Investigating histomorphometric relationships at the human femoral midshaft in a biomechanical context.

Justyna J. Miszkiewicz
Human Osteology Research Laboratory
School of Anthropology and Conservation
University of Kent
CT2 7NR
Canterbury, United Kingdom
E-mail: jm553@kent.ac.uk

ABSTRACT

Cortical bone histomorphometry utilised in human and animal bone biology studies has demonstrated that osteon densities and their geometric properties may be in a relationship with biomechanical load application. Further research is required to investigate mutual links between bone histological variables to elucidate their usefulness in future biomechanical studies. Here, a series of correlations exploring bone biology relationships at the human midshaft femur are performed using a large sample.

Mean intact, fragmentary, and total osteon population densities, Haversian canal diameter and area, osteon area, as well as osteocyte lacunae density were measured along the sub-periosteal cortex in sections removed from the posterior midshaft aspect of modern human male (n = 233) and female (n = 217) femora (total n = 450). Parametric and non-parametric correlations between the histology variables were sought in the entire sample, as well as within age and sex sub-groups.

Several significant positive and negative correlations explaining a large proportion of data variation were found. Haversian canal area, diameter, and osteon area were positively correlated. As the density of osteocyte lacunae increased, Haversian canals and osteons became smaller. As osteons increased in density, so did osteocyte lacunae, but Haversian canal and osteon area became smaller. Results were consistent across age and sex groups.

Findings suggest that an increased rate of bone remodelling is associated with a decrease in geometrical properties of osteons. An increased density of osteocyte lacunae and osteons indicates the involvement of bone maintenance cells in remodelling potentially induced by mechanical stimuli. Future histomorphometry studies will benefit from examining multiple bone histology variables due to many mutual bone biology relationships that exist at the human midshaft femur.

Keywords: histomorphometry, osteon, femur, biomechanics

Non-histomorphometric abbreviations:

AAD: age-at-death
YA: Young adult
MA: Middle-aged adult
F: Female
M: Male

1. INTRODUCTION

It is now well established that the process of bone remodelling is principal in bone functional adaptation [1, 2]. One methodological approach to gain insights into the relationship between biomechanical loading and long bone growth is histomorphometry, which has been mainly utilised in non-human animal (hereafter "animal") studies [e.g. 3 - 5], and less so using human samples [e.g. 6 - 8]. Bone microscopic structures studied as mechanically induced remodelling indicators can be categorised into two main groups.

The density (frequency count divided by section area) of (secondary) osteons and osteocyte lacunae can be estimated, providing insights into the amount of skeletal tissue present per section area [9, 10]. When totalled, the density of intact (N.On) and fragmentary (N.On.Fg) osteons indicates an overall osteon population density (OPD) that reflects bone remodelling dynamics. Early experimental studies on sheep radii [11], turkey ulnae [12], swine femora [13], and more recent research examining bear and rabbit bone [e.g. 14, 15] demonstrated that an increased number of osteons occurs in bone regions subjected to repetitive load application indicating new bone formation stimulated mechanically. Osteocyte lacunae density (Ot.Dn) has been studied as a proxy for osteocyte proliferation [16], and incorporated into biomechanical research due to the mechanosensing properties of osteocytes [17]. For example, increased values of Ot.Dn have been reported in mechanically strained salmon vertebrae [18], and mice limbs [19, 20].

The second group of histomorphometric variables includes parameter measurements (e.g. area, diameter, circumference) taken from osteons and their Haversian canals [9]. Studies have shown a link between these microscopic properties in animal and human long bone cortex with inferred or experimentally induced mechanical loading [e.g. 3 – 8, 21 - 23]. In general, smaller osteons and Haversian canals are associated with larger strains [23]. It is

currently not clear what the exact mechanism responsible for this inverse relationship is, but it is thought that localised clusters of smaller osteons offer a more structurally sound adaptation to repetitive loading that causes micro-damage [24].

Taking the above findings into account, the present study aimed to test whether mutual correlations between the described histomorphometry variables exist. This has not been previously tested utilising a large human sample. If certain negative or positive correlations are found, future studies may use such information as guidance in predicting bone micro-morphometric features, shedding new light on the current understanding of human cortical bone remodelling in a biomechanical context. Based on the principles of bone functional adaptation [2], it is hypothesised that positive correlations should exist between variables indicating increased remodelling, but these should be negatively correlated with osteon and Haversian canal size.

2. MATERIALS AND METHODS

2.1. Skeletal sample

The skeletal remains examined in the present study derive from a British archaeological collection (Canterbury, UK) dated to the late medieval period (early 11^{th} to 16^{th} centuries A.D.) [25], curated in the Human Osteology Research Laboratory in the School of Anthropology and Conservation (University of Kent, UK). The sample consisted of 450 adults, 233 of which were males and 217 were females (Table 1). The examination followed anthropological codes of ethics and practice (e.g. AAPA 2003 [26], BABAO 2010 [27]), and guidelines for invasive sampling (English Heritage 2013) [28]). Whilst there will be behavioural differences between these and contemporarily living humans, as this is a well preserved skeletal collection, it offers a large sample based insight into human bone biology. The range of physical activities represented by the examined individuals would have been

specific to the labour demands (e.g. according to social status stratification) of the Middle Ages in Britain [29], serving as a suitable inferred biomechanical setting for seeking universal (i.e. population-level) bone histomorphometric correlations.

2.2. Methods

2.2.1. Osteological assessment

Standard gross osteological procedures [30] were followed to estimate the sex (e.g. from sex-specific cranial and pelvic landmarks, and joint surface measurements) and age-at-death (AAD), applying multiple methods to increase the accuracy of biological profile reconstruction in each individual. Since OPD is considered a useful histological variable in AAD estimation from fragmentary skeletal remains (e.g. in forensics [31]), individuals were categorised into anthropological age ranges [30] (Table 1, see Table 2 for mean estimates) based on a standard macroscopic examination of cranial suture closure [32], dental wear on the permanent dentition [33], age-specific morphology of the pubic symphysis and auricular surface on the pelvis [34], allowing statistical analyses to be undertaken on separate age groups.

2.2.2. Sectioning

The femur was examined due to its weight-bearing capacities and involvement in lower limb movement. Using either a table-mounted bone holder (Dremel Muli-Vise®), or a hand holder (Irwin Quick-Grip Mini®), two parallel transverse cuts were made with a hand saw (Irwin BiMetal® 24PT, 12”/300mm) in the linea aspera posterior midshaft aspect. Longitudinal cuts were made using an electronic drill (Dremel Rotary Tool 230V – 50Hz 140W), resulting in approximately “C” shaped 1 cm ± 0.2 cm sections. The linea aspera region was deemed a suitable sectioning location because a) it is an insertion area for adductor muscles (although the present study does not ascertain or test a direct relationship between muscles, tendons,

and bone growth), and b) a preliminary analysis [35] where the most intra-section (i.e. anterior, posterior, medial, and lateral) histomorphometric variation was observed posteriorly when comparing femora of distinct robusticity indices in this sample.

2.2.3. Histological preparation

Standard histology preparation techniques were followed [36]. Sections were embedded in Buehler EpoxiCure® resin. Using a Buehler Isomet 1000 Precision Saw with a Buehler Diamond Wafering Blade (15.2 cm x 0.5 mm), each specimen was sectioned in half (from the medial towards the lateral end) reducing its (longitudinal) cortical thickness to 0.5 cm. Sections were mounted onto microscope glass slides, cut to 400 – 100 μm, ground (on a Buehler Eco-Met 300 Grinder-Polisher), polished (Buehler MicroPolish II 0.3 μm powder), washed, dried, and cleaned in an ultrasonic tub, dehydrated in 95% and 99 – 100% ethanol, cleared using histoclear, and sealed with cover slips.

2.2.4. Histomorphometric procedures

Using a digital camera (Olympus DP25) mounted on a high-powered microscope (Olympus BX51), images viewed under transmitted light were captured from six main regions of interest (ROIs) (Figure 1) and analysed in CELL® Live Biology Imaging software. The sub-periosteal region (i.e. cortical bone adjacent to the periosteum) (Figure 1) was examined because of the biomechanical context in the present study, and supported by previous experimental research where it had been shown that mechanically stimulated bone formation is formed sub-periostealy enhancing tissue strength [2]. The selection of ROIs within a section was largely determined by the visibility of osteons, sometimes obscured by localised diagenetic or taphonomic changes. Only those thin sections that displayed an almost entirely visible histology were examined, and if no confident identification of microscopic structures could be made, a variable was not recorded (hence minor disparities in sample size numbers

are reported in the results).There are currently no standardised guidelines as to ROI selection techniques [37], but it is recommended that a minimum of 25 to 50 intact osteons should be examined per cross-section [9]. Two ROIs (#1 and #6 as seen in Figure 1) were selected in the medial and lateral ends, two ROIs (#3 and #4) directly below the linea aspera region, one postero-medially (#2), and one postero-laterally (#5), allowing to examine a minimum of 60 and a maximum of 120 osteons (80 on average) per section:

1. One image viewed at 2X or 4X magnification was captured and used as a point of reference throughout the recording procedure.
2. Six images were captured within selected ROIs at 10X (~2.24mm^2 each).
3. Four images at 20X (~0.56mm^2 each), and 10 to 16 images at 40X (~0.13mm^2 each) were captured within each of the ROIs (point 2 as above).

The recorded variables were (a) counted (to estimate density), and (b) measured (in µm or µm^2) (see Table 3 for exact definitions, and Figure 2 for illustrations):

(a) Osteons and osteocyte lacunae were counted in micrographs captured at 10X and 40X respectively, using the point count technique [38].

(b) The area of intact osteons was measured in micrographs captured at 20X, whereas Haversian canal area and diameter [39] in micrographs captured at 40X.

2.2.5. Statistical analyses

The data were analysed in IBM SPSS® 20 (2012) and *R* (2.5.0)® (2007) at $p = .05$. Data normality was checked using a Kolmogorov-Smirnov test. Appropriate transformations were chosen for all not normally distributed variables. Intra-observer error was assessed by re-taking measurements on 10% (n = 45) of sections, and comparing them against original values by plotting primary and secondary data on graphs, performing paired parametric correlations, Bland-Altman plots, and paired samples *t*-tests [40]. Leg differences (right n = 367, left n = 83) were tested using an independent samples *t*-test. Correlations were sought

using Pearson's (normal distribution) or Spearman's (non-normal distribution) correlations. As there were only five elderly individuals, their data are excluded from the age-controlled analysis.

3. RESULTS

3.1. Intra-observer error, leg differences, data distribution

The data were not affected by observer bias (paired correlations: n = 90, r^2 = .867 - .990, p < .05; paired samples *t*-test: n = 90, p > .05). No differences in histology were observed between left (n = 83) and right femora (n = 367) (p > .05) allowing to pool the specimens for all analyses. Due to a lack of data normal distribution, N.On, N.On.Fg, OPD, On.Ar, and Ot.Dn were square rooted, and H.Ar and H.Dm were transformed using logarithm10. Where data distribution remained not-normal following these transformations, non-parametric testing was undertaken (as indicated in tables).

3.2. Correlations

Descriptive statistics for non-transformed data are given in Supplementary Tables 1 (entire dataset, sex groups), 2 (age groups), and 3 (combined age and sex groups). All results from the inferential statistical testing are given in Tables 4 to 8. Moderate to strong correlations (i.e. where r is > .35) appear underlined in tables, but are interpreted only where the coefficient of determination (r^2) is > .20 (i.e. explaining more than 20% of data variation) [41]. Scattergrams for the entire dataset, age groups (Figure 3.1), and the sexes (Figure 3.2) are provided in Figure 3, whereas the results for combined age and sex groups are given in Supplementary Figure 1 (females: 1.1, males: 1.2). Scattergrams illustrating correlations that were not consistently significant across the different age and sex groups are given in Supplementary Figure 2.

188 Osteon population density was excluded from correlations with N.On.Fg and N.On, because
189 these two variables formed OPD. All the remaining histology variables were found to
190 correlate significantly. Generally, osteon and Haversian canal geometric property data (i.e.
191 H.Ar, H.Dm, On.Ar) were negatively correlated with osteon and osteocyte lacunae densities
192 (i.e. N.On, N.On.Fg, OPD, Ot.Dn). The main finding was for six (i - vi) pairs of variables to
193 be consistently in a relationship (Figure 4): Haversian canal area changed with its diameter
194 (i), osteon area (ii), and osteocyte lacunae density (iii); Haversian canal diameter changed
195 with osteon area (iv), osteocyte lacunae density (v); and osteon area changed with osteocyte
196 lacunae density (vi), in a strong to moderately strong manner when analysing the entire
197 dataset (Table 4), males and females (Table 5), age groups (Table 6), and combined age and
198 sex groups (Tables 7 and 8).
199
200 Except for the above six, one additional moderately strong correlation (N.On. and On.Ar)
201 was identified in males (Table 5), middle-aged adults (Table 6), and middle-aged males
202 (Table 8). There were also two other moderately strong correlations identified within the
203 young male category (Table 8), and one within middle-aged males. Young males showed
204 weaker significant correlations between Ot.Dn and N.On.Fg, and Ot.Dn and OPD, whereas a
205 negative correlation between H.Ar and N.On.was observed within middle-aged males.

206 **4. DISCUSSION**

207 This study aimed to test whether commonly examined cortical bone histomorphometric
208 variables mutually correlated in a large human sample. Both negative and positive significant
209 correlations were identified, six of which were moderate to strong ($r^2 > .20$) and consistent
210 across the age and sex sub-groups (Figure 5). There was a trend for the measured variables
211 (i.e. H.Ar, H.Dm and On.Ar) to correlate negatively with the density variables (i.e. N.On,
212 N.On.Fg, OPD, Ot.Dn). Overall, the former increased in size when the latter decreased in

density. Although almost all of the reported correlations were statistically significant, many failed to explain large proportions of data variation, and therefore remain not interpreted in the discussion. Nevertheless, this study identified three main trends in the data, which form the following key findings from this study.

4.1. Haversian canal area, diameter, and osteon area are all positively correlated.

Small area of osteons was consistently associated with small Haversian canals in the present sample. This finding is in agreement with Skedros and colleagues' [42] work where Haversian canal and osteon surface area did not confirm positive (in ribs) and negative (in femora) allometric relationships. Extending the present result to a biomechanical context, it is also in line with past research where it has been demonstrated that small osteons and Haversian canals are in an inverse relationship with strain [e.g. 3 - 8, 21 - 23, 43]. Given that previous evidence mainly derives from smaller samples of animals and human cadaveric bone, results from the present study demonstrate strong bone histomorphometric relationships in a large human sample for the first time. They may indicate that Bone Multicellular Unit (BMU) activity responsible for generating osteons takes place in a manner influencing histomorphometry [44]. A strong correlation between the three measurements is illustrated in a three-dimensional scattergram in Figure 5.

4.2. As the density of osteocyte lacunae increases, Haversian canals and osteons become smaller.

Osteocyte lacunae are studied as a proxy for bone growth rates, because they represent the final stage of the osteoblast-osteocyte transformation process which is key in BMU-controlled bone remodelling [1, 2, 45]. Whilst the underlying mechanism of osteoblastic activity is complex, meaning that not all osteoblasts become incorporated into bone matrix as osteocytes [46], research has shown that osteocyte density reflects bone growth rates [16, 47]. However, it is stressed that skeletal growth on an inter-specific level is associated with

organismal life history, rather than sole biomechanical loading. Bromage et al [16] found that smaller animals with higher skeletal growth rates display higher osteocyte densities (negative relationship), but in the authors' (however small) human sample osteocyte lacunae density correlated positively with body mass. In the present study, increased densities of osteocyte lacunae were associated with smaller osteons and Haversian canals, matching the morphological and morphometric predictions of bone microstructural features associated with dynamic bone remodelling. The present finding is also in agreement with results from previous biomechanical studies where increased numbers of osteocytes have been noted in bone sites placed under high mechanical load in animals [e.g. 18, 19], and also when examining human pathological vs. healthy bone where remodelling is not balanced and, consequently, results in poor and weak bone mass characterised by a highly reduced amount of osteocytes [e.g. 48].

4.3. As osteons increase in density, so do osteocyte lacunae, but Haversian canal and osteon area become smaller.

This third finding was not universal in the present sample, but true in some data sub-sets. Firstly, higher density of intact osteons correlated with smaller osteon area in males, middle-aged adults and middle-aged males (and additionally with H.Ar in this sub-group) the strongest. Because intact osteons represent completed bone functional units, they provide an insight into the amount of bone tissue present per section area. It is likely that this correlation being specific to only a particular category of age and sex, highlights that age and sex controls are important when seeking correlations in large sets of bone histology data due to differences in bone metabolism. Other factors potentially affecting bone remodelling in the other sub-groups are addressed further below. However, the negative correlations between N.On and H.Ar, and On.Ar do still match the biomechanical and bone growth predictions for bone microstructure. In the light of bone remodelling principle, as osteocyte density

correlated positively with N.On.Fg and OPD in young males, it indicates that osteoblast proliferation is in a relationship with osteon deposition. A lack of strong correlations with intact osteons (N.On) implies an increased bone remodelling activity taking place in the young adult category (as evident from large numbers of fragmentary osteons).

4.4. Remarks on the complexity of bone growth.

Whilst the present results indicate that there are mutual changes in certain histological measurements that can be extrapolated to a biomechanical context, it is acknowledged that bone growth is a complex phenomenon. Several factors other than biomechanics influence bone remodelling, including age, diet, pathology, and genetic predispositions [2]. This study controlled for the effect of age by undertaking analyses on separate age groups, whereas the biomechanical context is supported by variable choice and sampling location. Dietary information available for this population is limited, but historical records [reviewed in 29] suggest that a portion (n = 40 in this study) of this sample consumed an overwhelmingly high protein diet. Evidence that bone remodelling may be affected (e.g. by an over-production of osteons) by blood changes (such as acidosis) associated with high protein consumption exists in the literature [49]. This dietary factor was here overcome by utilising a large sample size, meaning that only 8.9% of the data may be affected. Moreover, as the aim of this study was not to identify bone biology differences between specific behavioural groups, the universal relationships between histology variables remain valid. In order to account for pathology, it was ensured that bones with no abnormal macroscopic lesions and/or woven bone evidence observed microscopically were examined.

5. CONCLUSIONS

This study examined a variety of correlations between femoral bone histological indicators of bone remodelling. Small Haversian canals had small diameter values and tended to be

associated with small osteons. Large numbers of osteons were also associated with large numbers of osteocyte lacunae. However, it was observed that all measured variables decreased in size where an increase in density of osteons or osteocytes was noted. This result implies that dynamic bone remodelling (as indicated by large numbers of bone functional units) is associated with changes in the osteonal dimensional parameters. These results are in agreement with previous work demonstrating that bone undergoing high remodelling caused by experience of larger strains, exhibits smaller microscopic dimensions.

There are two implications arising from the presented analysis. Firstly, future studies that utilise bone histology in a biomechanical context will benefit from examining multiple variables (rather than selected few) due to the multiple bone biological relationships at the human midshaft femur. Secondly, it is demonstrated that bone remodelling in human bone influences the geometric properties of osteons, and confirmed that age and sex may have an effect on bone microstructure. This knowledge will be beneficial to forensic, anthropological, and clinical research.

ACKNOWLEDGMENTS

I thank Dr Patrick Mahoney for PhD research guidance and supervision, Prof Kate Robson Brown and Dr David Roberts for their helpful comments, Dr Simon Tollington for assistance with R, and the School of Anthropology and Conservation (University of Kent, UK) for PhD funding and facilitating access to the skeletal collection. My thanks are extended to the Editor-in-Chief, Associate Editor, and an anonymous reviewer whose invaluable comments and suggestions improved this paper.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY FIGURES 1 AND 2

REFERENCES CITED

1. van Oers RF, Ruimerman R, Tanck E, Hilbers PA, Huiskes R (2008) A unified theory for osteonal and hemi-osteonal remodeling. Bone 42: 250-259.
2. Robling AG, Castillo AB, Turner CH (2006) Biomechanical and molecular regulation of bone remodeling. Annu Rev Biomed Eng 8: 455-498.

3. Bourrin S, Ghaemmaghami F, Vico L, Chappard D, Gharib C, Alexandre C (1992) Effect of a five-week swimming program on rat bone: a histomorphometric study. Calcif Tissue Int 51: 137-142.
4. Skedros JG, Su SC, Bloebaum RD (1997) Biomechanical implications of mineral content and microstructural variations in cortical bone of horse, elk, and sheep calcanei. Anat Rec 249: 297–316.
5. Skedros JG, Sybrowsky CL, Parry TR, Bloebaum RD (2003) Regional differences in cortical bone organization and microdamage prevalence in Rocky Mountain mule deer. Anat Rec 274: 837–50.
6. Britz HM, Thomas CDL, Clement JG, Cooper DML (2009) The relation of femoral osteon geometry to age, sex, height and weight. Bone 45: 77–83.
7. Schlecht SH, Pinto DC, Agnew AM, Stout SD (2012) Brief communication: The effects of disuse on the mechanical properties of bone: What unloading tells us about the adaptive nature of skeletal tissue. Am J Phys Anth 149: 599-605.
8. Robling AG, Stout SD (2003). Histomorphology, geometry, and mechanical loading in past populations. In Agarwal SC, Stout SD (Eds) Bone loss and osteoporosis: an anthropological perspective. New York: Kluwer Academic/Plenum Publishers, pp. 189 – 206.
9. Stout S, Crowder C (2011) Bone Remodeling, Histomorphology, and Histomorphometry. In Crowder C, Stout SD (Eds) Bone Histology: An Anthropological Perspective, Boca Raton, FL: CRC Press, pp. 1–21
10. Wachter NJ, Krischak GD, Mentzel M, Sarkar MR, Ebinger T, Kinzl L, Claes L, Augat P (2002) Correlation of bone mineral density with strength and microstructural parameters of cortical bone in vitro. Bone 31: 90-95.
11. Lanyon LE, Goodship AE, Pye CJ, MacFie JH (1982) Mechanically adaptive bone remodeling. J Biomech 15: 141–54.
12. Lanyon LE, Rubin CT (1984) Static vs dynamic loads as an influence on bone remodeling. J Biomech 17: 897–905.
13. Raab DM, Crenshaw TD, Kimmel DB, Smith EL (1991) A histomorphometric study of cortical bone activity during increased weight-bearing exercise. J Bone Miner Res 6: 741– 749.
14. Wojda SJ, Weyland DR, Gray SK, Mcgee-Lawrence ME, Drummer TD, Donahue SW (2013) Black bears with longer disuse (hibernation) periods have lower femoral osteon population density and greater mineralization and intracortical porosity. Anat Rec 296: 1148-1153.
15. Yamada S, Tadano S, Fujisaki K (2011) Residual stress distribution in rabbit limb bones. J Biomech 44: 1285-1290.
16. Bromage TG, Lacruz RS, Hogg R, Goldman HM, McFarlin SC, Warshaw J, Dirks W, Perez Ochoa A, Smolyar I, Enlow DH, Boyde A (2009) Lamellar bone is an incremental tissue reconciling enamel rhythms, body size, and organismal life history. Calcif Tiss Int 84: 388-404.
17. Klein-Nulend J, Bakker AD, Bacabac RG, Vatsa A, Weinbaum S (2012) Mechanosensation and transduction in osteocytes. Bone 54: 190–182.
18. Totland GK, Fjelldal PG, Kryvi H, Løkka G, Wargelius A, Sagstad A, Hansen T, Grotmol S (2011) Sustained swimming increases the mineral content and osteocyte density of salmon vertebral bone. J Anat 219: 490–501.
19. Sugawara Y, Kamioka H, Ishihara Y, Fujisawa N, Kawanabe N, Yamashiro T (2013) The early mouse 3D osteocyte network in the presence and absence of mechanical loading. Bone 52: 189-196.

20. Nicolella DP, Moravits DE, Gale AM, Bonewald LF, Lankford J (2006) Osteocyte lacunae tissue strain in cortical bone. J Biomech 39: 1735-1743.
21. Skedros JG, Mason MW, Bloebaum RD (1994) Differences in osteonal micromorphology between tensile and compressive cortices of a bending skeletal system: Indications of potential strain-specific differences in bone microstructure. Anat Rec 239: 405-413.
22. Young DR, Niklowitz WJ, Brown RJ, Jee WSS (1986) Immobilization-associated osteoporosis in primates. Bone: 7: 109–117.
23. Van Oers RFM, Ruimerman R, Van Rietbergen B, Hilbers PAJ, Huiskes R (2008) Relating osteon diameter to strain. Bone 43: 476–82.
24. Martin RB (2002) Is all cortical bone remodeling initiated by microdamage?. Bone 30: 8-13.
25. Hicks M, Hicks A (2001) St. Gregory's Priory, Northgate, Canterbury Excavations 1988-1991 (p. Volume II). Canterbury: Canterbury Archaeological Trust Ltd.
26. Code of Ethics of the American Association of Physical Anthropologists (2003) http://physanth.org/documents/3/ethics.pdf
27. British Association for Biological Anthropology and Osteoarchaeology Code of Practice (2010) http://www.babao.org.uk/index/ethics-and-standards
28. Mays S, Elders J, Humphrey L, White W, and Marshall P (2013) Science and the Dead: guidelines for the destructive sampling of archaeological human remains for scientific analysis. Advisory Panel on the Archaeology of Burials in England. English Heritage.
29. Miszkiewicz JJ (2014). Ancient human bone histology and behaviour. PhD Thesis. University of Kent, UK.
30. Buikstra JE, Ubelaker DH (1994) Standards for data collection from human skeletal remains. Fayetteville: Arkansas Archaeology Survey.
31. Thomas CDL, Stein MS, Feik SA, Wark JD, Clement JG (2000) Determination of age at death using combined morphology and histology of the femur. J Anat 196: 463-471.
32. Meindl RS, Lovejoy CO (1985) Ectocranial suture closure: A revised method for the determination of skeletal age at death based on the lateral-anterior sutures. Am J Phys Anth 68: 57-66.
33. Miles AEW (1962). Assessment of the ages of a population of Anglo-Saxons from their dentitions. Proc R Soc Med 55: 881– 886.
34. Meindl RS, Lovejoy CO, Mensforth RP, Walker RA (1985) A revised method of age determination using the os pubis, with a review and tests of accuracy of other current methods of pubic symphyseal aging. Am J Phys Anth 68: 29-45.
35. Miszkiewicz JJ, Mahoney P (2012) Bone microstructure and behaviour in "gracile" and "robust" adult males from the Medieval Period, Canterbury, UK. Am J Phys Anth 147: 215-216 [abstract].
36. Bancroft JD, Gamble M (2008). Theory and practice of histological techniques. Elsevier Health Sciences.
37. Villa C, Lynnerup N (2010). Technical note: A stereological analysis of the cross-sectional variability of the femoral osteon population. Am J Phys Anth 142: 491–496.
38. Parfitt AM (1983) Steriologic basis of bone histomorphometry; theory of quantitative microscopy and reconstruction of the third dimension. In Recker RR (Ed) Bone histomorphometry: Techniques and interpretation. Boca Raton, FL: CRC Press, pp. 53–87.

39. Bell KL, Loveridge N, Power J, Garrahan N, Meggitt BF, Reeve J (1999) Regional differences in cortical porosity in the fractured femoral neck. Bone 24: 57-64.
40. Lynnerup N, Thomsen JL, Frohlich B (1998) Intra- and inter-observer variation in histological criteria used in age at death determination based on femoral cortical bone. Forensic Sci Int 91: 219–30.
41. Taylor R (1990) Interpretation of the correlation coefficient: a basic review. J Diagn Med Sonogr 6: 35-39.
42. Skedros JG, Knight AN, Clark GC, Crowder CM, Dominguez VM, Qiu S, Mulhern DM, Donahue SW, Busse B, Hulsey BI, Zedda M, Sorenson SM (2013) Scaling of Haversian canal surface area to secondary osteon bone volume in ribs and limb bones. Am J Phys Anth 151: 230–244.
43. Britz HM, Jokihaara J, Leppänen OV, Järvinen TL, Cooper DM (2012) The effects of immobilization on vascular canal orientation in rat cortical bone. J Anat 220: 67-76.
44. Smit TH, Burger EH, Huyghe JM (2002) A case for strain-induced fluid flow as a regulator of BMU-coupling and osteonal alignment. J Bone Miner Res 17: 2021–9.
45. Metz LN, Martin RB, Turner AS (2003) Histomorphometric analysis of the effects of osteocyte density on osteonal morphology and remodelling. Bone 33: 753-759.
46. Franz-Odendaal TA, Hall BK, Witten PE (2006) Buried alive: how osteoblasts become osteocytes. Dev Dynam 235:176–190.
47. Dai R, Ma Y, Sheng Z, Jin Y, Zhang Y, Fang L, Fan H, Liao E (2008) Effects of genistein on vertebral trabecular bone microstructure, bone mineral density, microcracks, osteocyte density, and bone strength in ovariectomized rats. J Bone Miner Metabol 26: 342-349.
48. Mullender MG, Tan SD, Vico L, Alexandre C, Klein-Nulend J (2005) Differences in osteocyte density and bone histomorphometry between men and women and between healthy and osteoporotic subjects. Calcif Tissue Int 77: 291-296.
49. Brandao-Burch A, Utting JC, Orriss IR, Arnett TR (2005) Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization. Calcif Tiss Int 77: 167-174.
50. Turner CH, Hsieh Y, Müller R, Bouxsein ML, Rosen CJ, McCrann ME, Donahue L R, Beamer WG (2001) Variation in bone biomechanical properties, microstructure, and density in BXH recombinant inbred mice. J Bone Miner Res 16: 206-213.