die each year due to stochastic processes (Manolagas and Parfitt, 2010), but in fact, the life span of an osteocyte has the potential to last for decades in interstitial primary bone (Jilka and O'Brien, 2016). However, in all other bony tissue that experience turnover, the age of the cell corresponds to the localized tissue age (Manolagas and Parfitt, 2013; Qiu et al., 2005) and not the chronological age of the individual. The long lived osteocytes are susceptible to a decrease in cellular function and other age related changes including

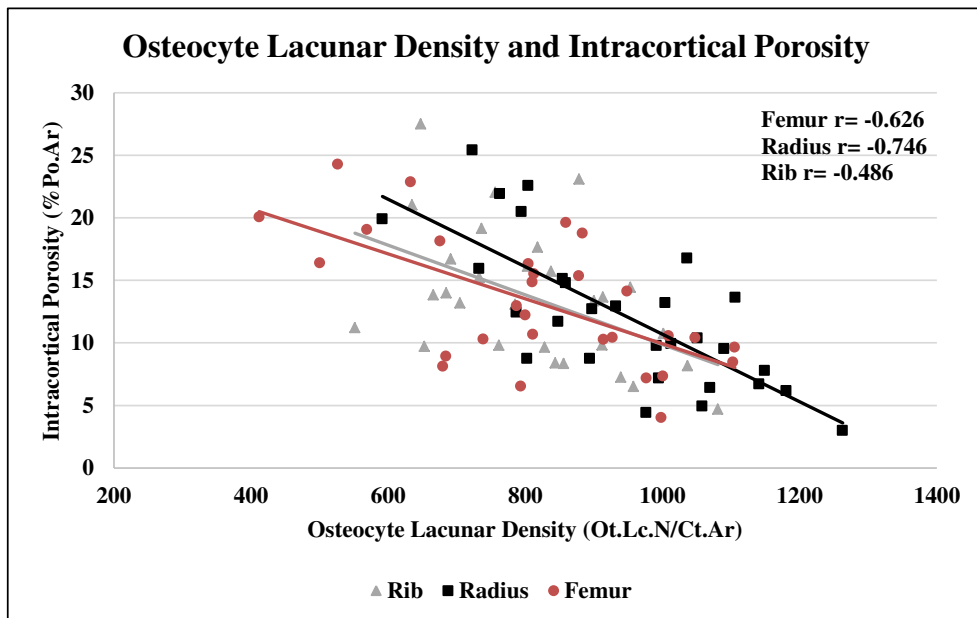


Fig. 4. Scatterplot demonstrating the significant negative correlation between osteocyte lacunar density (Ot.Lc.N/Ct.Ar) and amount of intracortical porosity (%Po.Ar) for each element.

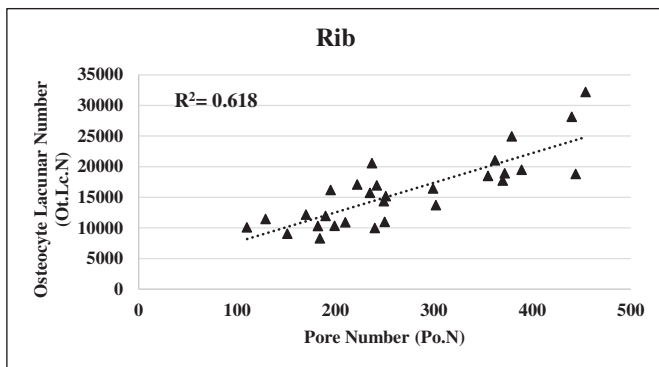


Fig. 5. Linear regression demonstrated the strong relationship between pore number and osteocyte lacunar number in the rib.

metabolic dysfunction (Almeida and O'Brien, 2013; Jilka et al., 2013). Although systemic pro-apoptotic factors such as hypogonadism, excess endogenous glucocorticoids and oxidative stress by reactive oxygen species (ROS) increase with age, their relationship with chronological age is tenuous and variable due to genetics and environmental factors. Chronological age does not always correspond to or capture the amount of allostatic or stress load (Crews and Ice, 2012). Thus, the weak explanatory power of chronological age on variation in osteocyte lacunar density in each skeletal element is not surprising. In our sample, age was only able to explain, at most, 20.8% of variation in osteocyte lacunar density decline in the femur. This inverse relationship, strongest in the femur may be a product of the age related decline in mobility or accumulating linear microdamage at a dynamic load bearing site in addition to the increase in the systemic factors discussed here and needs to be further explored. The cross-sectional nature of most human osteocyte density studies, including the results reported here, is a limitation in understanding the true effects of age on osteocyte lacunar density in human bone. Redefining age in future studies of osteocyte lacunar density as tissue age and quantifying senescence rather than chronological age may prove more informative to understanding these conflicting patterns.

Although the practice of using lacunae as a proxy for osteocytes themselves has been established in the literature, this is a limitation when working with cadaveric bone. It is possible that lacunar density overestimates osteocyte density as lacunar occupancy decreases with age (Frost, 1960b; Tomkinson et al., 1997). However, Qiu et al. (2002a) demonstrated that 97% of variation in osteocyte density was explained by lacunar density in human trabecular bone taken from transiliac biopsies, and Bloch et al. (2012) reported only 14% of lacunae were empty by age 80 years in the rib cortex. As the amount of time between osteocyte death and initiation of a remodeling event to resorb or initiate micropetrosis of the vacant lacunae is unknown, osteocyte lacunar density is at best an approximation of osteocyte density. The phenomenon of micropetrosis may also be dependent on some of the same influences proposed by Skedros et al. (2016) that affect osteocyte. Combining regional patterns of hypermineralized lacunae with osteocyte density may manifest in differential and measurable changes in bone quality that needs to be elucidated throughout the skeleton.

4.2. Effect of sex on osteocyte lacunar density

We did not find any significant sex differences in osteocyte lacunar density (Ot.Lc.N/B.Ar) or intracortical porosity (%Po.Ar). This relationship appears to hold across other studies investigating the femoral cortex (Busse et al., 2010; Carter et al., 2013; Vashishth et al., 2000) and iliac cortical bone (Bach-Gansmo et al., 2016). However, Vashishth et al. (2005) reported females with significantly higher osteocyte lacunar density (Ot.Lc.N/B.Ar) and number of lacunae per total area (Ot.Lc.N/Tt.Ar) than males but this was quantified in trabecular vertebral bone.

The lack of sex differences found in this study was unexpected as estrogen has anti-apoptotic effects on osteocytes and osteoblasts (Khosla et al., 2012; Sharma et al., 2012; Tomkinson et al., 1997) which would result in the maintenance of higher densities of occupied lacunae. Yet, in human iliac trabecular bone (metabolically more sensitive to estrogen withdrawal due to greater surface area and high rate of turnover), osteocyte density was found to decrease beginning shortly after skeletal maturity with no significant acceleration post-menopause (Qiu et al., 2002b). With advancing age, hypogonadism in both sexes could have deleterious effects on osteocyte viability (Bellido, 2014; Bellido and Hill, 2014) resulting in no significant differences in osteocyte lacunar density between males and females. In this sample, females range from 50 to 98 years old and can be expected to represent a post-menopausal population although hormone treatment status is unknown; thus, the lack of sex differences found here suggest that despite the inevitable estrogen withdraw in older females, other factors may play a larger role in osteocyte lacunar density decline. These results support Khosla et al. (2012) argument to shift the paradigm away from estrogen-centric explanations of bone quality.

4.3. Osteocyte lacunar density relationship with intracortical porosity

Intracortical porosity (%Po.Ar) was used here as a link between osteocyte lacunar density and the tangible effects of a reduction in lacunar density (Ot.Lc.N/Ct.Ar) on bone quality. A causal relationship has been previously established between intracortical porosity and osteocyte apoptosis (Jilka et al., 2013). Increasing amounts of cortical bone loss with age has emerged as a key factor in reductions in bone strength and potential increase in fracture risk (Nicks et al., 2012; Vilayphiou et al., 2016). The increasing intracortical porosity with age found in our study has been demonstrated numerous times in various anatomical sites (Bach-Gansmo et al., 2016; Cooper et al., 2007; Feik et al., 1997; Nishiyama et al., 2010; Thomas et al., 2005; Vilayphiou et al., 2016) and variation in this microstructural property has important consequences for fragility and biomechanical integrity (Agnew and Stout, 2012; Bell et al., 2000; Kazakia et al., 2011; Yeni et al., 1997; Zebaze et al., 2010; Zioupos, 2001). Osteocyte apoptosis has been spatially and temporally associated with linear microdamage resulting in targeted intracortical remodeling (Cardoso et al., 2009; Herman et al., 2010; Kennedy et al., 2012, 2014; O'Brien et al., 2013; Verborgt et al., 2000) as a preventative measure for gross failure. Both dysfunction of aged osteocytes or increased rates of apoptosis with age, whether due to microdamage, decreased mechanical loading, or endogenous systemic factors result in increased intracortical porosity, deficient mechanical properties of the tissue, altered geometry and poor bone quality (Plotkin, 2014).

In this study, intracortical porosity (%Po.Ar) increased with age and was strongly correlated ($r = -0.486$ to -0.790) with decreased osteocyte lacunar density at each anatomical site. Similarly, Vashishth et al. (2000, 2002) as well as Dong et al. (2014) demonstrated significant inverse relationships between intracortical porosity and osteocyte lacunar density in the midshaft femur. This relationship was also supported in the trabecular bone of the vertebral body (Vashishth et al., 2002) and the cortex of the femoral neck (Power et al., 2001). The significant relationship between the amount of intracortical porosity and osteocyte lacunar density in the midshaft femur found here includes the entirety of the cortex and encompasses all skeletal envelopes. This is the first study to offer evidence of decreasing osteocyte lacunar density and increasing intracortical porosity in the distal radius and midshaft rib. Our future work will include quantification of intra-individual variation in linear microdamage accumulation and its spatial relationship with osteocyte lacunae and intracortical porosity.

Skedros et al. (2016) highlighted the relative importance of available nutrition for determining and ultimately maintaining osteocyte density. Pore number (Po.N) was investigated here in relation to osteocyte lacunar number (Ot.Lc.N) for the femur, radius and rib as a proxy for the

available vascularity to which the osteocyte network could have access. We found that although the amount of intracortical porosity (%Po.Ar) and osteocyte lacunar density (Ot.Lc.N/B.Ar) demonstrated the weakest relationships with age ($R^2 = 0.115, 0.058$ respectively) in the rib, pore number was able to predict the greatest amount of variation in osteocyte lacunar number at this site ($R^2 = 0.618$). Measurements of intracortical porosity may not capture the true amount of bone loss experienced in the rib as a large portion is endocortical and the result of rapid cortical trabecularization (Dominguez and Agnew, 2016; Zebaze and Seeman, 2015) which may explain the weak relationships between %Po.Ar and age. Bach-Gansmo et al. (2016) did not find any significant relationships between cortical porosity and osteocyte lacunar density in iliac crest samples; however, they did find that cortical thinning with age, the similar phenomenon in the rib, explained more age related bone loss than intracortical porosity. Thus, cortical thinning may obscure the relationships between age related bone loss and osteocyte lacunar density resulting in weak or lack of relationships found at sampling sites such as the rib and iliac crest. However, in terms of availability of vasculature to support osteocyte cell populations, the rib demonstrates the greatest potential for elucidating this relationship which may be independent of age. The 2D histomorphometric methods employed in this study limit the investigation into vasculature and the branching behavior of existing vessels into new intracortical remodeling events being investigated by Maggiano et al. (2016). Future three dimensional micro-imaging methodology mapping vasculature in relation to surrounding osteocytes can further illuminate the basic relationships between pore number and osteocyte lacunar number demonstrated here.

4.4. Systemic trends in osteocyte lacunar density and intracortical porosity

To the authors' knowledge, this study is the first to examine intra-individual osteocyte lacunar density in elements representing both the axial and appendicular skeleton to establish if regional trends display a systemic relationship in human cortical bone. The 30 individuals included here demonstrated no significant differences between Ot.Lc.N/B.Ar in the midshaft femur and midshaft rib; however, the distal radius demonstrated significantly higher density than the other two sites. Qiu et al. (2003) reported a mean lacunar density of $848 \pm 129 \text{ mm}^{-2}$ which is slightly lower than the $939.23 \pm 132.33 \text{ mm}^{-2}$ reported here for the midshaft rib; however, this study only included osteocyte lacunae located within osteonal bone which may explain the discrepancy. As there are no comparative data for osteocyte lacunar density in the radius, nor for the entire femoral cross-section, it is difficult to contextualize these values. We suggest that there is likely a range of optimal densities each skeletal element is attempting to achieve to have both adequate regulation of the extracellular matrix while remaining energetically efficient. This may be affected by species, age, disease processes, medications, genetics, or other environmental factors and is likely site-specific. Differences in basal metabolic rates and nutritional demands of an organism may also play a role in osteocyte densities though there have been conflicting evidence reported (see Skedros et al., 2005, 2016 for a review).

Although a decline in lacunar density with age has been demonstrated in human bone, the rate of decline is unknown (Carter et al., 2013). The anatomically site specific osteocyte sensitivity and function cannot be ignored (Skedros et al., 2005) much as other aspects of bone quality such as extracellular material properties are site-specific (see Alliston, 2014 for a review). In this study, analyses of the standardized regression coefficients support a systemic trend in decreasing Ot.Lc.N/B.Ar with increasing age. The rate of decline between the femur (-0.456), radius (-0.337) and rib (-0.241) do not demonstrate any statistically significant differences in their 95% confidence intervals. However, there does appear to be a more rapid decline when comparing the femur to the rib for example. Additional indirect evidence for the systemic influence of age on Ot.Lc.N/B.Ar is the systemic pattern observed in the increase of

intracortical porosity (%Po.Ar) with age at these same sites. Due to the causal relationship between osteocyte apoptosis and initiating a targeted remodeling event, these data further support the biological and systemic link between Ot.Lc.N/B.Ar and %Po.Ar. It is possible that with the addition of younger individuals, the already varying rates of decline in osteocyte lacunar density between the femur, radius, and rib may result in statistically different trends between these sites. Incorporating more skeletal sites taken from the same individuals to control for systemic factors influencing density can further elucidate the presence of a systemic trend and the influence of mechanical environment on the rate of decline with age.

If apoptosis is influenced by both systemic and mechanical forces, and bones undergo various and complex mechanical loading, then we suggest there is a mechanically mediated component influencing organism wide decreases in osteocyte viability so that age related changes affect apoptosis differentially across the skeleton. In this study, mechanical microenvironment variation between the consistently loaded rib (Eleazer and Jankauskas, 2016) and the complex loading environments of the radius and femur may explain the slight variation in regression slopes between lacunar density (Ot.Lc.N/B.Ar) and age. Weight bearing mobility likely decreases more substantially with increasing age as evidenced by the highest rate of decline and greatest amount of variation in Ot.Lc.N/B.Ar in the midshaft femur. Meanwhile, the rib is thought to be cyclically loaded in bending (Agnew and Stout, 2012) and demonstrates the least amount of variation in osteocyte lacunar density due to a relatively consistent mechanical environment. Frost (1992) discussed an age related decline in mechano-sensitivity of the osteocyte whereby more strain is needed to elicit an anabolic response (Lara-Castillo et al., 2015). This effect is dependent on the mechanical environment in which the osteocyte cell population achieved those setpoints and varies across the skeleton (Klein-Nulend et al., 2015) and may affect their propensity to apoptose under alteration of such environment. The stronger relationships between lacunar density, age, and intracortical porosity in the femur and radius could be a result of the altered mechanical environment associated with decreasing mobility and activity amplified by proapoptotic systemic factors and decreased mechanosensitivity. Cellular senescence due to systemic factors encourages delayed responses in remodeling which allows for micropetrosis and hypermineralization of bone (especially in older interstitial lamellar bone) (Busse et al., 2010) and resulting decrease in osteocyte detection of normal magnitude loads (Nicolella et al., 2006). We posit that a combination of changes in material properties of the bone and the variation in loading environments experienced in the appendicular skeleton could result in stronger relationships between lacunar density and age in the radius and femur over the rib. The natural heterogeneity in osteocyte populations between elements (Buenzli and Sims, 2015; Hesse et al., 2015) may be a product of this phenomenon and must be considered when comparing data across anatomical sites.

5. Conclusions

The strong case for considering osteocyte density as an additional indicator of bone quality has been made and supported repeatedly (Ma et al., 2008). Recently, Mader et al. (2013) have called for more quantitative and standardized measures of osteocyte lacunae and their properties. Current issues in establishing normal variation of osteocyte lacunar density and its gross effect on bone quality include the incongruity of sampling sites, bone type, and small sample sections (Carter et al., 2013; Skedros et al., 2016). The stage has been set for better understanding the physiological processes that link influences at the organism and tissue levels to the cellular level after which osteocytes respond appropriately to affect change that maintains or alters the bone as a whole (tissue level). Understanding patterns of inter-individual variation in lacunar density with age and between anatomical locations allows for interpretations of data falling outside of these trends and investigation into consequences for differential fracture risk. This

study will add to the body of work being done to position the osteocyte into a dominant role in maintaining bone quality and function as the first to examine multiple skeletal elements from within individuals.

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