Osteocyte lacunocanalicular microstructure across the midshaft femur in adult males from Medieval England.

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ABSTRACT

Archaeological human bone histology can reveal well-preserved osteocyte lacunae, which are indicators of bone remodeling activity. Analyses of these lacunae can be useful when reconstructing past human mechanical loading histories or metabolic fluctuations from bone microstructure. However, the relationship between osteocyte lacunae and bone anatomical variation within archaeological samples is largely unknown. We examined osteocyte lacunocanalicular network morphology in Medieval human femora to test if osteocyte lacunae change with anatomical site location. Osteocyte lacunae density (Ot.Dn) data were analyzed statistically in ten middle-aged (35-50 years old) males dated to the 11th-16th centuries AD (Canterbury, England). A subsequent case study was conducted using two well-preserved samples from which canaliculi number per lacuna (Ci.N) and canaliculi-rich lacunae density (Ci.Dn) were preliminarily examined descriptively. The data were collected from cortical bone regions encompassing intra-cortical to sub-periosteal midshaft femur bone, comparing anterior, posterior, medial, and lateral locations inter- and intra-individually. Results show that Ot.Dn varied significantly between the four anatomical regions ($p = 0.001$), with the medial and lateral femur regions showing the highest median Ot.Dn. The median of Ci.N was also the highest on the medial aspect, but Ci.Dn did not change largely across all four bone aspects. The combination of these results suggests that midshaft femur anatomical location, which undergoes morphological change with biomechanical load, affects the expression of bone microstructure at the osteocyte lacuna level. This knowledge will benefit future osteoarchaeological methods that infer past behavior from the human femur.

Key words: behavior; osteocyte lacunae; canaliculi; femur; histology

1. INTRODUCTION

Osteoarchaeologists typically reconstruct ancient human behavior and lifestyle from external morphology, morphometry, and robusticity of limb bones (Licata et al., 2019; Meyer et al., 2011; Ruff, 2008; Ruff & Larsen, 2011; Wanner et al., 2007; Villotte & Knüsel, 2013). However, when internal bone structures are well-preserved , behavioral inferences can also be achieved through histological methods (e.g. Miszkiewicz & Mahoney, 2016; Stout, 1978; Robling & Stout, 2003). Microscopic indicators of bone remodeling can reflect the way living bone adapts to mechanical stimuli (Miszkiewicz, 2016; Robling et al., 2006). Histological features typically examined in archaeological human bone include Haversian canals and secondary osteons (e.g. Miszkiewicz & Mahoney, 2016; Pfeiffer et al., 2006; Robling & Stout, 2003), which are discussed in relation to strenuous physical activities associated with mechanical load variation (van Oers et al., 2008). Osteocyte lacunae, cavities that house osteocytes in live bone, have been less often studied in osteoarchaeology despite their broad application in the palaeobiology of fossil bone form and function (e.g. Cullen et al., 2014; Grunmeier & D'Emic, 2019; Miszkiewicz et al., 2020).

In modern human bone, research has reported osteocyte lacunae densities (Ot.Dn) reflect metabolism and body mass (Bromage et al., 2009; 2016). Human and animal experimental research has found that filopodia are the central mechanosensory components of osteocytes and are distributed within bone according to nutrient accessibility and biomechanical load (Bonewald, 2011; Kerschnitzki et al., 2013; Thi et al., 2013; Verbruggen et al., 2014). However, our understanding of variation in osteocyte lacunae, and the number of osteocyte filopodia across different regions of bone, remains poorly understood overall, particularly for archaeological human samples. Improving this understanding is important to assist with osteoarchaeological inferences of bone functional adaptation (Crowder & Stout, 2011).

1.1. Osteocytes and the osteocyte lacunocanalicular network

In living bone, metabolic activity is essentially governed by osteocytes, and executed by osteoblasts and osteoclasts. These cells play a key role in regulating the internal adaptability of bone to biomechanical load (Burger et al., 2003; Sims & Vrahnas, 2014; Qiu et al., 2005). During bone remodeling, osteoclasts absorb bone matrix, osteoblasts synthesize and secrete new matrix, and osteocytes become embedded within the matrix to perform communicatory and regulatory roles (Bonewald, 2011; Burger et al., 2003; Qiu et al., 2005; Sims & Vrahnas, 2014). External stimuli and demand for bone adaptation activate signals in the osteoblast and transform its circular shape into a smaller, stellate morphology. This transformed cell is then labelled an osteocyte (Bonewald, 2011). Osteocytes in living tissue comprise a cell body, single nucleus, organelles (Golgi apparatus, free ribosomes, endoplasmic reticulum, and mitochondria), and projections called filopodia (Heckman et al., 2013; Sugawara et al., 2008; Uda et al., 2017). The osteocyte cell body is maintained in a cavity called a lacuna and its filopodia project into long canals called canaliculi (Marotti et al., 1995). These lacunae and canaliculi connect with other neighboring lacuna-canaliculi complexes to form the osteocyte lacunocanalicular network – a communication system as complex as the neuronal network in the human brain (Buenzli & Sims, 2015; Franz-Odendaal et al., 2006). Current literature suggests that, outside of transporting nutrients and maintaining bone homeostasis (Bonewald, 2011; Kerschnitzki et al., 2016; Marotti et al., 1995), the network is responsible for stimulus recognition and response (Judex et al., 2010).

1.2. Osteocyte lacunocanalicular network and behavior

Previous studies considering the lacunocanalicular network focused on its micro-morphological trends and quantification in cell lines, animal models, and fresh human bone (Buenzli & Sims, 2015; Kartsogiannis & Ng, 2004; Zhang et al., 2019). For example, it has

been suggested that osteocyte cells are spatiotemporally distributed according to nutrient accessibility and response demand (Marotti et al., 1995; Kerschnitzki et al., 2013). Marotti et al. (1995) noted that this was also a trend for osteocyte lacunae, whereby canaliculi presence was non-uniform within bone and directionality correlated to nearby nutrient reservoirs. Kerschnitzki et al. (2013) suggested that the network may therefore sense stimulus and transport nutrients to sites with high bone-remodeling demand.

Biomechanical load is recognized through changes in fluid pressure, sheer stress, and hydrostatic pressure in a process called mechanosensation (Uda et al., 2017; Judex et al., 2010). It has been suggested that the majority of the mechanosensation occurs with polarity at the filopodia of the osteocyte (Thi et al., 2013; Verbruggen et al., 2014). Ongoing research suspects that the filopodia form gap junctions, which transform a signal into a biological cue in a process called mechanotransduction (Uda et al., 2017; Heckman et al., 2013). The mechanosensation and mechanotransduction processes are also known to vary between skeletal elements and different species (Van Hove et al., 2009). For example, the human femur experiences a different pattern of biomechanical load compared to the human tibia due to its central weight-bearing role (Drapeau & Streeter, 2006; Miszkiewicz, 2016; Van Hove et al., 2009). Rudman et al. (2006) explored this and noted that high modulus was present in areas of high bone density and allowed for the development of an optimized strain pattern that is characteristic only of the human femur. Tayton et al. (2010) supported this finding and noted that strain also varied at different bone sites.

While the principle of bone remodeling and re-distribution as part of bone functional adaptation is the premise of osteoarcheological research into behavior (see Ruff et al., 2006 for review), osteocyte lacunae are yet to be studied properly within archaeological human assemblages. The

140 osteocyte relationship to load has already been shown using ovine, dog, extant birds, and
141 dinosaur samples, confirming different loading patterns, osteocyte distributions, and bone
142 turnover rates in comparison to humans (Canè et al., 1982; Cullen et al., 2014; Grunmeier &
143 D'Emic, 2019; Kerschnitzki et al., 2013). Therefore, where access to femoral cross sections in
144 archaeological human samples is available, even when osteocytes themselves do not preserve,
145 their lacunae are evidence of osteocyte existence, and thus can shed light on localized bone
146 functional adaptation and anatomical location (Bromage et al., 2009; Miszkiewicz & Mahoney,
147 2016; 2019).
148
149 Given the relationships between biomechanical load and the osteocyte lacunocanalicular
150 network, we can predict that osteocyte lacunae and their canaliculi, should vary between
151 different anatomical regions (anterior, posterior, medial, lateral) of a single bone as per stress
152 distribution of the bone shaft. Therefore, we examined osteocyte lacunocanalicular network
153 morphology in pre-prepared histological slides of Medieval human midshaft femora
154 (Miszkiewicz, 2016; Miszkiewicz & Mahoney, 2012; 2016) to address if density of osteocyte
155 lacunae (Ot.Dn) could indicate whether or not the mechano-sensory cells of bone are uniformly
156 distributed throughout the bone cross-section. We also conducted a subsequent 'case study'
157 using this sample to test whether the number of osteocyte canaliculi per lacuna (Ci.N), and the
158 density of canaliculi-rich osteocyte lacunae (Ci.Dn), also change with anatomical region. This
159 could provide a preliminary insight into osteocyte role in sensing and responding to varying
160 biomechanical load, and should explain the distribution and preservation of the communication
161 network within bone (Rolvien et al., 2018).
162
163 **2. MATERIALS AND METHODS**
164 The human midshaft femur samples analyzed in this study were randomly selected from the
165 Hard Tissue Histology collection of thin sections housed as part of the Biological

Anthropology Collection at the School of Archaeology and Anthropology (Australian National University, Canberra). The samples are dated to the 11th – 16th centuries AD and represent a larger British osteological collection curated at the Skeletal Biology Research Centre (University of Kent, UK) (see Miszkiewicz & Mahoney, 2017). There were ten individuals represented in the study, each having four anatomical regions available for analysis (medial, lateral, anterior, posterior). As reported previously, following standard anthropological methods (Buikstra & Ubelaker, 1994), each individual was estimated to be a middle-aged (35-50 years old) male (Miszkiewicz & Mahoney, 2012). The femora were previously cross sectioned in the transverse plane into 1 cm ± 0.2 cm femur segments, which were later processed into thin sections (~100 µm) following standard methods (Miszkiewicz & Mahoney, 2017). This involved embedding the femur samples in epoxy resin, sectioning on a Buehler IsoMet 1000 precision saw, grinding, polishing, dehydrating in ethanol baths, clearing in Histoclear®, and covering with glass microscope slide covers.

2.1. Histomorphometric analysis

The thin sections were analyzed using an Olympus BX53 high-powered microscope with a DP74 camera. From the thin sections, images representing regions of interest (ROIs) were multi-layer captured using the Olympus CELL® Live Biology Imaging software that allows z-plane stacking while live imaging. These were taken from the mid-point of each sample mainly within the sub-periosteal area of bone [**Figure 1**]. However, in some cases the ROIs crossed into the intra-cortical space where overlapping of ROIs in the sub-periosteal region was unavoidable. All ROIs contained at least an approximate 50% of one secondary osteon captured at 40x magnification [**Figure 1**]. For reference purposes and to avoid repeated capture, 10x or 20x captures were also taken for each anatomical region that contained the associated ROIs. In the case where extensive network was evident, higher magnifications, such as 60x were used

191 to aid in the microstructural analysis. The "multi-point" tool of ImageJ® (vol. 1.52) software
192 was used to manually count the osteocyte lacunae and their canaliculi in each capture for an
193 area of ~0.13mm^2, so that Ot.Dn, Ci.N, and Ci.Dn could be computed. Based on the overall
194 preservation within the sample, a threshold for inclusion in the count of "canaliculi-rich"
195 lacunae was five primary projecting canaliculi. Where ambiguity was apparent, additional tools
196 were employed to aid in the analysis of the captures at 40x. For example, increased
197 magnification at 60x was used to clarify whether each canaliculus was primary or secondary,
198 and also whether they projected from a neighboring lacuna. All ambiguous, branching, or
199 neighbor-originating canaliculi were excluded from the manual counts. **Figure 1B** provides an
200 example of the final canaliculi count for a single isolated lacuna.
201
202 For the main goal of the study, a minimum of five regions of interest (ROIs) were captured
203 (aiming for 20 ROIs per individual = 200 in total) to compute Ot.Dn in each anatomical region.
204 However, two ROIs showing poorly identifiable osteocyte lacunae on the posterior aspect were
205 evident in two different individuals. This reduced the total ROI number to 198 from which data
206 could be collected. The densities were calculated as number of lacunae per image area, that is
207 per ~0.13mm^2 (Miszkiewicz, 2016; Miszkiewicz & Mahoney, 2019). Any osteocyte lacuna in
208 the capture with the appropriate morphology based on that outlined by Marotti et al. (1995)
209 and Bonewald (2011) was included. Lacunae on the image borders were also counted.
210
211 For the subsequent case study, we undertook manual counts of extremely minute canaliculi
212 (previously reported diameter range is 0.13 – 0.39µm, You et al., 2004). This meant we could
213 only examine samples that were of pristine preservation at the osteocyte lacuna level. The two
214 best preserved samples (Individuals SK2 and SK9) were selected for Ci.N and Ci.Dn analyses
215 **[Figure 2]**. Ci.N was the number of all visible primary canaliculi protruding directly out of

each lacuna, whereas Ci.Dn was the number of osteocyte lacunae with rich canaliculi divided by image area. 'Canaliculi-rich lacunae' were defined as those with more than five extending canaliculi. For the Ci.N and Ci.Dn calculations, we aimed to capture six ROIs per anatomical region (anterior, posterior, medial, and lateral). SK2 had six ROIs measured for Ci.N counts, but five ROIs for Ci.Dn because six single ROIs of each anatomical region could not be clearly examined due to the close proximity of the limited accessible canaliculi-rich lacunae. Furthermore, the calculation of Ci.Dn was not possible in two ROIs on the anterior aspect in SK9 due to a lack of identifiable canaliculi, that is, Ci.N and Ci.Dn were zero. We could not find all six ROIs with suitably preserved lacunae, so only five ROIs were included in the analysis of Ci.N for SK9.

2.3. Statistical procedures

The statistical analysis was conducted in IBM SPSS® statistical software (2019). The sample size warranted the use of non-parametric inferential tests, but this was only applicable to the main goal of the study where all ten individuals were examined. For the purpose of repeatability checks, intra- and inter-observer tests were performed on randomly selected 20% of the images. The data were re-counted by two co-authors, and then compared with the original counts using a Wilcoxon-Signed Rank test.

The descriptive analysis of all data was conducted by reporting the median, minimum, maximum, and interquartile range (Q1 at 25%, and Q2 at 75%) for each anatomical region for all three parameters (Ot.Dn, Ci.N, Ci.Dn). For the intra-individual descriptives, we only report the median, minimum, and maximum as the interquartile ranges are not truly meaningful per one individual.

To address the main goal of the study using inferential statistics, Ot.Dn was analyzed using a non-parametric ANOVA (Related-Samples Friedman's Two-Way ANOVA) with a Related-Samples Wilcoxon Signed Rank post-hoc test to determine if the median distribution was equal. If the reported p was < 0.05, the data were deemed statistically significant. The four anatomical regions were compared across all ten individuals first. This was followed by an intra-individual analysis where Ot.Dn data were compared between the anatomical regions belonging to each individual. We also performed Spearman's *Rho* correlations from each region to test for possible femoral side mutual relationships in an increase or decrease of Ot.Dn. In addition to $p < 0.05$, the strength of correlations was interpreted from the *Rho* value (*Rho* > 0.68 = strong correlation, see Miszkiewicz, 2016).

3. RESULTS

The error tests of osteocyte lacunae identification returned statistically insignificant differences between the original and repeated counts (intra-observer: $p = 0.180$, W = 12; inter-observer: $p = 0.343$, W = 15).

3.1. Osteocyte lacunae densities

There was a statistically significant difference in Ot.Dn values between anterior, posterior, medial, and lateral femur regions in the whole sample ($p = 0.001$) [**Table 1, Figure 3A**]. Descriptively, the medial and lateral aspects of the bone had the highest median Ot.Dn, whereas the anterior and posterior aspects had relatively lower median values. This was further supported statistically. Post-hoc comparisons indicated that the medial and lateral Ot.Dn consistently differed significantly from the anterior and posterior bone aspects ($p < 0.05$). The medial and lateral sections of the femoral midshaft also had the highest values in the 25% (Q1) and 75% (Q2) quartiles, despite the maximum lateral data point being the lowest when

compared to the remaining anatomical locations. The Spearman's *Rho* correlations also demonstrated a strong positive relationship between Ot.Dn from the medial and lateral aspects (*Rho* = 0.715, $p < 0.001$, n = 50), but a weak one when anterior and posterior regions were considered (*Rho* = 0.380, $p = 0.008$, n = 48) [**Figure 3B**].

Once the intra-individual analysis was conducted, the density of osteocyte lacunae differed significantly across the anterior, posterior, medial, and lateral femoral midshaft sections in all individuals ($p < 0.05$) except for one individual (SK5) **[Tables 2, 3, Figure 4]**. However, there was inconsistency in which pairs of midshaft locations differed from each other. For example, some individuals had similar data across almost all anatomical location comparisons, except for only one pair being statistically significantly different (e.g. medio-lateral in SK1, antero-posterior in SK4). Out of all the anatomical pairs tested, the antero-posterior and medio-lateral comparisons were the most consistently statistically significantly different with six and five individuals having $p < 0.05$, respectively. Overall, it is apparent that the majority of our individuals showed variation in Ot.Dn between the anterior, posterior, medial, and lateral bone regions.

3.2. Canaliculi number per osteocyte lacuna and canaliculi-rich osteocyte lacunae density case study

Descriptively, the median of Ci.N was highest on the medial, but lowest on the posterior anatomical region in SK2 [**Figure 4**]. The Ci.N data were almost consistent across the femoral regions in individual SK9. In SK9, the highest median Ci.N was observed in the lateral region and the lowest median was apparent in the medial region. Thus, in a similar manner to the Ot.Dn results presented for the entire sample [**Tables 1, 2**], these data suggest that some

individuals showed variation in Ci.N, whereas others did not, though a larger sample size will validate these findings in the future. Descriptively, the highest median Ci.Dn was observed in the medial and anterior regions, and lowest in the posterior region in individual SK2.

4. DISCUSSION

Very limited prior osteoarcheological research has considered osteocyte lacunae and linked them to behavior. One example includes 11th-16th centuries medieval English samples (also the subjects of our study, Miszkiewicz, 2016). Osteocyte lacuna characteristics were also previously examined in an Iron Age (3rd-5th BC, Alfedena and Sulmona, Italy) sample, describing diagenesis at the microscopic level (Capasso & Tota, 1993). Results from our study indicate that the Medieval individuals had statistically significant variation in osteocyte lacunae among the anterior, posterior, medial, and lateral anatomical regions, and thus expand the limited data currently available in the osteoarchaeological literature.

4.1. Behavioral links

Prior preliminary research on this sample indicated femoral morphology to range from gracile to robust, with some associations to histology, hinting at bone structure hierarchical effects of functional adaptation in this sample (Miszkiewicz & Mahoney, 2012). When comparing the bone histology with anatomical region, we found that there were more consistent regions for trends in Ot.Dn, namely the medial and lateral regions. Both had the highest median osteocyte lacunae, and both differed significantly from the posterior and anterior aspects, where osteocyte lacunae median was lower. This suggests that there is less paired variation in Ot.Dn among individuals in these portions of the femoral midshaft. These results suggests that midshaft femur anatomical location, which undergoes morphological changes with biomechanical load, affects the expression of bone microstructure at the osteocyte level.

Our main finding that osteocyte lacunae increase on both the lateral and medial aspects of the femur, while the anterior and posterior sides of the bone are not as influenced by this increase, is consistent with conclusions drawn in the literature. For example, a histological study by Gocha and Agnew (2015) exploring osteon population density variation in the human femoral midshaft, determined that the lateral and antero-lateral regions of the femoral transverse cross-sections experience the highest strain magnitude and tensile strain resulting in higher presence of secondary osteons in these regions. Their finding is further supported by previously reported positive correlations between secondary osteon population densities and osteocyte lacunae densities (Miszkiewicz, 2016). A study by Stigler et al. (2019) explored the distribution of osteocytes across cranial, axial, and limb areas of the skeleton to report that cortical bone showed variation among anatomical sites, whereas trabecular bone did not. This was consistent with what was discovered in our study, however, our degree of variation among anatomical sites was not always statistically significant.

The preliminary case study considering the distribution variation of canaliculi-rich osteocyte lacunae observed in our samples suggests that the more outer (closer to the sub-periosteal bone) regions of the femur may require more nutrients, communication, and/or bone remodeling (Bonewald, 2011) [**Figure 5**]. Previously, observations of canaliculi identified anatomical sites responsible for the generation of strain potentials (Cowin et al., 1995). Rolvien et al. (2018) suggested that there was a reduction in both canaliculi density and number per lacuna with age in the femur. Marotti et al. (1995) examining tibiae, also noted that canaliculi density was not significantly variable within secondary osteons, but instead found that there was a strong constitutive negative regulation of osteoclasts and positive regulation of osteoblasts by osteocytes through their canaliculi. A hypotheses worth constructing is that more canaliculi-rich osteocyte lacunae may be situated at the periosteal border [**Figure 5**], possibly linked to

343 increased biomechanically-induced remodeling demands. The fact that SK2 and SK9 also show
344 some differences in canaliculi-rich data suggest individual behavior possibly influencing the
345 morphological expression of osteocyte lacunae, which is worth exploring further.
346
347 **4.2. Remarks on limitations and multi-dimensional visualization of osteocyte morphology**
348 We did not consider bone porosity, which has been previously positively correlated with
349 osteocyte lacunae densities (Dong et al., 2013), suggesting that age and disease may lead to
350 their increase (Tiede-Lewis & Dallas, 2019). On the other hand, this would be inconsistent
351 with what is known about bone pathologies such as osteoporosis, osteoarthritis, osteomalacia,
352 osteopenia, and osteopetrosis, which are associated with deteriorating bone quality (Tatsumi et
353 al., 2007; Oliveira et al., 2016). Mullender et al. (1996) also suggest that osteocyte density
354 decreases with age, likely through micropetrosis. A study by Tiede-Lewis and Dallas (2019)
355 supplements these conclusions by reporting that although osteocyte density does not follow a
356 particular pattern throughout bone, the lacunocanalicular network of osteocytes does show
357 variation with ageing. Specifically, they identified that canaliculi reduced in densities, and
358 lacunae deformed and deteriorated with age (Tiede-Lewis and Dallas, 2019).
359
360 Limitations of our study include a sample size of ten. We also relied on the assumption that
361 one canaliculus contained one filopodia, which is a necessary methodological over-
362 simplification, though it is likely that more than one filopodia may have projected into the
363 canaliculus (Marotti et al., 1995). Future research should combine two-dimensional (2D) thin
364 sectioning with a three-dimensional (3D) approach such as micro-CT or laser confocal
365 scanning, as this will allow consideration of the lacunae shape and connectivity between
366 individual osteocytes (Andronowski et al., 2018). Our 2D approach only provides information
367 on a single orientation of the lacunocanalicular complex, which might have particularly

underlied some differences in Ci.Dn and Ci.N data between SK2 and SK9 in our case study. Where possible, if access to macroscopic information is available (e.g. bone robusticity or exterior morphology), it may help to improve understanding of the microscopic variation with femur size (see Miszkiewicz & Mahoney, 2019). Additionally, future studies would benefit from testing the distribution of canaliculi-rich lacunae statistically to determine whether location within the midshaft femoral cross section affects Ci.Dn and Ci.N.

5. CONCLUSION

This study reported intra-individual and inter-individual variation in the osteocyte lacunocanalicular network in the human femoral midshaft in a sample of Medieval males. The results showed that the medial and lateral femur regions had the highest densities of osteocyte lacunae when compared to anterior and posterior femoral aspects. The results correspond to what is known in literature in other species, as well as, in fresh human bone. The data also agree with the preservation of biomechanical loading patterns in humans, as well as, the lacunocanalicular network, which changes in morphology with age, disease, and/or behavior. This suggests that the reconstruction of past human behavior within osteoarchaeology could incorporate osteocyte lacunae analyses into their microscopic sampling and analysis protocols. Not only can this be used as a complementary method to the bone exterior shape and size data, but histological analyses can also be applied to fragmented human remains where the external anatomy is compromised (Crescimanno & Stout, 2012; Cuijpers, 2006; Cummaudo et al., 2019; Haas & Storå, 2015; Lemmers et al., 2020). On the basis of our data, future researchers may be able to estimate which anatomical region a midshaft femur fragment derives from.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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TABLE CAPTIONS

Table 1. Descriptive data for osteocyte lacunae densities across the whole sample of ten adult males, sub-divided by anatomical region of the femur, and including results from the inferential analysis. N ROIs – number of regions of interest, min. – minimum, max. – maximum, Q1 – lower quartile, and Q2 – upper quartile, and df – degrees of freedom. (Footnotes: *statistically significant $p < 0.05$, **excludes two ROIs with no Ot.Dn data due to preservation issues)

Table 2. Descriptive statistics for osteocyte lacunae density intra-individual variation across the four anatomical regions. N ROIs – number of regions of interest, df – degrees of freedom, min. – minimum data point, max. – maximum data point, Q1 – lower quartile, and Q2 – upper quartile.

Table 3. Results from the Related-Samples Friedman Two-Way ANOVA test with Related-Samples Wilcoxon Signed Rank Test comparisons evaluating osteocyte lacunae densities across femoral regions intra-individually. N ROIs – number of regions of interest (minimum five per anatomical location), df – degrees of freedom, std. – standardized, adj. – adjusted, A –

anterior, P – posterior, M – medial, and L – lateral. (Footnote: *statistically significant $p < 0.05$)

Table 4. Descriptive and Related-Samples Friedman Two-Way ANOVA test results for intra-individual variation in the number of canaliculi per osteocyte lacunae (Ci.N), and density of canaliculi-rich osteocyte (Ci.Dn) across four anatomical regions in individuals SK2 and SK9. Min. – minimum data point, Max. – maximum data point, SD – standard deviation, A – anterior, P – posterior, M – medial, L – lateral, and N ROIs – region of interest. (Footnote: *statistical significance $p < 0.05$)

FIGURE CAPTIONS

Figure 1. Sample selection and segmentation for histomorphometric analysis: A - femur cross section divided into anterior, posterior, medial, lateral sections; B - a 10x magnification image captured using transmitted light to show the borders (cement lines) of secondary osteons; C - a 40x magnification image captured for data collection. The squares with white dashed outlines indicate the bone regions from within which ROIs where captured.

Figure 2. Image A shows an example of the lacunocanalicular network in archaeological human bone captured within the anterior periosteal region. The image was taken at a 40x magnification. Osteocyte lacunae are marked with a white arrow, whereas the preserved projecting canaliculi are marked with a grey arrow. Image B, not related to Image A, shows a osteocyte lacuna magnified to 60x to illustrate manual counts of primary projecting canaliculi (the osteocyte lacuna is not to scale).

639 **Figure 3**. Box plots (lower, median, upper quartiles) summarizing the results of analysis in this
640 study. A illustrates osteocyte lacunae density variation with femoral region in the entire sample
641 where the lateral and medial aspects of the femur have the highest medians of osteocyte
642 lacunae, while the anterior and posterior sides are not as influenced by this increase. B shows
643 a scattergram correlating osteocyte lacunae densities in two pairs of anatomical regions (antero-
644 posterior, medio-lateral) further supporting our finding in A whereby the densities of osteocyte
645 lacunae align positively and stronger than in the antero-posterior femoral regions. Asterisks
646 and circular points are outliers.
647
648 **Figure 4.** Box plots (lower, median, upper quartiles) summarizing the results of analysis intra-
649 individually, showing examples of two selected skeletons (SK2, SK9) whose canaliculi were
650 examined. A illustrates osteocyte lacunae variation among femoral regions in the selected
651 individuals, whereas B shows the number of canaliculi per osteocyte lacuna variation with
652 femoral region in the two selected individuals. The circular points are outliers and asterisks
653 indicate statistical significance at $p < 0.05$.
654
655 **Figure 5.** Images illustrating variation in canaliculi-rich osteocyte lacunae in cortical bone of
656 individual SK2. Image A shows one of the regions of interest selected in the anterior (periosteal
657 border can be seen in the upper left corner of the image) section of the femur. Image B shows
658 the medial region of the femoral midshaft. Both images were captured under a magnification
659 of 40x. Image A shows 'richer' canaliculi connections in the bone than seen in Image B.
660