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1 **Osteocyte lacunocanicular microstructure across the midshaft femur in adult males**  
2 **from Medieval England.**

3

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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 11<sup>th</sup>-16<sup>th</sup> 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

65        **1. INTRODUCTION**

66        Osteoarchaeologists typically reconstruct ancient human behavior and lifestyle from external  
67        morphology, morphometry, and robusticity of limb bones (Licata et al., 2019; Meyer et al.,  
68        2011; Ruff, 2008; Ruff & Larsen, 2011; Wanner et al., 2007; Villotte & Knüsel, 2013).  
69        However, when internal bone structures are well-preserved , behavioral inferences **can also be**  
70        achieved through histological methods (e.g. Miskiewicz & Mahoney, 2016; Stout, 1978;  
71        **Robling & Stout, 2003**). Microscopic indicators of bone remodeling can reflect the way living  
72        bone adapts to mechanical stimuli (Miskiewicz, 2016; Robling et al., 2006). **Histological**  
73        **features typically examined in archaeological human bone include Haversian canals and**  
74        **secondary osteons (e.g. Miskiewicz & Mahoney, 2016; Pfeiffer et al., 2006; Robling & Stout,**  
75        **2003), which are discussed in relation to strenuous physical activities associated with**  
76        **mechanical load variation (van Oers et al., 2008). Osteocyte lacunae, cavities that house**  
77        **osteocytes in live bone, have been less often studied in osteoarchaeology despite their broad**  
78        **application in the palaeobiology of fossil bone form and function (e.g. Cullen et al., 2014;**  
79        **Grunmeier & D'Emic, 2019; Miskiewicz et al., 2020).**

80  
81        **In modern human bone, research has reported osteocyte lacunae densities (Ot.Dn) reflect**  
82        **metabolism and body mass (Bromage et al., 2009; 2016). Human and animal experimental**  
83        **research has found that filopodia are the central mechanosensory components of osteocytes**  
84        **and are distributed within bone according to nutrient accessibility and biomechanical load**  
85        **(Bonewald, 2011; Kerschnitzki et al., 2013; Thi et al., 2013; Verbruggen et al., 2014).**  
86        **However, our understanding of variation in osteocyte lacunae, and the number of osteocyte**  
87        **filopodia across different regions of bone, remains poorly understood overall, particularly for**  
88        **archaeological human samples. Improving this understanding is important to assist with**  
89        **osteoarchaeological inferences of bone functional adaptation (Crowder & Stout, 2011).**

90        **1.1. Osteocytes and the osteocyte lacunocanalicular network**

91 In living bone, metabolic activity is essentially governed by osteocytes, and executed by  
92 osteoblasts and osteoclasts. These cells play a key role in regulating the internal adaptability of  
93 bone to biomechanical load (Burger et al., 2003; Sims & Vrahnas, 2014; Qiu et al., 2005).  
94 During bone remodeling, osteoclasts absorb bone matrix, osteoblasts synthesize and secrete  
95 new matrix, and osteocytes become embedded within the matrix to perform communicatory  
96 and regulatory roles (Bonewald, 2011; Burger et al., 2003; Qiu et al., 2005; Sims & Vrahnas,  
97 2014). External stimuli and demand for bone adaptation activate signals in the osteoblast and  
98 transform its circular shape into a smaller, stellate morphology. This transformed cell is then  
99 labelled an osteocyte (Bonewald, 2011). Osteocytes in living tissue comprise a cell body, single  
100 nucleus, organelles (Golgi apparatus, free ribosomes, endoplasmic reticulum, and  
101 mitochondria), and projections called filopodia (Heckman et al., 2013; Sugawara et al., 2008;  
102 Uda et al., 2017). The osteocyte cell body is maintained in a cavity called a lacuna and its  
103 filopodia project into long canals called canaliculi (Marotti et al., 1995). These lacunae and  
104 canaliculi connect with other neighboring lacuna-canalicular complexes to form the osteocyte  
105 lacunocanalicular network – a communication system as complex as the neuronal network in  
106 the human brain (Buenzli & Sims, 2015; Franz-Odenaal et al., 2006). Current literature  
107 suggests that, outside of transporting nutrients and maintaining bone homeostasis (Bonewald,  
108 2011; Kerschnitzki et al., 2016; Marotti et al., 1995), the network is responsible for stimulus  
109 recognition and response (Judex et al., 2010).

110

111        **1.2. Osteocyte lacunocanalicular network and behavior**

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

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

121

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

136

137 While the principle of bone remodeling and re-distribution as part of bone functional adaptation  
138 is the premise of osteoarcheological research into behavior (see Ruff et al., 2006 for review),  
139 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; Miskiewicz & 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 (Miskiewicz, 2016; Miskiewicz & 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

166 Anthropology Collection at the School of Archaeology and Anthropology (Australian National  
167 University, Canberra). The samples are dated to the 11<sup>th</sup> – 16<sup>th</sup> centuries AD and represent a  
168 larger British osteological collection curated at the Skeletal Biology Research Centre  
169 (University of Kent, UK) (see Miskiewicz & Mahoney, 2017). There were **ten** individuals  
170 represented **in the study, each having four anatomical regions available for analysis (medial,**  
171 **lateral, anterior, posterior)**. As reported previously, following standard **anthropological**  
172 **methods (Buikstra & Ubelaker, 1994), each individual was estimated to be a middle-aged (35-**  
173 **50 years old) male** (Miskiewicz & Mahoney, 2012). The femora were previously cross  
174 sectioned in the transverse plane into 1 cm ± 0.2 cm **femur segments, which were later**  
175 **processed into thin sections (~100 µm) following** standard methods (Miskiewicz & Mahoney,  
176 2017). **This** involved embedding **the femur** samples in epoxy resin, sectioning on a Buehler  
177 IsoMet 1000 precision saw, **grinding, polishing,** dehydrating in ethanol baths, clearing in  
178 HistoClear®, and covering with glass microscope slide covers.

179

## 180 **2.1. Histomorphometric analysis**

181 The **thin sections** were analyzed using an Olympus BX53 high-powered microscope with a  
182 DP74 camera. **From the thin sections, images representing regions of interest (ROIs)** were  
183 multi-layer captured using the Olympus CELL® Live Biology Imaging software that allows z-  
184 plane stacking while live imaging. **These were taken from the mid-point of each sample mainly**  
185 **within the sub-periosteal area of bone [Figure 1]. However, in some cases the ROIs crossed**  
186 **into the intra-cortical space where overlapping of ROIs in the sub-periosteal region was**  
187 **unavoidable.** All ROIs contained at **least an approximate 50% of** one secondary osteon captured  
188 at 40x magnification [**Figure 1**]. For reference purposes and to avoid repeated capture, 10x or  
189 20x captures were also taken for each anatomical region that contained the associated ROIs. In  
190 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  $\sim 0.13\text{mm}^2$ , 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  $\sim 0.13\text{mm}^2$  (Miszkievicz, 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  $0.13 - 0.39\mu\text{m}$ , 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

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

226

### 227 **2.3. Statistical procedures**

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

234

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

240

241 To address the main goal of the study using inferential statistics, Ot.Dn was analyzed using a  
242 non-parametric ANOVA (Related-Samples Friedman's Two-Way ANOVA) with a Related-  
243 Samples Wilcoxon Signed Rank post-hoc test to determine if the median distribution was  
244 equal. If the reported  $p$  was  $< 0.05$ , the data were deemed statistically significant. The four  
245 anatomical regions were compared across all ten individuals first. This was followed by an  
246 intra-individual analysis where Ot.Dn data were compared between the anatomical regions  
247 belonging to each individual. We also performed Spearman's  $Rho$  correlations from each  
248 region to test for possible femoral side mutual relationships in an increase or decrease of Ot.Dn.  
249 In addition to  $p < 0.05$ , the strength of correlations was interpreted from the  $Rho$  value ( $Rho >$   
250  $0.68$  = strong correlation, see Miskiewicz, 2016).

251

### 252 3. RESULTS

253 The error tests of osteocyte lacunae identification returned statistically insignificant differences  
254 between the original and repeated counts (intra-observer:  $p = 0.180$ ,  $W = 12$ ; inter-observer:  $p$   
255  $= 0.343$ ,  $W = 15$ ).

256

#### 257 3.1. Osteocyte lacunae densities

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

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

270

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

282

### 283 **3.2. Canalliculi number per osteocyte lacuna and canalliculi-rich osteocyte lacunae**

#### 284 **density case study**

285

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

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

#### 294 295 **4. DISCUSSION**

296  
297 Very limited prior osteoarcheological research has considered osteocyte lacunae and linked  
298 them to behavior. One example includes 11<sup>th</sup>-16<sup>th</sup> centuries medieval English samples (also the  
299 subjects of our study, Miskiewicz, 2016). Osteocyte lacuna characteristics were also  
300 previously examined in an Iron Age (3<sup>rd</sup>-5<sup>th</sup> BC, Alfedena and Sulmona, Italy) sample,  
301 describing diagenesis at the microscopic level (Capasso & Tota, 1993). Results from our study  
302 indicate that the Medieval individuals had statistically significant variation in osteocyte lacunae  
303 among the anterior, posterior, medial, and lateral anatomical regions, and thus expand the  
304 limited data currently available in the osteoarcheological literature.

305

#### 306 **4.1. Behavioral links**

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

317

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

331

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

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

374

## 375 5. CONCLUSION

376

377 This study reported intra-individual and inter-individual variation in the osteocyte  
378 lacunocanicular network in the human femoral midshaft in a sample of Medieval males. The  
379 results showed that the medial and lateral femur regions had the highest densities of osteocyte  
380 lacunae when compared to anterior and posterior femoral aspects. The results correspond to  
381 what is known in literature in other species, as well as, in fresh human bone. The data also  
382 agree with the preservation of biomechanical loading patterns in humans, as well as, the  
383 lacunocanicular network, which changes in morphology with age, disease, and/or behavior.  
384 This suggests that the reconstruction of past human behavior within osteoarchaeology could  
385 incorporate osteocyte lacunae analyses into their microscopic sampling and analysis protocols.  
386 Not only can this be used as a complementary method to the bone exterior shape and size data,  
387 but histological analyses can also be applied to fragmented human remains where the external  
388 anatomy is compromised (Crescimanno & Stout, 2012; Cuijpers, 2006; Cummaudo et al.,  
389 2019; Haas & Storå, 2015; Lemmers et al., 2020). On the basis of our data, future researchers  
390 may be able to estimate which anatomical region a midshaft femur fragment derives from.

391

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400

#### 401 CONFLICT OF INTEREST

402 The authors declare no conflicts of interest.

403

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595

596 **TABLE CAPTIONS**

597

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

603

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

608

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

613 anterior, P – posterior, M – medial, and L – lateral. (Footnote: \*statistically significant  $p <$   
614 0.05)

615

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

622

623

#### 624 **FIGURE CAPTIONS**

625

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

631

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

638

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