# The effects of aging on bone macro- and microstructure of two populations in the Asia-Pacific region

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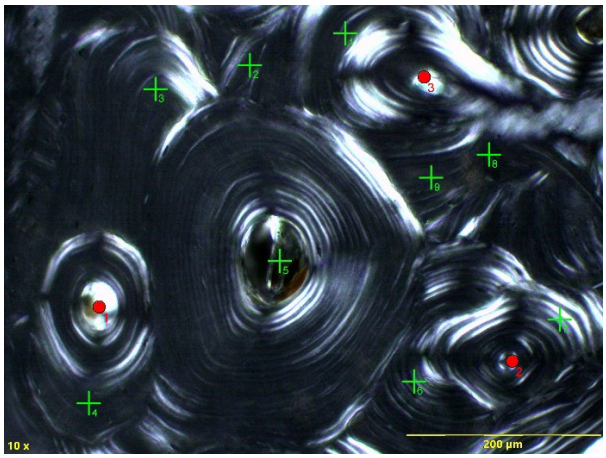
September 2023




A thesis submitted for the degree of Doctor of Philosophy, College of Medicine and Dentistry, James Cook University, Australia

## Declaration

This thesis is my own original work. Contributions by co-authors to papers are listed below in authorship declarations.

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Signed

Lucille T. Pedersen

## Foreword

The first two years of my PhD candidature were relatively tumultuous, with two major setbacks – the dreaded "C's" – cancer and COVID-19. Like for so many other people, the long-term cancellation of international travel due to the Covid pandemic, threw a spanner into my best laid plans. I was forced to make a total change in the topic of my thesis after a year of progress.

In January 2019 I had commenced my PhD with the topic "Chronological metamorphosis: An evaluation and revision of age-at-death estimation methods for human skeletal remains from a Southeast Asia population". In the first year of thesis writing, I had made good progress on the initial drafts of the first six chapters. I was due to fly to Thailand around the time of the initial outbreak of Covid, and for the next six months I was unsure if I would be allowed to travel overseas to complete data collection on the skeletal material held at Khon Kaen University (KKU) in northeast Thailand. By mid-2020 it became apparent that I would not be able to embark on my data collection phase whilst international travel was banned.

It was at this time that I began researching alternative PhD topics and commenced cancer treatment. I was fortunate that my primary supervisor, Associate Professor Kate Domett, was in the process of gaining ethics approval for a project that aimed to analyse quantitative data from modern Australian human bone histology samples prepared from skeletal tissue collected through the JCU Human Bequest program. Taking part in this project would allow me to continue my work on age-related skeletal degeneration, but with a focus mostly on microscopic analyses instead of macroscopic. Thus, began the metamorphosis of the PhD into "The effects of aging on bone macro- and microstructure of two populations in the Asia-Pacific region".

## Acknowledgments

Thank you to my fantastic supervisory team, Kate Domett, Anna Willis, and Nigel Chang - you kept me going through this very long project and lifted my spirits countless times. Thank you for your amazing feedback, advice, and encouragement. Justyna Miszkiewicz, you are the best histology guru that I know and am grateful for your advice and guidance, and your endless enthusiasm. Lit Chien Cheah, you saved me in the lab numerous times, and along the way we had fun trying to figure out our methods.

I would like to acknowledge the time and effort afforded by Professor Siân Halcrow who provided research support to facilitate communication with Khon Kaen University, Dr Nawaporn Techataweewan in selecting and processing the Thai bone sections, and Chelsea Morgan who put blood, sweat, and tears into turning these sections into histology slides – without those this thesis would offer much less, and I know how challenging that process is sister. Thank you to the wonderful James Cook University Anatomy Lab staff (Rod, Candi, Helen, Ash, Aggi, and Sharron) for welcoming me into the labs and showing me the ropes, and for your cheeky humour.

A big thank you to my family who have helped me through every step of my thesis and have always given me the most amazing encouragement. I bet they are just as relieved as I am that this is all over!

## Statement of Contribution of Others

This thesis includes previously unpublished data collected by others such as Associate Professor Kate Domett (James Cook University), and Associate Professor Nancy Tayles (University of Otago), and Afua Adjei (La Trobe University). I was responsible for the analysis and interpretation of the data for the final synthesis of the results.

Intellectual support

| | |
|---|---|
| Supervision | Assoc. Prof. Kate Domett was the primary advisor at James Cook University, Dr Anna Willis and Dr Nigel Chang were secondary advisors at James Cook University, Dr Justyna Miszkiewicz was an external secondary advisor from the University of Queensland. |
| Editorial Assistance and Manuscript preparation/ | Listed co-authors of publications included in the thesis provided intellectual input and relevant expertise to published manuscripts. |
| Sample preparation | Contributions are detailed below. |

Financial support

| | |
|---|---|
| | The candidate is a recipient of an Australian Government Research Training Program Scholarship<br><br>The candidate received 6 months extended study leave, competitive grants for conference fees and travel, and a College Top-up scholarship from the College of Medicine and Dentistry |

Research assistance

| | |
|---|---|
| | Dr Nawaporn Techataweewan provided support with ethics and sample selection and preparation for the Thai histology sample used in Chapters 5 and 6. Chelsea Morgan produced the histology slides from the Thai bone sections used in Chapters 5 and 6. |

## The Contribution of Authors to the Publications

**List of authors**

LP – Lucille Pedersen
KD – Kate Domett
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LS – Louise Shewan
DO – Dougald O'Reilly
CH – Charles Higham
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| **Thesis Chapter** | **Publication and Contribution.** |
|---|---|
| Chapter 2 | LP & KD. Adult age at death estimation: methods tested on Thai post-cranial skeletal remains. Adult age at death estimation: methods tested on Thai postcranial skeletal remains. *Anthropological Science*, 211-219. https://doi.org/10.1537/ase.211219 |

LP & KD conceived the study, LP performed the literature search and wrote the review. KD was responsible for review concept and editing.

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| Chapter 3 | LP, AA, GS, AW, NC, HB, SH, LS, DO, CH, KD. Vertebral osteophytosis in prehistoric northeast Thailand. (in preparation for submission 2023) |

LP & KD conceived the study, LP composed the overall question, conducted background research, and wrote the manuscript, conducted statistical tests, and interpreted the results, KD was responsible for editing, interpretation of the data, and collection of data for the two samples. KD, AA, AW, NC, HB, SH, CH: critically reviewed the manuscript and refined arguments. LS and DO secured grant funding.

| | |
|---|---|
| Chapter 5 | LP, JM, LC, AW, & KD. Age-dependent change and intraskeletal variability in secondary osteons of elderly Australians (in review 2023). |

LP: conceived the study, composed the overall question, background research, and wrote the manuscript, prepared the sample, conducted statistical tests, and interpreted the results, KD was responsible for conception and editing. JM provided expert advice on content and input into interpretation of the data and editing the manuscript. LC: provided expertise in sample preparation and laboratory procedures. AW provided critical review of the manuscript.

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| Chapter 6 | LP, JM, LC, NT, CM, AW & KD. Age-related histomorphometric variation in the femoral midshaft of modern Thai and Australian samples (in review). |

LP: conceived the study, composed the overall question, background research, and wrote the manuscript, conducted statistical tests, and interpreted the results, KD was responsible for conception and editing. JM provided expert advice on content and input into interpretation of the data and editing the manuscript. LC: provided expertise in sample preparation and laboratory procedures. NT and CM prepared the Thai sample. AW provided critical review of the manuscript.

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## Abstract

Interpopulation and intraindividual variation in adult bone remodelling make age at death estimation a particularly problematic step in the identification of unknown human skeletal remains. Age estimation methods have traditionally been developed using a reference population of northern American or Western European ancestry. However, application of these methods to other populations that have their own unique ancestry continues to cause considerable error and bias. Age estimation also remains more difficult in mid-aged and older adults as skeletal degeneration escalates with age and becomes more highly variable. These issues are only exacerbated by a world-wide shortage of suitable skeletal and human bone histological collections with documented age and sex, and equal representation of younger and older adults. The primary aim of this thesis was to increase the understanding of age-related adult skeletal degeneration of two ancestral groups within the Asia-Pacific region. To achieve this, firstly, the shortfalls of using Western population-based methods on Thai individuals was assessed through a review of the literature.

Secondly, bioarchaeological analysis of age-progressive vertebral osteophytosis (VO) formation was evaluated in relation to environmental and sociocultural changes from the Neolithic to the Late Iron Age in two populations from northeast Thailand. Lastly, histological analysis of secondary osteons in cortical bone was conducted to evaluate intraskeletal and interpopulation variation, using recently established Australian and Thai human bone histology collections. The key findings confirmed that Western-based methods incur considerable error rates and wide age ranges that are often far removed from a Thai individual's chronological age. The temporal evaluation of VO formation revealed a prevalence that did not conform to the hypothesised pattern expected from the archaeological and biological evidence for an increase in intensive manual labour from the Neolithic to the Iron Age. Histological analyses determined that bone size and type of bone influence the underlying age-related

histomorphometric features in elderly Australian males. Interpopulation comparisons showed a statistically significantly higher osteon population density, and larger osteon area in the Thai sample compared to the Australians, depending on sex and age group. These results support the popular view that age estimation methods should be specific to each population group to account for their unique genetic, environmental, and socio-cultural influences. This thesis contributes to our understanding of intraskeletal and interpopulation variation, particularly in the elderly and the Asia-Pacific ancestry groups – which has repercussions for the future development of adult age estimation methods.

## List of Abbreviations Used

| | |
|---|---|
| BMD | Bone mineral density |
| BMU | Bone multicellular units |
| BNW | Ban Non Wat |
| CMU | Chiang Mai University |
| FASE | Forensic Anthropology Society of Europe |
| Fem | Femur |
| FORC | Forensic Osteology Research Centre |
| H.Ar | Haversian canal area |
| Hum | Humerus |
| JCUHSHC | James Cook University human skeletal histology collection |
| KKUHSHC | Khon Kaen University human skeletal histology collection |
| OPD | Osteon population density |
| On.Ar | Osteon area |
| NBJ | Non Ban Jak |
| PVO | Pathological vertebral osteophytosis |
| RC | Resorption cavity |
| RI | Robusticity index |
| ROI | Region of interest |
| SD | Standard deviation |
| SE | Standard error |
| SEE | Standard error of the estimate |
| VO | Vertebral osteophyte/osteophytosis |

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# Chapter 1 Introduction and Background

## 1.1 Introduction

When a bioarchaeologist examines excavated ancient burials, or a forensic anthropologist arrives on a crime scene to assess skeletal remains, their expertise is relied upon to ascertain individual identity and interpret trauma and pathology. Age at death is an important element for determining population demographics from the burials representing a premodern population. This is of significance to understanding population growth, morbidity and mortality, and social organisation, among other features of human settlement (Bocquet-Appel & Bar-Yosef, 2008). In forensic cases, being able to reliably estimate the age at death of an individual, along with ascertaining biological sex, stature and ancestry, is crucial for identification of the deceased (Cattaneo, 2007). The success of age estimation methods largely depends on understanding the degree of individual and population variability in skeletal age indicators (Cappella et al., 2017). However, even with such importance placed on age estimation, there are still many challenges to accurately correlating age to skeletal maturation features.

Most of the age estimation methods that skeletal analysts rely on have been developed on large reference collections from North American and European populations, and inaccuracy in age estimates are known to be introduced when these methods are used on population groups from other regions of the world (Gocha et al., 2015; Schmitt, 2004). This thesis addresses the under representation of Asia-Pacific population groups by using new data from Thai archaeological samples, and two newly established human bone histological reference collections, the Khon Kaen University human skeletal histology collection (KKUHSHC) in

northeast Thailand, and the James Cook University human skeletal histology collection in northern Australia. This thesis uses the combined methods of bioarchaeological and histological analyses to evaluate both macroscopic and microscopic skeletal age indicators to address two main issues in adult age estimation – interpopulation variation between different ancestry groups and understanding the large variability in older adults.

An understanding of population-specific traits is critical as bone is a living and dynamic tissue that encapsulates a physical record of bone macro- and micro-structure as it changes, not only in relation to advancing age, but also in response to biomechanical stress, diet, disease, and trauma (Kobyliansky et al., 2000). This is known to influence the aging rate in skeletal tissue differently between individuals of the same population and between population groups (Garvin et al., 2012).

The documented age ranges for each method are often biased by an unequal representation of each biological sex and age group in the reference sample used to develop the method (Cox, 2000). A sample biased toward young adults will lower the mean age for each assigned age phase generated in the method (Bocquet-Appel & Masset, 1982). This creates a problem whereby older adults in a target sample tend to have their age underestimated (Cox, 2000; Gocha et al., 2015). As individuals age, the degree of variability tends to become more intense (Bertsatos et al., 2021), and assigned age ranges will become wider (Buckberry, 2015). These factors all contribute to older adults commonly being assigned into an unrealistic and unhelpful 50+ age category (Milner & Boldsen, 2012). This has led to a longstanding misconception that not many adults in prehistoric populations reached old age (Cave & Oxenham, 2016; Gowland, 2007), or that males lived longer than females (Cave & Oxenham, 2017). Obtaining better age categories for elderly adults is critical for forensic investigations as the number of adults globally aged over 65 years is steadily increasing each decade, rising by 16% by 2050 (United Nations Department of Economic and Social Affairs, 2022). Population statistics project that by 2030, 20% of the Australian population will be over 65 years of age (United Nations

Department of Economic and Social Affairs, 2022), and approximately 25% of the Thai population will be over 60 years of age (Knodel & Chayovan, 2014). This makes it essential to document the range of age-related skeletal variation in this demographic.

Advances in methods are constantly being introduced as research increases in depth and breadth to potentially improve adult age estimation. The correlation of vertebral osteophyte (VO) prevalence with age is one such method that is showing potential (Chiba et al., 2022; Kacar et al., 2017; Listi & Manhein, 2012; Praneatpolgrang et al., 2019; Watanabe & Terazawa, 2006) but it still requires rigorous testing and development. Histological methods have not been scrutinised for as long or to the same degree as macroscopic analysis, but there is an increasing interest in histological methods which examine bone microstructure, particularly in situations where macroscopic methods cannot be used to estimate age of human remains that are incomplete or highly fragmented (Franklin, 2010). This benefit negates the cost, time, and ethical challenges of this destructive technique (Bertsatos et al., 2021).

## 1.2 Aim of This Study

This study aims to quantify the range of temporal, individual, and population variability in age-related skeletal changes that occur in Australian and Thai populations, which have yet not been adequately investigated. Sample age distribution skewed toward elderly adults in this study will help to improve the understanding of skeletal degeneration in adults beyond 50 years of age. The range of intra- and inter-skeletal variability quantified in this study will ultimately expand our knowledge of human variation and thus contribute to the development of a higher standard of accuracy in age at death estimations for the Australian and Thai populations in the fields of forensics and bioarchaeology.

## 1.3 Objectives

*Objective 1: Appraise how commonly used age estimation methods, developed on American and European populations, are subject to complications from interpopulation-based skeletal variation, when applied to a genetically distant (northeast Thai) population.*

*Objective 2: Validate using osteophytes as age indicators, by evaluating how vertebral osteophyte formation rates differ between two pre-modern Thai populations (Neolithic to Iron Age), and considering possible contributing factors, such as different lifestyle/physical activities.*

*Objective 3: Within a northern Australian population, evaluate intra-skeletal variation of the three most common histomorphometric features used in age estimation methods.*

*Objective 4: Contrast cortical bone remodelling of the femoral mid-shaft between Thai and Australian samples. Identify if there are significant inter-population variations that justify the need for population-specific age estimation methods.*

*Objective 5: Based on the findings from the previous objectives, evaluate whether the currently adopted age estimation methods require reconsideration.*

## 1.4 Background and Significance

### The Importance of Age Estimation

Chronological age is measured in calendar years since the time of birth, whereas an individual's physiological age is an estimate, assessed by how much growth or subsequent degeneration and remodelling has progressively occurred in the bodily tissues (teeth and bones) with advancing age (Falys & Lewis, 2011; Rogers, 2016). Assuming good health and a stable diet and environment, this regular pattern of change occurs in relative synchrony with chronological age stages and developmental milestones in subadults (Scheuer & Black, 2000). However, once adulthood is reached, age estimation becomes reliant on highly variable stages of skeletal and dental remodelling and degeneration (Franklin, 2010). This requires that different methods be used when estimating the age of subadults as opposed to adults. Macroscopic observations of morphological characteristics of the bone or joint surface (Figure 1-1) are compared with published standards of criteria-based phases that have been correlated to a chronological age range (Garvin et al., 2012; Gocha et al., 2015).

These osseus changes in adults all have a much broader relationship with chronological age compared to subadults. They become more unpredictable as bone tissue degenerates as an individual advances in age (Buckberry, 2015; Falys & Lewis, 2011). For example, where subadult age is usually quite accurate due to rapid incremental changes in dental and skeletal growth providing a narrow estimated age range within 0.5 to 2 years (Cunha et al., 2009), in adults the provided age range is at least 10-20 years, or the range may only be described in general categories of young adult, mid aged adult, or older adult (Calce, 2012).

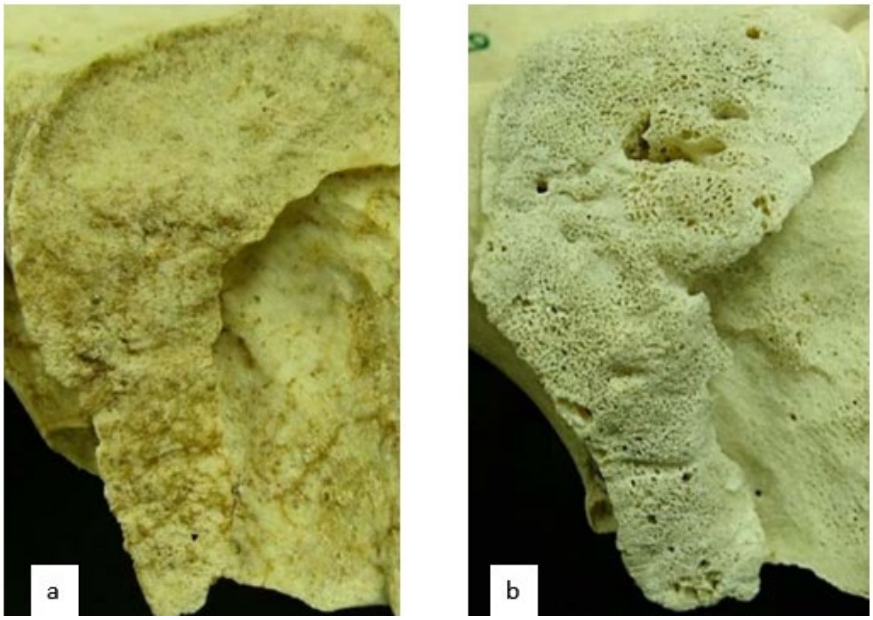



*Figure 1-1 Example of the stages of degeneration used to macroscopically assess age. Auricular surface of the ilium; (a) a 47-year-old female exhibiting bone with a smooth and with no obvious porosity; (b) 72-year-old female with degeneration of the bone surface in the form of porosity and erosion (Singsuwana et al., 2012:209).*

The choice of which of the adult age estimation methods to use is dependent on several factors, and each individual skeleton needs to be assessed on a case-by-case basis. Observers are required to determine which skeletal elements are present for analysis (usually the pelvis, clavicle, cranium, and/or ribs are used), and the level of completeness and preservation of these elements. The most relied upon techniques focus on scoring the degree of gross-morphological degenerative changes to joint articular surfaces in correlation with age at death (Brooks & Suchey, 1990; Buckberry & Chamberlain, 2002; İşcan et al., 1984, 1985; Lovejoy et al., 1985b; McKern & Stewart, 1957; Osborne et al., 2004; Todd, 1920, 1921). Each method has its own limitations with regard to achieving a broad or narrow age range, and efficiency and ease of use (Cunha et al., 2009).

### Age Bias of the Elderly

In the analysis of prehistoric and historic burials, limitations in age estimation methods means that older adults are most often classified together into a 45+ or 50+ years of age category

(Haughton & Powlesland, 1999; Milner et al., 1989; Oliveira et al., 2006; Stojanowski et al., 2002; Walker et al., 1988; Wyatt & Miszkiewicz, 2019) which contributes to the lack of recognition of the true age of the elderly. This means that adults in their 60's, 70's, 80's or above appear to be invisible in the mortality profiles of the population (Appleby, 2011). This has prompted critical discussion on the ramifications of a general misconception that prehistoric adults had a much shorter lifespan, an average of 45+, compared to 70+ of modern adults with a similar life course (Cave & Oxenham, 2016; Gowland, 2007). Age bias of the reference sample (many more young adults than older adults) on which an age at death estimation method has been developed, leads to the underestimation of age in older adults and overestimating the age of young adults (Gocha et al., 2015). Another critical issue of importance is that biological sex and age biases in bone preservation are known to introduce error into past demographic profiles (Walker et al., 1988) and fertility estimates (Paine & Harpending, 1998). Bone tissue, weakened by a loss in bone mineral density in older adults, often more so in females during and post-menopause or those with osteoporosis (Warming et al., 2002), are suggested to contribute to a lack of preservation in the bones of older adults (Biehler-Gomez et al., 2022). This includes fragile features such as the pubic symphysis and the auricular surface of the ilium, as well as porous and less dense bones such as sternal rib ends and vertebrae, which are all key skeletal elements for age estimation (Gowland, 2007).

## Standards in Age Estimation Methods

Garvin and Passalacqua (2012) conducted a survey of current practices by forensic anthropologists and found that the age estimation method chosen by scientists was dictated by personal experience, familiarity with certain methods, as well as a lack of awareness of newer techniques, or uncertainty with how to accurately use recently developed methods. However, rather than relying on methods and procedures that are familiar, constant revision of methods or development of new techniques should be the normative practice, particularly as greater understanding of physiological processes is reached via new clinical research, and

as new skeletal samples become available for developing and testing methods. Several studies (Buckberry, 2015; Franklin, 2010; Garvin & Passalacqua, 2012; Marquez-Grant, 2015) are critical of the lack of standardisation in the training of forensic and biological anthropologists between institutions and countries. The *Daubert* ruling (Solomon & Hackett, 1996) from a 1993 US Supreme Court case demands that scientific evidence be attained from quantitative methods with known error rates or an acceptable standard deviation range, and methods must also be reliable and easily replicable. Following this ruling, Ritz-Timme et al. (2000, p. 129) determined that age estimation methods, especially in a forensic context, should fulfill the following specific demands: (i) they must have been presented to the scientific community through peer-reviewed publication; (ii) their accuracy must have been tested using valid statistical procedures and described by clearly defined terms; and (iii) the method must be accurate enough for routine forensic application. Solheim and Sundnes (1980) have suggested an error range of <±10 years is acceptable for forensic dentistry, while >±15 years is unsatisfactory. However, Christensen and Crowder (2009, p 1214) have pointed out that the courts will not dismiss a method if it is not highly reliable, they only require forensic anthropologists to relay *how reliable* the techniques they used are by reporting the known error or potential error rates.

The established macroscopic age methods have become standard practice due to their ease of use and decades of testing and revision on several different populations. However, most skeletal data used to develop these methods was collected from reference samples around 50 years to almost a century ago (for example, McKern & Stewart, 1957; Moorrees et al., 1963; Todd, 1920). Secular change in growth and development, occurring in response to genetic control and environmental factors over a number of generations, is widely reported (Cardoso et al., 2010; Rousset et al., 2003), this in turn affects the accuracy of these methods when used on modern populations, especially in a forensic context (Petaros et al., 2021). Introducing modern population data into the research is important to account for more diverse genetic admixture as the world's populations becoming increasingly mobile (Uren et al., 2020).

## Age Estimation and Population Differences

There are several concerns surrounding age estimation research that suggest that there needs to be more of a focus on developing methods using reference samples that, firstly, reflect the same regional location and environment as the individual for which age is being estimated (Wärmländer & Sholts, 2011), secondly, represent the same time period (Cox, 2000), and thirdly have a similar demographic and genetic profile (Hoppa & Vaupel, 2008). However, it is challenging to find large enough, ethically sourced human bone histology collections with documented age and sex, especially samples with equal numbers of both sexes, and with all age groups represented (Andronowski & Taylor, 2022).

In their review of scientific literature, Khan et al. (2017) noted the numerous studies that indicated regional and ancestry differences in bone microstructure, which suggests population-specific age estimation regression equations are required. The most commonly used age at death estimation methods have been established using skeletal reference samples mostly of European or African American ancestry, and to a much lesser extent other genetically distinct populations including Native American (Ubelaker, 1999), Black South African (Oettle & Steyn, 2000), Japanese (Igarashi et al., 2005), Portuguese (Rissech et al., 2006), and Thai (Kampan et al., 2014).

In 2017 the Forensic Anthropology Society of Europe (FASE) provided an open-source interactive Google map of human skeletal collections available for scientific research. As of February 2023, the map listed 151 documented skeletal collections located in 61 different countries (http://forensicanthropology.eu/osteological-collections/). This is a valuable resource that informs researchers of the number of individuals in the collection, population/s represented, and web address of where it is located.

Asia-Pacific populations, such as Australia and Thailand, are grossly underrepresented in the standard age estimation methods that have so far been developed. Any genetic and physiological differences between the sample population and reference population that the method was derived from compromises the accuracy of age estimation methods (Komar & Grivas, 2008). The following chapter in the thesis highlights the consistent underestimation of age in older adults and overestimation in age of younger adults from northeast Thailand populations, that was likely in part due to inherent variability in physiological patterns of ageing compared to the Western reference samples the age estimation methods were developed on. In this case, when having to use non-population specific methods on a Southeast Asian sample, the Scientific Working Group for Forensic Anthropology (SWGANTH) recommends that methods with greater variance in standard deviation in estimated age are the best option (SWGANTH, 2013). This would mean accepting a broader age range. Too broad an estimated age range increases the chances of including an individual's true age somewhere in that range, however, is not helpful in forensic cases where a narrow age range better assists in identifying a missing person (Garvin et al., 2012). This leads into the second part of the thesis which addresses the dearth of information on variability in bone remodelling in older Australian adults and Thai populations, using histology.

### Histomorphometric Methods

Histological analysis of bone has been used to estimate age at death since the method's initial conception by Kerley (1965) (Figure 1-2). A number of different methods have since been developed but there is still a need for further testing and refinement on different populations. They examine the different stages of formation of secondary osteons (Figure 1-2) from the initial resorption of old or damaged bone, followed by a reversal stage after which new bone is deposited appositionally in layers around a central vascular (Haversian) canal. The methods most commonly involve quantifying a combination of histomorphometric features such as intact and fragmentary osteons, osteon size and area, Haversian canal size and area, type of

osteon (Type I, Type II), percentage of osteonal bone, and cortical thickness. These histomorphometric features are discussed in greater detail in Chapter Four.

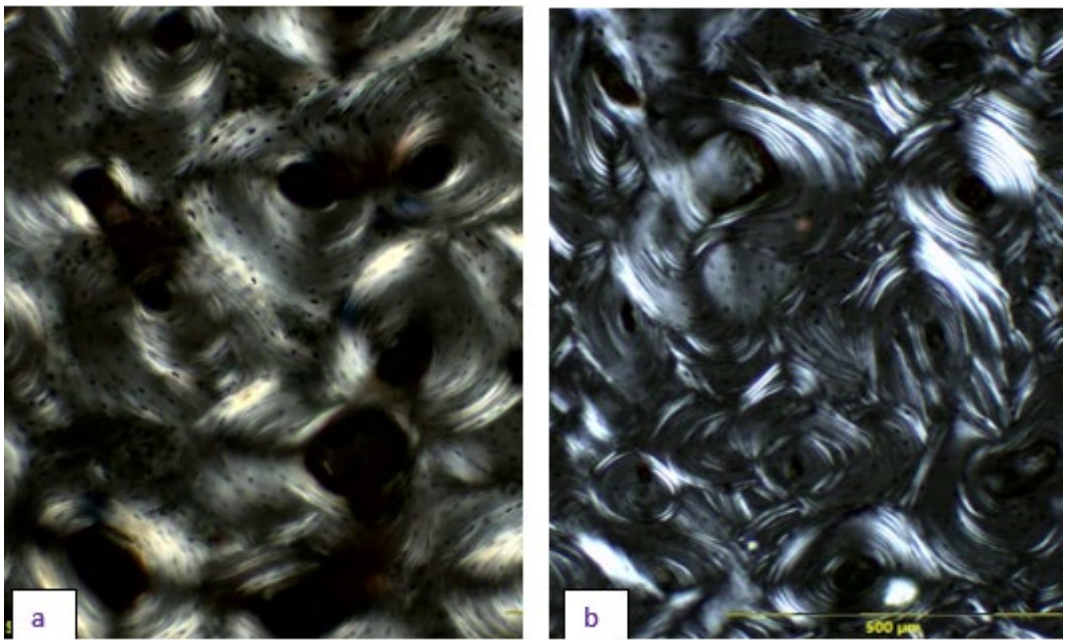



*Figure 1-2 Example of histological changes in bone microstructure. Image (a) is from a 46 year old Australian female, showing lower OPD and larger osteons compared to a 74 year old Australian female (b).*

The majority of histological age estimation methods have been developed using the femur (Ahlqvist & Damsten, 1969; Crowder & Dominguez, 2012; Ericksen, 1991; Fangwu, 1983; Kerley, 1965; Kerley & Ubelaker, 1978; Maat et al., 2006; Samson & Branigan, 1987; Singh & Gunberg, 1970; Thompson, 1979; Watanabe et al., 1998) and less often the tibia (Kerley, 1965; Kerley & Ubelaker, 1978; Singh & Gunberg, 1970; Thompson & Galvin, 1983; Uytterschaut, 1985), fibula (Kerley, 1965; Kerley & Ubelaker, 1978), humerus (Iwamoto et al., 1978; Rother, 1978; Thompson, 1979; Yoshino et al., 1994), rib (Cho et al., 2002; Stout et al., 1994; Stout & Paine, 1992), ulna (Thompson, 1979), cranium or mandible (Cool et al., 1995; Drusini & Businaro, 1990; Singh & Gunberg, 1970), clavicle (Stout et al., 1996), or a metacarpal (Kimura, 1992). These methods were, for the most part, developed using individuals of Caucasian ancestry from the United States or Europe (Netherlands, Germany,

Italy), while only a few were developed using Japanese, Chinese, or South American populations.

The main drawback is that studying cortical bone remodelling requires the removal of small portions of bone from individuals that must undergo a long preparation process before histomorphometric features can be examined under a microscope. Firstly, the bone portions removed from cadavers need to be macerated in a mix of warm water and liquid detergent with enzymes to break down the soft tissue. This is followed by drying of the bone sections, embedding in epoxy, cutting, mounting on microscope slides, grinding, and polishing. Chapter Four explains this process in greater detail. The preparation process is destructive, time consuming, requires specialised training and equipment, and the cost too prohibitive to process large skeletal samples (Buckberry, 2015). However histological methods offer a quantitative way to develop age estimation methods, instead of relying on visual interpretation of macroscopic skeletal feature which tends to be highly subjective between observers (Franklin, 2010; Kerley, 1965). Researchers have very few options to access already prepared human bone histology slides and digital images, and these are described below.

### World-wide Histological Collections of Human Bone for Research

It is very rare for curators of human skeletal collections to grant permission for researchers to conduct the destructive sampling procedures necessary for histological analyses. Therefore, across the world there remains a scarcity of ethically sourced and documented (known age and sex) human skeletal reference series specifically for use in histological research. Unlike the FASE open-source online map of the 153 contemporary and historical human skeletal collections held in institutions around the world (Petaros et al., 2021), there is not yet a similar resource for locating curated human bone histology collections. It is important for the advancement of science and collaborative research that such collections gain visibility. If researchers are not aware of the existence of collections, or how to access them, then the

opportunity to expand the boundaries of current knowledge is limited. Andronowski and Taylor (2022) tried to address this issue by publishing a summary of three of the documented human bone histology collections, as well as introducing their own newly established collection held at the Memorial University of Newfoundland in Canada. The Faculty of Medicine, Dentistry and Health Sciences at the University of Melbourne, Australia curates the *Melbourne Femur Research Collection*. It has supported over 80 research projects since its establishment in 1991 (Blanchard et al., 2019; Thomas & Clement, 2011). Various smaller tissue collections which combined make up the *National Museum of Health and Medicine Skeletal Tissue Collections* in the United States (Spatola et al., 2011) have been studied for decades. Very little information has so far been published on the *Texas State Comparative Histology Collection* that was initiated in 2019 (Gocha et al., 2022).

It is likely that there are far more ethically sourced human bone histology collections that have been created around the world, such as in forensic institutes, universities, or medical museums, however, their data is not easily retrievable or remains unpublished. For ethical reasons digital data files and histology images need to be kept on secure servers and the histologic and bone samples in secure facilities with access via authorisation from the curator (Andronowski & Taylor, 2022). These collections may not be open to all, or the curators of such collections may still need to overcome ethical or legal concerns related to their use in research and teaching. It is anticipated that the number of histology collections available for scientific research will gradually increase as more people are trained in the preparation of thin section slides and age estimation via histological methods becomes more routine. The Australian and Thai histology samples used in this dissertation will also be available for further research projects.

| Texas State Comparative Histology Collection (TXSTCHC) | |
|---|---|
| **Location:** | United States, Forensic Anthropology Centre at Texas State (FACTS) at Texas State University |
| **Established:** | 2019 |
| **Age at death range:** | Not published |
| **Ancestry/population:** | (% of the 710 donors in the FACTS skeletal sample) White (90%), Hispanic (4.5%), Black (3%), mixed ancestry (2%), Asian or Native American (<1%) |
| **Histology samples:** | Undecalcified thin section slides of mid-thoracic rib taken from 235 individuals and the midshaft femora and metatarsal from 6 individuals. Transmitted and polarised images of each slide. Dental histology slides from 30 individuals |
| **Additional information:** | Basic histomorphological and histomorphometric data |
| **Reference:** | Gocha et al. (2022) |

| Andronowski Skeletal Collection for Histological Research (ASCHR) | |
|---|---|
| **Location:** | Canada, Memorial University of Newfoundland |
| **Established:** | 2017 |
| **Age at death range:** | 15 – 105 years |
| **Ancestry/population:** | Body donors form Canada and United States |
| **Histology samples:** | >1200 bone samples (mid-shaft 6th rib and mid-shaft femur) from 621 individuals, complemented by imaging files (including X-Ray Photoelectron Spectroscopy, Confocal Laser Scanning Microscopy, micro–Computed Tomography). Also, cranial bones from 100 individuals and various other skeletal elements |
| **Additional information:** | Occupation, drug use history, general health information, cause and manner of death |
| **Reference:** | Andronowski and Taylor (2022) |

| Melbourne Femur Research Collection (MFRC) | |
|---|---|
| **Location:** | Australia, University of Melbourne |
| **Established:** | 1991 |
| **Age at death range:** | 1 – 95 years |
| **Ancestry/population:** | Australian |
| **Histology samples:** | > 600 femur bone sections from cadavers, and surgical specimens. 1000's of imaging files |
| **Additional information:** | CT/microCT/PQCT scans, microradiographs and associated data for 300 images |
| **Reference:** | Andronowski and Taylor (2022), Thomas and Clement (2011) |

| Ericksen Femur Collection (EFC) | |
|---|---|
| **Location:** | The Ohio State University, Texas State University, and the Tarrant County Medical Examiner's Office in Fort Worth, Texas, USA |
| **Established:** | |
| **Age at death range:** | 14 – 97 years |
| **Ancestry/population:** | European (95%), Dominican Republican or Chiléan (5%) |
| **Histology samples:** | Mid-shaft femur bone section and histological slides from 328 individuals |
| **Additional information:** | Catalogued bone tumour pathology and bone diseases, patient medical history and treatment, radiographs |
| **Reference:** | Andronowski and Taylor (2022) |

| National Museum of Health and Medicine Skeletal Tissue Collections (NMHMSTC) | |
|---|---|
| **Location:** | United States, Armed Forces Institute of Pathology, Washington |
| **Established:** | 1862 as the Army Medical Museum |
| **Age at death range:** | Subadults and adults |
| **Ancestry/population:** | |
| **Histology samples:** | A combination of several different collections with >10,000 histological slides of stained and undecalcified bone and joint sections (tibia, fibula, femur) |
| **Additional information:** | Catalogued bone tumour pathology and bone diseases, patient medical history and treatment, radiographs |
| **Reference:** | Andronowski and Taylor (2022), Spatola et al. (2011) |

### The Australian and Thai histological Collections Analysed in this Thesis

The Australian and Thai human bone histology collections used in this dissertation are newly established (since 2021) and are yet to be introduced to the scientific community. Below is a summary of their profiles. The collections are somewhat limited in size due to their newness, particularly the Australian sample. However, the collection is actively being expanded with more bone samples, histology slides, images, and new research projects. Figure 1-3 illustrates the geographical locations for the five currently available human bone histology collections.

## James Cook University Human Skeletal Histology Collection (JCUHSHC)

The Human Bequest program at James Cook University (JCU) College of Medicine and Dentistry accepts human donors for the purpose of teaching and research in anatomy and pathology. In 2021, ethics permission was granted (JCU Human Research Ethics approval #H8352) to initiate the JCUHSHC, which is to serve as a hard tissue reference database available for researchers from fields encompassing anthropology, the biological and forensic sciences, and medicine. The Australian histological bone sample used in this study is unique in that sections of bone have been collected from three different bones intra-individually for this population. Midshaft anterior or posterior bone sections or full transverse cross-sections were removed from the femur, humerus, and 7$^{th}$ rib from each embalmed cadaver. Each bone sample was then processed into thin section slides for microscopic analyses. From each thin section a series of digital images were produced using both transmitted and polarised light to establish a permanent record of bone microarchitecture properties. The collection currently consists of older/elderly adults with an average age at death of 82 years. The body donations were received from hospitals and morgues from within a 400 km radius of the regional city of Townsville in north Queensland, Australia. Potential donors supplied basic medical information via the donor consent form which asks for their current and previous medical conditions and surgeries. The ancestry of donors was not recorded but a donor could indicate if they are of Aboriginal or Torres Strait Islander origin. To the best of knowledge there are currently no Indigenous Australian donors included in the JCUHSHC. The Australian Bureau of Statistics defines ancestry as the ethnic and cultural background that individuals most self-identify with and can relate to the cultural group or place of birth of their relatives going back around three generations (abs.gov.au). The City of Townsville 2021 census (Australian Bureau of Statistics 2021) provides a record of the cultural diversity of the population that the donors came from. In the census, 79% of respondents indicated they were born in Australia and the majority identified their ancestry as either English or Australian (74% of the responses), followed by either Irish or Scottish ancestry (21%), German or Italian (9.5%),

Aboriginal and/or Torres Strait Islander (8.8%), Filipino or Dutch (2.8%). The Human Bequest registrant screening process excludes donors with notifiable infectious diseases, and any of the following factors that can hinder the embalming process: excessive body mass index, recent surgeries, or time of death greater than 48 hours before embalming is initiated. The cause of death and contributing medical conditions are obtained from the Death Certificate (Form 9). Working with embalmed rather than fresh bone reduces biological hazards and tissue deterioration, and there is no indication that the embalming process interferes with cortical bone microstructure (Melnyk et al., 2021).


*Figure 1-3 Location of the worldwide human skeletal histology collections. Blue circles are the newly established collections used in this study; red circles represent previously established collections.*

## Khon Kaen University Human Skeletal Histology Collection (KKUHSHC)

The modern Thai histological sample was produced from bone samples removed from cadavers bequeathed to the Department of Anatomy, Faculty of Medicine, Khon Kaen University (KKU), northeast Thailand. Some cadavers were embalmed, while others remained

unembalmed (Techataweewan et al., 2018). Information obtained from the Body Donation Unit records and a survey of donors by Techataweewan et al. (2018) showed the average age at death of male and female donors was close to 70 years, but significantly more male body donations were received compared to female. Upon registering, donors were asked to provide information about their health, previous hospitalisations, income, occupation, level of education, ancestry, religion, and marital status. Stated occupations included public servants, businesspeople, farmers, labourers, monks, nuns, housewives, and retirees (Techataweewan et al., 2018). Just over half of registered donors revealed that they had an underlying disease (cancer, heart disease, diabetes, hypertension or a form of bone or joint disease) (Techataweewan et al., 2018).

Donors were excluded if they had a communicable disease, limb amputation, history of severe injury, or their weight was outside the range of 40 to 100kg (Techataweewan et al., 2018). The donors to the program were individuals that had resided in the Isan region, northeast Thailand. There has been a complex genetic interplay over time in the region, with an admixture of indigenous populations and migrants including Khmer, Suay, Phu Tai, and Vietnamese (Kutanan et al., 2011; McCargo & Hongladarom, 2004). However, the majority of donors are suggested to be from the Tai-Lao ethnic group (Techataweewan et al., 2017) who have a close genetic relationship with southern Chinese populations (Muisuk et al., 2020). A large portion of the donors come from rural areas, towns, and villages, while the remainder are from the major cities of Khon Kaen and Nakhon Ratchasima in the Isan region (Techataweewan et al., 2018).

Both the modern populations from northeast Thailand and Australia each have a unique genetic admixture, hence the need to verify how, and to what extent, bone microstructural properties differ within and between these populations, and this will be explored further in Chapters Five and Six.

## 1.5 Significance of this Research

Estimation of the age of deceased adults by measuring skeletal degeneration is one of the most critical components to building a population demographic profile or biological identity of an individual. Yet, there is still limited research with a focus on age-related skeletal metamorphosis for Australian and Thai populations. This leaves forensic anthropologists and bioarcheologists having to rely on age estimation methods developed on genetically and regionally distant populations, even though ancestral and demographic sample differences have been widely discussed as creating over- or underestimation of age and wide age ranges. This study uses macro- and microscopic analyses of age dependent traits from Australian and Thai skeletal samples, to address the dearth of research within the Asia-Pacific region. A common limitation of traditional macroscopic methods is the placement of older adults into an open-ended 50+ age category, mostly due to inadequate number of older samples. The bias toward older adults of the Australian and Thai histology collections in this study will help to address the lack of understanding of the wide range of variability in older adults. The more research expands into genetically and regionally distant populations, the better we can understand and account for human variation. Incorporating this knowledge into age estimation methods is a step toward the goal of providing reliable, replicable, and accurate biological identity to unknown human remains. This is crucial for the future of age estimation in forensic investigations and palaeodemographic and palaeoepidmiological analyses.

## 1.6 Thesis Structure

This dissertation is compiled from a series of published and in-review manuscripts, forming a thesis by publication. Through a review of the scientific literature, *Chapter Two* defines the inherent issues affecting accuracy and precision in age at death estimation standards when interpopulation variation is taken into consideration. This chapter is published:

Pedersen, L. T., & Domett, K. (2022). Adult age at death estimation: methods tested on Thai postcranial skeletal remains. *Anthropological Science*, 211-219. https://doi.org/10.1537/ase.211219.

*Chapter Three* examines the correlation between age and one form of skeletal degeneration - vertebral osteophytosis. VO prevalence was compared between two prehistoric populations in northeast Thailand, spanning the Neolithic to the Late Iron Age (1750 BC – 820 AD) to investigate the correlation of VO rates to age and sex, and take into consideration possible contributing factors such as different lifestyle and physical activity patterns. The relationship between age and the prevalence of VO in Thai populations can be incorporated into future development of population specific age estimation methods. The paper for this chapter has been prepared for publication but not yet submitted:

Pedersen, L. T., Adjei, A., Stannard, G., Willis, A., Chang, N., Buckley, H., Halcrow, S., Shewan, L., O'Reilly, D., Higham, C., Domett, K. (n.d). Vertebral osteophytosis in prehistoric northeast Thailand.

*Chapter Four* introduces the basics of bone histology and identifies the histomorphometric features analysed and discussed in the following two chapters.

*Chapter Five* is a histological analysis of intraskeletal variation between the femur, humerus, and rib of elderly Australians, which has not been previously defined in Australian elderly individuals. This chapter is published:

Pedersen, L.T., Miszkiewicz, J., Cheah, L.C., Willis, A. & Domett, K.M. (2024) Age-dependent change and intraskeletal variability in secondary osteons of elderly Australians. Journal of Anatomy, 00, 1–15. Available from: https://doi.org/10.1111/joa.14010

*Chapter Six* is a comparative histological analysis of the effects of aging in bone microstructure in two genetically distinct populations in the Asia-Pacific region. The paper for this chapter has been submitted for review to the Journal of Bone and Mineral Metabolism: L.T. Pedersen, J. Miszkiewicz, Lit Chin Cheah, N.Techataweewan, C. Morgan, A. Willis, K. M. Domett (n.d). Age-related histomorphometric variation in the femoral midshaft of modern Thai and Australian samples.

*Chapter Seven* concludes this study by discussing significant differences in aging related degeneration in bone macro and microstructure between Australian and Thai populations, and the implications for age at death estimation in forensic and archaeological contexts.

# Chapter 2 Adult Age at Death Estimation: Methods Tested on Thai Postcranial Skeletal Remains

While working on archaeological excavations in Southeast Asia and analysing the skeletal remains for trauma and disease, the lack of population specific age estimation methods became frustrating. This provided the impetus for this thesis, starting with a literature review which documents the shortfalls in using age estimation methods developed on reference populations that are different from Southeast Asian populations on many levels –culture, genetics, health, and their environment – all of which impacts skeletal development. This chapter has been published, and a copy is provided in Appendix A. In this chapter the article has been reformatted into the style of the thesis.

Lucille T Pedersen and Kate Domett (2022), Adult age at death estimation: methods tested on Thai postcranial skeletal remains. *Anthropological Science*, *130*(2), 147-159, DOI: 10.1537/ase.211219.

## 2.1 Introduction

Age estimation is a crucial element in the analysis of human skeletal remains when building a biological profile, either to identify an individual in forensic cases or to establish mortality profiles of past populations. However, it is argued that reliability of methods is too dependent on the demographic profile of the Western reference samples that the method was developed on. The rate of bone remodeling and degeneration is known to be different between European, African and Asian populations (Aiello & Molleson, 1993; Schmitt et al., 2002), yet Southeast Asian skeletal research has not yet received the same amount of consideration as Western

populations (Cho, 2019; Go et al., 2019). There are several scenarios that have created increasing pressure to ensure that skeletal age estimation methods are sufficiently accurate and reliable for a Thai population. These include that in Thailand, each year, on average, at least 200 unidentified human remains are registered at government agencies (Central Institute of Forensic Science, n.d.) and, as of 2008, the Thai Tsunami Victim Identification and Repatriation Centre was still trying to identify almost 400 unidentified remains from the 2004 Boxing Day tsunami (United Press International (UPI), 2008). There has also been a recent upsurge of the number of archaeological excavations conducted in Southeast Asia and an interest in the mortality and health of these individuals.

Age estimation of unidentified adult human remains relies on standards that have used a reference population of known age, sex, and ancestry to correlate various signs of skeletal degeneration and remodeling to different life stages and their associated chronological age ranges. The most accurate age estimations will always be achieved using standards developed on a reference sample that is the same as the study (target) population, as skeletal growth and degeneration are non-uniform across time and regions due to complex relationships with genetics, environment, socioeconomics, and behavioral influences (Gocha et al., 2015; Schmitt, 2004). However, most adult age estimation methods universally in use today were originally developed on skeletal collections in Europe, North America, and South Africa. These methods still require further validation to test their reliability and accuracy on other populations, especially those geographically isolated from the reference sample, such as Southeast Asian populations.

Over the past six decades a number of studies have tested these adult age at death estimation methods on Thai skeletal remains. This collation of age estimation studies from domestic and international scientific journals and unpublished theses provides a quick reference guide for forensic and bioarchaeological experts to determine the effectiveness and reliability of each

technique when used on Thai individuals, and in particular which methods are best for young adults or older adults, and each sex.

The Thai studies have drawn their samples from the modern population, within there exists great genetic diversity due to high rates of migration and distinct ethnic indigenous groups (Benjavongkulchai & Pittayapat, 2018). This population's biological and cultural diversity, and largely agricultural economy, means that it is likely that skeletal maturation and degeneration will vary in relation to other geographically and genetically distant populations, hence the need to verify the reliability of the age estimation methods. The Thai studies use samples consisting of either skeletal remains from curated collections or autopsied cadavers (Table 2-1). Thailand has two large modern skeletal research collections with documented age and sex. The first is the Forensic Osteology Research Centre (FORC) at the Faculty of Medicine, Chiang Mai University (CMU) in northern Thailand, and the second is the Khon Kaen University (KKU) Human Skeleton Research Centre (HSRC) which has body donors from the rural Isan region, northeast Thailand. Both skeletal collections represent individuals who had mostly lived in the 20$^{th}$ to early 21$^{st}$ centuries and were from low to middle socioeconomic groups (Techataweewan et al., 2018; Techataweewan et al., 2017; Traithepchanapai et al., 2016). These curated skeletal collections are the first modern skeletal representations of this size, geographic location, and ancestry, available for research. They, therefore, represent an important opportunity to thoroughly test, develop and revise traditionally used age estimation methods that were developed on genetically distant populations.

Reports of accuracy and reliability for age estimation methods currently remain without a clear set of standards and this somewhat limits the comparability of results between studies (Garvin et al., 2012) (Table 2-2). Some methods present the results in terms of bias (the mean over- or under-estimation of age) and inaccuracy (a measure of the mean sampling error when comparing estimated age to known age); others report in confidence intervals, standard

deviations (SD) from the mean or the percentage of individuals for which known age fell within the SD of the mean, standard errors (SE) or correlation coefficients (differences between the estimated and known ages).

Table 2-1 Sample used to test methods and investigate age-related skeletal characteristics (in order of publication date).

| **Reference** | **Sample population** | **Total sample size (Male/Female)** | **Age range (years)** | **Mean age (years) Male/Female** |
|---|---|---|---|---|
| Schmitt (2004) | Thai - FORC skeletal collection | 66 (37/29) | 20 - 60+ | * |
| Namking et al. (2008) | Thai - HSRC skeletal collection | 200 (120/80) | 18 - 94 | * |
| Chanapa and Mahakkanukrauh (2011) | Thai - FORC skeletal collection | 200 (139/61) | 35 - 95 | 71 |
| Singsuwana et al. (2012) | Thai - FORC skeletal collection | 210 | 21 - 96 | * |
| Tipmala (2012) | Thai - FORC skeletal collection | 236 | 20 - 96 | * |
| Gocha et al. (2015) | Thai - HSRC skeletal collection | 88 (44/44) | 20 - 97 | 48/53 |
| Khomkham et al. (2017) | Thai - FORC skeletal collection | 48 (34/14) | 20 - 89 | * |
| Iamsaard et al. (2017) | Thai - HSRC skeletal collection | 454 (254/200) | * | 61/60 |
| Suwanlikhid et al. (2018) | Thai - FORC skeletal collection | 250 (125/125) | 22 - 89 | 58/62 |
| Chompoophuen et al. (2019) | Thai - CMU cadavers | 71 (49/22) | 25 - 92 | 52 |
| Monum et al. (2019) | Thai - CMU cadavers | 40 (24/16) | 16 - 88 | 58/54 |
| Praneatpolgrang et al. (2019) | Thai - FORC skeletal collection | 400 (262/138) | 22 - 97 | 66/66 |
| Singsuwan et al. (2019) | Thai - FORC skeletal collection | 200 (98/102) | 22 - 90 | 63/63 |

*information not provided or not included in English abstract. HSRC = Human Skeleton Research Centre (held at Khon Kaen University, northeast Thailand), FORC = Forensic Osteology Research Center (held at Chiang Mai University (CMU), northern Thailand).

Table 2-2 Comparison of measures of accuracy in each study

| Measure of accuracy | Reference | Bone region | Method | Accuracy |
|---|---|---|---|---|
| Regression correlation & standard error | Monum et al. (2019) | Femur (aspartic amino acid racemization) | Benešová et al. (2004) | Male age = SEE of 8.07 yrs (r = 0.912, $r^2$ = 0.8322). Combined sex age SEE 11.01 yrs (r = 0.8316, $r^2$ = 0.6916). Female age = SEE of 15.77 years (r = 0.716, $r^2$ = 0.5136). |
| | Chompoophuen et al. (2019) | Femur (histology) | Adapted from Yoshino et al. (1994), Martrille et al. (2009) & Pfeiffer (1998b) | r = 0.906, SEE = 8.26 (using combination of Pm.H.Ar, COL.B & Lm.B.Ar). Pm.H.Ar stood out as being the individual variable most closely correlated with age ($r^2$ = 0.733) with the lowest SEE of 9.91 years. |
| | Praneatpolgrang et al. (2019) | Cervical, thoracic & lumbar vertebrae | Snodgrass (2004), Watanabe and Terazawa (2006) & developed a modified scoring system | r = 0.801, $r^2$ = 0.642, SEE 9.506 ($p$<0.01) = highest accuracy with scoring method of Snodgrass (2004) using female mean lumbar score. |
| | Suwanlikhid et al. (2018) | Lumbar vertebrae | Adapted from Kacar et al. (2017), Van Der Merwe et al. (2006), Watanabe and Terazawa (2006) | $r^2$= 0.408 with a SEE of 11.686 years, $p$ = 0.000 (Highest accuracy using degree of osteophyte formation on the inferior surface of L1). |
| Accuracy & standard deviation | Gocha et al. (2015) | Auricular surface | Osborne et al. (2004) | 93.0% M, 88.1% F known age within ± 2 SD of assigned phase mean (males had highest correlation $r_s$ = 0.581, $p$-value 0.000). |
| | | Pubic symphysis | Suchey-Brooks (1990) | 88.6% M, 78.0% F known age within ± 2 SD of assigned phase mean (males had highest correlation $r_s$ = 0.907, $p$-value 0.000). |
| | | Auricular surface | Buckberry and Chamberlain (2002) | 81.4% M, 76.2% F known age within ± 2 SD of assigned score mean (females had highest correlation $r_s$ = 0.643, $p$-value 0.000). |
| | | Sternal end 4th rib | Iscan et al. (1984 & 1985) | 66.7% M, 48.0% F known age within ± 2 SD of assigned phase mean (males had highest correlation $r_s$ = 0.565, $p$-value 0.001). |
| | Schmitt (2004) | Pubic symphysis | Suchey-Brooks (1990) | 36.1% M, 37.9% F accurately classified within ±1 SD of the reference phase mean. |
| Accuracy | Tipmala (2012)<br>Singsuwan et al. (2019) | Pubic symphysis<br>Acetabulum | Modified Suchey-Brooks (1990)<br>Rissech et al. (2006) | 86.4% left os pubis, 85.2% right os pubis.<br>71% accuracy estimated age within 12 yrs of known age. 66% accuracy within 10 yrs. |
| | Singsuwana et al. (2012) | Auricular surface | Modified Lovejoy et al. (1985b) & Buckberry and Chamberlain (2002) & developed regression equations | 56.4% accuracy with SE 11 yrs (using left side). 67.8% accuracy with SE 10.6 yrs (using right side) |
| | Schmitt (2004) | Auricular surface | Lovejoy et al. (1985b) | 7% of individuals accurately classified within Lovejoy's 5-year classes. |

Yrs = years, SD = standard deviation, SE = standard error, SEE = standard error of estimate, $r^2$ = coefficient of determination = coefficient of determination, r & $r_s$ = correlation coefficient.

## 2.2 Review by Skeletal Region

### Pelvis (pubic symphysis, auricular surface, and acetabulum)

Several different techniques for estimating age via the pubic symphysis and auricular surface have been developed over the decades, some of which have been tested on Thai samples, including the Suchey-Brooks method (Brooks & Suchey, 1990), which was developed on a reference sample of predominantly North American ancestry with a minority from European, South American or Asian ancestry. Also tested on Thai samples was the original Lovejoy et al. (1985b) auricular surface method which has gone through several revisions by Buckberry and Chamberlain (2002) and Osborne et al. (2004). The Lovejoy method was developed using the prehistoric (8th – 11th century AD) North American Libben Cemetery skeletal sample, cadavers from several North American forensic cases, and also the Hamann-Todd skeletal collection, which is comprised of African Americans and European Americans from a historic to modern period. The acetabulum has also recently shown promise for use to estimate age, and a method developed by Rissech et al. (2006) on a Portuguese skeletal collection was tested recently on Thais (Khomkham et al., 2017).

#### Pubic Symphysis

Schmitt (2004) was the first to apply the Suchey-Brooks pubic symphysis method (Brooks & Suchey, 1990) to a Thai sample. Schmitt (2004) reported that the results for the 20 – 39 year age cohorts should be disregarded for both methods as the sample size, especially for females, was inadequate. Schmitt (2004) found the Suchey-Brooks method tended to overestimate the age of Thai adults less than 40 years of age and underestimated age for older adults (Table 2-3). Brooks and Suchey (1990) noted that when they tested their original method on a modern North American sample it was more reliable for young adults (up to 40

years of age), after this age they observed a wide range of individual variability which produced wide age distributions. Brooks and Suchey (1990) also noted that female standard deviations were greater than males by 0.5 to 1.3 years, and standard deviations got progressively greater as age progressed (up to 12.4 years ±1 SD). Similarly, in Schmitt's study, bias and inaccuracy values tended to be higher for Thai females compared to males. Inaccuracy for adults aged less than 60 years ranged from 2 years to 17 years, however, in adults over 60 years of age, inaccuracy was as high as 32.2 years for females and 27.2 years for males. Even given that the Suchey-Brooks method has such broad and overlapping age ranges for each phase, Schmitt noted that in only 37% of the Thai sample did known age fall within one standard deviation of the assigned phase mean age.

The 2012 thesis by Tipmala (2012) (written in Thai with an English abstract) also tested the Suchey-Brooks method, however Tipmala (2012) used multinomial logistical regression analysis to produce new age ranges for each phase (Table 2-3) with the intention of increasing age estimate accuracy for Thai individuals. Side asymmetry was also tested, with accuracy determined to be 86.4% for the left os pubis and 85.2% for the right side. Compared to the age ranges per phase developed in the original Suchey-Brooks method, these newly adapted Thai age intervals for each phase are narrower and without overlap. This study shows that Thai skeletal maturation is delayed in the later phases where they reach phases 3 to 5 later compared to the North American Whites that the method was developed on.

When testing the Suchey-Brooks method, Gocha et al. (2015) reported similar results to Schmitt (2004) wherein adults over 40 years tended to have age underestimated, and individuals under 40 years were usually observed with lower values of bias and inaccuracy, and had age overestimated (apart from females 20 – 29 years in the Gocha et al. (2015) sample, and no females in this age group were present in Schmitt's sample). In both studies, the overall results show females have greater bias and inaccuracy than males (Table 2-3). On average, age estimates differed from known age by approximately 10 years or more. Bias and

inaccuracy increased to 25.3 years for females in the 70+ age group, but this was still less than the bias and inaccuracy of up to 32.2 years reported by Schmitt (2004) for adults over 60 years.

### Auricular Surface

Schmitt (2004) tested the Lovejoy auricular surface 8-phase method and observed that male age estimation performed with less bias and inaccuracy compared to females, and age tended to be underestimated once individuals were over 30 years of age. With the auricular surface, the rate of bias and inaccuracy for adults over 40 years of age was higher than when the Suchey-Brooks method was tested (except for females over 60 years of age), with inaccuracy reaching a peak of 31.9 years for males (30.4 for females) aged over 60 years. Schmitt (2004) established that only a very small number of Thai individuals (7%) were assigned to the correct age range, with the majority of the sample being incorrectly placed into younger age phases (20 – 49 years), even though almost 85% of the Thai sample had a chronological age of over 40 years. When Lovejoy et al. (1985b) tested their own method on a combined sexes sub-sample of the North American Hamann-Todd skeletal collection they observed inaccuracy ranging from just 3.2 years for young adults, up to a maximum inaccuracy of 11.1 years (but with a bias of just 1.9 years) in the over 50 age group. Schmitt (2004) determined that repeatability of the method was exacerbated by the difficulty the observers faced in interpreting the description of some features as outlined in the original methods, particularly for the auricular surface. This problem has previously been discussed as an issue (Merritt, 2013).

In a 2012 conference paper, Singsuwana et al. (2012) presented a new age estimation scoring system and quadratic regression equations using the auricular surfaces from a Thai skeletal sample. The authors utilized a selection of the features used in the Lovejoy et al. (1985b) method (transverse organization, surface texture, microporosity, apical change and retroauricular area activity), in combination with a composite score similar to Buckberry

and Chamberlain (2002). Their first step was to individually assess each of the five features of the auricular surface to obtain a combined composite score for both the left and right auricular surfaces to then develop regression equations specifically for a Thai population. They found some of the feature descriptions developed by Lovejoy et al. (1985b) (microporosity and density) were difficult to evaluate in the Thai sample, just as Schmitt (2004) had reported. Singsuwana et al. (2012) believe this was due to a difference in morphological characteristics between the Thai sample population and the Western reference population that the method was developed on. Singsuwana et al. (2012) noted that this sample was represented by more older adults than young. Using composite scores from the left side and the right side, they tested the new equations (Table 2-3) on a sample of 60 individuals. No statistically significant differences were observed between the left and right os coxae. Accuracy of the new equations was slightly greater when tested on the right side, at 67.8% with a standard error of 10.6 years. The left side produced an accuracy of 56.4% with a standard error of 11 years.

When the auricular surface of the ilium was examined by Gocha et al. (2015) using the method developed by Osborne et al. (2004), they found underestimation of age occurred in adults over 50 years of age. The least amount of bias was observed in the 40 – 49 age group (overestimation by 0.2 years for females and 2.2 for males) and the highest amount of bias was seen in the 70+ age group (underestimation by 27.8 years for females and 23.4 for males). The overall results show that the level of inaccuracy was the same for both sexes (12.2 years) with age tending, on average, to be underestimated by 4.4 years for males and by 5.7 years for females. In comparison, when the Buckberry and Chamberlain method was also tested on the Thai sample by Gocha et al. (2015), age was underestimated from 50 years of age for females, but not until 70 years of age for males. The overall results of the Buckberry and Chamberlain method showed a similar level of inaccuracy between males and females, but overall bias was noticeably lower in females than males (Table 2-3). Gocha et al. (2015) observed a decrease in inaccuracy and bias of adults older than 50 years, particularly in

comparison to their results from testing both the Suchey-Brooks and the Osborne methods, and also the Lovejoy method tested by Schmitt (2004) on a Thai sample. Gocha et al. (2015) reported that the Suchey-Brooks and Osborne methods were more reliable to estimate age in younger Thai adults (<50 years), and the Buckberry and Chamberlain method was more reliable for older adults.

Gocha et al. (2015) went on to test several multifactorial combinations, including averaging point estimates from all three pelvic methods (combination A), and a combination of average point estimates from the Suchey-Brooks method and one of the auricular surface methods, which required using the Osborne method on younger adults (if the Suchey-Brooks method indicated the pubis was in phases I-IV), or the Buckberry and Chamberlain method for older adults (if the pubis was in phase V-VI) (combination B).Gocha et al. (2015) determined that combination B produced the least bias and inaccuracy than any of the individual methods tested alone. With both combinations, A and B, there was a tendency for overestimation of age for adults under 50 years and underestimation of age for adults above this age. Overall, both combinations also achieved marginally improved results for males compared to females. Gocha et al. (2015) found that combination A provided a reasonable level of accuracy between the ages of 40 and 59 years in both sexes, whereas combination B worked to a reasonable level of accuracy for adults up to 69 years of age, except for females 30-39 years whose bias and inaccuracy was almost double that for males. They also tried combining the average point estimates from all six methods, but the sample size was drastically reduced due to insufficient skeletal elements in some individuals. This combination was found to perform sufficiently only on adults in the 40 – 49-year age group. Testing on a larger sample may see an improvement in results. There were several hurdles faced by Gocha et al. (2015) in this study. They were constrained to a data collection period of just one week, restricting the sample size to 88 individuals, and leaving no time to test for intra- and inter-observer errors.

### Acetabulum

Khomkham et al. (2017) examined morphological features of the acetabulum as they changed with age using the steps outlined in the age estimation method of Rissech et al. (2006), to observe and score seven features on both left and right sides of each individual. The authors did not observe any statistically significant differences in scores between the sexes or sides. For three of the features, they did find a significant correlation with age. These were the acetabular groove (the most significant correlation was in the left female acetabulum ($r = 0.61$)), acetabular rim porosity and apex activity (most significant correlation in the left male acetabulum, ($r = 0.59$ and $r = 0.62$ respectively)). To Khomkham et al. (2017) these results suggested there are at least some similarities in timing and changes to morphological features between this Thai sample and the reference sample of Portuguese males that Rissech et al. (2006) developed their method on. However, the other four features are weakly correlated with age and highlight that there are some population differences in growth and degeneration in this part of the pelvis.

The Rissech et al. (2006) acetabular method was also applied to a Thai sample by Singsuwan et al. (2019). A preliminary test with 88 individuals determined that there were no significant side differences, so the method was comprehensively tested using a sample of 200 individuals. Singsuwan et al. (2019) recorded no significant sex differences. In comparison to Khomkham et al. (2017), they observed significant correlation with known age for all seven morphological variables. Results showed that overestimation of age occurred in adults younger than 66 years, and underestimation occurred in adults over this age. Low levels of bias (over or under estimation to a maximum of 4.4 years) were seen in young and mid-aged adults (21-46 years) and older adults (61-75 years). But age was underestimated by 11.38 years in adults 86-90 years. Inaccuracy reached a maximum of almost 12 years for the 56-60 and 86-90 years age groups. Singsuwan et al. (2019) recorded an accuracy of 66% when estimating age to within 10 years of known age, up to a maximum accuracy of 71% within 12

years of known age. In comparison, accuracy was higher for the Portuguese sample that Rissech et al. (2006) used to develop the method on, in their sample accuracy reached 89% when estimating age to within 10 years of known age. Singsuwan et al. (2019) suggested refining the scoring system, finding in the Thai sample, some differences in the degree of change in some features compared to that reported by Rissech et al. (2006), such as inconsistencies in density of acetabular fossa activity and deeper grooves surrounding the rim. They could see that apex activity showed a clear progression of change with age whereas the acetabulum groove showed high overlap between ages.

## Thorax (sternal rib ends, clavicle, and vertebrae)

Observing age related changes in the sternal ends of the ribs at the costochondral joint was conceived as an alternative to using methods developed on the pelvis or cranial sutures. Cranial sutures are not included in this review since many studies have shown this method to be highly inaccurate (Brooks, 1955; Hershkovitz et al., 1997; Ruengdit et al., 2020; Singer, 1953). The method that has been tested on a Thai sample is the İşcan nine-phase system (İşcan et al., 1984, 1985) using the 4$^{th}$ rib of white male and female cadavers from an American Medical Examiner Office. Several pilot studies used the Thai samples to observe age related changes to the medial articular surface of the clavicle or vertebrae to evaluate their potential to estimate age, and several other studies calibrated age estimation regression equations for the Thai population using a combination of different scoring systems.

### Sternal Rib Ends

Gocha et al. (2015) were the only researchers to have directly applied the İşcan method (İşcan et al., 1984, 1985) on a Thai sample to estimate age via changes to the sternal ends of ribs. Low levels of bias and inaccuracy in the earlier phases showed that the method was more accurate overall for Thai males and young adults. However, adults of both sexes above the

age of 40 (phase 5 onwards) consistently had their age underestimated. İşcan and colleagues (İşcan et al., 1984, 1985) noted that their method was most reliable for young to mid-aged white North Americans up to 40 years of age. After this age the SD from the mean reached up to 11 years for males and 15 years for females, with very wide age ranges per phase. Gocha et al. (2015) did not recommend this method for use in a forensic context for Thais or other Southeast Asian populations due to a poor correlation between observed and documented chronological ages, particularly apparent for individuals >40 years. Above this age both bias (underaging) and inaccuracy was as high as 37.5 years for females in the 70+ age group, whilst for males it reached 30.6 years in the 60 – 69 years age group.

Differences between reference and target sample size and distributions would have an impact on mean ages and standard deviation rates (Loth, 1995; Yavuz et al., 1998). The small Thai sample size (55 individuals) in the study of Gocha et al. (2015) hampered a thorough examination of the performance of this technique as the number of individuals examined per sex/age group ranged from just one (females 20-29) up to a maximum of eight (females 40-49). The İşcan method would benefit from further testing and modification on Thai sample of larger size, with an even representation of males and females in all age groups.

### Clavicle

Iamsaard et al. (2017) seriated 454 clavicles from northeastern Thais of the HSRC skeletal collection to closely observe and record surface typography of the medial articular surface of the clavicle as a way of providing a population-specific learning aid for medical and paramedical students. The age range of the sample was not provided but the average age of the sample was 60.69 years (±14.36 years). Surface typography, as well as porosity and osteophyte formation, is an important feature to record in a population-sample as once epiphyseal fusion is completed in young adults, changes of the medial surface of the clavicle provide another way to estimate age for older adults (Falys & Prangle, 2015). The study by

Iamsaard et al. (2017) did not record porosity or osteophyte formation, and instead chose to focus only on assessing surface topography, which Falys and Prangle (2015) determined was the trait most closely correlated with age. Therefore, the Falys and Prangle composite score method still needs to be tested on a Thai sample to ascertain accuracy of age ranges, means, and standard deviations for use on a Thai or other Southeast Asian population. The pilot study of Iamsaard et al. (2017) could only confirm that the types of medial articular surface (smooth, slight granulation, coarse granulation, nodule formation, undulating and degenerative) observed in the European reference sample of Falys and Prangle were also observed in the Thai sample. Another study by Traithepchanapai (2014) confirmed that commencement of osteophyte growth occurred on the margin of the medial articular surface of the clavicle of Thai individuals of at least 39 years of age in both males and females (cited in Traithepchanapai et al. (2016)).

### Vertebrae

Four studies focused on observing the prevalence and severity of vertebral osteophytes in Thai samples to aid in identifying potential symptoms in clinical cases, and to investigate their potential to estimate age. In the first of these studies, Namking et al. (2008) examined cervical, thoracic, and lumbar vertebrae to determine that osteophyte prevalence significantly correlates with increasing age and does so more significantly in males than females. Most frequently, osteophytes were observed in the lumbar vertebrae (73% of L4, 70% of L5 and 69% of L3), followed by the thoracic (50.5% of T11 and 49.5% of T10), and cervical (46% of C5, 44% of C6, and 38% of C4). The most prominent osteophytes were located on the anterosuperior aspect of the rim of lumbar vertebrae L3, L4 and L5.

Chanapa and Mahakkanukrauh (2011) studied only the cervical vertebrae in their northern Thai sample, recording the highest prevalence of osteophyte formation in vertebral bodies (49%), followed by facet joints (35%), and foramen (16%) of cervical vertebrae (C3 to C7).

Chanapa and Mahakkanukrauh (2011) concluded that osteophyte length significantly correlated with age, but not significantly to sex. The average length of C3 osteophytes were longer than on any other cervical vertebrae, but the maximum length of an osteophyte was recorded on the superior facet of a C4 vertebra (13mm). The greatest osteophyte prevalence was observed in cervical (C5) vertebrae (83%), followed by C6 (77%), C4 (74%), C7 (65%) and C3 (64%). These prevalence values are much greater than that observed by Namking et al. (2008) in the northeastern Thai sample (C5 (46%), C6 (44%) and C4 (38%)).

Suwanlikhid et al. (2018) used linear regression to estimate age from degenerative changes to lumbar vertebrae by observing and scoring three morphological features including changes to the cortical surface of the lumbar body, and the degree of osteophyte formation and macroporosity on the superior and inferior borders and endplates of the lumbar vertebrae. Suwanlikhid et al. (2018) produced an adaptation of several previously developed vertebral osteophyte scoring systems (Kacar et al., 2017; Van Der Merwe et al., 2006; Watanabe & Terazawa, 2006), as well as developing a new scoring system on the Thai sample for macroporosity and resorption of the cortical surfaces. Eight grades were used to score the degree of osteophyte formation with or without bridging and projections. Four grades were used to determine the degree of macroporosity on the superior and inferior surface of the vertebral body, and four grades were used to determine the degree of roughness with porosity on the cortical surface.

Suwanlikhid et al. (2018) found that all three features had moderate correlation with age, with the prevalence of osteophytes having the highest correlation of the three features, particularly on the inferior surface. Osteophyte formation was observed to commence around 26 years of age. The scores for each feature were used to develop new age estimation equations for each of the five lumbar vertebrae, producing twenty-five equations in total, with standard errors ranging from 11.7 to 14.5 years. The highest level of accuracy was gained from observing osteophyte formation on the inferior surface of the first lumbar vertebra ($r^2$= 0.408 with a

standard error of 11.7 years), but even this was a weak correlation between actual age and estimated age.

Praneatpolgrang et al. (2019) calibrated age estimation equations based on examining vertebral osteophyte formation in a Thai sample using the 5-grade scoring system developed by Snodgrass (2004) and the 4-grade system designed by Watanabe and Terazawa (2006), as well as developing their own new 6-grade scoring system focused on changes to the rugosity of the surface of the inferior and superior margins of the vertebral body, osteophyte length, and fusion of adjacent vertebrae. Praneatpolgrang et al. (2019) separately scored cervical, thoracic, and lumbar vertebrae for three groups (males, females, and combined sexes). A significant correlation was found between known age and the scores for all parts of the vertebral column (cervical, thoracic, and lumbar). The mean lumbar score had the best correlation with age for all three groups in each of the three scoring systems. The correlation coefficient (*r*) tended to be valued above 0.75 (strong positive correlation), the $r^2$ values were consistently between 0.53 and 0.64 (moderate positive correlation), and the standard error of estimates for mean lumbar scores were consistently between 9 – 11 years (*p*-value <0.01).

This is less error than recorded by Suwanlikhid et al. (2018) (SEE = 11.7 to 14.5 years) discussed above. Praneatpolgrang et al. (2019) found that the results for all three scoring systems were similar and were suitable for use in Thai forensic cases. They reported that their new 6-stage scoring system was more objective and faster to use than the Snodgrass and the Watanabe and Terazawa methods. However, the scoring system of Snodgrass (2004) overall produced the best results on this sample, with the most accurate of these regression equations recorded in the female mean lumbar score (**Error! Reference source not found.**) with a standard error of 9.506 ($p$<0.01), r = 0.801, $r^2$ = 0.642. Weakest correlation between age and vertebral osteophyte formation was generally found when using individual cervical or thoracic vertebrae in all three of the scoring systems.

Table 2-3 Age estimation methods tested on pelvic region.

| | | | | | | Overall Bias/Inaccuracy (years) | | Minimum inaccuracy (years) [age group] | |
|---|---|---|---|---|---|---|---|---|---|
| **Reference** | **Bone region** | **Method** | **Accuracy** | **Age over-estimated** | **Age under-estimated** | **Males** | **Females** | **Males** | **Females** |
| Schmitt (2004) | Pubic symphysis | Suchey-Brooks (1990) | 36.1% M, 37.9% F known age within ±1 S.D of the assigned phase mean. | ≤ 39 | ≥ 40 | -14.5 / 17.2 | -16.1 / 18.8 | 2.4 [20-29] | 6.7 [30-39] |
| | Auricular surface | Lovejoy et al. (1985b) | 7% of individuals accurately classified within Lovejoy's 5-year age classes. | ≤ 29 | ≥ 30 | -17.8 / 18.3 | -20.0 / 20.0 | 2.0 [20-29] | 6.3 [30-39] |
| Gocha et al. (2015) | Pubic symphysis | Suchey-Brooks (1990) | 88.6% M, 78.0% F known age within ± 2 SD of assigned phase mean. More reliable in males & younger adults (<50). | n/a | ≥ 40 | -7.8 / 9.2 | -8.7 / 12.5 | 2.8 [30-39] | 6.2 [20-29 & 40-49] |
| | Auricular surface | Osborne et al. (2004) | 93.0% M, 88.1% F known age within ± 2 SD of assigned phase mean. More reliable in younger adults (<50). | ≤ 49 | ≥ 50 | -4.4 / 12.2 | -5.7 / 12.2 | 5.6 [40-49] | 2.9 [20-29] |
| | Auricular surface | Buckberry and Chamberlain (2002) | 81.4% M, 76.2% F known age within ± 2 SD of assigned score mean. More reliable for older adults (>50). | ≤ 49 | ≥ 50 | 11.2 / 14.5 | 5.1 / 15.4 | 5.8 [60-69] | 10.7 [60-69] |
| Singsuwan et al. (2019) | Acetabulum | Rissech et al. (2006) | 71% accuracy estimated age within 12 yrs of known age. 66% accuracy within 10 yrs. | ≤ 65 | ≥ 66 | -0.17 / 8.55 sexes combined | | 1.25 [31-35] sexes combined | |
| Tipmala (2012) | Pubic symphysis | Modified Suchey-Brooks (1990) | 86.4% accuracy left os pubis, 85.2% accuracy right os pubis. New age ranges for Thais: Phase 1 = age range ≤ 21 years, Phase 2 = age range 22 -28 years, Phase 3 = age range 29 -34 years, Phase 4 = age range 35 -43 years, Phase 5 = age range 44 -54 years and Phase 6 = age range ≥ 55 years. | | | | | | |
| Singsuwana et al. (2012) | Auricular surface | Modified Lovejoy et al. (1985b) & Buckberry and Chamberlain (2002) & developed regression equation | 56.4% accuracy with SE 11 yrs for left side: age = $-0.465CS_L^2 + 14.65CS_L - 29.67$. 67.8% accuracy with SE 10.6 yrs for right side: age = $-0.59CS_R^2 + 16.86CS_R - 36.8$. | | | | | | |

M = male, F = female, SE = standard error, SD = standard deviation, yrs = years.

Table 2-4 Age estimation methods tested on the thorax.

| Reference | Bone region | Method | Accuracy | Age over-estimated | Age under-estimated | Overall Bias/Inaccuracy (years) | | Minimum Inaccuracy (years) [age group] | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Males | Females | Males | Females |
| Gocha et al. (2015) | Sternal end 4th rib | İşcan et al. (1984) & (İşcan et al., 1985) | 66.7% M, 48.0% F known age within ± 2 SD of assigned phase mean. More reliable for males, especially < 40yrs. | ≤ 39 | ≥ 40 | -10.0 / 12.9 | -13.01 / 18.5 | 3.8 [20-29] | 6.4 [30-39] |
| Namking et al. (2008) | Cervical, thoracic, and lumbar vertebrae | n/a | Osteophyte prevalence significantly correlates with increasing age, and more significantly in males than females. | | | | | | |
| Chanapa & Mahakkanukrauh (2011) | Cervical vertebrae | n/a | Osteophyte length significantly correlated with age, but not significantly to sex | | | | | | |
| Suwanlikhid et al. (2018) | Lumbar vertebrae | Adapted from Kacar et al. (2017), Van Der Merwe et al. (2006), Watanabe and Terazawa (2006) | Highest accuracy using degree of osteophyte formation on the inferior surface of L1 ($r^2$ = 0.408 with a standard error of 11.686 years). | | | | | | |
| Praneatpolgrang et al. (2019) | Cervical, thoracic & lumbar vertebrae | Snodgrass (2004), Watanabe and Terazawa (2006) & developed a modified scoring system | Mean lumbar score most accurate in all three scoring methods. Highest accuracy with scoring method of Snodgrass (2004) using female mean lumbar score Age y=32.308+15.994x, with SE 9.506 ($p$<0.01), r = 0.801, $r^2$ = 0.642. | | | | | | |

SD = standard deviation, SE = standard error, yrs = years, $r^2$ = coefficient of determination, r = correlation coefficient.

### Femur

All the previously discussed adult age estimation methods have been made via non-destructive qualitative or quantitative macroscopic observations. Histological methods and aspartic amino acid racemization are generally avoided as they require destructive sampling of bone, albeit a very small piece of the femur, humerus or rib, usually approximately 2cm², to observe microstructural changes in the cortical bone.

A study by Chompoophuen et al. (2019) is the only one to examine histomorphometric age estimation using cortical bone sections of femora in a Thai sample. They used decalcified and stained bone sections, image analysis (Image J®), and the computer program MATLAB to determine the correlation between age and pixel density of histological variables. Chompoophuen et al. (2019) used linear regression analysis to calibrate established predictive formulae to estimate age from five variables, and to develop one new variable for quantifying collagen measurements in bone (COL.B). They found that collagen in males had higher correlation with age ($r$ = 0.800) compared to females ($r$ = 0.467). In the combined sex sample, measuring the perimeter of the haversian canals (Pm.H.Ar) produced the highest correlation coefficient with age ($r$ = 0.856), and equations produced from regression analysis showed that Pm.H.Ar stood out as being the individual variable most closely correlated with age ($r^2$ = 0.733) with the lowest standard error (9.91 years) (Table *2-5*). Stepwise multiple regression showed that overall, a combination of three variables, Pm.H.Ar, COL.B, and Lm.B.Ar (percentage of lamellar bone area), provided the most accurate predictor of age (correlation coefficient of 0.906).

Monum et al. (2019) evaluated the aspartic amino acid racemization procedure suggested by Ohtani et al. (1998) and Ohtani and Yamamoto (2005) to predict age from femoral bone samples using aspartic amino acid racemization. Monum et al. (2019) found that the Dextro/Levo (D/L) ratio was highly correlated with known age in the Thai sample, with males

showing better correlation ($r$ = -0.912) than females ($r$ = 0.716), but there were no statistically significant differences between rate of racemization and sex. The combined sex sample produced a standard error of 11.01 years. Monum et al. (2019) used the racemization results and linear regression to calculate age estimation equations for each sex and sexes combined (Table 2-5).

### Computed Tomography Scans

It should also be noted that data collected from routine computed tomography (CT) scans have shown great potential in evaluating age related morphological changes in several Thai studies. Pattamapaspong et al. (2015) assessed the timing of fusion of the medial clavicle in CT scans of Thai patients to calibrate and slightly modify the classification methods of Schmeling et al. (2004) and Kellinghaus et al. (2010).They found that Stage 4 of fusion best represents Thai individuals over 18 years of age. Pattamapaspong et al. (2019) then used a new cinematic volume render to produce 3D CT images of the os pubis and auricular surface of individuals from the CMU skeletal collection to test the Suchey-Brooks and Buckberry-Chamberlain methods of age estimation. The authors found that the new technique has a high success rate when assessing features of the os pubis, however, most auricular surface features cannot be clearly seen in the CT scans and are best assessed in dry bone. A major limitation of CT scanning is that such technology is often only accessible in large laboratories or hospitals and is expensive. Technical training is required to use specialized and expensive imaging equipment and to assess images with specialized computer software. However, as CT scans can be collected from live patients of all ages, it overcomes the sample bias seen in all skeletal collections in which the very young and the very old are underrepresented.

Table 2-5 Age estimation methods tested on the femur.

| Reference | Bone region | Method | Accuracy |
|---|---|---|---|
| Chompoophuen et al. (2019) | Femur (histology) | Adapted from Yoshino et al. (1994), Martrille et al. (2009) & Pfeiffer (1998b) | Age = (-28.199 + 0.0138 (Pm.H.Ar) + 0.00005 (COL.B) + 9.312 (Lm.B.Ar )), r = 0.906, SEE 8.26. Pm.H.Ar stood out as being the individual variable most closely correlated with age ($r^2$ = 0.733) with the lowest SEE (9.91 years). |
| Monum et al. (2019) | Femur (aspartic amino acid racemization) | Ohtani et al. (1998) & Ohtani and Yamamoto (2005) | Combined sex age = (Ln(1+*D*/*L*)/(1–*D*/*L*)–0.0192))/0.0005), with SEE 11.01 yrs, r = 0.8316, $r^2$ = 0.6916. Male age = (Ln(1+D/L)/ (1–D/L)–0.0155))/0.0005), with SEE of 8.07 yrs, r = 0.912, $r^2$ = 0.8322. Female age = (Ln(1+D/L)/(1–D/L)–0.0236))/0.0004), SEE of 15.77 years (r = 0.716, $r^2$ = 0.5136). |

SEE = standard error of estimate, $r^2$ = coefficient of determination, r = correlation coefficient.

## 2.3 Discussion

Several of the Thai studies (Gocha et al., 2015; Schmitt, 2004) tested for sexual dimorphism by measuring bias and inaccuracy. These studies showed that techniques using the pelvis and ribs tended to be more reliable for males and younger adults. For instance, overall bias was almost always observed to be less in males than for females (1 – 6 years difference), the exception to this was for the Buckberry and Chamberlain (2002) auricular surface method where overall female bias was 6 years less than for males. The overall degree of inaccuracy was also observed to be lower in males than females (1 – 6 years difference). Males and females will often experience bone remodeling at different rates (Cho et al., 2006; Lewis & Roberts, 1997) and females could have been exhibiting a greater degree of morphological variation of age indicators (Djurić et al., 2007). The use of a larger female sample might have assisted in better phase placement.

Bias and inaccuracy increase considerably with age for all methods tested on Thai samples. Methods that were more reliable for younger Thai adults (<40 years) included all the methods using the pubic symphysis, acetabulum, rib ends, and auricular surface. The exception to this was the Buckberry and Chamberlain (2002) auricular surface method which was more reliable for Thai adults over 50 years of age. This method was established using a reference group with a higher proportion of adults aged over 60 years so it is not surprising that they performed better on older Thai individuals. Whereas the original methods of Suchey-Brooks pubic symphysis method, Lovejoy auricular surface method and the İşcan rib method were developed on younger reference groups and have proven to be more accurate on adults less than 40 years (Berg, 2008; Merritt, 2013).

There was a prevailing tendency for bias, where age was over-estimated in young adults and under-estimated in older adults in the Thai studies. Research verifies this trend is a persistent limitation for methods using regression-based models to correlate morphological data with age

(Aykroyd et al., 1999; Berg, 2008; Getz, 2020; Schmitt et al., 2002). Disagreement in size, age structure and mean age between the reference group and target sample can amplify this bias (Bocquet-Appel & Masset, 1982). The youngest adults and those over 60 years of age at death are particularly underrepresented in the original methods (Lucy et al., 2002; Martrille et al., 2007; Merritt, 2013; Miranker, 2016). Most of the Thai samples had a mean age of between 50 – 60 years and under-representation of some age groups, usually 20 -29 years and 70+ years. There is also often unequal representation of males and females in the Thai target samples, with a bias towards more males. The Thai sample demographics have been impacted by the number of body donations and suitable autopsied cadavers accessible from university forensic departments and hospitals from which the study samples were obtained.

Gocha et al. (2015) determined a method's accuracy by assessing the percentage of the sample whose documented age was within ±2 SD of the mean age reported for the phase or stage. The İşcan rib method showed the weakest correlation with known age, with high bias and a maximum accuracy of 67%, with Gocha and colleagues listing this the least preferred skeletal indicator to use on Thai individuals. It must, however, be noted that the study was hindered by a small sample size in which young females in particular were underrepresented (Gocha et al., 2015). Greater accuracy (76% - 93%) was achieved in methods utilizing the pubic symphysis and auricular surface. Whilst 93% accuracy of the Osborne method sounds impressive it must be noted that the age ranges assigned to an individual with ±2 SD are very broad with overlap between successive stages, making the estimated age ranges too impracticable to be of real use (Rogers, 2016). Wide age categories for each phase are usually required to capture the range of individual morphological variability experienced within a population (Berg, 2008; Djurić et al., 2007). The advantage of a wider age range is that fewer individuals are placed in the incorrect phase for age (Bocquet-Appel & Masset, 1982; Brooks & Suchey, 1990).

Schmitt (2004) preferred to use estimates ±1 SD within the mean to obtain a maximum accuracy of nearly 38% for the Suchey-Brooks method. But this confidence level also suffers from the limitation that it may produce too narrow and precise an age range that cannot account for all individual morphological variation in the Thai sample (Rogers, 2016). This increases the probability that an individual will be placed in an age phase that is above or below the one in which their documented age actually falls (Garvin et al., 2012). Just 7% of individuals were correctly placed in the assigned age phase when Schmitt (2004) tested the Lovejoy auricular surface method. The low level of accuracy achieved with this method is a product of the 5-year age ranges published in the Lovejoy method that are far too narrow to capture the full range of morphological variation of the auricular surface(Osborne et al., 2004). Even when Singsuwana et al. (2012) developed population-specific regression equations derived from Thai auricular surfaces, the maximum accuracy observed was nearly 68% but with a 10 year standard error.

Correlation coefficient values were highest when regression equations were derived from the Thai samples using either histological and biochemical (amino acid racemization) techniques or vertebral osteophyte prevalence in the lumbar vertebrae ($r$ = >0.8 ) with standard errors ranged from 8 – 16 years. Monum et al. (2019) states that a standard error of 11 years is acceptable, whereas Rösing and Kvaal (1998) argue that standard errors that exceed 5 – 7 years should not be applied to forensic cases, nor archaeological contexts. Correlation over 0.8 is usually accepted as a strong level of correlation between morphological indicators and age, although Bocquet-Appel and Masset (1982) argued that values under 0.9 are likely to introduce considerable risk of error to age estimations.

Almost all the authors of the Thai studies in this review argued that inter-population variation greatly reduced the reliability of the methods. Most either did not recommend the methods for use on a Thai population, particularly not for a forensic setting, or only if applied with a high degree of caution. This is not a unique situation to just this population. Current research

predominantly indicates that bone degeneration and remodeling occur at non-uniform rates between individuals of Asian, African, and European origin (Buckberry & Chamberlain, 2002; Mays, 2012; Schmitt et al., 2002; Shilpa et al., 2013) due to varying genetic and environmental factors experienced between regionally diverse populations (Berg, 2008; Garvin et al., 2012). These studies argue that age estimation standards developed on one sample population therefore cannot adequately reflect the individual and population level rate of bone remodeling and degeneration found in another population, necessitating population-specific standards to ascertain biological age.

In contrast, other researchers suggest using one large reference group made up of individuals from a number of genetically distant populations to improve the reliability of methods by capturing a greater level of skeletal variation (Brooks & Suchey, 1990; Zhang et al., 2009). Furthermore, some studies stress that ancestry is not the main cause of skeletal age variation as there should be similar skeletal growth and degeneration rates between populations when health and environmental conditions are similar (Schmeling et al., 2000). It is suggested that a combination of factors, including health and hormones, and socioeconomic status linked to body mass index (Ferguson et al., 1982; Mays, 2015; Schmeling et al., 2000) have more of an effect on the timing and rate of bone turnover rather than population affiliation. This is usually not clearly discussed as a consideration when a method of age estimation is chosen by anthropologists.

There is a paucity of studies that compare how socioeconomic status, health, and physical activity impact the reliability of current age estimation methods between different populations. An individual's level of physical activity and mechanical loading on the skeleton will affect the rate of bone turnover and bone mass (Adami et al., 2008), however, recent research on individuals of European and African American descent suggests that high levels of repetitive physical activity has little significant impact on rates of degenerative change to age related features in load bearing joints of the pelvis (Bertsatos et al., 2021; Campanacho et al., 2012;

Winburn, 2019). Merritt (2015) was the first to show a clear relationship between underestimation of skeletal age and short stature combined with low body mass, whereas overestimation of age occurred in tall people with high body mass, with evidence of increased bone surface degeneration in relation to weight increase (see also Wescott & Drew, 2015). Additionally, Merritt (2017) determined which age estimation methods were most reliable for smaller-bodied individuals and for larger-bodied individuals.

Health statistics in Thailand have identified a secular trend for height/weight increase correlating with improved living conditions since records began in 1975 (Jaruratanasirikul & Sriplung, 2015). Lower socioeconomic status in rural areas has caused delayed bone age in young Thai rural children compared to their peers from more affluent regions such as the United States and urban middle class Thailand (Bailey et al., 1984). High-income (modern) Western populations are largely sedentary and their levels of physical inactivity are twice as high compared to low-income countries (Guthold et al., 2018). Clinical research has established there are significant differences in the relationship between skeletal muscle mass and age among Hispanic, African American, White American, and Asian (Chinese, Indian, Korean, and Japanese) individuals (Silva et al., 2010). Iamsaard et al. (2017) argued that the pattern of degenerative changes seen in the older age Thai adults of the KKU skeletal sample from rural northeast Thailand may be influenced by activities in labour-intensive manual rice agriculture, an occupation that a majority of the individuals from this low to mid-socioeconomic region are likely to have participated in (Techataweewan et al., 2017). Similarly, Tayles and Halcrow (2015) have considered the biomechanical forces involved in rice planting, which requires repetitive flexing at the hip joint, and its effects on age-related change in the auricular surface, as well may there be influences from the cultural practice of a full squatting position commonly adopted by Thai people, and other Asian populations. The impact that these factors have on skeletal age estimation in the Thai population is still poorly understood and needs additional research.

## 2.4 Chapter Summary

Ultimately, this chapter informs forensic anthropologists and bioarchaeologists of the most appropriate age estimation methods to use on Southeast Asian skeletal remains by evaluating studies that have tested the replicability and accuracy of methods on Thai target samples. The demographic profile of the Western reference samples that most methods were developed on contributed to the overestimation of age of young adults and overestimation of age of older adults in the Thai sample. High bias and inaccuracy mean the majority of methods should be used with caution in Thai forensic investigations. The best way forward to understand the wide range of morphological variation in humans is for future studies to investigate genetically diverse populations. Studying the skeletal remains of past populations offers a way to broaden and diversify the current pool of research, which is limited by a worldwide lack of ethically sourced and curated skeletal collections. The next chapter utilizes the well-preserved skeletal remains of a prehistoric population from northeast Thailand to record and evaluate age-related macroscopic features of the vertebrae.

# Chapter 3 Vertebral Osteophytosis in Prehistoric Northeast Thailand

The previous chapter addressed the lack of population-specific age estimation methods available to forensic anthropologists and bioarchaeologists involved in the analysis of Southeast Asian skeletal remains. The best way to increase our understanding of the considerable morphological variation in humans is to evaluate a range of genetically diverse populations and examine different skeletal elements. The use of vertebral osteophytosis (VO) formation to estimate the age at death of adults is still relatively new and there is currently a limited understanding of interpopulation variation of VO formation rates. Therefore, this chapter compared VO prevalence between two prehistoric populations in northeast Thailand, spanning the Neolithic to the Late Iron Age (1750 BC – 820 AD) to investigate the correlation of VO rates to age and sex, and take into consideration possible contributing factors such as different lifestyle and physical activity patterns. This chapter is currently in preparation for submission:

> Lucille Pedersen, Afua Adjei, Georgia Stannard, Anna Willis, Nigel Chang, Hallie Buckley, Siân Halcrow, Louise Shewan, Dougald O'Reilly, Charles Higham, Kate Domett, *Vertebral osteophytosis in Prehistoric Northeast Thailand*.

## 3.1 Introduction

Vertebral osteophytosis (VO) involves the development of abnormal growth or deposits of bone on the superior and inferior rims of the vertebral centrum (Klaassen et al., 2011). The formation of VO often has a direct relationship to the narrowing of intervertebral disc space, degeneration of the nucleus pulposus and annulus fibrosus, and herniation of the

intervertebral disc from compressive forces over time (Bridges, 1992; Chanapa & Mahakkanukrauh, 2011; Klaassen et al., 2011; Wilke et al., 2006). Due to its positive correlation with advancing age, several studies have scored osteophytes based on degree and severity of formation on vertebral centrum rims, in order to develop age at death estimation methods (Chiba et al., 2022; Kacar et al., 2017; Listi & Manhein, 2012; Praneatpolgrang et al., 2019; Suwanlikhid et al., 2018; Watanabe & Terazawa, 2006). These studies show that there is significant individual and population variation in the distribution and timing of VO formation, arising from multiple aetiologies. There is a need to increase the breadth of research of VO prevalence within different populations to gain insight into possible sociocultural and lifestyle aetiologies to consider physical loading and environmental effects on vertebral osteophytosis prevalence.

Clinical heredity studies on same-sex twins have identified the significant role of genes and shared familial environment on the rate and extent of vertebral degeneration (Battié et al., 2004; Sambrook et al., 1999; Videman et al., 2006). Certain genes can interfere with normal bone mineralisation and growth, accelerating degeneration, thus affecting the ability of a vertebral body to resist compressive and flexion forces (Kalichman & Hunter, 2008). Obesity interrupts energy storage and bone formation processes during bone metabolism, and chronic inflammation can affect bone resorption (Cao, 2011; Thijssen et al., 2015). Additionally, osteophyte formation can be influenced by an array of metabolic bone diseases and congenital disorders, such as DISH, Paget's disease, osteoporosis, osteomalacia, and osteosclerosis, to name just a few, which contribute to abnormal bone turnover and vertebrae development as well as interfering with movement and posture (Adams, 2005; Kumar et al., 1988).

Numerous clinical reports focus on the relationship between VO development and activity-related stress in physically demanding occupations that place a high mechanical load on the vertebral column. Although a direct comparison between prehistoric and modern skeletal remains is not possible, these clinical studies can be used to test hypotheses on the types of

activities and behaviours that may have contributed to VO development within archaeological populations. Modern Thai rubber farmers, for example, experience long-term lumbar strain and lower back pain from repetitive trunk flexion and extension movements over prolonged periods, particularly tree-tapping below knee-level, and sustained spinal loading from lifting and carrying heavy buckets of rubber sap (Udom et al., 2016). Interviews with Thai rice farmers undertaking the hand-planting of rice, indicated that lower back pain was the most frequently reported issue (Puntumetakul et al., 2014). This is due to the adherence of traditional forms of hand-planting rice, which places the most stress on lumbar vertebrae, and in severe cases causing microfractures in the vertebral endplates and intervertebral disc (IVD) herniation (Puntumetakul et al., 2014). Likewise, Chanapa (2016) linked the very high prevalence of lumbar osteophytes (97.2%) found in Thai workers within agricultural and manufacturing sectors to activities requiring repetitive trunk flexion and lateral bending over long periods of time.

Carrying heavy loads of bundled firewood or water on the head is a common practice in some parts of rural Africa (Porter et al., 2013) and Southeast Asia (De Young, 1963). This activity places increased axial compressive forces on vertebrae, accelerating degeneration, particularly in the upper cervical vertebrae (Jumah & Nyame, 1994). The most profound changes observed are a reduction of height in vertebral bodies and IVD space narrowing (Echarri & Forriol, 2002). Osteophyte frequency increased in post-menopausal female bearers, particularly among those who engaged in the activity for a prolonged period (Echarri & Forriol, 2002). However, in these studies even people in the control groups not involved in the head bearing activities still experienced vertebral degeneration and cervical osteophytes, albeit at a significantly lower prevalence and severity, thus it is evident that heavy load bearing cannot be the only factor in vertebral degeneration; age, hormones, and genetics play a role too.

The aim of this study was to assess if there is any variation in osteophyte prevalence between the sexes and among age groups in two prehistoric Thai populations that span from the Neolithic to the Late Iron Age, to enhance the understanding of osteophyte development in correlation to age while considering other contributing factors such as increased physical labour with shifting subsistence strategies. This is of interest as evidence drawn from paleoenvironmental studies and archaeological contexts indicate there was an adoption of intensive wet-rice agriculture and moat construction to adjust to environmental stresses.

## 3.2 Palaeoenvironment and Cultural Context

Ban Non Wat (BNW) and Non Ban Jak (NBJ) (Figure 3-1) are two significant prehistoric sites that have been excavated in Southeast Asia. They are in very close in geography to each other, allowing regional variation to be controlled for. BNW is notable for its large number of burials (n= 696) and material culture representing periods of occupation spanning the Neolithic (1750 BC – 1050 BC), Bronze Age (1050 BC – 420 BC) (Higham & Higham, 2009) and Iron Age (420 BC – AD 600) (Higham et al., 2019). Isotopic evidence suggests that the population of BNW remained genetically homogonous and maintained low levels of long-distance immigration over a span of ~2300 years (King et al., 2015). NBJ (AD 300 – AD 820) (Higham et al., 2019) is important for its well-preserved human remains (n= 137) that provide insight into the Late Iron Age and early historic periods of northeast Thailand. These sites offer the critical opportunity to observe social, economic, and environmental change over the millennia.

The Neolithic period at BNW incorporated dry-land rice cultivation, and domesticated pigs and cattle (Higham, 2011) to supplement the dependable supply of wild food resources within the Khorat Plateau. During this period, BNW had an established ceramic industry, and developed exchange networks with maritime communities, as evidenced by cowrie shells as mortuary offerings (Higham & Kijngam, 2011).

Dietary shifts in the Bronze Age indicate a higher reliance on rice (King et al., 2015), and copper-based metallurgy provided an impetus for increasing economic and social complexity (Higham, 2011). Analysis of the mortuary ritual suggests that some early Bronze Age burials at BNW displayed more obvious mortuary wealth compared to others, and over several generations. Lower levels of linear enamel hypoplasia and increasing stature suggests that health improved from the Neolithic into the Middle Bronze Age at BNW, but these indicators of stress later reversed to indicate a general decline in health during the Late Bronze Age, interpreted within the interplay of subsistence, environment, and sociocultural influences (Clark et al., 2013).

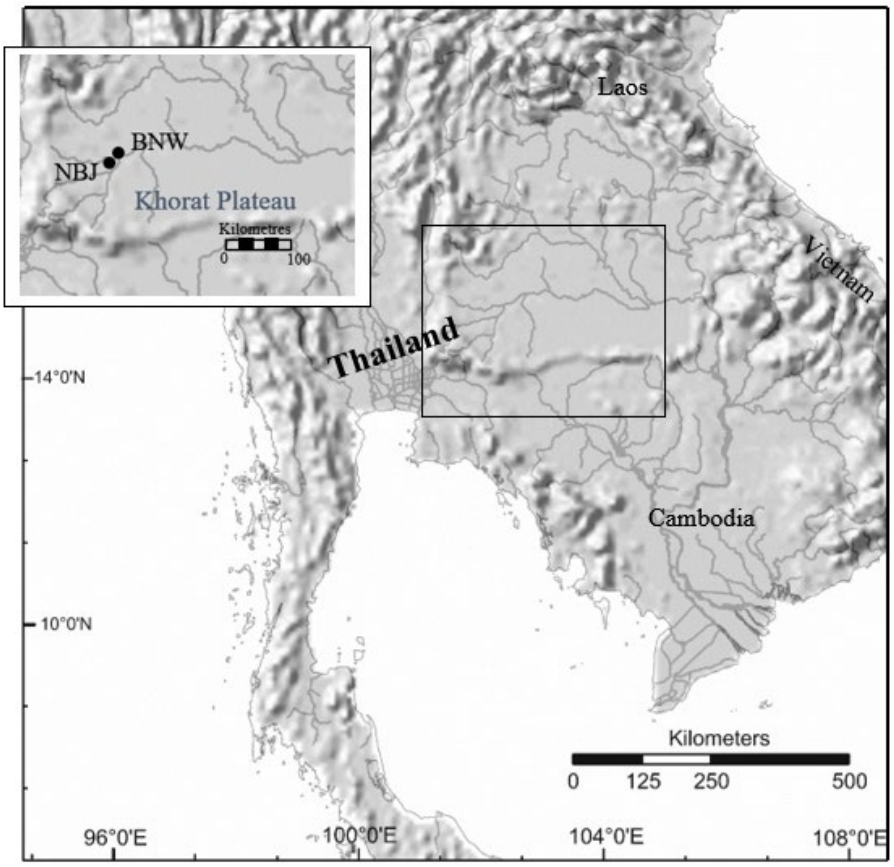



Figure 3-1 Map of Thailand with the boxed area highlighting the Khorat Plateau in northeast Thailand and the two sites in this study, Ban Non Wat (BNW) (1750 BC – AD 600) and Non Ban Jak (NBJ) (AD 300 – AD 820).

The monsoon seasons during the Iron Age were becoming highly unpredictable, and aridity was increasing in northeast Thailand (Higham et al., 2019). Populations adapted to this significant change in climate and landscape with the construction of multiple moats around most settlements to sustain the adoption of wet-rice agriculture (Castillo et al., 2018; Duke et al., 2016; Fochesato et al., 2021). There were some rapid developments in technology and social organisation, particularly with small-scale industrialisation in salt-production, metal smithing and smelting, new ploughing technology, forging of iron tools and weapons, and construction of clay-lined floors, as well as concreted walls representing residential structures at NBJ (Higham et al., 2019). The mortuary context, as well as health disparities between the sexes at NBJ, supports the suggestion for a degree of social inequality (Fochesato et al., 2021; Ward et al., 2019). Analysis of skeletal trauma at NBJ and BNW indicates risk of injury was mostly from occupational activities and lifestyle, such as falls and interactions with large animals, and some trauma from interpersonal violence, possibly from inter- or intra-community conflict (Pedersen et al., 2019).

## 3.3 Materials and Methods

Osteophytes were observed and recorded from the skeletal material of two prehistoric sites located in the Upper Mun River Valley in northeast Thailand; the Late Iron Age period site of NBJ (A.D. 300 – 820) (Higham & Kijngam, 2020) and BNW, located approximately 10km to the northeast of NBJ, and occupied from at least 17500 B.C. to A.D. 600 (and likely into later protohistoric periods) (Higham & Higham, 2009). This study focuses on the detailed examination of osteophyte development around the vertebral centrum as this has not yet been analysed for these populations. To accurately examine the progressive growth of osteophytes over an adult lifespan, all individuals with vertebrae were assessed for VO, but only those with the required preservation were included in this analysis. All data was collected by one observer (K.Domett).

Archaeological samples are rarely complete due to taphonomic insults in the burial environment, such as acidic soil, animal, insects, and tree root activity. Given this study focuses on a discussion of the impact of VO on individuals and regions of the vertebrae within individuals (cervical, upper thoracic, lower thoracic, and lumbar), this study includes only individuals with at least 50% of rims preserved in at least one vertebral region. For example, at least five out of the possible ten rims (superior or inferior) in the cervical region (C3-C7) or the lumbar region (L1-L5) were assessable, or at least six out of the possible twelve rims in the upper thoracic region (T1-T6) or the lower thoracic region (T7-T12). The first (atlas) and second (axis) cervical vertebrae were not included due to their unique morphology. For a vertebral centrum rim to be assessable for VO it was required to have at least 75% preserved.

### Individual Prevalence by Sex, Age, and Vertebral Region

Within the assessable skeletal sample from BNW and NBJ, individuals that had at least one rim with VO (any grade, Table 3.1) were included in analyses of individual prevalence by sex and age group. Also, as each vertebral region is functionally different, it was useful to then assess individual prevalence of VO by sex and age group within each of the four vertebral regions. To be counted, individuals were required to have VO in at least one rim from at least one vertebral region. Next, the prevalence of pathological VO (graded ≥2:2) was compared between the assessable samples of NBJ and BNW by vertebral region, sex, age at death, and time period.

### Grading of Vertebral Osteophytes

The superior and inferior centrum rims of all adequately preserved vertebrae were graded for osteophyte development using a scale according to the assessment protocol in Buikstra and Ubelaker (1994). Each vertebral centrum rim was given a grade that combined the degree of

osteophyte development and extent of the rim affected (Table 3-1). For example, the superior centrum rim from the third cervical vertebrae (C3) was graded '3:1' if extensive spicule formation (grade 3) was observed on less than one third of the margin (grade 1).

*Table 3-1 Grading system for each vertebral body margin (rim).*

| Marginal osteophyte development | |
|---|---|
| Degree of formation | Extent of vertebral rim affected (if degree of formation 1 or above) |
| 0 = no change | |
| 1 = barely discernible | 1 = <1/3 of rim affected |
| 2 = sharp ridge, some curled with spicule | 2 = between 1/3 to 2/3 of rim affected |
| 3 = extensive spicule formation | 3 = >2/3 of rim affected |
| 4 = ankylosis | |

Possible grades (pathological grades in boxed area):

| | | |
|---|---|---|
| 1:1 | 1:2 | 1:3 |
| 2:1 | 2:2 | 2:3 |
| 3:1 | 3:2 | 3:3 |
| 4:1 | 4:2 | 4:3 |

## Grading of Pathological Vertebral Osteophytosis

Individuals were assessed to have pathological vertebral osteophytosis (PVO) if they had at least one vertebral body with extensive changes to a rim, such as sharp ridges, spicule formation, or ankylosis (degree of formation ≥2), and the amount of rim affected was greater than 1/3 (extent grading ≥2) (Buikstra & Ubelaker, 1994). Preservation of vertebral rims in archaeological assemblages is often an issue so that the extent of PVO in a sample is likely to be underrepresented. Therefore, if a poorly preserved rim had degree of formation ≥3 and extent grading ≥1 it was also assessed as having PVO. This was because severe pathology affected a minimum of 1/3 of the rim, and if there was more of the rim preserved, it is likely it would have been recorded to have an extent grading ≥2 (Adjei, 2022). Inter-site comparisons were made by sex, age, and time period.

## The Skeletal Assemblages

Fifty-five adult burials were excavated from NBJ, of which thirty-one had sufficient preservation of their vertebrae to be included in the assessable sample. There was a slightly higher representation of males (51.6%) than females (45.2%) (Table 3-2), and more mid-aged adults made up the sample (38.7%) than older (32.3%) and younger (25.8%) adults but none of these differences were statistically significant. One female was of indeterminate age, and one mid-aged adult was of indeterminant sex.

Of the 469 adult burials excavated from BNW, seventy-nine had the required vertebral preservation to be included in the assessable sample, representing three time periods, Neolithic (n =10), Bronze Age (n = 68), and the Iron Age (n = 1). There were no significant differences in the number of females compared to males in any of the time periods. In the Neolithic and Bronze Age periods there were proportionately more mid-old age adults compared to young adults, the same pattern as NBJ, but, as with NBJ, this difference was not statistically significant (Table 3-3). The Iron Age BNW sample size was affected by poor skeletal preservation, with only one adult in the assessable sample, and therefore the BNW Iron Age was excluded from comparison to the Late Iron Age NBJ burials.

Only individuals 20 years of age or older were included in this study as this is usually the latest age at which the vertebral epiphyseal rings have fused and the earliest age osteophytes may start to develop (Nathan, 1962). The commonly used age and sex estimation standards of Buikstra and Ubelaker (1994) were followed, using the methods known to currently be the most reliable for Southeast Asian populations (Pedersen & Domett, 2022). Pelvic and cranial morphology was used to estimate biological sex, and age at death was estimated using the pubic symphysis and auricular surface morphology and dental wear seriation. Ordinal age categories (e.g. young, mid, and older adult) are commonly relied upon in palaeodemography (Buckberry, 2015), as current age at death estimation methods rely on evaluating

degenerative characteristics of bone which has too much individual variation to provide chronological age ranges, especially when dealing with fragmentary and poorly preserved skeletal features. The NBJ skeletal remains had been assigned into ordinal age categories of young adult, mid-aged adult, and older-aged adult (Buckley et al 2020), whereas the BNW sample had been originally assigned into a number of different age categories, including young, young adult, young-mid, middle aged, mid-old, and old adult (Tayles & Halcrow, 2015). The BNW vertebral osteophytosis data used in this study was reported by Adjei (2022) who, for more meaningful analysis, combined the first three age categories into one category of young adult (YA), and the other categories were combined into the category of mid-old adult (MOA). To allow for inter-site comparison of pathological vertebral osteophytosis prevalence, the NBJ mid-aged adult and older aged adult samples were also combined into one sample (MOA).

Table 3-2 Representativeness of the Non Ban Jak assessable sample[a].

| Age group | Female n (%) | Male n (%) | ?Sex n (%) | Total n/31 (%) |
|---|---|---|---|---|
| Young adult (YA) | 4 (50.0) | 4 (50.0) | 0 (0) | 8 (25.8) |
| Mid-age adult (MA) | 4 (33.3) | 7 (58.3) | 1 (8.3) | 12 (38.7) |
| Older adult (OA) | 5 (50.0) | 5 (50.0) | 0 (0) | 10 (32.3) |
| ?Age | 1 (100.0) | 0 (0) | 0 (0) | 1 (3.2) |
| Total | 14 (45.2) | 16 (51.6) | 1 (3.2) | 31 (100.0) |

[a]individuals with at least 50% of the rims assessable in at least one vertebral region (cervical, upper thoracic, lower thoracic, lumbar); n = number of adults in that sex/age group from the assessable sample (and % of the sub total); ?Age/?Sex = individuals of indeterminate age & sex
FET *p* value females vs males (0.7997)
FET *p* value young adults vs mid-aged adults (0.4155)
FET *p* value young adults vs older adults (0.7802)
FET *p* values mid-aged adults vs older adults (0.7911)

Table 3-3 Representativeness of the Ban Non Wat assessable sample (n = 79[a]) by time period.

| | Female<br>n (%) | Male<br>n (%) | Total<br>n (%) |
|---|---|---|---|
| Neolithic | | | |
| Young adult (YA) | 4 (66.7) | 0 (0.0) | 4/10 (40.0) |
| Mid-old adult (MOA) | 2 (33.3) | 4 (100.0) | 6/10 (60.0) |
| Sub-Total (n/N) | 6/10 (60.0) | 4/10 (40.0) | 10 (100.0) |
| Bronze Age | | | |
| Young adult (YA) | 15 (46.9) | 15 (41.7) | 30/68 (44.1) |
| Mid-old adult (MOA) | 17 (53.1) | 21 (58.3) | 38/68 (55.9) |
| Sub-Total (n/N) | 32/68 (47.1) | 36/68 (52.9) | 68 (100.00) |
| Iron Age | | | |
| Young adult (YA) | 0 (0.0) | 0 (0.0) | 0/1 (0.0) |
| Mid-old adult (MOA) | 1 (100.0) | 0 (0.0) | 1/1 (100.0) |
| Sub-Total (n/N) | 1/1 (100.0) | 0/1 (0.0) | 1 (100.0) |

Data from Adjei (2022); [a]individuals with at least 50% of the rims assessable in at least one vertebral region (cervical, upper thoracic, lower thoracic, lumbar); n = assessable subsample. FET *p* value females vs males: Neolithic (0.6563), Bronze Age (0.6071) (Iron Age sample too small for statistical analyses), FET *p* value young adults vs mid-older adults: Neolithic (0.6563), Bronze Age (0.2298)

## Statistical Analysis

Statistical analyses were performed using Graph Pad Prism (version 9.4.1). Statistical significance of sex and age differences in the severity of vertebral osteophytes were analysed in each of the vertebral regions for the NBJ sample. Prevalence of pathological vertebral osteophytosis was compared between the NBJ and BNW samples to identify significant differences between the sexes, age groups, vertebral regions, and time periods. Due to the small sample sizes, Fisher's Exact Tests (FET) were used to test for statistically significant differences. A level of significance was set at 0.05.

## 3.4 Results

### Vertebral Osteophytosis in the Non Ban Jak Sample

The grading of VO recorded in the NBJ sample ranged from 1:1 (barely discernible osteophyte development on less than one third of the rim) to 3:3 (extensive spicule formation on greater than two thirds of the rim). No vertebral rims were observed with a grade of 4 (ankylosis).

Out of the 31 adults in the assessable NBJ sample, 58.1% (n = 18) were recorded with vertebral osteophytosis of any grade on at least one vertebral rim (Figure 3-2).

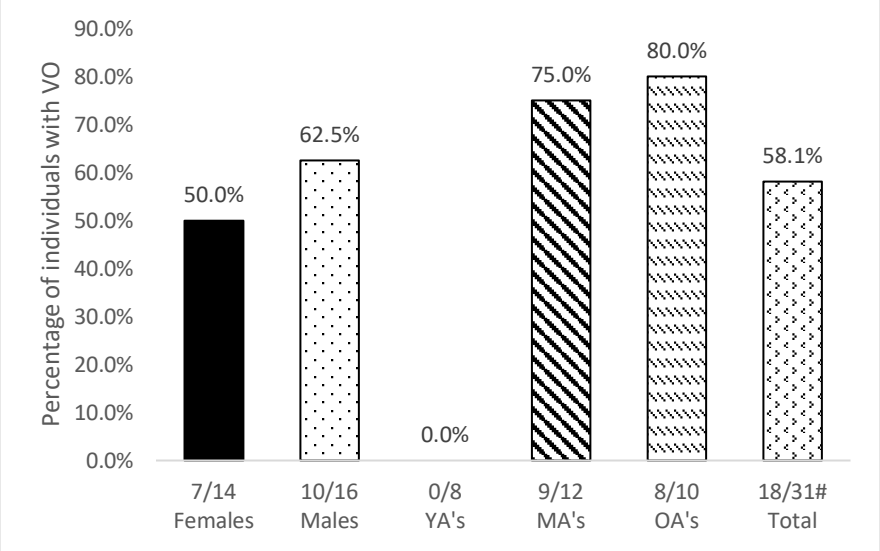



*Figure 3-2 Prevalence of VO by sex and age group in the NBJ sample. YA = young adult, MA = mid-aged adult, OA = older adult.*

### NBJ Prevalence by Sex

A higher proportion of the NBJ males experienced VO (62.5%, 10/16) compared with the number of females affected (50.0%, 7/14) ($p$ = 0.7131) (Figure 3-2).

### NBJ Prevalence by Age

None of the young adults (n = 8) had VO, while a statistically significantly higher number of mid-aged adults (75.0%, 9/12) ($p$ = 0.0014), and older adults (80.0%, 8/10) ($p$ = 0.0011) had VO (Figure 3-2), although the sample sizes are small. There was no significant difference in the prevalence of VO between mid-aged adults and older adults ($p$ = >0.9999).

### NBJ Prevalence by Vertebral Region

There was no significant differences in the prevalence of VO between the sexes within any of the vertebral regions (Table 3-4, Figure 3-3). In the cervical region, more females had VO (37.5%) compared with males (25.0%). In the upper thoracic region, only marginally more males were affected (28.6%) compared with females (22.2%), and it was similar in the lower thoracic region (males 50.0%, females 44.4%), but in the lumbar region the difference between male prevalence of VO (72.7%) compared to female (57.1%) was more noticeable, although not at a statistically significant level ($p$-value = 0.6267). In both sexes there was a gradual increase in the number of individuals affected by VO in their cervical region down to their lumbar region which had the highest prevalence (Figure 3-3).

When comparing prevalence by age group in each vertebral region, the number of mid-aged adults affected by VO gradually increased from the cervical region down to the lumbar region (Table 3-4 Prevalence of VO by vertebral region, sex, and age groups in the NBJ sample., Figure 3-4). Although more older adults had VO in their cervical vertebrae (50.0%) compared with mid-aged adults (33.3%) it was interesting that none of the older adults were observed with VO in their upper thoracic vertebrae, whereas 33.3% of mid-aged adults had VO present (Table 3-4 Prevalence of VO by vertebral region, sex, and age groups in the NBJ sample.). The prevalence of VO in mid-aged adults was only marginally higher than older adults in the lower thoracic region

(62.5% and 60.0% respectively) and lumbar region (87.5% and 83.3% respectively). No young adults had VO in any vertebral regions, but it was only in the lumbar vertebrae that there was statistically significantly higher prevalence of mid-aged (p value = 0.01) and older (p value = 0.04) adults with VO. The sample sizes were small in each age group, especially for the older adults. This increases the chance of Type I error (concluding there is a significant effect when there is not one) or Type II error (concluding there is not a significant effect when there really is one) (Akobeng, 2016). Small sample sizes are often unavoidable when dealing with premodern human skeletal remains, but any information gained is valuable if interpreted with care.

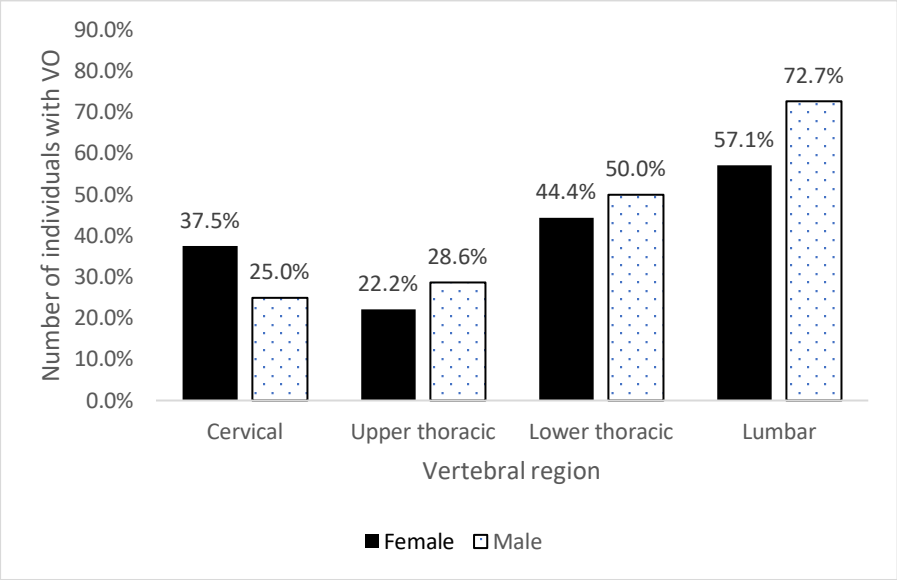



*Figure 3-3 Prevalence of VO by vertebral region and sex in the NBJ sample.*

## Other Vertebral Degenerative Pathologies at NBJ

There were 14 individuals from the NBJ sample with degenerative vertebral conditions other than VO (45.2%, 14/31) (Table 3-5). Two out of the twelve mid-aged adults experienced Schmorl's nodes (16.7%), which was a higher prevalence compared with younger adults

(12.5%, 1/8) and older aged adults (10%, 1/10). Schmorl's nodes were also slightly more prevalent in males (18.8%, 3/16) than females (14.3%, 2/14). However, both comparisons by age and sex were not statistically significantly different (Table 3-5). Preliminary analysis of osteoarthritis in this sample showed that four mid-aged adults (33.3%, 4/12) and one adult of unknown age had pathological osteoarthritis of the zygapophyseal joints, with a total of eleven vertebral joints affected among these individuals. Four of the individuals had a compression fracture or wedging/collapse of a vertebral body. Two of the older adults had a possible case of *pars interarticularis*.

Table 3-4 Prevalence of VO by vertebral region, sex, and age groups in the NBJ sample.

| | Cervical | | Upper thoracic | | Lower thoracic | | Lumbar | |
|---|---|---|---|---|---|---|---|---|
| | a/n | (%) | a/n | (%) | a/n | (%) | a/n | (%) |
| Female | 3/8 | (37.5) | 2/9 | (22.2) | 4/9 | (44.4) | 4/7 | (57.1) |
| Male | 3/12 | (25.0) | 2/7 | (28.6) | 4/8 | (50.0) | 8/11 | (72.7) |
| YA | 0/6 | (0.0) | 0/3 | (0.0) | 0/4 | (0.0) | 0/4 | (0.0) |
| MA | 3/9 | (33.3) | 3/9 | (33.3) | 5/8 | (62.5) | 7/8 | (87.5) |
| OA | 2/4 | (50.0) | 0/4 | (0.0) | 3/5 | (60.0) | 5/6 | (83.3) |
| ?Age | 1/1 | (100.0) | 1/1 | (100.0) | 1/1 | (100.0) | 0/0 | (0.0) |
| Total for Region | 6/20 | (30.0) | 4/17 | (23.5) | 9/18 | (50.0) | 12/18 | (66.7) |

a = number of adults with at least one rim with VO in that vertebral region; n = number of adults (of that sex and age group) with the required number of assessable rims for that vertebral region; * statistically significant

FET *p* values Female vs male: cervical (0.6424), Upper thoracic (>0.9999), Lower thoracic (>0.9999), Lumbar (0.6267)

FET *p*-values YA vs MA: cervical (0.2286), Upper thoracic (0.5091), Lower thoracic (0.0808), Lumbar (*0.0101)

FET *p*-values p values YA vs OA: cervical (0.1333), Upper thoracic (>0.9999), Lower thoracic (0.1667), Lumbar (*0.0476)

FET *p*-values p values MA vs OA: cervical (>0.9999), Upper thoracic (0.4965), Lower thoracic (>0.9999), Lumbar (>0.9999)

FET *p*-values p values total for region: Cervical vs Upper thoracic (0.7246), Cervical vs Lower thoracic (0.3201), Cervical vs Lumbar (*0.0496), Upper thoracic vs Lower thoracic (0.1642), Upper thoracic vs Lumbar (*0.0176), Lower thoracic vs Lumbar (0.4998).

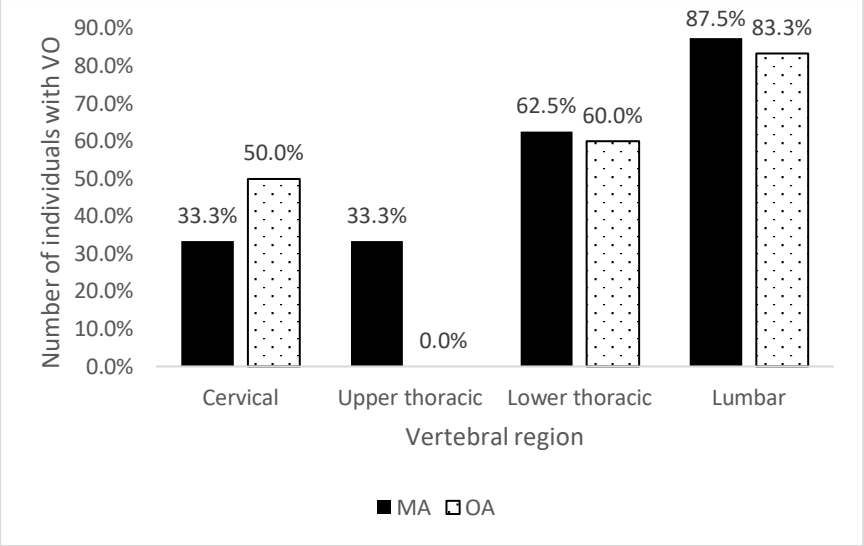



*Figure 3-4 Prevalence of VO by vertebral region and age in the NBJ sample.*
*MA = mid-aged adult, OA = older adult. Note: none of the young adults had VO.*

Table 3-5 NBJ individuals with vertebral degenerative conditions other than VO.

| Age | Sex | Burial | Pathological vertebral osteoarthritis | Schmorl's nodes | Other |
|---|---|---|---|---|---|
| YA | F | B43 | | | Extensive lytic changes T6-T12, T6 collapsed body, neural arches ankylosed T6-T8 |
| YA | F | B162 | | L2 | |
| YA | M | B81 | | | wedge fracture L1 vertebral body |
| MA | F | B84 | 3 x joints (1x cervical, 1 x upper thoracic, 1 x lumbar) | | L5 centrum somewhat compressed |
| MA | M | B108 | 3 x lumbar joints | | |
| MA | M | B120 | 1 x lumbar joint | | |
| MA | M | B136 | | L2 | |
| MA | M | B176 | | L2 | |
| OA | F | B64 | 2 x lumbar joints | | L4 spondylolysis, L5 PVO |
| OA | F | B199 | | | wedge fracture of L1 vertebral body |
| OA | M | B77 | | | L4 lytic lesion, L5 pars interarticularis? |
| OA | M | B82 | | | T10-T11 vertebral bodies almost ankylosing |
| OA | M | B134 | | L2 | wedge fracture of T10 vertebral body |
| ?Age | F | B105 | 2 x cervical joints | T9 | |

Adjei (2022), Buckley et al. (2020), and Domett (unpublished).
FET *p* values Prevalence of Schmorl's nodes: YA vs MA (>0.9999),
YA vs OA (>0.9999), MA vs OA (>0.9999), Males vs Females (>0.9999).

## Pathological Vertebral Osteophytosis in the Non Ban Jak and Ban Non Wat Samples

To gain an understanding of how vertebral osteophyte development may change over time in correlation to age and different activity patterns, VO in the NBJ Late Iron Age sample was compared to the BNW Neolithic and Bronze Age samples. Here the focus was on severe (pathological) VO (PVO) which was classed as a grade of 2:2 or above and would be likely to have had an impact on quality of life.

### Non Ban Jak

Of the 31 adults from the NBJ sample, just over half (51.6%) had PVO (Table 3-6). There was no significant difference in prevalence between the sexes ($p$ = 0.7152), with just over half the males (56.3%) and just under half the females (42.9%) affected. When examined by age group, none of the young adults were observed with PVO, whereas the combined mid-aged and older adults (MOA) had statistically significantly more PVO (68.2%, $p$ = 0.0022).

### Ban Non Wat

In total, forty-one of the 78 adults (52.6%) from BNW had PVO. All four of the Neolithic males (100%) had PVO compared with one third of the females (33.3%) (Table 3-6). Almost all mid-older Neolithic adults had PVO (83.3%) compared with a quarter of the young adults (25%) ($p$ = 0.1905), however the small sample sizes inhibit a meaningful comparative analysis. Bronze Age adults had a marginally higher proportion of males (55.6%) affected by PVO than females (46.9%) ($p$ = 0.6273), and statistically significantly more mid-older adults (63.2%) than young adults (36.7%) ($p$ = 0.0499).

## Temporal Comparison of Pathological Vertebral Osteophytosis

The total proportion of individuals with PVO was similar over the time periods (Figure 3-5). BNW Neolithic adults experienced a marginally higher prevalence of PVO (60%) (but this was also the smallest sample), while adults of the BNW Bronze Age (51.5%) and the NBJ Late Iron Age (51.6%) remarkably had the same prevalence. None of the comparisons of prevalence rate between time periods were statistically significant (Table 3-6).

### Temporal Prevalence by Sex

During all time periods, males had a greater prevalence of PVO compared to females, and the sex difference was most notable within the small BNW Neolithic sample (Table 3-6), Figure 3-5). When comparing the proportion of females with PVO between the time periods, it can be seen that a similar proportion of females experienced PVO in the BNW Bronze Age (46.9%) and the NBJ Late Iron Age (42.9%), whereas one third of females in the BNW Neolithic period (33.3%) had PVO. In the males, the prevalence was similar between BNW Bronze Age (55.6%) and NBJ Late Iron Age (56.3%), while 100% of BNW Neolithic males had PVO (n = 4). None of these differences were statistically significant (Table 3-6).

Table 3-6 Prevalence of PVO in sex and age groups.

| | [a]BNW Neolithic a/n (%) | BNW Bronze Age a/n (%) | NBJ Late Iron Age a/n (%) |
|---|---|---|---|
| Female | 2/6 (33.3) | 15/32 (46.9) | 6/14 (42.9) |
| Male | 4/4 (100.0) | 20/36 (55.6) | 9/16 (56.3) |
| Total | 6/10 (60.0) | 35/68 (51.5) | 16/31^ (51.6) |
| YA | 1/4 (25.0) | 11/30 (36.7) | 0/8 (0.0) |
| MOA | 5/6 (83.3) | 24/38 (63.2) | 15/22 (68.2) |
| Total | 6/10 (60.0) | 35/68 (51.5) | 16/31[b] (51.6) |

a = number of adults affected by PVO in at least one vertebral rim, n = number of adults in the assessable sample; [a]Adjei (2022); ^Total includes one adult of unknown sex with PVO; [b]Total includes one adult of unknown age with PVO; LIA = Late Iron Age, Neo = Neolithic, BA = Bronze Age; * = statistically significant

FET *p*-values total sample: NBJ LIA vs BNW Neo (0.7269); NBJ LIA vs BNW BA (>0.9999); BNW Neo vs BNW BA (0.7404)

FET *p*-values females: NBJ LIA vs BNW Neo (>0.9999); NBJ LIA vs BNW BA (>0.9999); BNW Neo vs BNW BA (0.6724)

FET *p*-values males: NBJ LIA vs BNW Neo (0.2487); NBJ LIA vs BNW BA (>0.9999); BNW Neo vs BNW BA (0.1362)

FET *p*-values females vs males (intrasite): NBJ (0.7152); BNW Neo (0.0762); BNW BA (0.6273)

FET *p*-values YA: NBJ LIA vs BNW Neo (0.3333); NBJ LIA vs BNW BA (0.0765); BNW Neo vs BNW BA (>0.9999)

FET *p*-values MOA: NBJ LIA vs BNW Neo (0.6399); NBJ LIA vs BNW BA (0.7831); BNW Neo vs BNW BA (0.6467)

FET *p*-values YA vs MOA (intrasite): NBJ (0.0022*); BNW Neo (0.1905); BNW BA (0.0499*)

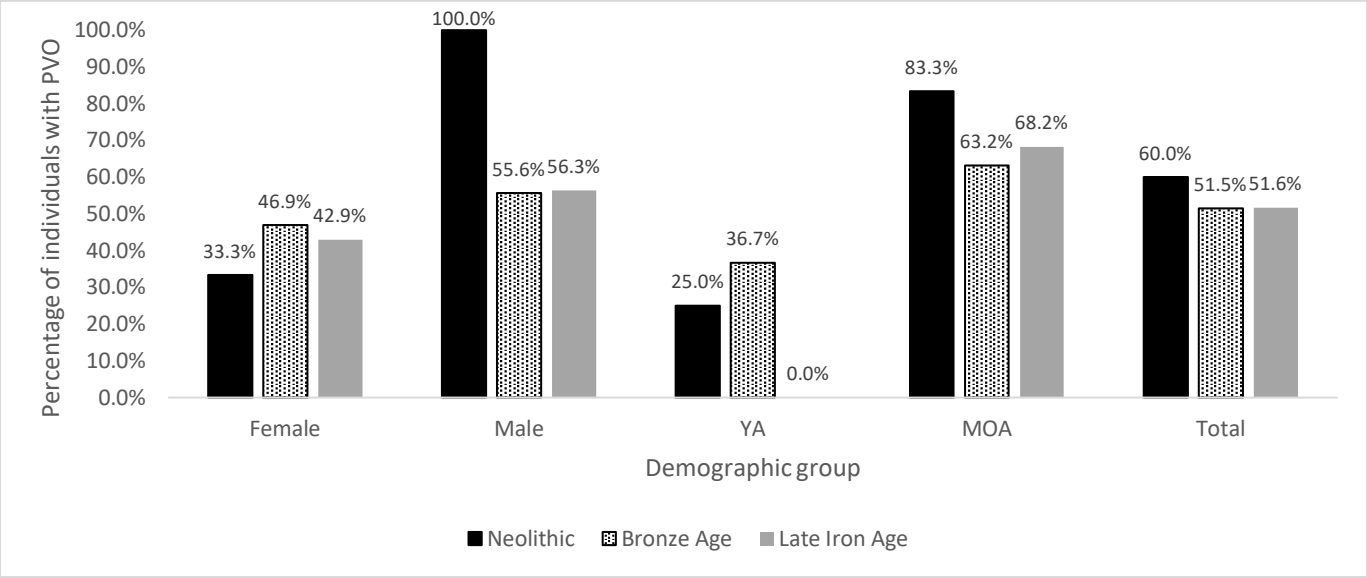



*Figure 3-5 Temporal prevalence of PVO by sex and age group.*

### Temporal Prevalence by Age

As expected, during all time periods PVO prevalence was greater in mid-older adults than young adults (Table 3-6). When comparing just the young adult samples over time, the prevalence of PVO was highest in the BNW Bronze Age (36.7%) compared with BNW Neolithic (25%), and the NBJ Late Iron Age (0%) young adults. However, comparisons between the mid-older adult samples showed the highest prevalence of PVO was in the BNW Neolithic (83.3%), and the prevalence was notably similar between Late Iron Age (65.2%) and Bronze Age (63.2%) mid-older adults. None of the results were statistically significant (Table 3-6).

### Temporal Sex Comparison by Vertebral Region

A comparison of the male samples over time showed a higher proportion of BNW Neolithic males were affected by PVO than BNW BA males and NBJ LIA males in all vertebral regions. BNW BA males had the second highest prevalence of PVO in all regions, and NBJ LIA males the least (the lumbar region was the only exception). Only in the cervical region did NBJ males have statistically significantly lower prevalence of PVO compared to BNW Neolithic males ($p$ = 0.0071) and BNW BA males ($p$ = 0.0466) (Table 3-7).

No significant differences were observed in any of the vertebral regions for the female over time (Table 3-7, Figure 3-6), but a higher proportion of BNW BA females were affected by PVO in all but the lumbar region, here the highest prevalence was observed in NBJ LIA females. BNW Neolithic females were only observed with PVO in their lumbar region and NBJ Late Iron Age females only had PVO in the lumbar and cervical regions.

### Temporal Age Comparison by Vertebral Region

A comparison of the young adult samples over time showed that PVO prevalence in every vertebral region (except the lumbar) was highest in BNW BA young adults (Table 3-7, Figure 3-7). However, this was due to none of the NBJ LIA young adults having PVO, and BNW Neolithic females were only observed with PVO in their lumbar vertebrae, and the female sample sizes were small in both these time periods in comparison to the BNW BA.

The temporal comparison of the mid-older aged adult samples showed that for every vertebral region a higher prevalence was always observed in the BNW Neolithic sample, and the lowest prevalence was always observed in the NBJ LIA. But no significant differences were found. The small BNW Neolithic sample size could have affected the results.

Table 3-7 Prevalence of PVO by vertebral region, sex, and age groups.

| | Cervical | | | Upper thoracic | | | Lower thoracic | | | Lumbar | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BNW Neo a/n (%) | BNW BA a/n (%) | NBJ a/n (%) | BNW Neo a/n (%) | BNW BA a/n (%) | NBJ a/n (%) | BNW Neo a/n (%) | BNW BA a/n (%) | NBJ a/n (%) | BNW Neo a/n (%) | BNW BA a/n (%) | NBJ a/n (%) |
| Female | 0/5 | 8/24 | 2/8 | 0/3 | 2/10 | 0/9 | 0/5 | 3/16 | 0/9 | 3/4 | 8/17 | 4/7 |
| Male | 3/4 | 9/32 | 0/12 | 1/2 | 3/15 | 1/7 | 2/2 | 9/22 | 2/8 | 2/2 | 15/23 | 8/11 |
| YA | 0/4 | 2/24 | 0/6 | 0/1 | 4/15 | 0/3 | 0/3 | 4/21 | 0/4 | 2/3 | 8/23 | 0/4 |
| MOA | 3/6 | 15/32 | 1/13 | 1/4 | 1/10 | 1/13 | 2/4 | 8/17 | 3/13 | 3/3 | 15/17 | 12/14 |
| Total for Region a/n | 3/9 | 17/56 | 2/20^ | 1/5 | 5/25 | 1/17^ | 2/7 | 12/38 | 3/18^ | 5/6 | 23/40 | 12/18 |
| % | | | | | | | | | | | | |
| Female | 0.0 | 33.3 | 25.0 | 0.0 | 20.0 | 0.0 | 0.0 | 18.8 | 0.0 | 75.0 | 47.1 | 57.1 |
| Male | 75.1 | 28.1 | 0.0 | 50.0 | 20.0 | 14.3 | 100.0 | 40.9 | 25.0 | 100.0 | 65.2 | 72.7 |
| YA | 0.0 | 8.3 | 0.0 | 0.0 | 26.7 | 0.0 | 0.0 | 19.0 | 0.0 | 66.7 | 34.8 | 0.0 |
| MOA | 50.0 | 46.9 | 7.7 | 25.0 | 10.0 | 7.7 | 50.0 | 47.1 | 23.1 | 100.0 | 88.2 | 85.7 |
| Total for region | 33.3 | 30.4 | 10.0 | 20.0 | 20.0 | 5.9 | 28.6 | 31.6 | 16.7 | 83.3 | 57.5 | 66.7 |

^ Total NBJ sample includes adult of unknown age with PVO; a = number of adults affected with PVO in at least one rim in that vertebral region; n = number of adults with the required number of assessable rims for that vertebral region; * statistically significant

FET *p*-values total adults NBJ/BNW Neo: cervical (0.2872), UT (0.4113), LT (0.5968), Lumbar (0.6287)

FET *p*-values total adults NBJ/BNW BA: cervical (0.0809), UT (0.3739), LT (0.3381), Lumbar (0.5726)

FET *p*-values total adults BNW Neo/BNW BA: cervical (>0.9999), UT (>0.9999), LT (>0.9999), Lumbar (0.3802)

FET *p*-values females NBJ/BNW Neo: cervical (0.4872), UT (>0.9999), LT (>0.9999), Lumbar (>0.9999)

FET *p*-values females NBJ/BNW BA: cervical (>0.9999), UT (0.4737), LT (0.2800), Lumbar (>0.9999)

FET *p*-values females BNW Neo/BNW BA: cervical (0.2832), UT (>0.9999), LT (0.5489), Lumbar (0.5865)

FET *p*-values males NBJ/BNW Neo: cervical (0.0071*), UT (0.4167), LT (0.1333), Lumbar (>0.9999)

FET *p*-values males NBJ/BNW BA: cervical (0.0466*), UT (>0.9999), LT (0.6722), Lumbar (>0.9999)

FET *p*-values males BNW Neo/BA: cervical (0.0980), UT (0.4265), LT (0.1993), Lumbar (>0.9999)

FET *p*-values YA NBJ/BNW Neo: cervical (>0.9999), UT (>0.9999), LT (>0.9999), Lumbar (0.1429)

FET *p*-values YA NBJ/BNW BA: cervical (>0.9999), UT (>0.9999), LT (>0.9999), Lumbar (0.2855)

FET *p*-values YA BNW Neo/BNW BA: cervical (>0.9999), UT (>0.9999), LT (>0.9999), Lumbar (0.5385)

FET *p*-values MOA NBJ/BNW Neo: cervical (0.0709), UT (0.4265), LT (0.5378), Lumbar (>0.9999)

FET *p*-values MOA NBJ/BNW BA: cervical (0.0165*), UT (>0.9999), LT (0.2595), Lumbar (>0.9999)

FET *p*-values MOA BNW Neo/BNW BA: cervical (>0.9999), UT (0.5055), LT (>0.9999), Lumbar (>0.9999)

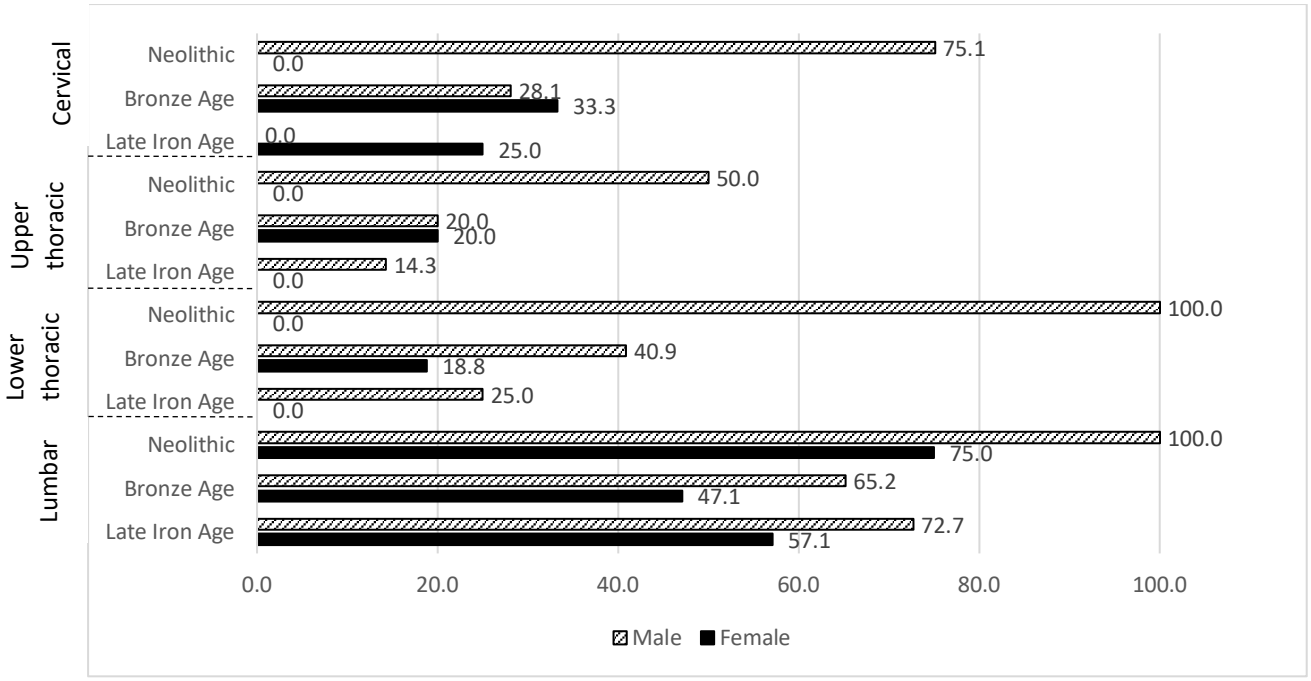



*Figure 3-6 Percentage of individuals with PVO by vertebral region and sex.*

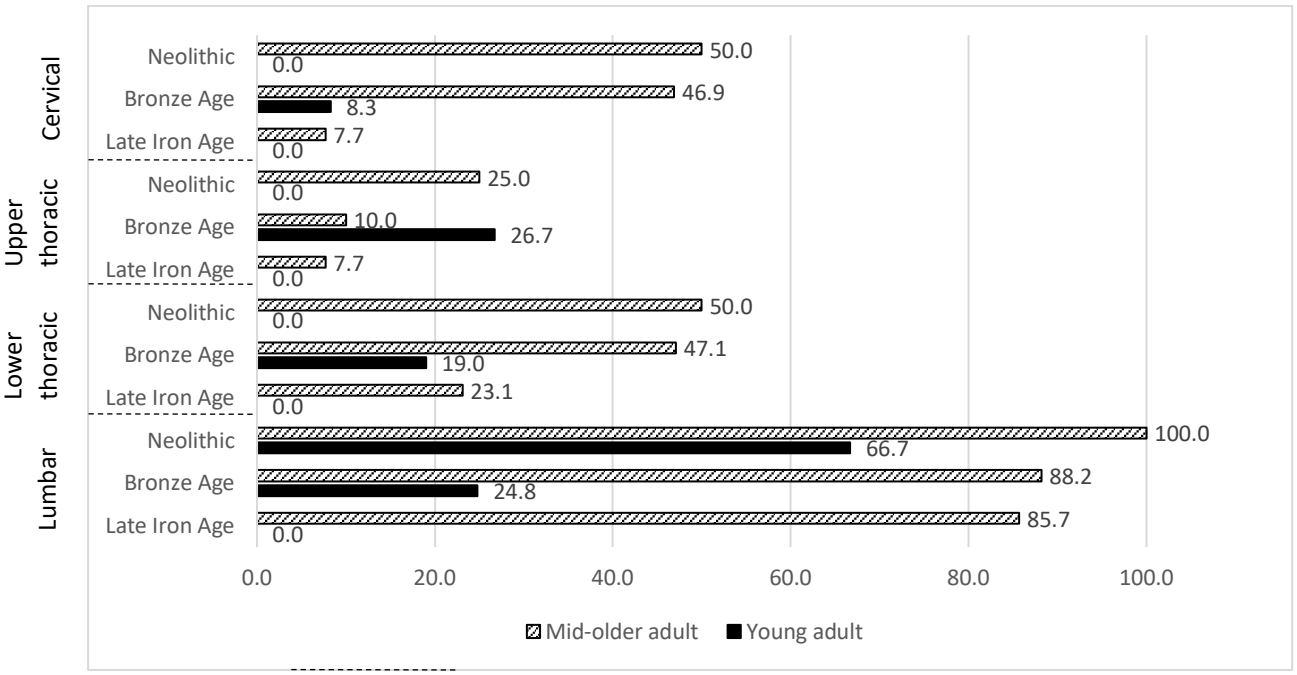



*Figure 3-7 Percentage of individuals with PVO by vertebral region and age.*

## 3.5 Discussion

This discussion is in two parts. First is an in-depth evaluation of VO, of any severity, by age and sex in the NBJ sample, and the possible contributing factors. The second part focuses on the impact of severe (pathological) VO on quality of life between the two sites of BNW and NBJ.

### Non Ban Jak

The main findings for the NBJ population were that 1) no young adults were observed with vertebral osteophytosis; 2) as expected, osteophyte prevalence increased with advancing age, but even so, the prevalence was similar between mid-aged and older aged adults; 3) the greatest prevalence and severity of VO was found in the lumbar vertebrae; and 4) the overall prevalence of VO was higher in males than females.

#### Age

Clinical and archaeological studies indicate that vertebral osteophytes are not commonly recorded in teenagers or young adults for several reasons. VO is associated with skeletal degeneration over a number of decades, and likely develops well after epiphyseal fusion of the vertebral epiphyseal rings which occurs around 20 years of age (Nathan, 1962). Vertebral columns of younger individuals are more likely to be healthy and robust, and therefore more resistant to compression forces, in addition, the intervertebral discs are not yet likely to have begun to deteriorate. This could be partly the reason why none of the NBJ young adults were observed with vertebral osteophytes. This is in agreement with the study of a modern northern Thailand population in which osteophytes were not seen to form in lumbar vertebrae until 36 years of age in adults identified as manual labourers involved in the agriculture and manufacturing sectors (Chanapa, 2016). However, the results of that study is in contradiction

with Suwanlikhid et al. (2018) who, using a similar Thai sample, observed that lumbar vertebrae had already started forming osteophytes and microporosities on the inferior and superior vertebral endplates by 26 years of age, and thinning of cortical bone on the lumbar body was observed at the earliest age of 22 years. Suwanlikhid et al. (2018) suggested that a genetic link to bone loss or influences from either poor posture or increased BMI are possible aetiologies for the vertebral degeneration. The different results between these two studies could largely come down to their very different methods of recording osteophytes, highlighting how non-standardisation of methods makes comparisons difficult.

In contrast, clinical and archaeological studies show adolescents and young adults affected with VO, albeit at a low incidence. For instance, osteophytes were already forming in the vertebrae of 20 year old Turkish individuals (Kacar et al., 2017), and in Caucasian and African Americans (Nathan, 1962). Nathan (1962) highlighted that young adults may develop osteophytes when compressive forces on the vertebral endplates exceed normal capacity of bone resistance, citing intense physical labour as an example. Igbinedion and Akhigbe (2011) report 15.8% of Nigerian young adults 21-30 years of age, were affected with VO, and particularly at higher risk are individuals with less formal education who are generally more likely to be involved in low-paying, strenuous employment. In a pre-modern Korean population sample ($15^{th}$ – $19^{th}$ century), young adults were observed to have a 91% prevalence of osteophytes in their thoracic vertebrae (Kim et al., 2012). The young adults in their study may have participated in strenuous activities such as carrying heavy loads on the back, which placed pressure on certain vertebrae, particularly those situated furthest from the line of gravity where biomechanical pressure is greatest (Kim et al., 2012).

These population differences in VO prevalence and activity patterns suggest that young adults at NBJ engaged in different sociocultural roles and behaviour to those from other communities. There was also no osteoarthritis or Schmorl's nodes observed in the vertebrae of NBJ young adults. These young adults likely remained within a healthy weight range and had not engaged

in activities that placed excessive compressive forces on the vertebral column or experienced repetitive flexion and torsion movements, at least not for prolonged periods of time.

Osteophyte prevalence increased with advancing age, but even so, the prevalence was quite similar between mid-aged and older aged adults at NBJ (75% and 80% respectively). This prevalence rate is lower than the 92% in cervical vertebrae of mid-older aged modern Thais (92%) (Chanapa & Mahakkanukrauh, 2011), mid-aged and older adult Nigerians (80-100%) (Igbinedion & Akhigbe, 2011), and Caucasian and African Americans (100%) (Nathan, 1962). In the NBJ population, a higher proportion of older adults had VO in their cervical vertebrae compared to mid-aged adults, perhaps due to undertaking physical activities that placed greater flexion and extension forces on the neck (Chanapa & Mahakkanukrauh, 2011). Interestingly, osteophytes were noticeably absent from the upper thoracic region of older adults, and this age group had a marginally lower prevalence of VO in the lower thoracic and lumbar regions compared to mid-aged adults.

Few studies have reported the prevalence of osteophytes to be highest in the thoracic vertebrae compared to vertebrae in the cervical region (Jurmain, 1990), and in even fewer cases more than the lumbar region (Cvijetić et al., 2000). Reasons for this vary from less range of movement in the thoracic vertebrae due to the stabilising effect of the connecting ribs (Hollinshead, 1969), less weight-bearing requirements than lumbar vertebrae (Bridges, 1994) as stresses from the weight of internal organs and ribs placed on the thoracic vertebrae is transferred away through the line of gravity (Okamoto et al., 2015). However, there are contradictory factors. Thoracic vertebrae are reported to be more susceptible to rotational actions whereas the larger cross-section in lumbar vertebrae protects them from torsional force (Kapandji, 1974). Load bearing activities can place tremendous strain on the mid-vertebral region. The close similarity in overall prevalence between the NBJ mid-aged and older adults suggest that quality of life did not significantly deteriorate in older adults, at least in terms of vertebral degeneration.

## Sex

The overall prevalence of VO was higher in NBJ males than females and this is consistent with the majority of studies (Clark & Delmond, 1979; Liu et al., 1997; O'Neill et al., 1999; Shao et al., 2002). This is commonly argued to be attributable to be the result of sex differences in physical labour and activities. It is suggested that males may experience more VO development due to traditionally engaging in more strenuous physical activity or occupational stress (Kim et al., 2012; Lai & Lovell, 1992). Differences in anatomical structure and applied biomechanics, such as variances in muscle mass and strength affecting spinal stability (Sinaki et al., 2001) also means that the expression of osteophyte prevalence and DJD is different between each vertebral region and between the sexes (Bogduk, 2012).

Although some researchers, based on Western ideas, reason that females have a lower likelihood of engaging in heavy manual labour, this is contradicted by the pattern of VO reported by Chapman (1972) who noted female skulls in the prehistoric Mexican *Tlatilco* population were robust and often had strong muscle attachments visible on the posterior aspect of the skull that they suggested could be correlated to the rigorous physical activity of maize cultivation. Of note, Burial 105, a female of unknown adult age from NBJ, had the highest individual prevalence of VO, including pathological osteophytes in the cervical and upper thoracic vertebrae. This female was also recorded with a Schmorl's node and lytic destruction on several cervical vertebrae. Schmorl's nodes occur when the nucleus pulposus of the intervertebral disc material herniates through the inferior or superior endplate and invades the vertebral body, forming a lesion (Kyere et al., 2012). Population prevalence can vary tremendously, making it difficult to understand its aetiology (Dar et al., 2009), but frequency may be determined by physical activity patterns and postures (Faccia & Williams, 2008), and Schmorl's nodes are reported to be highly heritable and positively correlated with lumbar disc disease (Williams et al., 2007). The adult prevalence of Schmorl's nodes in the NBJ assessable sample was just 9.7% (3/31) and the prevalence was slightly higher for mid-

aged adults and males. NBJ females also had a higher prevalence (21.4%) of pathological vertebral osteoarthritis than did the males (14.3%). Burial 82, an old age male, was observed to have the single most severe incident of VO, where the 10$^{th}$ thoracic vertebrae was very close to ankylosing with the 11$^{th}$ thoracic vertebrae. The 2$^{nd}$ lumbar vertebral centrum inferior rim also had a large osteophyte. The presence of osteophytes, osteoarthritis, and Schmorl's nodes can alter the vertebral column's ability to withstand mechanical loading. These individuals would have experienced back pain and loss of movement, and the effects can be debilitating and limit participation in daily activities.

The only vertebral region in which a higher proportion of NBJ females had VO compared with males was in the cervical vertebrae. One possibility is that NBJ females may have carried burdens on their heads, an activity that subjects the cervical vertebrae to considerable compressive force and axial loading through the intervertebral disc (IVD). Echarri and Forriol (2002) studied the effect of axial loading on the cervical spine in young adult and mid-aged adult female Congolese woodbearers. Compared to non-woodbearers, these individuals experienced hypertrophy of the trapezius muscle in the neck, significantly higher prevalence of intervertebral disc space narrowing, reduced vertebral body height, and a higher risk of listhesis. A significantly higher prevalence of vertebral osteophytes was positively correlated to the post-menopause period, short stature, and the number of years engaged in this activity (Echarri & Forriol, 2002). Ichchou et al. (2010) found increased prevalence in degenerative diseases (osteoporosis, osteophytes, and intervertebral disc-space narrowing) in the lumbar vertebrae of 88% of post-menopausal women in their sample that they correlated to age, sex, and obesity. The effect of reduced testosterone on bone health in aging males is not as clear-cut as the effects of menopause in females (Golds et al., 2017). However, it is known that a decline in testosterone will increase the risk of low bone mineral density and osteoporosis (Shigehara et al., 2021).

In the NBJ sample, osteophyte formation was most pronounced in the lumbar vertebrae, but in comparing the sexes it was found that males experienced more VO in the thoracic and lumbar regions than did females (although not to a significantly different level). This could perhaps be because males tend to have much greater body weight than females which would place more pressure on the vertebral column (Suwanlikhid & Mahakkanukrauh, 2019). The five lumbar vertebrae, at the base of the vertebral column, are under the most biomechanical stress and are subject to the most musculoskeletal disorders and degenerative change. They have the largest centra of all the vertebrae but are highly flexible and provide a range of motion including trunk rotation, lateral bending, flexion and extension (Tanaka et al., 2001). In contrast to the NBJ population, Cvijetić et al. (2000) studied radiographs of thoracic and lumbar vertebrae of older Croatian adults (>45 years of age) and observed VO prevalence was higher in females compared to males. Cvijetić et al. (2000) suggested that this sex difference may have resulted from females in this population being more affected by vertebral deformities, which was significantly associated with vertebral osteophyte formation. However, the NBJ females were not significantly more affected by vertebral degenerative conditions such as spondylolysis, lytic lesions, or compressed vertebrae than the males.

Clinical studies on Thai populations illustrate how certain activities can increase the risk of osteophytes and lower back pain. The typical Asian practice of deep squatting, low to the ground, or sitting on the floor with no back support, also places abnormal pressure on the vertebral column and lower extremities (Cho et al., 2012; Lu et al., 2020). Thai females with greater than 10 years spent repetitively engaged in squatting activities such as cooking and latrine use, were particularly vulnerable to lower back pain and degenerative changes in the lumbosacral region (Wajanavisit et al., 2009). Chanapa (2016) observed a 100% prevalence of VO in the lumbar region of modern Thai adults aged over 36 years engaged in agricultural and manufacturing jobs which involved repetitive heavy lifting, and imposed flexion and lateral bending on the lumbar region. Chanapa et al. (2014) also discovered that osteophyte length

has a strong correlation to advancing age, and prevalence was greater in males compared to females.

Roles and responsibilities in the past may be similar to those experienced by present day communities in a similar environment, or allocated roles for males and females may have changed considerably since the prehistoric period, dependent on a number of factors such as social and family relationships, age, health, education, personal experience, and traditional values. Modern studies are often used to reconstruct prehistoric lifestyles, but this should always be considered with caution. A sexual division of labour in the modern Thai rural subsistence economy has been noted. For the most part, males are considered to conduct the majority of heavy lifting over the course of daily activities. However, females will work in the fields at crucial times such as planting and harvesting (Singhanetra-Renard & Prabhudhanitisarn, 1992; Weir, 2007). Furthermore, modern rural Thai female roles and responsibilities are reported to commonly include fishing, weaving and sowing, carer, household chores, and child rearing (Sa-idi et al., 1993). Females are more often noted to take on economic roles to support the household, whereas village leadership and political positions tend to be roles reserved for males (Sa-idi et al., 1993; Weir, 2007).

Ward et al. (2019) observed that social inequality between the sexes at NBJ is suggested by a decrease in stature seen in females from the later mortuary phases compared to those in earlier mortuary phases, and there were high levels of infant mortality, indicating that females experienced greater stress than males. Males also received greater quantities of grave goods than females (Ward et al., 2019). This suggests that differences in social status could at least partly explain the pattern of VO prevalence in this population.

## Temporal Comparison of PVO: Comparing BNW and NBJ

The key results for the comparison of pathological (grade of >2:2) vertebral osteophytosis prevalence between the Neolithic, Bronze Age, and the LIA indicates that 1) overall prevalence was marginally higher in BNW Neolithic adults (60%), while both BNW Bronze Age and NBJ Late Iron Age adults notably had the same, slightly lower, rate (51%); 2) in all three time periods males had a higher prevalence than females, and mid-older age adults were more adversely affected by PVO than young adults; 3) in young adults, PVO prevalence increased from the Neolithic to the Bronze Age, and was notably absent in the LIA.

It was hypothesized there would be an increase in prevalence of PVO from the Neolithic to the Iron Age, largely to do with the physical labour burdens of land clearance, moat and residential building construction, and agricultural intensification. Yet there are no statistically significant differences in PVO prevalence between sex or age groups over time. There was also a lack of significant sex or age differences in osteoarthritis prevalence in the major synovial limb joints from the BNW Neolithic to the Bronze Age (Domett et al., 2017), or in the zygapophyseal joints in the Late Iron Age (Adjei, 2022).

### Age

The lack of PVO in the Late Iron Age young adults at NBJ was in stark contrast to the earlier time periods at BNW, where PVO prevalence in young adults increased from the Neolithic to the Bronze Age but was still less than that experienced by mid-older adults. The clinical and archaeological literature supports the observation of higher prevalence of PVO within older individuals, due in part to a longer period of time spent conducting physical activities that contribute to osteophyte development (Gellhorn et al., 2013; Maat et al., 1995; Weiss & Jurmain, 2007). The distribution of PVO also varied between vertebral regions. Interestingly, Neolithic young adults were only observed with PVO in their lumbar vertebrae. Whereas

Bronze Age young adults were more severely affected, having PVO in all vertebral regions, with the highest prevalence in the lumbar region. Bronze Age mid-older adults had significantly higher prevalence of cervical PVO compared to those in the Late Iron Age. Pathological VO has a multifactorial aetiology, it is likely that physical activity associated with agricultural intensification during the Bronze Age contributed in some part to the increase of young adults of both sexes and mid-older age females with PVO from the Neolithic into the Bronze Age. However, why there is then an absence of PVO in the vertebrae of Late Iron Age young adults is intriguing. This suggests that their quality of life may have improved over time, perhaps linked to greater access to resources or participating in less labour-intensive roles compared to in the Bronze Age; even though heavy and intense physical labour was required for large-scale moat development for water management during the Iron Age (Duke et al., 2016). Interestingly, mid-older age adults in the Neolithic were less affected by PVO than in the latter two periods in which the prevalence was very similar, but it is likely that the much smaller Neolithic sample is biasing the results somewhat.

### Sex

Sex differences in PVO prevalence was reasoned to correlate somewhat with a sexual division of labour and/or social inequality. Both of these factors can determine an individual's access to resources. This has a bearing on diet and health, and those involved in mechanically strenuous labour will have experienced greater musculoskeletal stress and degenerative joint disease (Klaus et al., 2009; Pechenkina et al., 2011; Schrader, 2015). Throughout the Neolithic and Bronze Age time periods, young adult and mid-old adult males exhibited higher rates of PVO compared to female young adults and female mid-old adults. In the Late Iron Age, the prevalence of PVO was also higher in males than females but both sexes experienced similar rates to the Bronze Age. Whilst mid-older age males were less affected by PVO in the Bronze Age than in the Neolithic, it is likely that the much smaller Neolithic sample is biasing the results somewhat. Other degenerative pathologies, such as vertebral

osteoarthritis, in other Southeast Asian sites also differ in the ratios of pathology and sex (Zhang et al., 2017), and it seems degenerative pathologies are not always reflective of environmental conditions as sites with similar environmental contexts reflect differing results of males and females with degenerative pathologies (Adjei, 2022). The overall prevalence of trauma increased from the Neolithic to the Late Iron Age, and within all three time periods the highest proportion of injuries was always higher in males compared to females (Pedersen, 2017). Trauma and a sexual division of labour are often correlated with one another and can be used to explore the physical and cultural environment of a society (Domett & Tayles, 2006; Molnar et al., 2011). A BNW study on entheseal changes, osteoarthritis levels, and robusticity scores indicated that the intensity of labour remained consistent over the Neolithic and Bronze Age, however, the pattern of skeletal and joint change suggested that males and females engaged in different activities over time (Foster, 2011). Clinical observations, in particular identical twin studies, have identified genetic predisposition as a stronger risk factor to intervertebral disc degeneration and osteophyte development than age and physical activity (Battié et al., 2009; Battié et al., 2004; Sambrook et al., 1999). Many different gene forms have been discussed as causal factors, such as those associated with the vitamin D receptor which influences bone metabolism (Colombini et al., 2013; Videman et al., 2001), or genotypes responsible for inflammation or degradation of connective tissues (Kawaguchi, 2018) leading to disc narrowing and bulging, vertebral endplate deterioration, annular tears, herniations, and osteophytes.

The relationship between diet and sex and associated procurement strategies of food sources have been suggested to correlate with each other in previous studies in Southeast Asia (Domett, 2004; King et al., 2013; Larsen, 2015; Okazaki et al., 2013). Dental wear has been used to explore sexual difference of diet at BNW (Domett, 2004) and NBJ (Heap, 2022). Males at Late Iron Age NBJ had significantly higher rates of advanced dental wear than did females. At BNW, there was a significant difference in stable carbon isotope values from dental enamel between males and females during the Bronze Age 2-3A periods, with males presenting with

a higher prevalence of advanced wear associated with a coarser diet (King et al., 2013; Newton, 2014). The difference in stable carbon isotope values and the rate of advanced wear suggests a difference in diet between males and females.

There is a period in the Early Bronze Age where the mortuary context suggested some individuals enjoyed greater wealth and social status than others, but by the Late Bronze Age there was no such displays of wealth (Higham, 2011) and generally health had declined, although not to an extreme level (Clark, 2014). Even though that by the Iron Age there was a subsistence shift to a less varied diet and a higher reliance on rice (King et al., 2013), bone growth evidence in infants and children did not show a significant decline in health at BNW (Dhavale et al., 2017). It is quite possible that during the transition from Bronze Age to Iron Age the population simply exchanged one set of activities for another, of different nature, but with a similar intensity of effects on the spine. Early wet-rice agriculture, for example, may have continued to employ simple seed casting and general weeding, rather than the labour-intensive nursery beds and transplanting practices of more recent centuries. Male PVO prevalence was consistently higher than that of the females suggesting that a sexual division in labour, habitual activities, and diet pervaded throughout the span of the two settlements (1800 B.C. to A.D. 820). Vertebral degeneration does however have a multifactorial aetiology and the influence from genetic factors is largely unknown in prehistoric populations.

Finally, it is interesting that there is no indication of increasing variability of PVO within the Late Iron Age period. If there was an increase in social inequality oat this time, with lower status individuals assigned the more labour-intensive work, then we would expect to see a sharp contrast between those with and those without, presumably with a large proportion of the population more heavily affected by VO. This does not seem to be the case.

## 3.6 Conclusion

The pattern of VO formation in the Late Iron Age people of NBJ differed to earlier time periods in the BNW sample and many other archaeological and modern populations, in that no young adults were observed with any grade of vertebral osteophytes; yet it is also similar to BNW and a majority of other populations in that osteophyte prevalence was highly correlated with advancing age, more males were affected than females, and the greatest prevalence and severity of VO was in the lumbar vertebrae compared to the cervical and thoracic. Contextual evidence and the pattern of pathological vertebral osteophytosis in the different vertebral regions could suggest that the quality of life declined for females from the Neolithic to the Bronze Age in particular, but for males there was a definite improvement from the Neolithic, while young adults may have enjoyed less physical stress in the Late Iron Age. However, absent is an expected overall significant increase in vertebral osteophytosis and osteoarthritis from the Neolithic Period to the Late Iron Age. Instead, there is a remarkable similarity in prevalence between the Bronze Age and Late Iron Age, which defies the expectation for vertebral degeneration to intensify under the pressures of increased physical labour, and dietary changes that had the potential to exacerbate vertebral and joint degeneration over an individual's lifetime. This suggests that genetics may be a stronger risk factor than activity and environment. Or, perhaps more controversially, that there was actually no particular increase in intensity of physical labour from one era to the next. Perhaps the first centuries of wet-rice agriculture in northeast Thailand represented a marginal increase in labour intensity, rather than a revolutionary step. The lack of young adults with any VO might support this model. Future VO studies on other Southeast Asian prehistoric populations could continue to gather information on diet, health, social stratification, and physical activities, that will allow for a more precise evaluation of factors affecting the progression of vertebral degeneration with advancing age.

## 3.7 Chapter Summary

This chapter evaluated the degenerative pattern of VO between the archaeological sites of NBJ and BNW, which were in close geographical proximity but from different time periods and sociocultural contexts. These key archaeological sites spanning from the Neolithic to the Late Iron Age allowed the relationship between vertebral osteophyte formation and age to be considered alongside other contributing factors such as increased physical labour with shifting subsistence strategies. The main aim of this chapter, however, was to record interpopulation prevalence of VO to assess if there was a clear correlation between formation rates and age to clarify its usefulness as an adult age indicator. There was significantly greater prevalence of VO in mid-aged adults and older adults than young adults, which was to be expected, but there were no significant differences observed between mid-age adults and older adults. PVO rates were significantly higher in mid to older aged adults than younger adults in both the Bronze Age and Late Iron Age periods, but no significant differences were found between the different age groups in the Neolithic sample. These results indicate that, in these samples, vertebral osteophytosis rates may have limited usefulness as a means to estimate adult age. Future VO studies on Southeast Asian populations should incorporate larger samples and clinical studies for a more precise evaluation of factors (e.g. diet, genetics, physical activity, and health) affecting the progression of vertebral degeneration with advancing age.

# Chapter 4 Bone Remodelling and Histology

The focus of this study now moves to the investigation of age dependant changes in intracortical remodelling using histological methods. Before proceeding to the histomorphometric analyses in the following two chapters, it is beneficial to introduce the cells and processes involved in cortical bone remodelling, which will be discussed here. Histologic features examined in this study are also defined. This is a necessary step as the definition of features used in age estimation methods lacks standardisation and introduces interobserver error into analyses (Crowder et al., 2022). Being able to correctly identify intact versus fragmentary osteons, Type I from Type II osteons, resorption cavities, and branching events with the aid of concise and precise definitions and images provides an important guide that not only reduces observer error but improves replicability.

## 4.1 Bone Modelling and Remodelling

### Cortical Bone and Cancellous Bone

The human skeleton is 80% cortical bone and 20% cancellous bone, both of which are modelled and remodelled over an individual's lifetime (Katsimbri, 2017). It is the microscopic properties within remodelled bone that provide the means to estimate age. Cancellous (trabecular) bone is found in the medullary cavity of bones, long bone metaphyses, vertebral centra, and the flat bones of the cranium. It has a light, honeycomb or spongy structure made up of a three-dimensional porous network of interconnecting spaces (Parfitt, 1987).

Cortical bone is denser and provides an outer protective wall of varying thickness, depending on the bone, including the vertebrae, cranium, and long bone diaphyses (shaft). During different stages of an individual's life, several types of cortical bone can be seen under microscopic examination: primary (woven) bone that matures into primary circumferential lamellar bone, which in turn is gradually replaced over time by the remodelling process with osteonal lamellar bone (Liu et al., 2000; Pfeiffer, 2006). This study focuses on the quantitative histomorphology of cortical bone microstructure and its correlation with age.

### Woven Bone and Lamellar Bone

Woven and lamellar bone are found in both cortical and cancellous bone tissue. Woven bone is immature bone that first develops in the embryonic skeleton and is present in young children, up to approximately two years of age (Pfeiffer, 2006). Mineralised collagen fibril bundles are rapidly deposited in a disorganised fashion, giving the bone its woven appearance. This bone is weaker but more flexible than the lamellar bone that eventually replaces it in adult cortical bone. Woven bone is usually only found in adults in response to pathologies such as metabolic bone disease and bone tumours, or to repair skeletal trauma (fracture) (Bailey et al., 2022; Frost, 1992). This interferes with age estimates as the area of immature bone around pathological lesions has a decreased mean tissue age in comparison to the individual's actual age (Gocha et al., 2019).

Lamellar bone has a more complex structure that is formed more slowly compared to woven bone (Pfeiffer, 2006). It is a collagen fiber matrix uniformly laid down by osteoblasts in microscopically thin sheets of mineralized fibrils (lamellae) on existing bone or cartilage (Mitchell & van Heteren, 2016). It is the type of bone that is more prevalent in the bone cortex of adults (Pfeiffer, 2006). The way remodelling leaves its mark within lamellar bone provides the key to assessing adult age.

The organization of lamellae was originally viewed to be like the layers in laminated plywood, each layer tending to have collagen fibers laid on a slightly different angle (Gebhardt, 1906). Alternatively, many later studies ascribed to the suggestion that lamellae are collagen-rich tissue alternating with layers of collagen-poor tissues. In their review of the numerous models of lamellae organization that have been proposed over the decades, Mitchell and van Heteren (2016) show there is still a general lack of consensus. Regardless of the model followed, the lamellae are easily viewed microscopically as alternating light and dark bands under polarized light (birefringence), depending on whether the fibres are perpendicular or parallel to the light source (Bromage et al., 2003). Lamellae, in particular those of secondary osteons, are an important feature of remodelling events.

Lamellar bone can be primary (primary osteons and circumferential lamellae) or secondary (secondary osteons). Primary circumferential lamellar bone is deposited in highly organised parallel layers on an existing bone surface, just under the periosteum as well as on the endosteal edge of the cortex (Chang & Liu, 2022). Contained within this matrix are primary osteons and non-Haversian canals. The area of circumferential lamellar bone decreases with age; in some elderly adults it will be completely remodelled by secondary osteons, while in other adults small portions may still remain unremodelled up to 95 years of age (Kerley, 1965).

Circumferential lamellae of secondary osteons are laid centripetally to infill resorption spaces within the cement line boundary, replacing primary bone (Mitchell & van Heteren, 2016). Primary bone still exists in the space between the secondary osteons as interstitial lamellae, until eventually most is remodelled (Hage & Hamade, 2014). The histomorphometric features of secondary osteons are most useful in estimating age and will be discussed later in this chapter.

## Growth and Modelling

Growth of bone begins within the first few weeks post-conception, either via endochondral ossification, where cartilage buds of long bones, vertebrae, and the pelvis, calcifies and is then replaced by bone (Rauner et al., 2012); or via intramembranous ossification in flat bones and some irregular bones (Martin et al., 2015). In subadults, long bones grow in length as calcium deposition and bone formation continues onto the metaphyses and growth plates (secondary ossification centres) (Shapiro 2008). This growth occurs incrementally at rates known to correlate with chronological age so that measurements of subadult long bone lengths can be compared to published standards to estimate age (Buikstra & Ubelaker, 1994; Scheuer & Black, 2000).

Long bones continue to grow until cartilage deposition ceases in the growth plates (epiphyses) which then start fusing to the metaphysis at each end of the diaphysis (Shapiro 2008). This prevents the bone from continuing to grow in length. The timing of this fusion varies between each bone, and between the sexes, and is another way to estimate age (Cardoso et al., 2014). Once bone growth ceases, other methods need to be used to estimate the age of the mature skeleton. Typically, this involves scoring macroscopic characteristics of bone degeneration and remodelling of the pubic symphyses (Brooks & Suchey, 1990), auricular surface of the ilium (Buckberry & Chamberlain, 2002), and sternal rib ends (İşcan & Loth, 1986a, 1986b).

Modelling is also described as sculpting (Roberts et al., 2006; Streeter, 2005), whereby the shape and size of skeletal elements is accomplished by bone being laid down in some areas and removed in others (but not at the same time) as an individual grows from a child into an adult. Modelling accounts for growth in width and density of the long bone diaphysis as osteoblastic activity adds bone to the outer periosteal surface and osteoclastic activity appositionally works to remove bone from the inner endosteal surface (Katsimbri, 2017). This coordinated effort can lead to cortical drift of the bone cortex which changes its shape (such

as the curvature), or its cross-section size relative to its central axis (Gocha et al., 2019; Goldman et al., 2009). Cortical drift can erase evidence of lamellae deposited at a young age as it is 'modelled out' (Stout & Crowder, 2012). This leads to the actual mean tissue age of packets of adult cortical bone being of younger age than the cortex's chronological age (Frost, 1987b; Stout & Lueck, 1995).

### Remodelling

Bone is constantly being turned over by the resorption of old or damaged bone and its eventual replacement with new osteoid matrix that mineralizes into bone (Parfitt et al., 1997). It is different to modelling in that it does not change the shape or size of the skeletal element (Katsimbri, 2017). Remodelling occurs throughout life, but mostly as an adult, in response to biomechanical stresses, injury, and to maintain bone mineral homeostasis and strength (Katsimbri, 2017). Remodelling occurs at different rates depending mostly on the age of the individual, their biological sex (Miszkiewicz et al., 2022a), and genetics (Dominguez & Agnew, 2016). Eventually, by the time an individual reaches old age, most or all traces of primary bone have usually been replaced by secondary bone (Mitchell & van Heteren, 2016).

Remodelling is a coordinated effort of bone multicellular units (BMU's) with most of the work done by bone resorbing osteoclast cells and bone forming osteoblast cells. Any imbalances in this relationship can cause age-related metabolic bone diseases, which can affect the normal activity of bone resorbing and forming cells. The most common of these diseases is osteoporosis which is also menopause-related and occurs when bone is resorbed faster than it is being formed and the cortex reduces in volume (Manolagas & Parfitt, 2010; Seeman, 2013). Other diseases cause excessive bone formation (such as Paget's disease) (Seitz et al., 2009), or diminished bone resorption (osteopetrosis) whereby bone density increases (Stark & Savarirayan, 2009). Osteomyelitis actively destroys bone, creating a periosteal

reaction of rapidly remodelled woven bone that usually has higher levels of osteocytes with an increased proliferation of cancellous bone in the medullary cavity (Sybenga et al., 2020).

## 4.2 Formation of the Secondary Osteon

The histomorphometric study of bone microstructure is used as an essential tool for investigating bone remodelling and its relationship with age through the development and functioning of multiple generations of basic structural units (BSU's, or secondary osteons) in mature cortical bone. This is achieved by documenting, counting, and measuring the features of secondary osteons (also known as Haversian systems), including their central Haversian canals, lamellae, osteocytes, lacunae (Figure 4-1) and the number of remodelling events per square millimetre, or their geometric properties (area, diameter, circularity). These are the features observed on the transverse plane either in high-resolution 3D micro-CT images, or via microscopy using thin-section slides.

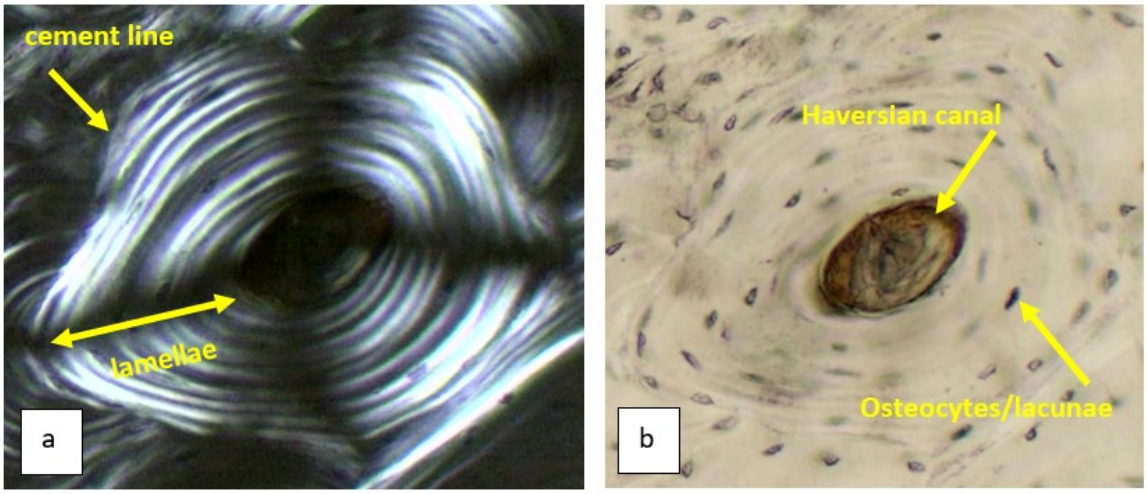



*Figure 4-1 Secondary osteon features (not-to-scale).*
*Midshaft femur: (Image a, polarised light) intact osteon with concentric lamellae and a cement (reversal) line around the perimeter of the osteon; (Image b, transmitted light) osteocytes housed in lacunae are sandwiched between the lamellae that surround a central Haversian (vascular) canal.*

## The Bone Multicellular Unit

To be able to identify the features of a secondary osteon it is necessary to understand the different stages in its lifecycle that are formed by bone multicellular units (BMU) that operate in consecutive phases of activation, resorption, reversal, and formation (Figure 4-2). This cycle completes in an average of 3-6 months (Sommerfeldt & Rubin, 2001). A BMU is a 3-dimensional feature that travels through the cortex by using a cutting cone at its leading edge where mononuclear cells and osteoclasts are activated to resorb small packets of damaged or old bone, bit-by-bit creating a narrow channel around a central vascular (Haversian) canal (Robling & Stout, 1999). In a long bone, the BMU proceeds through the diaphysis in a longitudinal orientation, likely in response to the major forces of biomechanical loading (van Oers et al., 2008). As the cutting cone progresses, it leaves behind a scalloped-edged resorption cavity on which bone-lining cells create a reversal (cement) line.

Bone-forming osteoblast cells can then lay down a new surface of osteoid on the cemented line. New bone is appositionally deposited on the sides of the resorbed channel in rings of lamellar bone, until eventually the concentric rings of lamellae fill the cavity to surround the central Haversian canal, at which point the secondary osteon (BSU) is structurally mature (Parfitt, 1994). 3D micro-CT scanning has been used to map the network of Haversian systems that permeate mature cortical bone to record lengths varying from ~ 1.5 mm to > 1cm (Cooper et al., 2006; Maggiano et al., 2016). They can branch off laterally at which point the canal can either widen or narrow (Jaffe, 1929) and is observed as a branching event of a transverse cross-section.

The Haversian systems can travel either unidirectionally (distally or proximally), or in both directions at once with a cutting cone at each end (Cooper et al., 2006). They are not perfectly cylindrical, instead width varies along the axis by ~2 to 3 mm, and they often divide into several

branches or are clustered (Chang & Liu, 2022). The point at which a canal branches can either widen or narrow and will become surrounded by its own concentric lamellae (Jaffe, 1929) which can be observed on a transverse cross-section as a branching event. Neighbouring Haversian systems are interconnected to each other and to the periosteal vascular system on the outer bone layer by transversely tunnelling Volkmann's canals, ensuring nutrient homeostasis throughout bone tissue.

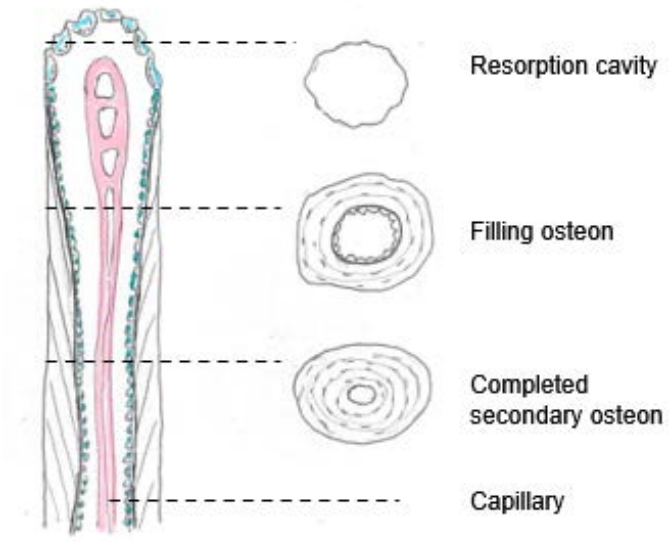



*Figure 4-2 Longitudinal view of bone multicellular unit. Stages of osteon formation typically captured in a transverse cross-section.*

A typical 100 µm thick transverse cross-section of cortical bone in an adult will show a snapshot of multiple generations of osteons, each captured at a particular stage of their life cycle, in the envelope of bone that the cross-section has intersected (Figure 4-3). Active resorption cavities created by the opening cone at the resorption stage may be visible, while a neighbouring osteon may be seen at its reversal stage just after the cement line is formed around the edges of the resorption cavity; others will be captured at their formation stage, with a complete cement line and a number of concentric lamellae rings either in the process of

filling or having completely filled the cavity until just the central Haversian canal remains, at which point the secondary osteon is complete (the closing cone end of the BMU). A completed osteon viewed in cross-section is roughly circular and typically has a diameter between 200 to 300 µm (Sommerfeldt & Rubin, 2001). The cutting cones of new BMU's can intercept existing canals so that a cross-section will show resorption spaces and newly formed osteons overlapping, or embedded within, the reversal line of older osteons until only fragments of the latter are seen.

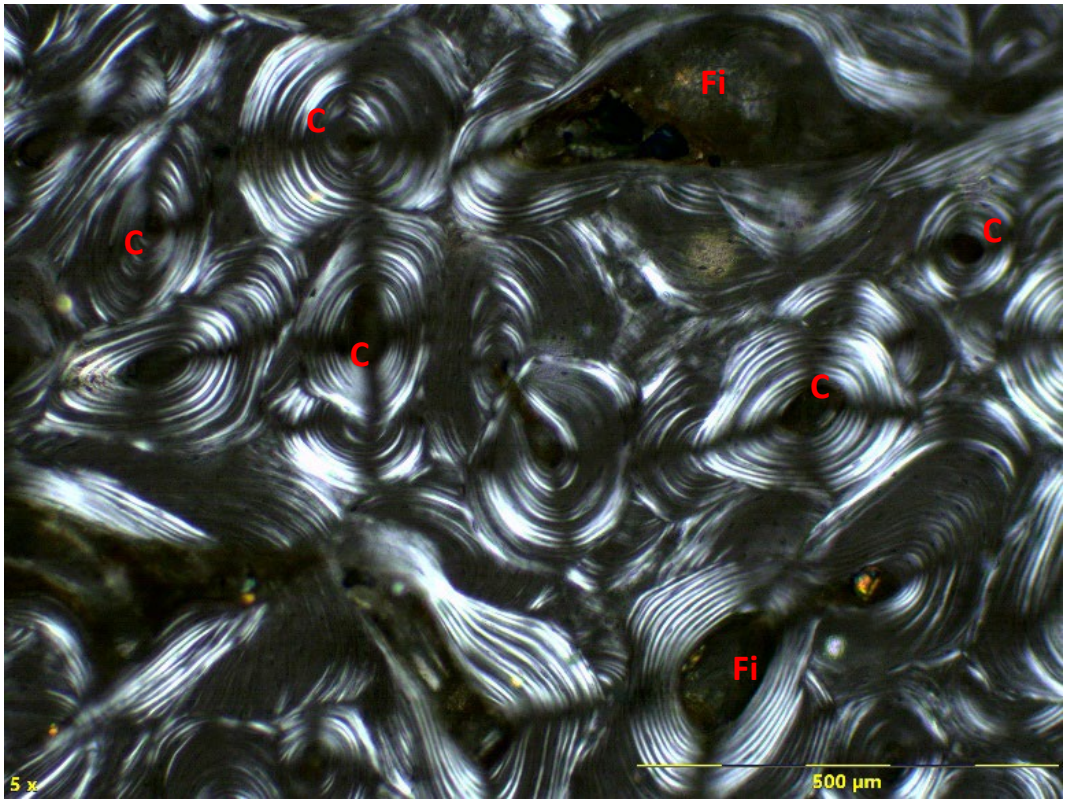



*Figure 4-3 Femur midshaft cross-section.*
*Showing osteons of various sizes and at different stages of in-filling. C = completed osteon, Fi = osteon still infilling with concentric lamellae to fill the resorption space.*

## 4.3 Definitions of Histomorphological Features of Remodelled Bone

### Primary Osteons

Unlike secondary osteons, primary osteons are not involved in the remodelling of bone, therefore it is important to be able to visually differentiate them. During bone development, primary osteons are formed around vascular canals or spaces (Hennig et al., 2015). They are microscopically distinguished from secondary osteons by the lack of a cement line, they have a smaller central vascular canal, and are only surrounded by very few concentric lamellae, or none at all (non-Haversian canal) (Pfeiffer, 2006). The circumferential lamellae usually appear to flow around the vascular canal rather than encircle it (Ericksen, 1991). Under polarized light the birefringence of the collagen in the many lamellae of the secondary osteon easily distinguishes them from primary osteons (Skedros et al., 2009). As primary osteons do not remodel, they will not intersect other osteons as secondary osteons do (Skedros et al., 2009).

### Secondary Osteon Variants

Secondary osteons develop from remodelling of pre-existing bone or a primary osteon (Chang & Liu, 2022). Formation of osteons by the BMU's during the remodelling process is stimulated by internal factors (bone mineral homeostasis and hormones (Chen et al., 2015), metabolic bone disease (Price et al., 1994)) and external factors (biomechanical stresses) (Miszkiewicz, 2016), as well as natural variation (Cooke et al., 2021). Under these influences different osteon morphologies develop in bone cortex that are important features in age estimation methods. These are described below.

### Type I Osteon

This is the most common type of secondary osteon that is characterised by concentrically deposited lamellae surrounding a central Haversian canal (Figure 4-4). Each osteon is contained within a cement line that separates it from the surrounding interstitial tissue and adjacent osteons (Frost, 1963). The cement line is a product of the change from BMU resorption to formation. The number of lamellae can vary but is typically no more than twenty (Sommerfeldt & Rubin, 2001). The shape of the osteon can vary between circular and elliptical and this can be age dependent (Hennig et al., 2015), and the Haversian canal may be off-centered (Hillier & Bell, 2007). A completed osteon may have no evidence left of a resorption cavity, and the lamellae will all be mineralised. An active osteon will have a resorption cavity still infilling with new lamellae of freshly deposited osteoid that gradually mineralizes (Sommerfeldt & Rubin, 2001).

### Type II Osteon

These are osteons that have formed by radial erosion within the cement line of an existing Type I osteon (Robling & Stout, 1999), which is why it is also called an embedded osteon (Raguin & Streeter, 2018). There are suggestions the BMU's of Type II variants are attracted to areas of cortex previously remodelled by older osteons and thus seek to resorb their well-mineralised matrix (Maggiano et al., 2016; Richman et al., 1979). The Type II osteon is surrounded by its own cement line which distinguishes its lamellae from those of the pre-existing osteon that it is embedded within (Raguin & Streeter, 2018). The lamellae of the two osteons will never be perfectly aligned, which further identifies them as two separate osteons. When observed on a transverse cross-section (Figure 4-4) the structure will be counted as one intact osteon (Type II) and one fragmentary osteon (Type I). Type II osteons are more commonly found in older adults compared to younger adults (Maggiano et al., 2016), possibly

as a means to maintain mineral homeostasis as an individual ages (Ericksen, 1991; Yoshino et al., 1994). However, Richman et al. (1979) found no association of Type II osteons with age and suggested a high protein diet in their American and Inuit sample populations was the main contributing factor.

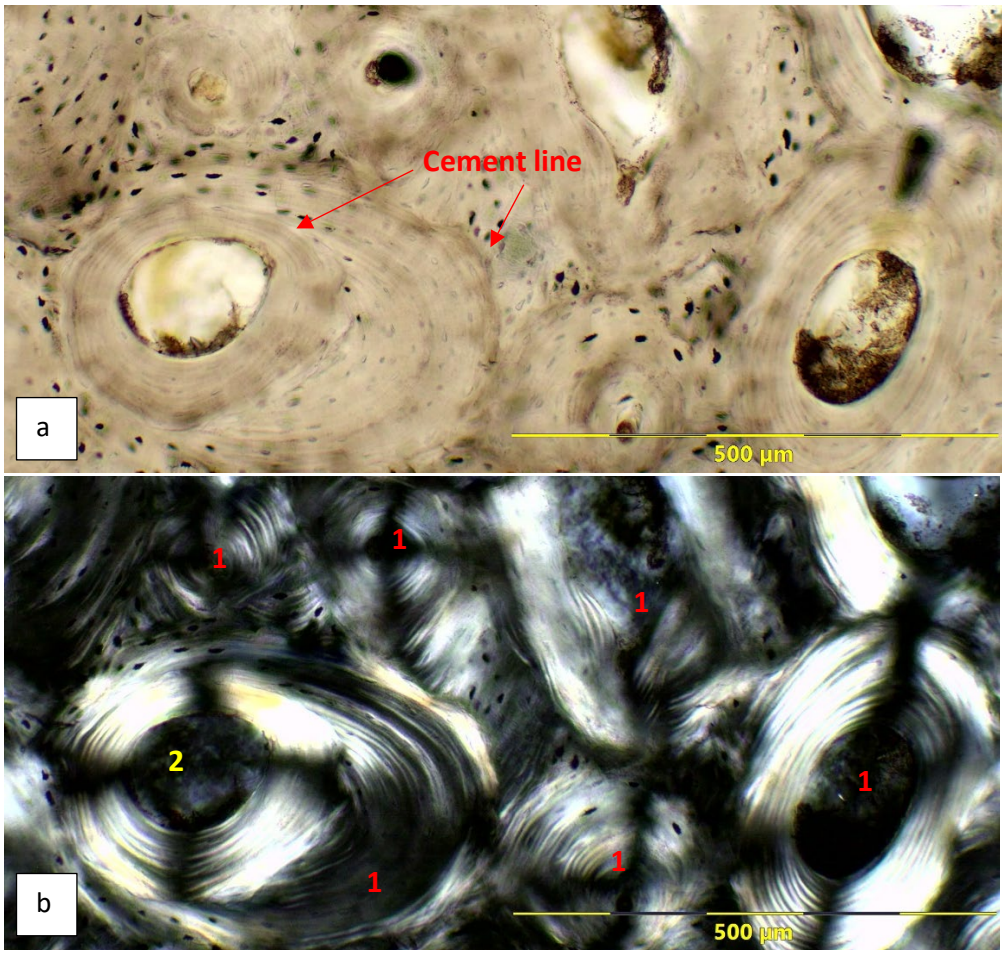



*Figure 4-4 Examples of Type I and Type II osteons.*
*Midshaft femur. Image a = transmitted light. Image b = polarised light. 1 = Type I osteon; 2 = embedded (Type II) osteon, note the cement line and lamellae of the embedded osteon are distinct from those of the pre-existing (Type I) osteon beneath it (now a fragment).*

## Double Zonal Osteon

Within the lamellae of some osteons appear a hypercalcified ring, where the bone matrix has been temporarily arrested; and mineralisation has suddenly increased during the formation stage of a BMU (Raguin & Streeter, 2018) (Figure 4-5). There is evidence that the ring may be visible due to a change in the orientation of the lamellae collagen fibres (Raguin & Drapeau, 2020). The hypercalcified rings are best viewed on microradiographs (Raguin & Streeter, 2018; Robling & Stout, 1999) but can also be viewed under polarized light (Raguin & Drapeau, 2020). Compared to Type I osteons, double zonal osteons tend to be significantly smaller and have a smaller Haversian canal (Raguin & Drapeau, 2020). The hypercalcified ring should be smooth, dense, and continuous, distinguishing it from the scalloped edge of the cement line surrounding Type I and II osteons (Raguin & Streeter, 2018). They are not thought to correlate with age or bone pathology as do Type I and drifting osteons (Raguin & Streeter, 2018; Yoshino et al., 1994).

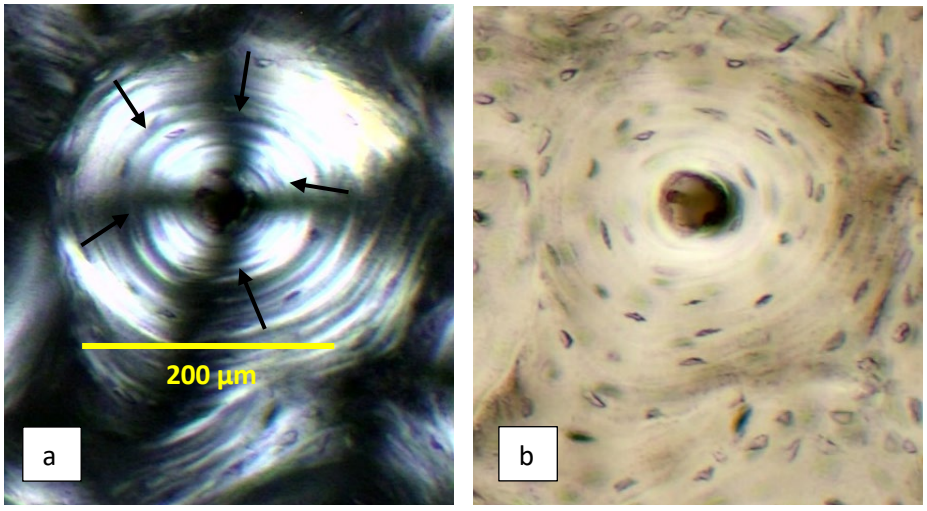



*Figure 4-5 Double zonal osteon.*
*Image a under polarized light the hypercalcified ring is visible and almost looks like an embedded osteon. Image b under transmitted light shows no cement line is visible which would have identified it as an embedded osteon instead of a double zonal osteon.*

### Drifting Osteon

Osteons that move in both a transverse plane and a longitudinal plane, as their lamellae are laid down over time, appear to be ‘waltzing’ (Frost, 1964) or ‘drifting’ (Epker & Frost, 1965) through the cortex (Figure 4-6). The successively deposited lamellae look like a tail trailing behind the Haversian canal, as the transverse angle of travel typically changes by up to 90 degrees, while the osteon is forming along its longitudinal axis (Robling & Stout, 1999). The hemicyclic drift movement is the result of resorption on one edge of an active osteon and opposed by formation on the opposite edge (Epker & Frost, 1965). Intermittently, the transverse travel may temporarily cease and the osteon takes on a Type I morphology before changing drift direction again (Robling & Stout, 1999). In a drifting BMU, resorption is not restricted to just the cutting cone, as is the case in a typical BMU of a Type I osteon, but instead resorption extends along its longitudinal wall (Schnitzler & Mesquita, 2013).

Under the microscope, the tail of a drifting osteon may be misinterpreted as overlapping osteon fragments. The lack of a cement line in the drifting osteon’s tail should distinguish it (Robling & Stout, 1999), and their Haversian canals tend to be more eccentrically shaped (Streeter, 2010). Drift has been suggested to result from localized stresses, such as repair of a microcrack (Cooper et al., 2011) or to maintain mineral homeostasis, particularly as subadults are experiencing rapid growth (Coutelier, 1976; Streeter, 2010).

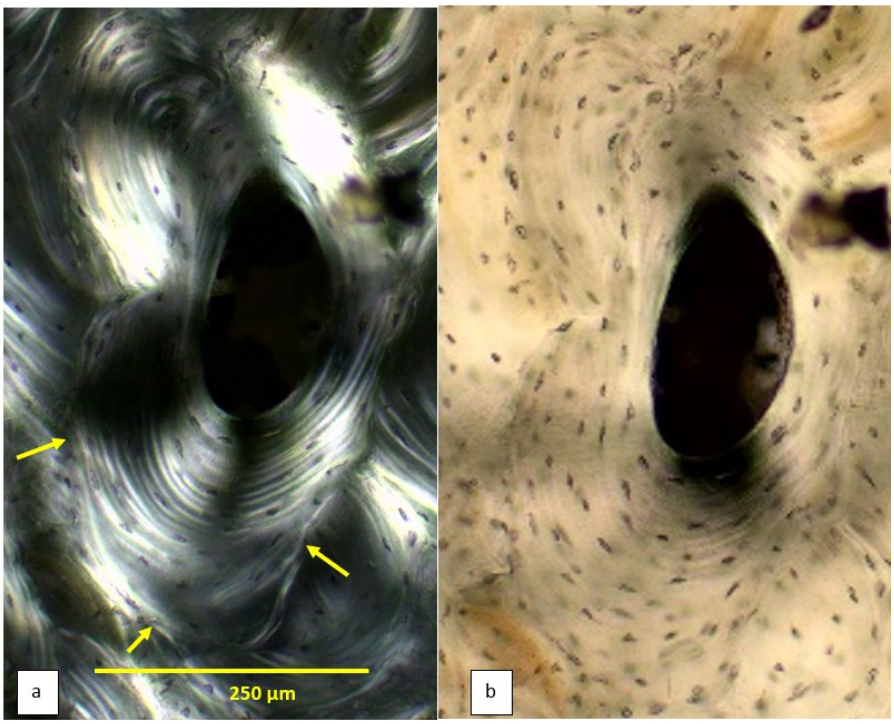



*Figure 4-6 Example of a drifting osteon.*
*Image a polarised light, arrows point to lamellae of the tail that could be confused with the fragments of a pre-existing osteon overlapped by a new osteon. Image b indicates no cement line is visible under transmitted light, indicating it is one intact osteon. This osteon has ceased drifting and has taken on Type I morphology.*

### Intact Osteon

An intact osteon is one that is surrounded by an uninterrupted cement line and has an intact Haversian canal, and has not been remodelled by newer formed osteons or resorption cavities (Ericksen & Stix, 1991) (Figure 4-7). These osteons may be mature, whereby they have completed filling, or they may still be in the process of filling in the space around the vascular canal created by the resorption cavity.

### Fragmentary Osteon

This is a pre-existing osteon that has been remodelled by a resorptive cavity or the lamellae of a newly formed osteon (Crowder et al., 2022) (Figure 4-7). In most cases its outer perimeter (cement line) will be breached during remodelling unless the new resorption cavity or

secondary osteon is embedded wholly within the boundaries of its cement line. If its Haversian canal has been completely breached, then partial lamellae may be all that remains. The osteocytes embedded within lacunae in its lamellae will distinguish it from the surrounding interstitial lamellar matrix (Crowder et al., 2022).

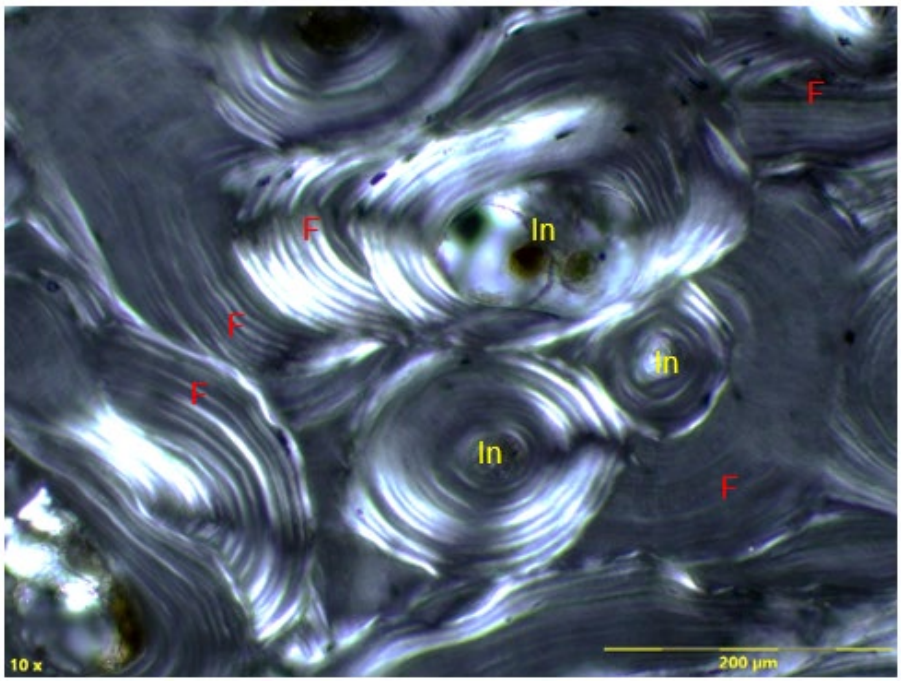



*Figure 4-7 Rib cross-section, In = intact osteon; F = fragmentary osteon.*

## Resorption Cavity

This is also referred to as the cutting cone of a BMU. It is an area of bone cortex resorbed by osteoclasts during the initial phase of the remodelling process (Figure 4-8). The border of a resorption cavity is identified as having a scalloped edge that is a result of the pits (Howship's lacuna) formed by individual osteoclasts during a burst of remodelling activity as they are producing the cutting cone (Delaisse et al., 2021). Resorption cavities are eventually infilled by new bone deposited by osteoblasts to become completed secondary osteons (Ericksen, 1991).

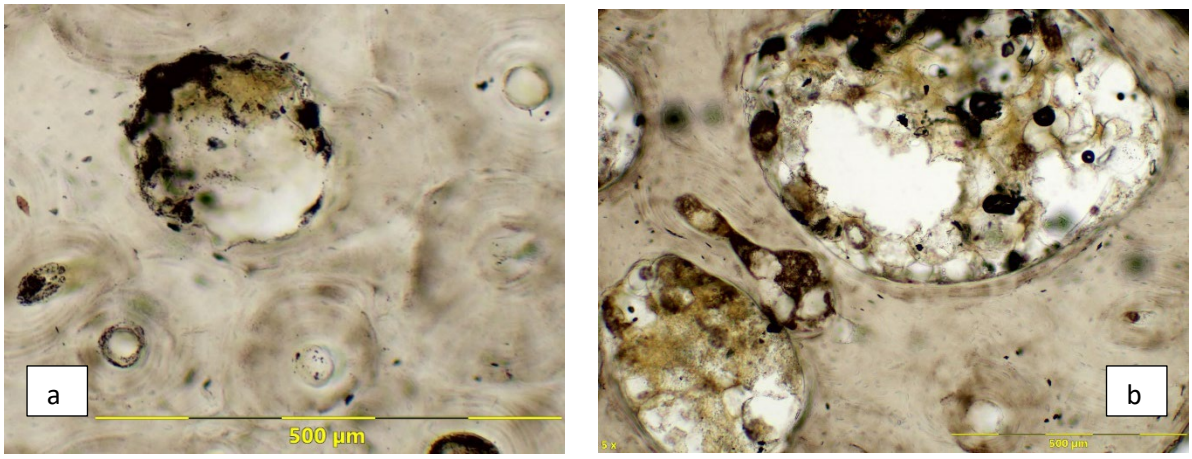



*Figure 4-8 Examples of resorption cavities.*
*Image a midshaft humerus, resorption cavity breaching pre-existing osteons; Image b resorption cavities of varying size.*

## Super Osteon

The term ‘super osteon’ was coined by Bell et al. (2001) with reference to “the merging of spatially clustered remodelling osteons resulting in the formation of deleteriously large cavities” (Bell et al., 2001: 378) (Figure 4-9). Bell and colleagues determined that when the Haversian canals of adjacent osteons merge into one giant canal with a diameter >385 µm (in the femur) it was classified as a super osteon (Bell et al., 2001). Gocha and Agnew (2016) caution that canals associated with age-related intracortical bone loss could be as large, or larger, in diameter.

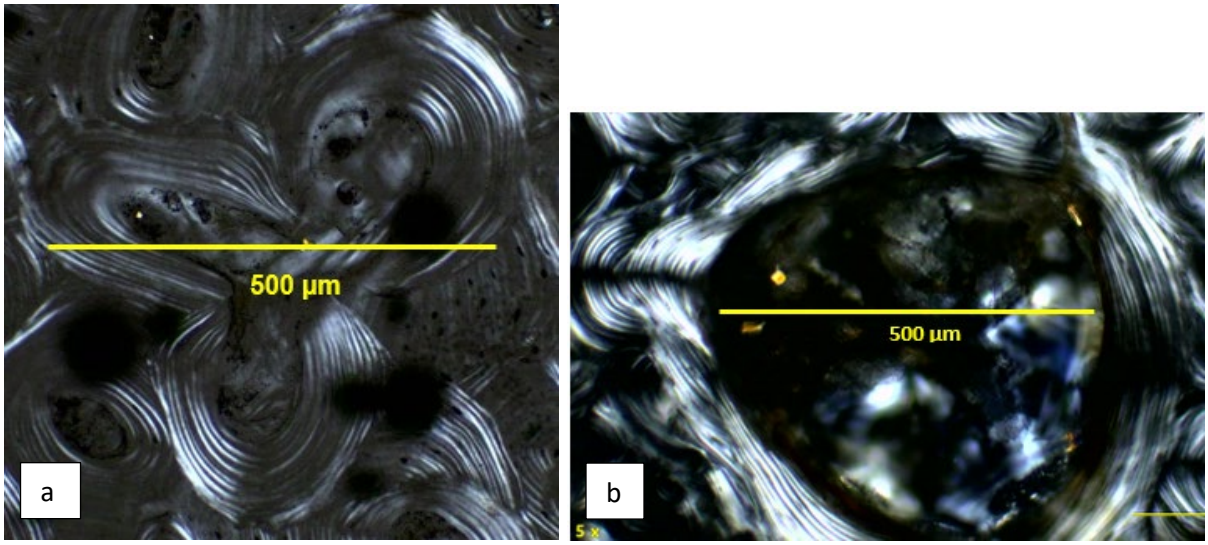



*Figure 4-9 Cluster of osteons merging into one giant osteon ('super osteon').*
*Image a and b from midshaft femur*

## BMU Branching Event

Stout et al. (1999) first described 'dumb bell' shaped osteons as being the result of a cross-section dissecting the point at which a Haversian canal is morphing (branching) into two separate canals. Maggiano et al. (2016) classified two types of branching: dichotomous branches (where two new branches form from one) tend to be very similar in size and shape, whereas bifurcating branches are of two different sizes in a cross-section (a smaller branch shoots off the larger parent branch). In a transverse section, two or more branching osteons may intercept to merge their central Haversian canals or share a cement line, and these are counted as one intact osteon (Figure 4-10) and not as individual osteons or fragments (Crowder et al., 2022).

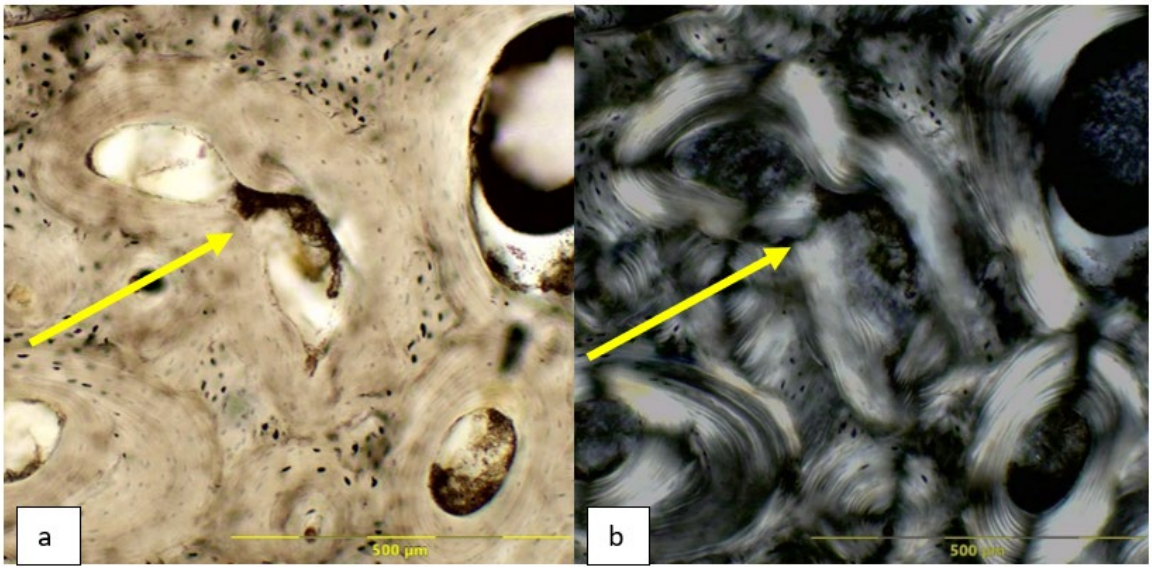



*Figure 4-10 Example of a branching event.*
*Midshaft femur. Image a = transmitted light. Image b = polarised light. Arrow points to where two parallel branches of the Haversian network have intersected so that their canals are merging into one.*

### Volkmann's Canal

These are new vascular channels that travel on an irregular transverse or sometimes oblique path through the cortex to perforate the longitudinally orientated Haversian canals in a long bone (Jaffe, 1929) (Figure 4-11). They interconnect Haversian canals to one another, and some Volkmann's canals connect to the periosteum on the bone surface and to the marrow cavity to aid the transmission of nutrients and blood through the bone cortex. Their diameter tends to be no more than 20 µm (Hazenberg et al., 2006), and they are more commonly found in young cortical bone (Maggiano et al., 2016). They can be distinguished from branching events of a single secondary osteon as they are not surrounded by concentric lamellae (Jaffe, 1929). For this reason, adjacent osteons joined by a Volkmann's canal are counted as two individual osteons.

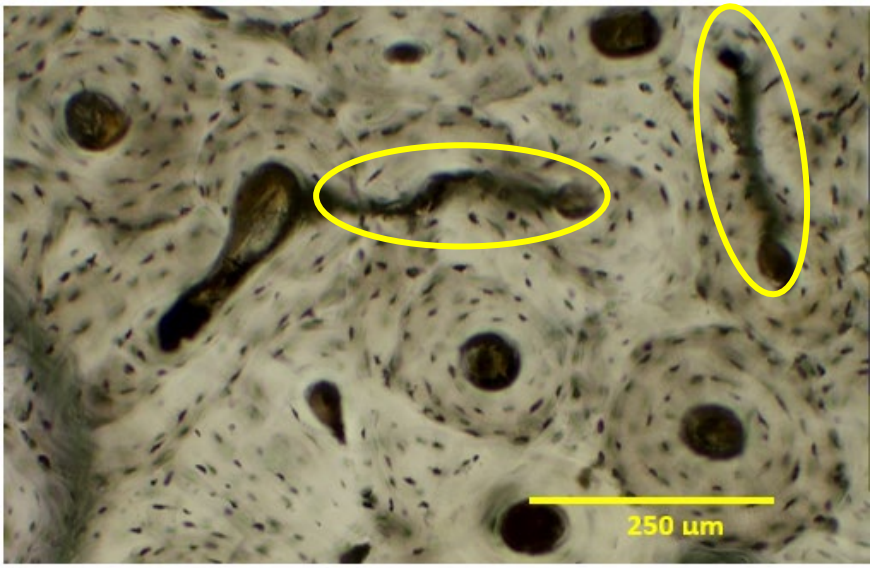



*Figure 4-11 Example of Volkmann's canals.*
*Midshaft femur. Volkmann's canals (encircled) inter-connecting the Haversian canals of adjacent osteons. Note that the Volkmann canals are not surrounded by their own concentric lamellae.*

## 4.4 Bone Remodelling and Age

Age progression has several quantitative effects on bone microarchitecture (Figure 4-12**)**. In a normal, healthy individuals, osteon population density increases, osteon size (area and diameter) decreases, osteon circularity increases (Britz et al., 2009), the size and population density of Haversian canals increases, lacuna density decreases (Katsimbri, 2017) and non-Haversian canal population density decreases (Karydi et al., 2022). Histomorphometric features are investigated to capture the range of individual and population variability (Gocha et al., 2019; Pfeiffer et al., 2006; Trammell & Kroman, 2013).

Histomorphometric features progress in stages that approximately align with the advancement of age. Remodelling rates differ between younger and older adults. For example, under the microscope, the structure of young adult cortical bone has large areas of unremodelled (primary) bone and non-Haversian canals (Keough et al., 2009). The shape of osteons in

young adults tends to be more irregular, appearing to travel, or drift, transversely as well as longitudinally in long bones and are therefore larger than the more circular osteons in older adults (Currey, 1964). Remodelling slows down in older adults and there is increased bone resorption compared to bone formation (Miszkiewicz et al., 2022a). Filogamo (1946) reported that older adults have Haversian systems of shorter length that are less likely to branch. A more recent study that used 3D microCT scanning suggested that increased porosity in females 60 - 80 years of age caused them to have significantly shorter canals than males of the same age (Cooper et al., 2006). Young adult bone is more metabolically active so they also have a higher variability in Haversian canal size than in older adults whereas remodelling slows down in older adults (Pfeiffer, 2006).

Age estimation methods show that older adults will usually experience a decrease in the size of their osteons and an increase in their population density (Britz et al., 2009; Cho et al., 2002; Currey, 1964; Frost, 1987b; Stout & Paine, 1992). This is suggested to be the effect of the medullary cavity expanding with advancing age and reducing the relative area of cortical bone as bone resorption (trabecularisation) becomes more pronounced in the endosteal region compared to the periosteal region (Chang & Liu, 2022; Cooper et al., 2006). The endosteal region usually contains osteons that are larger and of lower population density compared to the periosteal region, so a loss of endosteal bone may affect osteon counts and area measurements (Chang & Liu, 2022). The decrease in osteon area as an individual advances in age usually leads to a higher OPD due to a greater number of smaller, intact osteons per unit area able to be counted (Britz et al., 2009) and less larger (and eccentrically shaped) osteons able to be included when the majority of their area lies outside of the field of view of each region of interest examined under the microscope (Goliath et al., 2016).

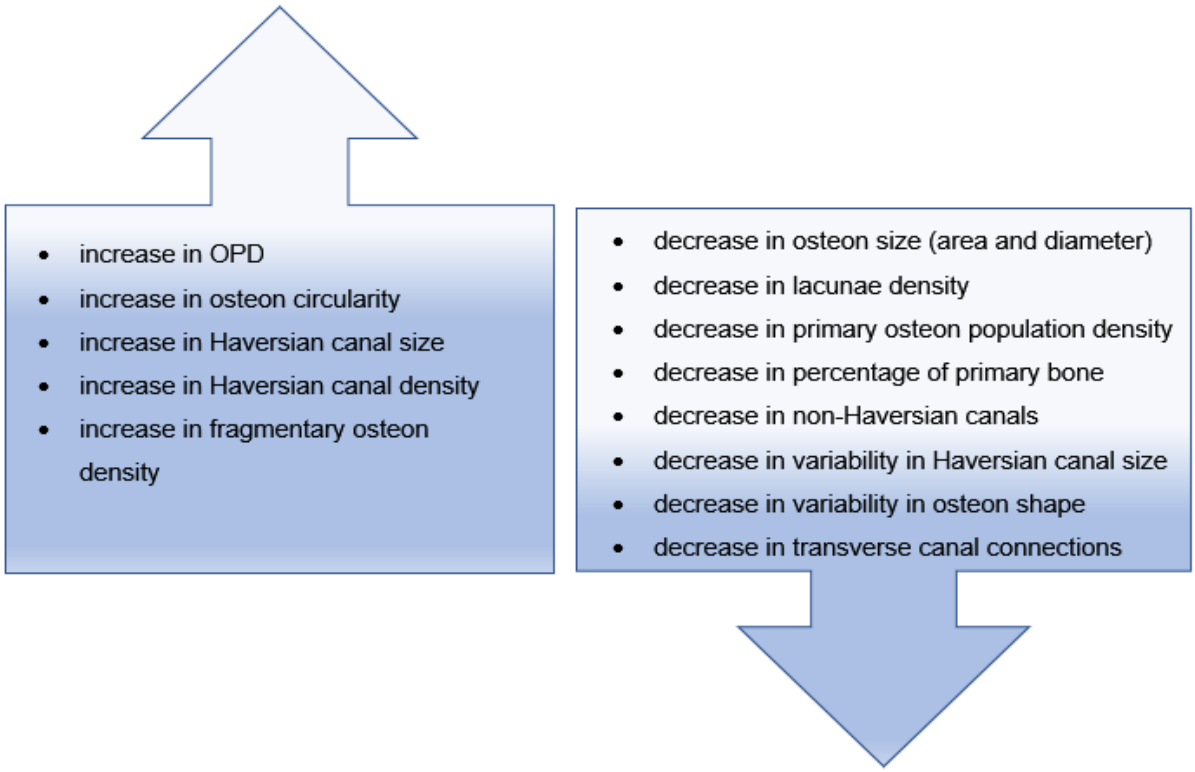



*Figure 4-12 Correlation trends with advancing age.*

## 4.5 The Impact of OPD Asymptote

As an individual advances in age, a greater proportion of bone undergoes remodelling leading to multiple generations of osteons, both complete and fragmentary. Older adults have so much bone turnover that often no un-remodelled primary bone is visible. Asymptote is reached whereby newer osteons remove most traces of older osteons, at which point the OPD value will remain constant even as bone continues to be remodelled (Frost, 1987b). There is currently conflicting accounts of the degree to which asymptote affects the ability to effectively use OPD or percentage of remodelled bone to estimate the age of elderly adults. This has led to a general consensus that histological age estimation methods are less effective for individuals over 60 years of age (Cho et al., 2002). The fact that there is still little evidence for

the age at which asymptote occurs in different populations and different bones means, for the short term, it will remain a limitation of histological age estimation.

The OPD value at which asymptote is reached varies by age, between skeletal elements, and between different regions of a bone (anterior, posterior, lateral, medial, or cortical third (periosteal, middle, or endosteal cortex) (Stout & Crowder, 2012). Due to the greater cortical area in the femur, it is more likely to be saturated with fragmentary osteons at a later age, and thus asymptote is delayed in comparison to the faster remodelling of the thin cortex of the rib (Dominguez & Mavroudas, 2019). Asymptote in the rib tends to be reported to occur between the fifth and sixth decades of life (Crowder, 2005; Stout & Paine, 1994). In comparison, Gocha and Agnew (2014) observed that in the lateral and anterolateral regions of the midshaft femur, OPD asymptote of 55/mm² will not occur until approximately 80 years of age in some adults. Karydi et al. (2022) recorded that even individuals of 95 years of age continued to have increasing OPD counts in the femur, tibia, and fibula. Frost (1987b) calculated that asymptote is reached when an OPD count in the femoral midshaft reaches 50/mm², although they did not state at which age this occurs.

### 4.6 Intraskeletal Variation

When age is controlled for, evidence argues for more variability of osteon size (osteonal area in mm²) in cortical bone exposed to greater localized biomechanical forces (Crowder & Rosella, 2007). Targeted remodelling events will be experienced as bones respond to microdamage from trauma (Andronowski & Cole, 2021) and bone mass will vary between bones, depending on the amount of mechanical loading endured (Peck & Stout, 2007). Goldman et al. (2014) observed that slender bones are prone to having fewer and smaller osteons than did more robust bones.

Smaller osteons are observed in the ribs (Pfeiffer, 1998b) which are under constant low-strain mechanical loading over the course of respiration (Skedros et al., 2013). Ribs are more sensitive to hormonal changes and experience more metabolic bone loss than other long bones such as the femur and humerus (Eleazer & Jankauskas, 2016). This is why it is suggested that the femur produces stronger correlations with age compared to the rib (Khan et al., 2017). Britz et al. (2009) used body weight as a proxy of mechanical loading to confirm that osteon area and diameter had a negative relationship with abnormal weight loading on the femur.

Dietary isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) have a profound impact on remodelling rates between elements. Using stable isotope analysis, Fahy et al. (2017) confirmed that skeletal elements with the fastest bone turnover (ribs, humerii, and metacarpals) had significantly higher levels of nitrogen compared to the other seven bones they tested (femur, pelvis, clavicle, radius, thoracic vertebrae, and the occipital bone). Olsen et al. (2014) only found significant intraskeletal isotopic variation in bones with interference from certain metabolic diseases or fracture and only when sampling close to a lesion and non-pathological individuals had very little intraskeletal variability in isotopic ratios.

### 4.7 Variation by Bone Region

Sampling sites located within a cross-section of single bone may affect age estimation as variable rates of remodelling are reported between the anatomical axes (anterior, posterior, medial, and lateral) (Chan et al., 2007). Mechanical axes (affected by maximum and minimum moments) have been shown to exhibit less remodelling variability than the anatomical axes, and the endosteal cortex tends to show a greater percentage of remodelling compared to subperiosteal cortex (Pfeiffer et al., 1995). Other studies concluded that regional variability is not significant, and therefore the sampling site selected may not affect correlation with age. For example, using a modern Australian femur sample, Maggio and Franklin (2021)

demonstrated that age-related decreases in size and shape of osteons and Haversian canals were similar in both the anterior and posterior cortex of the femur. In another study, Gocha and Agnew (2016) demonstrated that OPD was not uniform throughout the femoral midshaft, and instead tended to be greatest in the lateral and anterolateral regions of the periosteal third. The authors have shown this could be due to the phenomena of modelling drift, where higher remodelling rates in this area of the midshaft femur leads to greater areas of young primary tissue compared to the medial and posterior regions which are under greater mechanical strain (Gocha & Agnew, 2014). Although there are no standardised sampling criteria to account for variability of remodelling activity in various fields, a minimum analysis of 50mm² of cross-sectional bone per individual, suggested by Frost (1969) has often been adhered to. However, minimally invasive sampling requirements often limit sampling to a small wedge of bone (~20mm²).

Using global information system (GIS) mapping, Gocha and Agnew (2016) found that OPD varied between the eight quadrants of the femoral midshaft (anterior (A), anteromedial (AM), medial (M), posteromedial (PM), posterior (P), posterolateral (PL), lateral (L), and anterolateral (AL)). They found that osteon density was consistently higher in the lateral and anterolateral regions for adults >45 years of age. OPD counts were highest in the periosteal third, and lowest in the endosteal third when evaluating the P, PL, L, and AL regions, however they found that OPD was highest in the mid cortex in the A and M regions. Gocha and Agnew (2016) surmised that appositional cortical drift in the modelling stage of long bone diaphysis development as a subadult meant that the AL cortex had older primary bone which has had more time to be remodelled extensively and have a higher OPD compared to other areas of the femoral cortex of an adult.

Gocha and Agnew (2016) calculated that OPD for an entire cross section was different to an OPD from just one of the four regions. No entire cross section had a count higher than 31/mm². However, when assessing density in a localised area an OPD of > 50/mm² was observed, but

not until adults were of at least 80 years of age, with a peak OPD of 55/mm² recorded in the lateral and anterolateral regions (Gocha & Agnew, 2016). This led them to suggest that if an entire cross-section of the femur can be used to assess OPD, then age estimation would be useful up to the 10th decade of life. However, caution must be used if only a portion of the cortical bone is available for sampling as Gocha and Agnew (2016) have proven the difference within the femoral midshaft of one individual can be >20/mm².

Ribs have small cross-sections and a thin cortex so it is normal to sample across the mid-section (Stout et al., 1994; Stout & Paine, 1992). OPD counts are not significantly different between the 6th mid-thoracic rib and ribs 3 – 7, suggesting that any of these vertebral-sternal ribs can be used for age estimation (Crowder & Rosella, 2007).

## 4.8 Biological Sex Differences

Differences in remodelling rates between the sexes is often reported, but the results between studies varies. Britz et al. (2009) recorded significantly smaller osteons (area and diameter) in the midshaft femur when body mass index increased in females compared to males. However, they found no significant sex differences in measures of osteon circularity. Menopause is a likely factor as a decrease in oestrogen levels sees females experiencing more rapid bone loss and slower bone formation compared to males, causing a steady decline in bone density (Clarke, 2008). Bone loss in the endosteal layer due to cortical thinning and trabecularisation is more pronounced in older females compared to older males (Britz et al., 2009; Rehman et al., 1994). Cooper et al. (2006) found no resorption cavities in females over 81 years of age due to the extensive trabecularisation of the cortical bone. Differences in activity levels, such as a sexual division in labour, has been the suggested reason for larger osteons and smaller Haversian canals in prehistoric Native American females (Burr, 2002) and medieval Nubian females (Mulhern & Van Gerven, 1997). Other studies have found no significant correlation between sex and osteon area (Pfeiffer, 1998b; Pfeiffer et al., 2006).

## 4.9 Interpopulation Variation

Current histological age estimation methods are mainly based on reference samples sourced from European and North American populations, leaving a clear gap in the ability to fully understand variability in human bone microstructure between genetically distinct populations (Cho et al., 2002; Pratte & Pfeiffer, 1999). Studies have observed significant interpopulation differences in osteon size and density, and the relative cortical area of the rib between individuals of European American and African American ancestry (Cho et al., 2006). European Americans compared to African Americans tend to have faster bone remodelling rates, lower bone mass, a larger number of pores contributing to increased cortical porosity (therefore higher OPD), and a larger mean osteon area (Bell et al., 1995; Cho et al., 2002; Weinstein & Bell, 1988). In comparison, 'black' Africans have a close affinity to European Americans in regard to bone turnover rates (Schnitzler, 1993). Pratte and Pfeiffer (1999) identified increased inaccuracy in histological age estimation methods that have been developed on a Midwestern North American reference sample of predominantly European ancestry when applied to South African individuals. How greatly genetic and environmental factors attribute to cortical bone remodelling rates is still so greatly underexplored in populations from the Asia-Pacific region.

## 4.10 Method of Counting and Measuring Osteons

The following two chapters investigate the correlation of age with OPD, On.Ar, and H,Ar, within two population samples. This section provides a summary and pictorial diagram of the sample preparation (Figure 4-13) and how these three histomorphometric features were measured and counted (Figures 4.14 – 4.16). More detail is given in the methods section in the following two chapters.

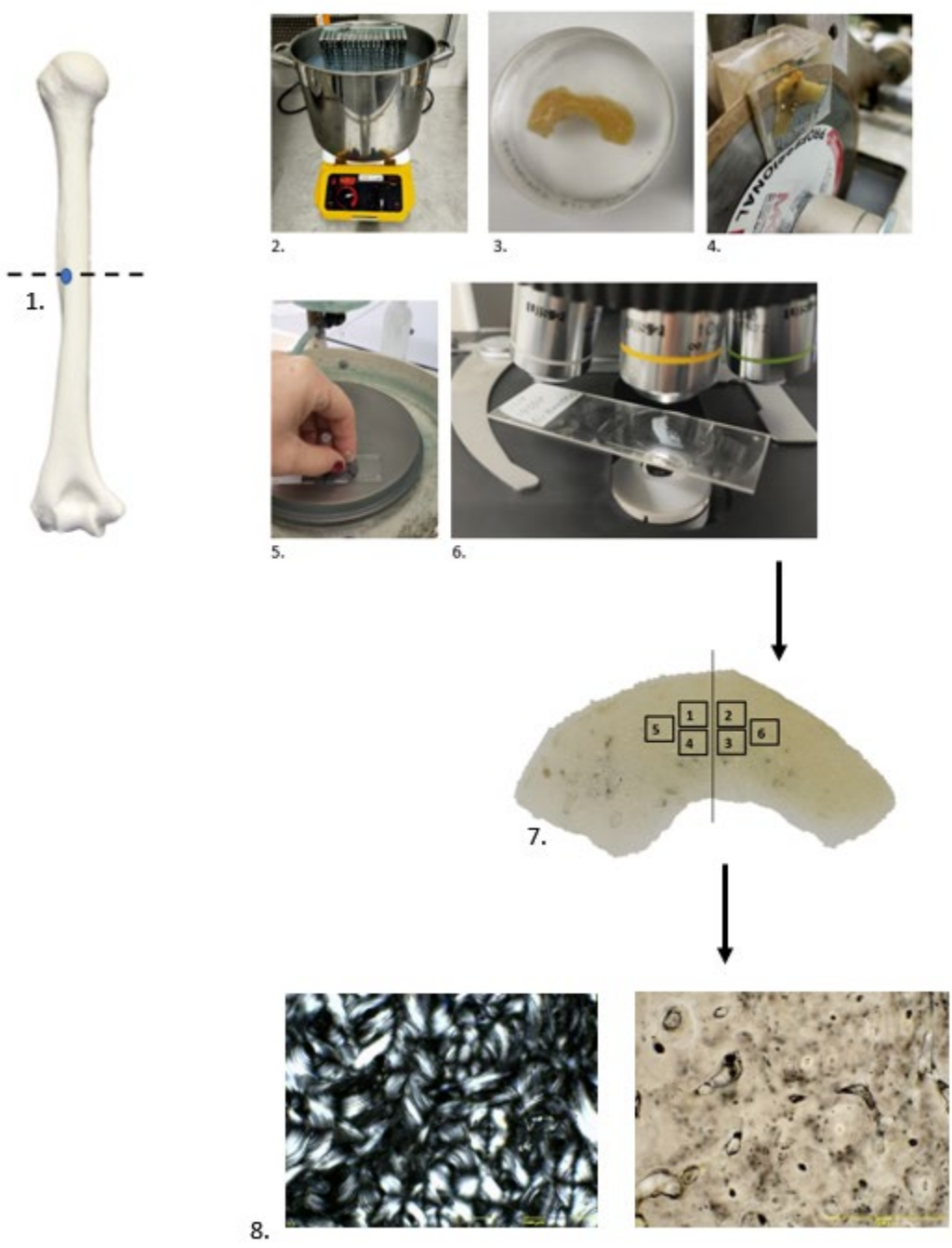



*Figure 4-13 Overview of sample preparation.*
*(1.) From the long bone midshaft a 1-2 cm² piece of bone was removed with a reciprocating bone saw and stored in a sampling jar preserved in 70% ethanol solution; (2.) to remove soft tissue, the bone sections were gently macerated for 12 hours in warm water with liquid laundry detergent containing enzymes; (3.) after dehydration via a graded alcohol series and xylene, each bone section was embedded in epoxy resin; (4.) a ~400 µm thick section was removed using a precision low-speed diamond-blade saw; (5.) thin sections were ground and hand polished to ~50 - 100 µm thickness, before undergoing cleaning and dehydration; (6.) then adhered to a microscope slide; (7.) images of six regions of interest were taken (8.) using polarised and transmitted light.*

Using a microscope mounted camera, six regions of interest (ROIs) were selected (Figure 4-13, step 7.) within the mid-cortical region. Each digital image was analysed using ImageJ Fuji® software to record and label each image with the drawing and multipoint counting tools. Features were identified using the definitions in this chapter. The polarised light image was used to label and count each intact and fragmentary osteon. The x/y co-ordinates of each label were recorded on an excel spreadsheet along with a count. Osteon population density (OPD) per image was calculated from the total number of intact and fragmentary osteons/mm$^2$. Osteons cut-off by the image border were not included in the count if it could not be ascertained that they were intact, unless they were clearly overlapped by another osteon, in which case they were counted as a fragmentary osteon (Figure 4-14).

Each intact osteon was traced around its perimeter (Figure 4-15) which was identified by its cement line, and the area was recorded in μm$^2$ in an excel spreadsheet. The area measurements were totalled, and the mean recorded. Under transmitted light (Figure 4-16) the Haversian canal perimeter was distinguishable from the lamellae of each osteon and was traced and the area recorded in the same way as for the intact osteons. Any canal that was intact (its perimeter uninterrupted) was included in the count.

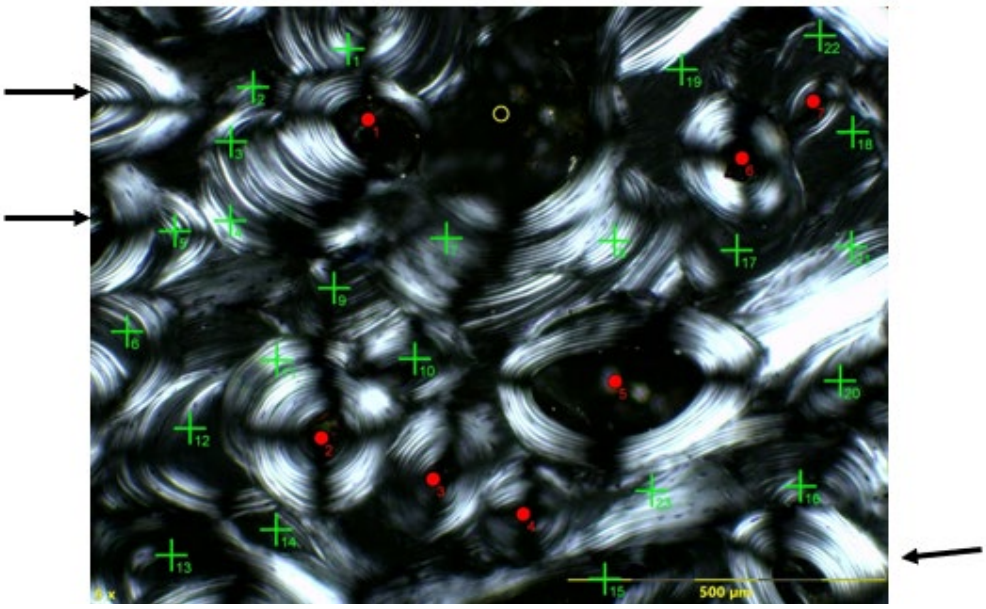



*Figure 4-14 Example of counted osteons and resorption cavities.*
*Femur -solid red dot = intact osteon; green cross = fragmentary osteon; yellow circle = resorption cavity; black arrow = osteons intersected by the border that are not included in the count.*

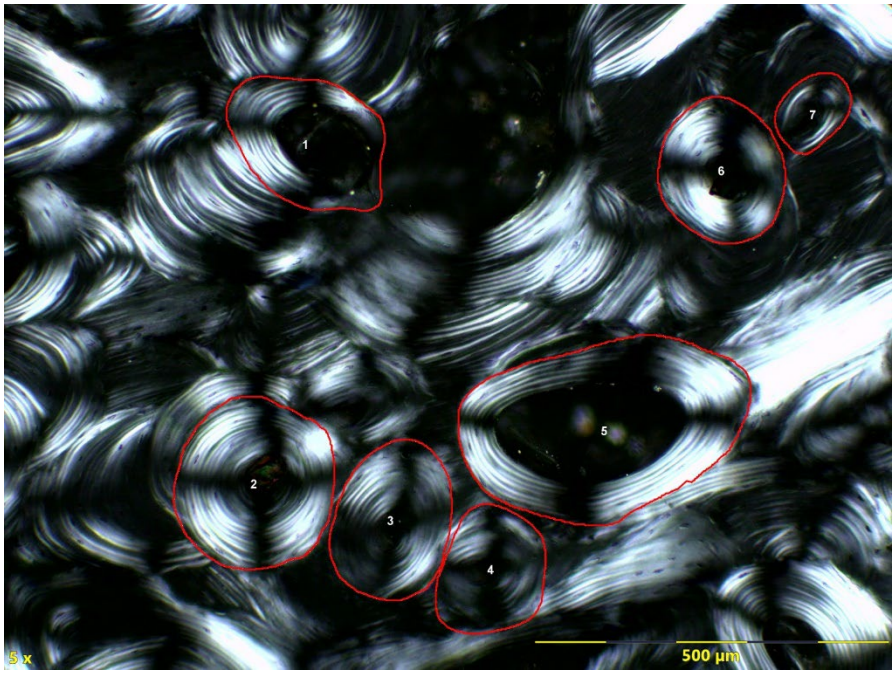



*Figure 4-15 Example of tracing osteon area.*
*Each intact osteon was traced around its perimeter (cement line) and the area recorded in µm².*

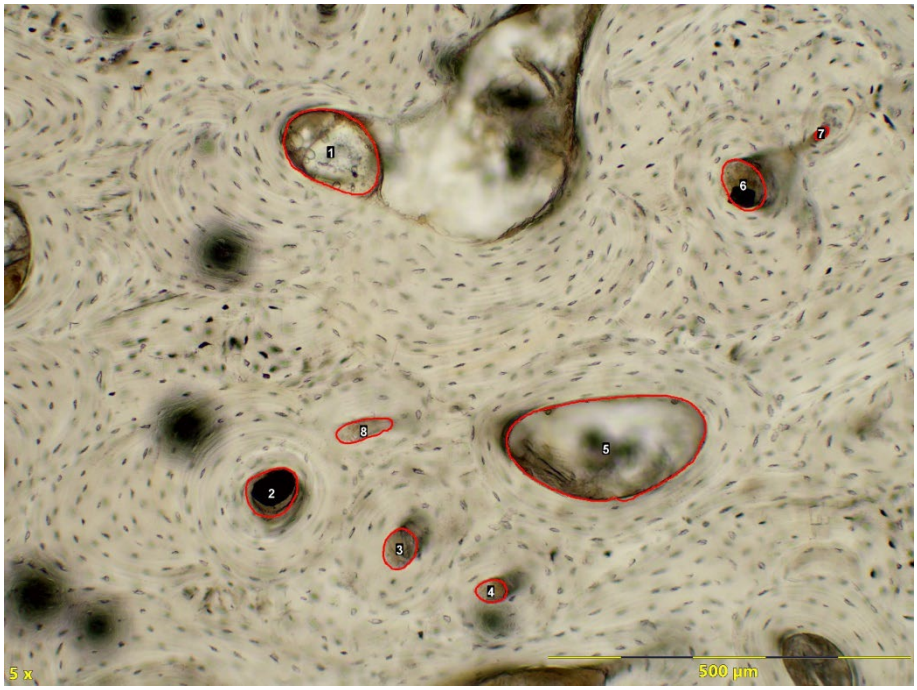



*Figure 4-16 Example of tracing Haversian canal area.*
*The perimeter of each complete Haversian canal was traced on a transmitted light image. (Note, black shadows are an artefact of sample processing)*

## Chapter Summary

This chapter introduced the different type of bone in humans, the bone multicellular units that coordinate remodelling, and the features of the basic structural units (secondary osteons) that can be used to investigate bone remodelling and its relationship with an individual's age. This chapter has discussed how remodelling is not homogenous between different population groups, age and sex groups, intraskeletally between bones, or even between regions of the same bone. Variations in remodelling are further influenced by pathology, genetics, mechanical loading, bone mass and robusticity. Each of these factors has the potential to interfere with the accuracy and precision of histological age estimation methods. Using the newly established James Cook University Human Bone Histology Collection, the following chapter focuses on evaluating intraskeletal differences in the size of Haversian canals and osteons and their population density, between the femur, humerus, and rib in elderly male Australians. These are the three histomorphometric features that are most frequently used in

age estimation methods and it is crucial to understand how their correlation with age may differ between bones.

# Chapter 5 Age-Dependent change and intraskeletal variability in secondary osteons of elderly Australians

The development and improvement of accurate adult age estimation using microscopic features of secondary osteons is reliant on understanding remodelling rates which can vary between different bones within an individual. In this chapter intraskeletal variability of three histomorphometric features, which are commonly used in age estimation methods, are examined using the femur, humerus, and rib midshaft region in a new Australian sample. This has not yet been evaluated in this population before. This chapter has been published, and a copy is provided in Appendix B.1. In this chapter the article has been reformatted into the style of the thesis.

Pedersen, L.T., Miszkiewicz, J., Cheah, L.C., Willis, A. & Domett, K.M. (2024) Age-dependent change and intraskeletal variability in secondary osteons of elderly Australians. Journal of Anatomy, 00, 1–15. Available from: https://doi.org/10.1111/joa.14010

## 5.1 Introduction

Forensic anthropologists and bioarchaeologists have been embracing quantitative bone histology to establish age at death by utilizing the changes in bone microstructure that are known to correlate to advancing age (Crowder & Dominguez, 2013; Khan et al., 2017). Basic structural units in bone, secondary osteons, are evidence of bone remodelling processes. These osteons are the key feature of most histological age estimation methods that typically use regression analyses to correlate independent histological variables, such as the tendency with advancing age to see an increase in the size of vascular pores and secondary osteon

population density, and a corresponding decrease in overall osteon size (Ahlqvist & Damsten, 1969; Ericksen, 1991; Kerley, 1965; Stout & Paine, 1992; Yoshino et al., 1994).

Remodelling does not occur uniformly throughout the skeleton so age estimation methods are developed on specific bones, most commonly the femur and the rib (Crowder & Dominguez, 2013; Gocha et al., 2019). Varying rates of remodelling are reported not only between different bones of an individual skeleton (Cole et al., 2022; Karydi et al., 2022), but also within different regions of a bone (Chan et al., 2007; Dominguez et al., 2020; Gocha & Agnew, 2016) depending on biomechanical loading regimes and metabolic activity. Increasing knowledge of intraskeletal histomorphometric variation is critical to improving accuracy in age at death estimation, particularly in older adults (Stout & Crowder, 2012).

Curated Australian human skeletal histological material accessible to researchers has been largely limited to the Melbourne Femur Research Collection (MFRC), which was established in 1991 at the University of Melbourne in Victoria (Thomas & Clement, 2011). Over the years, many national and international research articles have used this collection to document histomorphometric properties of the femur. However, what has been lacking is a skeletal reference collection that includes several different bones to evaluate intraskeletal variability in a modern Australian population. Documenting intraskeletal variability in bone remodelling within different populations is a critical step in understanding individual and regional trends, with implications for developing population-specific age estimation techniques. This will be the first study to evaluate sections from three different bones that have been collected intra-individually from an Australian sample.

The Australian histological bone sample used in this study was recently established at James Cook University in Townsville, Australia in 2021 (The James Cook University Human Skeletal Histology Collection (JCUHSHC)). This present study is a unique opportunity to assess age-progressive features of cortical bone remodelling by measuring secondary osteon population

density (OPD), and size of Haversian canals and intact secondary osteons in the femur, humerus, and rib of elderly Australians. It is hypothesized that localised biomechanical forces and metabolic activity would cause variation in remodelling. This study will contribute to a greater understanding of the range of histological variation that is yet to be fully explored in an Australian population.

Research on the femora of modern Australians has been relatively prolific, largely due to the large femoral sample held at the MFRC which is complemented by cadaveric samples from the Victorian Institute of Forensic Medicine (VIFM). Key findings for age-dependent histological changes in the femur show that there are similarities and contradictions between these studies. Britz et al. (2009) found that age statistically significantly correlated with increasing osteon circularity, and they observed decreasing osteon size in the femoral midshaft, with females having statistically significantly smaller osteons compared to males. Hennig et al. (2015) was able to confirm that osteon circularity increases with age in the anterior femoral midshaft of females, however they did not find an age associated decrease in osteon size. Two-dimensional microradiograph analysis did not identify significant correlations between cortical porosity in the midshaft femur and an individual's height or weight (Stein et al., 1999). Porosity refers to the vascular canal network in cortical bone which is linked to the remodelling process and bone loss (Cooper et al., 2016). Later 3D analysis showed that adults with lower body weight had significantly larger mean pore diameter (Cooper et al., 2007). Both studies found the most significant correlation to porosity to be age, and the differences in significance for weight may well be down to the very different methods of measuring porosity. It has also been observed that, compared to younger Australians, older individuals had a substantially thinner cortical zone in the upper femoral neck (Mayhew et al., 2005) and a tendency for new osteons to remodel within previously formed Haversian systems (Maggiano et al., 2016).

The Kerley (1965) age estimation equations derived from the midshaft anterior region continues to be popular with anthropologists (García-Donas et al., 2016) and many age-related histology studies have continued to focus just on sampling bone from just the anterior region; this has resulted in far less research of age-related remodelling in the other three anatomical regions (posterior, medial, and lateral). The posterior femur has received less attention than the anterior, and there are conflicting reports on which region is affected by the most variability in remodelling, particularly in relation to age. Most of these Australian studies focus on remodelling in the anterior midshaft region of the femur, while this present study will record changes in the posterior region, in addition to the rib and the humerus, which increases the current knowledge on inter-bone variability. This is key to better inform future archaeological and forensic efforts of age at death estimation from incompletely preserved and fragmentary human remains, where only some bones may be available for examination. Further, it is important to know which regions of bone will produce different OPD counts within an individual as this will impact age at death estimations (Dominguez et al., 2020). For example, statistically significant differences in OPD are observed between the anterior and posterior regions of transverse mid-shaft cross-sections of ribs (Dominguez et al., 2020). In contrast, only a non-significant difference in OPD was discovered between the anterior and posterior femoral midshaft (Maggio & Franklin, 2021), whereas the anterolateral region recorded statistically significantly higher OPD, presumably depending on the degree of bending stress and axial loading placed on each region (Gocha & Agnew, 2016).

The current lack of research into age-related changes to secondary osteons in bones other than the femur in the Australian population is concerning for the future development of population-specific of age at death estimation methods. Especially as it is well-documented that different bones are under varying degrees of metabolic stress and mechanical loading which tandemly effect cortical remodelling (Eleazer & Jankauskas, 2016). The femur usually experiences the greatest magnitude of biomechanical loading to support body weight in bipedal motion and it is subjected to compression and torsional forces with movement (Pfeiffer

et al., 2006; Ruff et al., 1993).These forces instigate microscopic damage to bone which is then targeted for repair by remodelling (Robling & Stout, 2003). In comparison, the humerus is under less weight-bearing loading but experiences axial loading forces from manipulative movements of the hands and arms (Sumner & Andriacchi, 1996); and, in turn, the ribs are under consistent, but low-level loading from breathing (Skedros et al., 2013). Ribs are more sensitive to hormonal changes and experience more metabolic bone loss than the femur and humerus, which can have an effect on remodelling rates (Eleazer & Jankauskas, 2016). These three skeletal elements also vary in cortical width and length. Human and non-human animal studies have determined that intracortical bone remodelling responds to bone size (Currey, 2003). Bone robusticity, an increase in diaphyseal width relative to length, is viewed as a naturally programmed response to compensate for increased porosity from metabolic activity (Eleazer & Jankauskas, 2016; Goldman et al., 2014). Short and wide bones will generally have an increased cortical region and more remodelling events compared to long and thin bones (Currey, 2003; Jepsen et al., 2015). More robust bones will have a higher OPD and larger osteons and Haversian canals than bones that are more gracile (Goldman et al., 2014). These studies highlight just how important it is to account for gross bone size when measuring microscopic geometric features within its cortex, yet it is not commonly accounted for (Miszkiewicz & Mahoney, 2019). This study will test for the effects of bone robusticity and aims to address the uncertainty of intraskeletal variation between the microstructure of the femur, humerus, and rib within a modern Australian sample, and which of the histological variables (OPD and osteon and Haversian canal size) have the highest correlation to age, specifically in males that have reached their $7^{th}$ or $8^{th}$ decade of life.

## 5.2 Materials and Methods

### Skeletal Sample

Bone samples examined in the present study derive from human cadavers received as donations in the Human Bequest Program at James Cook University (JCU), Townsville, Queensland, Australia. Ethics protocols were filed and approved (JCU #H8352 and University of Queensland #2022/HE000860) to obtain and analyse bone samples from individual donors that had been embalmed and used for medical training in the JCU College of Medicine and Dentistry. The sample in this study consisted of 17 adults, four of whom were female and 13 of whom were male (Table 5-1). Age at death ranged from 67 to 93 years (mean 82.4 years ± S.D. 6.04 years). The females ranged in age from 67 – 88 years (mean age 80.5 years ± S.D. 9.95 years), and male age range was 75 – 93 years (mean age 83 years ± S.D. 4.73 years). Body donors in this study were bequeathed between 2014 and 2019, from within a 400km radius of the regional city of Townsville in north Queensland. Donor information on record primarily includes sex, date of birth, age at death, and cause of death. No individuals of Aboriginal or Torres Strait Islander ancestry were included in this study.

Table 5-1 JCU sample size subdivided by sex.

| | *n* | Age range (years) | Mean age (years) | SD |
|---|---|---|---|---|
| **All adults** | 17 | 67-93 | 82.4 | 6.04 |
| **Females** | 4 | 67-88 | 80.5 | 9.95 |
| **Males** | 13 | 75-93 | 83.0 | 4.73 |

## Bone robusticity Measurements and Sectioning

Three sampling sites were chosen from each donor, incorporating a lower limb (femur), an upper limb (humerus), and the thorax (rib). The soft tissues surrounding the sites of interest were first dissected using a scalpel and knife, and the joints disarticulated at the knee and hip for the femur, and at the elbow and shoulder for the humerus. A standard tape measure was used to obtain the midshaft circumference, and maximum bone length from the distal-most point to the proximal-most point of the femur and humerus (cm) (Buikstra & Ubelaker, 1994). This enabled the robusticity index (RI = circumference/bone length x 100) (Ruff et al., 1993) to be calculated for the femur and humerus and used to account for the effect of bone size on histology data. A limitation of this study was that RI could not be estimated for the rib as circumference and length measurements for this bone could not be obtained at the time of sample collection.

For sampling to be minimally invasive, yet sufficient, only a 1-2 cm length wedge section of cortical bone was removed (for example, Maat et al., 2006; Mays et al., 2013) from (1) the anterior midshaft of the humerus, incorporating the lower aspect of the deltoid tuberosity, (2) the posterior midshaft of the femur, incorporating the linea aspera, and (3) a cross-section from the midshaft of the 7$^{th}$ rib (**Error! Reference source not found.**). Two individuals did not have ribs available for sampling, therefore a total of 49 bone sections were extracted from the 17 individuals. Sections were consistently taken from the right bone, with the exception of one individual (JCU 197-17) who only had bones on their left side available for sampling. Sections were removed using a hand-held electric rotary autopsy saw and then immediately fixed in a 70% ethanol solution and placed in cold storage (4°C) until further processing (Ries, 2003).

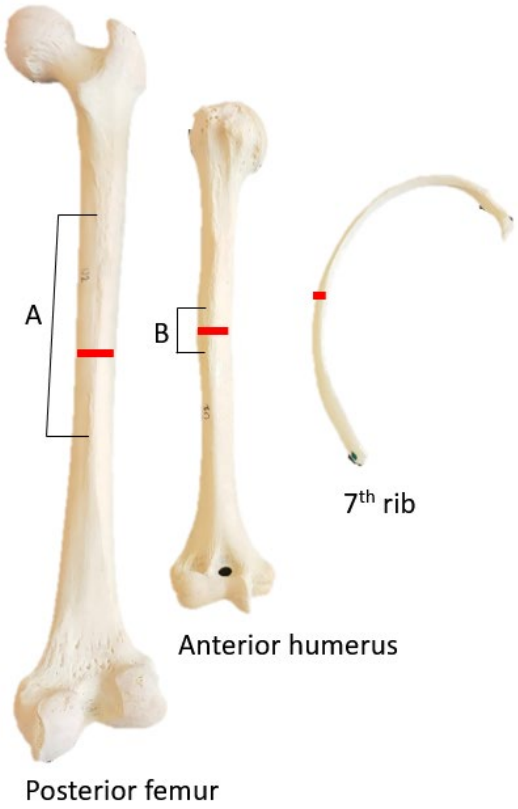



*Figure 5-1 Midshaft sample locations from right bone. The samples incorporated the linea aspera (a) on femora and the deltoid tuberosity of the humerus (b).*

## Histological Preparation

Standard histological methods were followed to prepare thin sections (Bancroft & Gamble, 2008). Using a portable hotplate (ThermoFisher Scientific Cimarec®), extracted bone sections were gently macerated in a warm water and enzymatic laundry detergent mix over low heat (55 ± 5° C) for approximately 12 hours, with the water and detergent mix replenished three times (Uhre et al., 2015). This is a quick, effective, and gentle process that does not damage the bone microstructure. Adhering soft tissue was then removed with the assistance of a plastic spatula and soft toothbrush. The undecalcified bone samples were placed in a fume cabinet to air dry for 24 hours before being dehydrated in a graded ethanol series and cleared with xylene (An et al., 2003). Each section was embedded in resin (Buehler EpoxiCure™2). Sections of ~400µm thickness were cut from the embedded blocks using a Buehler®

IsoMet™ Low Speed Precision Cutter with a diamond blade, to obtain a transverse cross-section (Bancroft & Gamble, 2008). Each section was ground using a Gemmasta™ GF4 faceting machine with a diamond lap, and then hand-polished to 100 ±25μm thickness using diamond paste and a polishing cloth, before being washed, dried, cleaned in an ultrasonic bath, dehydrated, and mounted onto a glass microscope slide and sealed with a cover slip (Bancroft & Gamble, 2008).

### Selection of Regions of Interest

Each slide was viewed under a high-powered microscope (Olympus BX43) and images of six regions of interest (ROIs) (Figure 5-2) were taken with a mounted microscope camera (Olympus EP50 with 0.5 camera adapter) (1) under transmitted light and (2) with a polarising filter. The technique used to select ROIs greatly varies between studies and lacks standardisation (Villa & Lynnerup, 2010b). Studies will vary in the placement of ROIs, size of fields of view, and circumferential sampling region (subperiosteal, midcortical, endosteal), depending on the research question. In this study, ROIs were selected within the midcortical region to avoid overlapping the insertion area for the linea aspera muscle, as this study is not evaluating a direct relationship between histomorphometric features and biomechanical loading. The endosteal region was avoided as in older adults there is a tendancy for it to have significantly higher cortical porosity than the subperiosteal and midcortical regions (Andreasen et al., 2020). In this study, ROIs were selected within the mid-cortical region. For the femur and humerus, the positions of ROI 1 and 2 were selected to be slightly offset either side of an arbitrary midline of the peri-curve (Figure 5-2), ROIs 3 and 4 were parallel to ROIs 1 and 2 but positioned toward the endosteum), ROI 5 was adjacent to ROIs 1 and 4, and ROI 6 was adjacent to ROIs 2 and 3. For the ribs, ROI 1 was selected on the cutaneous side toward the costal groove (if identifiable), then moving in an anti-clockwise direction ROI 2 was positioned in the mid-section, ROIs 3 and 4 opposite each other on the superior edge, ROI 5 opposite

ROI 2, and ROI 6 opposite ROI 1. The position of each ROI was consistent to ensure replicability and to prevent osteons from one ROI being included in an adjacent ROI. This ensured that the same osteons would not be counted twice. The humerus from several individuals had a narrow cortical width so the six ROI images had to be positioned side by side, mediolaterally, instead of the window pattern in Figure 5-2.

A x20 objective was used for the ribs, and a x10 objective for the humerus and femur (with 10 x oculars). With the camera adapter this produced a rectangular field of view (FOV) of 1.182 $mm^2$ for each ROI of a femur and humerus, and 0.285 $mm^2$ for each ROI of the rib. This gave a total area of 7.09 $mm^2$ for each femur and humerus bone section, and 1.71 $mm^2$ for each rib bone section. The rib cortical region is much narrower than larger bones such as the femur, so that the ROIs have to be positioned in a narrow space, and the magnification and FOV needs to be less to observe the whole rib cross-section. The average intact osteon count for sections of the femur was 30.6, humerus 28.8, and ribs 12.0. Bone size variability was accounted for by incorporating robusticity indices into the statistical analyses. Bone robusticity is a measure that is commonly used to interpret the allometric relationship between bone size and quantifiable characteristics of cortical bone remodelling (Miszkiewicz & Mahoney, 2019).

### Quantification of Histomorphometric Variables

Histomorphological features such as osteon cement lines and lamellae were most clearly observed on the polarised light images, while the perimeter of Haversian canals was best viewed on the transmitted light images. Features were recorded and labelled on corresponding images using imaging software (open access FIJI/ImageJ®) (Schindelin et al., 2015) with a range of drawing and point counting tools: the ‘multi-point’ and ‘freehand selection’ tools (Figure 5-3). Table *5-2* defines each histological variable. While this study focuses on the elderly, and it is known that the osteon population density (OPD) asymptote can erase real

OPD in individuals older than 50 (Crowder & Dominguez, 2013), OPD was recorded as part of the histomorphometric standards and included in analyses. However, the effect of OPD asymptote on OPD data will be taken into account when interpreting and discussing data. The number of intact and fragmented osteons per ROI was recorded on polarised light images and counts used to calculate OPD/mm² (#intact osteons + #fragmentary osteons divided by image area (mm²)). On transmitted light images, the perimeter of each Haversian canal was traced using a stylus and laptop, creating the variable Haversian canal area (H.Ar in µm²), while the perimeter of each intact osteon was traced on polarised images and recorded as secondary osteon area (On.Ar in µm²) (Maggio & Franklin, 2021).

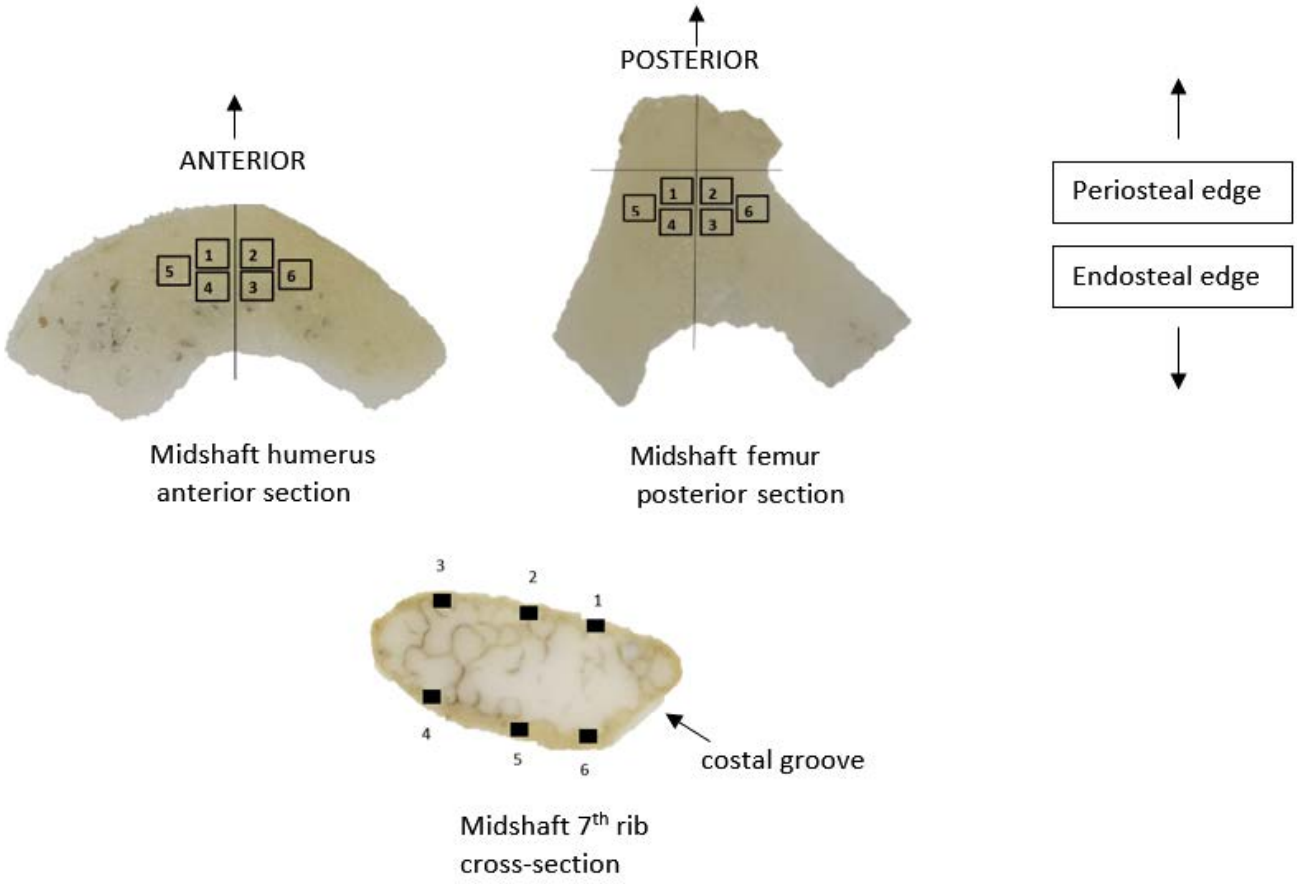
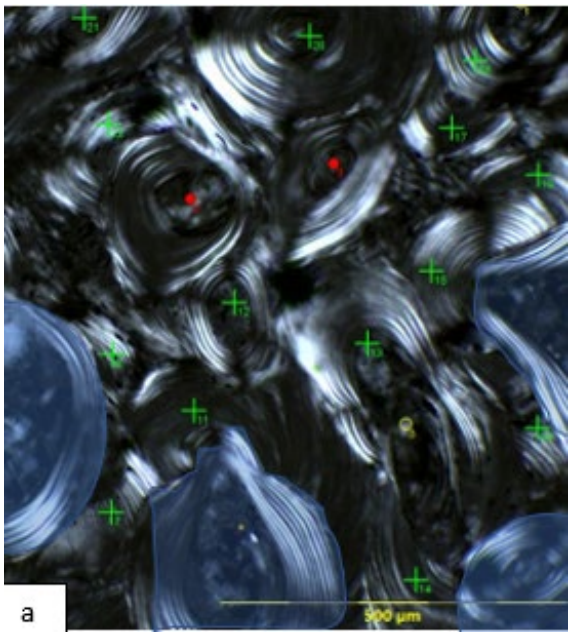
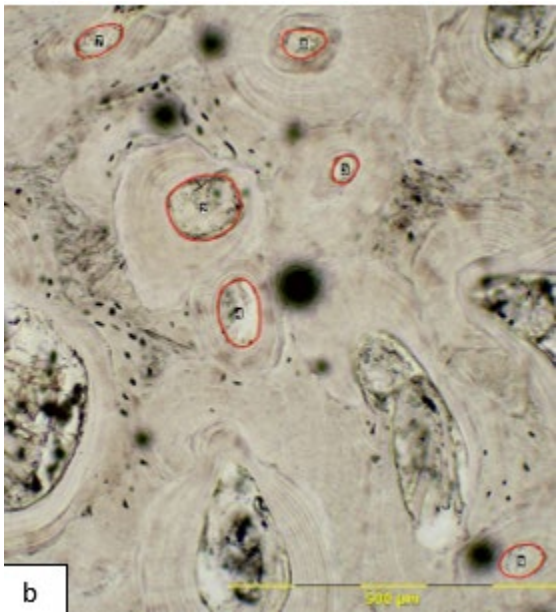



*Figure 5-2 Selection of the regions of interest (ROI).*
*Six ROIs were selected in the sub-periosteal layer of intracortical bone for the anterior midshaft of the humerus, posterior midshaft of the femur, and rib mid-shaft (bones not to scale and position of ROIs are approximate).*



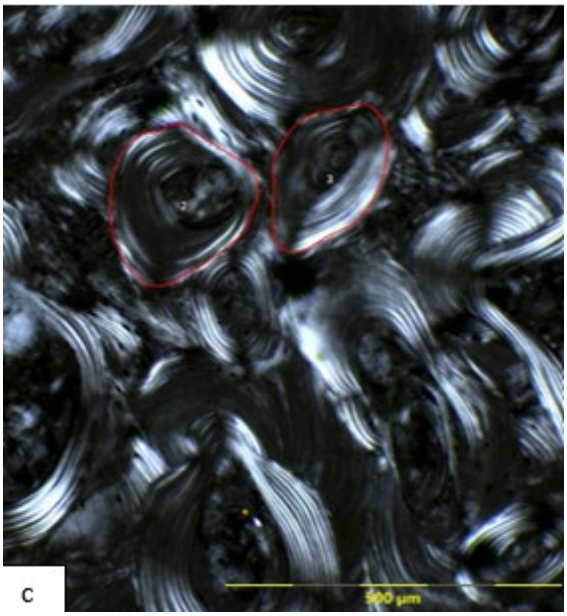




*Figure 5-3 Examples of histomorphometric features recorded on each ROI in this study (a) Count of intact osteons (red dot), fragmentary osteons (green cross), resorption cavity (yellow circle); osteons cut off by the image border were not counted if it could not be determined if they were intact or fragmentary (opaque blue); (b) Haversian canal area measurements (µm²) (red circles) (black dots are artefacts of sample processing); (c) intact osteon area* measurement (µm²) (red circles).

Table 5-2 Definitions of histological variables examined in this study.

| |
|---|
| **Intact osteons**<br>Osteon that is completely surrounded by a cement (reversal) line, its Haversian canal is intact, and it has not been intrupted by a resorption space/walls of a later osteon. This is a type I osteon.<br><br>If they follow the above criteria, included are: osteons that have either completed filling or are still being filled, osteons breached by a Volkmann's canal, and type II osteons (osteon embedded within the cement line of a pre-existing osteon).<br><br>If at least two osteons, in a transverse section, merge to share a Haversian canal and/ or are surrounded by a shared cement line, such as a branching event, then they are counted as one intact osteon. |
| **Fragmentary osteons**<br>A pre-existing osteon that has had its lamellae and/or the Haversian canal breached by subsequent generations of osteons or a resorption cavity. Concentric lamellae fragments that are clearly identifiable as previous osteons, i.e. have part of a cement line visible and osteocytes/lacunae between the lamellae, were included. |
| **Osteon population density (OPD)**<br>Total count of intact (N.On) and fragmentary osteons (N.On.fg) per region of interest (N.On + N.On.Fg/mm²) |
| **Resorption cavity (RC)**<br>An area of resorbed bone, bordered by scalloped edge of a Howship's lacuna. |
| **(mean) Osteon area (On.Ar)**<br>The reversal line of each intact secondary osteon was traced, and the internal area was calculated in µm². For each bone section, the mean On.Ar was used in analyses (summed On.Ar from all ROIs/number of measured osteons). |
| **(mean) Haversian canal area (H.Ar)**<br>Complete Haversian canals without any indication of resorption were traced, and the internal area in µm² was calculated. For each bone section, the mean H.Ar was used in analyses (summed H.Ar from all ROIs/number of measured canals.) |

Modification of Crowder et al. (2022) and Cho et al. (2002)

## Statistical Analyses

The data were analysed in IBM SPSS® 29.0, at $p = 0.05$. Intra-observer error was assessed by re-taking measurements on 10% of ROIs and comparing them against original values by performing paired non-parametric correlations (Wilcoxon signed-rank test). As this was a relatively small sample, non-normal distribution was assumed, and thus non-parametric testing was chosen (DePuy & Pappas, 2004). As there were only four females, sex differences in histology were not tested for. Instead, correlations were first tested on the pooled sex group and then on the male only group. A Spearman's rank-order correlation tested the relationship between age and three histological variables (OPD, On.Ar, and H.Ar). To account for robusticity of the femur and humerus, and the allometric/isometric effect of bone size on the histological variables, the Spearman's rank-order tests were also recalculated with the histology variables adjusted by the robusticity index (RI) (e.g., H.Ar $_{Lg10}$).

Next, the significance of the relationship of each of the histological variables was tested between the three bones (femur, humerus, and rib) using Friedman two-way analysis of variance by ranks test. This was followed by post hoc Wilcoxon signed-rank tests (with the Bonferroni correction for multiple comparisons) to determine which of the bones was most sensitive to remodelling. Two of the males did not have ribs available for sampling (JCU195/17 and JCU197/17) so were removed from this part of the analysis, this reduced the entire dataset to 15 individuals, and the male dataset to 10 individuals for Friedman two-way and post hoc analyses.

Correlations between age groups were not sought as most of the sample fit into a narrow age at death range between 75 to 88 years (n = 15). A 93-year-old male (JCU141/14) was excluded from the analyses as this was the only individual in their 9$^{th}$ decade of life and their OPD was a strong outlier. This elderly male will be discussed as an individual case. Therefore,

the results of this study can best be used to interpret the bone microstructure of elderly adult Australian males in their 7th and 8th decade of life.

## 5.3 Results

### Intra-Observer Tests and the Effects of Age

There were no statistically significant intra-observer differences between the original and repeated measures (paired correlations, n = 51, z = -2.722 to -1.255, $p$ = >0.05). Spearman's test of correlations within the pooled sex group, showed no statistically significant relationships between age and each of the histological variables (OPD, On.Ar, and H.Ar). A moderate positive correlation was observed between age and Haversian canal area in the rib ($r_s$ = 0.442, $p$ = 0.099) which is illustrated in Figure 5-4. The rest of the correlations for the pooled sexes were weak and are presented in Appendix B. 2.

For the male group, scatter plots illustrating the four strongest relationships with moderate correlations are provided in Figure 5-5 and all other correlations (weak) are illustrated in Appendix B. 3. There was a statistically significant positive relationship between age and OPD of the femur ($r_s$ = 0.603, $p$ = 0.038), and the Haversian canal area in the rib ($r_s$ = 0.646, $p$ = 0.043). A moderate positive relationship was recorded between age and OPD of the humerus ($r_s$ = 0.529, $p$ = 0.077) along with a corresponding moderate negative correlation with Haversian canal area in the humerus ($r_s$ = -0.423, $p$ = 0.170).

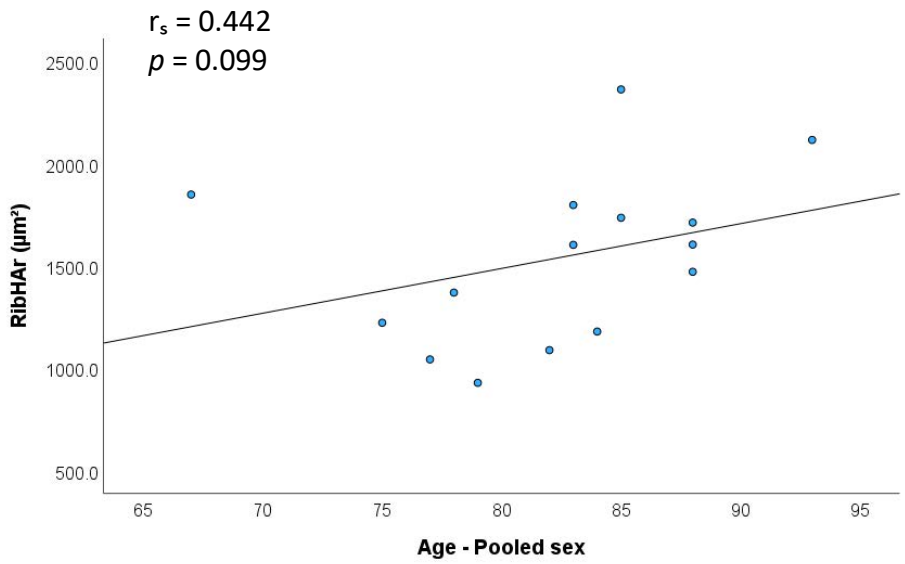
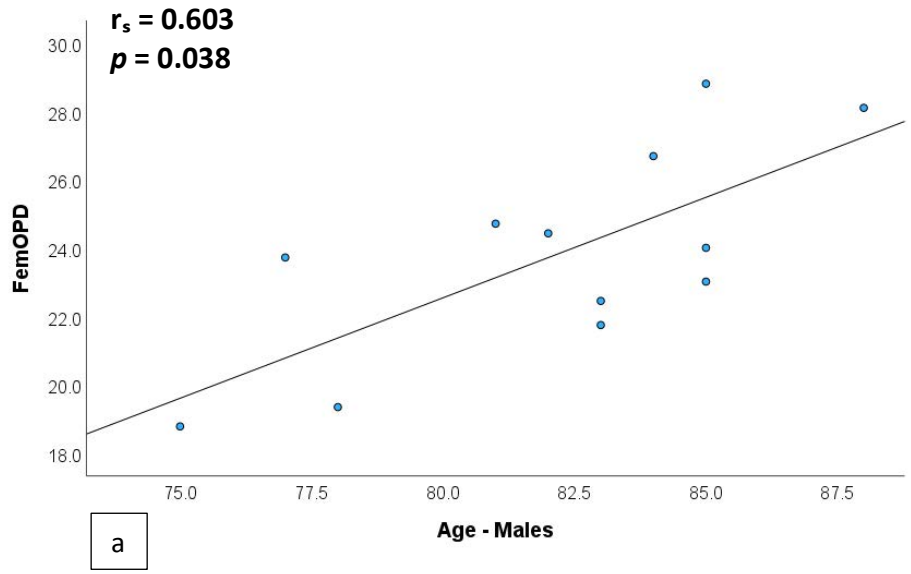
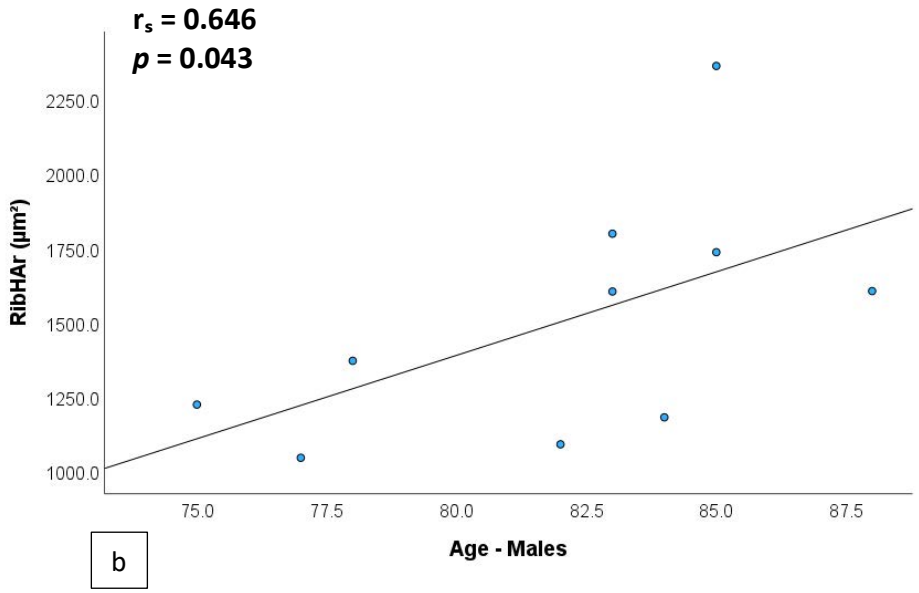


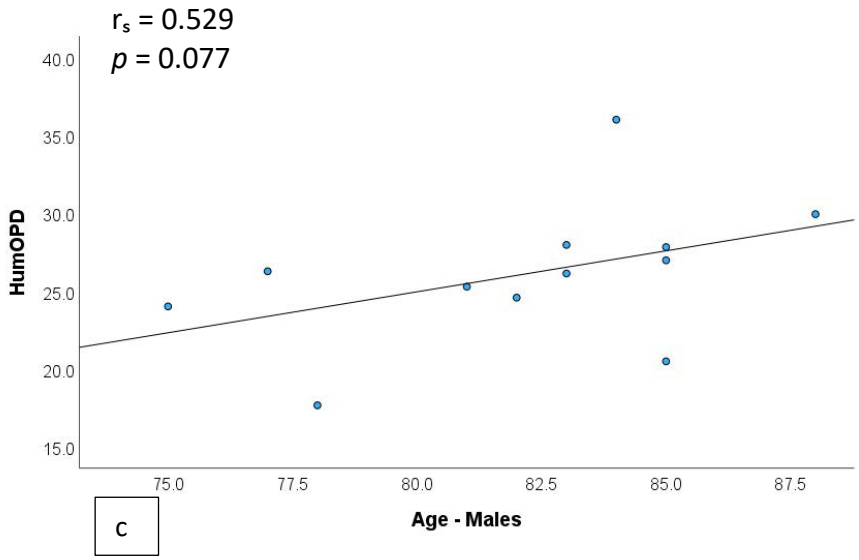

*Figure 5-4 Scatter plot with line of best illustrating the moderate positive relationship between age and Haversian canal size of the rib within the pooled sex group.*


$r_s$ = 0.603
*p* = 0.038
FemOPD
30.0
28.0
26.0
24.0
22.0
20.0
18.0
75.0
77.5
80.0
82.5
85.0
87.5
Age - Males

a


$r_s$ = 0.646
*p* = 0.043
RibHAr (µm²)
2250.0
2000.0
1750.0
1500.0
1250.0
1000.0
75.0
77.5
80.0
82.5
85.0
87.5
Age - Males

b



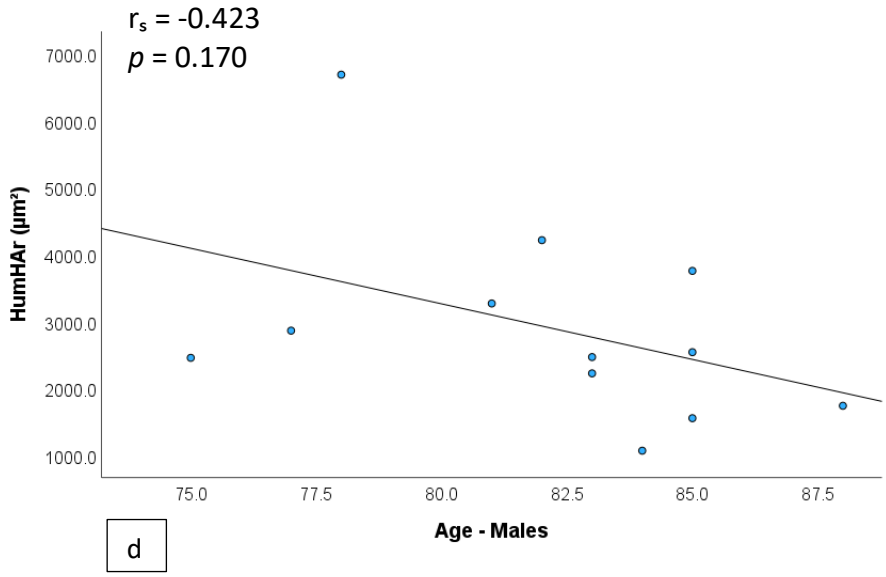




*Figure 5-5 Examples of the four best correlations with age within the male sample: statistically significant positive relationships (bold-faced) with OPD of the femur (a) and Haversian canal area of the rib (b); moderate positive correlation with humerus OPD (c), and moderate negative correlation with humerus Haversian canal size (d).*

## Intra-Skeletal Differences

The Friedman test identified statistically significant differences in OPD count, osteon area, and Haversian canal area (pooled sample: $\chi^2$ = 14.533 – 19.600, $p$ <0.001, n= 15) (male sample: $\chi^2$ = 9.800 – 12.800, $p$ = 0.007 – 0.002, n = 10) between at least two of the bones (Table *5-3*). Subsequent corresponding post hoc analysis on the entire dataset identified that the rib had statistically significantly more osteons, as well as statistically significantly smaller osteons and Haversian canals, in comparison to both the femur and the humerus (Table *5-4*). In males, the rib had a significantly higher osteon count than the femur (Z = -2.803, $p$ = 0.005), and rib Haversian canals were statistically significantly smaller than in the humerus (Z = -2.803, $p$ = 0.005). No significant differences were found between the femur and humerus for any of the histological variables. Descriptive statistics (Appendix B. 4) show that the mean for OPD, osteon area, and Haversian canal area are always similar between the femur and humerus, but the rib is different.

Table 5-3 Friedman test of significant relationships between bones by sample.

| Pooled sex n = 15 | $\chi^2$ | Sig. | Males n = 10 | $\chi^2$ | Sig. |
|---|---|---|---|---|---|
| OPD | 19.600 | <0.001 | OPD | 12.800 | 0.002 |
| On.Ar | 14.533 | <0.001 | On.Ar | 9.800 | 0.007 |
| H.Ar | 18.533 | <0.001 | H.Ar | 11.400 | 0.003 |

Table 5-4 Post hoc Wilcoxon test.

| Pooled sex n = 15 | Z | *Sig. | Males n = 10 | Z | *Sig. |
|---|---|---|---|---|---|
| Humerus OPD - Femur OPD | 2.385[a] | 0.017 | Humerus OPD - Femur OPD | 2.293[a] | 0.022 |
| Rib OPD - Femur OPD | 3.408[a] | **<0.001** | Rib OPD - Femur OPD | 2.803[a] | **0.005** |
| Rib OPD - Humerus OPD | 3.237[a] | **0.001** | Rib OPD - Humerus OPD | 2.497[a] | 0.013 |
| Humerus On.Ar - Femur On.Ar | 1.136 | 0.256 | Humerus On.Ar - Femur On.Ar | 0.968[b] | 0.333 |
| Rib On.Ar - Femur On.Ar | 3.010[b] | **0.003** | Rib On.Ar - Femur On.Ar | 2.497[b] | 0.013 |
| Rib On.Ar - Humerus On.Ar | 3.237[b] | **0.001** | Rib On.Ar - Humerus On.Ar | 2.701[b] | 0.007 |
| Humerus H.Ar - Femur H.Ar | 0.966[b] | 0.334 | Humerus H.Ar - Femur H.Ar | 1.172[b] | 0.241 |
| Rib H.Ar - Femur H.Ar | 3.408[b] | **<0.001** | Rib H.Ar - Femur H.Ar | 2.803[b] | **0.005** |
| Rib H.Ar - Humerus H.Ar | 3.124[b] | **0.002** | Rib H.Ar - Humerus H.Ar | 2.293[b] | 0.022 |

[a] based on negative ranks; [b] based on positive ranks; *Bonferroni adjusted p value of 0.006; statistically significant results are in bold.

## RI Adjusted Data for the Femur and Humerus

### The Effects of Age

When bone size of the femur and humerus were taken into account by adjusting the histological values with RI, there was a change in correlation strength (

Table *5-5* and Table *5-6*). Spearman's test of correlations between age and the three adjusted histological variables within the pooled sex group showed all correlations were still weak. Scatter plots for these relationships are illustrated in Appendix B. 5, with the humerus showing the weakest relationship ($On.Ar_{Lg10}$, $r_s$ = -0.007, $p$ = 0.978), and the strongest relationship with age ($OPD_{Lg10}$, $r_s$ -0.219, $p$ = 0.398) compared to the femur.

In the male group, once bone robusticity was accounted for, some of the relationships with age became stronger (Haversian canal size in the femur and humerus), while others became weaker (OPD in the femur and humerus, and humerus osteon area), or remained similar

between the original and RI adjusted values (femur osteon area). Moderate correlations between all three adjusted histological variables and age were observed in the femur (OPD $_{Lg10}$, $r_s$ = 0.476, $p$ = 0.118; On.Ar$_{Lg10}$ $r_s$ = -0.360, $p$ = 0.251; and H.Ar $_{Lg10}$, $r_s$ = -0.360, $p$ = 0.256) (

Table *5-5*). Whereas in the humerus the only moderate correlation with age was in Haversian canal size (H.Ar $_{Lg10}$, $r_s$ = -0.536, $p$ = 0.072) (and this was also the strongest of all correlations with age). Scatter plots for each relationship are illustrated in Appendix B. 6.

### Intra-Skeletal Differences

When the variable of bone size was removed there was no longer a statistically significant difference in OPD between the femur and humerus for individuals in the pooled sample (Table *5-6*), however, small sample size would have an effect on statistical power. Male individuals were found to have statistically significantly smaller Haversian canals in their humerus compared to their femur (Z = -2.201, $p$ = 0.028). No other relationship between the femur and humerus was statistically significant.

Table 5-5 Spearman's correlation tests comparing results of original values to RI adjusted values for the femur and humerus.

| Pooled sex | **Original values** | | **RI adjusted values** | |
|---|---|---|---|---|
| | $r_s$ | Sig. | $r_s$ | Sig. |
| **FemOPD** | -0.053 | 0.840 | -0.189 | 0.469 |
| **FemOn.Ar** | -0.010 | 0.970 | -0.166 | 0.523 |
| **FemH.Ar** | -0.087 | 0.738 | -0.169 | 0.517 |
| **HumOPD** | 0.229 | 0.376 | 0.211 | 0.417 |
| **HumOn.Ar** | -0.078 | 0.767 | -0.007 | 0.978 |
| **HumH.Ar** | -0.239 | 0.355 | -0.219 | 0.398 |
| Males | Original values | | RI adjusted values | |
| | $r_s$ | Sig. | $r_s$ | Sig. |
| **FemOPD** | **0.603*** | **0.038** | 0.476 | 0.118 |
| **FemOn.Ar** | -0.339 | 0.282 | -0.360 | 0.251 |
| **FemH.Ar** | -0.159 | 0.622 | -0.360 | 0.256 |
| **HumOPD** | 0.529 | 0.077 | 0.174 | 0.819 |
| **HumOn.Ar** | -0.243 | 0.446 | -0.109 | 0.735 |
| **HumH.Ar** | -0.423 | 0.170 | -0.536 | 0.072 |

underlined values are moderate correlations ($r_2$ = 0.36 - 0.67)
Taylor (1990);the only significant relationship with age (p≤0.05) is bold-faced*.

Table 5-6 Wilcoxon signed ranks test comparing significance of original values and RI adjusted values for the femur and humerus.

| Pooled sex | **Original values** | | **RI adjusted values** | |
|---|---|---|---|---|
| | Z | Sig. | Z | Sig. |
| **HumOPD - FemOPD** | **-2.107[a]*** | **0.035** | -1.112[a] | 0.266 |
| **HumOn.Ar - FemOn.Ar** | -0.639[b] | 0.256 | -1.254[b] | 0.210 |
| **HumH.Ar - FemH.Ar** | -0.254[b] | 0.334 | -1.538[b] | 0.124 |

| Males | **Original values** | | **RI adjusted values** | |
|---|---|---|---|---|
| | Z | Sig. | Z | Sig. |
| **HumOPD - FemOPD** | **-1.961[a]*** | **0.050** | -1.083[a] | 0.279 |
| **HumOn.Ar - FemOn.Ar** | -0.157[b] | 0.875 | -1.433[b] | 0.152 |
| **HumH.Ar - FemH.Ar** | -1.569[b] | 0.177 | **-2.201[b]*** | **0.028** |

[a] based on negative ranks; [b] based on positive ranks; significant differences (p≤0.05) are bold-faced*.

## 5.6 Discussion

In this study, the secondary osteons of elderly modern Australians are examined to 1) evaluate the relationship between age and measures of cortical bone remodelling (OPD and size of osteons and Haversian canals); 2) intra-skeletally compare remodelling between different bones of the axial (rib) and appendicular (femur and humerus) skeleton that are subject to different rates of metabolic and biomechanical stresses; 3) examine the relationship between a skeletal element's macroscopic size and the microarchitecture within its cortical bone. In aging research, it is more common for studies to evaluate histological geometric variables without adjusting for bone size, but this study will discuss the results using both the 'raw' data and the 'adjusted' data which takes into account the effect of bone size on the underlying histological features.

In the pooled sex sample age only weakly affected OPD, osteon area, and Haversian canal area within all three bones, with the exception of a moderate increase in Haversian canal size within the rib. Even when bone robusticity was accounted for, the correlation of age with each of the three histological variables remained weak within the femur and humerus. These results are likely an effect of combining the sexes, as rates of bone remodelling are reported to differ between males and females (Abdullah et al., 2018; Ericksen, 1991), but sample size could also have an effect, therefore, future studies with a larger sample should explore this further.

Descriptive statistics of the JCU sample show that females have larger mean osteon area in all three bones compared to males, however the relatively small female sample size meant that significance between the sexes could not be reliably tested. Some studies have observed only weak sexual dimorphism in osteon area (Pfeiffer, 1998a; Pfeiffer et al., 2006), while others reported that males had statistically significantly smaller osteons than females, attributable to

greater body mass in males and the likelihood to have participated in more physically strenuous activities (Mulhern & Van Gerven, 1997). Britz et al. (2009) also found an inverse relationship between body weight and osteon size, but they observed that Australian females have statistically significantly smaller osteons than males in the anterior midshaft of the femur, with menopause likely impacting the rate of remodelling in females (Cho & Stout, 2011). In this present study, female mean Haversian canal area is also larger in all three bones compared to males. The females are all over 67 years of age and would be post-menopause. Estrogen deficiency causes an imbalance in bone metabolism and remodelling (Tobias & Compston, 1999), with greater bone resorption (Riggs, 2000), and an increased rate of cortical thinning (Seeman, 2013). The mean OPD is lower in females than males in the femur and humerus, but not the rib. Sexual dimorphism in hormones, diet, body mass, and genetics (Cho & Stout, 2011) would likely explain the different pattern of remodelling observed in sexes.

As there were only four females in the sample, the remainder of analyses was restricted to males who comprised three quarters of the sample. With increasing age, male femora showed a statistically significant increase in OPD as did Haversian canal area in the rib. This means that as males age, they generally accumulated more osteons in the femur compared to their humerus and rib. Vascular canals in the rib get larger as remodelling activity becomes out of balance and the filling of osteons is arrested (Pfeiffer et al., 2006). For a given loading environment the rib is expected to be under the most metabolic influence, the femur minimal, and the humerus intermediate (Eleazer & Jankauskas, 2016). Minimal bending forces and dynamic loading are experienced by the rib (Robling & Stout, 2003) and at a rate that is relatively uniform across individuals (Bonicelli et al., 2022). These factors affect vascular porosity, visible in the form of larger Haversian canals and greater variability in size of the canals (Pfeiffer et al., 2006). On the other hand, the literature generally reports the femur to be placed under greater biomechanical loading (Ruff et al., 1991), causing locally intensified remodelling to aid microcrack repair (Wasserman et al., 2008). This generally constrains the size of vascular pores and more of the smaller osteons remodel the space (Miszkiewicz, 2016), generating a higher OPD. Alternatively, some studies report declined OPD in the femur

compared to the rib which is under less mechanical strain and therefore is better able to reflect mineral homeostasis and hormonal responses (Skedros et al., 2013).

Males in the present study also showed a moderate positive relationship between age and OPD of the humerus along with a corresponding moderate inverse relationship with Haversian canal area in the humerus. Biomechanics affect the humerus to a lesser extent than the femur but this is still likely confounding the remodelling process (Skedros et al., 2013), whereby as the males age there is an accumulation of osteons in the humerus accompanied by smaller Haversian canals. Upper limb bones such as the humerus are typically non-weightbearing, but muscle pull and strain from the wide-range of motion in the arm and shoulder (Santos et al., 2018) subjects the limb to axial loading and bending and torsional forces (Trinkaus et al., 1994). Descriptive statistics (Appendix B. 4) show that the means for OPD, osteon area, and Haversian canal area are similar between the femur and humerus, but the rib is different. OPD in the rib tends to be higher and osteon and canal size lower compared to the long bones. This shows that the rib in the axial skeleton has a unique pattern of remodelling compared to the femora and humeri in the appendicular skeleton. Robusticity indices were used to account for the effect of bone size on remodelling rates (Miszkiewicz & Mahoney, 2019). In this study the robusticity of the rib could not be accounted for as length and width measurements were not collected at the time of sampling. Adjusting for bone robusticity in the femora and humeri changed the strength of the relationships between age and the three histological variables in males, but the overall pattern of remodelling remained valid. OPD tended to increase with age, while the size of osteons and Haversian canals tended to decrease with age. With the effect of bone size removed, it appears that bone tissue in the femur and humerus has similar quality within one individual despite the leg and the arm being used in slightly different ways. The only statistically significant result was that Haversian canals were smaller in the humerus compared to the femur, whereas OPD and size of osteons were relatively similar between the long bones. This shows there is a tight anatomical relationship between bone macro and micro size.

The mean age of the male sample in this present study was 82 years, with age at death ranging from 75 to 88 years, therefore the results best represent elderly Australian males. Most age-related cortical bone loss occurs after the age of 60 years (Seeman, 2013), with a decrease in vascular pore numbers corresponding with an increase in pore size (Bousson et al., 2001). Age-related increase in pore size in older adults may be relatable to the coalescence of pre-existing Haversian canals and new resorption cavities (Andreasen et al., 2020; Seeman, 2013). There is much individual variability, especially in the elderly who are at increased risk of metabolic bone diseases such as osteoporosis (Kulminski et al., 2006) and a lifestyle with less physical activity and reduced muscle mass (Cvecka et al., 2015). Increasing levels of physical inactivity and nutrient deficient diet in the elderly are regularly reported in clinical literature for their detrimental effects on bone health and interruption to remodelling processes (Bonjour et al., 2009). Additionally, many elderly seek therapeutic treatment for osteoporosis and metastatic bone diseases and cancers that deliberately target bone resorption and formation by inhibiting bone resorption and/or formation (Skjødt et al., 2019).

One male of 93 years age at death was removed from the male sample as their OPD counts made them a strong outlier. The remodelling in this individual's femur was unique compared to the other males. This individual has the lowest mean OPD count for the femur but also the largest mean osteon area (except for JCU 247/19, an 82-year-old male). Porosity in this individual's femur was more pronounced than the younger males. Their large resorption cavities had removed evidence of earlier osteons and therefore reduced the OPD count and interfered with osteon area measurements. The intact osteons are visibly smaller than the remnants of fragmentary osteons. This is consistent with studies that report osteon size decreasing with age (Dominguez & Agnew, 2016; Maggio & Franklin, 2021; Narasaki, 1990).Takahashi et al. (1965) made the point that the well-documented decrease in osteon area could largely be due to an increased likelihood of the largest and most irregularly shaped osteons being remodelled, with only smaller intact osteons left to measure. This male also has

one of the lowest OPD counts for the humerus and rib, compared to all the other males who are younger, and almost the lowest mean canal area in both the humerus and rib. A factor to consider is that the age of OPD asymptote may have been reached in at least one of the bones in this individual. Asymptomatic values indicate that the cortical bone has been completely remodelled, in which case any new secondary osteons will simply be replacing already remodelled bone that is saturated with fragmentary osteons (Frost, 1987a). This means that the mean annual OPD will not increase and will no longer correlate with age (Frost, 1987b). Fewer intact osteons with an uninterrupted cement line will remain to measure osteon area, and this issue is compounded by the narrowing of cortical bone with age, especially in the rib, which further decreases the number of intact and fragmentary osteons to count and measure (García-Donas et al., 2021). The age at which asymptote occurs varies between bones depending on osteon size, bone turnover rate, and cortical area (Andronowski & Crowder, 2019). For example, asymptote is recorded to occur in the 5$^{th}$ or 6$^{th}$ decade of life in ribs at an OPD of 30/mm² (Cho et al., 2002; Stout & Paine, 1994), likely due to the narrow cortical area being completely remodelled before the thicker and denser cortex of the femur (Frost, 1987a). Asymptote in the femoral midshaft is documented to be reached when OPD count is approximately 50-55/mm², after the 8$^{th}$ decade of life (Gocha & Agnew, 2014). The sex and ancestry have an effect on bone remodelling and thus are likely to each have an effect on OPD asymptote but this requires further investigation on more genetically diverse populations to fully understand the correlation between OPD, sex and ancestry (Pfeiffer, 1992).

In the present study, males showed few statistically significant relationships between age and the three histological variables, and in the intraskeletal comparisons, but this still shows some potential for the use of histology in aging research. However, even the non-significant relationships have provided a useful account of the remodelling processes within this modern Australian sample. To summarise this present study, OPD in elderly males tended to increase with age within the femur and humerus but decline within the rib. This is most likely tied in with

higher targeted mechanical loading placed on the posterior femur and anterior humerus, compared to the less strenuous but constant loading placed on ribs. This study suggests that research on the Australian population would benefit from focusing on the rib because it will not be as impacted by biomechanical stress as the femur and may therefore have greater potential for age estimation via histomorphometry. Osteon and canal size tended to decrease with age in the femur and humerus whereas they increased in the rib with advancing age, and this could be accounted by ribs experiencing greater porosity with age due to greater metabolic influences than the bones from the appendicular skeleton are subjected to. Differences in bone size, and therefore the amount of cortical bone area, may be confounding raw histology measurements. Accounting for bone size in males tended to weaken most of the relationships between age and histomorphometric values within the femur but tended to strengthen more of them within the humerus. Significantly smaller Haversian canals were observed in the humerus compared to the femur, whereas osteon and canal size were relatively similar between the two bones. These results support prior work on other populations which has shown that bone size has an underlying effect on its internal microarchitecture (Goldman et al., 2014; Miszkiewicz & Mahoney, 2019).

## 5.7 Limitations

The sampling was restricted by the scarcity of young adult and female donors. Differences between the sexes, and between young and older adults could therefore not be examined. The age and sex biases reflect the nature of body donation for medical education and research within Australia. Studies have found that even when an individual expresses their willingness to donate, there are psychological (i.e. emotional), religious, cultural, or social factors that may hinder family members from providing final consent for body donation. Thus, this study does not reflect the full extent of histomorphometric variation in the Australian population. It is expected that as the JCUHSHC grows over time it will gradually gain more female and younger donors. Future studies can then build upon this study with a larger and more representative

sample. Adjustments for robusticity could not include the rib, as the maximum length and midshaft circumference measurements were only obtained for the femur and humerus before bone samples were taken from the donors. As the amount of research on the JCUHBHC increases, the sampling procedures will also be refined to include more measurements. It is well-documented that genetics, health, metabolic processes, and biomechanical loading influence osteon geometric properties (Stout et al., 2019), and the effect of one may mask or mimic the effect of the other (Eleazer & Jankauskas, 2016). This information has not been collected from the donors who self-report medical and personal information. Donors are not required to provide details such as occupation and physical activity levels, so it is difficult to determine how these are affecting interpretations of remodelling rates. It is possible that future ethics applications may be adjusted to allow for this data to be collected from donors.

## 5.8 Conclusion

The motivation for this study was to enhance the understanding of intra-skeletal variation of cross-sectional geometric properties, and in particular, how these properties correlate to age in a modern Australian sample. The sex and age biases in this sample means that the results best measure cortical remodelling of elderly Australian males. This study confirms that bone size has an underlying effect on histology, changing the strength of the correlation with age. It is recommended that future studies in age estimation research should consider bone macro- and micro-measurements together. With robusticity accounted for, the one statistically significant result in males (statistically significantly smaller Haversian canals in the humerus compared to femur) shows some potential for the use of histology in aging research. This study suggests that the rib will not be as impacted by biomechanical stress as the femur and may therefore have greater potential for age estimation via histomorphometry.

## 5.9 Chapter Summary

This chapter addressed the gap in research for intraskeletal variation of bone microstructure within the Australian population. It evaluated the range of variability of histomorphometric features between different bones within an individual, and the effects of bone robusticity on bone remodelling rates. These results contribute to the understanding of age-related changes in older adults which will allow refinement of age at death estimation methods for this demographic. The next chapter compares the Australian sample to a northeastern Thai sample to evaluate interpopulation variation in age-related cortical bone histology.

# Chapter 6 Interpopulation Variation of Age-Related Histomorphometric Features in the Femoral Midshaft

In this chapter three histomorphometric features of bone remodelling are compared between northern Australian and northeastern Thai samples. Our current knowledge of age-related histomophology is limited for both these populations. This chapter is a journal article that is under review for publication:

> L.T. Pedersen, J. Miszkiewicz, Lit Chin Cheah, N.Techataweewan, C. Morgan, A. Willis, K. Domett. *Age-related histomorphometric variation in the femoral midshaft of modern Thai and Australian samples.*

## 6.1 Introduction

Remodelling of cortical bone by bone multicellular units (BMUs) is a continuous process that occurs throughout the skeleton for almost all of the human lifespan. The products of BMUs, secondary osteons (hereafter ‘osteons’), are histologically correlated with age when counted and measured such that predictable changes to the osteons and their central Haversian canal can be made (Stout et al., 1994). There are conflicting reports in the literature of significant interpopulation variability in measures of relative cortical area, osteon population density (OPD), and size and circularity of osteons (Botha et al., 2020; Cho et al., 2002; Karydi et al., 2022; Pratte & Pfeiffer, 1999). Just how much variation exists in bone microarchitecture in modern Australians compared to populations from geographically and genetically distinct regions, such as Southeast Asia, has not been explored as much as it has with American and European populations. This study uses two recently created human skeletal histology

collections from northeastern Thailand and northern Australia to investigate interpopulation variability and the implications of this on age estimation reliability and accuracy.

Most of the age at death estimation studies based on bone histomorphometry have been developed using modern skeletal samples from the United States and Europe (Andronowski & Cole, 2021), which can produce large standard errors when applied to genetically distinct populations from other regions of the world, such as Greek (García-Donas et al., 2016), Chinese (Fangwu, 1983), and ethnic Dominican (Ubelaker, 1986). Age estimation is documented to be more reliable and accurate when the reference sample that the method was developed on is representative of the target population for which age is being estimated (Stout & Gehlert, 1980). It is recommended that that the reference and target samples are similar in ancestry and genetic background (Cho et al., 2006), age and sex distribution (Bouvier & Ubelaker, 1977; Kim et al., 2007), health and social conditions (Pratte & Pfeiffer, 1999; Ritz-Timme et al., 2000), and the samples be from the same time period (archaeological or modern) to account for secular changes (Aiello & Molleson, 1993; Martin & Armelagos, 1985). Dissimilarities are known to contribute to over- or under-estimation of age with wide error rates of ±5 to 19 years in both macroscopic (Pedersen & Domett, 2022) and microscopic methods (Aiello & Molleson, 1993; Pratte & Pfeiffer, 1999). An image processing technique used by Chompoophuen et al. (2019) to correlate age with different histological variables in decalcified femora bone sections is a rare example of age estimation equations developed on a Thai sample (reviewed in detail in Chapter 2). The authors claimed a multiple correlation coefficient of 0.906 (SE of 8.26) using Haversian canal perimeter, percentage of lamellar bone area, and bone collagen.

A number of specific genotypes contributing to osteoporosis have a great influence on bone mass, accounting for 50 to 90% of variability in bone mineral density (BMD) and bone turnover rates, depending on population ancestry (Anderson & Pollitzer, 1994; Mondockova et al., 2018; Recker & Deng, 2002). Comparison studies between groups of Caucasian, African, or

Asian ancestry have shown differences in a range of genetic polymorphisms, and specific genotypes, such as frequency variants in the vitamin D receptor (VDR) and B allele that influence BMD, osteoporosis risk, and bone remodelling rates (Leslie, 2012; Tokitan et al., 1996). Specific restrictions to the B allele genotype that can interfere with bone formation and levels of bone mineral have been observed in just 12% of Japanese females compared to 41% of females with European ancestry (Tokitan et al., 1996). African Americans are found to have a significantly lower rate of bone remodelling compared to European Americans, and this is correlated with greater bone mass and lower osteoporosis prevalence (Weinstein & Bell, 1988). Variation in cortical bone osteon characteristics has been reported to exist between different populations. Cho et al. (2002) observed lower OPD, smaller osteons, and lower number of Haversian canals (therefore less cortical porosity) in African Americans compared to European Americans. Botha et al. (2020) reported significant differences in OPD and osteon size between African and European origin South Africans and a Danish sample. However, there was a high degree of individual variability in Haversian canal size as age increased, leading to an overall similarity in canal size between the three groups (Botha et al., 2020).

Confounding the effects of ancestry is the multifactorial role played by age, sex, disease, hormones, diet, and environment on intra- and inter-population bone loss and BMD variation (Leslie, 2012). Nutritional deficiencies interrupt mineral homeostasis, affecting the bone resorbing and bone forming cells and impacting bone turnover rates (Keough et al., 2009; Martin & Armelagos, 1985). Dietary deficiencies can disrupt metabolic processes and affect the quality of bone. This is suggested to be a significant factor for interpopulation variability in remodelling rates, and therefore reduces accuracy of histological age at death estimation methods (Paine & Brenton, 2006; Pratte & Pfeiffer, 1999). The functional adaptation of bone to repetitive strain loading from strenuous activities can increase targeted cortical bone remodelling response to facilitate fatigue repair (Burr et al., 1985; Robling et al., 2006). This contributes to increased osteon wall thickness and population density (Burr et al., 1990), and

smaller Haversian canal size (diameter and circumference) of increased population and density (Miszkiewicz et al., 2022b; Mulhern & Van Gerven, 1997).

A better understanding of the histological expression of unique population differences in cortical bone microarchitecture within the Asia-Pacific region is needed to assist in revising current methods, such as those reviewed by Andronowski and Cole (2021) to ensure greater accuracy and reliability for these populations. This study therefore aimed to evaluate regional trends in human osteon measurements in relation to age and sex between modern Thai and Australian samples of distinct ancestry.

## 6.2 Materials and Methods

This project received ethics approval at James Cook University (#H8352), and the University of Queensland (#2022/HE000860) in Australia, in addition, ethical approval was obtained from the Khon Kaen University Ethics Committee, Thailand (HE611495). All histology slides in this study are of the posterior midshaft region of the femur, incorporating the linea aspera, from individuals representing two modern donor populations with documented age and sex (Table 6-1). Bone sections were not stained or decalcified. The first sample, from northeast Thailand is derived from the Body Donation Unit in the Faculty of Medicine's Department of Anatomy at Khon Kaen University (Techataweewan et al., 2018). The demographic composition of the donors is most representative of the ethnic and migrant groups from the Isan region, which is an area largely associated with relatively low socioeconomic status and an agricultural economy (Techataweewan et al., 2017). A collection of thin section histology slides of the right posterior femoral midshaft exists for 93 individuals from this sample. For this study, 69 of these femoral slides, from 29 females (46 to 93 years of age, mean 68.66 ± 13.35 years) and 40 males (35 to 85 years, mean 68.9 ±10.97 years) were selected with a sampling protocol based on adequately representing the sexes and age ranges given the bias toward males and older

adults in the sample. The pooled sex sample is 35 to 93 years of age (mean 68.8 ± 11.94 years).

Table 6-1 Thai and Australian samples subdivided by sex.

| Thai | *n* | Age range | Mean age | S.D | Australian | *n* | Age range | Mean age | S.D |
|---|---|---|---|---|---|---|---|---|---|
| Female | 29 | 46-93 | 68.7 | 13.35 | Female | 4 | 67-88 | 80.5 | 9.95 |
| Male | 40 | 35-85 | 68.9 | 10.97 | Male | 13 | 75-93 | 83.0 | 4.73 |
| Pooled sex | 69 | 35-93 | 68.8 | 11.94 | Pooled sex | 17 | 67-93 | 82.4 | 6.04 |

The second sample is composed of the femora of 17 Australian individuals that made up the newly established James Cook University Human Skeletal Histology Collection (JCUHSHC), formed from body donations bequeathed to the James Cook University Human Bequest Program for teaching and research in anatomy, Townsville, northern Australia. Individuals were included if there were no signs of recent trauma or bone pathologies, other than age-related changes such as osteoarthritis, which was to be expected in an elderly sample. All the individuals were body donors that died between 2014 and 2019 within 400km of Townsville. Occupation of the donors was not documented. Cause of death was known for all individuals. Age at death ranged from 67 to 93 years (mean age of 82.4 ± 6.04 years). Much of the sample consists of males 75 to 93 years of age (n = 13, mean 83.0 ± 4.73 years), and four females 67 to 88 years of age (mean 80.5 ± 9.95 years).

## Sample Preparation

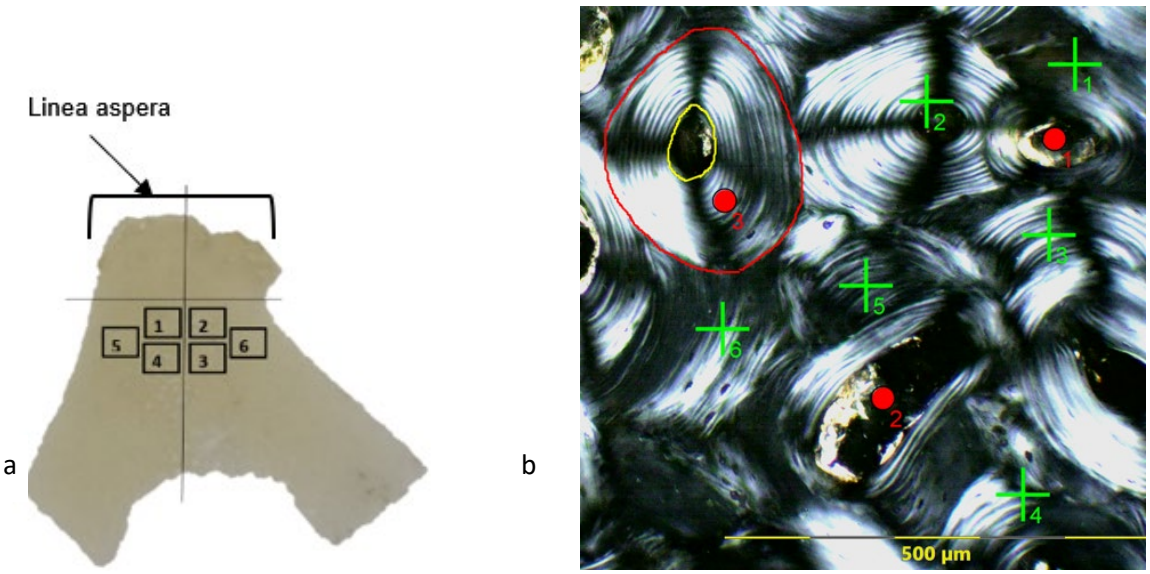
Revised histology standards were used as a guide for sample preparation (Garcia-Donas et al., 2017). A hand-operated rotary autopsy bone saw was used to remove a 1-2cm bone section, transverse to the long axis of the bone, from the right femora of embalmed cadavers, except for one male for which the left side was used. Soft tissue was easily removed from the bone sections after soaking in a warm water and liquid enzyme detergent solution at low

temperature overnight (see Chapter Five for a more details). This was followed by 24 hours of air drying, then dehydration in graded alcohol soaks (90%, 95%, and 100%, and a xylene rinse). Bone sections were embedded in epoxy resin (Buehler EpoxiCure™2) according to manufacturer specifications. A Buehler® IsoMet™ low speed, gravity fed diamond bladed cutter was used to reduce each section to a thickness of ~400µm which was then glued to a glass slide. Machine grinding with a Gemmasta™ GF4 lapping machine, followed by hand polishing was used to evenly reduce each section to ~100µm. This was followed by cleaning via ultrasonic bath, graded alcohol dehydration, and coverslip application (Bancroft & Gamble, 2008).

Transmitted and polarised light images were taken of each slide with an Olympus EP50 camera with 0.5 camera adapter mounted on an Olympus BX43 high-powered microscope (10x objective, 10 x oculars). Digital images were captured of six regions of interest (ROIs) within the mid-cortical region. A total of 7.09mm² was imaged per slide, with each ROI capturing a 1.182mm² area. ROIs were positioned either side of an arbitrary midline through the linea aspera, with ROIs 1 and 2 posteriorly and ROIs 3 and 4 anteriorly positioned, while ROI 5 was adjacent to ROIs 1 and 4, and ROI 6 was adjacent to ROIs 2 and 3 (Figure 6-1). Enough separation was kept between the ROIs to ensure no intact or fragmentary osteon was captured by more than one ROI. If an osteon was interrupted by the image border it was not included in the count/measurements if it could not be determined if they were intact or fragmentary. Osteons were counted and measured without prior knowledge of sex or age. Using Image J software, intact (regardless of their deviation from circularity) and fragmentary osteons were counted with the multipoint tool to measure OPD/mm², and via a tablet, the freehand area tool and a stylus was used to trace the perimeter of osteons with an intact cement line and the perimeter of intact Haversian canals, with the area of each feature calculated in µm². All imaging and measurements were taken by one observer (LTP). Time constraints during data collection meant that focus was placed on the three most common histomorphometric variables to be used in age estimation methods. Open access

FIJI/ImageJ® (Schindelin et al., 2015) was used to record these three variables within six ROI images per bone section for all individuals (adapted from Cho et al. (2002) and Goliath et al. (2016)):

1) Average osteon population density (OPD) per femur (#/mm²): per unit area, the number of intact osteons (completely surrounded by a cement line and containing a complete Haversian canal) and fragmentary osteons (osteons with their cement line breached by subsequent osteons or resorption cavities),
2) Mean osteon area (On.Ar) per femur: the average area within the cement line of intact osteons, including the Haversian canal, calculated in µm²,
3) Mean Haversian canal area (H.Ar) per femur: the average area within the Haversian canals of intact osteons, calculated in µm².




a b

*Figure 6-1 a) Posterior femur section with site of each of the six ROIs b) example of the histomorphometric features recorded per ROI: intact osteons (red dot), fragmentary osteons (green cross), osteon area (red outline), Haversian canal area (yellow outline).*

## Statistical Analysis

All statistical analyses were performed using SPSS 29.0 (IBM SPSS Statistics Inc.) with the significance level set at $p \leq 0.05$ or $p \leq 0.01$. Intra-observer measurement error was calculated from repeat measurement of all three histological variables on the ROIs of 10% of Thai individuals (n = 7) using a paired-samples *t*-test. Original and repeated measurements were conducted at least one month apart. Inter-observer tests were not necessary as all measurements and counts were conducted by a single observer (LTP).

Tests for the assumption of linearity and homogeneity of distribution were conducted on each sample. Normal distribution of each of the three histological variables was tested using the Kolmogorov-Smirnov test normality for the Thai pooled sex group (n = >50), and the Shapiro–Wilk test for the smaller female and male sub-samples (n = <50) (Razali & Wah, 2011). Non-parametric tests (Spearman's correlation and Mann-Whitney *U* test) were chosen due to their suitability for samples that violate normal distribution and are relatively small (Nachar, 2008). As age is known to influence all three histologic variables in the femur (Crowder et al., 2022), Spearman's rank correlation tests were used to determine if these relationships were significant in three groups [pooled sex, females, and males (all ages), and elderly males (75 to 88 years)] within the Thai sample. Mann-Whitney U tests (2-tailed) were performed to determine whether OPD, On.Ar, and H.Ar differed by sex or age group within the Thai sample, and between the Thai and Australian samples. Significance based on the asymptotic distribution (approximation of the true distribution) of the sample (*Asymp. Sig*) is reported unless the sample size is small (<40) or is not normally distributed, in which case the exact significant (*Exact Sig*.) is a better indicator of statistical significance (Nachar, 2008).

## 6.3 Results

### Thai Intra-Population Analyses

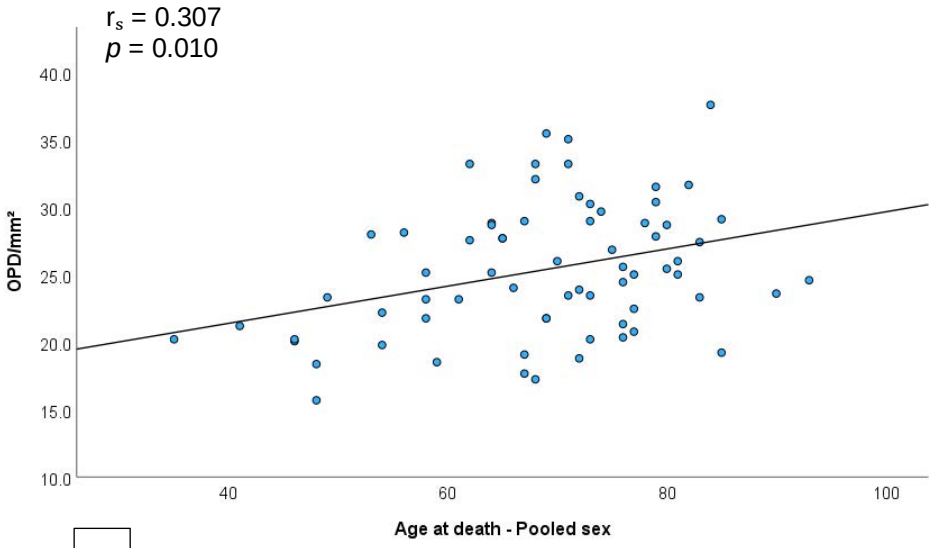
#### Intra-Observer Checks

Intra-observer measurement error was calculated from repeat count/measurement of all three histological variables on 42 ROIs (six ROIs per the seven individuals - 10% of sample). A paired-samples *t*-test determined that there was no statistically significant difference between the first and repeated observations (OPD $t = 0.633$, $p = 0.530$; On.Ar $t = -0.215$, $p = 0.831$; H.Ar $t = -1.608$, $p = 0.115$).

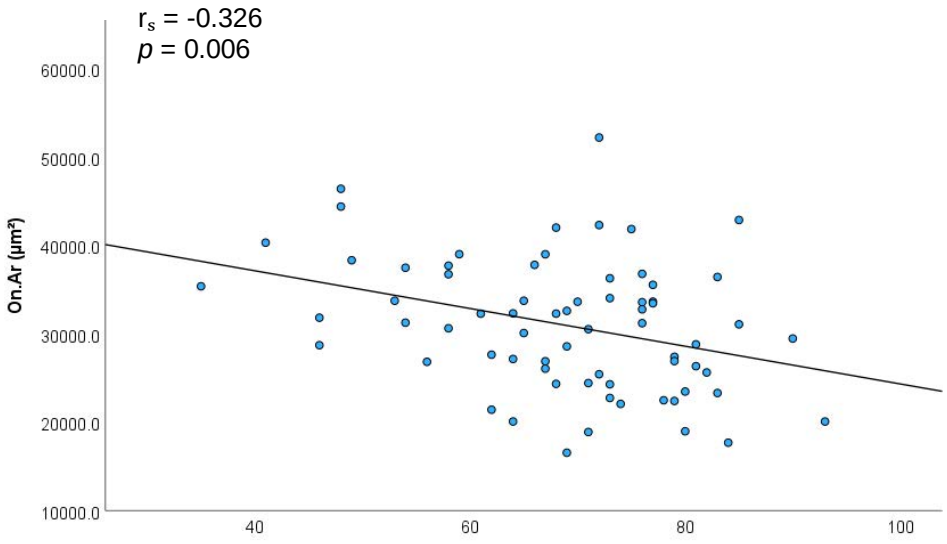
#### The Effects of Age on Bone Histomorphometric Features

Kolmogorov-Smirnov and Shapiro–Wilk tests of normality indicated that H.Ar was not normally distributed within the Thai pooled sex group ($p = 0.021$). All other histological variables within each of the groups (pooled sex, female, and male) were observed to have a linear relationship with normal distribution ($p > 0.05$). There were no outliers within the groups. Spearman's correlations revealed three statistically significant relationships between age and either OPD or osteon area (Table 6-2), which are illustrated via scatter plots (Figure 6-2). The scatter plots of non-significant correlations are presented in Appendix C.1. The results showed that in the pooled sex group there was a statistically significant, weakly positive relationship between age and OPD ($r_s = 0.307$, $p = 0.010$). However, when the sexes were observed separately, both male and female groups showed a similar weak strength of correlation between age and OPD (female $r_s = 0.336$, $p = 0.075$; male $r_s = 0.287$, $p = 0.073$). On.Ar decreased with age in the pooled sex, male, and female groups, with a statistically significant smaller osteon are for females ($r_s = -0.439$, $p = 0.017$) and the pooled sexes ($r_s = -0.326$, $p = 0.006$). For the male group, there was an absence of statistically significant correlations between age and any of

the histological variables. Descriptive statistics (Appendix C.2) show that H.Ar has the most individual variability compared to OPD and On.Ar. In the female group, Haversian canal area weakly increased with age ($r_s$ = 0.064, $p$ = 0.740), whereas in male group, canal area weakly decreased with age ($r_s$ = -0.292, $p$ = 0.067).




a




b

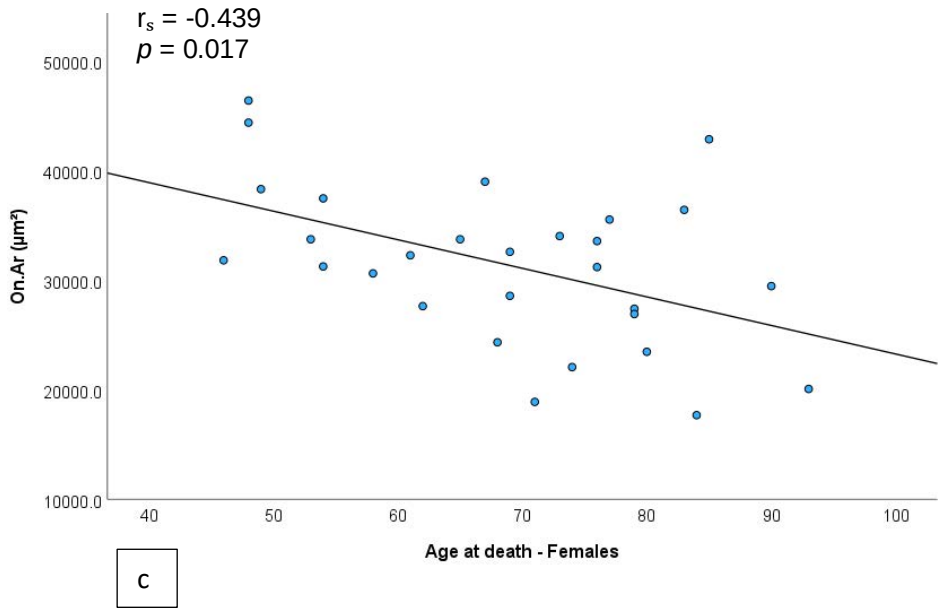



*Figure 6-2 Scatter plots for the Thai sample, with line of best fit showing statistically significant Spearman's correlations between age and a) pooled sex OPD; b) pooled sex On.Ar; and c) female On.Ar.*

Table 6-2 Spearman's correlations with age – Thai sample.

| Pooled sex n = 69 | variable | $r_s$ | Sig. (2-tailed) |
|---|---|---|---|
| | OPD | **0.307*** | **0.010** |
| | On.Ar | **-0.326**** | **0.006** |
| | $H.Ar_{Lg10}$ | -0.108 | 0.377 |
| Females n = 29 | variable | $r_s$ | Sig. (2-tailed) |
| | OPD | 0.336 | 0.075 |
| | On.Ar | **-0.439*** | **0.017** |
| | H.Ar | 0.064 | 0.740 |
| Males n = 40 | variable | $r_s$ | Sig. (2-tailed) |
| | OPD | 0.287 | 0.073 |
| | On.Ar | -0.240 | 0.137 |
| | H.Ar | -0.292 | 0.067 |

Statistically significant correlations are bold-faced; *correlation is significant at the 0.05 level; ** correlation is significant at the 0.01 level.

### Interpopulation Comparisons Between Thai and Australian Samples

Mann-Whitney U tests showed that distributions of the OPD counts and On.Ar were not similar between the Thai and Australian pooled sex groups, as assessed by visual inspection. Results indicate that there was a statistically significantly higher OPD in the Thai sample (25.000/mm²) compared to the Australian sample (22.740/mm²), $p = 0.023$ (Table 6-3, with U and z scores). The Thai sample had statistically significantly larger osteon area (31219.236 µm²) as opposed to the Australian sample (26416.040 µm²), $p = 0.021$. Haversian canal area was larger (but not statistically significantly) in the Australian sample (3497.315 µm²) compared to the Thai sample (3419.439 µm²), $p = 0.807$.

When the samples were sub-divided by sex, Mann-Whitney U tests showed that OPD was statistically significantly higher in Thai females (23.305/mm²) compared to Australian females (16.526/mm²), $p = 0.012$. On.Ar and H.Ar were similar between the two female samples. Thai males also had statistically significantly higher OPD (25.777/mm²) compared to JCU males (23.729/mm²), $p = 0.042$. Osteon area was also statistically significantly higher in Thai males (30322.788 µm²) than in Australian males (24269.700 µm²), $p = 0.037$.

The Australian male group consisted entirely of elderly males, so further tests were conducted using a Thai male group with the same age range (75 to 88 years) to see if removing younger Thai males changed the strength of relationships (Table 6-4, with U and z scores). Mann-Whitney U tests revealed that OPD was still statistically significantly higher for elderly Thai males (25.989/mm²) compared to the Australian elderly males (23.729/mm²), $p = 0.008$. Osteon area was no longer significantly different between the two samples ($p = 0.204$).

Table 6-3 Mann-Whitney U tests for interpopulation differences in mean distribution of each histological variable.

| | Pooled sex | | | Female | | | Male | | |
|---|---|---|---|---|---|---|---|---|---|
| | OPD/mm² | On.Ar µm² | H.Ar µm² | OPD/mm² | On.Ar µm² | H.Ar µm² | OPD/mm² | On.Ar µm² | H.Ar µm² |
| Thai | 25.000 | 31219.236 | 3419.439 | 23.305 | 31840.195 | 3537.111 | 25.777 | 30322.788 | 3036.276 |
| Australian | 22.740 | 26416.040 | 3497.315 | 16.526 | 28488.915 | 4465.422 | 23.729 | 24269.700 | 3302.633 |
| *U* | 796.500 | 800.000 | 564.000 | 14.000 | 45.000 | 70.000 | 358.500 | 361.000 | 251.000 |
| *z* | 2.277 | 2.315 | -0.244 | -2.428 | -0.717 | 0.662 | 2.037 | 2.088 | -0.186 |
| *p* | **0.023**[a] | **0.021**[a] | 0.807[a] | **0.012**[b] | 0.505[b] | 0.540[b] | **0.042**[a] | **0.037**[a] | 0.852[a] |

Correlations are significant at the 0.05 level (bold face). Significance is displayed as [a]asymptotic or [b]exact.

Table 6-4 Mann-Whitney U tests for interpopulation differences in distribution of each histological variable within elderly males.

| | Male (75 – 88 years) | | |
|---|---|---|---|
| | OPD/mm² | On.Ar µm² | H.Ar µm² |
| Thai | 25.989 | 28819.539 | 2417.957 |
| Australian | 23.729 | 24269.470 | 3302.633 |
| *U* | 136.500 | 110.000 | 66.000 |
| *z* | 2.668 | 1.308 | -0.949 |
| *p* | **0.006**[b] | 0.204[b] | 0.362[b] |

Correlations are significant at the 0.05 level (bold face).
Significance is displayed as [a]asymptotic or [b]exact.

## 6.4 Discussion

This study has identified significant differences in osteon area and population density between two genetically and geographically distant Asia-Pacific populations. Histological features have not previously been compared between skeletal samples from northeastern Thailand and northern Australia. It is important to document interpopulation variation in bone remodelling as differences are known to complicate the global application of histological age estimation methods used by forensic anthropologists and archaeologists. Inter-population comparisons revealed the greatest differences were in OPD and On.Ar. Overall, the Thai sample had significantly higher population densities of osteons, as well as larger osteons. It is likely that the elderly Australian sample had higher intracortical porosity (Laval-Jeantet et al., 1983) than the Thai sample which had a younger mean age by 13.6 years (SD 5.9 years).

Osteon size can be linked to OPD (Nor et al., 2014). An increase in the population density of osteons is possible because of an inverse reduction in osteon size with age, allowing more osteons to populate the field of view (Currey, 1964). Likewise, if many large osteons take up the cortical bone then there will be fewer osteons to count for a given area (Pratte & Pfeiffer, 1999). However, this does not fit the pattern seen in the Thai sample which has greater osteon area and mean OPD than the Australian sample. The size of osteons is also suggested to be controlled by the amount of cortical bone initially resorbed by osteoclasts, often in response to repair of microcracks of different sizes (Hazenberg et al., 2009). Likewise, low OPD counts are found in individuals with low bone mass (Pratte & Pfeiffer, 1999). In this present study, cortical area was not measured and therefore it is not known what effect this may have had on either sample.

When the samples were sub-divided by sex, it was observed that Thai males had significantly higher mean OPD and On.Ar compared to Australian males. The mean population density of osteons was also found to be significantly greater for Thai females than Australian females,

but On.Ar proved to be relatively similar between the two female samples. The results would have been influenced in part by a difference in mean age, and the age range of each group, especially for females. There were only four females, all over the age of 66 years, in the Australian sample, whereas almost a third of the Thai females were younger than 67 years. Over two-thirds of Thai males were under 75 years of age, whereas the entire Australian male sample was aged over 74 years.

Further sub-dividing the Thai samples into younger (≤ 74 years) and older groups (≥ 75 years) was possible. The older Thai male group demonstrated a significant inverse relationship between age and osteon area. It is normal in older adults for osteons to decrease in size and develop a more circular shape (Goliath et al., 2016). In contrast, younger adults have more well-organised primary bone which has a low OPD (Pfeiffer, 2006), but osteon numbers continue to increase with age, and again dip to lower levels in older adults as bone remodelling becomes less efficient and more chaotic in appearance (Currey, 1964). This could explain the lower mean OPD in the older Australian sample. Clinical studies suggest that metabolic and biomechanical factors, such as a lack of physical activity (Moreira et al., 2014), medication, and fluctuating levels of sex hormones (Seeman, 2008) in elderly adults, can significantly increase the risk of cortical bone thinning and higher porosity levels. Significant relationships between age and higher OPD and smaller osteons were found in the younger Thai female group. This could be related to relative cortical area. Cho et al. (2002) noted that larger cortical bone area is normal in young adults and equates to more space between osteons and therefore fewer osteons to measure per region of interest.

The Thai sample followed the expected age-correlated pattern of smaller osteons and higher OPD, whereas in the Australian sample, OPD actually decreased with age, as did On.Ar (examined in Chapter Five of this thesis). Some researchers could not detect any significant age-related change in osteon area between European and South African samples (Pfeiffer, 1998b; Pfeiffer et al., 2016). Jowsey (1966) observed a decrease in osteon size (diameter) in

the rib but no change in osteon size in the femur. However, a significant decrease in osteon size (diameter, area, and perimeter) with age has been reported in Malaysian upper and lower limb bones (Nor et al., 2014), Australian femora (Britz et al., 2009), and European American femora and ribs (Dominguez & Agnew, 2016; Goliath et al., 2016), while Japanese humeri showed a non-significant negative correlation between age and osteon size (average area) (Yoshino et al., 1994). Britz et al. (2009) were the first to identify the significant influence body weight (loading strain) has in decreasing the size of femoral osteons. They also acknowledge the role that endocrine regulation of bone, muscle, and fat metabolism likely has. Osteon size may be regulated by the amount of cortical bone available, with smaller osteons corresponding to an increase in relative cortical porosity and reduction in percent cortical area (Dominguez & Agnew, 2016). Mechanical load and strain are attributed to producing considerable influence on osteon size, with higher compression forces producing smaller osteons (Miszkiewicz & Mahoney, 2019). Bones under greater biomechanical strain, such as the femur from body weight and locomotive forces, have strain-restricted remodelling (Miszkiewicz et al., 2022b). In comparison, ribs are under minimal biomechanical forces, mainly from cyclic bending strain from lung expansion during breathing (Agnew & Stout, 2012). Crowder and Rosella (2007) have indicated that OPD can notably vary between the vertebral-sternal and vertebra-costal (floating) ribs within an individual due to biomechanical differences. Variable cortical area has also been proposed as a reason for this inter-rib variability (Frost, 1969).

Human groups with nutritional deficiencies and health issues as a result of experiencing social inequality and inequity in access to various economic resources are at a greater risk of chronic disease (Dowd et al., 2009), lower bone mineral density (Arabi et al., 2004), and compromised bone remodelling (Miszkiewicz & Cooke, 2019). Other studies found no significant differences in OPD between ancestral groups if they experienced a similar socio-economic background (Pratte & Pfeiffer, 1999). In northeastern Thailand body donors, low socio-economic status is suggested to affect the majority of donors whom have low-paying occupations (monk/nun, farmer, labourer) (Techataweewan et al., 2017), or a lower level of education (Techataweewan

et al., 2018). Individuals only list their occupation at the time of donor registration, and this does not necessarily represent the work they have been involved in for the bulk of their life. In this instance, occupation may be of little relevance to assess socio-economic status and its effect on bone remodelling. Occupation and socio-economic status of the Australian donors was not recorded. According to the Australian Bureau of Statistics, the resident population in the local government area of Townsville ranks above average (7$^{th}$ decile) in the Socio-Economic Indexes for Areas (SEIFA) (ABS, 2021). This ranking is based on census data on education, income level, occupation/employment, housing, and family structure. It is difficult to compare what effect of socio-economic status and lifestyle has on the rate of bone remodelling between the two populations when this personal information is not recorded.

In contrast to OPD and On.Ar, scatter plots and Spearman's correlations illustrate that age does not have a significant influence on H.Ar in either the Thai or Australian samples. Canal area in this study was highly variable, with a weak inverse relationship to age in both samples, so that H.Ar was only marginally higher in the Australian sample. Pfeiffer (1998b) also reports great individual variability in H.Ar with no significant relationship to age and sex, or ancestry (Pfeiffer et al., 2006). There are several possible reasons why individuals would have a high proportion of larger Haversian canals. They may have been experiencing increased metabolic activity at the time of their death (Pfeiffer et al., 2006), increased bone resorption in response to disease (Yajima et al., 2007), or osteocytes may have prematurely signalled osteoblasts to stop secreting bone matrix (Qiu et al., 2003) to ensure Haversian canals are large enough to provide adequate nutrients to osteocytes throughout the osteon (Metz et al., 2003).

The mean H.Ar was larger in the Thai and Australian female population groups compared to each male group. Greater porosity in females has been correlated to an age and hormone related decline in three factors: cortical thickness (endocortical resorption), bone mass and bone mineral index (Andreasen et al., 2020; Seeman, 2013; Thompson, 1980). Women undergoing menopause experience fluctuating oestrogen levels, causing bone to be resorbed

faster than it is produced (Ji & Yu, 2015). This initiates a steady decline in bone density, which is why an increase in porosity (osteoporosis) is more commonly seen in postmenopausal women compared to men of the same age, or younger females (< 35 years) (Gulsahi, 2015; Ji & Yu, 2015).

There are no standardised criteria for choosing microscope settings and fields of view (FOV), and methods differ in how they discern between intact and fragmentary osteons (Villa & Lynnerup, 2010a), which only exacerbates inter-study variability. To evaluate mean osteon size and circularity, some methods call for only osteons with an intact cement line to be measured, others will include osteons with some (10%) overlap from subsequently forming osteons. As Takahashi et al. (1965) pointed out, larger and more irregularly shaped osteons have a higher likelihood of being remodelled by subsequent osteons than do smaller osteons, and therefore are often excluded from evaluation of mean osteon area (On.Ar). Age estimation will thus be impacted by chosen methods, and the effect of osteon size and shape. In the present study it was possible to mitigate against such differences as all histological variables in the two population samples were measured by one observer using the same method and field of view. However, the FOV for each ROI in this study (1.182 $mm^2$) is smaller compared to those used elsewhere, for example 2.06 $mm^2$ (Karydi et al., 2022). A smaller field of view increases the likelihood of larger osteons being cut off by the image border and therefore excluded from intact osteon area measurements.

The effects of age, sex, and ancestry on bone remodelling were considered in this study, but it is difficult to pinpoint if differences between the Thai and Australian samples are predominantly due to genetic diversity or environmental influences. There is a wealth of research documenting multifactorial influences including socioeconomic status, health, disease, nutrition, body mass, and lifestyle (Beresheim et al., 2018; Cho et al., 2006; Keough, 2007; Miszkiewicz, 2019; Miszkiewicz & Cooke, 2019; Paine & Brenton, 2006; Richman et al., 1979; Schultz, 2001) that could not be controlled for in this study. There were inevitably also

contributing effects from differences in population demographics between the two samples, namely very few females and no young adults in the Australian sample. This is a limitation commonly seen in ethically sourced skeletal collections that are produced from body donations (Boulware et al., 2004; Rokade & Gaikawad, 2012). Regardless of the exact cause of the histomorphometric variability observed, this study supports the recommendation for the development of population-specific age estimation methods.

## 6.5 Summary and Conclusion

This study demonstrated significant age and sex related histomorphological variation between northeastern Thai and northern Australian samples in OPD and On.Ar. Between the two populations, the Thai sample exhibited significantly higher OPD and increased osteon area in both the pooled sex and male groups, as well as significantly higher OPD in the females. There appears to be greater individual variability in Haversian canal area regardless of population, with the Australian sample having only marginally increased H.Ar than the Thai sample. Significant positive correlation between age and OPD was observed within the Thai pooled sex and within the Australian male groups, while On.Ar and age have a significant negative correlation within Thai pooled sex, female, and older male (≥75 years of age) groups. These results are largely consistent with studies on other populations which document a trend of increasing OPD and decreasing osteon area with age, and the most individual variability in Haversian canal area. This study importantly provides validation of histomorphometric variation between these two populations that furthers the argument that population-specific age estimation methods are necessary. Nutrition, disease, and physical activity are also major influences on bone maintenance and mineral homeostasis but are harder to measure as this data is usually not provided by donors. Future research with a larger Australian sample, and a better representation of females and young adults for both populations are desirable to expand on these results and to fully explore age-related changes in remodelling. Expanding future analyses to include other age-related histomorphometric variables, and incorporating

different bones, and different regions of bone, will build on the foundation that has been laid by this present study.

## 6.6 Chapter Summary

This chapter documented the range of variation of age-related histomorphometric features between two population samples which each have their own distinct ancestry, environment, and sociocultural history. This is built on the previous chapter which found significant intraskeletal variation within the individuals of one population. The purpose of these studies was to increase the information based on human variability in cortical bone remodelling and understand why these differences exist. Next, the concluding chapter brings together the key findings of the individual studies within this thesis.

# Chapter 7 Discussion and Conclusion

In this concluding chapter of the thesis, the key findings and significance of the research are summarised in relation to each of the five objectives, followed by a discussion of the limitations and recommendations for future research. The aim of this thesis was to improve our understanding of intraindividual and interpopulation variation in skeletal degeneration to assist in improving the development and revision of adult age estimation methods. This was conducted using Australian and Thai population samples, both of which have not been explored as much as American and European populations have in the development of adult age estimation methods (Go et al., 2019).

Adult age estimation via the skeleton relies on measuring relatively predictable degenerative macroscopic changes to articular surfaces (such as the auricular surface or pubic symphysis of the pelvis, and sternal rib ends) (Garvin et al., 2012) or microscopic changes to cortical bone (including, osteon population density and size) that accumulate over time (Kerley, 1965). As alluded to in the introduction chapter, a major issue in adult age estimation is that the rate and severity of degenerative change is heavily influenced by individual variation, sexual dimorphism, older age, and population-based biological and sociocultural factors (Aiello & Molleson, 1993; Gocha et al., 2015; Schmitt et al., 2002). The multifaceted relationship between age and bone degeneration and remodelling makes it challenging for any biological anthropologist or bioarchaeologist to produce an estimated age range that matches closely to an individual's actual age at the time of their death.

Even though age estimation measured through macroscopic skeletal changes has been used for over a century (Todd, 1920), and histological methods for almost 60 years (Kerley, 1965),

there is still so much to discover about the complexities of intra-skeletal and inter-population variation for genetically distinct population groups world-wide. Part of the problem centres on the reliance of (mainly) American and European skeletal collections, that have been readily accessible to researchers for a number of decades, for the development of age estimation methods (Kimmerle et al., 2008). Meanwhile, other countries are still establishing ethically sourced human skeletal collections from body donors and forensic cases. Human bone histology collections are even rarer than skeletal collections, limiting how we gain valuable data on individual- and population-specific variation.

## 7.1 Addressing the Objectives

To tackle these issues the first objective of the thesis was to evaluate how interpopulation skeletal variability impacts the reliability of age estimation methods when applied to a northeast Thai sample. This was conceived to be useful as a reference to human skeletal analysts to select which method would provide the best age estimates for a Thai target sample. Once the first objective had been established, the subsequent objectives aimed to build on the current understanding of human skeletal variation. This was achieved by focusing on two populations from the Asia-Pacific region to document macroscopic changes to the vertebrae and microscopic changes to cortical bone that can be useful as age predicting tools. This present study was made possible by the establishment of human bone histology collections using body donations from northeastern Thailand and northern Australia, and macroscopic analysis of several pre-modern northeastern Thai skeletal samples. The key findings and significance of each objective is summarised below.

*Objective 1: Appraise how commonly used age estimation methods, developed on American and European populations, are subject to complications from interpopulation-based skeletal variation, when applied to a genetically distant (northeast Thai) population.*

Forensic anthropologists and bioarchaeologists analysing skeletal remains of individuals of Southeast Asian ancestry, such as Thai, are faced, by necessity, with having to use age estimation methods that represent Western skeletal maturation rates (Gocha et al., 2015). Many individual studies have tested these Western ancestry-based methods on Thai skeletal samples with varying degrees of success. What was lacking was a cohesive guide that brought all the individual studies together in the one reference, making it easier for skeletal analysts to decide the best method to use based on the sex, age, and ancestry profile of their Thai target sample. Due to the admixture of genes and migration in the Southeast Asian region, this reference should also be applicable to other Southeast Asian populations.

This study confirmed that the commonly relied upon age estimation techniques, which have for the most part been developed on northern American and European physiologies, produce a wide range of standard errors when applied to the skeletal remains of individuals from the northeast of Thailand. This was established by reviewing and synthesising the results of individual studies that had each tested age estimation methods on Thai post-cranial skeletal remains. This chapter was published in 2022 as *Adult age at death estimation: methods tested on Thai postcranial skeletal remains*. L.T. Pedersen and K. Domett, *Anthropological Science*, 211-219. https://doi.org/10.1537/ase.211219.

This study found that bias and inaccuracy tended to steadily worsen in the age estimation of Thai adults as they got older, with errors between estimated age and chronological age reaching as high as 32.2 years for females and 27.2 years for males. For Thai adults younger than 40 years of age, it was established that the Suchey–Brooks pubic symphysis method

(Brooks & Suchey, 1990), the methods of Lovejoy (Lovejoy et al., 1985a), and Osborne (Osborne et al., 2004) using the auricular surface, and the Rissech acetabulum method (Rissech et al., 2006) produce lower bias and higher accuracy. Suchey-Brooks was the most widely adopted method, and it produced age estimates with the highest accuracy (up to 93%) compared to other methods. However, this accuracy was only achievable with age estimates within ±2 SD, which produced age estimates that were, at a minimum, approximately 10 years from known age in younger adults (Gocha et al., 2015). Accuracy dropped to just 36% for Thai males, and 38% for Thai females within ±1 SD (Schmitt, 2004). The success of this method could have been influenced by the diverse populations represented in the reference sample (European, North and South American, and Asian) (Brooks & Suchey, 1990). The sternal rib end method (İşcan et al., 1984, 1985) showed the weakest correlation with chronological age, therefore it is recommended that it be avoided as a stand-alone method of age estimation.

Overall, bias and inaccuracies were lower for males than females within almost all age groups. This highlights how the sex and age demography of the reference sample, used to develop each method, are affecting how successful it was to estimate the age of Thai samples. As expected, it was found that methods developed with a reference population biased toward younger adults and males, such as the Suchey-Brook method, would tend to be less accurate for females, and underestimate age of older Thai adults. In comparison, the Buckberry–Chamberlain auricular surface method (Buckberry & Chamberlain, 2002) was developed on a sample biased toward older adults, so it was most reliable for Thai adults aged over 50 years. This reiterates how important it is that the chosen method has been developed on a reference sample with a sex and age profile that closely matches that of the target sample. Older adults are often under-represented in the reference populations, so estimating age of older adults is often inadequate. The bias toward older individuals in the Thai and Australian samples used in this study was advantageous toward establishing if vertebral osteophytes and histology would be useful age indicators well beyond the usual 50-year limit of most current methods, and this was assessed in the following objectives.

When lesser-known techniques were tested, such as vertebral osteophyte prevalence or histologic and biochemical methods (on femora), the small Thai sample sizes showed good correlation with age but will have to be tested on larger samples to account for individual variability. It is a recommendation of this study that caution be exercised when using the above methods to estimate age for forensic contexts, due to the lack of precision when applied to individuals of Thai ancestry. It would be best to use a combination of different methods based on different skeletal elements, rather than rely on age estimates from just a single method. It is important to use a method, where possible, in which the reference sample is similar in age and sex demographics to the target sample. Further research into the correlation between age and variability in degenerative characteristics of bone is necessary, particularly between different ancestral groups, to produce meaningful age estimates. This was the impetus for the next objective, stemming from the opportunity to compare age-related vertebral degenerative characteristics between two pre-modern Thai archaeological samples, which are discussed below.

> *Objective 2: Validate using osteophytes as age indicators, by evaluating how vertebral osteophyte formation rates differ between two pre-modern Thai populations (Neolithic to Iron Age), and considering possible contributing factors, such as different lifestyle/physical activities.*

There have been several studies that have assessed VO formation and distribution in relation to age in modern Thai samples. But a lack of standardisation in methods meant that the way VO was described, and its formation scored, was very different between studies. A common finding was that males had a higher prevalence of VO compared to females (Chanapa et al., 2014; Namking et al., 2008; Suwanlikhid et al., 2018). This conflicted with Chanapa and Mahakkanukrauh (2011) who found no sexual dimorphism in VO prevalence. However, they had limited their study to cervical vertebrae only. There was conflicting evidence of the age at which osteophytes started forming in samples from northern Thailand. Chanapa et al. (2014)

observed this to occur no earlier than 35 years of age, compared to 26 years of age in another study (Suwanlikhid et al., 2018). Both samples had a similar mean age, however the discrepancy could come down to the very different scoring systems. Lumbar vertebrae were shown to have the most significant correlation with age compared to other vertebral regions (Chanapa et al., 2014; Praneatpolgrang et al., 2019; Suwanlikhid et al., 2018). What is missing in these Thai studies is a critical discussion of the possible influence of environmental and socio-cultural influences on VO prevalence in different age groups.

The development of osteophytes is known to have multifactorial influences, not only correlating with advancing age, prevalence of VO also has a relationship to working lifestyles (activity patterns), body mass, genes, health, nutrition, and disease (Battié et al., 2004; Chanapa, 2016; Echarri & Forriol, 2002; Park, 2017; Puntumetakul et al., 2014). This shows how important a multifactorial approach is to evaluate the significance of osteophyte prevalence.

This thesis used a comparison of pathological VO prevalence between two prehistoric Thai populations, both of which occupied the same geographical region of northeast Thailand but in different time periods: Ban Non Wat (BNW – Neolithic to Bronze Age) and Non Ban Jak (NBJ – Late Iron Age). This allowed VO formation to be examined in the context of cultural and technological innovations over many generations. This chapter is in preparation for submission to a journal: *Vertebral osteophytosis in prehistoric northeast Thailand*, Lucille Pedersen, Afua Adjei, Georgia Stannard, Anna Willis, Nigel Chang, Hallie Buckley, Siân Halcrow, Louise Shewan, Dougald O'Reilly, Charles Higham, Kate Domett.

The most noteworthy results from this study showed that none of the Late Iron Age young adults were observed with VO. In comparison to the Late Iron Age, a significantly higher prevalence was recorded in young adults of the Neolithic (25%) and Bronze Age (36.7%). Clinical and archaeological studies show that osteophytes can form in individuals as young as

20 years, albeit at a low incidence (Igbinedion & Akhigbe, 2011; Kim et al., 2012; Nathan, 1962). These studies suggest that mechanical stress from repetitive load bearing activities, strenuous sports, or obesity had a bearing on the formation of VO in the young adults. Degenerative bone disorders (osteoarthritis), or degenerative conditions (Schmorl's nodes) were noted to be absent in these young adults. One possibility is that Late Iron Age young adults experienced less mechanical stress on their vertebrae, but it is unknown what additional effects on osteophyte development there may have been from genetics, nutrition, and metabolic factors as these could not be measured in this study.

Another key finding of this study was that there was not the expected significant increase in prevalence of pathological VO from the Neolithic to the Late Iron Age. Instead, there was the same prevalence (51%) in both the Bronze Age and Late Iron Age individuals, and (insignificantly) more in the Neolithic (60%). Examination of pathological VO by vertebral region and sex show there is a similar prevalence pattern within the Neolithic, Bronze Age, and Late Iron Age periods: prevalence was highest in males compared to females, and highest in the lumbar region compared to the thoracic and cervical regions. Sexual division of labour is often expressed as one possible explanation for sex bias in VO prevalence (Cho et al., 2012; Sofaer Derevenski, 2000; Wilson, 1993). However, trying to identify from the archaeological record the activity-related roles that males and females participated in can be difficult, and this view needs to be regarded with some caution. The main aim of this study was to build on the current knowledge of the influences on osteophyte formation and pave the way for future development and refinement of age estimation techniques using VO formation to estimate age in a Thai population. The key findings however indicate that, in these samples, vertebral osteophytosis rates can be used to differentiate between young and older adults but have a limited usefulness as a means to differentiate between mid-aged and older adults, which has long been a problem in adult age estimation. The next two objectives moved away from macroscopic skeletal analysis and into histomorphometric analysis of human cortical bone, in order to address 1) animal and human research that has determined that bone size

effects remodelling events (Currey, 2003); 2) the lack of data on populations from the Asia-Pacific region and; 3) increase our knowledge of age-related microscopic changes in older adults.

*Objective 3: Within a northern Australian population, evaluate intra-skeletal variation of histomorphometric features used in age estimation methods.*

The current literature documents the need for histological age estimation methods to be developed on specific bones (Crowder & Dominguez, 2013; Gocha et al., 2019). Studies have shown that, compared to bones in the upper body, the femur is placed under greater biomechanical loading regimes and torsional and compressive forces, and a different rate of metabolic activity (Pfeiffer et al., 2006; Ruff et al., 1993), both of which have an effect on cortical remodelling rates (Eleazer & Jankauskas, 2016). Ribs are reported to be under constant, but low-level loading from the breathing cycle (Skedros et al., 2013) but are also more sensitive to metabolic changes (Eleazer & Jankauskas, 2016). The humerus, in comparison, has less mechanical loading from body mass than the femur, but is under more torsional strain from manipulative arm and hand movements than the rib (Sumner & Andriacchi, 1996). Age-related histology studies on Australian individuals has largely been restricted to femoral anterior midshaft sections from the Melbourne Femur Research Collection (MFRC) (Thomas & Clement, 2011). Therefore, intraindividual comparisons between different bones have not been made for this population.

Animal and human research has determined that bone size effects remodelling events (Currey, 2003), so much so that short and wide (robust) bones tend to have more remodelling events and a larger cortical region compared to long and thin (gracile) skeletal elements (Currey, 2003; Jepsen et al., 2015). For example, a more robust femur will tend to have higher OPD and larger Haversian canals than a more gracile femur (Goldman et al., 2014). Yet, the effect of gross bone size on osteon counts and measures is not commonly accounted for

(Miszkiewicz & Mahoney, 2019), especially in age estimation techniques. To address these gaps in the literature, this study compared remodelling characteristics of OPD, osteon area (On.Ar) and Haversian canal area (H.Ar) in midshaft bone sections of the posterior femur, anterior humerus, and rib cross-section, and accounted for bone robusticity, within individuals of an Australian sample. These three variables are commonly used in histological age estimation methods. It was hypothesised that bone size, and localised biomechanical strain magnitude and metabolic activity would cause variation in remodelling between bones, which was yet to be fully explored in an Australian population.

Bone robusticity indices (circumference/bone length x 100) (Ruff et al., 1993) were calculated to account for the size difference between the femur and humerus. Cortical bone histology was used to address some of the challenges in using macroscopic methods to correctly estimate age in older adults, that were discussed in objective one. The average age of death of the Australian body donors was 82.4 years and there were only four females in the sample. The reasons for this are discussed in the limitations section at the end of this chapter. Due to the sample demographics, the results of this study are informative of remodelling occurring in Australian males in their $7^{th}$ or $8^{th}$ decade of life. The elderly sample allows the age of OPD asymptote to be investigated, for which there is currently both limited and conflicting evidence (Crowder et al., 2022; Crowder & Rosella, 2007; Gocha et al., 2019).

Key findings of this study showed that there were notable differences in remodelling patterns between the humerus, femur, and rib in Australian males (≥ 75 years). Statistically significant correlations were observed between age and a higher OPD count in the femur, and larger H.Ar in the rib. This shows a possible relationship to biomechanical strain, causing locally intensified remodelling in the posterior femur. The results showed that as males got older, porosity in the rib increased as in-filling of the osteon slowed or was halted, this could be in relation to the rib being under greater metabolic influence than the femur (Skedros et al., 2013). Intra-skeletal comparisons of the rate of remodelling between the three elements within

each male revealed that the size of Haversian canals was statistically significantly larger in the humerus when compared to the rib. Statistically significantly higher OPD counts were found in the rib when compared to the femur. Data were then adjusted for bone robusticity to reveal that remodelling characteristics between the femur and humerus were more similar than the previous analyses had indicated. This is despite the upper and lower limbs of an individual being subject to very different rates of biomechanical loading. As a result of this study, it is highly advisable that bone size should be accounted for by using robusticity indices when developing histological age at death estimations. It is evident that age estimation methods developed on a particular bone, most commonly the femur, are not necessarily applicable to bones of a different size. This helps to fill the gap in knowledge regarding senescence and bone remodelling, which is much needed in the quest to accurately estimate the age of older adults. The next objective was to then compare this Australian sample to a Thai sample to test if there were significant interpopulation differences in age-related histomorphometric features, that would warrant the need for population-specific age estimation methods to improve accuracy and reliability of age estimation methods, which has long been an issue.

> *Objective 4: Contrast cortical bone remodelling of the femoral mid-shaft between Thai and Australian samples. Identify if there are significant inter-population variations that justify the need for population-specific age estimation methods.*

The concept of histological age estimation methods is to use a reference population to quantify (relatively) predictable remodelling rates of cortical bone by bone maintenance units (Pfeiffer, 1992). Reference population samples are dominantly American (African American and European American ancestry groups), while far fewer are based on Japanese or South African ethnic groups, and only a few use Dutch, Korean, Italian, or Swiss sample populations (Andronowski & Cole, 2021). Population-specific histological adult age estimation methods have not been developed using an Australian sample, and only one using a Thai sample

(Chompoophuen et al., 2019). As discussed in Chapter One, this can partly be attributable to a previous lack of prepared human bone histology collections for these two populations. Objective three discussed how the current knowledge of normal histological age-related variation in the Australian population is largely based on the femoral anterior midshaft samples from the MFRC. What was yet to be analysed was how much variability there is in cortical bone remodelling between these two populations. Two recently established human bone histology collections representing Australian and Thai population samples enabled histomorphometric comparisons to be made in this study. The focus was on the posterior femoral midshaft region to identify the similarities and differences in remodelling. This has expanded the knowledge of individual and population variation to assist in determining if there is a need for population specific methods for the Asian-Pacific region.

The results illustrate that there are significant interpopulation differences in OPD and osteon area, whereas similarities were observed in mean Haversian canal area between the two populations. Firstly, sex and age groups were analysed to see how the three histomorphometric variables (OPD, On.Ar, and H.Ar) were affected by age. Three statistically significant correlations with age were observed within the Thai sample. The pooled sex group had significant correlation with increasing OPD ($p$ = 0.010) and decreasing On.Ar ($p$ = 0.006) with age. The Thai females had a significant inverse relationship between On.Ar and age ($p$ = 0.017). In the Australian sample, only the males had a significant correlation between age and OPD ($p$ = 0.038). These results largely reflect a relatively consistent global trend for OPD to increase with age, while osteon area or size (diameter or perimeter measurements) decreases (Britz et al., 2009; Goliath et al., 2016). Haversian canal area and size measurements seem to show less significant change with age (Pfeiffer, 1998b; Watanabe et al., 1998). Other studies showed no significant changes in osteon size in the femur with age (Jowsey, 1966).

The key results when comparing the two population samples showed that the Thai sample had a statistically significantly higher OPD count compared to the Australian sample, as well

as a statistically significantly larger osteon area. Compared to the Australian age and sex groups, a statistically significantly higher OPD was seen in Thai females and males (all ages group and elderly males (>75 years)). Thai males (all ages group) also had a statistically significantly larger osteon area. Australians had only marginally larger H.Ar than the Thai sample. The Thai sample consisted of both younger adults (35 to 66 years) and older adults (67 to 93 years), whereas the Australian sample comprised of adults that were all over the age of 67 years, and this age demographic difference would likely have a bearing on these comparisons. The small Australian sample size reduces the chances of detecting effects that might be there. This is discussed in greater detail in the limitation section of this chapter. The effects of age, sex, and ancestry were considered in this study, but other parameters could not be controlled for including occupation, body mass, nutrition, and disease; all of which clinical studies show to have a significant effect on histomorphometric parameters (Moreira et al., 2014; Seeman, 2008).

This study importantly provides a validation of histomorphometric variation between the northern Australian and northeastern Thai samples. The significant differences in remodelling rates highlight the need for population-specific age estimation methods. Incorporating this knowledge into age estimation methods is a step toward the goal of providing reliable, replicable, and accurate biological identity to unknown human remains.

*Objective 5: Based on the findings from the previous objectives, evaluate whether the currently adopted age estimation methods require reconsideration.*

This thesis began with a review of how the accuracy and reliability of traditional macroscopic adult age estimation methods developed on Western populations fluctuated considerably when applied to Thai samples, especially for older adults. This highlighted the need for the development of reference samples with a better representation of different ancestral groups, both biological sexes and all age groups (young, mid-aged, and older adults). Steps need to

be taken to document as much antemortem data (as ethically possible) on health and lifestyle to better understand the influence on skeletal aging traits.

The confirmation that VO prevalence had a significant relationship with age, sex, and vertebral region improves our understanding of degenerative changes within the human skeleton. This knowledge will assist in improving age estimation methods using the vertebrae and shows that it has relevance for discerning age of elderly Thai adults. The advantage of using archaeological samples from Neolithic to Late Iron Age time periods is that it allowed biocultural changes to be observed over a long period, which cannot be done within a modern population. This archaeological data can complement current clinical research in degenerative changes within the vertebrae and assist in the interpretation of quality of life in the past alongside studies of trauma, health, disease, and diet.

Significant interpopulation differences were found in cortical bone histomorphometric variables (OPD and osteon area) between northern Australians and northeastern Thais. This warrants the need for population-specific age estimation methods, and a recommendation that more histomorphometric variables be evaluated between these two populations to determine the extent of individual variability. The older age of the samples used in this thesis have shown that OPD continued to increase with age even in the elderly adults, so osteon asymptote did not appear to be a concern. This suggests that OPD would be useful for estimating age in adults over 50 years in these two populations, something that macroscopic methods struggle to do. This thesis has shown that in elderly Australian males, remodelling dynamics vary significantly between the femur, humerus, and rib within the one individual. The rib had significantly higher OPD than the femur but significantly lower Haversian canal area than the humerus, showing that anatomical site will have a bearing on age estimation. Differences in bone size are rarely considered in age-related histology studies. This thesis, however, established that bone robusticity has an underlying effect on histomorphometric features,

changing the strength of correlation with age. This study strongly recommends that future studies incorporate bone robusticity measures into micro-analysis.

## 7.2 Limitations

This study has several limitations. Firstly, the sex and age demographic, and size of each sample was restricted by several factors. Taphonomic processes affected the preservation and completeness of vertebral rims in the Neolithic to Late Iron Age Thai individuals observed for vertebral osteophytes in Chapter Three (objective two). This resulted in fragmented or low numbers of vertebrae able to be assessed for VO. The modern Australian and Thai skeletal samples for the histological analyses were mostly derived from human body donors, with the age and sex demographic of each sample limited by several factors. While there is a single national organ donation register in both Australia and Thailand (Nivatvongs et al., 2008; Rosenblum et al., 2012), bequeathing an entire body to science requires potential donors to contact universities in their local area that have a Human Bequest Program. Upon an individual's death, their next of kin (NOK) may choose to revoke consent for body donation (Jenkin et al., 2023). Numbers have not been reported on this, however, in the case of organ donation, in Australia there is a 40% revocation rate by NOK (Neate et al., 2015). This is similar to the 42% refusal rate in the United Kingdom (Hulme et al., 2016), but higher than that in the United States (25%) (Siminoff et al., 2013). A range of emotional, religious, and cultural belief factors all play a role in organ donation, particularly in a multi-cultural country such as Australia (Marck et al., 2015), and these factors likely have a similar effect on body donation rates. The average age of individuals at the time of registering for body donation was reported to be 72 years in Australia (McAndrew, 2015), and 50 years in the Khon Kaen region of Thailand (Techataweewan et al., 2018) which explains the lack of young adults in each sample. Donor rates are reported to be steadily rising each year, but expanding the donor pool to include more young adults for future studies may require a change in cultural mindset (Halldorson & Roberts, 2013) and educating the population on the importance of body

donation to medical research (Barcellos et al., 2005). The bias toward older aged adults, and in the case of the Australian population, the lack of females, means that these samples are not fully representative of the general population. The older age of the Australian female sample (>67 years, average 80.5 years) represents only postmenopausal individuals, while the Thai females had peri- and post-menopausal individuals (>45 years, average 68.7 years). Premenopausal patterns in bone micro- and macroscopic structure could not be examined. The larger Thai sample makes it less likely to detect relationships in the smaller Australian sample, even if the relationships were identical in both, or even if the effect was actually stronger in the Australian sample. The small Australian sample made it more difficult to detect real differences between Thai and Australian subjects, and difficult to detect differences between age and sex subgroups, even if the true differences in the population as a whole were really substantial. Future research on a much larger Australian sample can build on this study but it will take time to establish such a sample due to ethical considerations and donor limitations.

Ethical considerations mean that there is a limit to the type of antemortem information that can be collected from each individual in this study. It is known that osteophyte formation and bone remodelling are affected by a plethora of environmental, health, and cultural-specific lifestyle variables, including occupation, activity, trauma, disease, diet, and family history (Eleazer & Jankauskas, 2016; Leslie, 2012; Paine & Brenton, 2006; Recker & Deng, 2002). However, donors are not required to share such personal information, which makes it difficult to interpret and compare intra- and inter-population differences when the combined effect of these factors is unknown.

Lastly, to be as ethical and conservative as possible, bone samples removed from cadavers were comprised only of small midshaft sections ~2cm wide, taken from the posterior of the femur and the anterior of the humerus. This prevented bone remodelling rates to be compared between different regions within an entire cross-section of a single bone (anterior, posterior,

medial, lateral, anteromedial, anterolateral, posteromedial, posterolateral). Remodelling rates can vary considerably between different regions of bone if they experience notable differences in biomechanical loading, torsional, compressive, and flexion forces (Chan et al., 2007; Dominguez et al., 2020; Gocha & Agnew, 2016). This means that age estimation methods developed using one area of a bone (commonly the anterior femoral midshaft) usually cannot be applied to another area (such as the posterior femoral midshaft).

## 7.3 Future Research

The review of adult age estimation in Chapter Two of this study revealed a lack of standardisation of methods. There is a wide variation in descriptive terminology, identifying features of each age-indicator, age categories, and statistical analysis reporting, which limits comparison of results between studies (Falys & Lewis, 2011; Garvin et al., 2012; Garvin & Passalacqua, 2012). This is clearly a pressing matter that needs to be addressed in the near future as it directly influences the methods skeletal analysts choose to use and the outcome of age estimation.

Future analysis on Australian intra-individual skeletal variability could incorporate a larger sample that would build on the results presented in Chapter Five of this thesis. However, this is easier said than done, due to the restrictions associated with body donation from which bone samples are sourced, as discussed in the limitations. Future studies on how to increase body donor registration and family consent rates in Australia would allow the JCUHSHC to grow and thus document greater individual variation. This would be of enormous benefit to age estimation within this multi-cultural population. Future studies will need to navigate through ethical considerations before more personal information, such as occupation, income, ancestry, and physical activity levels, can be collected from donors. This information would further clarify degenerative processes and remodelling characteristics in relation to

environment, genetics, health, and sociocultural influences. Future Australian studies could explore intra-bone microstructure variation using the entire midshaft cross-sections that are now being collected from cadavers and added to the JCUHSHC. These can be used to evaluate if different regions within the femur, humerus, or rib, are likely to produce comparable age estimates or significant differences.

The original contributions of this thesis include conducting a systematic evaluation of age-related bone remodelling dynamics within and between two genetically and regionally distinct samples from northern Australia and northeastern Thailand. This demonstrated there are significant interpopulation and intraindividual differences. It illustrates some of the human variation that makes age estimation arguably the most difficult feature of a biological profile to achieve reliably and accurately. In this thesis the temporal patterns of vertebral osteophyte formation and prevalence challenges the notion that major sociocultural and lifestyle changes between the Neolithic period to the Late Iron Age would, through biomechanical and health stresses, contribute to increasing vertebral degeneration rates. The approach of investigating VO prevalence over a long period of time in two populations that remained relatively genetically stable assisted in understanding stress exposure in a way that cannot be viewed in the shorter timeframe of a modern population. Finally, the bias toward older individuals in the samples used in this thesis helps to broaden our understanding of bone senescence in this demographic, which is an important contribution in a world where life expectancies are improving and the percentage of people over 60 years of age will only continue to increase.

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# Appendices

## Appendix A Published paper (Chapter 2)

ANTHROPOLOGICAL SCIENCE

Advance Publication

*Review*

# Adult age at death estimation: methods tested on Thai postcranial skeletal remains

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Received 3 August 2021; accepted 19 December 2021

**Abstract** Scientific literature frequently reports that age-at-death estimation standards developed on European and North American populations are less effective when used on genetically distant populations. Ultimately, this paper aims to inform forensic anthropologists and bioarchaeologists of the most appropriate methods to use on Southeast Asian skeletal remains by evaluating studies that have tested the replicability and accuracy of adult age estimation methods on Thai target samples. Results show that methods using the pelvis recorded the highest accuracy of up to 93%, but only when broad age ranges are used (±2 SD). Most methods produced the least bias and inaccuracy in young adults but considerably underaged older adults. Overall biases and inaccuracies tended to be lower for males than females. The sternal rib end method showed the weakest correlation with chronological age. Methods that produced age prediction equations developed with regression analyses derived from the Thai samples produced standard errors ranging from 9.5 to 13.9 years (using vertebrae and femora). Most of these methods were deemed too imprecise to be useful in Thai forensic cases. The best way forward to understand the wide range of morphological variation is for future studies to evaluate the influence of body size, activity patterns, socioeconomic status, nutrition, and health on skeletal aging and how it differs between Thai and geographically distant populations.

**Key words:** age estimation, forensic anthropology, bioarchaeology, Thailand, interpopulation variation

## Introduction

Age estimation is a crucial element in the analysis of human skeletal remains when building a biological profile, either to identify an individual in forensic cases or to establish mortality profiles of past populations. However, it is argued that the reliability of estimation is too dependent on the demographic profile of the Western reference samples from which methods were generally developed. The rate of bone remodeling and degeneration is known to differ between European, African, and Asian populations (Aiello and Molleson, 1993; Schmitt et al., 2002), yet Southeast Asian skeletal research has not yet received the same amount of consideration as Western populations (Cho, 2019; Go et al., 2019). There are several scenarios that have created increasing pressure to ensure that skeletal age estimation methods are sufficiently accurate and reliable for a Thai population. These include that in Thailand, each year, on average, at least 200 unidentified human remains are registered at government agencies (Central Institute of Forensic Science, n.d.) and, as of 2008, the Thai Tsunami Victim Identification and Repatriation Centre was still trying to identify almost 400 unidentified remains from the 2004 Boxing Day tsunami (United Press International (UPI), 2008). There has also been a recent upsurge in the number of archaeological excavations conducted in Southeast Asia and an interest in the mortality and health of these individuals.

Age estimation of unidentified adult human remains relies on standards that have used reference populations of known age, sex, and ancestry to correlate various signs of skeletal degeneration and remodeling to different life stages and their associated chronological age ranges. The most accurate age estimations will always be achieved using standards developed on a reference sample that is the same as the study (target) population, as skeletal growth and degeneration are non-uniform across time and regions due to complex relationships with genetics, environment, socioeconomics, and behavioral influences (Schmitt, 2004; Gocha et al., 2015). However, most adult age estimation methods universally in use today were originally developed on skeletal collections in Europe, North America, and South Africa. These methods still require further validation to test their reliability and accuracy on other populations, especially those geographically isolated from the reference sample, such as Southeast Asian populations.

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Published online 24 May 2022
in J-STAGE (www.jstage.jst.go.jp) DOI: 10.1537/ase.211219

Table 1. Sample used to test methods and investigate age-related skeletal characteristics (in order of publication date)

| Reference | Sample population | Total sample size (male/female) | Age range (years) | Mean age (years) male/female |
|---|---|---|---|---|
| Schmitt (2004) | Thai—FORC skeletal collection | 66 (37/29) | 20–60+ | * |
| Namking et al. (2008) | Thai—HSRC skeletal collection | 200 (120/80) | 18–94 | * |
| Chanapa and Mahakkanukrauh (2011) | Thai—FORC skeletal collection | 200 (139/61) | 35–95 | 71 |
| Singsuwana et al. (2012) | Thai—FORC skeletal collection | 210 | 21–96 | * |
| Tipmala (2012) | Thai—FORC skeletal collection | 236 | 20–96 | * |
| Gocha et al. (2015) | Thai—HSRC skeletal collection | 88 (44/44) | 20–97 | 48/53 |
| Khomkham et al. (2017) | Thai—FORC skeletal collection | 48 (34/14) | 20–89 | * |
| Iamsaard et al. (2017) | Thai—HSRC skeletal collection | 454 (254/200) | * | 61/60 |
| Suwanlikhid et al. (2018) | Thai—FORC skeletal collection | 250 (125/125) | 22–89 | 58/62 |
| Chompoophuen et al. (2019) | Thai—CMU cadavers | 71 (49/22) | 25–92 | 52 |
| Monum et al. (2019) | Thai—CMU cadavers | 40 (24/16) | 16–88 | 58/54 |
| Praneatpolgrang et al. (2019) | Thai—FORC skeletal collection | 400 (262/138) | 22–97 | 66/66 |
| Singsuwan et al. (2019) | Thai—FORC skeletal collection | 200 (98/102) | 22–90 | 63/63 |

* Information not provided or not included in English abstract. HSRC, Human Skeleton Research Centre (held at Khon Kaen University, northeast Thailand); FORC, Forensic Osteology Research Center (held at Chiang Mai University (CMU), northern Thailand).

Over the past six decades a number of studies have tested these adult age-at-death estimation methods on Thai skeletal remains. This collation of age estimation studies from domestic and international scientific journals and unpublished theses provides a quick reference guide for forensic and bioarchaeological experts to determine the effectiveness and reliability of each technique when used on Thai individuals, and in particular which methods are best for young adults or older adults, and each sex.

The Thai studies have drawn their samples from the modern population, within which there exists great genetic diversity due to high rates of migration and distinct ethnic indigenous groups (Benjavongkulchai and Pittayapat, 2018). This population's biological and cultural diversity, and largely agricultural economy, means that it is likely that skeletal maturation and degeneration will vary in relation to other geographically and genetically distant populations, hence the need to verify the reliability of the age estimation methods. The Thai studies use samples consisting of either skeletal remains from curated collections or autopsied cadavers (Table 1). Thailand has two large modern skeletal research collections with documented age and sex. The first is the Forensic Osteology Research Centre (FORC) at the Faculty of Medicine, Chiang Mai University (CMU) in northern Thailand, and the second is the Khon Kaen University (KKU) Human Skeleton Research Centre (HSRC), which has body donors from the rural Isan region, northeast Thailand. Both skeletal collections represent individuals who had mostly lived in the 20th to early 21st centuries and were from low to middle socioeconomic groups (Traithepchanapai et al., 2016; Techataweewan et al., 2017, 2018). These curated skeletal collections are the first modern skeletal representations of this size, geographic location, and ancestry that are available for research. They, therefore, represent an important opportunity to thoroughly test, develop, and revise traditionally used age estimation methods that were developed on genetically distant populations.

Reports of accuracy and reliability for age estimation methods currently lack a clear set of standards and this somewhat limits the comparability of results between studies (Garvin et al., 2012) (Table 2). Some methods present the results in terms of bias (the mean over- or underestimation of age) and inaccuracy (a measure of the mean sampling error when comparing estimated age to known age); others report in confidence intervals, standard deviations (SDs) from the mean or the percentage of individuals for which the known age fell within the SD of the mean, standard errors (SEs), or correlation coefficients (differences between the estimated and known ages).

## Pelvis (pubic symphysis, auricular surface of the ilium and acetabulum)

Several different techniques for estimating age via the pubic symphysis and auricular surface have been developed over the decades, some of which have been tested on Thai samples, including the Suchey–Brooks method (Brooks and Suchey, 1990), which was developed on a reference sample of predominantly North American ancestry with a minority of European, South American, or Asian ancestry. Also tested on Thai samples was the original Lovejoy et al. (1985) auricular surface method which has gone through several revisions (Buckberry and Chamberlain, 2002; Osborne et al., 2004). The Lovejoy method was developed using the prehistoric (8th–11th century AD) North American Libben Cemetery skeletal sample, cadavers from several North American forensic cases, and also the Hamann–Todd skeletal collection, which is comprised of African Americans and European Americans from historic to modern periods. The acetabulum has also recently shown promise for use to estimate age, and a method developed by Rissech et al. (2006) on a Portuguese skeletal collection was tested recently on Thais (Khomkham et al., 2017).

### Pubic symphysis

Schmitt (2004) was the first to apply the Suchey–Brooks pubic symphysis method (Brooks and Suchey, 1990) to a Thai sample. Schmitt (2004) reported that the results for the

Table 2. Comparison of measures of accuracy in each study

| Measure of accuracy | Reference | Bone region | Method | Accuracy |
|---|---|---|---|---|
| Regression correlation and standard error | Monum et al. (2019) | Femur (aspartic amino acid racemization) | Benešová et al. (2004) | Male age SEE = 8.07 yrs ($r$ = 0.912, $r^2$ = 0.8322); combined sex age SEE = 11.01 yrs ($r$ = 0.8316, $r^2$ = 0.6916); female age SEE = 15.77 years ($r$ = 0.716, $r^2$ = 0.5136) |
| | Chompoophuen et al. (2019) | Femur (histology) | Adapted from Yoshino et al. (1994), Martrille et al. (2009), and Pfeiffer (1998) | $r$ = 0.906, SEE = 8.26 (using combination of Pm.H.Ar, COL.B, and Lm.B.Ar); Pm.H.Ar stood out as being the individual variable most closely correlated with age ($r^2$ = 0.733) with the lowest SEE of 9.91 years |
| | Praneatpolgrang et al. (2019) | Cervical, thoracic, and lumbar vertebrae | Snodgrass (2004), Watanabe and Terazawa (2006) and a modified scoring system | $r$ = 0.801, $r^2$ = 0.642, SEE = 9.506 ($P$ < 0.01) (highest accuracy with scoring method of Snodgrass (2004) using female mean lumbar score) |
| | Suwanlikhid et al. (2018) | Lumbar vertebrae | Adapted from Kacar et al. (2017), Van Der Merwe et al. (2006), and Watanabe and Terazawa (2006) | $r^2$ = 0.408 with an SEE of 11.686 years, $P$ = 0.000 (highest accuracy using degree of osteophyte formation on the inferior surface of L1) |
| Accuracy and standard deviation | Gocha et al. (2015) | Auricular surface | Osborne et al. (2004) | 93.0% male, 88.1% female known age within ±2 SD of assigned phase mean (males had highest correlation $r_s$ = 0.581, $P$ = 0.000) |
| | | Pubic symphysis | Suchey and Brooks (1990) | 88.6% male, 78.0% female known age within ±2 SD of assigned phase mean (males had highest correlation $r_s$ = 0.907, $P$ = 0.000) |
| | | Auricular surface | Buckberry and Chamberlain (2002) | 81.4% male, 76.2% female known age within ±2 SD of assigned score mean (females had highest correlation $r_s$ = 0.643, $P$ = 0.000) |
| | | Sternal end 4th rib | İşcan et al. (1984, 1985) | 66.7% male, 48.0% female known age within ±2 SD of assigned phase mean (males had highest correlation $r_s$ = 0.565, $P$ = 0.001) |
| | Schmitt (2004) | Pubic symphysis | Suchey and Brooks (1990) | 36.1% male, 37.9% female accurately classified within ±1 SD of the reference phase mean |
| Accuracy | Tipmala (2012) | Pubic symphysis | Modified Suchey and Brooks (1990). | 86.4% left os pubis, 85.2% right os pubis |
| | Singsuwan et al. (2019) | Acetabulum | Rissech et al. (2006) | 71% accuracy estimated age within 12 yrs of known age; 66% accuracy within 10 yrs |
| | Singsuwana et al. (2012) | Auricular surface | Modified Lovejoy et al. (1985) and Buckberry and Chamberlain (2002) and developed regression equations | 56.4% accuracy with SE = 11 yrs (using left side); 67.8% accuracy with SE = 10.6 yrs (using right side) |
| | Schmitt (2004) | Auricular surface | Lovejoy et al. (1985) | 7% of individuals accurately classified within Lovejoy's five-year classes |

yrs, years; SD, standard deviation; SE, standard error; SEE, standard error of estimate; $r^2$, coefficient of determination; $r$ or $r_s$, correlation coefficient.

20- to 39-year-old age cohorts should be disregarded for both methods as the sample size, especially for females, was inadequate. Schmitt (2004) found the Suchey–Brooks method tended to overestimate the age of Thai adults less than 40 years of age and underestimated age for older adults (Table 3). Brooks and Suchey (1990) noted that when they tested their original method on a modern North American sample it was more reliable for young adults (up to 40 years of age); after this age they observed a wide range of individual variability which produced wide age distributions. Brooks and Suchey (1990) also noted that female standard deviations were greater than males by 0.5–1.3 years, and standard deviations got progressively greater as age progressed (up to 12.4 years ± 1 SD). Similarly, in Schmitt's study, bias and inaccuracy values tended to be higher for Thai females compared with males. Inaccuracy for adults aged less than 60 years ranged from 2 years to 17 years; however, in adults over 60 years of age, inaccuracy was as high as 32.2 years for females and 27.2 years for males. Even given that the Suchey–Brooks method has such broad and overlapping age ranges for each phase, Schmitt noted that in only 37% of the Thai sample did known age fall within 1 SD of the assigned phase mean age.

The 2012 thesis by Tipmala (2012) (written in Thai with an English abstract) also tested the Suchey–Brooks method; however, Tipmala (2012) used multinomial logistical regression analysis to produce new age ranges for each phase (Table 3) with the intention of increasing age-estimate accuracy for Thai individuals. Side asymmetry was also tested, with accuracy determined to be 86.4% for the left os pubis and 85.2% for the right side. Compared to the age ranges per phase developed in the original Suchey–Brooks method, these newly adapted Thai age intervals for each phase are narrower and without overlap. This study shows that Thai skeletal maturation is delayed in the later phases where they reach phases III–V later than the North American Whites on whom the method was developed.

When testing the Suchey–Brooks method, Gocha et al. (2015) reported similar results to Schmitt (2004) wherein adults over 40 years tended to have age underestimated, and

Table 3. Age estimation methods tested on pelvic region

| Reference | Bone region | Method | Accuracy | Age overesti-mated | Age underesti-mated | Overall bias/inaccuracy (years) | | Minimum inaccuracy (years) [age group] | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Males | Females | Males | Females |
| Schmitt (2004) | Pubic symphysis | Suchey and Brooks (1990) | 36.1% males, 37.9% females known age within ±1 SD of the assigned phase mean | ≤ 39 | ≥ 40 | −14.5/17.2 | −16.1/18.8 | 2.4 [20–29] | 6.7 [30–39] |
| | Auricular surface | Lovejoy et al. (1985) | 7% of individuals accurately classified within Lovejoy's five-year age classes | ≤ 29 | ≥ 30 | −17.8/18.3 | −20.0/20.0 | 2.0 [20–29] | 6.3 [30–39] |
| Gocha et al. (2015) | Pubic symphysis | Suchey and Brooks (1990) | 88.6% males, 78.0% females known age within ±2 SD of assigned phase mean; more reliable in males and younger adults (<50 yrs) | n/a | ≥ 40 | −7.8/9.2 | −8.7/12.5 | 2.8 [30–39] | 6.2 [20–29 and 40–49] |
| | Auricular surface | Osborne et al. (2004) | 93.0% males, 88.1% females known age within ±2 SD of assigned phase mean; more reliable in younger adults (<50 yrs) | ≤ 49 | ≥ 50 | −4.4/12.2 | −5.7/12.2 | 5.6 [40–49] | 2.9 [20–29] |
| | Auricular surface | Buckberry and Chamberlain (2002) | 81.4% males, 76.2% females known age within ±2 SD of assigned score mean; more reliable for older adults (>50 yrs) | ≤ 49 | ≥ 50 | 11.2/14.5 | 5.1/15.4 | 5.8 [60–69] | 10.7 [60–69] |
| Singsuwan et al. (2019) | Acetabulum | Rissech et al. (2006) | 71% accuracy estimated age within 12 yrs of known age; 66% accuracy within 10 yrs | ≤ 65 | ≥ 66 | −0.17/8.55 sexes combined | | 1.25 [31–35] sexes combined | |
| Tipmala (2012) | Pubic symphysis | Modified Suchey and Brooks (1990) | 86.4% accuracy left os pubis, 85.2% accuracy right os pubis; new age ranges for Thais: phase 1 = age range ≤21 yrs, phase 2 = age range 22–28 yrs, phase 3 = age range 29–34 yrs, phase 4 = age range 35–43 years, phase 5 = age range 44–54 yrs, and phase 6 = age range ≥55 yrs | | | | | | |
| Singsuwana et al. (2012) | Auricular surface | Modified Lovejoy et al. (1985) and Buckberry and Chamberlain (2002) and developed regression equation | 56.4% accuracy with SE 11 yrs for left side: age = $-0.465CS_L^2 + 14.65CS_L - 29.67$. 67.8% accuracy with SE 10.6 yrs for right side: age = $-0.59CS_R^2 + 16.86CS_R - 36.8$ | | | | | | |

SE, standard error; SD, standard deviation; yrs, years.

individuals under 40 years were usually observed with lower values of bias and inaccuracy, and had age overestimated (apart from females 20–29 years in the Gocha et al. (2015) sample; no females in this age group were present in Schmitt's sample). In both studies, the overall results show females have greater bias and inaccuracy than males (Table 3). On average, age estimates differed from known age by approximately 10 years or more. Bias and inaccuracy increased to 25.3 years for females in the 70+ age group, but this was still less than the bias and inaccuracy of up to 32.2 years reported by Schmitt (2004) for adults over 60 years.

**Auricular surface**

Schmitt (2004) tested the Lovejoy auricular surface eight-phase method and observed that male age estimation showed less bias and inaccuracy compared with females, and age tended to be underestimated once individuals were over 30 years of age. With the auricular surface, the rate of bias and inaccuracy for adults over 40 years of age was higher than when the Suchey–Brooks method was tested (except for females over 60 years of age), with inaccuracy reaching a peak of 31.9 years for males (30.4 years for females) aged over 60 years. Schmitt (2004) established that only a very small number of Thai individuals (7%) were assigned to the correct age range, with the majority of the sample being incorrectly placed into younger age phases (20–49 years), even though almost 85% of the Thai sample had a chronological age of over 40 years. When Lovejoy et al. (1985) tested their own method on a combined-sexes subsample of the North American Hamann–Todd skeletal collection, they observed inaccuracy ranging from just 3.2 years for young adults, up to a maximum inaccuracy of 11.1 years (but with a bias of just 1.9 years) in the over 50 age group. Schmitt (2004) determined that repeatability of the method was exacerbated by the difficulty the observers faced in interpreting the description of some features as outlined in the original methods, particularly for the auricular surface. This problem has previously been discussed as an issue (Merritt, 2013).

In a 2012 conference paper, Singsuwana et al. (2012) presented a new age estimation scoring system and quadratic regression equations using the auricular surfaces from a Thai skeletal sample. The authors utilized a selection of the features used in the Lovejoy et al. (1985) method (transverse organization, surface texture, microporosity, apical change,

and retroauricular area activity), in combination with a composite score similar to that proposed by Buckberry and Chamberlain (2002). Their first step was to individually assess each of the five features of the auricular surface to obtain a combined composite score for both the left and right auricular surfaces to then develop regression equations specifically for a Thai population. They found some of the feature descriptions developed by Lovejoy et al. (1985) (microporosity and density) were difficult to evaluate in the Thai sample, just as Schmitt (2004) had reported. Singsuwana et al. (2012) believe this was due to a difference in morphological characteristics between the Thai sample population and the Western reference population on which the method was developed. Singsuwana et al. (2012) noted that this sample was represented by more older adults than young. Using composite scores from the left side and the right side, they tested the new equations (Table 3) on a sample of 60 individuals. No statistically significant differences were observed between the left and right os coxae. Accuracy of the new equations was slightly greater when tested on the right side, at 67.8% with a standard error of 10.6 years. The left side produced an accuracy of 56.4% with a standard error of 11 years.

When the auricular surface of the ilium was examined by Gocha et al. (2015) using the method developed by Osborne et al. (2004), they found underestimation of age occurred in adults over 50 years of age. The least amount of bias was observed in the 40- to 49-year-old age group (overestimation by 0.2 years for females and 2.2 years for males) and the highest amount of bias was seen in the 70+ age group (underestimation by 27.8 years for females and 23.4 years for males). The overall results show that the level of inaccuracy was the same for both sexes (12.2 years) with age tending, on average, to be underestimated by 4.4 years for males and by 5.7 years for females. In comparison, when the Buckberry and Chamberlain method was also tested on the Thai sample by Gocha et al. (2015), age was underestimated from 50 years of age for females, but not until 70 years of age for males. The overall results of the Buckberry and Chamberlain method showed a similar level of inaccuracy between males and females, but overall bias was noticeably lower in females than in males (Table 3). Gocha et al. (2015) observed a decrease in inaccuracy and bias of adults older than 50 years, particularly in comparison to their results from testing both the Suchey–Brooks and the Osborne methods, and also the Lovejoy method tested by Schmitt (2004) on a Thai sample. Gocha et al. (2015) reported that the Suchey–Brooks and Osborne methods were more reliable for estimating age in younger Thai adults (<50 years), and the Buckberry and Chamberlain method was more reliable for older adults.

Gocha et al. (2015) went on to test several multifactorial combinations, including averaging point estimates from all three pelvic methods (combination A), and a combination of average point estimates from the Suchey–Brooks method and one of the auricular surface methods, which required using the Osborne method on younger adults (if the Suchey–Brooks method indicated the pubis was in phases I–IV), or the Buckberry and Chamberlain method for older adults (if the pubis was in phases V–VI) (combination B). Gocha et al. (2015) determined that combination B produced the least bias and inaccuracy of any of the individual methods tested alone. With both combinations, A and B, there was a tendency for overestimation of age for adults under 50 years and underestimation of age for adults above this age. Overall, both combinations also achieved marginally improved results for males compared with females. Gocha et al. (2015) found that combination A provided a reasonable level of accuracy between the ages of 40 and 59 years in both sexes, whereas combination B worked to a reasonable level of accuracy for adults up to 69 years of age, except for females aged 30–39 years for whom the bias and inaccuracy was almost double that for males. They also tried combining the average point estimates from all six methods, but the sample size was drastically reduced due to insufficient skeletal elements in some individuals. This combination was found to perform sufficiently only on adults in the 40- to 49-year-old age group. Testing on a larger sample may see an improvement in results. There were several hurdles faced by Gocha et al. (2015) in this study. They were constrained to a data collection period of just one week, restricting the sample size to 88 individuals, and leaving no time to test for intra- and interobserver errors.

**Acetabulum**

Khomkham et al. (2017) examined morphological features of the acetabulum as these changed with age using the steps outlined in the age estimation method of Rissech et al. (2006), to observe and score seven features on both the left and right sides of each individual. The authors did not observe any statistically significant differences in scores between the sexes or sides. For three of the features, they did find a significant correlation with age. These were the acetabular groove (the most significant correlation was in the left female acetabulum ($r = 0.61$)), acetabular rim porosity, and apex activity (most significant correlation in the left male acetabulum ($r = 0.59$ and $r = 0.62$, respectively)). To Khomkham et al. (2017) these results suggested there are at least some similarities in timing and changes to morphological features between this Thai sample and the reference sample of Portuguese males on which Rissech et al. (2006) developed their method. However, the other four features are weakly correlated with age and highlight that there are some population differences in growth and degeneration in this part of the pelvis.

The Rissech et al. (2006) acetabular method was also applied to a Thai sample by Singsuwan et al. (2019). A preliminary test with 88 individuals determined that there were no significant side differences, so the method was comprehensively tested using a sample of 200 individuals. Singsuwan et al. (2019) recorded no significant sex differences. In comparison to Khomkham et al. (2017), they observed significant correlation with known age for all seven morphological variables. Results showed that overestimation of age occurred in adults younger than 66 years, and underestimation occurred in adults over this age. Low levels of bias (over- or underestimation to a maximum of 4.4 years) were seen in young and mid-aged adults (21–46 years) and older adults (61–75 years). However, age was underestimated by 11.38 years in adults aged 86–90 years. Inaccuracy reached a maximum of almost 12 years for the age groups 56–60 and 86–

90 years. Singsuwan et al. (2019) recorded an accuracy of 66% when estimating age to within 10 years of known age, up to a maximum accuracy of 71% within 12 years of known age. In comparison, accuracy was higher for the Portuguese sample on which Rissech et al. (2006) developed their method; in their sample accuracy reached 89% when estimating age to within 10 years of known age. Singsuwan et al. (2019) suggested refining the scoring system, finding in the Thai sample some differences in the degree of change in certain features compared to that reported by Rissech et al. (2006), such as inconsistencies in density of acetabular fossa activity and deeper grooves surrounding the rim. They could see that apex activity showed a clear progression of change with age, whereas the acetabulum groove showed high overlap between ages.

## Thorax (sternal rib ends, clavicle, and vertebrae)

Observing age-related changes in the sternal ends of the ribs at the costochondral joint was conceived as an alternative to using methods developed on the pelvis or cranial sutures. Cranial sutures are not included in this review because many studies have shown this method to be highly inaccurate (Singer, 1953; Brooks, 1955; Hershkovitz et al., 1997; Ruengdit et al., 2020). The method that has been tested on a Thai sample is the İşcan nine-phase system (İşcan et al., 1984, 1985) using the fourth rib of White male and female cadavers from an American medical examiner's office. Several pilot studies used the Thai samples to observe age-related changes to the medial articular surface of the clavicle or vertebrae to evaluate their potential to estimate age, and several other studies calibrated age estimation regression equations for the Thai population using a combination of different scoring systems.

### Sternal rib ends

Gocha et al. (2015) were the only researchers to have directly applied the İşcan method (İşcan et al., 1984, 1985) on a Thai sample to estimate age via changes to the sternal ends of ribs. Low levels of bias and inaccuracy in the earlier phases showed that the method was more accurate overall for Thai males and young adults. However, adults of both sexes above the age of 40 years (phase V onwards) consistently had their age underestimated. İşcan et al. (1984, 1985) noted that their method was most reliable for young to mid-aged White North Americans up to 40 years of age. After this age the SD from the mean reached up to 11 years for males and 15 years for females, with very wide age ranges per phase. Gocha et al. (2015) did not recommend this method for use in a forensic context for Thais or other Southeast Asian populations due to a poor correlation between observed and documented chronological ages, particularly apparent for individuals >40 years. Above this age both bias (underaging) and inaccuracy was as high as 37.5 years for females in the 70+ age group, whilst for males it reached 30.6 years in the 60- to 69-year-old age group.

Differences between reference and target sample size and distributions would have an impact on mean ages and standard deviation rates (Loth, 1995; Yavuz et al., 1998). The small Thai sample size (55 individuals) in the study of Gocha et al. (2015) hampered a thorough examination of the performance of this technique as the number of individuals examined per sex/age group ranged from just one (females aged 20–29 years) up to a maximum of eight (females aged 40–49 years). The İşcan method would benefit from further testing and modification on a Thai sample of larger size, with an even representation of males and females in all age groups.

### Clavicle

Iamsaard et al. (2017) seriated 454 clavicles from northeastern Thais of the HSRC skeletal collection to closely observe and record surface typography of the medial articular surface of the clavicle as a way of providing a population-specific learning aid for medical and paramedical students. The age range of the sample was not provided but the average age of the sample was 60.69 years (± 14.36 years). Surface typography, as well as porosity and osteophyte formation, is an important feature to record in a population sample, as once epiphyseal fusion is completed in young adults, changes of the medial surface of the clavicle provide another way to estimate age for older adults (Falys and Prangle, 2015). The study by Iamsaard et al. (2017) did not record porosity or osteophyte formation, and instead chose to focus only on assessing surface topography, which Falys and Prangle (2015) determined was the trait most closely correlated with age. Therefore, the Falys and Prangle composite score method still needs to be tested on a Thai sample to ascertain accuracy of age ranges, means, and standard deviations for use on a Thai or other Southeast Asian population. The pilot study of Iamsaard et al. (2017) could only confirm that the types of medial articular surface (smooth, slight granulation, coarse granulation, nodule formation, undulating, and degenerative) observed in the European reference sample of Falys and Prangle were also observed in the Thai sample. Another study by Traithepchanapai (2014) confirmed that commencement of osteophyte growth occurred on the margin of the medial articular surface of the clavicle of Thai individuals of at least 39 years of age in both males and females (cited in Traithepchanapai et al. (2016)).

### Vertebrae

Four studies focused on observing the prevalence and severity of vertebral osteophytes in Thai samples to aid in identifying potential symptoms in clinical cases, and to investigate their potential to estimate age. In the first of these studies, Namking et al. (2008) examined cervical, thoracic, and lumbar vertebrae to determine that osteophyte prevalence significantly correlates with increasing age and does so more significantly in males than females. Most frequently, osteophytes were observed in the lumbar vertebrae (73% of L4, 70% of L5, and 69% of L3), followed by the thoracic (50.5% of T11 and 49.5% of T10), and cervical (46% of C5, 44% of C6, and 38% of C4). The most prominent osteophytes were located on the anterosuperior aspect of the rim of lumbar vertebrae L3, L4, and L5.

Chanapa and Mahakkanukrauh (2011) studied only the cervical vertebrae in their northern Thai sample, recording the highest prevalence of osteophyte formation in vertebral bodies (49%), followed by facet joints (35%), and foramen

(16%) of cervical vertebrae (C3–C7). Chanapa and Mahakkanukrauh (2011) concluded that osteophyte length significantly correlated with age, but not significantly with sex. The average length of C3 osteophytes were longer than on any other cervical vertebrae, but the maximum length of an osteophyte was recorded on the superior facet of a C4 vertebra (13 mm). Greatest osteophyte prevalence was observed in cervical (C5) vertebrae (83%), followed by C6 (77%), C4 (74%), C7 (65%), and C3 (64%). These prevalence values are much greater than that observed by Namking et al. (2008) in the northeastern Thai sample (C5, 46%; C6, 44%; and C4, 38%).

Suwanlikhid et al. (2018) used linear regression to estimate age from degenerative changes to lumbar vertebrae by observing and scoring three morphological features including changes to the cortical surface of the lumbar body, and the degree of osteophyte formation and macroporosity on the superior and inferior borders and endplates of the lumbar vertebrae. Suwanlikhid et al. (2018) produced an adaptation of several previously developed vertebral osteophyte scoring systems (Van Der Merwe et al., 2006; Watanabe and Terazawa, 2006; Kacar et al., 2017), as well as developing a new scoring system on the Thai sample for macroporosity and resorption of the cortical surfaces. Eight grades were used to score the degree of osteophyte formation with or without bridging and projections. Four grades were used to determine the degree of macroporosity on the superior and inferior surface of the vertebral body, and four grades were used to determine the degree of roughness with porosity on the cortical surface. All three features had moderate correlation with age, with the prevalence of osteophytes having the highest correlation of the three features, particularly on the inferior surface. Osteophyte formation was observed to commence around 26 years of age. The scores for each feature were used to develop new age estimation equations for each of the five lumbar vertebrae, producing 25 equations in total, with standard errors ranging from 11.7 to 14.5 years. The highest level of accuracy was gained from observing osteophyte formation on the inferior surface of the first lumbar vertebra ($r^2 = 0.408$ with a standard error of 11.7 years), but even this was a weak correlation between actual age and estimated age.

Praneatpolgrang et al. (2019) calibrated age estimation equations based on examining vertebral osteophyte formation in a Thai sample using the five-grade scoring system developed by Snodgrass (2004) and the four-grade system designed by Watanabe and Terazawa (2006), as well as developing their own new six-grade scoring system focusing on changes to the rugosity of the surface of the inferior and superior margins of the vertebral body, osteophyte length, and fusion of adjacent vertebrae. Praneatpolgrang et al. (2019) separately scored cervical, thoracic, and lumbar vertebrae for three groups (males, females, and combined sexes). Significant correlation was found between known age and the scores for all parts of the vertebral column (cervical, thoracic, and lumbar). The mean lumbar score had the best correlation with age for all three groups in each of the three scoring systems. The correlation coefficient ($r$) tended to be valued above 0.75 (strong positive correlation), the $r^2$ values were consistently between 0.53 and 0.64 (moderate positive correlation), and the standard error of estimates for mean lumbar scores were consistently between 9 and 11 years ($P < 0.01$). This is less error than recorded by Suwanlikhid et al. (2018) (standard error of estimate (SEE) = 11.7–14.5

Table 4. Age estimation methods tested on the thorax

| Reference | Bone region | Method | Accuracy | Age overesti-mated | Age underesti-mated | Overall bias/inaccuracy (years) | | Minimum inaccuracy (years) [age group] | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Males | Females | Males | Females |
| Gocha et al. (2015) | Sternal end 4th rib | İşcan et al. (1984, 1985) | 66.7% males, 48.0% females known age within ±2 SD of assigned phase mean; more reliable for males, especially <40 yrs | ≤39 | ≥40 | −10.0/12.9 | −13.01/18.5 | 3.8 [20–29] | 6.4 [30–39] |
| Namking et al. (2008) | Cervical, thoracic, and lumbar vertebrae | N/A | Osteophyte prevalence significantly correlates with increasing age, and more significantly in males than females | | | | | | |
| Chanapa and Mahakkanukrauh (2011) | Cervical vertebrae | N/A | Osteophyte length significantly correlated with age, but not significantly with sex | | | | | | |
| Suwanlikhid et al. (2018) | Lumbar vertebrae | Adapted from Kacar et al. (2017), Van Der Merwe et al. (2006), and Watanabe and Terazawa (2006) | Highest accuracy using degree of osteophyte formation on the inferior surface of L1 ($r^2 = 0.408$ with an SE of 11.686 yrs) | | | | | | |
| Praneatpolgrang et al. (2019) | Cervical, thoracic, and lumbar vertebrae | Snodgrass (2004), Watanabe and Terazawa (2006), and developed a modified scoring system | Mean lumbar score most accurate of all three scoring methods; highest accuracy with scoring method of Snodgrass (2004) using female mean lumbar score age $y = 32.308 + 15.994x$, with an SE of 9.506 ($P < 0.01$), $r = 0.801$, $r^2 = 0.642$ | | | | | | |

SD, standard deviation; SE, standard error; yrs, years; $r^2$, coefficient of determination; $r$, correlation coefficient.

Table 5. Age estimation methods tested on the femur

| Reference | Bone region | Method | Accuracy |
|---|---|---|---|
| Chompoophuen et al. (2019) | Femur (histology) | Adapted from Yoshino et al. (1994), Martrille et al. (2009), and Pfeiffer (1998) | Age = (–28.199 + 0.0138(Pm.H.Ar) + 0.00005(COL.B) + 9.312(I.m.B. Ar)), $r = 0.906$, SEE = 8.26. Pm.H.Ar stood out as being the individual variable most closely correlated with age ($r^2 = 0.733$) with the lowest SEE (9.91 yrs) |
| Monum et al. (2019) | Femur (aspartic amino acid racemization) | Ohtani et al. (1998) and Ohtani and Yamamoto (2005) | Combined sex age = (ln(1 + D/L)/(1 – D/L) – 0.0192))/0.0005), with SEE = 11.01 yrs, $r = 0.8316$, $r^2 = 0.6916$; male age = (ln(1 + D/L)/(1 – D/L) – 0.0155))/0.0005), with SEE = 8.07 yrs, $r = 0.912$, $r^2 = 0.8322$; female age = (ln(1 + D/L)/(1 – D/L) – 0.0236))/0.0004), SEE = 15.77 yrs, $r = 0.716$, $r^2 = 0.5136$ |

D/L, dextro/levo; SEE, standard error of estimate; $r^2$, coefficient of determination; $r$, correlation coefficient.

years) discussed above. Praneatpolgrang et al. (2019) found that the results for all three scoring systems were similar and were suitable for use in Thai forensic cases. They reported that their new six-grade scoring system was more objective and faster to use than the Snodgrass and the Watanabe and Terazawa methods. However, the scoring system of Snodgrass (2004) overall produced the best results on this sample, with the most accurate of these regression equations recorded in the female mean lumbar score (Table 4) with a standard error of 9.506 ($P < 0.01$), $r = 0.801$, $r^2 = 0.642$. The weakest correlation between age and vertebral osteophyte formation was generally found when using individual cervical or thoracic vertebrae in all three of the scoring systems.

## Femur

All the previously discussed adult age estimation methods have been made via non-destructive qualitative or quantitative macroscopic observations. Histological methods and aspartic amino acid racemization are generally avoided as they require destructive sampling of bone, albeit a very small piece of the femur, humerus, or rib, usually approximately 2 $cm^2$, to observe microstructural changes in the cortical bone.

A study by Chompoophuen et al. (2019) is the only one to examine histomorphometric age estimation using cortical bone sections of femora in a Thai sample. They used decalcified and stained bone sections, image analysis (ImageJ), and the computer program MATLAB to determine the correlation between age and pixel density of histological variables. Chompoophuen et al. (2019) used linear regression analysis to calibrate established predictive formulae to estimate age from five variables, and to develop one new variable for quantifying collagen measurements in bone (COL.B). They found that collagen in males had higher correlation with age ($r = 0.800$) compared with females ($r = 0.467$). In the combined-sex sample, measuring the perimeter of the Haversian canals (Pm.H.Ar) produced the highest correlation coefficient with age ($r = 0.856$), and equations produced from regression analysis showed that Pm.H.Ar stood out as being the individual variable most closely correlated with age ($r^2 = 0.733$) with the lowest standard error (9.91 years) (Table 5). Stepwise multiple regression showed that, overall, a combination of three variables, Pm.H.Ar, COL.B, and Lm.B.Ar (percentage of lamellar bone area), provided the most accurate predictor of age (correlation coefficient of 0.906).

Monum et al. (2019) evaluated the aspartic amino acid racemization procedure suggested by Ohtani et al. (1998) and Ohtani and Yamamoto (2005) to predict age from femoral bone samples using aspartic amino acid racemization. Monum et al. (2019) found that the dextro/levo (D/L) ratio was highly correlated with known age in the Thai sample, with males showing better correlation ($r = –0.912$) than females ($r = 0.716$), but there were no statistically significant differences between rate of racemization and sex. The combined sex sample produced a standard error of 11.01 years. Monum et al. (2019) used the racemization results and linear regression to calculate age estimation equations for each sex and for sexes combined (Table 5).

## Computed tomography scans

It should also be noted that data collected from routine computed tomography (CT) scans have shown great potential in evaluating age-related morphological changes in several Thai studies. Pattamapaspong et al. (2015) assessed the timing of fusion of the medial clavicle in CT scans of Thai patients to calibrate and slightly modify the classification methods of Schmeling et al. (2004) and Kellinghaus et al. (2010). They found that stage 4 of fusion best represents Thai individuals over 18 years of age. Pattamapaspong et al. (2019) then used a new cinematic volume render to produce three-dimensional CT images of the os pubis and auricular surface of individuals from the CMU skeletal collection to test the Suchey–Brooks and Buckberry–Chamberlain methods of age estimation. The authors found that the new technique has a high success rate when assessing features of the os pubis; however, most auricular surface features cannot be clearly seen in the CT scans and are best assessed in dry bone. A major limitation of CT scanning is that such technology is often only accessible in large laboratories or hospitals and is expensive. Technical training is required to use specialized and expensive imaging equipment and to assess images with specialized computer software. However, as CT scans can be collected from live patients of all ages, it overcomes the sample bias seen in all skeletal collections in which the very young and the very old are underrepresented.

## Discussion

Several of the Thai studies (Schmitt, 2004; Gocha et al.,

2015) tested for sexual dimorphism by measuring bias and inaccuracy. These studies showed that techniques using the pelvis and ribs tended to be more reliable for males and younger adults. For instance, overall bias was almost always observed to be less in males than for females (1–6 years difference), the exception to this was for the Buckberry and Chamberlain (2002) auricular surface method where overall female bias was 6 years less than for males. The overall degree of inaccuracy was also observed to be lower in males than females (1–6 years difference). Males and females will often experience bone remodeling at different rates (Lewis and Roberts, 1997; Cho et al., 2006) and females could have been exhibiting a greater degree of morphological variation of age indicators (Djurić et al., 2007). The use of a larger female sample might have assisted in better phase placement.

Bias and inaccuracy increase considerably with age for all methods tested on Thai samples. Methods that were more reliable for younger Thai adults (<40 years) included all the methods using the pubic symphysis, acetabulum, rib ends, and auricular surface. The exception to this was the Buckberry and Chamberlain (2002) auricular surface method which was more reliable for Thai adults over 50 years of age. This method was established using a reference group with a higher proportion of adults aged over 60 years so it is not surprising that it performed better on older Thai individuals, whereas the original Suchey–Brooks pubic symphysis method, the Lovejoy auricular surface method, and the İşcan rib method were developed on younger reference groups and have proven to be more accurate on adults aged less than 40 years (Berg, 2008; Merritt, 2013).

There was a prevailing tendency for bias, where age was overestimated in young adults and underestimated in older adults in the Thai studies. Research verifies this trend is a persistent limitation for methods using regression-based models to correlate morphological data with age (Aykroyd et al., 1999; Schmitt et al., 2002; Berg, 2008; Getz, 2020). Disagreement in size, age structure, and mean age between the reference group and target sample can amplify this bias (Bocquet-Appel and Masset, 1982). The youngest adults and those over 60 years of age at death are particularly underrepresented in the original methods (Lucy et al., 2002; Martrille et al., 2007; Merritt, 2013; Miranker, 2016). Most of the Thai samples had a mean age of between 50 and 60 years and underrepresentation of some age groups, usually 20–29 years and 70+ years. There is also often unequal representation of males and females in the Thai target samples (Table 1), with a bias towards more males. The Thai sample demographics have been impacted by the number of body donations and suitable autopsied cadavers accessible from university forensic departments and hospitals from which the study samples were obtained.

Gocha et al. (2015) determined a method's accuracy by assessing the percentage of the sample whose documented age was within ±2 SD of the mean age reported for the phase or stage. The İşcan rib method showed the weakest correlation with known age, with high bias and a maximum accuracy of 67%, with Gocha et al. listing this as the least preferred skeletal indicator to use on Thai individuals. It must, however, be noted that the study was hindered by a small sample size in which young females in particular were underrepresented (Gocha et al., 2015). Greater accuracy (76–93%) was achieved in methods utilizing the pubic symphysis and auricular surface. Whilst 93% accuracy of the Osborne method sounds impressive, it should be noted that the age ranges assigned to an individual with ±2 SD are very broad with overlap between successive stages, making the estimated age ranges too impracticable to be of real use (Rogers, 2016). Wide age categories for each phase are usually required to capture the range of individual morphological variability experienced within a population (Djurić et al., 2007; Berg, 2008). The advantage of a wider age range is that fewer individuals are placed in the incorrect phase for age (Bocquet-Appel and Masset, 1982; Brooks and Suchey, 1990).

Schmitt (2004) preferred to use estimates ±1 SD within the mean to obtain a maximum accuracy of nearly 38% for the Suchey–Brooks method. However, this confidence level also suffers from the limitation that it may produce too narrow and precise an age range that cannot account for all individual morphological variation in the Thai sample (Rogers, 2016). This increases the probability that an individual will be placed in an age phase that is above or below the one in which their documented age actually falls (Garvin et al., 2012). Just 7% of individuals were correctly placed in the assigned age phase when Schmitt (2004) tested the Lovejoy auricular surface method. The low level of accuracy achieved with this method is a product of the 5-year age ranges published for the Lovejoy method, which are far too narrow to capture the full range of morphological variation of the auricular surface (Osborne et al., 2004). Even when Singsuwana et al. (2012) developed population-specific regression equations derived from Thai auricular surfaces, the maximum accuracy observed was nearly 68% but with a 10-year standard error.

Correlation coefficient values were highest when regression equations were derived from the Thai samples using either histological and biochemical (amino acid racemization) techniques or vertebral osteophyte prevalence in the lumbar vertebrae ($r = >0.8$) with standard errors ranging from 8 to 16 years. Monum et al. (2019) states that a standard error of 11 years is acceptable, whereas Rösing and Kvaal (1998) argue that standard errors that exceed 5–7 years should not be applied to forensic cases or archaeological contexts. Correlation over 0.8 is usually accepted as a strong level of correlation between morphological indicators and age, although Bocquet-Appel and Masset (1982) argued that values under 0.9 are likely to introduce considerable risk of error to age estimations.

Almost all the authors of the Thai studies in this review argued that interpopulation variation greatly reduced the reliability of the methods. Most either did not recommend the methods for use on a Thai population, particularly not for a forensic setting, or only if applied with a high degree of caution. This is not a situation unique to just this population. Current research predominantly indicates that bone degeneration and remodeling occur at non-uniform rates between individuals of Asian, African, and European origin (Buckberry and Chamberlain, 2002; Schmitt et al., 2002; Mays, 2012; Shilpa et al., 2013) due to varying genetic and

environmental factors experienced between regionally diverse populations (Berg, 2008; Garvin et al., 2012). These studies argue that age estimation standards developed on one sample population therefore cannot adequately reflect the individual and population-level rate of bone remodeling and degeneration found in another population, necessitating population-specific standards to ascertain biological age.

In contrast, other researchers suggest using one large reference group made up of individuals from a number of genetically distant populations to improve the reliability of methods by capturing a greater level of skeletal variation (Brooks and Suchey, 1990; Zhang et al., 2009). Furthermore, some studies stress that ancestry is not the main cause of skeletal age variation as there should be similar skeletal growth and degeneration rates between populations when health and environmental conditions are similar (Schmeling et al., 2000). It is suggested that a combination of factors, including health and hormones, and socioeconomic status linked to body mass index (Ferguson et al., 1982; Schmeling et al., 2000; Mays, 2015) have more of an effect on the timing and rate of bone turnover than population affiliation. This is usually not clearly discussed as a consideration when a method of age estimation is chosen by anthropologists. There is a paucity of studies that compare how socioeconomic status, health, and physical activity impact the reliability of current age estimation methods between different populations. An individual's level of physical activity and mechanical loading on the skeleton will affect the rate of bone turnover and bone mass (Adami et al., 2008); however, recent research on individuals of European and African American descent suggests that high levels of repetitive physical activity have little significant impact on rates of degenerative change to age-related features in load-bearing joints of the pelvis (Campanacho et al., 2012; Winburn, 2019; Bertsatos et al., 2021). Merritt (2015) was the first to show a clear relation between underestimation of skeletal age and short stature combined with low body mass, whereas overestimation of age occurred in tall people with high body mass, with evidence of increased bone surface degeneration in relation to weight increase (see also Wescott and Drew, 2015). Additionally, Merritt (2017) determined which age estimation methods were most reliable for smaller-bodied individuals and for larger-bodied individuals.

Health statistics in Thailand have identified a secular trend for height/weight increase correlating with improved living conditions since records began in 1975 (Jaruratanasirikul and Sriplung, 2015). Lower socioeconomic status in rural areas has caused delayed bone age in young Thai rural children compared with their peers from more affluent regions such as the United States and urban middle-class Thailand (Bailey et al., 1984). High-income (modern) Western populations are largely sedentary and their levels of physical inactivity are twice as high as those in low-income countries (Guthold et al., 2018). Clinical research has established there are significant differences in the relationship between skeletal muscle mass and age among Hispanic, African American, White American, and Asian (Chinese, Indian, Korean, and Japanese) individuals (Silva et al., 2010). Iamsaard et al. (2017) argued that the pattern of degenerative changes seen in the older age Thai adults of the KKU skeletal sample from rural northeast Thailand may be influenced by activities in labour-intensive manual rice agriculture, an occupation in which a majority of the individuals from this low to mid-socioeconomic region are likely to have participated (Techataweewan et al., 2017). Similarly, Tayles and Halcrow (2015) have considered the biomechanical forces involved in rice planting, which requires repetitive flexing at the hip joint, and its effects on age-related change in the auricular surface, and there may be influences from the cultural practice of a full squatting position commonly adopted by Thai people and other Asian populations. The impact that these factors have on skeletal age estimation in the Thai population is still poorly understood and needs additional research.

## Conclusion

The Thai studies follow similar trends often reported for other populations using these same methods in that bias and inaccuracy always increase with age (often dramatically), accuracy is dependent on wide age ranges, and some methods are more reliable than others for young adults. This really reflects the statistical analyses utilized (e.g. regression based) and age structure of the reference group on which the methods were originally developed. The Suchey–Brooks, Lovejoy, Osborne, and Rissech methods produce less bias and more accuracy on Thai adults younger than 40–50 years of age, whereas the Buckberry–Chamberlain method was more reliable for adults aged over 50 years. As such, the skeletal indicators with the highest accuracy were the pubic symphysis and the auricular surface, whereas the İşcan rib method was regarded as the least reliable.

However, when ±2 SD are used it should hardly come as a surprise that a method is deemed accurate, as the provided age ranges are so wide that 95% of estimated ages fall within the recommended phase or stage. Even with these weaknesses, well-established methods such as Suchey–Brooks, Lovejoy, and İşcan continue to be favoured due to their ease of use and popularity even though most were developed decades ago. Some of the newer or modified methods using the pubic symphysis, acetabulum, and rib ends still have not been tested on Thai samples. Lesser-known techniques such as vertebral osteophyte prevalence, histologic and biochemical methods have shown a high correlation with age in the Thai samples. But these methods are underutilized in current research and now is the time to further refine them on larger samples to discover their full potential. To fully understand morphological variation on an individual and population level, and to produce meaningful age estimates, emerging research needs to compare diet, activity levels, and health of Thais with other populations such as North American, European, and South African.

## Disclaimers

Nil

## Conflicts of interest

The authors declare no competing interests

## Author contributions

L.T.P. wrote the review; K.D. was responsible for review concept and editing.

## Acknowledgements

We are grateful to Dr. Nigel Chang and Dr. Anna Willis for providing critical review on early drafts of the manuscript. This study is supported by an Australian Government Research Training Program (RTP) Scholarship.

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Appendix B. 1 Published paper (Chapter 5)



Received: 20 September 2023 | Revised: 30 November 2023 | Accepted: 8 January 2024

DOI: 10.1111/joa.14010

Journal of Anatomy ANATOMICAL SOCIETY WILEY

ORIGINAL ARTICLE

# Age-dependent change and intraskeletal variability in secondary osteons of elderly Australians

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## Abstract

There is a need to fully understand intra-skeletal variability within different populations to develop and improve age-at-death estimation methods. This study evaluates age-related histomorphometric changes in three different bones intra-individually in a modern Australian sample. Four female and 13 male elderly Australian adult donors (67–93 years) were examined for osteon population density (OPD), osteon area (On.Ar), and Haversian canal area (H.Ar) of secondary osteons to compare between femora, ribs, and humeri and assess against age. In the pooled sex sample, no statistically significant correlations were observed between age and each histological variable. In the males, OPD of the femur increased significantly with age, as did porosity in the rib. In the male humeri, OPD increased moderately with age, while H.Ar was decreased moderately with age. Intra-bone comparisons showed that males had significantly higher osteon counts in their ribs compared to their femora, while their ribs showed statistically significantly less porosity than their humeri. When bone size was accounted for, by adjusting the femur and humerus histology data by robusticity indices, histology values were found to be similar between bones within the same individual. This is despite the upper and lower limbs receiving different ranges and types of biomechanical load. Our findings demonstrate that bone size influences histomorphometry, and this could confound age-at-death estimations that have not been adjusted for robusticity. Future studies would benefit from examining bone histomorphometry within a larger sample size and incorporating bone robusticity measures into histology analyses.

**KEYWORDS**
age-related histomorphometry, intra-skeletal variation, osteon, remodelling, robusticity index

## 1 | INTRODUCTION

Forensic anthropologists and bioarchaeologists have been embracing quantitative bone histology to establish age at death by utilising changes in bone microstructure that are known to correlate with advancing age (Crowder & Dominguez, 2013; Khan et al., 2017). Basic structural units in bone, secondary osteons, are evidence of bone remodelling processes. These osteons are the key feature of

most histological age estimation methods that typically use regression analyses to correlate independent histological variables, such as the tendency with advancing age to see an increase in the size of vascular pores and secondary osteon population density (OPD), and a corresponding decrease in overall osteon size (Ahlqvist & Damsten, 1969; Ericksen, 1991; Kerley, 1965; Stout & Paine, 1992; Yoshino et al., 1994).

Remodelling does not occur uniformly throughout the skeleton, so age estimation methods are developed on specific bones, most commonly the femur and the rib (Crowder & Dominguez, 2013; Gocha et al., 2019). Varying rates of remodelling are reported not only between different bones of an individual skeleton (Cole et al., 2022; Karydi et al., 2022) but also within different regions of a bone (Chan et al., 2007; Dominguez et al., 2020; Gocha & Agnew, 2016) depending on biomechanical loading regimes and metabolic activity. Increasing knowledge of intraskeletal histomorphometric variation is critical to improving accuracy in age-at-death estimation, particularly in older adults (Stout & Crowder, 2012).

Curated Australian human skeletal histological material accessible to researchers has been largely limited to the Melbourne Femur Research Collection (MFRC), which was established in 1991 at the University of Melbourne in Victoria (Thomas & Clement, 2011). Over the years, many national and international research articles have used this collection to document histomorphometric properties of the femur. However, what has been lacking is a skeletal reference collection that includes several different bones to evaluate intraskeletal variability in a modern Australian population. Documenting intraskeletal variability in bone remodelling within different populations is a critical step in understanding individual and regional trends, with implications for developing population-specific age estimation techniques. This will be the first study to evaluate sections from three different bones that have been collected intra-individually from an Australian sample.

The Australian histological bone sample used in this study was recently established at James Cook University in Townsville, Australia in 2021 (The James Cook University Human Skeletal Histology Collection [JCUHSHC]). This present study is a unique opportunity to assess age-progressive features of cortical bone remodelling by measuring secondary OPD, and size of Haversian canals and intact secondary osteons in the femur, humerus, and rib of elderly Australians. The aim is to test the degree to which histological features used in age estimation methods vary intra-skeletally within this population. It is hypothesised that localised biomechanical forces and metabolic activity would cause variation in remodelling. This study will contribute to a greater understanding of the normal range of histological variation that is yet to be fully explored in an Australian population.

Research on the femora of modern Australians has been relatively prolific, largely due to the large femoral sample held at the MFRC which is complemented by cadaveric samples from the Victorian Institute of Forensic Medicine (VIFM). Key findings for age-dependent histological changes in the femur show that there are similarities and contradictions between these studies. Britz et al. (2009) found that age statistically significantly correlated with increasing osteon circularity, and they observed decreasing osteon size in the femoral midshaft, with females having statistically significantly smaller osteons compared to males. Hennig et al. (2015) were able to confirm that osteon circularity increases with age in the anterior femoral midshaft of females; however, they did not find an age-associated decrease in osteon size. Two-dimensional microradiograph analysis did not identify significant correlations between cortical porosity (total and individual pore area, and number of pores) in the midshaft femur compared to an individual's height or weight (Stein et al., 1999), yet later 3D analysis showed that adults with lower body weight had significantly larger mean pore diameter (Cooper et al., 2007). Both studies found the most significant correlation to porosity to be age, and the differences in significance for weight may well be down to the very different methods of measuring porosity. It has also been observed that, compared to younger Australians, older individuals had a substantially thinner cortical zone in the upper femoral neck (Mayhew et al., 2005) and a tendency for new osteons to remodel within previously formed Haversian systems (Maggiano et al., 2016).

Most of these Australian studies focus on remodelling in the anterior midshaft region of the femur, while this present study will record changes in the posterior region, in addition to the rib and the humerus, which increases the current knowledge on inter-bone variability. This is key to better inform future archaeological and forensic efforts of age-at-death estimation from incompletely preserved and fragmentary human remains, where only some bones may be available for examination. Further, it is important to know which regions of bone will produce different OPD counts within an individual as this will impact age-at-death estimations (Dominguez et al., 2020). For example, statistically significant differences in OPD are observed between the anterior and posterior regions of transverse mid-shaft cross-sections of ribs (Dominguez et al., 2020). In contrast, only a statistically non-significant difference in OPD was discovered between the anterior and posterior femoral midshaft (Maggio & Franklin, 2021), whereas the anterolateral region recorded statistically significantly higher OPD, presumably depending on the degree of bending stress and axial loading placed on each region (Gocha & Agnew, 2016).

The current lack of research into age-related changes to secondary osteons in bones other than the femur in the Australian population is concerning for the future development of population-specific of age-at-death estimation methods, especially as it is well-documented that different bones are under varying degrees of metabolic stress and mechanical loading which tandemly effect cortical remodelling (Eleazer & Jankauskas, 2016; Stewart et al., 2021). The femur usually experiences the greatest magnitude of biomechanical loading to support body weight in bipedal motion, and it is subjected to compression and torsional forces with movement (Pfeiffer et al., 2006; Ruff et al., 1993). These forces instigate microscopic damage to bone which is then targeted for repair by remodelling (Robling & Stout, 2003). In comparison, the humerus is under less weight-bearing loading but

experiences axial loading forces from manipulative movements of the hands and arms (Sumner & Andriacchi, 1996), and, in turn, the ribs are under consistent, but low-level loading from breathing (Skedros et al., 2013). Ribs are more sensitive to hormonal changes and experience more metabolic bone loss than the femur and humerus, which can have an effect on remodelling rates (Eleazer & Jankauskas, 2016). These three skeletal elements also vary in cortical width and length. Human and non-human animal studies have determined that intracortical bone remodelling responds to bone size (Currey, 2003). Bone robusticity, an increase in diaphyseal length relative to width, is viewed as a naturally programmed response to compensate for increased porosity from metabolic activity (Eleazer & Jankauskas, 2016; Goldman et al., 2014). Short and wide bones will generally have an increased cortical region and more remodelling events compared to long and thin bones (Currey, 2003; Jepsen et al., 2015). More robust bones will have a higher OPD and larger osteons and Haversian canals than bones that are more gracile (Goldman et al., 2014). These studies highlight just how important it is to account for gross bone size when measuring microscopic geometric features within its cortex, yet it is not commonly accounted for (Miszkiewicz & Mahoney, 2019). This study will test for the effects of bone robusticity and aims to address the uncertainty of intraskeletal variation between the microstructure of the femur, humerus, and rib within a modern Australian sample, and which of the histological variables (OPD and osteon and Haversian canal size) have the highest correlation to age, specifically in males that have reached their 7th or 8th decade of life.

## 2 | MATERIALS AND METHODS

### 2.1 | Skeletal sample

Bone samples examined in the present study derive from human cadavers received as donations in the Human Bequest Program at James Cook University (JCU), Townsville, Queensland, Australia. Ethics protocols were filed and approved (JCU #H8352 and University of Queensland #2022/HE000860) to obtain and analyse bone samples from individual donors that had been embalmed and used for medical training in the JCU College of Medicine and Dentistry. The sample in this study consisted of 17 adults, four of whom were female and 13 of whom were male (Table 1). Age at death ranged from 67 to 93 years (mean 82.4 years ± SD 6.04 years). The females ranged in age from 67 to 88 years (mean age 80.5 years ± SD 9.95 years), and male age range was 75–93 years (mean age 83 years ± SD 4.73 years). Body donors in this study were bequeathed between 2014 and 2019, from within a 400 km radius of the regional city of Townsville in north Queensland. Donor information on record primarily includes sex, date of birth, age at death, and cause of death. No individuals of Aboriginal or Torres Strait Islander ancestry were included in this study.

TABLE 1 JCU sample size subdivided by sex.

| | n | Age range (years) | Mean age (years) | SD |
|---|---|---|---|---|
| All adults | 17 | 67–93 | 82.4 | 6.04 |
| Females | 4 | 67–88 | 80.5 | 9.95 |
| Males | 13 | 75–93 | 83.0 | 4.73 |

Abbreviation: JCU, James Cook University.

### 2.2 | Bone robusticity measurements and sectioning

Three bone sampling sites were chosen from each donor, incorporating a lower limb (femur), an upper limb (humerus), and the thorax (rib). The soft tissues surrounding the sites of interest were first dissected using a scalpel and knife, and the joints disarticulated at the knee and hip for the femur, and at the elbow and shoulder for the humerus. Maximum bone length and midshaft circumference were recorded (cm) using a standard tape measure to enable the robusticity index (RI) to be calculated for the femur and humerus. The robusticity index (RI = circumference/bone length × 100) (Ruff et al., 1993) was used to account for the effect of bone size on histology data. A limitation of this study was that RI could not be estimated for the rib as circumference and length measurements for this bone could not be obtained at the time of sample collection.

For sampling to be minimally invasive, yet sufficient, only a 1–2 cm thickness wedge section of cortical bone was removed (e.g., Maat et al., 2006; Mays et al., 2013; Miszkiewicz & Mahoney, 2019) from (1) the anterior midshaft of the humerus, incorporating the lower aspect of the deltoid tuberosity, (2) the posterior midshaft of the femur, incorporating the linea aspera, and (3) a cross-section from the midshaft of the 7th rib (Figure 1). Two individuals did not have ribs available for sampling, therefore a total of 49 bone sections were extracted from the 17 individuals. Sections were consistently taken from the right bone, with the exception of one individual (JCU 197-17) who only had bones on their left side available for sampling. Sections were removed using a hand-held electric rotary autopsy saw and then immediately fixed in a 70% ethanol solution and placed in cold storage (4°C) until further processing (Ries, 2003).

### 2.3 | Histological preparation

Standard histological methods were followed to prepare thin sections (Bancroft & Gamble, 2008). Using a portable hotplate (Thermo Fisher Scientific Cimarec®), extracted bone sections were gently macerated in a warm water and enzymatic laundry detergent mix over low heat (55 ± 5°C) for approximately 12 h, with the water and detergent mix replenished three times (Uhre et al., 2015). This is a quick, effective, and gentle process that does not damage the bone microstructure. Adhering soft tissue was then removed with

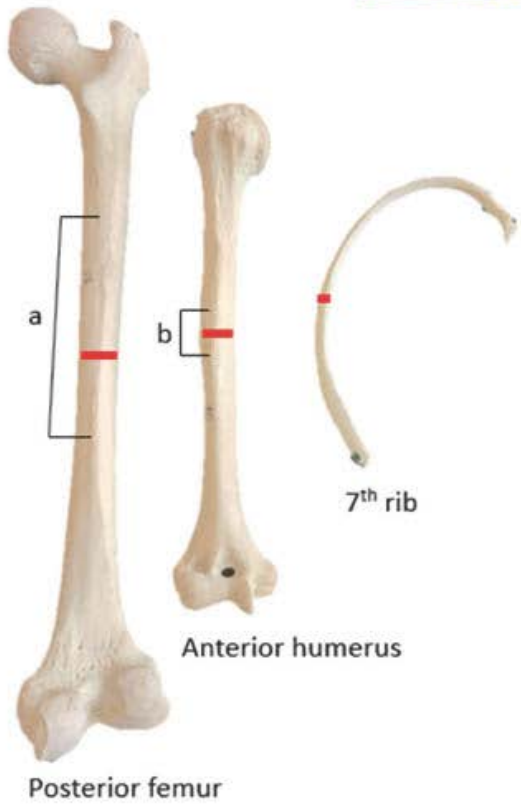


FIGURE 1 Midshaft sample locations from right bone. The samples incorporated the linea aspera (a) on femora and the deltoid tuberosity of the humerus (b).

the assistance of a plastic spatula and soft toothbrush. The undecalcified bone samples were placed in a fume cabinet to air dry for 24 h before being dehydrated in a graded ethanol series and cleared with xylene (An et al., 2003). Each section was embedded in resin (Buehler EpoxiCure™2). Sections of ~400 μm thickness were cut from the embedded blocks using a Buehler® IsoMet™ Low Speed Precision Cutter with a diamond blade, to obtain a transverse cross-section (Bancroft & Gamble, 2008). Each section was ground using a Gemmasta™ GF4 faceting machine with a diamond lap, and then hand-polished to 100 ± 25 μm thickness using diamond paste and a polishing cloth, before being washed, dried, cleaned in an ultrasonic bath, dehydrated, and mounted onto a glass microscope slide and sealed with a cover slip (Bancroft & Gamble, 2008).

## 2.4 | Selection of regions of interest

Each slide was viewed under a high-powered microscope (Olympus BX43), and images of six regions of interest (ROIs) (Figure 2) were taken with a mounted microscope camera (Olympus EP50 with 0.5 camera adapter) (1) under transmitted light and (2) with a polarising filter. The technique used to select ROIs greatly varies between studies and lacks standardisation (Villa & Lynnerup, 2010). In this study, ROIs were selected within the mid-cortical region. For the femur and humerus, the positions of ROI 1 and 2 were selected to be slightly offset either side of an arbitrary midline of the peri-curve (Figure 2), ROIs 3 and 4 were parallel to ROIs 1 and 2 but positioned toward the endosteum, ROI 5 was adjacent to ROIs 1 and 4, and ROI 6 was adjacent to ROIs 2 and 3. For the ribs, ROI 1 was selected on the cutaneous side toward the costal groove (if identifiable), then moving in an anti-clockwise direction ROI 2 was positioned in the mid-section, ROIs 3 and 4 opposite each other on the superior edge, ROI 5 opposite ROI 2, and ROI 6 opposite ROI 1. The position of each ROI was consistent to ensure replicability and to prevent osteons from one ROI being included in an adjacent ROI. This ensured that the same osteons would not be counted twice. The humerus from several individuals had a narrow cortical width so the six ROI images had to be positioned side by side, mediolaterally, instead of the window pattern in Figure 2.

A ×20 objective was used for the ribs, and a ×10 objective for the humerus and femur (with 10× oculars). With the camera adapter, this produced a rectangular field of view of 1.182 $mm^2$ for each ROI of a femur and humerus and 0.285 $mm^2$ for each ROI of the rib. This gave a total area of 7.09 $mm^2$ for each femur and humerus bone section and 1.71 $mm^2$ for each rib bone section. The average intact osteon count for sections of the femur was 30.6, humerus 28.8, and ribs 12.0. Bone size variability was accounted for by incorporating robusticity indices into the statistical analyses. Bone robusticity is a measure that is commonly utilised to interpret the allometric relationship between bone size and quantifiable characteristics of cortical bone remodelling (Miszkiewicz & Mahoney, 2019).

## 2.5 | Quantification of histomorphometric variables

Histomorphological features such as osteon cement lines and lamellae were most clearly observed on the polarised light images, while the perimeter of Haversian canals was best viewed on the transmitted light images. Features were recorded and labelled on corresponding images using imaging software (open access FIJI/ImageJ®) (Schindelin et al., 2015) with a range of drawing and point counting tools: the 'multi-point' and 'freehand selection' tools (Figure 3). Table 2 defines each histological variable. While this study focuses on the elderly, and it is known that the OPD asymptote can erase real OPD in individuals older than 50 (Crowder & Dominguez, 2013), OPD was recorded as part of the histomorphometric standards and included in analyses. However, the effect of OPD asymptote on OPD data will be taken into account when interpreting and discussing data. The number of intact and fragmented osteons per ROI was recorded on polarised light images

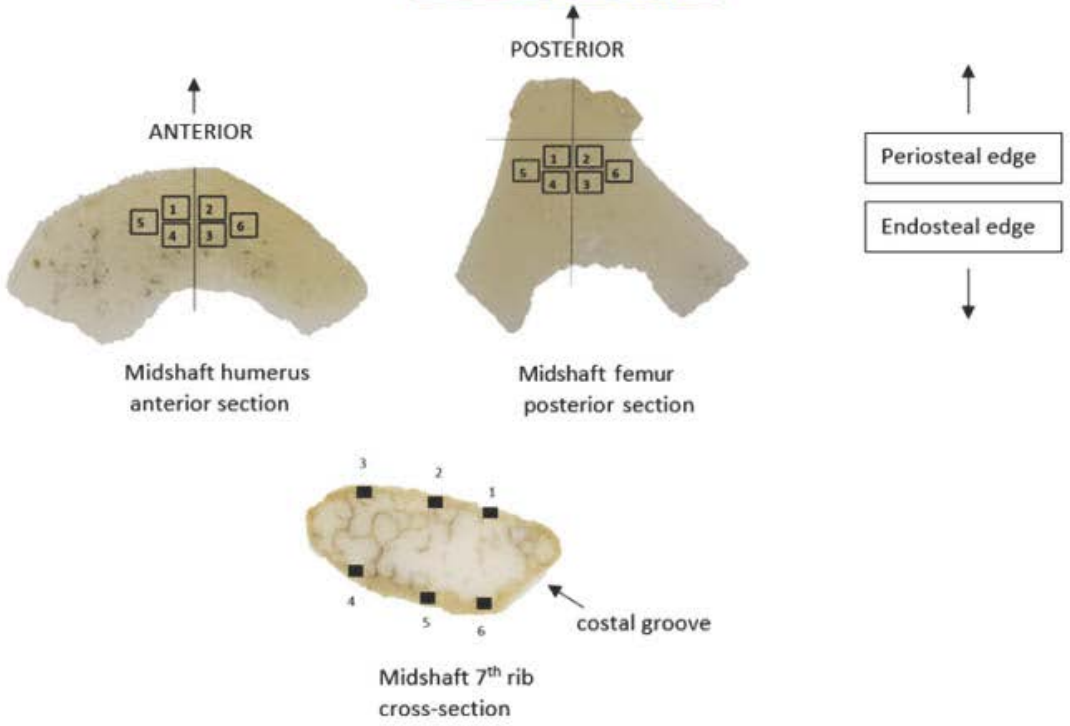


FIGURE 2 Selection of the regions of interest (ROI). Six ROIs were selected in the sub-periosteal layer of intracortical bone for the anterior midshaft of the humerus, posterior midshaft of the femur, and rib mid-shaft (bones not to scale and position of ROIs are approximate).

and counts used to calculate OPD/mm$^2$ (#intact osteons + #fragmentary osteons divided by image area [mm$^2$]). On transmitted light images, the perimeter of each Haversian canal was traced using a stylus and laptop, creating the variable Haversian canal area (H.Ar in μm$^2$), while the perimeter of each intact osteon was traced on polarised images and recorded as secondary osteon area (On.Ar in μm$^2$) (Maggio & Franklin, 2021).

## 2.6 | Statistical analyses

The data were analysed in IBM SPSS® 29.0, at $p = 0.05$. Intra-observer error was assessed by re-taking measurements on 10% of ROIs and comparing them against original values by performing paired non-parametric correlations (Wilcoxon signed-rank test). As this was a relatively small sample, non-normal distribution was assumed, and thus non-parametric testing was chosen (Depuy & Pappas, 2004). As there were only four females, sex differences in histology were not tested for. Instead, correlations were first tested on the pooled sex group and then on the male-only group. A Spearman's rank-order correlation tested the relationship between age and three histological variables (OPD, On.Ar, and H.Ar). To account for robusticity of the femur and humerus, and the allometric/isometric effect of bone size on the histological variables, Spearman's rank-order tests were also recalculated with the histology variables adjusted by the robusticity index (RI) (e.g., H.Ar Lg10).

Next, the significance of the relationship of each of the histological variables was tested between the three bones (femur, humerus, and rib) using Friedman two-way analysis of variance by ranks test. This was followed by post hoc Wilcoxon signed-rank tests (with the Bonferroni correction for multiple comparisons) to determine which of the bones was most sensitive to remodelling. Two of the males did not have ribs available for sampling (JCU195/17 and JCU197/17) so were removed from this part of the analysis, this reduced the entire dataset to 15 individuals, and the male dataset to 10 individuals for Friedman two-way and post hoc analyses.

Correlations between age groups were not sought as most of the sample fit into a narrow age at death range between 75 and 88 years ($n = 15$). A 93-year-old male (JCU141/14) was excluded from the analyses as this was the only individual in their 9th decade of life and their OPD was a strong outlier. This elderly male will be discussed as an individual case. Therefore, the results of this study can best be

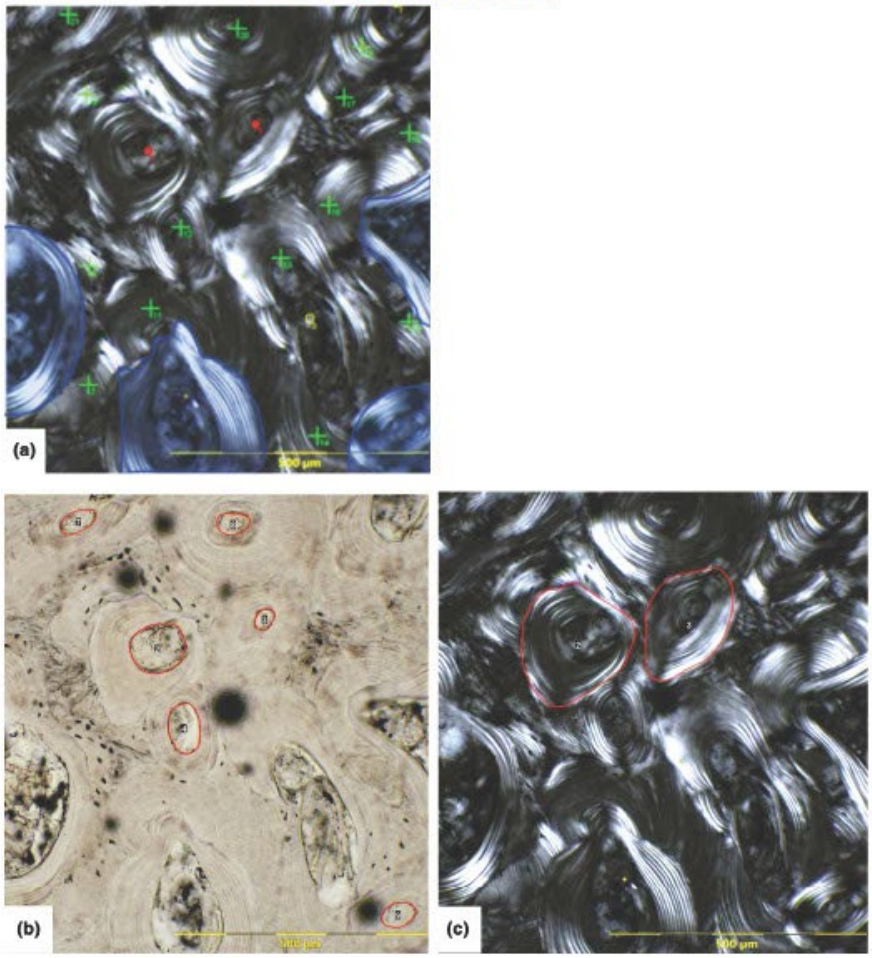


FIGURE 3 Examples of histomorphometric features recorded on each ROI in this study: (a) count of intact osteons (red dot), fragmentary osteons (green cross), resorption cavity (yellow circle); osteons cut off by the image border were not counted if it could not be determined if they were intact or fragmentary (opaque blue); (b) Haversian canal area measurements ($\mu m^2$) (red circles) (black dots are artefacts of sample processing); (c) intact osteon area measurement ($\mu m^2$) (red circles).

used to interpret the bone microstructure of elderly adult Australian males in their 7th and 8th decade of life.

## 3 | RESULTS

### 3.1 | Intra-observer tests and the effects of age

There were no statistically significant intra-observer differences between the original and repeated measures (paired correlations, $n=51$, $z=-2.722$ to $-1.255$, $p=>0.05$). Spearman's test of correlations within the pooled sex group showed no statistically significant relationships between age and each of the histological variables (OPD, On.Ar, and H.Ar). A moderate positive correlation was observed between age and Haversian canal area in the rib ($r_s=0.442$, $p=0.099$) which is illustrated in Figure 4. The rest of the correlations for the pooled sexes were weak and are presented in Figure S1.

For the male group, scatter plots illustrating the four strongest relationships with moderate correlations are provided in Figure 5 and all other correlations (weak) are illustrated in Figure S2. There was a statistically significant positive relationship between age and OPD of the femur ($r_s=0.603$, $p=0.038$), and the Haversian canal area in the rib ($r_s=0.646$, $p=0.043$). A moderate positive relationship was recorded between age and OPD of the humerus ($r_s=0.529$, $p=0.077$) along with a corresponding moderate negative correlation with Haversian canal area in the humerus ($r_s=-0.423$, $p=0.170$).

TABLE 2 Definitions of histological variables examined in this study.

| | |
|---|---|
| *Intact osteons* | Osteon that is completely surrounded by a cement (reversal) line, its Haversian canal is intact, and it has not been breached by a resorption space/walls of a later osteon. This is a type I osteon. If they follow the above criteria, included are osteons that have either completed filling or are still being filled, osteons breached by a Volkmann's canal, and type II osteons (osteon embedded within the cement line of a pre-existing osteon). If two or more osteons appear to share a Haversian canal and/ or share a cement line due to the plane of sectioning, including a branching event, then they are counted as one intact osteon. |
| *Fragmentary osteons* | A pre-existing osteon that has had its lamellae and/or the Haversian canal breached by subsequent generations of osteons or a resorption cavity. Concentric lamellae fragments that are clearly identifiable as previous osteons, that is have part of a cement line visible and osteocytes/lacunae between the lamellae, were included. |
| *Osteon population density (OPD)* | Total count of intact (N.On) and fragmentary osteons (N.On.fg) per region of interest (N.On+N.On.Fg/mm$^2$). |
| *Resorption cavity (RC)* | An area of resorbed bone, bordered by scalloped edge of a Howship's lacuna. |
| *(mean) Osteon area (On.Ar)* | The reversal line of each intact secondary osteon was traced, and the internal area was calculated in µm$^2$. For each bone section, the mean On.Ar was used in analyses (summed On.Ar from all ROIs/number of measured osteons). |
| *(mean) Haversian canal area (H.Ar)* | Complete Haversian canals without any indication of resorption were traced, and the internal area in µm$^2$ was calculated. For each bone section, the mean H.Ar was used in analyses (summed H.Ar from all ROIs/number of measured canals). |

*Note*: Modified definitions from Crowder et al. (2022) and Cho et al. (2002).

## 3.2 | Intra-skeletal differences

The Friedman test identified statistically significant differences in OPD count, osteon area, and Haversian canal area (pooled sample: $\chi^2$=14.533–19.600, $p$<0.001, $n$=15) (male sample: $\chi^2$=9.800–12.800, $p$=0.007–0.002, $n$=10) between at least two of the bones (Table 3). Subsequent corresponding post hoc analysis on the entire dataset identified that the rib had statistically significantly more osteons, as well as statistically significantly smaller osteons and Haversian canals, in comparison to both the femur and the humerus (Table 4). In males, the rib had a significantly higher osteon count than the femur ($Z$=−2.803, $p$=0.005), and rib Haversian canals were statistically significantly smaller than in the humerus ($Z$=−2.803, $p$=0.005). No significant differences were found between the femur and humerus for any of the histological variables. Descriptive statistics (Table S1) show that the mean for OPD, osteon area, and Haversian canal area are always similar between the femur and humerus, but the rib is different.

## 3.3 | RI-adjusted data for the femur and humerus

### 3.3.1 | The effects of age

When bone size of the femur and humerus were taken into account by adjusting the histological values with RI, there was a change in correlation strength (Tables 5 and 6). Spearman's test of correlations between age and the three adjusted histological variables within the pooled sex group showed all correlations were still weak. Scatter plots for these relationships are illustrated in Figure S3, with the humerus showing the weakest relationship (On.ArLg10, $r_s$=−0.007, $p$=0.978), and the strongest relationship with age (OPDLg10, $r_s$=−0.219, $p$=0.398) compared to the femur.

In the male group, once bone robusticity was accounted for, some of the relationships with age became stronger (Haversian canal size in the femur and humerus), while others became weaker (OPD in the femur and humerus, and humerus osteon area), or remained similar between the original and RI-adjusted values (femur osteon area). Moderate correlations between all three adjusted histological variables and age were observed in the femur (OPD Lg10, $r_s$=0.476, $p$=0.118; On.ArLg10, $r_s$=−0.360, $p$=0.251; and H.Ar Lg10, $r_s$=−0.360, $p$=0.256) (Table 5). However in the humerus, the only moderate correlation with age was in Haversian canal size (H.Ar Lg10, $r_s$=−0.536, $p$=0.072) (and this was also the strongest of all correlations with age). Scatter plots for each relationship are illustrated in Figure S4.

### 3.3.2 | Intra-skeletal differences

When the variable of bone size was removed, there was no longer a statistically significant difference in OPD between the femur and humerus for individuals in the pooled sample (Table 6). Male individuals were found to have statistically significantly smaller Haversian canals in their humerus compared to their femur ($Z$=−2.201, $p$=0.028). No other relationship between the femur and humerus was statistically significant.

## 4 | DISCUSSION

In this study, the secondary osteons of elderly modern Australians are examined to (1) evaluate the relationship between age and measures of cortical bone remodelling (OPD and size of osteons and Haversian canals); (2) intra-skeletally compare remodelling between different bones of the axial (rib) and appendicular (femur and humerus) skeleton that are subject to different rates of metabolic and biomechanical stresses; (3) examine the relationship between a skeletal element's macroscopic size and the microarchitecture within its cortical bone. In ageing research, it is more common for studies to evaluate histological geometric variables without adjusting for bone size, but this study will discuss the results using both the 'raw' data and the 'adjusted' data which takes

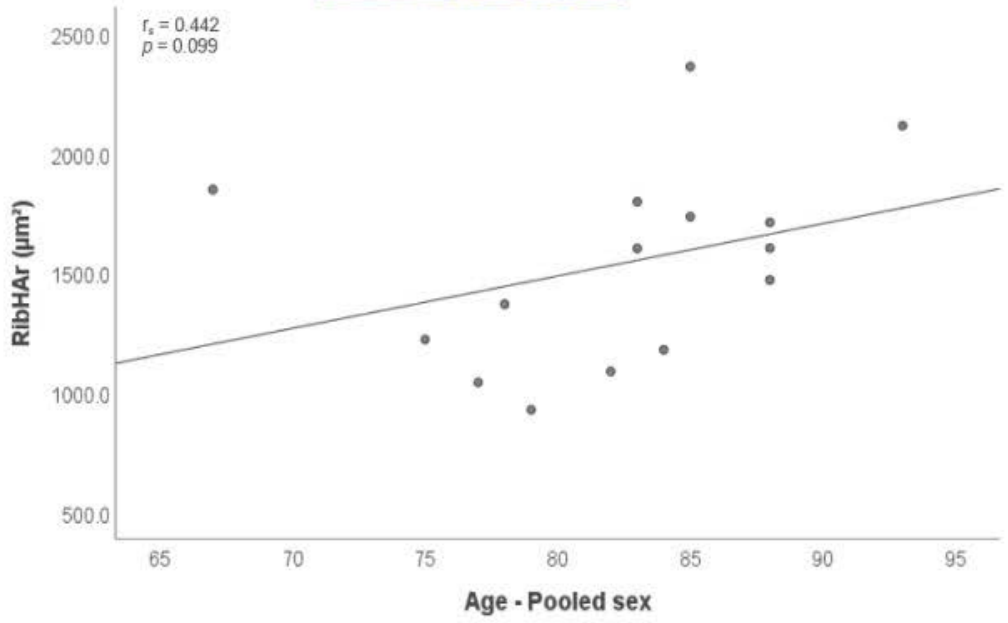


FIGURE 4 Scatter plot with line of best fit illustrating the moderate positive relationship between age and Haversian canal size of the rib within the pooled sex group.

into account the effect of bone size on the underlying histological features.

In the pooled sex sample, age only weakly affected OPD, osteon area, and Haversian canal area within all three bones, with the exception of a moderate increase in Haversian canal size within the rib. Even when bone robusticity was accounted for, the correlation of age with each of the three histological variables remained weak within the femur and humerus. These results are likely an effect of combining the sexes, as rates of bone remodelling are reported to differ between males and females (Abdullah et al., 2018; Ericksen, 1991). Descriptive statistics of the JCU sample show that females have larger mean osteon area in all three bones compared to males; however, the relatively small female sample size meant that significance between the sexes could not be reliably tested. Some studies have observed only weak sexual dimorphism in osteon area (Pfeiffer, 1998; Pfeiffer et al., 2006), while others reported that males had statistically significantly smaller osteons than females, attributable to greater body mass in males and the likelihood to have participated in more physically strenuous activities (Mulhern & van Gerven, 1997). Britz et al. (2009) also found an inverse relationship between body weight and osteon size, but they observed that Australian females have statistically significantly smaller osteons than males in the anterior midshaft of the femur, with menopause likely impacting the rate of remodelling in females (Cho & Stout, 2011). In this present study, female mean Haversian canal area is also larger in all three bones compared to males. The females are all over 67 years of age and would be post-menopause. Estrogen deficiency causes an imbalance in bone metabolism and remodelling (Tobias & Compston, 1999), with greater bone resorption (Riggs, 2000), and an increased rate of cortical thinning (Seeman, 2013). The mean OPD is lower in females than males in the femur and humerus, but not the rib. Sexual dimorphism in hormones, diet, body mass, and genetics (Cho & Stout, 2011) would likely explain the different pattern of remodelling observed in sexes.

As there were only four females in the sample, the remainder of analyses was restricted to males who comprised three-quarters of the sample. With increasing age, male femora showed a statistically significant increase in OPD as did Haversian canal area in the rib. This means that as males age, they generally accumulated more osteons in the femur compared to their humerus and rib. Vascular canals in the rib get larger as remodelling activity becomes out of balance and the filling of osteons is arrested (Pfeiffer et al., 2006). For a given loading environment, the rib is expected to be under the most metabolic influence, the femur minimal, and the humerus intermediate (Eleazer & Jankauskas, 2016). Minimal bending forces and dynamic loading are experienced by the rib (Robling & Stout, 2003) and at a rate that is relatively uniform across individuals (Bonicelli et al., 2022). These factors affect vascular porosity, visible in the form of larger Haversian canals and greater variability in size of the canals (Pfeiffer et al., 2006). On the other hand, the literature generally reports the femur to be placed under greater biomechanical loading (Ruff et al., 1991), causing locally intensified remodelling to aid microcrack repair (Wasserman et al., 2008). This generally constrains the size of vascular pores and more of the smaller osteons

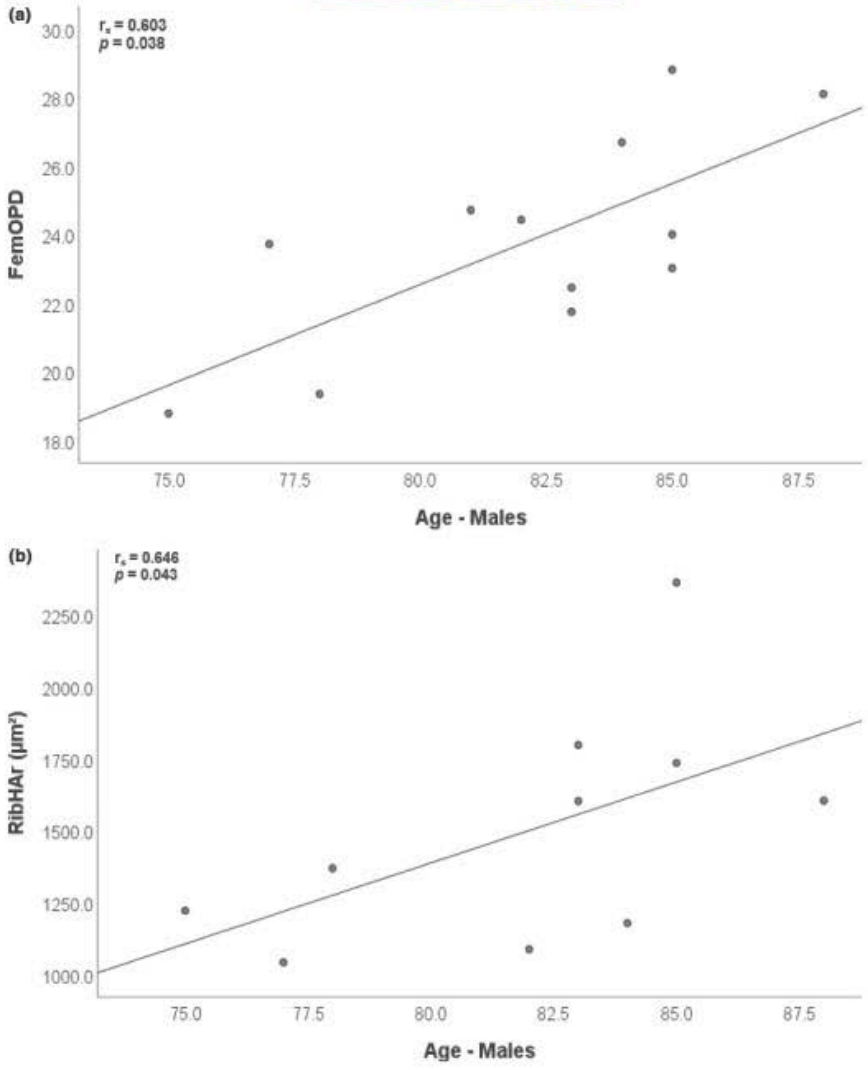


FIGURE 5 Examples of the four best correlations with age within the male sample: statistically significant positive relationships (bold-faced) with OPD of the femur (a) and Haversian canal area of the rib (b); moderate positive correlation with humerus OPD (c), and moderate negative correlation with humerus Haversian canal size (d).

remodel the space (Miszkiewicz, 2016), generating a higher OPD. Alternatively, some studies report declined OPD in the femur compared to the rib which is under less mechanical strain and therefore is better able to reflect mineral homeostasis and hormonal responses (Skedros et al., 2013).

Males in the present study also showed a moderate positive relationship between age and OPD of the humerus along with a corresponding moderate inverse relationship with Haversian canal area in the humerus. Biomechanics affect the humerus to a lesser extent than the femur but this is still likely confounding the remodelling process (Skedros et al., 2013), whereby as the males age there is an accumulation of osteons in the humerus accompanied by smaller Haversian canals. Upper limb bones such as the humerus are typically non-weightbearing, but muscle pull and strain from the wide range of motion in the arm and shoulder (Santos et al., 2018) subjects the limb to axial loading and bending and torsional forces (Trinkaus et al., 1994). Descriptive statistics (Table S1) show that the means for OPD, osteon area, and Haversian canal area are similar between the femur and humerus, but the rib is different. OPD in the rib tends to be higher and osteon and canal size lower compared to

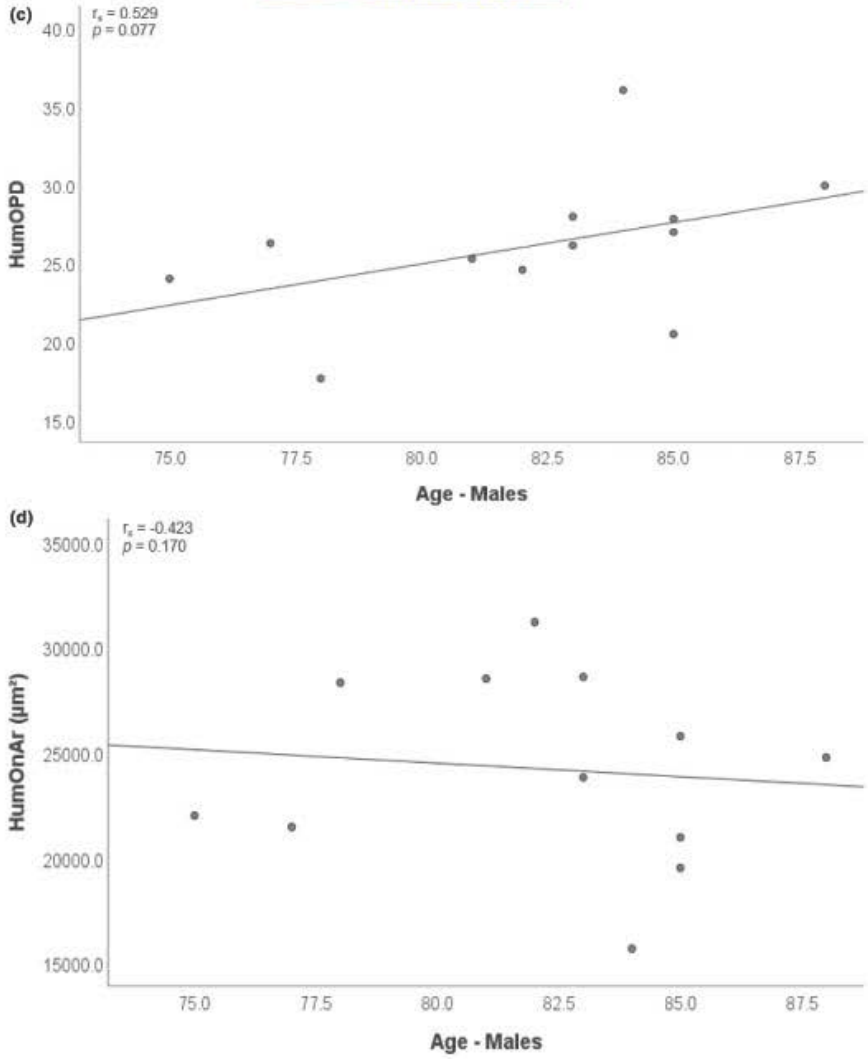


FIGURE 5 (Continued)

TABLE 3 Friedman test of significant relationships between bones by sample.

| Pooled sex $n=15$ | $\chi^2$ | Sig. | Males $n=10$ | $\chi^2$ | Sig. |
|---|---|---|---|---|---|
| OPD | 19.600 | <0.001 | OPD | 12.800 | 0.002 |
| On.Ar | 14.533 | <0.001 | On.Ar | 9.800 | 0.007 |
| H.Ar | 18.533 | <0.001 | H.Ar | 11.400 | 0.003 |

the long bones. This shows that the rib in the axial skeleton has a unique pattern of remodelling compared to the femora and humeri in the appendicular skeleton. Robusticity indices were used to account for the effect of bone size on remodelling rates (Miszkiewicz & Mahoney, 2019). In this study, the robusticity of the rib could not be accounted for as length and width measurements were not collected at the time of sampling. Adjusting for bone robusticity in the femora and humeri changed the strength of the relationships between age and the three histological variables in males, but the overall pattern of remodelling remained valid. OPD tended to increase with age,

TABLE 4 Post hoc Wilcoxon test.

| Pooled sex $n=15$ | Z | Sig.* | Males $n=10$ | Z | Sig.* |
|---|---|---|---|---|---|
| Humerus OPD—Femur OPD | 2.385[a] | 0.017 | Humerus OPD—Femur OPD | 2.293[a] | 0.022 |
| Rib OPD—Femur OPD | 3.408[a] | **<0.001** | Rib OPD—Femur OPD | 2.803[a] | **0.005** |
| Rib OPD—Humerus OPD | 3.237[a] | **0.001** | Rib OPD—Humerus OPD | 2.497[a] | 0.013 |
| Humerus On.Ar—Femur On.Ar | 1.136 | 0.256 | Humerus On.Ar—Femur On.Ar | 0.968[b] | 0.333 |
| Rib On.Ar—Femur On.Ar | 3.010[b] | **0.003** | Rib On.Ar—Femur On.Ar | 2.497[b] | 0.013 |
| Rib On.Ar—Humerus On.Ar | 3.237[b] | **0.001** | Rib On.Ar—Humerus On.Ar | 2.701[b] | 0.007 |
| Humerus H.Ar—Femur H.Ar | 0.966[b] | 0.334 | Humerus H.Ar—Femur H.Ar | 1.172[b] | 0.241 |
| Rib H.Ar—Femur H.Ar | 3.408[b] | **<0.001** | Rib H.Ar—Femur H.Ar | 2.803[b] | **0.005** |
| Rib H.Ar—Humerus H.Ar | 3.124[b] | **0.002** | Rib H.Ar—Humerus H.Ar | 2.293[b] | 0.022 |

[a]Based on negative ranks.

[b]Based on positive ranks.

*Bonferroni adjusted $p$ value of 0.006; statistically significant results are in bold.

TABLE 5 Spearman's correlation tests comparing results of original values to RI-adjusted values for the femur and humerus.

| | Original values | | RI-adjusted values | |
|---|---|---|---|---|
| Pooled sex | $r_s$ | Sig. | $r_s$ | Sig. |
| FemOPD | −0.053 | 0.840 | −0.189 | 0.469 |
| FemOn.Ar | −0.010 | 0.970 | −0.166 | 0.523 |
| FemH.Ar | −0.087 | 0.738 | −0.169 | 0.517 |
| HumOPD | 0.229 | 0.376 | 0.211 | 0.417 |
| HumOn.Ar | −0.078 | 0.767 | −0.007 | 0.978 |
| HumH.Ar | −0.239 | 0.355 | −0.219 | 0.398 |
| | Original values | | RI-adjusted values | |
| Males | $r_s$ | Sig. | $r_s$ | Sig. |
| FemOPD | *0.603** | **0.038** | *0.476* | 0.118 |
| FemOn.Ar | −0.339 | 0.282 | *−0.360* | 0.251 |
| FemH.Ar | −0.159 | 0.622 | *−0.360* | 0.256 |
| HumOPD | *0.529* | 0.077 | 0.174 | 0.819 |
| HumOn.Ar | −0.243 | 0.446 | −0.109 | 0.735 |
| HumH.Ar | *−0.423* | 0.170 | *−0.536* | 0.072 |

*Note*: Italicized values are moderate correlations Taylor (1990).

Abbreviation: RI, robusticity index.

*The only significant relationship with age ($p \leq 0.05$) is bold-faced.

TABLE 6 Wilcoxon signed ranks test comparing significance of original values and RI adjusted values for the femur and humerus.

| | Original values | | RI-adjusted values | |
|---|---|---|---|---|
| Pooled sex | Z | Sig. | Z | Sig. |
| HumOPD—FemOPD | **−2.107[a]*** | **0.035** | −1.112[a] | 0.266 |
| HumOn.Ar—FemOn.Ar | −0.639[b] | 0.256 | −1.254[b] | 0.210 |
| HumH.Ar—FemH.Ar | −0.254[b] | 0.334 | −1.538[b] | 0.124 |
| | Original values | | RI-adjusted values | |
| Males | Z | Sig. | Z | Sig. |
| HumOPD—FemOPD | **−1.961[a]*** | **0.050** | −1.083[a] | 0.279 |
| HumOn.Ar—FemOn.Ar | −0.157[b] | 0.875 | −1.433[b] | 0.152 |
| HumH.Ar—FemH.Ar | −1.569[b] | 0.177 | **−2.201[b]*** | **0.028** |

Abbreviation: RI, robusticity index.

[a]Based on negative ranks.

[b]Based on positive ranks.

*Significant differences ($p \leq 0.05$) are bold-faced.

while the size of osteons and Haversian canals tended to decrease with age. With the effect of bone size removed, it appears that bone tissue in the femur and humerus has similar quality within one individual despite the leg and the arm being used in slightly different ways. The only statistically significant result was that Haversian canals were smaller in the humerus compared to the femur, whereas OPD and size of osteons were relatively similar between the long bones. This shows there is a tight anatomical relationship between bone macro- and microscopic size.

The mean age of the male sample in this present study was 82 years, with age at death ranging from 75 to 88 years; therefore, the results best represent elderly Australian males. Most age-related cortical bone loss occurs after the age of 60 years (Seeman, 2013), with a decrease in vascular pore numbers corresponding with an increase in pore size (Bousson et al., 2001). Age-related increase in pore size in older adults may be relatable to the coalescence of pre-existing Haversian canals and new resorption cavities (Andreasen et al., 2020; Seeman, 2013). There is much individual variability, especially in the elderly who are at increased risk of metabolic bone diseases such as osteoporosis (Kulminski et al., 2006) and a lifestyle with less physical activity and reduced muscle mass (Cvecka et al., 2015). Increasing levels of physical inactivity and nutrient-deficient diet in the elderly are regularly reported in clinical literature for their detrimental effects on bone health and interruption to remodelling processes (Bonjour et al., 2009). Additionally, many elderly seek therapeutic treatment for osteoporosis and metastatic

bone diseases and cancers that deliberately target bone resorption and formation by inhibiting bone resorption and/or formation (Skjødt et al., 2019).

One male of 93 years age at death was removed from the male sample as their OPD counts made them a strong outlier. The remodelling in this individual's femur was unique compared to the other males. This individual has the lowest mean OPD count for the femur but also the largest mean osteon area (except for JCU 247/19, an 82-year-old male). Porosity in this individual's femur was more pronounced than the younger males. Their large resorption cavities had removed evidence of earlier osteons and therefore reduced the OPD count and interfered with osteon area measurements. The intact osteons are visibly smaller than the remnants of fragmentary osteons. This is consistent with studies that report osteon size decreasing with age (Dominguez & Agnew, 2016; Maggio & Franklin, 2021; Narasaki, 1990). Takahashi et al. (1965) made the point that the well-documented decrease in osteon area could largely be due to an increased likelihood of the largest and most irregularly shaped osteons being remodelled, with only smaller intact osteons left to measure. This male also has one of the lowest OPD counts for the humerus and rib, compared to all the other males who are younger, and almost the lowest mean canal area in both the humerus and rib. A factor to consider is that the age of OPD asymptote may have been reached in at least one of the bones in this individual. Asymptomatic values indicate that the cortical bone has been completely remodelled, in which case any new secondary osteons will simply be replacing already remodelled bone that is saturated with fragmentary osteons (Frost, 1987a). This means that the mean annual OPD will not increase and will no longer correlate with age (Frost, 1987b). Fewer intact osteons with an uninterrupted cement line will remain to measure osteon area, and this issue is compounded by the narrowing of cortical bone with age, especially in the rib, which further decreases the number of intact and fragmentary osteons to count and measure (García-Donas et al., 2021). The age at which asymptote occurs varies between bones depending on osteon size, bone turnover rate, and cortical area (Andronowski & Crowder, 2019). For example, asymptote is recorded to occur in the 5th or 6th decade of life in ribs at an OPD of 30/mm$^2$ (Cho et al., 2002; Stout & Paine, 1994), likely due to the narrow cortical area being completely remodelled before the thicker and denser cortex of the femur (Frost, 1987a). Asymptote in the femoral midshaft is documented to be reached when OPD count is approximately 50–55/mm$^2$, after the 8th decade of life (Gocha & Agnew, 2014). The sex and ancestry have an effect on bone remodelling and thus are likely to each have an effect on OPD asymptote but this requires further investigation on more genetically diverse populations to fully understand the correlation (Pfeiffer, 1992).

In the present study, males showed few statistically significant relationships between age and the three histological variables, and in the intraskeletal comparisons, but this still shows some potential for the use of histology in ageing research. However, even the non-significant relationships have provided a useful account of the remodelling processes within this modern Australian sample. To summarise this present study, OPD in elderly males tended to increase with age within the femur and humerus but decline within the rib. This is most likely tied in with higher targeted mechanical loading placed on the posterior femur and anterior humerus, compared to the less strenuous but constant loading placed on ribs. This study suggests that research on the Australian population would benefit from focusing on the rib because it will not be as impacted by biomechanical stress as the femur and may therefore have greater potential for age estimation via histomorphometry. Osteon and canal size tended to decrease with age in the femur and humerus whereas they increased in the rib with advancing age, and this could be accounted by ribs experiencing greater porosity with age due to greater metabolic influences than the bones from the appendicular skeleton are subjected to. Differences in bone size, and therefore the amount of cortical bone area, may be confounding raw histology measurements. Accounting for bone size in males tended to weaken most of the relationships between age and histomorphometric values within the femur but tended to strengthen more of them within the humerus. Significantly smaller Haversian canals were observed in the humerus compared to the femur, whereas osteon and canal size were relatively similar between the two bones. These results support prior work on other populations which has shown that bone size has an underlying effect on its internal microarchitecture (Goldman et al., 2014; Miszkiewicz & Mahoney, 2019).

## 5 | LIMITATIONS

The sampling was restricted by the scarcity of young adult and female donors. Differences between the sexes and between young and older adults could therefore not be examined. The age and sex biases reflect the nature of body donation for medical education and research within Australia. Studies have found that even when an individual expresses their willingness to donate, there are psychological (i.e., emotional), religious, cultural, or social factors that may hinder family members from providing final consent for body donation. Thus, this study does not reflect the full extent of histomorphometric variation in the Australian population. It is expected that as the JCUHSHC grows over time it will gradually gain more female and younger donors. Future studies can then build upon this study with a larger and more representative sample. Adjustments for robusticity could not include the rib, as the maximum length and midshaft circumference measurements were only obtained for the femur and humerus before bone samples were taken from the donors. Additionally, full shaft cross-section area of the femur and the humerus could not be included in robusticity calculations as the ethics protocol stipulated minimally invasive sectioning and CT scanning was not possible on the bones immediately pre-cremation. As the amount of research on the JCUHBHC increases, the sampling procedures will also be refined to include more measurements. It is well-documented that genetics, health, metabolic processes, and biomechanical loading influence osteon geometric properties (Stout et al., 2019), and the effect of one may

mask or mimic the effect of the other (Eleazer & Jankauskas, 2016). This information has not been collected from the donors who self-report medical and personal information. Donors are not required to provide details such as occupation and physical activity levels, so it is difficult to determine how these are affecting interpretations of remodelling rates. It is possible that future ethics applications may be adjusted to allow for this data to be collected from donors.

## 6 | CONCLUSION

The motivation for this study was to enhance the understanding of intra-skeletal variation of cross-sectional geometric properties, and in particular, how these properties correlate to age in a modern Australian sample. The sex and age biases in this sample mean that the results best measure cortical remodelling of elderly Australian males. This study confirms that bone size has an underlying effect on histology, changing the strength of the correlation with age. It is recommended that future studies in age estimation research should consider bone macro- and micro-measurements together. With robusticity accounted for, the one statistically significant result in males (statistically significantly smaller Haversian canals in the humerus compared to femur) shows some potential for the use of histology in ageing research. This study suggests that the rib will not be as impacted by biomechanical stress as the femur and may therefore have greater potential for age estimation via histomorphometry.

### AUTHOR CONTRIBUTIONS

LP: conceived the study, composed the overall question, background research, and wrote the manuscript, prepared the sample, conducted statistical tests, and interpreted the results; JM: provided expert advice on content and input into interpretation of the data and editing the manuscript, LC: provided expertise in sample preparation and laboratory procedures; AW provided critical review of the manuscript; KD: was responsible for conception and editing.

### ACKNOWLEDGEMENTS

The authors thank the anatomy laboratory staff at the James Cook University College of Medicine and Dentistry for their assistance in the collection of bone samples used in this study. This research was supported by an Australian Government Research Training Program (RTP) Scholarship. Open access publishing facilitated by James Cook University, as part of the Wiley - James Cook University agreement via the Council of Australian University Librarians.

### CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

### DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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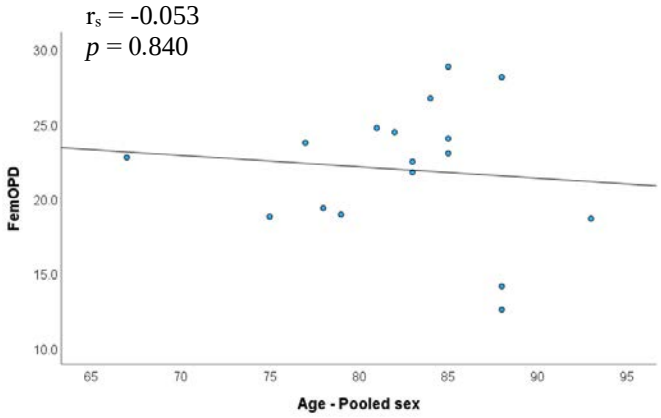
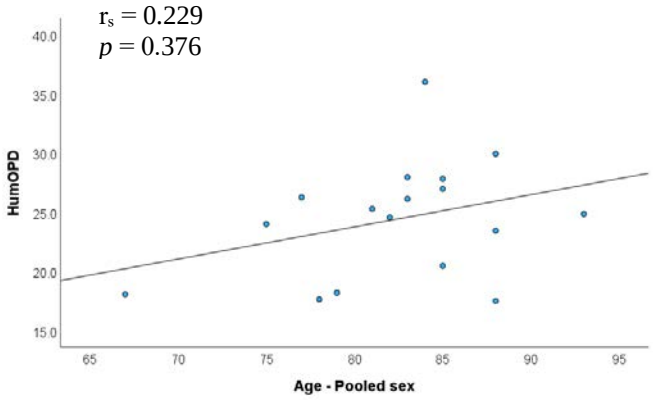
## SUPPORTING INFORMATION

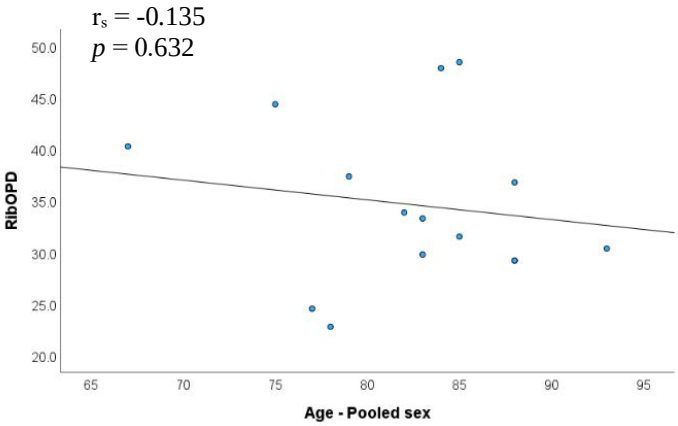
Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Pedersen, L.T., Miszkiewicz, J., Cheah, L.C., Willis, A. & Domett, K.M. (2024) Age-dependent change and intraskeletal variability in secondary osteons of elderly Australians. *Journal of Anatomy*, 00, 1–15. Available from: https://doi.org/10.1111/joa.14010

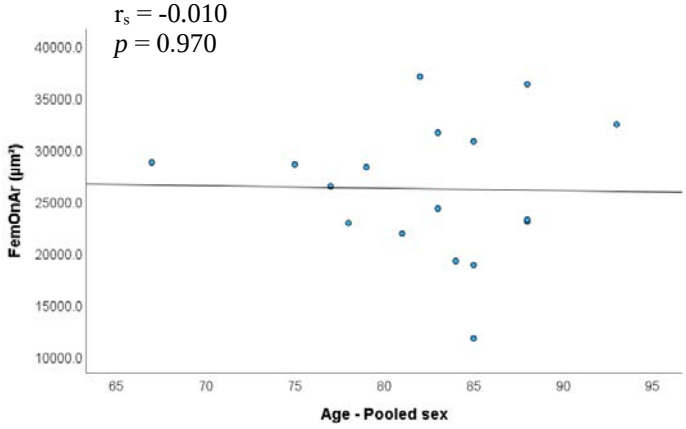
Appendix B. 2 . Scatter plots showing the weak correlations within the Australian pooled sex group, with line of best fit illustrating either a negative or positive relationship.



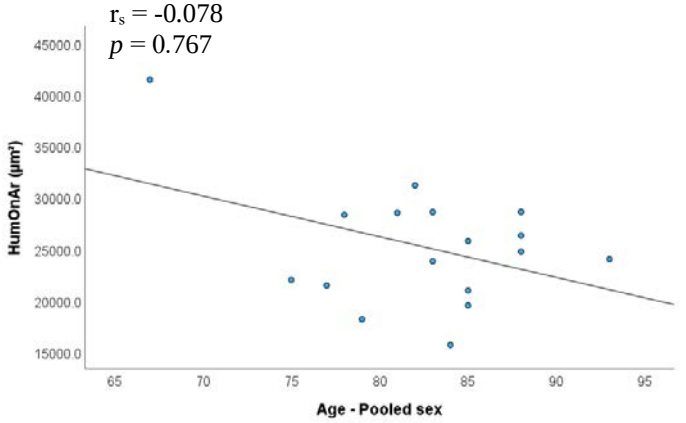



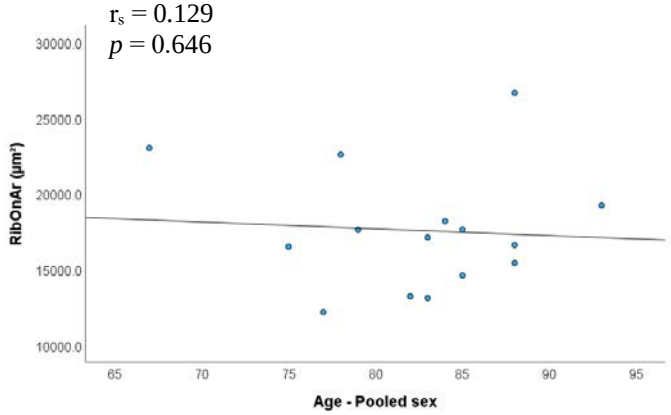
$r_s$ = -0.010
*p* = 0.970



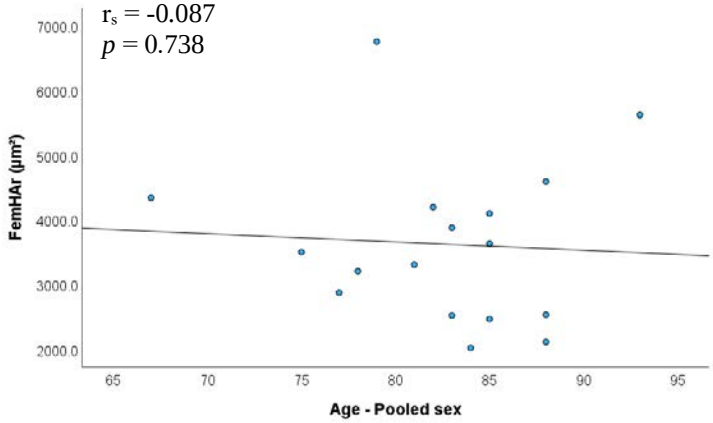

$r_s$ = -0.078
*p* = 0.767

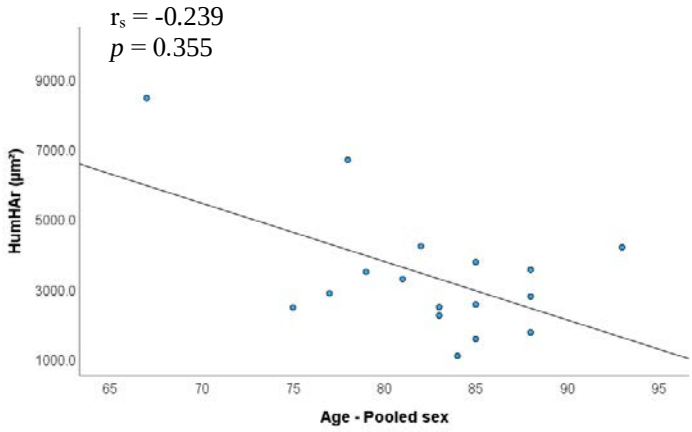



$r_s$ = 0.129
*p* = 0.646

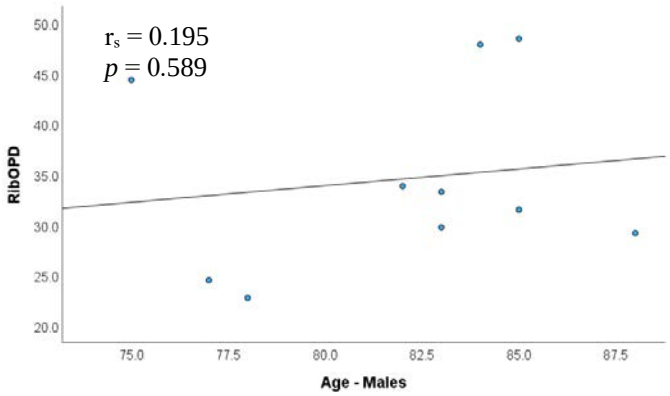


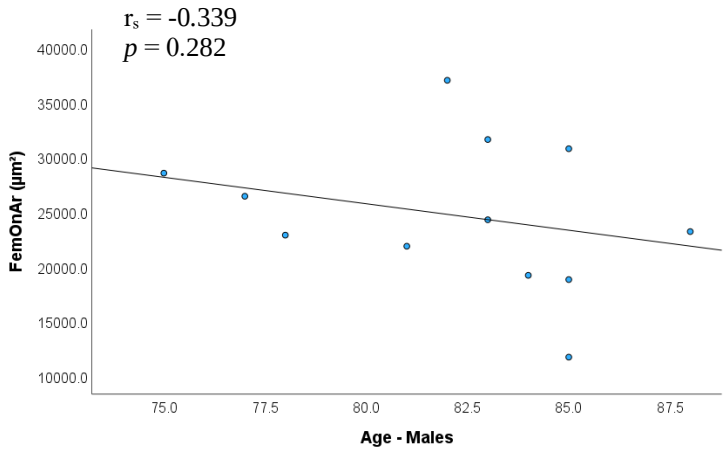

$r_s$ = -0.087
*p* = 0.738

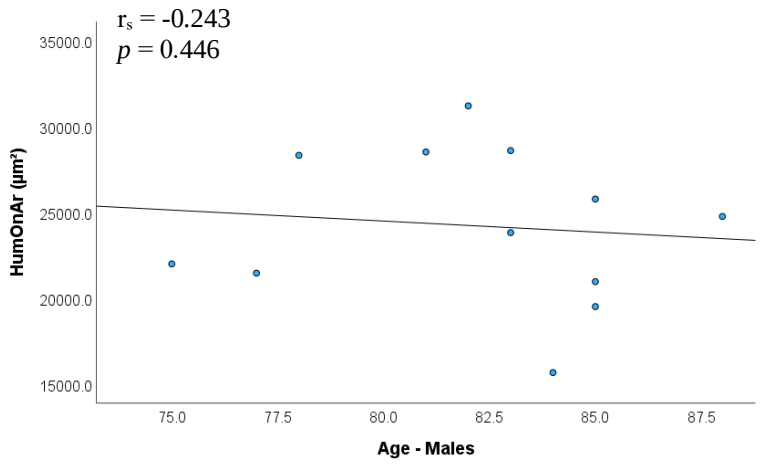


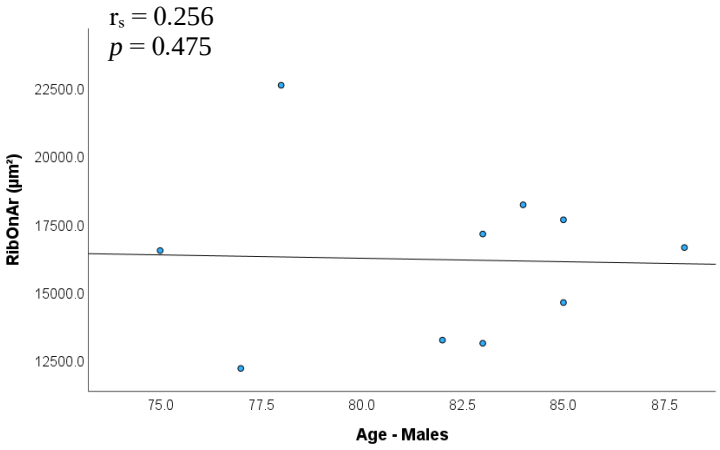

$r_s$ = -0.239
$p$ = 0.355
9000.0
7000.0
5000.0
3000.0
1000.0
HumHAr (µm²)
65
70
75
80
85
90
95
Age - Pooled sex

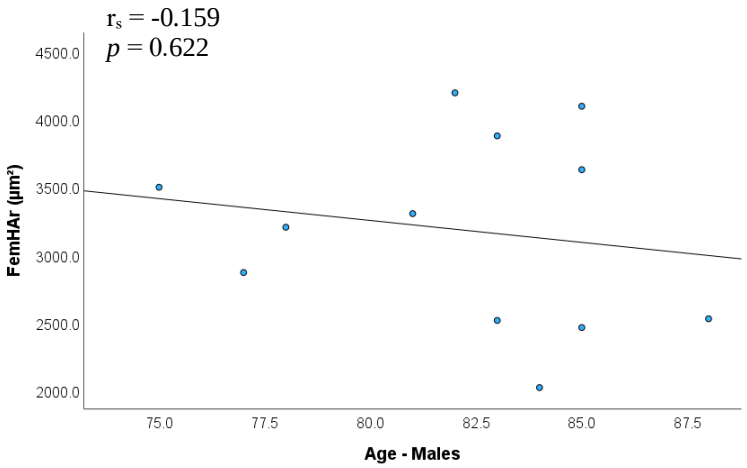
Appendix B. 3 Scatter plots showing the weak correlations within the Australian male, with line of best fit illustrating either a negative or positive relationship.




$r_s = 0.256$
$p = 0.475$
RibOnAr (µm²)
22500.0
20000.0
17500.0
15000.0
12500.0
75.0
77.5
80.0
82.5
85.0
87.5
Age - Males

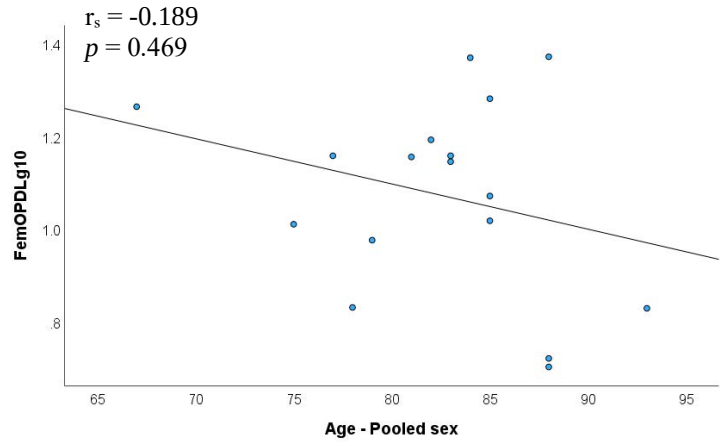

$r_s = -0.159$
$p = 0.622$
FemHAr (µm²)
4500.0
4000.0
3500.0
3000.0
2500.0
2000.0
75.0
77.5
80.0
82.5
85.0
87.5
Age - Males

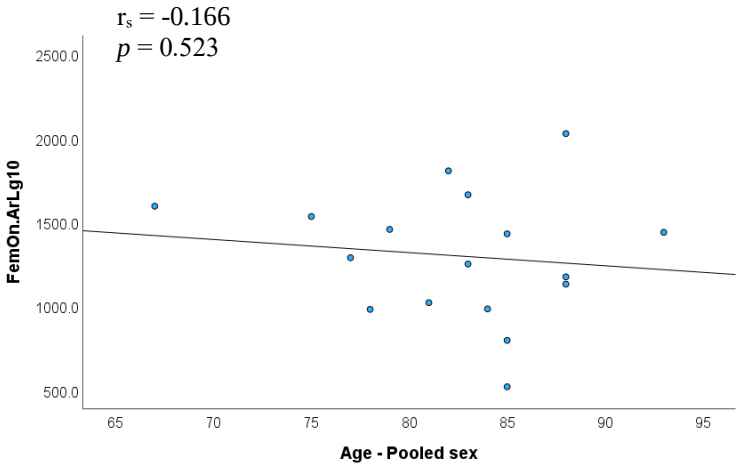
## Appendix B. 4 Descriptive statistics for Australian females and males.

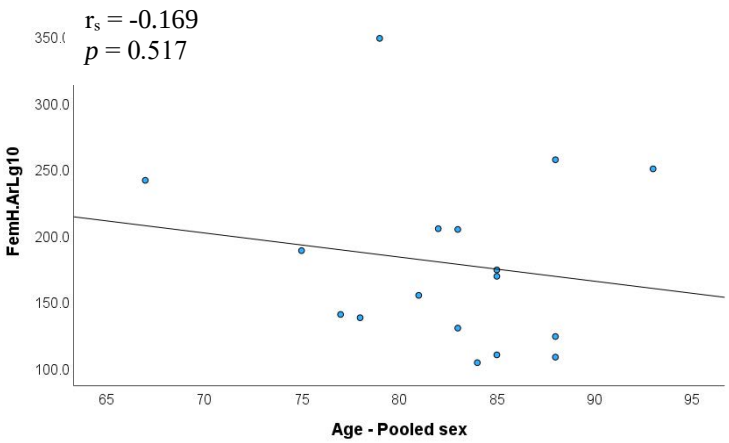
| | | OPD | | | On.Ar | | | H.Ar | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sex | Age at death | Femur | Humerus | Rib | Femur | Humerus | Rib | Femur | Humerus | Rib |
| Female | 67 | 22.740 | 18.079 | 40.280 | 28711.050 | 41484.250 | 23012.210 | 4338.540 | 8455.895 | 1848.569 |
| Female | 79 | 18.927 | 18.220 | 37.361 | 28266.780 | 18157.560 | 17623.260 | 6752.579 | 3475.306 | 929.253 |
| Female | 88 | 14.124 | 17.514 | 29.189 | 23024.420 | 28617.150 | 26651.380 | 2111.666 | 3533.931 | 1471.262 |
| Female | 88 | 12.571 | 23.446 | 36.778 | 36280.831 | 26318.820 | 15425.510 | 4592.304 | 2771.484 | 1711.691 |
| | **Mean** | **17.090** | **19.315** | **35.902** | **29070.770** | **28644.445** | **20678.090** | **4448.772** | **4559.154** | **1490.194** |
| | **Min** | **12.571** | **17.514** | **29.189** | **23024.420** | **18157.560** | **15425.510** | **2111.666** | **2771.484** | **929.253** |
| | **Max** | **22.740** | **23.446** | **40.280** | **36280.831** | **41484.250** | **26651.380** | **6752.579** | **8455.895** | **1848.569** |
| | **SD** | **4.637** | **2.771** | **4.731** | **5456.468** | **9665.137** | **5100.650** | **1897.572** | **2620.824** | **405.178** |
| Male | 75 | 18.785 | 24.011 | 44.367 | 28527.840 | 21997.623 | 16506.540 | 3497.315 | 2456.528 | 1222.451 |
| Male | 77 | 23.729 | 26.271 | 24.518 | 26416.041 | 21461.020 | 12179.320 | 2868.312 | 2862.510 | 1043.504 |
| Male | 78 | 19.350 | 17.655 | 22.767 | 22863.860 | 28321.111 | 22576.050 | 3202.536 | 6685.534 | 1369.828 |
| Male | 81 | 24.718 | 25.282 | 0.000 | 21849.730 | 28516.590 | 0.000 | 3302.633 | 3268.432 | 0.000 |
| Male | 82 | 24.435 | 24.576 | 33.859 | 37009.610 | 31199.310 | 13218.070 | 4193.715 | 4213.747 | 1088.894 |
| Male | 83 | 22.458 | 26.130 | 33.275 | 24269.470 | 28597.350 | 13102.660 | 2514.951 | 2467.933 | 1797.500 |
| Male | 83 | 21.751 | 27.966 | 29.772 | 31598.740 | 23819.850 | 17113.600 | 3876.290 | 2224.460 | 1602.921 |
| Male | 84 | 26.695 | 36.017 | 47.869 | 19191.070 | 15670.880 | 18186.620 | 2019.626 | 1069.611 | 1179.582 |
| Male | 85 | 24.011 | 20.480 | 0.000 | 18803.130 | 25774.030 | 0.000 | 4094.645 | 3755.052 | 0.000 |
| Male | 85 | 28.814 | 26.977 | 31.524 | 11716.710 | 19511.870 | 14597.630 | 2462.418 | 2540.930 | 2361.807 |
| Male | 85 | 23.023 | 27.825 | 48.453 | 30758.010 | 20963.620 | 17636.330 | 3626.663 | 1554.875 | 1735.230 |
| Male | 88 | 28.107 | 29.944 | 29.189 | 23188.460 | 24761.720 | 16617.170 | 2527.237 | 1740.316 | 1604.392 |
| Male | 93 | 18.644 | 24.859 | 30.356 | 32389.190 | 24024.340 | 19220.280 | 5617.920 | 4180.710 | 2115.252 |
| | **Mean** | **23.425** | **26.000** | **34.177** | **25275.528** | **24201.486** | **13919.559** | **3369.559** | **3001.588** | **1317.028** |
| | **Min** | **18.644** | **17.655** | **22.767** | **11716.71** | **15670.88** | **12179.320** | **2019.626** | **1069.611** | **1043.504** |
| | **Max** | **28.814** | **36.017** | **48.453** | **37009.61** | **31199.31** | **22576.050** | **5617.92** | **6685.534** | **2361.807** |
| | **SD** | **3.287** | **4.397** | **8.862** | **6806.162** | **4324.957** | **3056.431** | **956.034** | **1469.232** | **427.261** |

Zero values not included in the calculation of the descriptive statistics. The mean OPD count, and mean size of osteons and Haversian canals from the six ROIs were used.

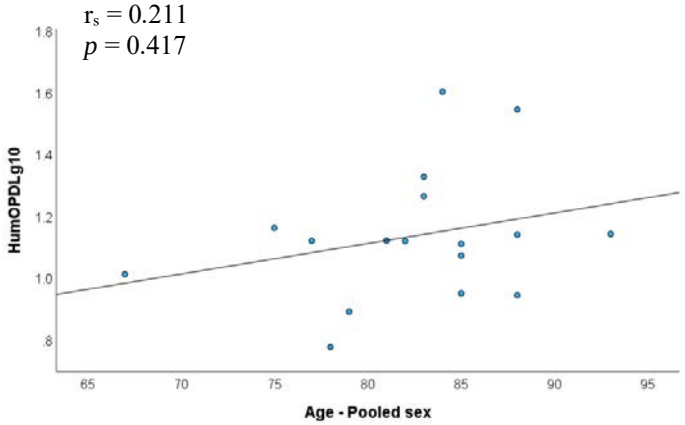
Appendix B. 5 Scatter plots showing the weak correlations within the Australian pooled sex group with RI adjusted values. Line of best fit illustrates either a negative or positive relationship.



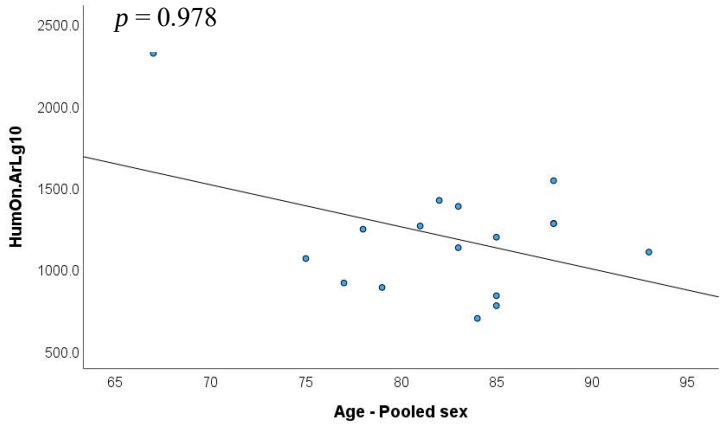



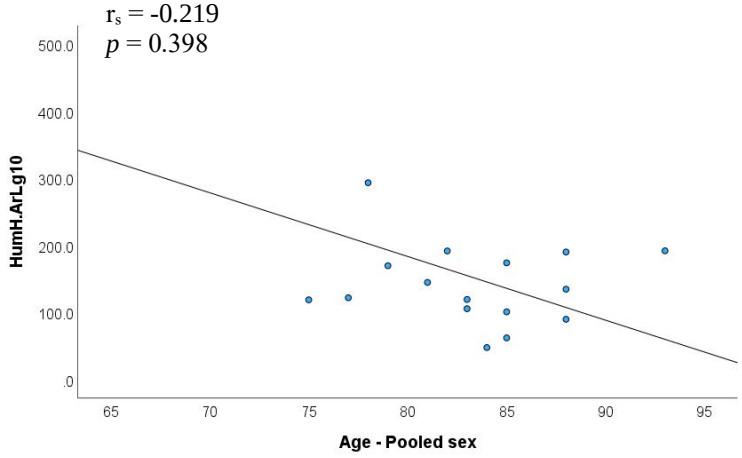
$r_s = 0.211$
$p = 0.417$




$r_s = -0.007$
$p = 0.978$

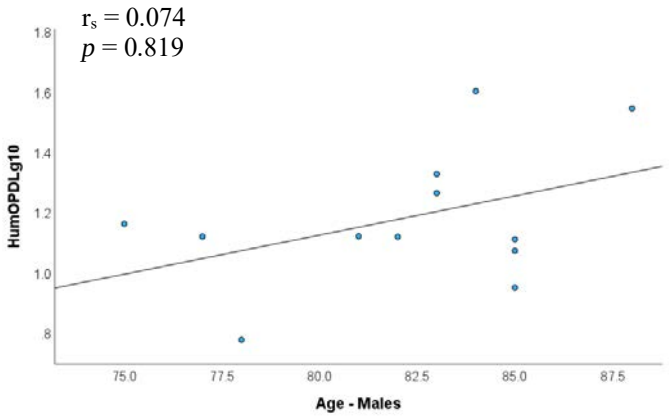



$r_s = -0.219$
$p = 0.398$

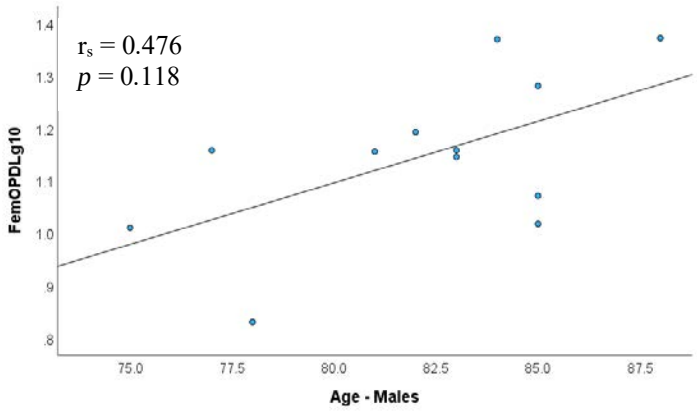


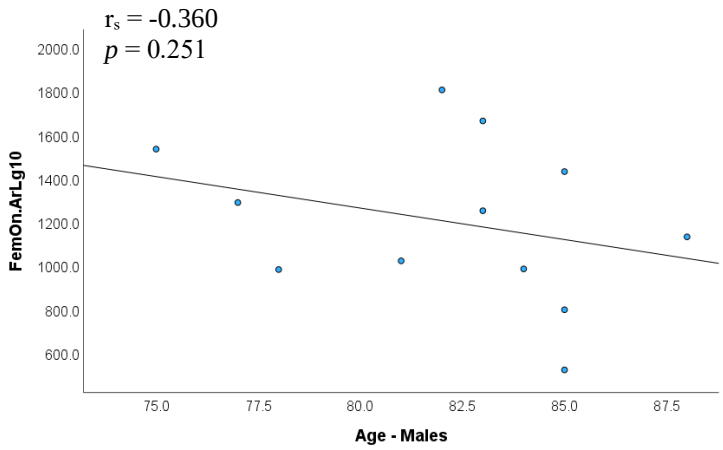
Appendix B. 6 Scatter plots showing the one weak correlation:
(a) (HumOPD Lg10), and moderate correlations (b) within the Australian male group with RI adjusted values. Line of best fit illustrates either a negative or positive relationship.

(a)

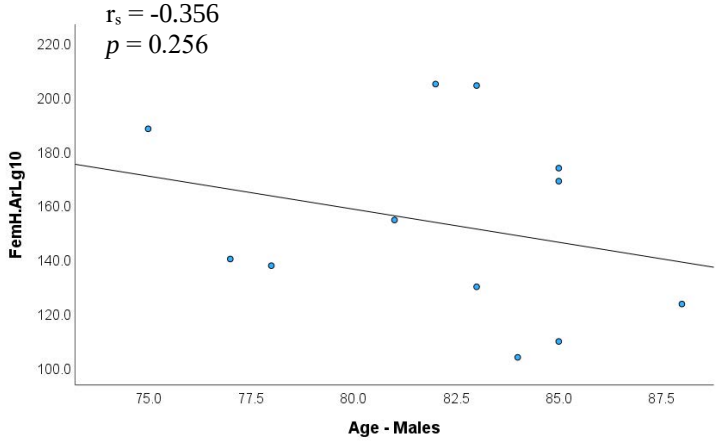



(b)

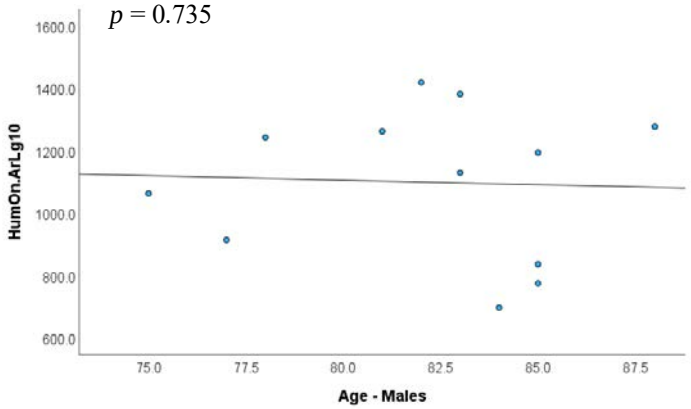


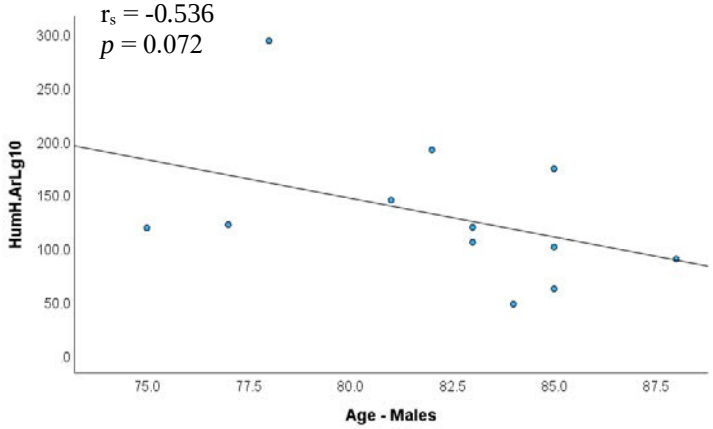
$r_s$ = -0.356
$p$ = 0.256



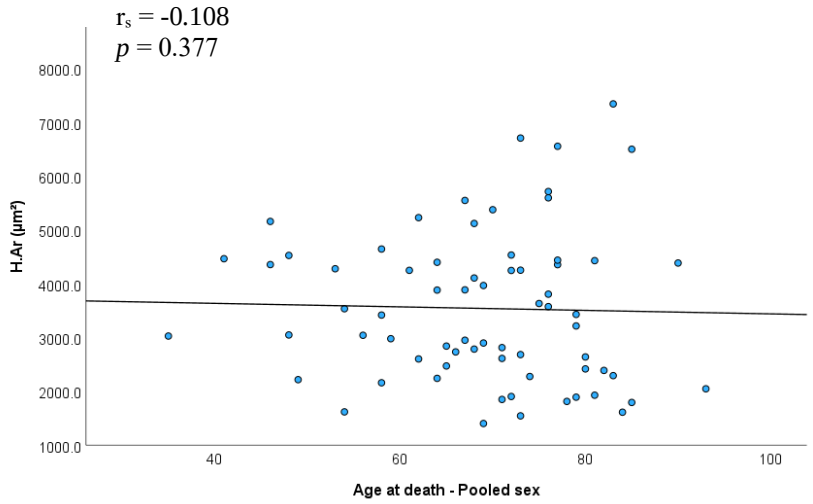

$r_s$ = -0.109
$p$ = 0.735

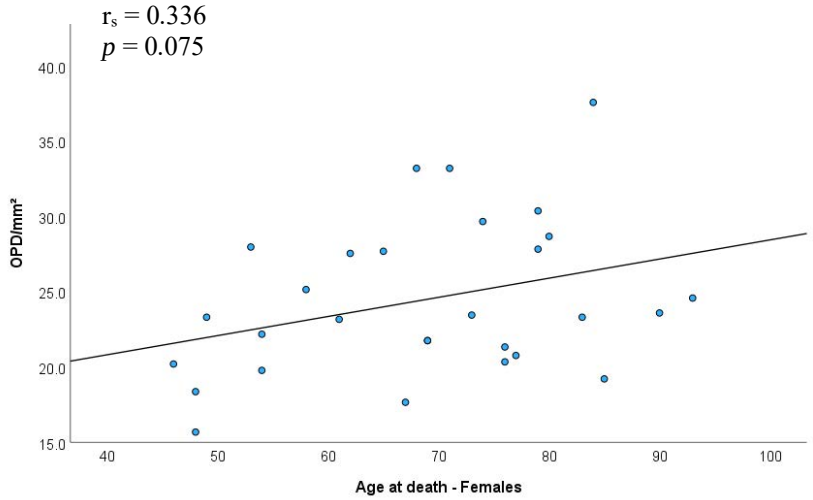


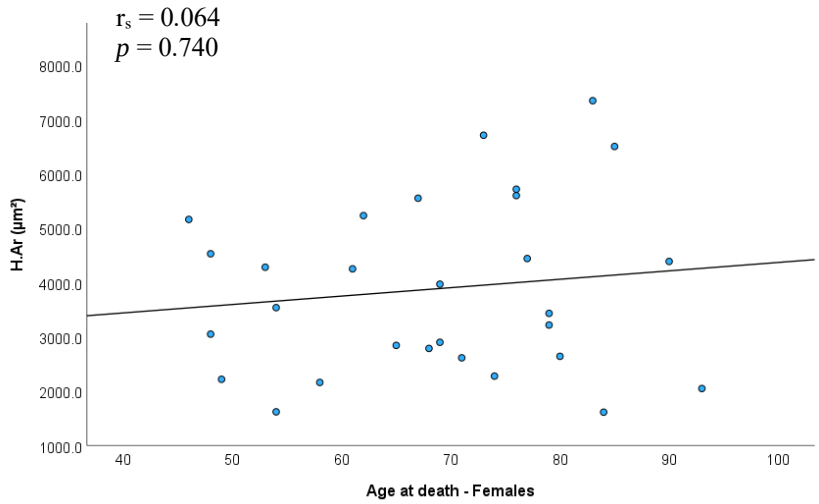

$r_s$ = -0.536
$p$ = 0.072

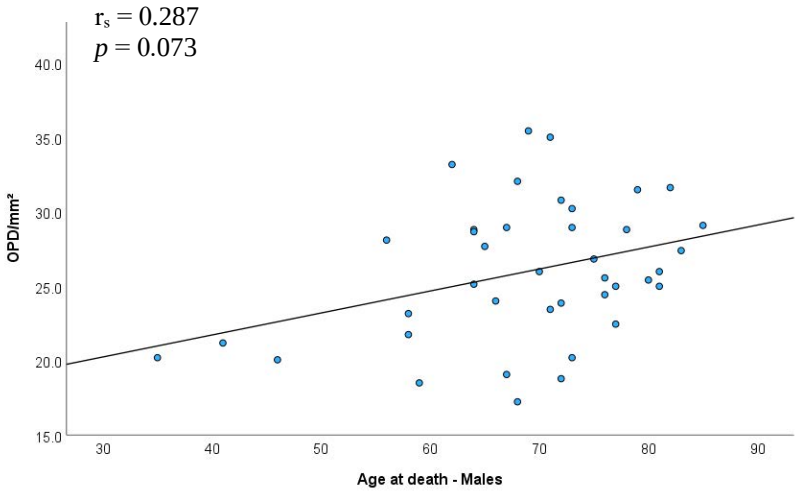


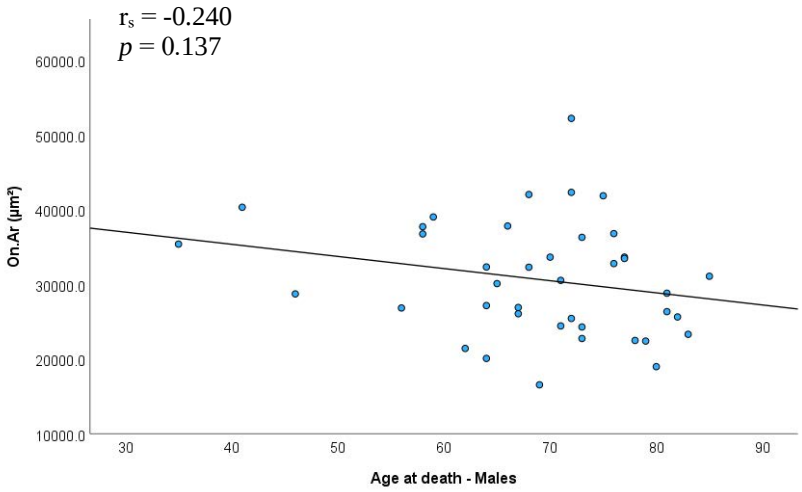
Appendix C. 1 Spearman's rank correlation scatter plots showing weak correlations within the Thai sex groups between age and histological variables for femora. A line of best fit illustrates either a positive or negative relationship.

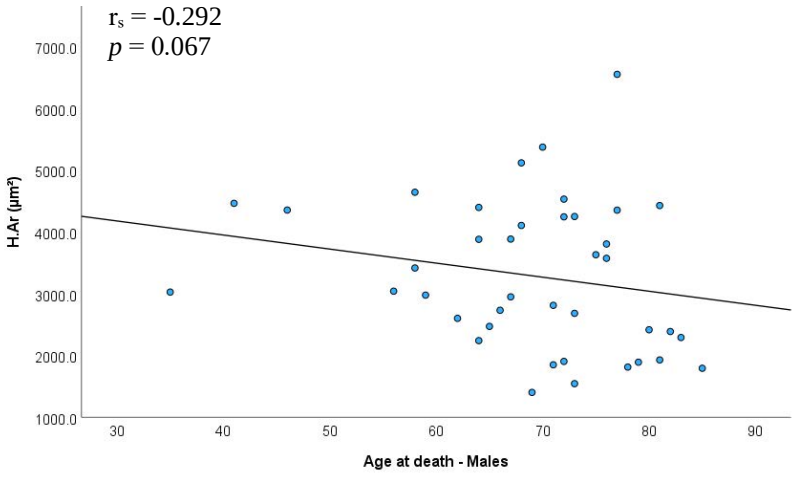



$r_s$ = 0.287
$p$ = 0.073
OPD/mm²
40.0
35.0
30.0
25.0
20.0
15.0
30
40
50
60
70
80
90
Age at death - Males


$r_s$ = -0.240
$p$ = 0.137
On.Ar (µm²)
60000.0
50000.0
40000.0
30000.0
20000.0
10000.0
30
40
50
60
70
80
90
Age at death - Males


$r_s$ = -0.292
$p$ = 0.067
H.Ar (µm²)
7000.0
6000.0
5000.0
4000.0
3000.0
2000.0
1000.0
30
40
50
60
70
80
90
Age at death - Males

## Appendix C. 2 Descriptive statistics for the Thai sample by sex.

| Sex | Age at death | OPD/mm² | On.Ar (µm²) | H.Ar (µm²) |
|---|---|---|---|---|
| Male | 35 | 20.198 | 35397.645 | 3028.978 |
| Male | 41 | 21.186 | 40336.412 | 4467.670 |
| Male | 46 | 20.057 | 28726.905 | 4358.447 |
| Male | 56 | 28.107 | 26844.779 | 3043.574 |
| Male | 58 | 23.164 | 36761.349 | 3419.439 |
| Male | 58 | 21.751 | 37732.825 | 4647.578 |
| Male | 59 | 18.503 | 39033.764 | 2979.616 |
| Male | 62 | 33.192 | 21421.346 | 2603.275 |
| Male | 64 | 28.814 | 27164.146 | 2242.081 |
| Male | 64 | 25.141 | 20084.670 | 3884.279 |
| Male | 64 | 28.672 | 32331.359 | 4401.121 |
| Male | 65 | 27.684 | 30106.276 | 2472.733 |
| Male | 66 | 24.011 | 37825.307 | 2734.143 |
| Male | 67 | 28.955 | 26906.688 | 2951.558 |
| Male | 67 | 19.068 | 26070.119 | 3888.459 |
| Male | 68 | 32.062 | 32301.928 | 4108.874 |
| Male | 68 | 17.232 | 42041.971 | 5123.427 |
| Male | 69 | 35.452 | 16548.935 | 1402.445 |
| Male | 70 | 25.989 | 33650.937 | 5378.920 |
| Male | 71 | 35.028 | 24436.494 | 1849.978 |
| Male | 71 | 23.446 | 30539.301 | 2814.191 |
| Male | 72 | 18.785 | 52242.976 | 4536.100 |
| Male | 72 | 23.870 | 42330.815 | 4248.978 |
| Male | 72 | 30.791 | 25436.332 | 1904.907 |
| Male | 73 | 30.226 | 22750.349 | 1544.420 |
| Male | 73 | 20.198 | 36305.430 | 4254.047 |
| Male | 73 | 28.955 | 24296.432 | 2684.356 |
| Male | 75 | 26.836 | 41875.681 | 3634.100 |
| Male | 76 | 25.565 | 32789.138 | 3808.386 |
| Male | 76 | 24.435 | 36805.920 | 3576.521 |
| Male | 77 | 25.000 | 33658.645 | 4357.027 |
| Male | 77 | 22.458 | 33472.271 | 6558.809 |
| Male | 78 | 28.814 | 22491.499 | 1813.369 |
| Male | 79 | 31.497 | 22409.391 | 1892.020 |
| Male | 80 | 25.424 | 18978.388 | 2417.957 |
| Male | 81 | 25.000 | 26350.868 | 1929.030 |
| Male | 81 | 25.989 | 28819.539 | 4431.778 |
| Male | 82 | 31.638 | 25638.528 | 2389.702 |
| Male | 83 | 27.401 | 23313.146 | 2292.648 |
| Male | 85 | 29.096 | 31091.491 | 1793.955 |
| **n = 40** | **Mean** | **25.992** | **30683.000** | **3296.722** |
| | **Min** | **17.232** | **16548.935** | **1402.445** |

| Sex | Age at death | OPD/mm² | On.Ar (µm²) | H.Ar (µm²) |
|---|---|---|---|---|
| Female | 46 | 20.198 | 31840.195 | 5161.072 |
| Female | 48 | 18.362 | 44423.590 | 3049.341 |
| Female | 48 | 15.678 | 46437.974 | 4528.921 |
| Female | 49 | 23.305 | 38344.488 | 2217.896 |
| Female | 53 | 27.966 | 33759.417 | 4281.004 |
| Female | 54 | 22.175 | 37498.873 | 3537.111 |
| Female | 54 | 19.774 | 31277.530 | 1618.538 |
| Female | 58 | 25.141 | 30642.441 | 2160.105 |
| Female | 61 | 23.164 | 32296.095 | 4251.359 |
| Female | 62 | 27.542 | 27651.487 | 5231.671 |
| Female | 65 | 27.684 | 33758.396 | 2842.243 |
| Female | 67 | 17.655 | 39024.301 | 5551.567 |
| Female | 68 | 33.192 | 24345.418 | 2786.370 |
| Female | 69 | 21.751 | 28589.296 | 2899.535 |
| Female | 69 | 21.751 | 32613.727 | 3969.037 |
| Female | 71 | 33.192 | 18896.709 | 2612.947 |
| Female | 73 | 23.446 | 34055.445 | 6711.531 |
| Female | 74 | 29.661 | 22078.108 | 2276.621 |
| Female | 76 | 21.328 | 33596.977 | 5600.157 |
| Female | 76 | 20.339 | 31219.236 | 5717.319 |
| Female | 77 | 20.763 | 35564.489 | 4440.945 |
| Female | 79 | 30.367 | 27411.355 | 3431.651 |
| Female | 79 | 27.825 | 26929.148 | 3217.262 |
| Female | 80 | 28.672 | 23478.125 | 2640.503 |
| Female | 83 | 23.305 | 36450.859 | 7347.523 |
| Female | 84 | 37.571 | 17686.771 | 1612.335 |
| Female | 85 | 19.209 | 42904.481 | 6504.311 |
| Female | 90 | 23.588 | 29479.757 | 4387.612 |
| Female | 93 | 24.576 | 20081.204 | 2047.795 |
| **n = 29** | **Mean** | **24.455** | **31459.858** | **3883.941** |
| | **Min** | **15.678** | **17686.771** | **1612.335** |
| | **Max** | **37.571** | **46437.974** | **7347.523** |
| | **SD** | **5.151** | **7255.456** | **1587.380** |

| | | | |
|---|---|---|---|
| **Max** | **35.452** | **52242.976** | **6558.809** |
| **SD** | **4.642** | **7665.481** | **1204.680** |

| Pooled sex | | **OPD/mm²** | **On.Ar (µm²)** | **H.Ar (µm²)** |
|---|---|---|---|---|
| **N = 69** | **Mean** | **25.346** | **31009.506** | **3543.524** |
| | **Min** | **15.678** | **16548.935** | **1402.445** |
| | **Max** | **37.571** | **52242.976** | **7347.523** |
| | **SD** | **4.886** | **7451.546** | **1398.263** |