Histological age-at-death estimation in human bone: Assessment of inter-population variation

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Declaration

I, Deona Botha, declare that this thesis is my own work, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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Dedication

"The purpose of anthropology is to make the world safe for human differences."

Ruth Benedict

(American anthropologist, 1887-1948)

For my daughter, Clara

Presentations arising from this research project

1. Botha D, Bhagwandin A, Lynnerup N, Steyn M. Applying stereology to histological age estimation: method discussion and preliminary results. Anatomical Society of Southern Africa (ASSA) Annual Conference, Langebaan, Western Cape, 23-26 April 2017. Podium presentation.
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Publications arising from this research project

1. Botha D, Bhagwandin A, Lynnerup N, Steyn M. 2018. The use of stereological methods in the histomorphometric assessment of bone for age-at-death estimation. Forensic Science International 290:353.e1-353.e7.
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Contributions of the author

Paper 1: Principal author performed planning of project, collection of the data, assessment of variables, statistical analysis, evaluation of results and writing of the article. Second co-author performed scanning of bone sections for analysis. Third and fourth co-authors assisted with project planning and revised the draft manuscript in order to make suggestions for improvement.

Paper 2: Principal author performed planning of project, assisted with DXA scanning procedures, performed statistical analysis, evaluated the results and were responsible for writing of the article. Second and third authors revised the manuscript in order to make suggestions for improvement.

Paper 3: Principal author performed planning of project, collection of data, assessment of variables, statistical analysis, evaluation of results and writing of the article. Second and third co-authors assisted with project planning and revised the draft manuscript in order to make suggestions for improvement.

Paper 4: Principal author performed planning of project, assessment of variables, statistical analysis, evaluation of results and writing of the article. Second and third co-authors assisted with project planning and revised the draft manuscript in order to make suggestions for improvement.

Abstract

Age-at-death estimates in skeletal remains are hampered by observer bias and error, as well as individual and population variation. This study aimed at improving accuracy and reliability of age estimates obtained from applying methods involving the assessment of histomorphometric variables of bone and bone mineral density (BMD).

The assessment of osteon population density (OPD) and size (length, surface area and volume) of Haversian systems were investigated in three populations by means of stereology. Bone slides prepared from South African black (n = 99), South African white (n = 94) and Danish white (n = 30) individuals were analysed using MicroBrightField's StereoInvestigator software. ANCOVA results revealed a statistically significant difference ($p < 0.001$) between the three groups in terms of OPD. No statistically significant difference was seen in the size of secondary osteons between the groups. Linear regression analysis was used to construct population-specific formulae for age-at-death estimation in South African white and black individuals. The Danish sample was used as a comparative group for white South African standards established. Age mimicry appeared to play a role in the over-estimation of age in the Danish individuals, after which the problem was corrected by combining the two samples in order to achieve normal age distribution within the larger sample.

Secondly, DXA scans of the proximal femur were done for a subgroup of South African black (n = 64) and white (n = 59) individuals for estimation of age-at-death from bone mineral density. Results displayed a significant difference between white and black groups for total and neck BMD. White males and females differed in total and neck BMD, although black males and females differed only in terms of neck BMD. Age could be significantly correlated with BMD in the white population, but not in the black population. Regression analysis was also done for the complete sample (white and black individuals pooled), as ancestry is unknown in some cases. A significant correlation was seen for age versus neck BMD in the total sample and in sex-specific groups.

The outcome of this study suggested that the three population samples are somewhat dissimilar in bone microstructure, with differences related to OPD and BMD present between the groups. Based on this outcome, it should be emphasized that age estimation

standards should be adapted to accommodate combined groups and create to more generally applicable standards. Further research involving various disciplines is needed to better understand the underlying reason(s) for these differences between these populations.

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List of abbreviations

Avg_Frags	Average number of fragments per grid area
Avg_Hav_Ar	Average Haversian canal surface area
Avg_Hav_L	Average Haversian canal length (width)
Avg_Hav_Vol	Average Haversian canal volume
Avg_OPD	Average number of osteons per grid area
Avg_Ost_Ar	Average osteon surface area
Avg_Ost_L	Average osteon length (width)
Avg_Ost_Vol	Average osteon volume
BMD	Bone mineral density
BMI	Body mass index
DOB	Date of birth
DXA	Dual x-ray absorptiometry
FH	Femoral head
IUR	Isotropic uniform random
OPD	Osteon population density
ROI	Region of interest
VUR	Vertical uniform random

PART 1: INTRODUCTION AND BACKGROUND

Chapter 1
GENERAL INTRODUCTION

1.1. Introduction

Age estimation from skeletal remains forms an essential part of constructing a biological profile for individuals of unknown identity. Forensic anthropologists have several methods available to use in order to obtain an age range. The choice of method(s) is based on the completeness of the skeleton and skeletal elements present. Although the majority of preferred methods are macroscopic, the use of dry bone histology for age estimation is useful especially in cases where limited skeletal material is available.

The life-long process of bone remodelling in which the number of secondary osteons increase within cortical bone areas had been noticed more than a century ago (Balthazard & Lebrun 1911). The use of bone histomorphometry for age estimation, however, only became a focus point after Kerley's pioneering publication (1965) on histological age-at-death estimation from cortical bone of the femur, tibia and fibula. Research on the subject subsequently increased with many studies expanding on Kerley's method (e.g., Singh & Gunberg 1970; Thompson 1979; Hauser *et al*. 1980; Ericksen 1991; Stout & Paine 1992) and testing the existing techniques on numerous skeletal locations such as the occipital bone (Cool *et al*. 1995), mandible (Singh & Gunberg 1970), ribs (Cho *et al*. 2002, Crowder 2013; Pfeiffer *et al*. 2016), bones of the upper limb (Thompson 1979; Yoshino *et al*. 1994) and clavicle (Stout *et al*. 1996).

Most of these studies are focused almost exclusively on specific population groups (e.g., Kimura 1992; Watanabe 1998; Maat *et al*. 2006), with few combining different groups to obtain more generally applicable standards (e.g., Ericksen 1991; Cho *et al*. 2002). Although most of the earlier studies focused on creating standards for specific population groups, later studies such as that of Pfeiffer *et al*. (2016) concluded that in future we should expand on sample sizes and aim to establish standards that may be more broadly applied.

The study sample itself may create inherent biological limitations when establishing age-at-death estimation methods. Biological variation in the skeleton are brought about by metabolic and environmental influences (Vigorita 2008; Gosman 2011). Variation in histological bone structures may be found between individuals of the same population group (intra-population variation), as well as between different groups (inter -population

variation). Differences in bone remodelling and density between sexes of the same group have also been reported (Wang & Dumas 2002; Goldman *et al*. 2003). Due to the presence of sex-related differences and, intra- and inter-population variation, the size and inclusion criteria of the study sample should be considered. Larger reference samples that include an equal number of individuals in each age cohort (thus uniformly distributed) tend to work best and result in less under- or overestimated cases as opposed to skewed reference samples.

In cases where the complete skeleton is present, macroscopic methods of age estimation are often preferred because it is less time consuming than microscopic methods. It is also non-destructive. Macroscopic methods are qualitative and rely on degenerative changes observed in areas such as the pelvis (e.g., Brooks & Suchey 1990; Buckberry & Chamberlain 2002; Rouge-Maillart *et al*. 2007), ribs (e.g., Oettlé & Steyn 2000; DiGangi *et al*. 2009) and cranium (e.g., Acsadi & Nemeskeri 1970). However, qualitative gross morphological methods are subjective as the degenerative changes recorded are influenced by the observer's knowledge, expertise and judgement (Ubelaker 1977). As age advances, the degree of degeneration becomes more variable and less accurate, with individuals older than 50 years often aged inaccurately (Ubelaker 1999).

The use of bone mineral density in estimating age has recently received some attention (Curate *et al*. 2013, Navega *et al*. 2018). A major advantage of this method is that it does not rely on observer bias or subjectivity, as it is solely based on DXA scan values. Also, it is a non-invasive technique that has performed fairly well for all ages (Paschall & Ross 2018).

Histological methods (histomorphometry) allows for the quantification of bone structures, producing distinct values that are less subjective to the observer's judgement and more likely to provide better age estimates for individuals older than 50 years (Ericksen 1991). However, microscopic methods are prone to bias from both the observer and method used. The use of stereology could potentially be a valuable tool in addressing this problem and was employed in this study. The importance of excluding bias should also not be overlooked, as it may seriously skew the study outcome. By employing unbiased stereological protocols for the selection of images, bias related to the method is excluded. Strict rules for counting/quantifying histological structures are

provided for the observer to ensure an accurate and precise outcome (Lynnerup *et al*. 2006; Howard & Reed 2010).

Quantitative bone histology and bone mineral density should not be overlooked, as it provides researchers with a less subjective method that offers accurate and precise results if bias is excluded. Ideally, both microscopic and macroscopic methods should be used where possible as this will ensure a more reliable outcome (Aiello & Molleson 1993).

1.2. Aim and objectives

The goal of this study was to perform histological age estimation on three diverse populations in order to investigate the degree of inter-population variation, as well as set up population specific standards for each group. Stereology was employed to assess whether it can provide a more objective means of scoring the bony features and reduce some of the problems related to inter-observer bias. Bone mineral density was also assessed in a subgroup of black and white South Africans to determine the difference between the groups and to provide regression equations using DXA values.

Objectives

The objectives of the project were as follow:

a) Histological features associated with increased age were assessed on sections of the anterior midshaft of the femur. Three population groups were studied.
b) Computer-based stereology was employed for analysis of bone slides. It was assessed whether this method provides advantages as far as accuracy and repeatability is concerned as compared to traditional 2D assessment.
c) Inter-population variation between black and white South Africans, as well as between South Africans and a Danish sample was assessed in terms of all histological structures examined.
d) DXA scans were performed for a subgroup of black and white South Africans. Bone mineral density (BMD) values of the total proximal area and femoral neck were correlated with age to create age estimation equations where possible.

1.3. Organization of the thesis

This study is presented as original published work and organized into nine chapters. A brief description of each chapter is provided here to provide information on the layout of the study.

Chapter 1 provides a general introduction and overview of the study. A short introduction, as well as the aim and objectives are given.

Chapter 2 presents an overview of relevant literature in terms of bone remodelling, histological age estimation and stereological principals and protocols.

Chapter 3 describes the materials and methods used. Details pertaining to the study sample, bone sectioning methods, variables assessed, and stereological protocols employed are given.

Chapters 4 to 7 represent original papers produced from this study. The first paper (Botha *et al*. 2018) deals with the use of stereology in histological age estimation and is based on the black South African group. Chapter 5 (paper submitted for review) reports on age estimation from BMD values of the femoral neck and provides standards for South African groups. Chapter 6 (paper submitted for review) deals with the inter-population variation of histomorphometric variables used in age-at-death estimation. The last paper (Chapter 7, submitted for review) serves as an example of age mimicry and provides histological age estimation standards based on white South African and Danish individuals.

Chapter 8 reviews the results and briefly discusses the outcome of the study.

Chapter 9 concludes with a summary of the findings and suggests future guidelines with regards to histological age estimation.

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Chapter 2
LITERATURE REVIEW

2.1. Introduction

The study of bone and documentation of its macroscopic and microscopic features have been carried out for more than a century by anatomists, pathologists, zoologists and biologists (Prudden 1881, 1891; Cooper *et al*. 1966, Garland 1993; Bilezekian *et al*. 2008). The resilience and strength of bone makes it, along with teeth, the longest surviving bodily tissue after death (Garland 1993). Some of the first histological examinations of bone were performed by Prudden at Yale University and dates back to the late 1800's. The first edition of his book, A Manual of Practical Normal Histology, was published in 1881 as a study guide for medical students, in which the section on bone describes the biological properties of bone, techniques on embedding bone tissue and general micromorphological features of bone. Prudden also noted that there is a difference in morphology between the osteocytes of younger and older individuals and stated that "in young bone the cells are not flat, but spherical or ovoidal" (Prudden 1891, p. 65).

Many studies on bone histology followed that of Prudden's, with earlier studies focusing mostly on palaeohistology and pathology (Moodie 1926; Graf 1949; Schultz 1997). The first study on bone and its histological application for age-at-death estimation was done in 1911 (Balthazard & Lebrun 1911) but produced poor results. Kerley's publication (1965) on age-at-death from long bones years later provided a starting point and set the benchmark for the requirements for accurate age estimation from histological slides, after which this field of study received much attention (Garland 1993).

Histology, i.e. the study of tissue on a microscopic level (Stout & Crowder 2012) includes two levels of differentiation. Histomorphology is based on the shape of cells, or the morphology of tissue. Histomorphometry describes the quantitative aspect of analysis, such as number of cells per area, surface area and size (Eriksen *et al*. 1994, Ott 2002; Dempster *et al*. 2009). The histomorphometric data of cells and other structures are often used to describe the histomorphology of a specific tissue by using variables such as minimum and maximum length to calculate a circularity index (Currey 1964; Goliath *et al*. 2016). The variables that are selected depend on the nature of the study, i.e whether it is designed to investigate static or dynamic aspects of bone microstructure. Dynamic

studies of bone are designed to observe cellular processes using histochemical methods and are conducted on wet bone specimens (Bassett *et al*. 2010; Maggiano *et al*. 2006). In contrast to what can be investigated on wet specimens, static variables such as volume, length, perimeter, diameter, area, circularity and number of secondary osteons are studied in dry bone (Robling & Stout 2008; Stout & Crowder 2012).

To understand bone histology in terms of the morphological changes that take place with the advancement of age, a thorough knowledge of bone biology and remodelling is essential (Stout & Crowder 2012). As this study is based on adult age estimation, bone biology and remodelling will be discussed in further detail in the following section. For additional information on bone physiology and bone modelling in subadults, refer to Karaplis (2008) and Kini and Nandeesh (2012).

2.2. Principles of bone biology and remodelling

2.2.1. Introduction

Bone is a connective tissue containing inorganic and organic components that plays a vital role in mineral homeostasis, growth and adaptation to the environment (Kular *et al*. 2012; Stout & Crowder 2012). In order for bone to fulfil its physiological role, it constantly undergoes reshaping to adapt to biomechanical forces and remodelling to replace old bone with new bone to maintain its strength and rigidity (Kini & Nandeesh 2012).

All bones have two constituents, namely cortical bone that makes up about 80% of the mass of the skeleton, and cancellous bone that contributes around 20% of bone mass (Eriksen *et al*. 1994). Cortical bone is dense and solid in nature and surrounds the marrow space. Periosteal cortical bone is responsible for appositional growth and fracture repair (formation surpasses resorption), whereas the endosteal part of cortical bone tends to have a higher level of remodelling where resorption exceeds formation, leading to the expansion of the marrow space as age advances (Peck & Stout 2009). Cancellous bone is composed of trabecular plates and rods forming a matrix with a honeycomb appearance. This matrix is intermingled with the bone marrow in the marrow space surrounded by cortical bone (Kini & Nandeesh 2012).

There are two types of cells involved in normal bone physiology. The interaction between osteoblasts (bone-forming) and osteoclasts (bone resorbing) is responsible for a

series of complex biochemical pathways that result in either bone modelling or remodelling. Bone modelling occurs in both the subadult and adult skeleton. In subadults, modelling is responsible for growth and adaptation to the biomechanical environment, whereas in adults, it is implemented for fracture repair (Peck & Stout 2009). Furthermore, two patterns of osteoid formation may be identified during modelling and remodelling. Woven bone is formed initially when osteoid is rapidly produced by osteoblasts for fracture repair or during the growth process of fetal bones. This unorganized matrix of bone is then gradually replaced by more parallel lamellar bone that is significantly stronger during the process of remodelling that occurs throughout life (Seeman 2008; Kini & Nandeesh 2012).

2.2.2. Bone remodelling

The process of bone growth and remodelling provides a framework for the study of age-related bone loss related to physical activity, altered nutrition, sex hormone deficiency, genetic mechanisms, as well as the normal advancement of age (Parfitt 2003). Bone remodelling is considered to be the process in which the internal microstructures continuously reconstruct in a constant state of turnover and is essential for maintaining the shape and density of bone (Parfitt 2003; Robling & Stout 2008). This process is regulated by interactive cellular activity, otherwise referred to as skeletal homeostasis, and may be influenced by biochemical and biomechanical factors.

Normal bone mass (i.e. after growth has come to an end) is maintained by the balance between osteoblastic (bone formation) and osteoclastic (bone resorption) activity (process of remodelling). Bone mass is quantified in terms of bone turnover, which is defined as the total volume of bone that is remodelled over a period of time and expressed as percentage/year (Parfitt 2002, 2004). A peak bone mass is reached around the third decade of life, after which a slow and steady loss of bone occurs (Grynpas 2003). On average, peak bone mass and the age at which it occurs differ between males and females. Males tend to achieve peak bone mass later than women and usually have a higher peak density than seen in females (Stini 2003).

According to Burr (2002) there are three goals of remodelling: mineral homeostasis maintenance, biomechanical adaptation of bone to the environment and repair of microcracks to prevent it from weakening and ultimately failing under mechanical pressures. Bone has the ability to locate the area of damage, detect the amount of repair

needed and to restore its microarchitecture (Parfitt 2002; Seeman 2008). This process is carried out by osteoblasts and osteoclasts combined into one functional unit, known as a basic multicellular unit (BMU) or a bone remodelling unit (BRU). The osteocytes within the bone matrix, bone lining cells (covering the bone surface) and capillary blood vessels (supplying nutrients and hormones) also forms part of this unit (Eriksen *et al*. 1994; Kular *et al*. 2012). The rate at which BMU's are formed is known as the activation frequency, which is an essential regulator of bone turnover (Parfitt 2002; Stout & Crowder 2012).

Remodelling consists of three distinct phases: activation (A), resorption (R) and formation (F). The process begins with the activation of osteoclast precursors (mononuclear cells) that originate within the bone marrow. These mononuclear cells then fuse together to form multinucleated pre-osteoclasts. The endosteum is then lifted off the bone surface to create a space for resorption. The pre-osteoclasts then migrate to this area and differentiate into mature cells which bind to the mineralized bone surface for resorption to take place (Clarke 2008; Kular *et al*. 2012).

Resorption by osteoclasts takes around two to four weeks to complete during a remodelling cycle (Kini & Nandeesh 2012). Initial resorption of bone requires an acidic microenvironment which is created by the secretion of hydrogen ions by the activated osteoclasts (Silver *et al*. 1988). The digestion of the organic bone matrix by the multinucleated osteoclasts results in the formation of Howship's lacunae (resorption pits) on the surface of the bone. Once the multinucleated cells are terminated by apoptosis, the resorption phase is completed by mononuclear osteoclasts (Eriksen *et al*. 1994; Reddy 2004; Clarke 2008).

Bone formation takes considerably longer to complete than bone resorption (approximately four to six months). Osteoblasts originate from mesenchymal stem cells which go through four stages (pre-osteoblast, osteoblast, osteocyte and bone-lining cell) to reach maturity (Kini & Nandeesh 2012). Given the appropriate microenvironment, pre-osteoblasts will differentiate into osteoblasts, which are responsible for the synthesis of new bone matrix and the mineralization thereof. Once bone formation has been completed, the osteoblasts are subjected to one of three possible outcomes. Between 50 and 70% of osteoblasts will undergo apoptosis (Lynch *et al*. 1998), some will differentiate into osteocytes (being trapped within the bone matrix) and others will

become bone-lining cells that cover the bone surfaces to prevent inappropriate resorption by osteoclasts (Kular *et al*. 2012).

The end product of remodelling in cortical bone is a secondary osteon or Haversian system, also known as a basic structural unit (BSU). These units are well-defined in cortical cross-sections, require no staining in order to be viewed, and are therefore reliable histological entities to study (Eriksen *et al*. 1994; Stout & Crowder 2012).

2.2.3. Factors that influence bone modelling and remodelling

The preservation of bone growth and remodelling is largely dependent on the delicate balance and availability of numerous cellular activities and factors. Disruption of cellular regulation of these processes may result in abnormal bone formation or degradation. There are several factors may influence bone ossification and maintenance. These factors form part of three major groups, i.e. lifestyle, life history and genetic predisposition. Factors most relevant to biological anthropology include health and disease, physical activity and nutrition (lifestyle), sex-related differences (life history), as well as genetic predisposition.

Health and disease

There are a number of long-term (chronic) metabolic disturbances and health-related problems that influence bone remodelling. Such conditions may either elevate or reduce bone remodelling rates and will therefore influence histological age estimates (Stout 1998; Keough 2007; De Boer & Maat 2012). The most frequently described conditions in literature to alter osteon counts include osteoporosis/osteopenia, hyperparathyroidism, Paget's disease, osteopetrosis and bone neoplasms (Ortner 2003; Tolar *et al*. 2004; Vigorita 2008; Feng & McDonald 2011).

Over time, bone mineral density tends to decrease, which is considered a normal process of aging. However, if the rate at which one's bone mineral density decreases beyond what is considered normal and severely affects bone structure to the point where spontaneous fractures may occur, it is considered abnormal. This condition is known as osteoporosis and is the most common metabolic bone disorder found in the western world. It is recognized by an abnormal decrease in bone mineral density whilst the outline of the cortex stays intact (Bruce & Stevenson 1990; De Boer *et al*. 2013). The loss of calcified bone as described in terms of radiographic assessment of osteon population density (OPD/mm^2) is the measure used to clinically diagnose osteoporosis

(Stout 1998; Kanis *et al*. 1994; WHO 1994). Several factors may lead to a decrease in OPD, including sex hormone (Manolagas 2000) and calcium-regulating hormone (Bikle *et al*. 1999; Kini & Nandeesh 2012) imbalances, immobilization (Feng & McDonald 2011) and nutritional deficiencies (Hu *et al*. 1993; New *et al*. 2000). Osteoporosis is thus a symptom of an underlying primary disease (i.e. a condition with genetic factors contributing to its effect) or physiological process (e.g., menopause), rather than a disease itself.

Other conditions characterized by a decrease in overall bone mineral density include diabetes mellitus, osteoclastic bone neoplasms and hyperparathyroidism (Stout 1998; Kini & Nandeesh 2012). Paget's disease, however, results in increased numbers of osteoblasts and osteoclasts and the formation of unorganized bone on a histological level, which ultimately leads to increased bone thickness due to abnormally high bone formation rates (Keough 2007; Seitz *et al*. 2009).

Physical activity

Several clinical studies have linked physical activity to the acquisition of bone mass (e.g., Van der Meulen & Carter 1999; Beck & Snow 2003). The magnitude of physical activity exerted by an individual can be positively correlated with an increase in bone mineral density, specifically during pubertal years up to about 30 years of age when peak bone mass is acquired (Katzman *et al*. 1991; Välimäki *et al*. 1994; Sundberg *et al*. 2001). If the form and function of bone changes, the microarchitecture of that bone will change, which ultimately will alter its shape and strength. This phenomenon is known as Wolff's Law (Frost 1994; Ruff *et al.* 2006). Further research into the field of bone mechanics and architecture revealed that bone will remodel over time to become stronger based on the amount of physical/mechanical load it is placed under (Frost & Schönau 2000; Frost 2001). The biomechanical process by which this achieved is known as mechanotransduction. Bone will, however, only adapt to become stronger if a continuous increased load is experienced (Duncan & Turner 1995).

A study by Bailey *et al*. (1999) investigated the influence of physical activity on bone mineral content in adolescent boys and girls in a longitudinal study spanning six years. Dietary intake was recorded in addition to anthropometric data. Results indicated a 9% and 17% greater total body bone mineral content for active boys and girls, respectively, over their inactive teenage peers. They also estimated that about 26% of adult total body

bone mineral content is accumulated during the two years surrounding the peak velocity rate of bone mineral acquisition that occurs in the young adult years. The bone density and structural features achieved during the growth phase is retained as the skeletal system reached maturity, however, a slow and constant decrease of skeletal density is then observed as age progresses and physical activity becomes less intense (Gosman 2012).

Variation in physical activity among different ancestral groups should also be mentioned. Significantly greater bone mineral densities have been reported for American black individuals, compared to their white peers (Kleerekoper *et al*. 1994; Nelson *et al*. 2000; Barondess *et al*. 1997). However, physical activity was not the only factor that contributed to these differences. Nutrition, socio-economic status and geographical location were also taken into account. In studies performed on whites and blacks from the same geographical areas in both South Africa (Patel *et al*. 1992; Daniels *et al*. 1995) and Gambia (Prentice *et al*. 1990; Aspray *et al*. 1996) bone mineral densities of blacks did not exceed that of whites. Both physical activity and nutrition were similar for these two groups, which most likely contributed largely to the outcome of the study.

Nutrition

Nutrition undoubtedly plays a role in bone growth and formation. Although many nutrients play a vital part in human growth and development, a few selected nutrients are essential for healthy bone formation. These include calcium, magnesium, phosphorus, fluoride, zinc, vitamin D and protein (Anderson 1999; Feldman *et al*. 2005; Palacios 2006). Clinical studies have shown that calcium and protein alone do not provide the necessary building blocks for normal bone mineral acquisition, and that the lack of only calcium from the complete list of nutrients needed for bone growth does not prevent growth during adolescence. However, calcium supplementation to one's diet may increase bone mineral density for the period that it is taken, although this may not be a lasting effect once supplementation is stopped, as other factors such as hormone imbalances or metabolic disease may play a role in the acquisition of bone mineral density (Lloyd & Cusatis 1999).

Nutritional deficiencies leading to certain diseases often affect the architecture of bone and may have a profound effect on histological aging techniques. Lifestyle habits such as alcoholism have been linked to increased rates of osteoporosis and reduced absorbency of vitamin C, which is essential for osteoid production, formation of

connective tissues and blood clotting (Santolaria *et al*. 2000; White & Folkens 2000; Brickley & Ives 2008).

Rickets (in children) and osteomalacia (in adults) result due to a deficiency of vitamin D and/or calcium (and manifest as bone deformation due to poor mineralization of newly deposited bone (Favus 1999; White & Folkens 2000; Brickley & Ives 2008). It should, however, be kept in mind that several metabolic disorders may also cause riskets/osteomalacia, such as coeliac disease, renal tubular acidosis or malabsorption syndrome that all bring about malnutrition. Softened bone leads to a bowed appearance of the long bones in children, and pathological fractures, stature reduction and bending of bones in adults (Favus 1999). Histologically, bone shows a failure to mineralize with the retention cartilage matrixes that weakens the bone structure (Whyte & Liberman 2011).

Initially, it was thought that vitamin D deficiency isn't common in African populations due to adequate sunlight exposure, as compared to countries that experience colder temperatures and fewer hours and less intensity of sunlight (Aufderheide & Rodriques-Martin 1998; Gloth *et al*. 1999). However, the latest research shows that despite adequate daily sunlight, vitamin D deficiency and insufficiency are extremely common in population groups living in Africa and the Middle East (Green *et al*. 2015). Numerous factors associated with specific groups have been identified to contribute to vitamin D deficiency and insufficiency, including dark skin colour and seasonality in South African children (Poopedi *et al*. 2011), traditional or religious dress in Africa and the Middle East (Sanchez *et al*. 1987; Glew *et al.* 2010), indoor lifestyle (Glew *et al*. 2010), exclusive breastfeeding in Nigerian children (Sanchez *et al*. 1987;) and chronic disease in Egyptian adults (Gannagé-Yared *et al*. 2009). Research thus suggest that vitamin D deficiency and insufficiency may be more common than originally anticipated and that it often is a multi-factorial condition shaped by environmental, cultural and biological factors.

Furthermore, an individual's vitamin D status may also be determined by genetic factors. The vitamin D endocrine system, which controls calcium and bone homeostasis, is enabled by the vitamin D receptor (VDR). Variation in this gene (polymorphisms) is responsible for certain diseases surfacing more frequently in some population groups than in others (Valdivielso & Fernandez 2006) as well as predisposing a specific population to vitamin D deficiency or insufficiency (Wang *et al*. 2010).

Stout and Paine (1992) mentioned that in their study individuals suffering from nutritional disorders and related diseases were underestimated by about 30 years in age due to larger secondary osteons and Haversian canal diameter.

Sex-related differences

It has been shown that the degree of mineralization in the femoral midshaft differ significantly between the sexes (Goldman *et al*. 2003). Although the overall degree of mineralization decreases for males and females as age advances, males usually show a higher bone turnover rate than females and thus males display an increase in the variation coefficient (a measure of the rate and efficiency of bone renewal) with age.

It is expected that there will be sex-specific variation due to the fact that skeletal maturation and timing of growth cessation differ between males and females (Keough 2007). Although early studies investigating histological age estimation stated that there were no significant sex differences in their samples (Kerley 1965; Ahlqvist & Damsten 1969; Singh & Gunberg 1970), numerous studies that followed suggested the need for sex specific standards (Thompson 1979; Burr *et al*. 1990; Ericksen 1991). Ericksen (1991) and Thompson (1979) made use of the cortical bone of the femur and were able to establish sex-specific standards. Other studies using the rib and clavicle (Stout & Paine 1992; Stout *et al*. 1994) found no sex difference and therefore made use of pooled data. It is unclear what caused the amount of variation between males and females among different bones, but it was assumed that remodelling rates, cortical area and biomechanical stress may have played a role (Stout 1998).

In a study by Maat *et al*. (2006), results indicated that there is no statistically significant difference between the sexes when exploring unremodelled bone. These results are expected if one takes into consideration the statement from Goldman *et al*. (2003) saying that male bone turnover rates (influenced by testosterone) are higher than that of females. This suggests that sex differences will most likely be observed in remodelled, rather than unremodelled bone.

A study by Cole and colleagues (2015) examined ethnic and sex differences in skeletal maturation among boys and girls (black and white) from nine to 20 years. Hand-wrist radiographs were used to construct individual and mean growth curves of bone maturity for comparison. The results indicated that black boys matured later but at the same rate as white boys. Black girls, however, matured at the same age as white girls, but at a

faster rate. Overall, girls matured earlier than boys by around two years. It was clearly demonstrated that there are differences in maturation in terms of ethnicity and sex.

2.3. The development of histological age estimation techniques

2.3.1. Introduction

Age related bone loss occurs throughout the skeleton. Histological studies have identified several microstructures that show age-related changes. For example, an increase in mean diameter of Haversian systems, as well as increased intracortical porosity is observed with age (Martin *et al*. 1980; Grynpas 2003). As age advances, the number of secondary osteons, as well as osteon fragments in the cortex also tends to increase in number (Robling & Stout 2008). Thus, lamellar (unremodelled) bone is found in greater quantities in younger individuals, and as a person ages, this becomes replaced by remodelled bone (mature Haversian systems with osteons and osteonal fragments). The identification of histological variables (such as secondary osteons) have been found to be reliable and when applied correctly, may yield relatively accurate age estimates (Stout & Paine 1992; Lynnerup *et al*. 1998; Lynnerup *et al*. 2006; Maat *et al*. 2006; Keough *et al*. 2009).

2.3.2. The use of bone histology in age-at-death estimation

The first published report on bone histomorphometry and using it to estimate age-at-death appeared over a century ago (Balthazard & Lebrun 1911). This method made use of type I osteon diameter and was tested by Deslypere and Baert (1958) but produced poor results. The first notable publication on age estimation from bone histomorphometry was produced by Kerley (1965), who based his method on observations reported by Amprino and Bairati (1936) and Jowsey (1960). This method delivered considerable better results than previous publications on the subject.

Many studies have been published since Kerley's (1965) pioneering paper. These methods are based on a number of different variables and focus on numerous skeletal locations. A summary of the most prominent studies that contributed to the development and improvement of histological methods for estimating age-at-death is found in Table 2.1.

The most popular skeletal locations for histomorphometric estimation of age include the long bones of the upper and lower limb, clavicle and ribs (Stout & Paine 1992; Robling

& Stout 2008; Streeter 2012; Crowder 2013). Varying degrees of accuracy were reported by studies published in the past five decades with standard error estimates ranging from approximately 3 to 16 years. A profile summary of these different techniques may be found in Robling & Stout (2008).

There is some variation in the definition of variables used by different authors. For example, the criteria used to include or exclude secondary osteons is different in Kerley's (1965) study than in Stout's (1986). The former included secondary osteons for which 80% or more of the original lamellar area is visible, while the latter only counted secondary osteons for which at least 90% of the structure was visible. The criteria employed for each study thus varies largely and is dependent on observer interpretation.

Overall, results from various studies have revealed a general trend indicating that with the advancement of age, the number of osteons tend to increase (e.g., Ericksen 1991, Kimura 1992, Lynnerup *et al*. 2006; Keough *et al*. 2009), whereas osteon and Haversian canal size decreases (e.g., Iwamoto *et al*. 1978; Cho *et al*. 2002; Pfeiffer *et al*. 2016). Also, the number of osteons seem to be the most reliable indicator of age-at-death (Lynnerup *et al*. 2006).

While most studies focus on remodelled bone, Maat et al. (2006) employed a different approach and assessed the percentage unremodelled bone. Sections were taken from the anterior femoral cortex at the midshaft. The sample comprised of 162 Dutch individuals with an age distribution ranging from 15 to 96 years. Three $1mm^2$ areas of bone were measured, from which they demonstrated that unremodelled bone declines as age advances. No statistically significant difference between males and females were seen.

An interesting study on the application of age estimation methods in a taphonomic setting was published by Absolonova and colleagues (2013), which investigated the use of histology for age-at-death estimation in burned remains. Compact bone samples were divided into 4 sections, which were burned at 700, 800 and 1000ºC. Based on the microstructural changes observed in the bone, regression equations for age estimation from burned bone were constructed. This study shows that there are additional contexts in which histological age-at-death estimation methods may be used, specifically with regards to post-mortem changes to skeletal remains.

2.3.3. Problems and limitations in histological age estimation

In terms of histological age estimation, sex and population heterogeneity, as well as intra-individual variation has been addressed and taken into consideration when performing analyses. In the past, the establishment of population standards were focused on when developing new methods. This may, however, lead to problems such as age mimicry where samples are too small and/or skewed in terms of its distribution. As with the development of any other method for age-at-death estimation, histological analyses require the use of multiple variable analysis and advanced statistical procedures (Seber & Lee 2003; Lang & Altman 2013).

Problems related to methodology include mistakes with the application of a technique, inter- and intra-observer error, instrument errors and computer related errors in terms of data capturing (Kouchi *et al*. 1999; Ousley & Hollinger 2012). There may also be problems related to the microscopic technique itself, such as sampling site (Simmons 1985). One must be cautious as to apply only a single indicator of age to obtain an estimate. Some authors recommend that multiple sections be taken if only one skeletal site is used (Stout 1998), whilst others recommend the use of various skeletal locations to calculate a mean age estimate for an individual (Stout & Paine 1992).

Complications such as observer error, observer bias and the loss of information of the objects under study related to two-dimensional images (ultimately resulting in the need to take multiple sections from the same site of investigation) may be avoided by employing stereological principles. Stereology provides an alternative way to quantify 3D objects/cells based on its appearance in 2D sections/images (Mouton 2005).

Furthermore, skeletal material as such has its own limitations. The lack of clinical or biological information often accompany skeletal remains poses a problem, as the nutritional and health status of the individual is unknown. The reference sample used for the establishment of a method should, ideally, not contain individuals with pathology. Any individual with macroscopically visible changes due to disease or metabolic condition may easily be excluded from the reference sample. However, malnutrition may alter bone microscopically (Brickley & Ives 2008; Raubenheimer 2008), therefore often appearing healthy enough to be included in the reference sample. It is thus very difficult to account for the inclusion of individuals that suffered from less than optimal nutrition, especially when working with individuals of low socio-economic status.

Table 2.1. Summary of most prominent histological age estimation studies.

Study	Bone location	Variables	Accuracy	Population
Kerley 1965	Midshaft cross-sections of femur, tibia and fibula	OPD, osteon fragments, non-Haversian canals, % unremodelled bone	Femur: SE = 9.39-13.85 Tibia: SE = 6.69-13.62 Fibula: SE = 5.27-10.85	White and black individuals combined (n = 126)
Ahlqvist & Damsten 1969	Cross-sections of the femur, positioned to avoid the linea aspera	% remodelled vs unremodelled bone	SEE = 6.71	Autopsy specimens (no further information given)
Singh & Gunberg 1970	Cross-sections of the anterior midshaft of the femur and tibia. Posterior border of the mandibular ramus.	OPD, average number of lamellae per osteon, average Haversian canal diameter	Femur: SEE = 3.24-5.01 Tibia: SEE = 3.02-4.59 Mandible: SEE = 2.55-3.83	US Cadavers (no further information given)
Iwamoto *et al*. 1978	Femur	OPD/mm^2, lamellae/mm^2, average osteon size, average Haversian canal diameter	73.4% of estimates were within ±10 years of the actual age	Modern Japanese (n = 96)
Thompson 1979	Humerus, ulna, femur	19 variables	SEE = 7.1-8.6 (pooled sexes)	New England white individuals (n = 116)
Hauser *et al*. 1980	Femur	OPD, mean min. diameter of Haversian canals, % surface of Haversian canals	SEE = 10.7-11.4	Modern Europeans (n = 96)
Thompson & Galvin 1983	Tibia	8 variables	Mean difference between actual and estimated age = ±7 yrs	US white and black individuals combined (n = 53)
Eriksen 1991	Anterior cortex of the femur at midshaft	8 variables	SEE = 10.0-12.2	Mixed sample of white, oriental, black and Hispanic individuals (n = 328)

Kimura 1992	2nd metacarpal	Cortical bone density, cortical thickness, OPD, osteon fragments	SEE = 9.99-14.82	Modern Japanese (n = 227)
Stout & Paine 1992	6th rib	OPD	Mean difference between actual and estimated age = ±4 yrs	US white and black individuals combined (n = 40)
Yoshino et al. 1994	Microradiographs of proximal humerus (compact bone)	OPD, resorption spaces, Haversian canals, osteon area	SEE = 6.1-8.8	Modern Japanese (n = 40)
Cool *et al*. 1995	Occipital – outer and inner cortex investigated	Fractional volume of primary osteons, secondary osteons, osteon fragments and unremodelled bone	$r^2 = 0.10$ (sec. osteons) $r^2 = 0.44$ (fragments) $r^2 = 0.32$ (lamellar bone)	Caucasian males (n = 18)
Stout *et al*. 1996	Cross-section of the midshaft of the clavicle	OPD	$r^2 = 0.85$	US white and black, 19th century Swiss individuals combined (n = 123)
Lynnerup *et al*. 1998	Anterior cortex of the femur at midshaft	Secondary osteons, Haversian canals, osteon fragments	Inter- and intra-observer variation assessment	Danish (n = 29)
Watanabe 1998	Anterior cortex of the femur at midshaft	Osteon perimeter, Haversian canal length, number of osteon fragments	SEE = 6.39	Japanese (n = 93)
Cho *et al*. 2002	6th rib	OPD, N.On, N.On.Fg, On.Ar, Ct.Ar/Tt.ar	RMSE = 12.22	US white (n = 51) and black (n = 103) individuals
Maat *et al*. 2006	Anterior cortex of the femur at midshaft	% unremodelled bone	SEE = 10.06	Contemporary Dutch (n = 162)
Lynnerup *et al*. 2006	Anterior cortex of the femur at midshaft	Secondary osteons	r = 0.493; Barlett $\chi^2 = 5.72$, p = 0.017	Danish (n = 24)
Keough *et al*. 2009	Anterior cortex of the femur at midshaft	10 variables	SEE = 13.31	South African black individuals (n = 146)

Absolonova *et al*. 2013	3rd to 10th ribs	Burned vs Unburned: 28 variables	Unburned: SEE = 13.92 Burned @ 700ºC: SEE = 16.25 Burned @ 800ºC: SEE = 14.90	Central European (n = 13 to 60 sections per group)
Pfeiffer *et al*. 2016	Mid-thoracic ribs	OPD, On.Ar, Ct.Ar/Tt.Ar (test of Cho *et al*. 2002 method)	SEE = ±12.22	Coloured, black and white South Africans

OPD – osteon population density
N.On – intact osteon population density
N.On.Fg – fragmentary number of osteons
On.Ar – secondary osteon area
Ct.Ar/Tt.Ar – relative cortical area

2.4. Stereology and its application to the microarchitecture of bone

2.4.1. Introduction

Stereology as a modern scientific discipline was established in the 1960's, although some of the principles it relies on date back as far as the 17th century. The complicated matter of understanding and quantifying the three-dimensional shape of objects based on looking at sections in a two-dimensional plane led researchers from fields of biology, geology, mathematics and engineering to combine their methods and officially create a new approach for the analysis of 3D objects/structures (Mouton 2005). Following the first meeting in 1961, the International Society for Stereology (ISS) was established in 1962, providing a platform for the development, discussion and implementation of practical methods to assess objects/structures/cells (Haug 1987; Mouton 2005). The name has recently (2017) been changed to International Society for Stereology and Image Analysis (ISSIA).

Modern stereology is defined as a set of methods that allows for the evaluation of solid structures in three dimensions in order to study the object profiles in terms of volume, surface (i.e. surface of a shape that isn't flat), length and number of solids (Mouton 2011; West 2012). It is based on systematic random sampling that ensures that every section made within a specimen has an equal opportunity for being selected (uniform random), making it well suitable for histological sections cut along a single axis (West *et al*. 1996; West 2012).

To ensure that the results of a study are unbiased (i.e. accurate), sampling should be uniformly random (orientation of the sections is selected at random) and examination of the sample should be structured in a way as not to ask biased questions. A violation of either one of these aspects, i.e. sampling bias or systematic bias will lead to biased (inaccurate) results. Accuracy is measured in terms of how similar the results are in comparison to the actual population mean and should not be confused with precision. Results may be precise (having a small standard deviation with values clustering close to one another), but inaccurate/biased (estimated mean far away from the true population mean). Precise results may thus appear to be accurate if bias is not taken into consideration (Mouton 2005; Howard & Reed 2010). Therefore, the most desirable outcome is unbiased results as it reveals the true nature of the structures studied.

Estimates obtained from analysing biological structures are, however, not exact, as biological differences between individuals/specimens account for variation. Biological factors such as the environment, genotype and evolution underwrite the largest foundation of variation in biological samples/populations. The way in which sampling of a population is performed is thus of the utmost importance to ensure that the results display an accurate proportion of variation (Schmitz *et al*. 1999; Mouton 2005).

Ideally, a large population should be sampled to reduce the amount of variation observed in the data. However, it often happens that a sample is slightly smaller than planned, leading to more variation portrayed by the data than is actually present within the larger population, known as sampling error (Mouton 2005). Statistically, sampling error is described in terms of the coefficient of error (CE). The CE value provides an indication of when an optimal number of sections has been reached to obtain an accurate and reliable estimate, i.e. the point where additional sampling will not have a major influence on the calculated estimate (Glaser & Wilson 1999; Schmitz & Hof 2000). It is therefore recommended that a pilot study be performed to establish an acceptable CE value.

In order to better understand how to create sampling processes optimized for maximal efficiency, the principle of "do more, less well" was proposed (Gundersen & ØSterby 1981; DC Sterio 1984). This principle addresses the question of how many individuals/sections should be studied, as well as the intensity with which each individual should be looked at. This means that an optimal number of sections should be analysed to produce reliable estimates (over-counting should be avoided), but that the intensity of studying each section should not exceed the point where a reliable estimate has been reached, i.e. over-analysing of sections. Over-counting and/or over-analysing sections leads to a proportionally large amount of work and time spent analysing in relation to the minimum number needed to obtain a reliable estimate. To assist with achieving optimal sample sizes and calculating appropriate CE values, design-based stereology was implemented.

2.4.2. Design-based stereology

2.4.2.1. Introduction

The term "design-based" refers to assumption- or model-free methods. Earlier stereological methods relied upon cell geometry for correction formulas, thus making assumptions regarding the shape and size of the object. An estimate may then give an

estimate of assumption (a result based on prior knowledge regarding information on the shape, size or orientation of the object), rather than determining an estimate based purely on mathematical and statistical calculations (Geuna 2000; West 2001).

Modern design-based stereological methods that have been developed are rather complex and require the used of computer-interfaced microscopes and imaging equipment. Analysis of these images are then performed using semi-automated computer-based stereology systems such as CAST (Visiopharm, Denmark), Digital Stereology (Kinetic Imaging, UK) and StereoInvestigator (MicroBrightField, USA). Stereology systems rely on a three-axis motor-driven specimen stage connected to a computer to acquire information from histological structures, after which specific probes are selected for analysis. Although some stereological analyses may be performed using basic microscopes and reduced computer support, the use of computer-based analysis is recommended as it allows for optimization in terms of data collection, storage and analysis (Schmitz & Hof 2005).

In current practices, stereology is mainly employed in clinical and research studies working on tissues such as that of the lung (Ochs *et al*. 2004; Schneider & Ochs 2013; Mühlfeld *et al*. 2013) and brain (Pakkenberg & Gundersen 1997; Schmitz & Hof 2007). These studies focus on the quantification of certain areas within the organ and/or the complete organ, either for the comparison with other studies or diagnostic purposes (Price *et al*. 2001; Ochs 2006). Several papers have also reported on the quantification of trabecular/cancellous bone (Vesterby 1993; Fyhrie *et al*. 1993; Odgaard 1997; Thomsen *et al*. 2005), although no extensive studies exist on the assessment of cortical bone using design-based stereology.

2.4.2.2. Considerations for quantification of structures

There are several factors that may adversely affect the outcome of a study applying stereology. Analyses performed based on intuition may be biased, misinterpreted or inaccurate. It is thus essential that conceptual planning is done, and a pilot study is performed to determine the precise parameters of the stereological workflow, as well as a minimum number of individuals. Stereology software allows for the study design to be carried out step-by-step, providing the researcher with the appropriate tools for acquiring images, selecting parameters and field-of-view sampling (Hsia *et al*. 2010; Tschanz *et al*. 2014).

Apart from systematic pitfalls and errors, tissue preparation should also be considered. Chemicals used for embedding tissue may cause distortion or shrinkage and lead to disproportional and anisotropic changes (Dorph-Petersen *et al*. 2001). There are several recommendations for avoiding tissue deformation in soft tissue (Dorph-Petersen *et al*. 2001; Ochs *et al*. 2004; Mühlfeld *et al*. 2013). Hard tissue such as bone and teeth are much less prone to shrinkage and distortion, and may be embedded using chemicals such as epoxy resin or glycol methacrylate (Gerrits *et al*. 1992; Dorph-Petersen *et al*. 2001).

2.4.3. Stereology probes

2.4.3.1. Introduction

Stereological probes are virtual shapes (e.g. lines, balls, etc.) that are used to estimate features of biological tissue. Based on the number of times a shape interacts with the structure of interest (countable events), an estimate is generated through a predetermined mathematical formula that has been built into the software (Tschanz *et al*. 2011; Sagheb & Moudi 2014). Probes are used in conjunction with systematic random sampling (SRS), which is essential to ensure a lack of bias (Mouton 2002; West 2012).

First order probes are used to estimate number, length, surface area and volume (Howard & Reed 2010). Second order probes are used to determine distribution and connectivity of structures, thus estimating the relationship among features, for instance how close two structures are to each other (Odgaard & Gundersen 1993). Only first-order stereology will be discussed further as it is used in this study.

For first order stereology to be applied, it is important that either the tissue examined, or the probe used is isotropic (characteristically being identical in all directions). In cases where the tissue is not isotropic (such as cross-sections of bone) it is best to select an isotropic probe, which will guarantee that the orientation of the probe does not affect the mean of the estimate (Jensen & Gundersen 1989; Herculano-Houzel *et al*. 2015).

A large number of modern unbiased stereological probes are available to quantify features of biological tissue in the fields of biological and medical research. A summary of the most commonly used probes is given in Table 2.2. The probes relevant to this study have been labelled bold in this table and will be discussed in detail.

2.4.3.2. Number estimation

The total number of particles or cell population may be estimated using the counting frame in conjunction with systematic random sampling. The optical disector or fractionator is often used to estimate the number of objects in a flat region/cross-section. The disector and fractionator differs in terms of how the total number is calculated. When the total area is known, the number estimate is multiplied by the reference area to obtain the total number estimates (Disector). For the fractionator, the number estimate is extrapolated to obtain an estimate for the area investigated (Howard & Reed 2010). The optical fractionator will be discussed in detail as it is used in this study.

The concept of the optical fractionator probe is based on the principle of the Optical Disector first described by Gundersen (1986) and was designed for thick tissue sections. It serves as a modification of the Disector method which uses two physical sections placed adjacent to one another for counting. The original disector method states that objects appearing in the first section, but not in the second, are counted. This ensures that objects are not counted twice. However, instead of using adjacent sections, the computer-based system generates a series of focal planes that are superimposed to build an image stack through which the z-plane may be viewed. The optical fractionator is designed to estimate the total number of cells/structures through systematic random sampling in a set of counting spaces in three directions (X, Y and Z planes) and is appropriate to use for tissue sectioned in any orientation (Gundersen *et al*. 1999). Number estimates for the entire region of interest (ROI) are generated by extrapolating a subset of the total number of objects counted (Howard & Reed 2010).

2.4.3.3. Length estimation

Estimating the length of an object relies heavily on the orientation of the object. For isotropic sections, an image plane may be used to count the number of times the object intersects with the plane, i.e. the more intersections, the longer the object is in length. Depending on the orientation (isotropy), as well as tissue thickness of the section under investigation, different probes may be used which included space balls (Mouton *et al*. 2002; West 2018), image planes (Evans *et al*. 2004) or perimetrics (Howard & Reed 2010).

In cases where the nucleator is used to estimate cell volume, the length of an object being assessed is used in the calculation for volume and surface area and is produced as part of

the output. The length estimations used in this study was obtained as such, and therefore no additional probes were used for the estimation of secondary osteon and Haversian canal length.

2.4.3.4. Surface area estimation

In order to estimate surface area, it is essential that the combination of the tissue and a probe be isotropic as to prevent over-estimation of the surface area. It is best to make use of thick tissue sections, which will allow one to use the isotropic fakir that is classified as an isotropic probe (Kubínová & Janacek 1998). In cases where only thin sections are available, cycloids (non-isotropic) may be used, although isotropy of the tissue must then be guaranteed (Baddeley *et al.* 1998).

If the nucleator is used for cell volume estimation, the surface area of the objects assessed is incorporated into the calculation for volume and is thus produced as part of the output. Surface estimations used in this study were obtained from the nucleator data output, and therefore no additional probes were used to estimate secondary osteon and Haversian canal surface area.

2.4.3.5. Volume estimation

Volume estimation may be done for either the region or the cell/particle assessed. To calculate an estimated volume of a specific region, the Cavalieri method (Gundersen & Jensen 1987) or area fraction fractionator (Howard & Reed 2010) are typically used.

To estimate the volume of particles, the nucleator is often used (Gundersen *et al*. 1988). It is used in conjunction with number-weighted sampling (i.e. the optical fractionator) and requires thick sections. The nucleator may be used either as an isotropic probe (IUR sections used) or a vertical probe (VUR sections). The IUR (isotropic uniform randon) tissue sections may be orientated according to its nature and the interest of the specific study when making use of the isotropic nucleator. However, when using VUR (vertical uniform random) sections, the orientation must be specified.

The nucleator functions by placing an arbitrary point inside the boundaries of the particle, from which radiating lines are then drawn to intersect with the boundary. Each length of these radiating lines is then squared/cubed, from which a mean is calculated and multiplied by π or $4/3\pi$ to produce the final estimated surface area and volume, respectively (Evans *et al*. 2004).

Table 2.2. Summary of commonly used unbiased stereology probes

Probe	Isotropic probe	Tissue preparation	Tissue thickness	Reference
		Number estimation		
Optical fractionator	**Yes**	**Any orientation**	**Thick (>20µm)**	**Gundersen 1986** **West *et al*. 1991** **Antunes 1995**
Physical fractionator	Yes	Any orientation	Thin (<20 µm, paired)	Gundersen 1986 Howard & Reed 2010
Fractionator	Yes	Monolayer	n/a	Gundersen 1986 Geiser *et al*. 1990
		Length estimation		
Space balls	Yes	Any orientation	Thick (>20µm)	Mouton *et al*. 2002 West 2018
Image plane	No	Isotropic sections	Thin (<20 µm)	Evans *et al*. 2004 Howard & Reed 2010
Petrimetrics	Yes	Monolayer	n/a	Howard & Reed 2010
		Surface area estimation		
Isotropic fakir	Yes	Any orientation	Thick	Kubínová & Janacek 1998
Cycloids	Yes	Vertical sections	Thin	Baddeley *et al*. 1998 Howard & Reed 2010
		Regional/component volume estimation		
Cavalieri point estimator	Yes	Any orientation	Thick or thin	Gundersen & Jensen 1987
Area fraction fractionator	Yes	Any orientation	Thick or thin	Howard & Reed 2010
		Cell volume estimation		
Isotropic nucleator	**Yes**	**Any orientation***	**Thick**	**Gundersen 1988** **Gundersen *et al*. 1988**
Vertical nucleator	Yes	Vertical sections	Thick	Gundersen 1988 Gundersen *et al*. 1988 González-Villa *et al*. 2017

*Orientation of tissue needs to be taken into consideration if not isotropic; orientation has to be customized according to type of tissue used as isotropy using the nucleator is achieved via a combination of the probe and tissue.

Probes in bold indicate those used in this study.

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Chapter 3
MATERIALS AND METHODS

3.1. Samples and population demographics

The sample of bone specimens used in this study included individuals from three diverse population groups. White and black South African males and females were selected from the Pretoria Bone Collection (L'Abbé *et al.* 2005) and the University of the Witwatersrand's student bone collection (Dayal *et al.* 2009). Both of these collections comprise of cadaver-derived skeletons and contain a mixture of unclaimed and donated remains. The majority of black remains in the collections originated from unclaimed bodies and largely represent individuals of low socio-economic status, whereas the remains of white individuals mainly epitomise bequeathed bodies and represent individuals across all socio-economic classes. Both these collections represent modern human remains and no archaeological material was used for the purpose of this study.

A comparative sample of 30 bone slides was selected from the Department of Forensic Pathology (Copenhagen University). All Danish sections originated from adult individuals, of which 14 were male and 16 female. The sections were produced in the Department of Forensic Pathology as part of routine forensic analyses performed during autopsy procedures.

Bone slides made for a previous study at the University of Pretoria (black males, n = 60 and black females, n = 39; Keough, 2007) were used to limit resampling and unnecessary destruction of bones. A total of 94 femora from white individuals were sampled from the Pretoria Bone Collection, of which 50 were male and 44 female. In total, the South African sample comprised of 110 males and 83 females.

A random sampling approach was employed for all skeletal samples. Random sampling was performed within each age category to obtain a specific number of individuals per age cohort, as it was essential to create a normal age distribution for the samples. The size, sex and age distribution of the complete sample used for bone sectioning is given in Tables 3.1 and 3.2. All individuals with visible pathology were excluded.

According to the Human Tissue Act 65 of 1983 and the National Health Act 61 of 2003, an institution may use tissues for research from an individual whom donated his/her body for this purpose. Permission to use the bone samples from the University of Pretoria was

obtained from the custodian of the material (Appendix 1). Ethical clearance for the project was granted through the University of the Witwatersrand waiver that states that all research on cadaveric material in the School of Anatomical Sciences does not require clearance from the Human Research Ethics Committee (Medical). This waiver was certified by Prof. P. Cleaton-Jones (Appendix 2).

Table 3.1. Sample size and sex distribution.

	University of Pretoria (South African samples)		University of Copenhagen (Danish sample)	**TOTAL**
	B	**W**	**W**	
Males	60	50	14	124
Females	39	44	16	99
TOTAL	99	94	30	**223**

B – black individuals
W – white individuals

Table 3.2. Age distribution of the total sample.

Age cohort	RSA white				RSA black				Danish white			
	M	**Mean**	**F**	**Mean**	**M**	**Mean**	**F**	**Mean**	**M**	**Mean**	**F**	**Mean**
20 – 29	-	-	2	21.5	7	24.3	4	22.3	5	23.8	5	24.6
30 – 39	1	36.0	1	39.0	9	35.3	5	36.8	3	34.3	3	33.3
40 – 49	4	43.8	2	47.0	10	45.1	10	44.3	2	42.0	2	42.5
50 – 59	11	53.7	4	55.3	11	54.3	7	52.4	1	50.0	2	53.5
60 – 69	11	63.2	14	65.8	11	64.2	8	62.8	-	-	1	67.0
70 – 79	11	74.5	13	76.2	10	74.4	3	71.7	2	73.5	-	-
80 +	12	85.1	8	85.0	2	81	2	80	1	82.0	3	85.3
TOTAL	**50**	**66.7**	**44**	**67.9**	**60**	**52.5**	**39**	**50.3**	**14**	**41.8**	**16**	**46.1**

3.2. DXA scans

Dual-energy X-ray absorptiometry (DXA, previously known as DEXA) was performed on a white and black South African sample to quantify bone mineral density (BMD). Scanning was performed at DPHRU (MRC/Wits research entity: Developmental Pathways for Health Research Unit) situated at Chris Hani Baragwanath Hospital using the Hologic Discovery DXA system (array mode).

This radiological technique is based on two X-ray beams of different energy levels that are passed through the selected area of bone tissue and measures BMD according to the absorption of each beam by bone. The DXA report provides values for bone mineral density (BMD; g/cm^2), as well as a T- and Z-scores for the total area assessed. The T-score compares the individual's BMD to that of a healthy 30-year old individual and is used for the diagnosis of osteoporosis. The range for normal bone mineral density is -1 and above. A score between -1 and -2.5 indicates low bone density (osteopenia), whereas a score of -.2.5 and below depicts osteoporosis. The Z-score matches the individual's BMD to what is considered normal in someone of the same age, sex and population group (WHO 2007; Miller 2008).

The left proximal femur of 59 SA white (29 males and 30 females) and 64 SA black individuals (34 males and 30 females) were scanned (Table 3.3). In order to analyse the scans, information regarding the stature (cm), weight (kg), BMI (kg^2/cm) and age (years) was required for each individual.

Biometric data were recorded in the cadaver books for only 65 of the 113 individuals used for scanning. All individuals lacking biometric data were black South Africans. Before scanning commenced, stature (cm) and weight (kg) were calculated for individuals lacking this information by using regression formulae constructed by Lundy and Feldesman (1987) for stature estimation of black South African males and females with correction factors revised by Raxter *et al*. (2006):

Males: *Stature = (2.403)(femur physiol. length in cm) + 45.721*

= _____ cm + correction factor of 12.2 cm

Females: *Stature = (2.769)(femur physiol. length in cm) + 27.424*

= _____ cm + correction factor of 11.4 cm

Weight estimation was calculated using formulae for pooled sexes by McHenry (1992) using the maximum diameter of the femoral head (FH) based on four sample means of North American males and females, African pygmies and Khoi-San, as there are no population specific formulae for weight estimation of South African individuals:

$$Body\ mass = (2.239)(FH) - 39.9$$
$$= \_\_\_\_\_\ kg$$

Due to the DXA program being used mainly for obtaining clinical data, it automatically calculates the age of an individual based on the date of birth (DOB). As all individuals investigated in this study were already deceased for a number of years, an adapted DOB was calculated for each individual by subtracting the age from the year in which analysis was performed (2016/2017).

Table 3.3. Summary of South African individuals scanned for BMD.

Age cohort (years)	**White males**			**White females**			**Black males**			**Black females**		
	N	Mean age	SD	N	Mean age	SD	N	Mean age	SD	N	Mean age	SD
20-29	-	-	-	2	21.5	0.7	5	23.8	1.6	2	24.0	2.8
30-39	-	-	-	-	-	-	4	34.0	2.9	7	34.7	3.2
40-49	5	43.0	3.7	-	-	-	6	45.3	3.1	6	45.3	3.6
50-59	8	53.9	3.3	2	57.0	2.8	6	53.0	2.3	5	51.6	3.1
60-69	10	63.1	2.8	11	66.5	2.4	6	63.2	2.5	8	63.1	3.0
70-79	2	77.0	1.4	10	75.5	2.1	6	74.7	3.5	1	70.0	-
80 +	4	87.0	4.6	5	84.8	4.7	1	80.0	-	1	80.0	-
Total	**29**	**61.3**	**14.1**	**30**	**68.9**	**15.2**	**34**	**51.5**	**17.7**	**30**	**49.2**	**14.4**

N – number of individuals
SD – standard deviation

3.3. Bone slide preparation

3.3.1. South African samples

The bone slides made for South African black individuals from the femoral mid-shaft (Keough 2007) was prepared by manual grinding as outlined in Maat *et al.* (2002), which caused sections to vary slightly in thickness, ranging between 100 and 200 µm.

For the white South African sample, cross-sections with a thickness of 2 - 4 mm were made 2 - 3 cm below the midshaft of the anterior surface of the femur (as not to affect bone measurements in future) using the EXAKT 312 pathology saw fitted with a diamond blade (Bone Research Laboratory, WITS), resulting in a half moon-shaped bone section (illustrated in Fig.3.1).

The sections were manually grinded by hand until it reached a thickness of around 1 mm according to the Manual for the Preparation of Ground Sections for the Microscopy of

Bone Tissue (Maat *et al.* 2002). This method is more cost-effective than machine grinding, but does not ensure a uniform thickness throughout the section.

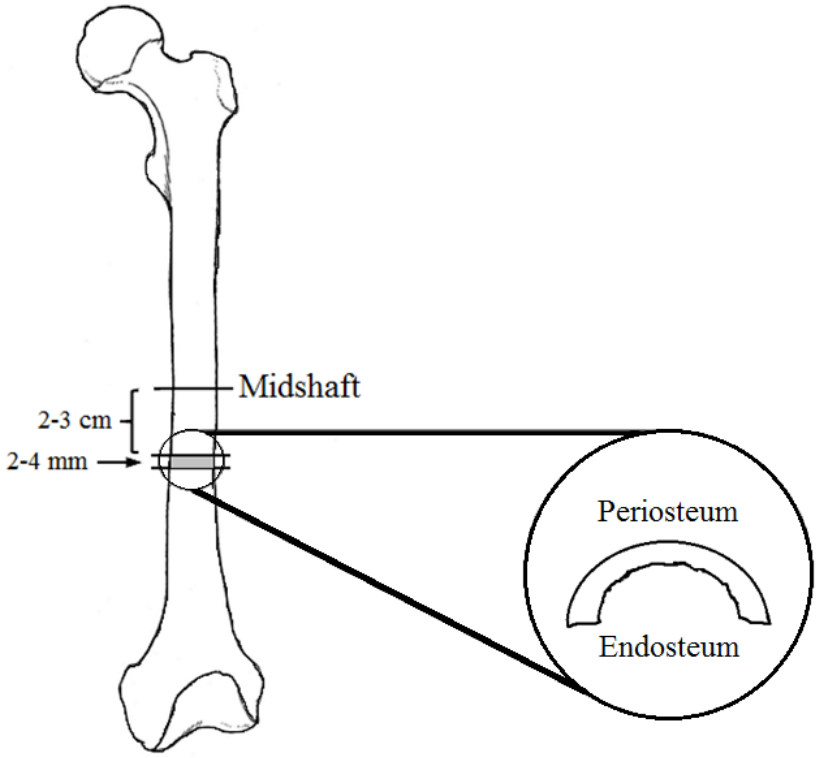

Figure 3.1. Location of cross-sections on the anterior surface of the left femur.

Manual grinding entails the use of abrasive paper wetted by a few drops of water. Grinding is then performed by placing the index and middle fingers onto the one side of a thick bone section and applying moderate pressure, performing a rotating motion to grind the other side of the section on the abrasive area (Keough 2007; Maat *et al.* 2002). Alternatively, "Frost's gripping device" may be used to assist in grinding (Frost 1958). This requires folding a strip op grinding paper (with the abrasive side outward) around a glass microscope slide and joining the two free ends together on top to hold the slide horizontally in place for grinding to proceed. The two free ends of the paper are gripped by the thumb and index finger, placed onto the bone section and rotated in the same way as one would do using only the index and middle fingers.

During the grinding process, sections were alternated between sides to ensure evenly distributed thickness and evenness as far as possible. Once the section reached the

desired thickness, it was washed in distilled water and allowed to dry completely before mounting.

In order to ensure a smooth and uniform surface area, sections were further grinded to a thickness of ± 150 µm using a surface grinding machine. To prepare for this process, sections were mounted onto perspex slides measuring 25 x 75 x 1.5 mm using EXAKT Technovit 7210 VLC precision adhesive and grinded to a minimum thickness of 100 µm (thicknesses ranged between 100 to 200 µm, depending on the brittleness of the bone) with the EXAKT 400 CS surface grinder (Bone Research Laboratory, WITS) using P500 grinding paper. All sections were unstained.

3.3.2. Danish sample

Cross-sections from the femoral mid-shaft were made for a large number of autopsies at the Department of Forensic Pathology, University of Copenhagen from 1988 to 1990. Sections were prepared by boiling complete sections of the anterior midshaft of the femora (sectioned to be ± 4 cm in length) in water and detergent for a few hours, before 100 µm slices were cut using a Buehler Isomet low speed diamond water saw and mounted on glass slides without staining (Villa & Lynnerup 2009).

3.4. Stereological analysis of histological variables

3.4.1. Scanning of bone samples

Scanning of the slides was performed using the Zeiss Z2 vario axioimager and Stereo Investigator (MBF, version 11.08.1, 64-bit) system. This equipment is available at the School of Anatomical Sciences, WITS. The area used for analysis (region of interest) was selected as such to include the outer, middle and inner cortex.

A number of histological variables were then selected for study using MBF Bioscience's Stereo Investigator software. Stereo Investigator provides a large number of tools for the advanced analysis of histological structures and allows for the quantification of cell/structure number, length, surface area and volume using different probes in a 3D environment.

3.4.2. Probe and variable description

The variables selected for studying age related features of bone, the associated stereological classification and probes are summarized in Table 3.4. Previous studies

(e.g. Ericksen 1991; Keough 2007) included variables such as the number of lamellae per osteon and resorption spaces. These variables, however, showed a weak correlation with age and was not considered for analysis in this study.

Table 3.4. Stereological classification and probes of variables under study.

Classification	**Variable**	**Probe**
Number (0D)	1. Average number of Haversian systems 2. Average number of osteon fragments	Optical fractionator
Length (1D)	3. Average Haversian system width/diameter 4. Average Haversian canal width/diameter	Nucleator
Surface (2D)	5. Average Haversian system area 6. Average Haversian canal area	Nucleator
Volume (3D)	7. Average Haversian system volume 8. Average Haversian canal volume	Nucleator

Probe descriptions:

1. **Optical fractionator:** this design-based stereological method is used to estimate the number of objects in a specified region of an organ/tissue. It involves a two-stage systematic sampling method combining the optical disector with the fractionator sampling method and is typically use when the population of cells/objects is too large to count exhaustively. Number estimates for the entire region of interest (ROI) are the generated by extrapolating a subset of the total number of objects counted (Howard & Reed 2005).

   Before using the optical fractionator, a number of parameters are set that include the counting frame size, grid size, guard zone size, optical disector height, section increment (1 in this study as only one section per individual is used) and section thickness.

   The surface of tissue sections are often irregular, and because it is essential for counting of particles to take place within a known sample volume, guard zones are set in place at the top and bottom surfaces which place the counting frame just below the top surface of the section (Fig. 3.2).

The number of counting frames generated based on the region of interest (ROI) for all slides examined was set at a minimum of 35 sampling sites (as the minimum recommended number of sampling sites/counting frames should be no less than 20 in order to count between 100-300 objects; Gundersen 1986; Howard & Reed 2005).

Details regarding all parameters set for scanning of the sections are summarized in Table 3.5. The section sub-fraction for all sections was one, due to only one section being available per individual. The disector height, average mounted section thickness, guard zone height and CE values were reported as ranges, due to the section thicknesses differing slightly between individuals.

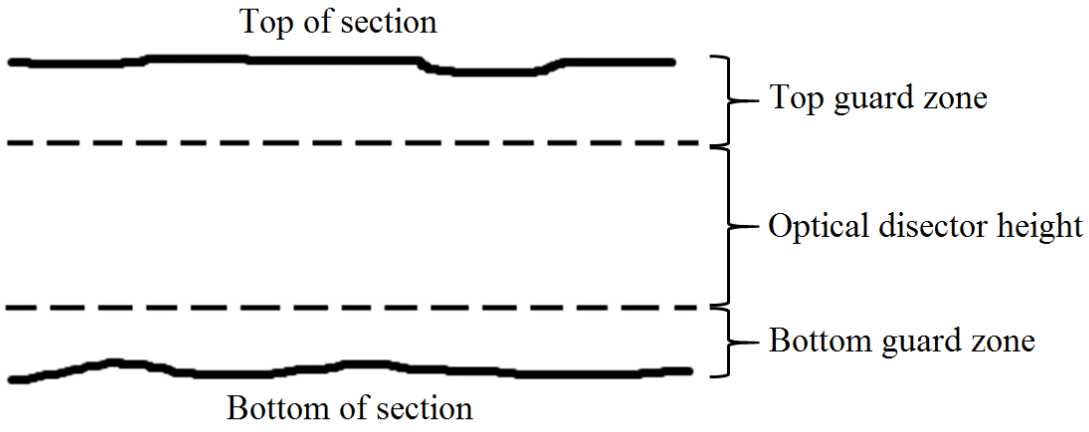

Figure 3.2. Illustration of guard zones and optical disector height used to establish sample volume.

Once the guard zones, sample volume and counting frame size have been established, counting of particles (structure of interest, i.e. Haversian systems and fragments) was done after the grid had been randomly placed on the ROI (Fig. 3.3). The osteon cement lines and Haversian canal boundaries were used as the leading edge (unique "point" on the object brought into focus) for defining the Haversian systems for counting. Counting was performed according to the following rules (Gundersen 1977; Howard & Reed 2005):

a) every particle is marked with one unique point, as to avoid over counting.
b) particles are only counted once its unique point comes into focus.

c) particles that lie within the counting frame are counted according to the rules depicted by the inclusion / exclusion lines. Particles situated completely within the counting frame, not touching any lines, are counted. Particles crossing the exclusion (red) lines are not counted, whereas those touching the inclusion (green) lines are counted.

Table 3.5. Summary of parameter details using the optical fractionator for all sections analysed.

Parameter	**RSA black**	**RSA white**	**Danish white**
Section sub-fraction	1	1	1
Area sub-fraction: • counting frame area • sampling grid area	1000 x 1000 = 1000000 µm^2 1250 x 1250 = 1562500 µm^2	1000 x 1000 = 1000000 µm^2 1250 x 1250 = 1562500 µm^2	1000 x 1000 = 1000000 µm^2 1250 x 1250 = 1562500 µm^2
Magnification	5X	5X	5X
Height sub-fraction: • disector height range • average mounted section thickness range	150 – 215 µm 195 – 250 µm	200 – 270 µm 250 – 300 µm	180 – 270 µm 220 – 280 µm
Guard zone height range	7.5 – 10 µm	10 µm	
CE used and its value	Gundersen, m = 1; 12 – 13%	Gundersen, m = 1; 12 – 13%	Gundersen, m = 1; 12 – 13%

2. **Nucleator:** this probe is used for estimating volume and is usually employed in conjunction with number-weighted sampling, i.e. the optical fractionator, and requires a thick tissue section. To make use of this probe, the tissue examined should be either isotropic or vertical in nature. The Nucleator may be set in either IUR (independent uniform random) or VUR (vertical uniform random) mode for isotropic or vertical sections, respectively. In this case the IUR mode was used that allowed for all directions in space to have the same chance of being chosen from the reference point, making the method unbiased.

A number of rays is then selected that will intersect with the boundary of the particle. In this study, five rays were used, each length starting at the reference point (arbitrary point at the centre of the Haversian canal) and projecting outward to intersect with the boundary (i.e. cement line) of the particles examined (Fig. 3.3).

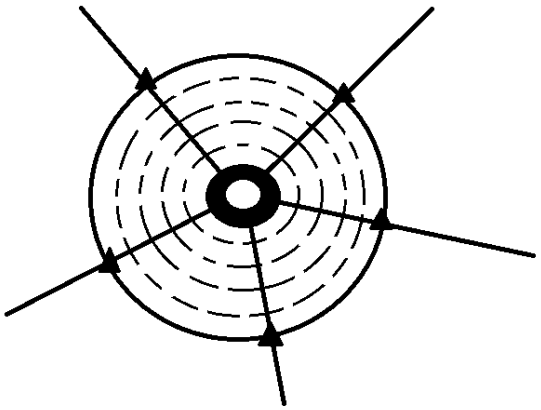

Figure 3.3. Illustration of five lengths (rays) from an arbitrary point in the centre of the Haversian canal intersecting with the boundary of the osteon. The intersections are marked by the solid triangles (▲).

Two referencing (arbitrary) points were marked for each Haversian system: one to define the boundaries of the osteon and the second to mark the boundaries of the Haversian canal.

The nucleator allows for the assessment of irregular particles due to it using an adapted version of the original formula for volume estimation (Méndez *et al.* 2007):

Original formula: $V = \frac{4}{3}\pi \bullet l^3$

Adapted formula: $V_N = \frac{4}{3}\pi \bullet l_n{}^3$

Where V = volume estimate

V_N = mean volume estimate

l = length of radiating line / intercept

$l_n{}^3$ = mean of the cubed lengths of the rays

N = number of particle volumes

n = number of radiating lengths

The lengths of the radiating lines (from arbitrary point to the intercept with the osteon boundary) are cubed and the average calculated before being multiplied by 4/3π to gain a mean volume estimate of the osteon.

An area estimate was also done using the Nucleator, as the surface area formula also uses the length of the radiating lines. The lengths are squared instead of cubed for this estimate, after which the mean length is multiplied by π to obtain a mean surface area estimate of the osteon:

$$S_N = \pi \bullet l_n{}^2$$

Where S_N = mean surface area estimate

l = length of radiating line / intercept

n = number of radiating lengths

Variable descriptions:

1. **Average number of Haversian systems:** this count included all identifiable, intact Haversian systems. All (a) complete secondary osteons, (b) incomplete secondary osteons with intact Haversian canals and (c) Type II (embedded) osteons were counted (Fig. 3.4). Osteons that were significantly obscured by Volkmann's canals, presented with unclear/obscured Haversian canals or affected by microfractures in the bone were also counted, but excluded for measurement purposes.

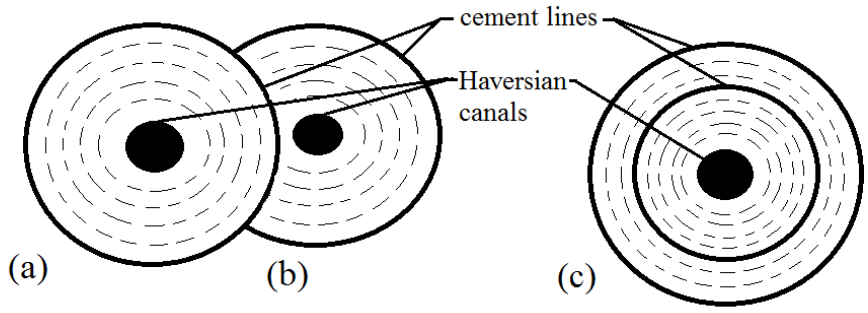

Figure 3.4. Illustration of Haversian systems included in total count; (a) complete secondary osteon, (b) incomplete secondary osteon with intact Haversian canal and (c) Type II secondary osteon.

All countable Haversian systems/osteons lying entirely within or touching the inclusion lines of the counting frame were counted. Osteons touching the exclusion lines of the counting frame were not counted (Fig. 3.5).

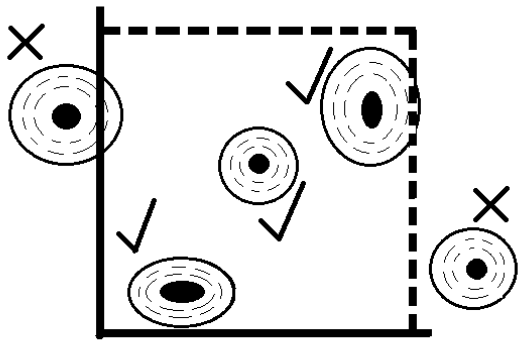

Figure 3.5. Illustration of osteons included or excluded in stereological count (solid lines = exclusion barrier; dashed lines = inclusion barrier).

The total number of Haversian systems counted was then divided by the number of counting frames with counts to produce an average number of Haversian systems per grid area (1562 500 $\mu m^2 \approx 1.563\ mm^2$).

2. **Average number of osteon fragments:** secondary osteon fragments were identified as remnants of original osteons that remained after the remodelling of new osteons covered the old osteons. Fragments counted included (a) osteon remnants and (b) osteons of which the Haversian canal is incomplete and already obscured by the newly formed osteon, located outside the cement line of the intact (new) osteon (Fig. 3.6).

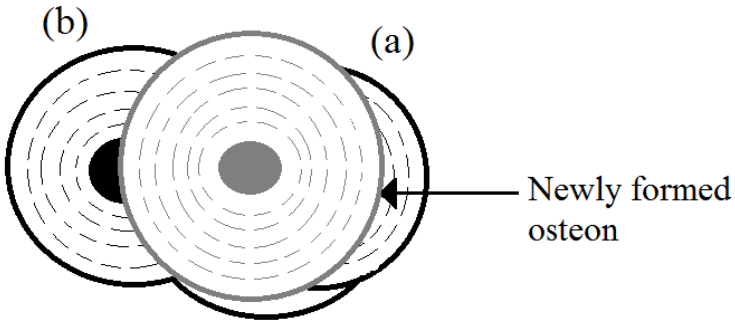

Figure 3.6. Illustration of secondary osteon fragments included in total count; (a) osteon fragment with obliterated Haversian canal and (b) osteon fragment with incomplete Haversian canal.

All countable fragments lying entirely within or touching the inclusion lines of the counting frame were counted. Fragments touching the exclusion lines or located entirely outside of the counting frame were not counted (Fig. 3.7).

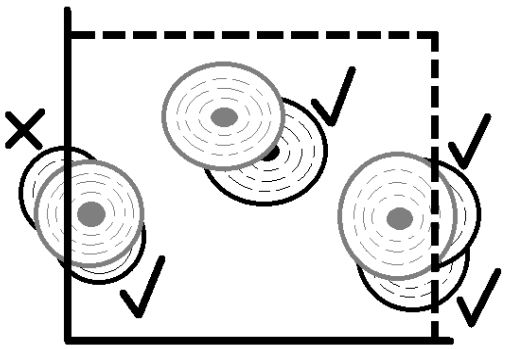

Figure 3.7. Illustration of fragments included in the stereological count (solid lines = exclusion barrier; dashed lines = inclusion barrier).

The total number of fragments counted was then divided by the number of counting frames with counts to produce an average number of fragments per grid area (1562 500 $\mu m^2 \approx 1.563\ mm^2$).

3. **Average osteon width (µm):** given as the average diameter of all osteons measured (Haversian systems with intact/clear perimeters) for each individual. The diameter

estimates for the region of interest (ROI) were added together. The total sum was then divided by the number of osteons measured to calculate an estimated osteon diameter average.

4. **Average Haversian canal width (µm):** given as the average diameter of all Haversian canals measured (Haversian systems with intact/clear canal perimeters) for each individual. The diameter estimates for the region of interest (ROI) were added together. The total sum was then divided by the number of Haversian canals measured to calculate an estimated canal diameter average.

5. **Average Haversian system area (μm^2):** given as the average area of all Haversian systems measured (Haversian systems with intact/clear canal perimeters) for each individual. The surface area estimates for the region of interest (ROI) were added together. The total sum was then divided by the number of Haversian systems measured to calculate an estimated Haversian system area average.

6. **Average Haversian canal area (μm^2):** given as the average area of all Haversian canals measured (Haversian systems with intact/clear canal perimeters) for each individual. The surface area estimates for the ROI were added together. The total sum was then divided by the number of Haversian canals measured to calculate an estimated canal area average.

7. **Average Haversian system volume (μm^3):** given as the average volume of all Haversian systems measured (Haversian systems with intact/clear canal perimeters) for each individual. The volume estimates for the ROI were added together. The total sum was then divided by the number of volume estimates measured to calculate an estimated Haversian system average.

8. **Average Haversian canal volume (μm^3):** given as the average volume of all Haversian canals measured (those with clear and intact perimeters) for each individual. The volume estimates for the ROI were added together. The total sum was then divided by the number of volume estimates measured to calculate an estimated Haversian canal average.

3.5. Statistical analysis

3.5.1. Linear and multiple regression analyses

Regression analysis was employed to observe the strength of the relationship between known age of the individuals and the variables assessed. This form of statistical modelling provides a method used to predict one variable, given that the value of the other variable is known. A scatter plot is created in which the predictor/response/dependent variable is usually plotted on the y-axis and the explanatory/regressor/independent variable on the x-axis. A straight line ($y = mx + c$) is then fitted through the data points to demonstrate the best possible linear relationship between the variables. The strength of the linear relationship is given by the correlation coefficient (r; $0 \leq r \leq 1$), where $r = \pm 1$ represents perfect linear correlation and $r = 0$ shows no correlation or correlation of a non-linear nature (Madrigal 1998; Seber & Lee 2003; Montgomery *et al.* 2012).

Once the correlation coefficient (r) has been established, the coefficient of determination (r^2) is calculated which gives the proportion of variation (fluctuation) in the response variable that can be explained by the variation in the regressor variable. R^2 ($0 \leq r^2 \leq 1$) is often given as a percentage and provides a measure of how well the regression line fits the data (Seber & Lee 2003; Montgomery *et al.* 2012).

Linear and multiple regression analyses were done for all independent variables vs. age (dependent variable) in pooled and unpooled sex groups of each population using SPSS (version 23) statistical software.

Results from the regression analysis showed whether the relationship between age and the variables concerned is valid and meaningful. The analyses for which the p-values were significant were viewed as meaningful because changes in the predictor variable (age) was related to changes in the response (independent) variables (Seber & Lee 2003).

The results were then used to construct single and multiple variable regression formulae for the estimation of age in the different sex and population groups based on the following equation derived from the basic equation for a straight line ($y = mx + c$):

Age = (slope x variable) + intercept ± SEE (standard error of the estimate)

Where m = slope = $\Delta y/x$

c = intercept

SEE = a measure of the accuracy of the predictions

For the assessment of data obtained from the DXA scans, an independent-samples t-test was used to compare BMD values for black and white South African groups and to explore sex differences. BMD values were correlated with age using a linear regression model to explore the relationship between age and total/neck BMD for ancestry/sex specific groups.

3.5.2. ANOVA / ANCOVA analyses

To test for significant differences between means, analysis of variance is employed. This method compares the variance between different groups to explain the mean differences observed. To accomplish this, the inter-group variability (called the mean square effect) is assessed. The difference in variance is then tested for statistical significance and if found to be significant, a noteworthy difference between the groups is present (Hill & Lewicki 2006).

One-way ANOVA involves analyses based on one level of the factor variable (e.g., comparing means of pooled sex populations with one or more response variables), while two or more levels of the factor variable may be assessed using two-way analysis (e.g., comparing group differences with the distinction between males and females).

ANOVA (F test) is used to compare groups of a factor based on a single continuous response variable. ANCOVA is similar to ANOVA, but is described as a univariate general linear model and used to detect a difference in the means of three or more independent groups where one needs to control for a scale covariate, in this case age (Hill & Lewicki 2006; Deviant 2010). Analysis of covariance (ANCOVA) was performed in order to determine the relationship between RSA white, RSA black and Danish population means, with the influence of age for all histological variables used in the estimation of age-at-death.

3.5.3. Repeatability tests

Repeatability of the proposed method was done by conducting intra-observer error tests. The Danish sample (n = 30; 14 males and 16 females) was re-analysed. Variability of the observed data was demonstrated by the intraclass correlation coefficient (ICC), which is

measured on a scale of 0 to 1. One represents perfect reliability where no error is present and the measurement from the first and second round/observer are identical. Zero indicates that no reliability exists (Rankin & Stokes 1998; Stemler & Tsai 2008).

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PART 2: RESULTS/PAPERS

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Forensic Anthropology Population Data

The use of stereological methods in the histomorphometric assessment of bone for age-at-death estimation

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ABSTRACT

Stereological examination of the anterior femur was done for the estimation of age-at-death. The aim of this study was to assess particular bone microstructures that change with advancing age and use these variables to create revised regression formulae applicable to the black population of South Africa. A sample of 99 bone sections (n = 60 males and n = 39 females) that had previously been analysed using 2D methods, were re-analysed using the optical fractionator and nucleator sampling methods. Single and multiple regression analyses were performed to assess the strength of the relationship between known age and all independent variables. For sex-pooled data, the average number of osteons per grid area (Avg_OPD) showed the highest correlation with age ($r = 0.528$; $r^2 = 0.278$), followed by average osteon volume ($r = -0.383$; $r^2 = 0.146$). The remaining variables reflected a low correlation with age. Pooled, as well as sex-specific single regression formulae were constructed. Multiple regression formulae were constructed for pooled sexes only, as there were no significant difference between males and females overall. Although the employment of stereological methods ensured that the results are accurate and unbiased, the outcome was on par with previously reported SEE's and SD's for this population.

1. Introduction

Estimation of age-at-death in adult skeletal remains is one of the most challenging tasks in a forensic setting, as skeletal changes related to age in adults are less precise than in juveniles and inter-individual variation is high [1–3]. Macroscopic adult age estimation methods suffer from several problems, including wide ranges and inaccurate estimates [4–6]. Another disadvantage of macroscopic methods is that one requires nearly complete skeletal elements in order to narrow down the estimated age range.

In cases where skeletal remains are incomplete or fragmentary, histological techniques for age estimation provide a valuable alternative to macroscopic methods [2,3,7]. Methods for the estimation of age from histological bone structures have been used for over 40 years and have been proven to be fairly accurate in several populations, although age ranges with standard error rates of between 10 and 15 years seem to have become the norm [3,8–10]. Many studies have made use of the cortex of the anterior femur for producing bone slides [2,3,8–14]. A major advantage of constructing population standards based on the femur is that this bone is usually well preserved and is often present in skeletal remains as it is the largest bone in the body.

Inter-population variation of bone microstructures has been reported by a few authors [15–17] and advances in bone biology and histology over the past 40 odd years provide information that allows for more accurate interpretation of microscopic data retrieved from bone. Factors that influence bone remodeling and turnover (i.e. nutrition, disease, genes, physical activity) have been discussed in detail [3,18–21], many of which have not been known by early pioneers of histological age estimation methods.

Histological studies have identified several microstructures that show age-related changes. For example, a decrease in mean diameter of Haversian systems, as well as increased intracortical porosity is observed with age [22,23]. As age advances, the number of secondary osteons, as well as osteon fragments in the cortex also tend to increase in number [20].

When considering histological methods for assessing microscopic structures, stereology has been proven to be unbiased and provide more accurate results for counting and studying microstructures compared to two-dimensional methods due to the fact

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that it is based on random sampling and incorporates the three-dimensional space in which the structures of interest are found [24]. Stereology is a set of methods that allows for the evaluation of solid structures in terms of volume, surface (i.e. surface of a shape that is not flat), length and number of solids [25,26]. Systematic random sampling ensures that every section made within a specimen has an equal opportunity of being selected, making it well suitable for histological sections cut along a single axis [26,27].

The aim of this study was to assess specific bone micro-structures (variables) shown to change with advancing age, using computer-based stereology in a black South African population. All variables were correlated with age to set up revised population-specific regression formulae for the estimation of age. The results were compared to a previous study done by Keough et al. [3] on black South Africans using 2D histological analysis. A comparison of the two methods in general, their similarities/differences in age ranges and standard error estimates obtained are discussed.

2. Materials and methods

2.1. Sample size and bone slide preparation

The bone samples used originated from a previous study by Keough et al. [3] and represent adult individuals of black South African descent. Femora were selected for sectioning from the student bone collection housed in the Department of Anatomy at the University of Pretoria. The majority of remains of black South Africans in the collection originated from unclaimed bodies that subsequently ended up as cadaver-derived skeletons in the bone collection. Demographic details such as age, sex, ancestry and stature were recorded for all skeletons. Due to the fact that unclaimed remains include people such as migrant workers and destitute individuals, the collection largely represents individuals of low socio-economic status of whom very little is known about their general health and nutritional status [28].

Bone sections were removed from the anterior cortex of the femur in the region of the mid-shaft. Bone slides were prepared by manual grinding according to the protocol suggested by Maat et al. [29]. This method does, however, result in sections differing slightly in thickness. The section thickness for the sample is therefore given as a range (Table 2).

The sample selected for this study comprised of 60 male and 39 female individuals with ages ranging between 21 and 82 years for males (mean age = 52.5 years) and females ranging between 20 and 80 years (mean age = 50.3 years). This difference between the sample sizes of males and females reflects the demographics of the Pretoria Bone Collection which is dominated by males and older individuals. The total sample of n = 99 had a mean age of 51.6 years (Table 1).

Table 1
Age distribution of the sample.

Age cohort	Male			Female			Total		
	n	Mean	SD	n	Mean	SD	n	Mean	SD
20–29	7	24.3	1.6	4	22.3	2.1	11	23.5	2.0
30–39	9	35.3	2.6	5	36.8	2.7	14	35.9	2.6
40–49	10	45.1	2.8	10	44.3	3.5	20	44.7	3.1
50–59	11	54.3	3.1	7	52.4	2.9	18	53.6	3.1
60–69	11	64.2	3.2	8	62.8	3.2	19	63.6	3.2
70–79	10	74.4	3.5	3	71.7	2.9	13	73.8	3.4
80+	2	81	1.4	2	80	0.0	4	80.5	1.0
Total	60	52.5	17.1	39	50.3	15.5	99	51.6	16.4

n — number of individuals.
SD — standard deviation.

2.2. Stereological analysis

For the quantification of Haversian systems, an unbiased design-based systematic random sampling stereological protocol was employed. We used a MicroBrightfield (MBF) (Colchester, Vermont, USA) system with three plane motorised stage, Zeiss.Z2 Vario Axioimager and StereoInvestigator software (MBF, version 11.08.1; 64-bit). Initially we measured the thickness of bone sections and obtained an average to assist in maintaining consistency with stereological parameters. Pilot studies were then conducted to optimise sampling parameters, such as the counting frame and sampling grid sizes, and achieve a coefficient of error (CE) below 0.1 [30,31]. At this point we would like to acknowledge that while every effort was made to obtain a CE below 0.1, this was not always achieved mostly due to a limited number of countable sections in those instances. In addition, we measured the tissue section thickness at every sampling site and the vertical guard zone was determined according to tissue thickness to avoid errors/biases due to sectioning artefacts [31]. We decided to maintain consistency amongst sampling parameters and employed a single-blind procedure, to reduce unfavourable stereological estimation biases. Table 2 provides a detailed summary of the parameters that were used in the current study.

To estimate the total number of Haversian systems within a consistently defined region of interest (ROI), we used the 'Optical Fractionator' probe and the following equation [31]:

$$N = Q/(SSF \times \mathrm{ASF} \times \mathrm{TSF}) \quad (1)$$

where N was the total estimated secondary osteon number, Q was the number of Haversian systems counted, SSF was the fraction of the sections sampled, ASF was the area sub-fraction (which is calculated by the ratio of the size of the counting frame to the size of the sampling grid), and TSF was the thickness sub-fraction (which is calculated by the ratio of the disector height relative to the section thickness).

One ROI (region of interest) per bone section/individual was selected, yielding a minimum of 35 counting frames. It is generally recommended that no less than 20 counting frames be used in order to get reliable results [24]. The number of counting frames differed among sections and ranged between 35 and 75 frames, depending on the size/width of the cortical bone area.

A number of variables were assessed using the optical fractionator workflow in conjunction with the 'nucleator' probe. The nucleator probe allows for estimation of volume in thick sections [30]. An arbitrary point is placed at the centre of the Haversian canal from which five radiating lines then intersect with the boundary of the object, i.e. the cement line. The software then calculates an estimated volume based on the length of these lines.

Variables were selected based on the strength of their correlation with age from the results of previous studies [3,9,32–34] and included the average number of osteons, osteon width, Haversian canal width, osteon surface area and volume, as well as Haversian canal surface area and volume. The variable descriptions are summarized in Table 3.

Counting of particles (structure of interest, i.e. Haversian systems and fragments) was strictly done in concordance to counting rules outlined by Gundersen [35] and Howard and Reed [24]:

a) every particle is marked with one unique point, to avoid over counting.
b) particles are only counted once its unique point comes into focus.
c) particles that lie within the counting frame are counted according to the rules depicted by the inclusion/exclusion lines. Particles situated completely within the counting frame, not touching any lines, are counted. Particles crossing the

Table 2
Summary of the parameter details for scanning of sections.

Counting frame size	Sampling grid size	Disector height	Average mounted section thickness	Guard zones	Average number of sampling sites	Average CE (Gundersen, m = 1)
1000 × 1000 = 1,000,000 μm^2	1250 × 1250 = 1,562,500 μm^2	150–215 μm	195–250 μm	7.5–10 μm	55	0.12

Table 3
Description of the histological variables assessed.

Stereological classification	Variable name	Variable abbreviation	Description
Number (0D)	Average number of osteons per grid area	Avg_OPD	The total number (estimated number using number-weighted section thickness) of all intact, identifiable Haversian systems divided by the number of counting frames with counts to produce the average number of Haversian systems per grid area in each individual
Length (1D)	Average osteon width (μm)	Avg_Ost_L	The average diameter of all osteons measured in each individual
	Average Haversian canal width (μm)	Avg_Hav_L	The average diameter of all Haversian canals measured in each individual
Surface area (2D)	Average osteon surface area (μm^2)	Avg_Ost_Ar	The average surface area of all osteons measured in each individual
	Average Haversian canal area (μm^2)	Avg_Hav_Ar	The average surface area of all Haversian canals measured in each individual
Volume (3D)	Average osteon volume (μm^3)	Avg_Ost_Vol	The average volume of all osteons measured in each individual
	Average Haversian canal volume (μm^3)	Avg_Hav_Vol	The average volume of all Haversian canals measured in each individual

exclusion (red) lines are not counted, whereas those touching the inclusion (green) lines are counted.

2.3. Statistical analysis

After the stereological analysis had been completed, regression analyses were done using SPSS (version 23) statistical software to observe the strength of the relationship between known age of the individuals and the variables assessed. The strength of this relationship is given by the correlation coefficient (r; $0 \leq r \leq 1$), where $r = \pm 1$ represents perfect linear correlation and $r = 0$ shows no correlation or correlation of a non-linear nature [36–38].

Once the correlation coefficient (r) has been established, the coefficient of determination (r^2) is used to indicate the proportion of variation (fluctuation) in the response variable that can be explained by the variation in the independent variable. R^2 ($0 \leq r^2 \leq 1$) is often given as a percentage and provides a measure of how well the regression line fits the data [37,38].

Previous studies have demonstrated that the rate of bone turnover is significantly different in males and females [39,40], and therefore linear and multiple regression analyses were done for all independent variables vs. age (dependent variable) in pooled and unpooled sex groups.

The results were then used to construct single and multiple variable regression formulae for the estimation of age in pooled and unpooled sex groups based on the following equation:

$$Age = (slope \times variable) + intercept \pm SEE \quad (standard\ error\ of\ the\ estimate) \qquad (2)$$

The significance of the linear model was tested and explained by the F-value (the ratio of the mean regression sum divided by the mean error sum of squares). This test statistic may range from 0 (indicating that the model has no predictive capability) to any large number (null hypothesis rejected). All regression models which show non-significant F-ratios should not be used for the prediction of age.

3. Results

In order to establish the strength of the relationship between age and the independent variables, the correlation coefficients and coefficients of determination for all single variables for pooled and unpooled sexes were used (Table 4). A positive correlation with age was seen only for the average number of osteons per grid area. All other variables were negatively correlated with advancing age.

When considering pooled data, a moderate correlation was observed for two of the variables, which were the average number of osteons per grid area ($r = 0.528$; $r^2 = 0.278$) and average osteon volume ($r = -0.383$; $r^2 = 0.146$). Low correlation was observed for average osteon length ($r = -0.339$; $r^2 = 0.115$), average osteon area ($r = -0.320$; $r^2 = 0.103$) and average Haversian canal length ($r = -0.302$; $r^2 = 0.091$). Very little correlation with age was seen for average Haversian canal volume ($r = -0.230$; $r^2 = 0.053$) and average Haversian canal area ($r = -0.170$; $r^2 = 0.029$).

Separate sex group analyses demonstrated that males had a slightly stronger correlation with age than females in terms of average number of osteons per grid area (Fig. 1) and average osteon volume (Fig. 2). Males also showed a higher correlation with age than females for average osteon length (M: $r = -0.414$, F: $r = -0.266$), average osteon area (M: $r = -0.398$, F: $r = -0.236$) and average Haversian canal area (M: $r = -0.195$, F: $r = -0.161$). However, females showed a higher correlation with age than males for average Haversian canal length (F: $r = -0.355$, M: $r = -0.314$) and volume (F: $r = -0.256$, M: $r = -0.243$).

Independent-sample t-tests were performed to compare male and female values for all variables assessed. A significant difference was observed only in the values for average Haversian canal length ($t(97) = 2.407$, $p = 0.018$) between males (mean = 32.67, SD = 4.48) and females (mean = 30.59, SD = 3.73). These results indicate that sex-specific regression formulae should only be used when estimating age using average Haversian canal length, and that it

Table 4
Coefficient of correlation (r) and coefficient of determination (r^2) for all single variables plotted against age.

Variable	Correlation coefficient (r)			Coefficient of determination (r^2)		
	Pooled	Male	Female	Pooled	Male	Female
1. Avg_OPD	0.528	0.547	0.488	0.278	0.299	0.239
2. Avg_Ost_L	−0.339	−0.414	−0.266	0.115	0.171	0.071
3. Avg_Hav_L	−0.302	−0.314	−0.355	0.091	0.099	0.126
4. Avg_Ost_Ar	−0.320	−0.398	−0.236	0.103	0.158	0.056
5. Avg_Hav_Ar	−0.170	−0.195	−0.161	0.029	0.038	0.026
6. Avg_Ost_Vol	−0.383	−0.409	−0.389	0.146	0.167	0.151
7. Avg_Hav_Vol	−0.230	−0.243	−0.256	0.053	0.059	0.065

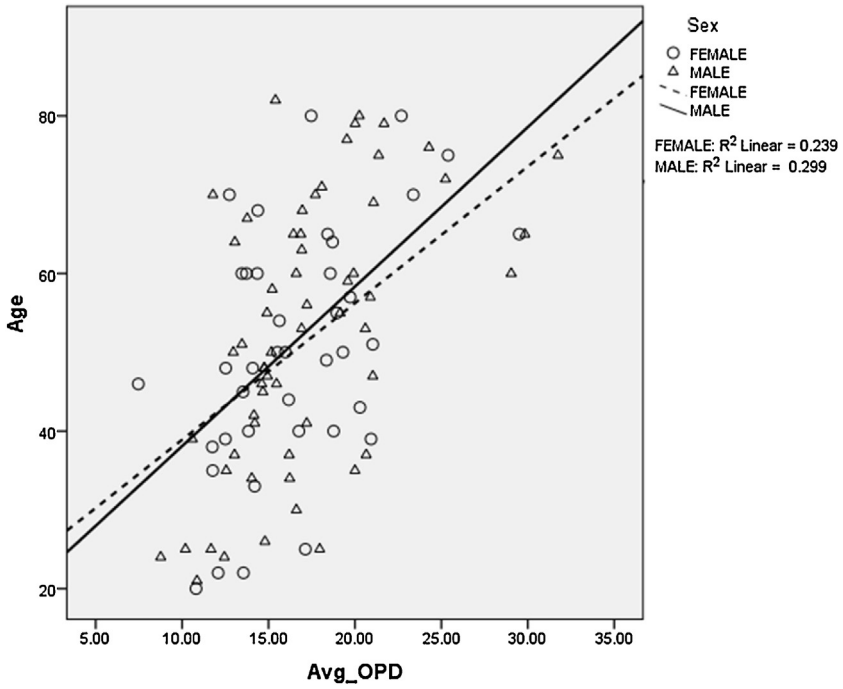

Fig. 1. Correlation for age vs. average number of osteons per grid area for males and females.

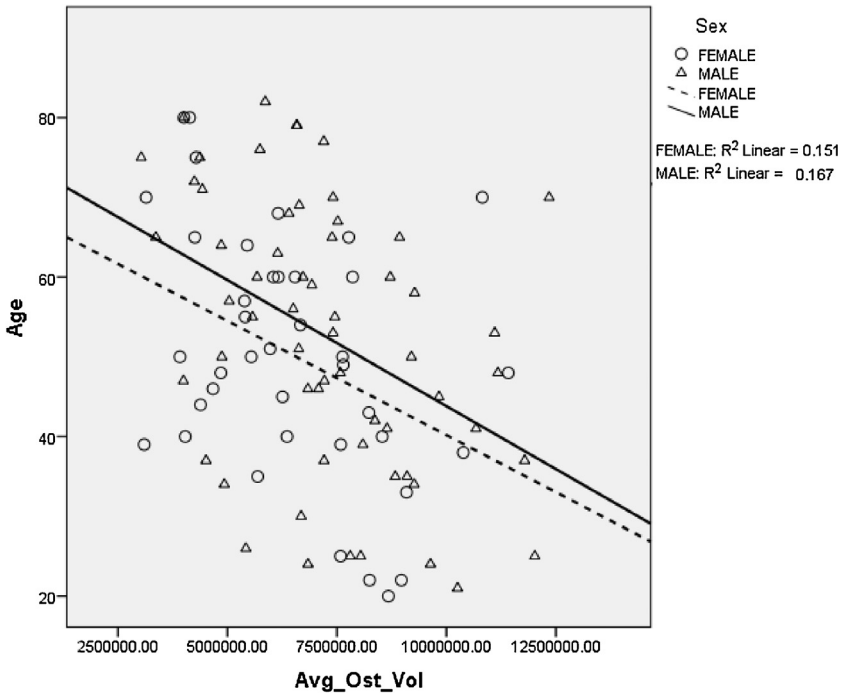

Fig. 2. Correlation for age vs. average osteon volume for males and females.

is possible to use pooled data regression formulae if the sex is unknown for all other variables.

A simple linear regression was calculated to predict age based on each of the seven variables selected (Table 5). A significant regression equation (sex-pooled data) was found for the first six variables in descending order: average number of osteons per grid area ($F(1, 97) = 37.429$, $p < 0.01$), average osteon volume ($F(1, 97) = 16.638$, $p < 0.01$), average osteon length ($F(1, 97) = 12.569$, $p < 0.01$), average osteon area ($F(1, 97) = 11.091$, $p < 0.01$), average Haversian canal length ($F(1, 97) = 9.709$, $p < 0.01$) and average Haversian canal volume ($F1, 97) = 5.437$, $p < 0.05$). Average Haversian canal area, however, showed unpredictable changes with age and could thus not be used to produce a significant regression equation for predicting age.

Sex-specific significant regression equations were also calculated for the first five variables (Avg_OPD, Avg_Ost_Vol, Avg_Ost_L, Avg_Ost_Ar and Avg_Hav_L). However, female regression equations for average osteon length and area proved to be non-significant.

In order to perform multiple regression analyses, preliminary tests were performed to ensure that there was no violation of the

Table 5
Linear regression analysis of age using single histomorphometric variables (in descending order of highest to lowest correlation with age).

Variable	Group	Slope	Intercept	SEE	F Ratio
Avg_OPD	P	1.925	19.103	14.015	**37.429****
	M	2.024	17.865	14.410	24.717**
	F	1.733	21.582	13.693	11.593**
Avg_Ost_Vol	P	$-2.870e^{-006}$	71.563	15.243	**16.638****
	M	$-3.160e^{-006}$	75.431	15.707	11.627**
	F	$-2.863e^{-006}$	68.818	14.460	6.578*
Avg_Ost_L	P	−0.497	105.429	15.524	**12.569****
	M	−0.636	122.446	15.667	11.977**
	F	−0.378	90.171	15.128	2.815
Avg_Ost_Ar	P	−0.001	77.136	15.629	**11.091****
	M	−0.001	86.188	15.788	10.916**
	F	0.000	68.053	15.250	2.180
Avg_Hav_L	P	−1.150	88.236	15.730	**9.709****
	M	−1.197	91.575	16.337	6.354*
	F	−1.474	95.333	14.669	5.343*
Avg_Hav_Vol	P	$-3.742e^{-005}$	60.420	16.055	**5.437***
	M	$-3.776e^{-005}$	61.899	16.693	3.640
	F	$-4.795e^{-005}$	60.531	15.170	2.592
Avg_Hav_Ar	P	−0.003	61.232	16.258	2.894
	M	−0.003	63.877	16.879	2.290
	F	−0.002	59.193	15.487	0.989

Bold values signifies the pooled group significance values.
P — pooled sexes.
M — males.
F — females.
* $p < 0.05$.
** $p < 0.01$.

Table 7
Result summary of multiple regression analyses.

Variables	Coefficient (β)	SE	t	Sig.	95% CI
1. Intercept	37.267	21.356	1.745	0.084	−5.131 to 79.664
Avg_OPD (x_1)	0.478	0.385	4.528	0.000	0.978–2.506
Avg_Ost_L (x_2)	−0.086	0.167	−0.755	0.452	−0.457 to 0.205
Avg_Hav_L (x_3)	−0.012	0.429	−0.106	0.916	−0.897 to 0.806
$Age = 37.27 + 0.478x_1 - 0.09x_2 - 0.01x_3 \pm 14.10$					
2. Intercept	24.582	12.606	1.950	0.054	−0.444 to 49.607
Avg_OPD (x_1)	0.512	0.367	5.093	0.000	1.140–2.596
Avg_Ost_Area (x_2)	−0.141	0.000	−1.333	0.186	−0.001 to 0.000
Avg_Hav_Area (x_3)	0.119	0.002	1.150	0.253	−0.001 to 0.005
$Age = 24.58 + 0.51x_1 - 0.14x_2 + 0.119x_3 13.99$					
3. Intercept	30.360	10.583	2.869	0.005	9.351– 51.370
Avg_OPD (x_1)	0.460	0.375	4.469	0.000	0.933–2.423
Avg_Ost_Vol (x_2)	−0.168	0.000	−1.577	0.118	0.000–0.000
Avg_Hav_Vol (x_3)	0.044	0.000	0.437	0.663	0.000–0.000
$Age = 30.36 + 0.46x_1 - 0.17x_2 + 0.04x_3 \pm 13.98$					
4. Intercept	29.295	11.941	2.453	0.016	5.593–52.996
Avg_OPD (x_1)	0.484	0.356	4.954	0.000	1.058–2.472
Avg_Ost_Area (x_2)	−0.094	0.000	−0.961	0.339	−0.001 to 0.000
$Age = 29.30 + 0.48x_1 - 0.09x_2 \pm 14.02$					
5. Intercept	31.848	9.978	3.192	0.002	12.041–51.655
Avg_OPD (x_1)	0.450	0.364	4.508	0.000	0.918–2.363
Avg_Ost_Vol (x_2)	−0.152	0.000	−1.526	0.130	0.000–0.000
$Age = 31.85 + 0.45x_1 - 0.15x_2 \pm 13.92$					

assumption of normality, linearity and multicollinearity required to obtain accurate results using this statistical method. To conduct multiple regression analysis the sample should be large (>20 entries for each variable; in this case a pooled-sex sample was used of n = 99 with a maximum of three independent variables) and the presence of possible outliers was investigated using Cook's distance (no outliers were detected). Both Kolmogorov–Smirnov and Shapiro–Wilk test statistics were non-significant, indicating normality. A scatter plot (standardized residual vs. standardized predicted value) demonstrated an acceptable linear relationship between the dependent (age) and independent variables. The data were thus found to be suitable for employing multiple regression analyses.

Correlations between independent variables indicated that multicollinearity (when one predictor variable is strongly correlated with another, i.e. one variable can predict another, resulting in redundant information) was present between average osteon volume/area, volume/length and area/length, as well as between average Haversian canal volume/area, volume/length and area/length. Therefore, only multiple regression analyses containing either average volume, area or length of the specific objects studied (i.e. osteon and Haversian canal) were included in combination with the average number of osteons per grid area.

A standard multiple regression analysis was conducted to evaluate the overall relationship between age and a combination of variables. The linear combination of variables for all models was significantly related to age as demonstrated by the ANOVA results (Table 6). The multiple correlation coefficients for the five models ranged between 0.53 and 0.55, indicating that approximately 53–55% of variance in age may be accounted for by the combination of different variables when predicting age. When looking at the R^2 and adjusted R^2 values it was clear that the combination of Avg_OPD and Avg_Ost_Vol has the highest correlation with age on a linear scale. Since the sample size is large and the data normally distributed, it was deemed acceptable to interpret either the coefficient of determination (R) or the adjusted R^2, both of which indicated an average correlation strength with age. The results and regression equations for predicting age form the combination of different variables are summarized in Table 7.

Although variables associated with the microstructure of bone show no more than average correlations with age, the use of additional regression formulae such as these provided here, could add value to the age range estimated in cases where unidentified remains are incomplete.

Table 6
Model summary and ANOVA results of multiple regression analyses.

Combination of variables	R	R^2	Adjusted R^2	SEE	ANOVA
1. Avg_OPD Avg_Ost_L Avg_Hav_L	0.534	0.285	0.262	14.101	$F(3,95) = 12.600$, $p < 0.001$
2. Avg_OPD Avg_Ost_Area Avg_Hav_Area	0.543	0.295	0.273	13.997	$F(3,95) = 13.258$, $p < 0.001$
3. Avg_OPD Avg_Ost_Vol Avg_Hav_Vol	0.545	0.297	0.275	13.979	$F(3,95) = 13.374$, $p < 0.001$
4. Avg_OPD Avg_Ost_Area	0.534	0.285	0.270	14.020	$F(2,96) = 19.162$, $p < 0.001$
5. Avg_OPD Avg_Ost_Vol	0.544	0.297	0.281	13.920	$F(2,96) = 20.135$, $p < 0.001$

4. Discussion

Assessment of changes in bone microstructure with advancing age have been shown to be a reliable indicator of age in cases where macroscopic analyses are not possible [1,10,20]. Although some of the earliest studies have reported correlation coefficients of up to 0.96 with standard error estimates ranging from six to 10 years for variables such as total osteon count [8,11,12], later studies suggest that the correlation between age and histological variables of bone may be somewhat lower than initially reported [2,3,9,34]. Several factors may have contributed to this outcome, of which the first is observer bias and error. In some studies it is unclear how specific areas for osteon counting were decided on and if all observers were completely unbiased in terms of the region of interest (ROI) pertaining to each section in the sample under study [8,11,12,41]. Later studies have adapted and improved on the methodology of early studies in order to increase accuracy and reliability of the outcome, and suggested that the correlation of certain variables with age are moderate to low [3,9,34,42]. In an attempt to overcome bias related to the selection of ROI's and quantification of variables within the ROI, stereology was employed in this study. As stereology relies on sampling procedures (probes) that have been meticulously planned based on strict protocols to avoid biased conclusions, the results are believed to be unbiased and represent an accurate reflection of the relationship between age and histomorphometric variables. Furthermore, employing stereology allows for the extraction of three-dimensional quantitative information from two-dimensional measurements. By making use of the nucleator probe, all cross sections could be compared in terms of length, area and volume of Haversian systems and canals. For the reason that this probe essentially treats objects as isotropic, it allowed for the volume estimate of sections of slightly different height to be compared.

In this study, all single variables in the pooled sex sample were found to change significantly with age except for the average Haversian canal area. The average number of osteons per sampling grid (Avg_OPD) had the highest correlation with age ($r = 0.528$, with an SEE of 14.02) and was classified as being moderately associated with age. Previous studies have also reported the total number of osteons/osteon population density to be the most reliable variable of estimating age at death [33,34]. Keough [2,3] reported a correlation of 0.504 for this variable (pooled sexes, $n = 146$) with an SEE of 13.31. Although the results are believed to be accurate and unbiased, it did not give a significantly better outcome in comparison to other studies, as reflected by the SEE. The reason for this outcome most likely lies with the intrinsic bone biology, i.e. the histological structure of bone acts as the restraint on achieving narrower age range estimates.

When considering the other variables, average osteon and Haversian canal volume and area decreased with advancing age as expected and also showed moderate correlation with age. Multiple regression analyses demonstrated that these variables are useful in combination with the average number of osteons per grid area to obtain a better correlation with age. Although moderate correlations were only slightly increased by combining variables, all multivariate regressions were statistically significant.

The results of the current study and that of Keough [2,3] are similar and both show that high variability exists in the black South African population. The presence of high inter-individuals variation within this population is most likely influenced by the large amount of variation in the population's gene pool, as genes have been proven to contribute significantly to intra-cortical remodelling [43]. However, lifestyle factors undoubtedly also contributes fundamentally to inter-individual variation of bone histomorphometry and is driven by bone turnover rates, which may differ between individuals and populations. The rate of bone turnover is affected by several factors including nutrition, lifestyle and physical activity. In other words, socio-economic status may set one population apart from another in terms of osteon population density and size measured at a certain age, thus contributing to inter- and intra-population variation. Paine and Brenton [44] argued that health status and diet contributes significantly to bone turnover rates and that a number of nutritional disorders and metabolic diseases may cause an increased/decreased number of secondary osteons per volume of bone, given what is expected for a given age. In this study all individuals with recorded or macroscopically visible signs of disease were excluded from the sample. However, it is very difficult to account for nutritional status and diet, given only skeletal remains. Low socio-economic status is often linked to poor nutrition, although it should be kept in mind that this may not be true for all individuals present within the collection. It should be taken into account that the majority (if not all) of individuals in the skeletal collection are from a low socio-economic status and possibly suffered from malnutrition, suggesting that the regression standards derived here may not be appropriate to use in cases where the individual may be of high socio-economic status.

5. Conclusion

The current study showed that certain bone microstructures, when correlated with age, may produce reliable regression formulae for the estimation of age in a specific population. The results were similar to other studies reporting moderate to low correlation of variables with age and acceptable age ranges and standard errors, suggesting that the use of histology in estimating age has not yet surpassed that of macroscopic methods.

The results of this study demonstrated the high amount of variation present within populations such as the South African blacks. Many factors, including nutrition, physical activity, health and genes play a significant role in bone turnover rates, which can unfortunately not always be accounted for when dealing with skeletal remains. However, it is recommended that these formulae should be used when dealing with individuals of the same socio-economic status where possible.

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Age estimation using bone mineral density in South Africans

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ABSTRACT

The use of bone mineral density (BMD) to predict age-at-death in skeletal remains provides a usable alternative to other methods because the values obtained are not observer-dependent. The aim of this study was to investigate the usability of BMD to estimate age in South African populations, and to assess inter-population variation and sex-specific differences in BMD values from the proximal end of the femur. In order to estimate age, regression analysis was done for the construction of population dependent formulae. The sample comprised of a total of 123 femora of black and white South Africans. DXA scans were performed using the Hologic Discovery system. Data analysis was done by employing independent-samples t-tests and correlation/regression analyses. The results indicated a statistically significant difference between black and white South Africans. Male groups were also significantly different from one another, but black and white females showed no significant differences. Linear regression analysis revealed a significant correlation between BMD values and age for the white population and the combined sample, but not for the black population. Bootstrapping were employed to confirm validity of the results. In conclusion, this study showed that the use of DXA measurements of the femur for estimating age may be used for the estimation of age-at-death in white South Africans, but more research is needed to better understand the relationship between bone mineral density and age in black South Africans.

1. Introduction

Accurate age estimation in unidentified adult skeletons remains one of the most challenging aspects in establishing a biological profile [1–3]. Macroscopic methods for estimating age in adults rely on the observation of degenerative changes in the skeleton and often present with observer-related challenges, i.e. bias and subjectivity leading to poor reproducibility [4,5]. Furthermore, inter-individual variation itself causes inconsistencies between biological and chronological age, giving rise to low accuracy in adult age estimates. This variation is brought on by environmental factors (e.g. physical activity and nutrition), sex-related differences, genetic make-up and chronic metabolic disturbances [3,6].

Due to these challenges, anthropologists are constantly exploring new approaches to reduce the number of problems encountered and increase accuracy and repeatability of age estimation methods. Recently, the use of bone mineral density (BMD) to estimate age has been proposed [7–10]. A major advantage of using BMD for estimating age is that observer-related errors and problems may be excluded, as the BMD values are obtained by the scanner and are not observer-dependent. Using BMD as a method for age estimation is based on the principle of a constant decline in bone mass as one ages. Once an individual's peak bone mass is reached, constant bone remodelling has to take place to maintain a healthy bone microstructure [11]. As age advances, the bone's ability to remodel successfully declines due to impairment of function at a cellular level. This leads to bone resorption rate slowly surpassing bone formation rate, causing a decline in bone mineral density [12,13]. Reduction in bone mass over time with age has been reported comprehensively, with studies showing BMD variation between sexes, ancestry groups, individuals and geographical regions [10,14,15]. Based on this knowledge, specific methods may be generated to estimate age in human skeletal remains by correlating BMD values with age that are specific to a skeletal location and population.

Previous studies have managed to successfully correlate BMD and age as a predictor of age-at-death in adult skeletal remains. Paschall and Ross [10] performed DXA scans on a modern North American population using cranial fragments from 32 adult individuals, as well as 41 adult crania and associated femora. The results showed no significant age-related association with the cranium, but femoral neck BMD for pooled sexes was significantly

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correlated with age. A regression equation for estimating age from femoral neck BMD was derived with a root mean standard error of 13 years. A different statistical approach using BMD values obtained from DXA scans to estimate age-at-death was reported by Navega et al. [9]. The sample comprised of 100 adult female femora from the Coimbra Identified Skeletal Collection. BMD values of the proximal femur were used to predict age-at-death using a modified general regression neural network approach, from which an online web application called DXAGE was produced. The mean difference between known and estimated age were reported to range between 9.2 and 13.5 years.

The aim of this study was to explore the inter-population variation between South African black and white groups, in addition to considering sex-related differences using BMD values obtained from the proximal end of the femur. The data were then used to construct regression equations for estimating age from femoral neck and total BMD values of the proximal femur.

2. Materials and methods

The sample comprised of a total of 123 left femora of South African males and females (64 black and 59 white individuals), with 110 femora selected from the Pretoria Bone Collection [16] and 13 from the Raymond A. Dart Collection of Human Skeletons [17] and represents contemporary/modern remains. Only individuals for which specific age-at-death and ancestry were recorded and for which no visible pathology or trauma was present were used. The sample of black South Africans included 34 males and 30 females with ages ranging between 21 and 80 years. The sample of white South Africans included 29 males and 30 females with an age range of 21–91 years (Table 1). There is a relatively low representation of white South Africans for the younger ages, as both these collections have limited numbers in the younger age categories.

DXA (dual-energy x-ray absorptiometry) scans were done according to standard procedures at the DPHRU (MRC/University of the Witwatersrand research entity: Developmental Pathways for Health Research Unit) situated at Chris Hani Baragwanath Hospital using the Hologic Discovery DXA system (array mode). BMD values of the total proximal area and neck region were used in this study.

For scans to be analysed, information regarding the stature (cm), weight (kg), BMI (kg^2/cm) and age (years) was required for each individual to allow for BMD calculation. Biometric data were recorded in the cadaver books for most individuals. However, a number of black individuals lacked stature and weight recordings (n = 31). Before scanning commenced, stature (in cm) was calculated for individuals lacking this information by using regression formulae constructed by Lundy and Feldesman [18] for stature estimation of black South African males and females with corection factors revised by Raxter et al. [19]. Weight estimation was calculated using a formula for pooled sexes by McHenry [20] using the maximum diameter of the femoral head (FH) based on four sample means of North American males and females, African pygmies and Khoi-San, as there are no population specific formulae for weight estimation of South African individuals.

Table 1
Age distribution of the sample.

Age cohort	SA black		SA white		TOTAL
	Male	Female	Male	Female	
20–29	5	2	–	2	9
30–39	4	7	–	–	11
40–49	6	6	5	–	17
50–59	6	5	8	2	21
60–69	6	8	10	11	35
70–79	6	1	2	10	19
80+	1	1	4	5	11
TOTAL	34	30	29	30	123

An independent-samples t-test was done to compare BMD values for SA black and white groups, as well as to assess for differences between males and females. The relationship between age and total/neck BMD for ancestry/sex groups and for the total group (black and white individuals pooled) was explored using a linear regression model.

Bootstrapping was performed [21] for regression outcomes that showed a statistically significant correlation between age and BMD to assign a measure of accuracy to the method. All analyses were done in SPSS (v.24).

3. Results

The descriptive statistics are summarized in Table 2. The t-test results revealed significant differences between the black and white groups for both total BMD ($t(121) = -4.045$, $p < 0.05$) and neck BMD values ($t(121) = 2.901$, $p < 0.05$). For total BMD, the white group had a significantly higher mean bone density (1.15 g/cm^2) than the black group (1.03 g/cm^2), but the opposite was true for neck BMD (White: 0.68 g/cm^2, Black: 0.75 g/cm^2).

When comparing black males and females, a significant difference was seen in neck BMD ($t(62) = -2.858$, $p < 0.05$), with males having a higher mean BMD (0.78 g/cm^2) than females (0.70 g/cm^2). White males and females differed significantly in total BMD ($t(57) = -3.060$, $p < 0.05$) and neck BMD ($t(57) = -2.252$, $p < 0.05$), with males showing higher mean BMD values for both skeletal areas. A significant difference was also seen between black and white males for total BMD ($t(61) = -6.459$, $p < 0.05$) and neck BMD values ($t(61) = 2.349$, $p < 0.05$), with the white male mean value for total BMD (1.21 g/cm^2) surpassing that of black males (1.02 g/cm^2). However, the opposite was seen for neck BMD mean values, where black males (0.78 g/cm^2) scored higher than white males (0.72 g/cm^2). Female groups showed no significant differences.

Linear regression analysis was done to assess the relationship between age and BMD of the total area and neck region. Although a decrease of BMD with age could be seen for all sex/population groups (Figs. 1–6), no statistically significant change could be linked to advancing age by making use of linear regression for the black sample. This suggest that the changes in BMD may not be consistent enough to use it for age estimation in black South Africans with the current study sample.

The white population (pooled sexes), however, did show a significant decrease in BMD with age for total BMD ($F(1, 57) = 6.682$, $p < 0.05$) and neck BMD ($F(1, 57) = 10.061$, $p < 0.05$). For sex-specific groups, neck BMD correlated with age showed significant results only in white males ($F(1, 27) = 6.599$, $p < 0.05$).

It is often difficult to estimate ancestry in cases where remains are fragmentary or incomplete. To aid in the estimation of age for such individuals/cases, linear regression was performed for the total sample, as well as for pooled males and females. A statistically significant correlation with age was seen for the total sample ($F(1, 121) = 15.657$, $p < 0.05$), pooled females ($F(1, 58) = 7.253$, $p < 0.05$) and pooled males ($F(1, 61) = 7.153$, $p < 0.05$).

Regression equations for the estimation of age from total and neck BMD values were derived and are summarized in Table 3. Standard error estimates (SEE) ranged between 13 to 14 years for the white sample and 16–17 years for the total/sex-pooled groups.

Table 4 gives a summary of the bootstrap results for regression analyses with a p-value of < 0.05. The regression outcome of the white sample (total and neck BMD), white males (neck BMD), total sample (neck BMD), pooled females (neck BMD) and pooled males (neck BMD) was tested using a bias corrected and accelerated (BCa)

Table 2
Descriptive statistics of the BMD values for both populations.

	BM	BF	WM	WF	BG	WG
Age range (mean)	21–80 (52)	22–80 (49)	40–91 (61)	21–91 (69)	21–80 (52)	21–91 (65)
	Total BMD (g/cm^2)					
Mean	1.02	1.05	1.21	1.08	1.03	1.15
SD	0.11	0.17	0.13	0.19	0.14	0.18
	Neck BMD (g/cm^2)					
Mean	0.78	0.70	0.72	0.64	0.75	0.68
SD	0.11	0.12	0.12	0.14	0.12	0.14

BM — black males.
BF — black females.
WM — white males.
WF — white females.
BG — black group.
WG — white group.

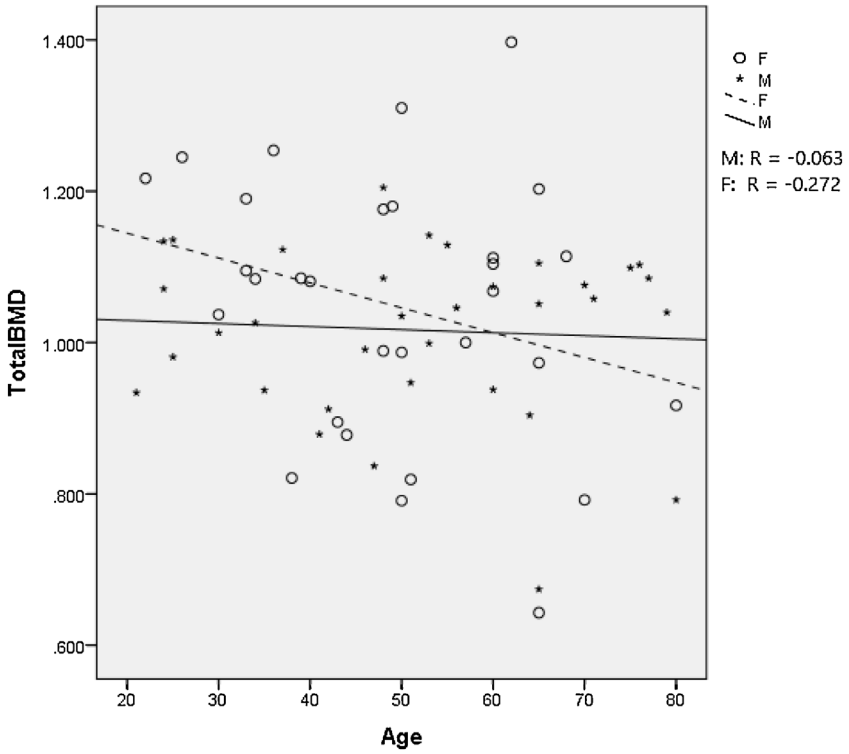

Fig. 1. Total BMD values vs. age for black males and females.

bootstrap estimation approach (BCa 95% confidence interval) with 1000 samples. The bootstrap for the Pearson correlation confirmed that the regression outcomes for all groups are significantly correlated with age.

4. Discussion

Studies from various disciplines (i.e. clinical, molecular, epidemiological and anthropological) have established that bone mineral density tends to decline as age advances, and that this a normal senescence-related phenomenon [22,23]. However, this relationship between age and BMD is not perfectly linear, as much inter-individual and inter-population variation exists. An abnormal decline in BMD which may be caused by a number of factors may result in osteopenia or osteoporosis [24]. Osteopenia (one standard deviation from the norm) or osteoporosis (two standard deviations from the norm) account for a large amount of variation that is observed as some populations are more osteoporotic than others [23,25].

The results from this study indicated a significant difference between black and white groups for total proximal femur area and neck bone mineral density values. Overall, bone mineral density of the femoral neck was lower in whites than in blacks, which is in agreement with the results from previous studies done on South African groups [14,26]. However, mean total BMD was observed to be higher in white than in black South Africans, which was unexpected and not in accordance with previous studies [27]. A better understanding of age-related bone loss of the two populations in relation to one another may be gained from looking at Figs. 5 and 6. These graphs illustrate that BMD in white individuals seem to be relatively high in comparison to that of black individuals during young adulthood, but that bone loss over time occurs much more rapidly than what is seen in the black group. Black individuals seem to maintain a higher BMD

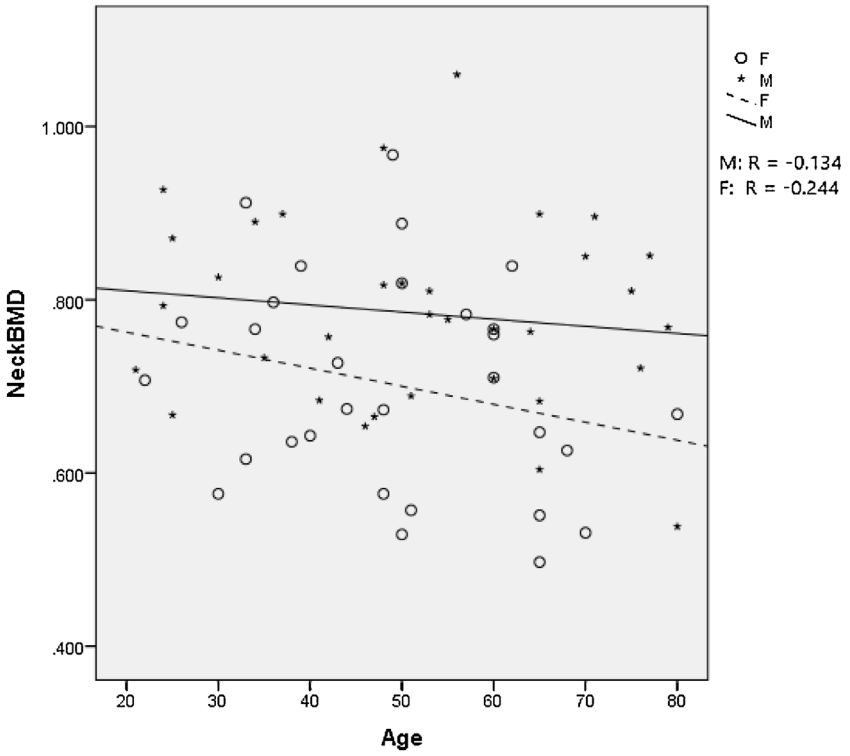

Fig. 2. Neck BMD values vs. age for black males and females.

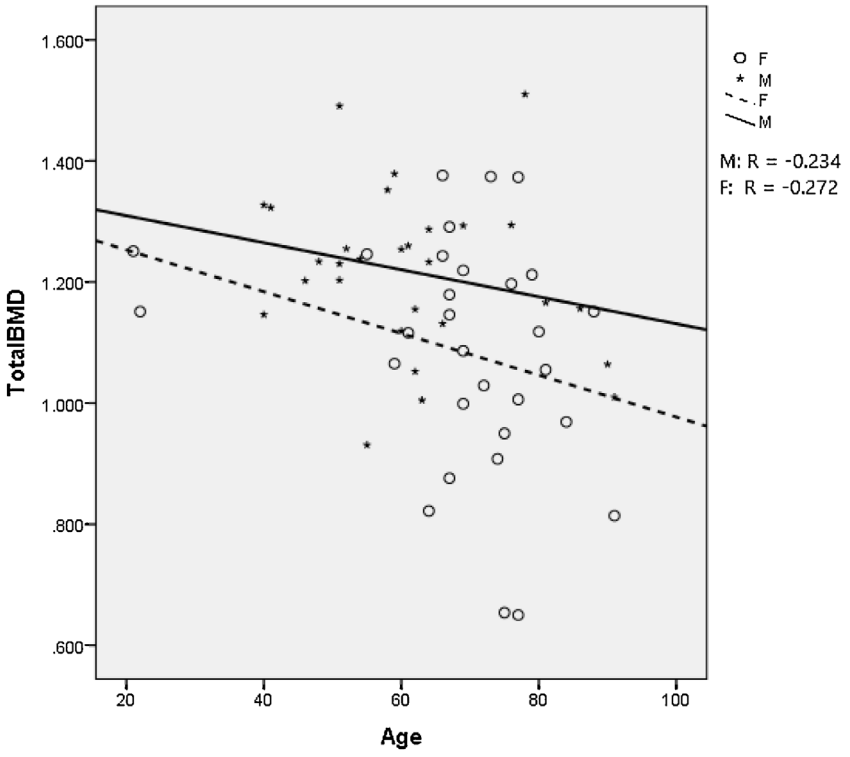

Fig. 3. Total BMD values vs. age for white males and females.

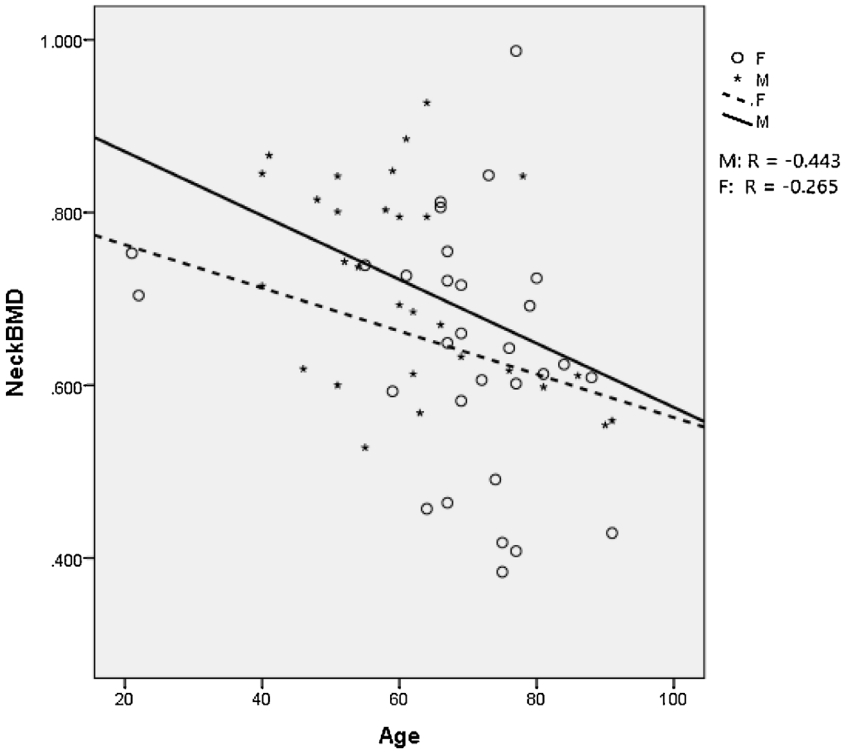

Fig. 4. Neck BMD values vs. age for white males and females.

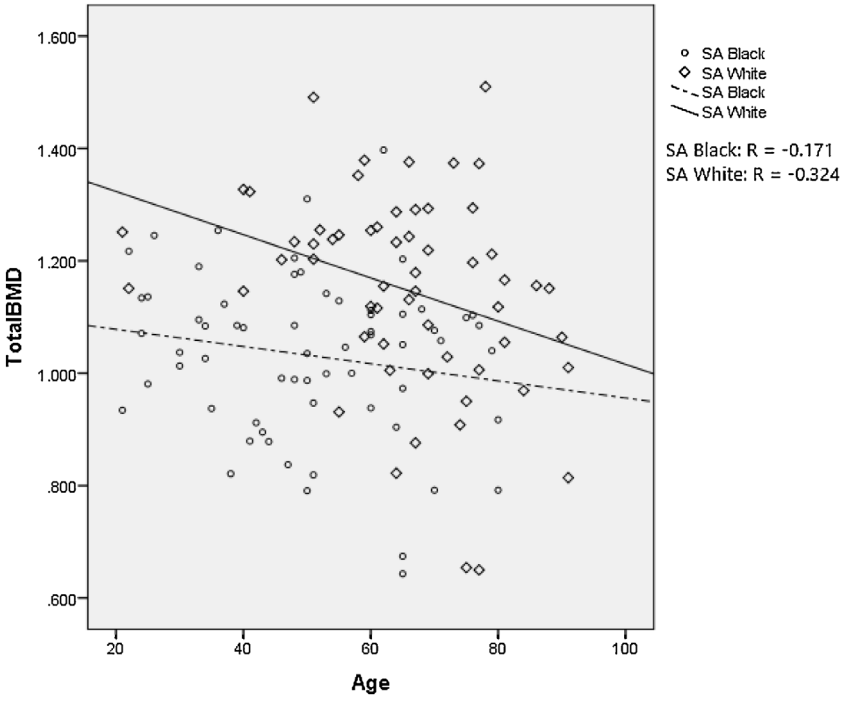

Fig. 5. Total BMD values vs. age for black and white populations.

throughout life than their white counterparts. However, reassessment using a larger sample may possibly alter this result.

Sex-related analyses indicated a significant difference between black males and females only for neck BMD. In contrast to what was found by Paschall and Ross [10], white males and females differed significantly for total area and neck BMD values, with males having higher BMD values than females. This may indicate that sex-related variation in bone mineral density is larger in South Africans than in North American individuals [10].

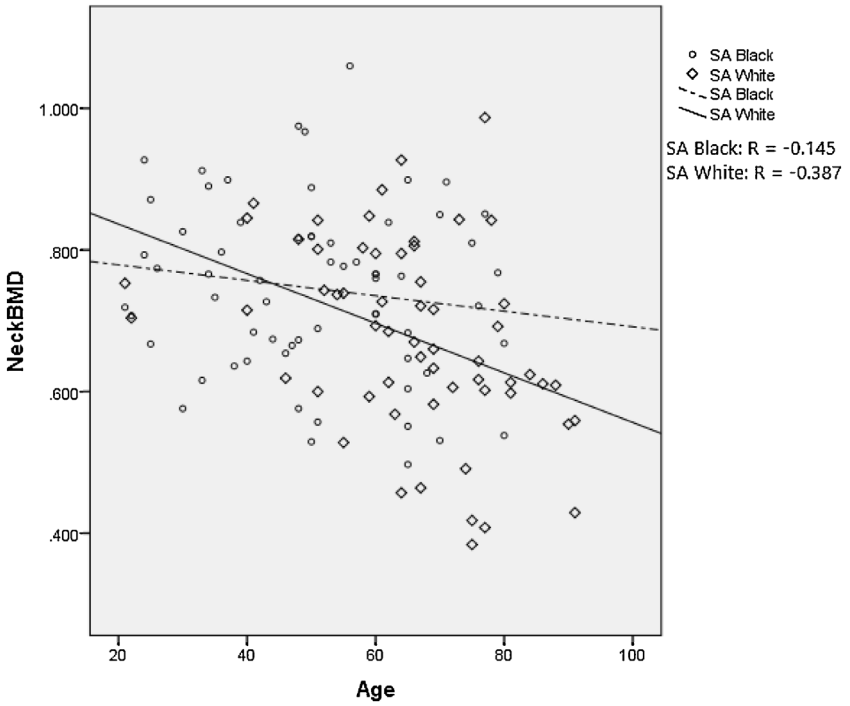

Fig. 6. Neck BMD values vs. age for black and white populations.

Table 3
Regression equations for the estimation of age-at-death.

Population/sex group	Sample size N	Regression equation
White population (pooled sexes)	59	Age = 96.6 – 27.3 (total BMD) ± 14
White population (pooled sexes)	59	Age = 94.3 – 42.8 (neck BMD) ± 14
White population (males only)	29	Age = 99.4 – 53.1 (neck BMD) ± 13
Total population (all individuals)	123	Age = 88.9 – 43.9 (neck BMD) ± 16
Females only (black and white)	60	Age = 88.3 – 43.5 (neck BMD) ± 17
Males only (black and white)	63	Age = 91.1 – 46.4 (neck BMD) ± 16

Table 4
Bootstrap result summary of the regression analyses ($p < 0.05$).

	Pearson correlation	BCa 95% CI for Pearson correlation		95% CI for coefficients		BCa 95% CI for coefficients	
		Lower	Upper	Lower	Upper	Lower	Upper
White population							
1. Total BMD vs. Age	−0.324	−0.495	−0.157	−48.430	−6.150	−46.542	−12.607
Constant				71.987	121.165	77.243	122.607
2. Neck BMD vs. Age	−0.387	−0.555	−0.219	−69.888	−15.795	−66.914	−21.421
Constant				75.558	112.966	78.023	111.549
White males							
Neck BMD vs. Age	−0.443	−0.706	−0.114	−95.502	−10.686	−98.728	−11.223
Constant				68.621	130.257	64.767	136.558
Total population							
Neck BMD vs. Age	−0.338	−0.465	−0.191	−65.991	−21.978	−63.645	−23.715
Constant				72.932	104.864	74.427	102.597
Pooled females							
Neck BMD vs. Age	−0.333	−0.514	−0.126	−75.835	−11.169	−69.486	−14.640
Constant				66.128	110.393	69.005	106.116
Pooled males							
Neck BMD vs. Age	−0.324	−0.534	−0.091	−81.165	−11.718	−82.696	−12.364
Constant				64.571	117.526	68.024	114.727

As the majority of white females in the sample are older than 50 years of age, the effect of sex hormone deficiency (post-menopausal stage) should also be considered. South African white females have been reported to show a faster decline in femoral bone density post-menopausally than South African black females [14]. Estrogen deficiency during this stage may lead to increased osteoclast production which fuels excessive bone loss [28]. Clinical studies support the notion that estrogen deficiency is one of the leading causes of osteoporosis [29–31]. It is thus possible that the difference seen between white males and females in this study is driven to some extent by an alteration in female sex hormones after menopause.

Significant differences were also seen between male groups for both regions. Considerable population variation in the proximal femur structure and BMD among older men has previously been reported by other studies [27,32]. It has been suggested that black men tend to have a more solid cortical bone structure and a higher BMD in the region of the femoral neck, contributing to them experiencing lower hip fracture rates due to osteoporosis [27]. The results from this study also indicated that black males have a significantly higher mean femoral neck BMD than white males and is thus in agreement with that of Marshall et al. [27].

Reasons for black males showing higher femoral neck BMD values than white males may possibly be linked to genetic/molecular mechanisms. Schnitzler et al. [26] reported that black individuals have thicker iliac crest trabeculae than whites and that their bone architecture seem to stay undisrupted for longer. This may suggest that black individuals have higher bone turnover rates helping to maintain their bone microstructure, which is most likely linked to genetic make-up.

Furthermore, a difference in age at which peak bone mass is reached between black and white groups has also been reported. Daniels et al. [14] found that peak bone mass is reached later in life in the black group and that an earlier peak bone mass mean age in whites aids in the bone being subjected to decreased BMD earlier in life. Although it is difficult to establish the exact reason(s) for this phenomenon, bone adaptation to physical activity should be considered. The amount of mechanical load generated by physical activity, particularly during the sub-adult/young adult years, plays a vital role in achieving optimal bone mineral density and microarchitecture. The higher the amount of mechanical load placed on the skeleton continuously over a long period of time, the less prone the individual will be to developing osteopenia or osteoporosis [33,34]. Black individuals in South Africa have in the past largely been living in low socio-economic conditions and therefore forced to accept work opportunities involving hard physical labour. The amount of physical labour performed by adolescent boys and young men could have contributed greatly to increased BMD values (as compared to whites) and a delayed peak bone mass mean age seen in black South Africans.

Scatter plots with linear regression best fit lines (Figs. 1–6) showed a negative correlation between age and BMD for all population/sex groups. Although linear regression results indicated no significant age-related change for the black population, a significant correlation was found in the total sample. This suggests that BMD is potentially valuable in estimation age-at-death for all groups investigated. Larger sample sizes may be required in detecting a significant regression with age for the black population.

A large number of studies have been carried out in the past to establish various methods for more accurate and reliable estimation of age. Popular macroscopic techniques of dry bone include degenerative changes observed in the pubic symphysis [35], sternal rib ends [36–38], osteophytic spurs of the vertebrae [39] and the auricular surface [40,41]. Standard deviations reported for these methods are similar, ranging between 3 and 16 years. Recent studies on histological age estimation [42–44] using the femur have reported estimates with SEE values around 13–14 years. Age estimation using BMD is on par with histological outcomes, reporting SEE values of around 13 years [9,10]. The current results (SEE of 13–17 years) are thus similar to that of age estimation methods from BMD and histology. Although macroscopic methods such as the ones mentioned above reported standard deviation as low as 3 years, narrow age estimates should be avoided and conservative age ranges are preferred as biological variation should be accommodated for. Based on the bootstrap results, it should be noted that a low neck BMD value may result in a large age range. It is therefore recommended that neck BMD values of lower than 0.5 be considered to be consistent with older individuals.

The use of bone mineral density (DXA) for estimating age provides measurements/values that are not restricted by observer subjectivity, are easily reproducible and reasonably accurate, which makes it a usable method. Also, it is extremely valuable in cases where remains are somewhat incomplete. In cases where the remains are incomplete to the extent that ancestry cannot be estimated, the regression equation for the total/combined sample should be implemented. It should also be kept in mind that bone mineral density may be altered by post-mortem degradation/taphonomic processes leading to increased brittleness such as in the case of archaeological material [45,46]. It is thus recommended that this method be applied to well preserved forensic/modern skeletal material, and not to poorly preserved modern skeletons or archaeological material.

5. Conclusion

A negative correlation between advancing age and BMD values of the proximal femur was demonstrated for black and white South African populations. However, the changes with age in the black sample were not statistically significant. A significant difference between the two populations was observed, as well as between white males and females. The use of DXA measurements for estimating age was used to estimate age-at-death using the femur in the white South Africans. Formulae for the combined group were less accurate. Further research and expansion of the study sample is needed to obtain regression equations for estimating age in the black population, as well as to give new insights into the use of BMD for age estimation.

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ORIGINAL ARTICLE

Inter-population variation of histomorphometric variables used in the estimation of age-at-death

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Abstract

Population variation of several microscopic structures used in age-at-death estimation was assessed for three different population samples. The aim of the study was to determine if the need exists for population-specific standards when dealing with individuals of African and European origin. A total sample 223 bone sections from the anterior cortex of the femur ($n = 99$ black South Africans, $n = 94$ white South Africans and $n = 30$ Danish individuals) were analysed using a stereological protocol. Variables assessed included the average number of osteons per grid area (OPD), osteon size and Haversian canal size. ANCOVA was employed for assessment of statistically significant differences. The results indicated that OPD differed significantly between the three groups, but that osteon size was similar for all individuals. Haversian canal size showed unpredictable changes with age and high levels of variation, making it unsuitable to use for age estimation as a single factor. As there are conflicting opinions in the literature on whether to use population-specific equations for the estimation of age-at-death or not, this paper provided additional insight into the use of specific variables and its related variation between groups.

Keywords Femur · Stereology · OPD · Osteon size · Haversian canal size

Introduction

Several microscopic features of bone change with age (e.g., an increase in osteon number and decrease in secondary osteon size) and have been used to develop age estimation techniques from bone histology [1–5]. These age estimation techniques have been developed for a large number of different populations, but with most studies taking only one specific population into account. For example, population-specific studies have been published for the Dutch [6], American white and black groups [1, 3], Danish [7, 8] and black South Africans [9], to mention a few. Bone mineral density and strength are established during childhood and early adulthood (up to the point where skeletal maturity is reached). Various studies have found that differences in bone turnover rates and/or strength between different ancestral and geographical groups exist from childhood and continue into adulthood [10–12]. Although skeletal growth and establishment of bone strength are a complicated process affected by many factors, a general trend indicating bone mass acquisition and loss may be observed. Once peak bone mass (skeletal maturity) is reached around 30 years of age, the almost-linear relationship between age and bone mineral density begins to weaken as the effect of variation sets in.

Variation seen within bone mineral density and variables used for age estimation may be linked to factors influencing skeletal growth, maturity, and maintenance. One should keep in mind that variation in bone microstructure is often multi-factorial and that no single cause should be accredited as the sole factor for its existence. Differences, specifically between black and white groups, have been reported in bone mass/density [13–16], muscle mass and obesity [13, 17, 18], remodelling rates [19, 20], bone histomorphometry [21, 22], bone geometry [13, 23], calcium metabolism [10, 24, 25] and other metabolic occurrences [20, 26, 27]. Some of the observed differences are due to heredity [28, 29], but may also be the result of environmental factors.

Bone mass/density has been reported to be higher in black than in white individuals [13, 16, 30]. In some studies, muscle mass and obesity, like bone density, were also found to be higher

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in black than in white individuals and increased levels of body weight are known to stimulate bone formation, resulting in higher bone mass and strength [14, 27]. Bone remodelling rates are lower in black than in white groups [19]. A study by Qui et al. [31] stated that black individuals have longer bone formation periods and diminished osteoblast apoptosis.

When developing methods for age-at-death estimation, it is thus essential to consider population variation, as the factors causing variation may not only affect the time of development of specific skeletal features (e.g., osteon population density and osteon size) but may also alter its size and frequency. Socioeconomic status (SES) is frequently linked to many of the factors altering bone remodelling and maintenance. For example, people who are considered to be of low SES may suffer from malnutrition and poor health care [32]. In South Africa, people of low SES regularly travel away from home to find work. Job opportunities available in such cases often entail hard physical labour [33]. As these job opportunities provide a low income, these individuals are prone to less than optimal nutrition. Although increased physical activity for long time periods is considered healthy and is known to promote bone strength [34, 35], extreme physical labour can have the opposite effect, especially when combined with poor nutrition [36–38].

In contrast to South Africa, people living in European countries such as Denmark are mostly classified as being of moderate to high SES, with relatively few socioeconomic inequalities [39]. SES encompasses much more than only availability of food or adequate nutrition. A positive relationship between education, SES, health and nutrition exists [40–42], with education labelled as the most important social variable influencing nutrition and dietary habits [43]. Individuals of vastly different socioeconomic backgrounds can thus be expected to have different bone quality due to a variety of factors.

There are thus a number of factors related to SES (such as education, nutrition and health care), physical activity, environment (e.g. exposure to sunshine) and genetics that may account for differences seen in bone microstructure between population groups. The aim of this study was to assess the extent of inter-population variation in terms of ancestry and geographical location between three different population groups/samples with regard to histomorphometric variables of the anterior cortex of the femur used in age estimation. A better understanding of how histological variables vs. age differ between population groups may assist in determining if population-specific formulae are needed and which variables are reliable indicators of age.

Materials and methods

Sample size, demographics and slide preparation

Three study samples were selected for analysis. South African black and white samples were used for which age estimation standards based on histomorphometric variables of the anterior cortex of the femur have been established [44, 45]. These two groups reside in broadly the same geographical area (South Africa), but differ in terms of ancestry and probably also socioeconomic status. Black South African individuals are of "African origin," while white South Africans are predominantly of "European origin." A Danish sample was employed as comparative group that is geographically isolated from the South African samples, but are also obviously of European origin.

The black South African sample originated from a previous study by Keough et al. [44] selected from the Pretoria Bone Collection [46] and was used in this study to prevent re-sectioning of bone specimens. The sample comprised 60 males and 39 females (total $n = 99$). Ages ranged from 21 to 82 years. The majority of black South African remains in the collection originated from unclaimed bodies. If an individual dies in a public hospital and the body is not claimed within a certain period of time, it is transported to the relevant tertiary institution for medical training and research. As many of these unclaimed bodies include migrant workers and homeless individuals, these skeletons thus largely represent individuals of low socioeconomic status with no records regarding their general health and well-being (43). Details on bone slide preparation of this sample are described by Keough [9] and Keough et al. [44] and involved manual grinding of bone sections according to the protocol by Maat et al. [47].

The white South African sample, also selected form the Pretoria Bone Collection, comprised 50 males and 44 females (total $n = 94$) with ages ranging from 21 to 94 years. Many of the white remains in the collection originated form donated bodies and mostly represent older individuals, with very few young adults represented in the lower age categories [46]. They were mostly of lower or middle SES. Bone slides were prepared by making cross-sections with a thickness of 2–4 mm just below the midshaft of the anterior cortex of the femur (to not affect bone measurements in future) using an EXAKT 312 pathology saw fitted with a diamond blade. The sections were manually grinded by hand to a thickness of ± 1 mm according to the Manual for the Preparation of Ground Sections for the Microscopy of Bone Tissue [47]. This method does not ensure a uniform thickness throughout the section. Therefore, machine grinding was employed from this point forward to ensure a smooth and uniform surface area. Sections were mounted onto perspex slides and ground to a minimum thickness of 100 μm (thicknesses ranged between 100 and 200 μm, depending on the brittleness of the bone) with an EXAKT 400 CS surface grinder. All equipment used are situated in the Bone Research Laboratory at the University of the Witwatersrand.

The Danish sample originated from a number of autopsies performed between 1988 and 1990 at the Department of Forensic Pathology, University of Copenhagen. These

individuals represent a group of people of middle to higher SES, living in a region with relatively low UV light levels. Complete cross-sections from the femoral mid-shaft were made. Specimens were then boiled in water and detergent for a few hours, after which sections (± 100 μm) were cut with a Buehler Isomet water saw and mounted on glass slides without staining [7].

The total sample is shown in Table 1 and comprised 223 individuals. Their ages ranged from 21 to 94 years.

Stereological analysis

A MicroBrightField (MBF, Colchester, Vermont, USA) system with a three-plane motorised stage, Zeiss.Z2 Vario Axioimager was used for the scanning of sections. For quantification of secondary osteons and its related Haversian canals, StereoInvestigator software (MBF, version 11.08.1; 64-bit) was used. The optical fractionator and nucleator probes were employed.

Methodology and stereological criteria as described by Botha et al. [45] were used to analyse all sections. Stereology is preferred above traditional two-dimensional histological techniques because it allows for systematic random sampling, avoiding bias [45, 48]. Grid size was set at 1250 × 1250 μm^2 (1562 500 $\mu m^2 \approx 1.6\ mm^2$) with an average of 55 counting frames covering the entire section.

Variables assessed included average number of osteons per grid area (Avg_OPD), average osteon length (Avg_Ost_L), surface area (Avg_Ost_Ar) and volume (Avg_Ost_Vol), as well as average Haversian canal length (Avg_Hav_L), surface area (Avg_Hav_Ar) and volume (Avg_Hav_Vol).

Statistical analysis

ANCOVA (analysis of covariance), classified as a univariate general linear model, is similar to ANOVA (analysis of variance) but is used to detect a difference in the means of three or more independent groups for datasets where it is necessary to control for a scale covariate. If a covariate is present (i.e. age) that could influence the dependent variable, it should be controlled for. ANCOVA is a method that combines ANOVA with linear regression to point out group differences after accounting for the effects of the covariate [49].

ANCOVA was employed to detect statistically significant differences in variable number and size between the samples, whilst controlling for age. To better understand where the differences occurred between the groups, pairwise comparisons (post hoc tests) were used. Probabilities reported formed part of the ANCOVA analysis. The Bonferroni post hoc test was done to account for inflated error/results due to numerous tests being conducted. ANCOVA was performed for age subgroups 20–39 (young adults), 40–59 (middle aged adults) and 60+ (older individuals) years to assess the histomorphometric variation between groups across all life phases.

To illustrate the relationship between the independent variables and age, least squares regression was performed. Figures depicting the sample regression lines for each variable are given. All analyses were performed using SPSS (v. 24).

Results

The descriptive statistics for all variables are summarised in Table 2. Regression slopes are illustrated by Figs. 1, 2, 3, 4, 5, 6 and 7. Regression analysis indicated that a significant correlation exists between osteon number and size and age for all groups, but not between age and Haversian canal size.

In order to perform ANCOVA, preliminary checks (assumption testing) should be carried out to ensure that none of the covariates are highly correlated with one another, residuals are normally distributed, no outliers are present, homogeneity of regression slopes and equality of variance exist. Covariates were not highly correlated (all correlation values were smaller than 0.7). Kolmogorov-Smirnov and Shapiro-Wilk tests were non-significant, indicating normality. No outliers were detected (tested by means of Cook's distance). Levene's test was non-significant ($p > 0.05$) for all analyses, representing homogeneity of variance. A model for ANCOVA was performed to test the interaction between the independent variables and age. Interactions were found to be non-significant for the 20–39, 40–59 and 60+ age categories. There was thus no violation of the assumptions for ANCOVA the data were found to be suitable for its employment.

To establish if statistically significant differences exist between the three samples, ANCOVA (Table 3) and pairwise

Table 1 Sample size and age distribution

Age group	Age cohort	SA black			SA white			Danish white		
		n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD
Young adults	20–39	25	30.4	6.7	4	29.5	9.3	16	27.8	5.3
Middle aged adults	40–59	38	48.9	5.4	21	51.5	5.2	7	46.6	5.8
Older individuals	60+	36	69.1	7.0	69	74.3	9.0	7	78.6	8.0
	Total	99	51.6	16.4	94	67.3	14.9	30	44.1	21.8

Table 2 Descriptive statistics of single variables for black South African, white South African and Danish samples

Variable	Group	Mean	SD	SE	95% CI for mean	
					Lower	Upper
OPD	SAB	16.88	4.49	0.45	15.98	17.78
	SAW	25.78	6.27	0.65	24.50	27.07
	DAN	28.32	7.78	1.42	25.42	31.23
Avg_Ost_L	SAB	108.33	11.19	1.12	106.09	110.56
	SAW	106.16	14.22	1.47	103.70	109.52
	DAN	108.16	12.01	2.19	103.67	112.65
Avg_Hav_L	SAB	31.85	4.30	0.43	30.99	32.71
	SAW	30.32	6.17	0.64	28.96	31.49
	DAN	27.50	3.97	0.72	26.02	28.98
Avg_Ost_Ar	SAB	40,293.74	8295.19	833.69	38,639.29	41,948.18
	SAW	38,743.62	10,648.98	1098.36	36,562.49	40,924.74
	DAN	40,001.23	8609.64	1571.89	36,786.34	43,216.12
Avg_Hav_Ar	SAB	3832.51	1111.21	111.98	3610.88	4054.14
	SAW	3488.05	1638.89	169.04	3152.37	3823.73
	DAN	2800.45	820.09	149.73	2494.22	3106.68
Avg_Ost_Vol	SAB	6,958,100.87	2,188,730.01	219,975.64	6,521,566.37	7,394,635.38
	SAW	6,494,934.47	2,799,871.62	288,784.71	5,921,465.27	7,068,402.66
	DAN	6,828,080.53	2,130,795.58	389,028.27	6,032,428.39	7,623,732.68
Avg_Hav_Vol	SAB	235,831.84	101,061.07	10,157.02	215,675.56	255,988.12
	SAW	210,775.74	189,757.06	19,571.95	171,909.73	249,641.74
	DAN	143,285.63	64,189.14	11,719.28	119,317.01	167,254.24

comparisons (Table 4) were examined. A significant difference ($p < 0.001$) for Avg_OPD was observed between the three groups for all age classifications (subgroups 20–39, 40–59 and 60+ years). Mean values (Table 2) and Fig. 1 indicate that the black South African population presented with the least number of secondary osteons vs. age (± 17), followed by the white South African group (± 26). The Danish sample had the largest number of osteons vs. age (± 28). Pairwise comparisons suggested that significant differences exist between all age groups for this variable, except for SAB/SAW comparison in the young adult age group. The small sample size of the SAW group in the 20–39 years age cohort may account for this outcome, as there may be too few individuals to reach statistical significance.

No significant differences were observed in osteon size (Avg_Ost_L, Avg_Ost_Ar and Avg_Ost_Vol; Figs. 2, 3 and 4) between the samples for all age groups, except in average osteon volume in the young adult age group ($p < 0.01$). Pairwise comparisons suggested that a significant difference in Avg_Ost_Vol is found between SAW and DAN individuals, with white South Africans having larger average volumes than Danish individuals.

Quantification of the Haversian canal (Avg_Hav_L, Avg_Hav_Ar and Avg_Hav_Vol; Figs. 5, 6 and 7) showed significant differences ($p < 0.001$) between the groups for the young adult age cohort. Pairwise comparisons showed that these differences were present between black South Africans and Danish individuals, with black South African individual Haversian canal sizes being larger on average than that of the Danish. However, no significant difference was present for Haversian canal size in the middle and older aged groups, with the exception of Avg_Hav_L in middle aged individuals ($p < 0.01$). This difference was observed between SAW and DAN samples, with white South Africans showing larger Haversian canal lengths on average.

SAB South African black, *SAW* South African white, *DAN* Danish.

Discussion

Previous studies have demonstrated a relationship between histological variables and age [2, 3, 6, 8, 44, 50] and reported on inter- and intra-observer error [6, 51], as well as cross-sectional variability [7] related to these methods. However, inter-population/group variation has not received much attention. Some studies stated that differences between black and white individuals are present on a microstructural level [12, 19, 21], but these have not been explored in detail. Although these studies deal with bone microstructure differences between black and white groups, literature on differences between groups of the same ancestry, but geographically isolated

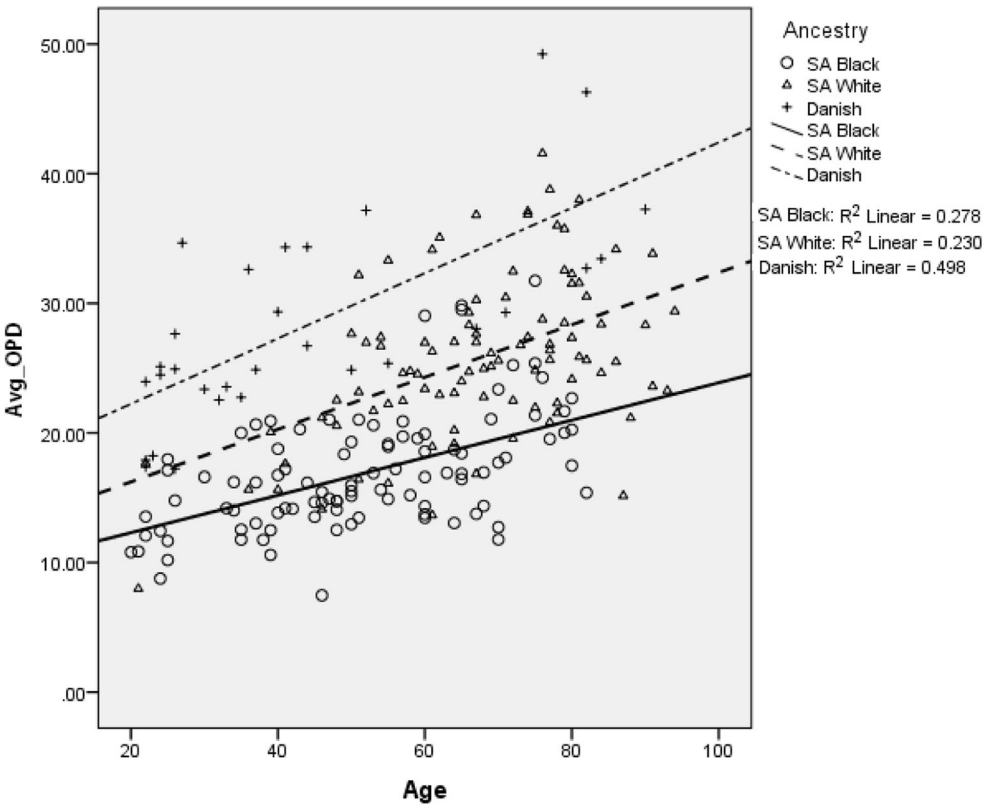

Fig. 1 Regression between average number of osteons per grid area and age

is almost non-existent. The intention of this study was to explore inter-population variation of histomorphometric variables of cortical bone used in age-at-death estimation between groups in terms of ancestry and geographical location.

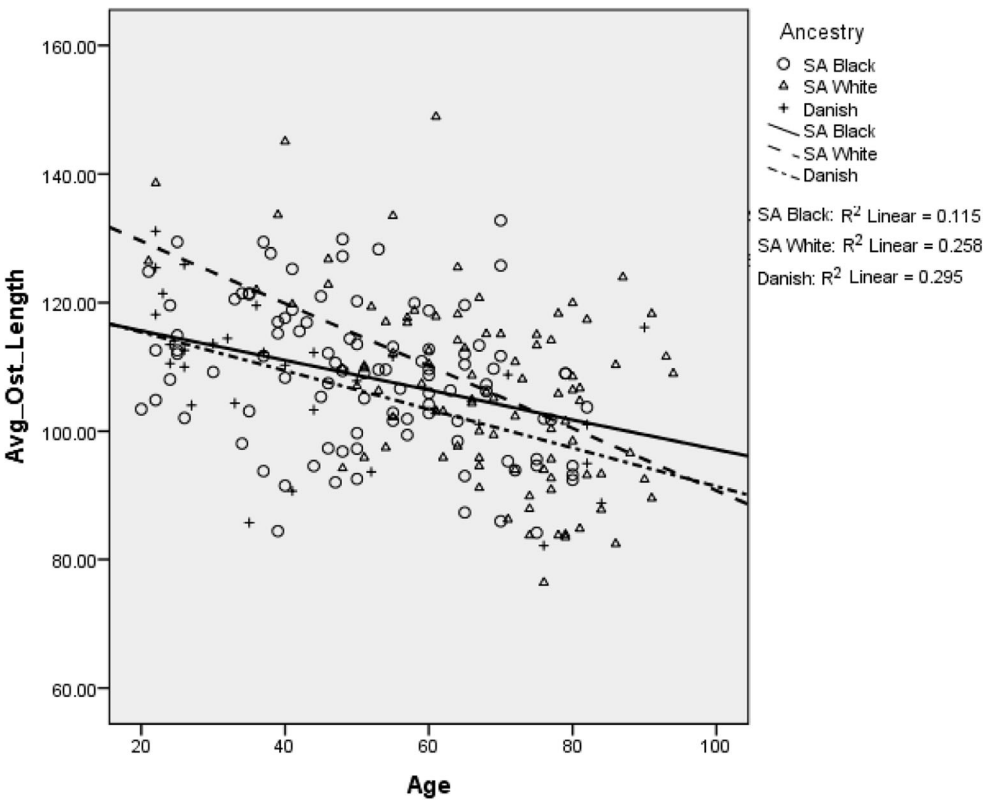

Fig. 2 Regression between average osteon length and age

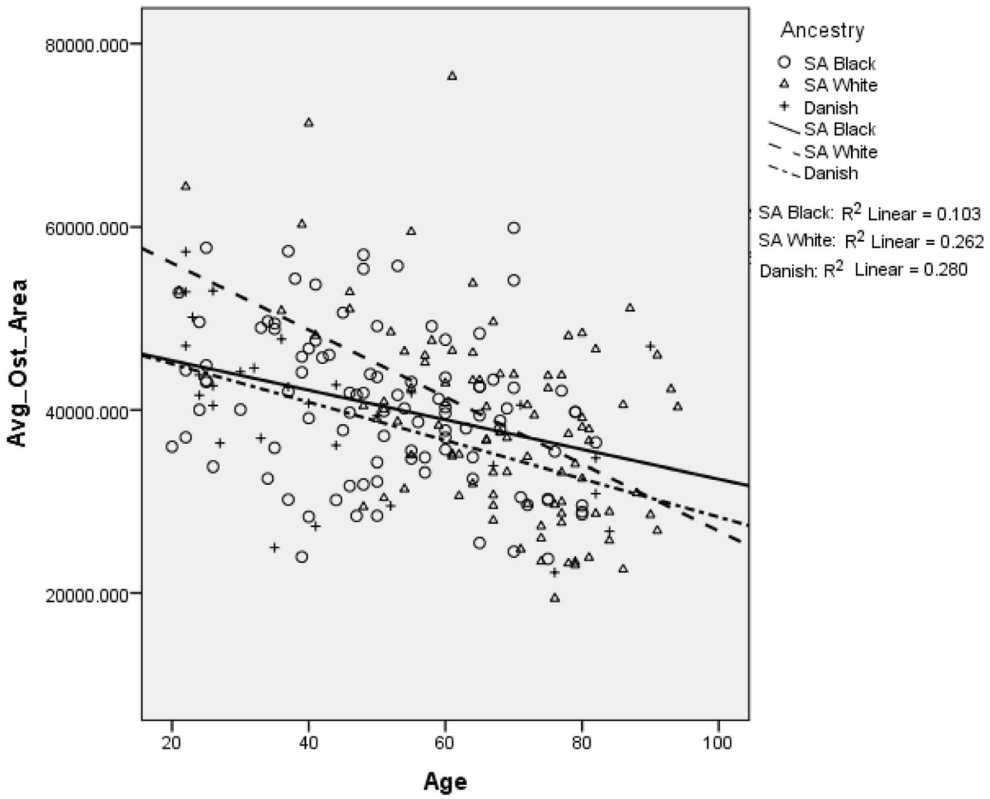

Fig. 3 Regression between average osteon surface area and age

When considering the average number of osteons (Avg_OPD), significant differences were observed in all of the different age cohorts. Black South Africans presented with the least number of osteons per grid area, followed white

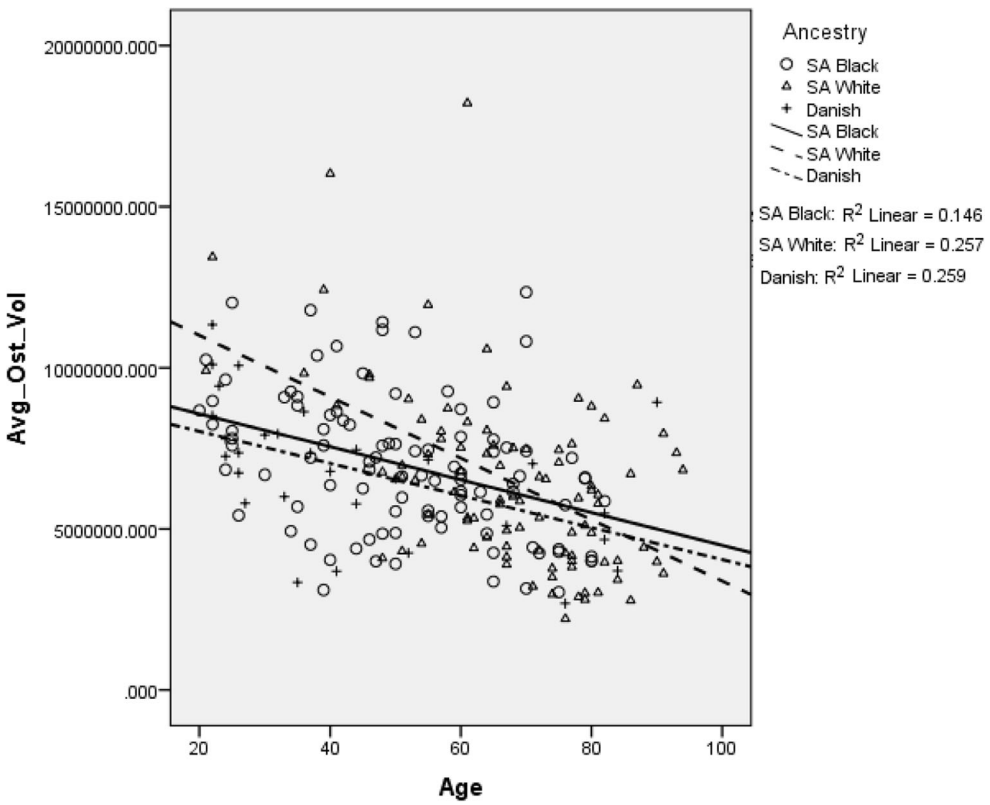

Fig. 4 Regression between average osteon volume and age

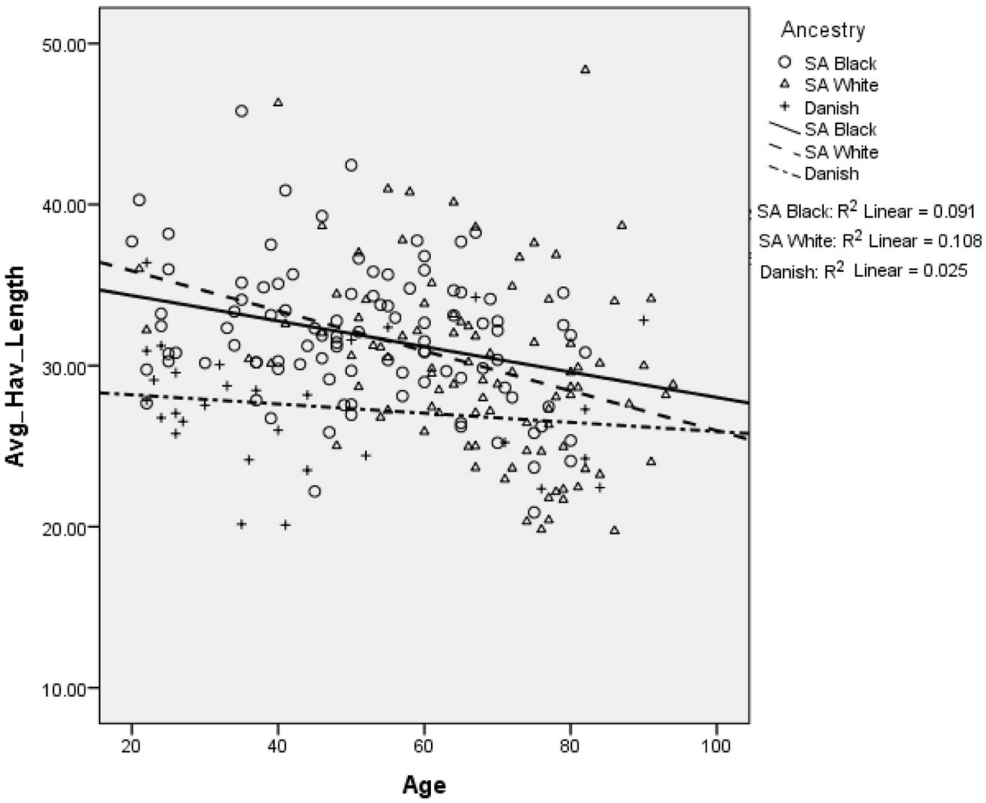

Fig. 5 Regression between average Haversian canal length and age

South Africans that had significantly higher OPD. Danish individuals showed the highest number of osteons per grid area. This outcome is supported by the results of other studies [19, 20] stating that bone remodelling rates are higher in white

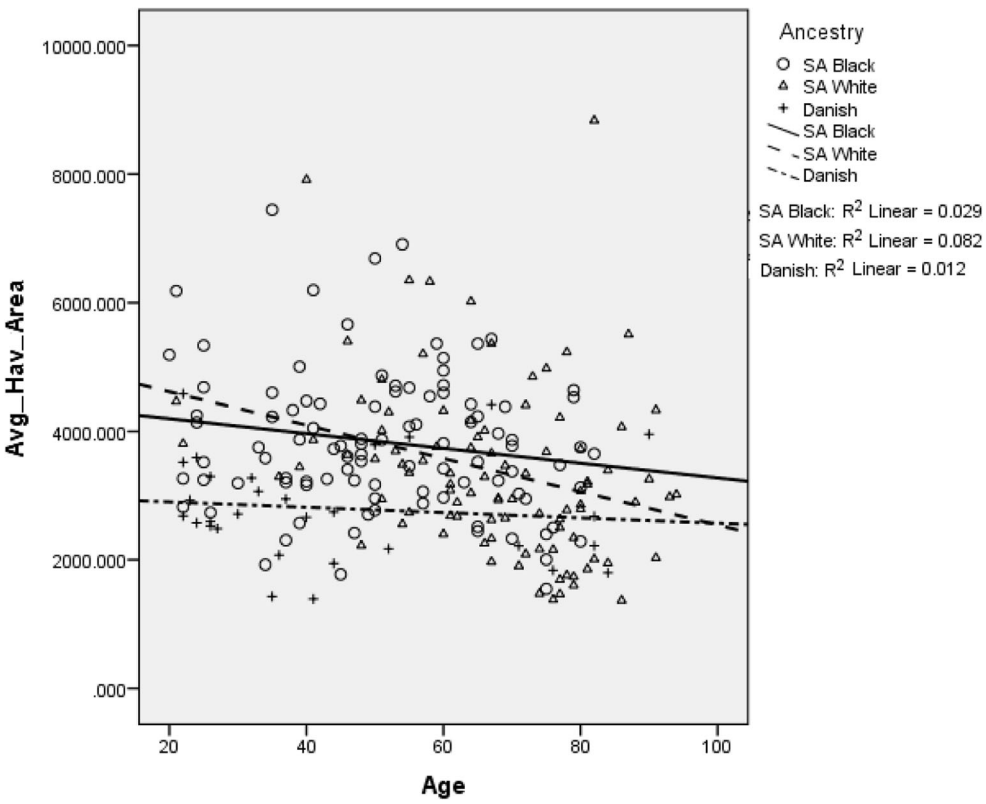

Fig. 6 Regression between average Haversian canal surface area and age

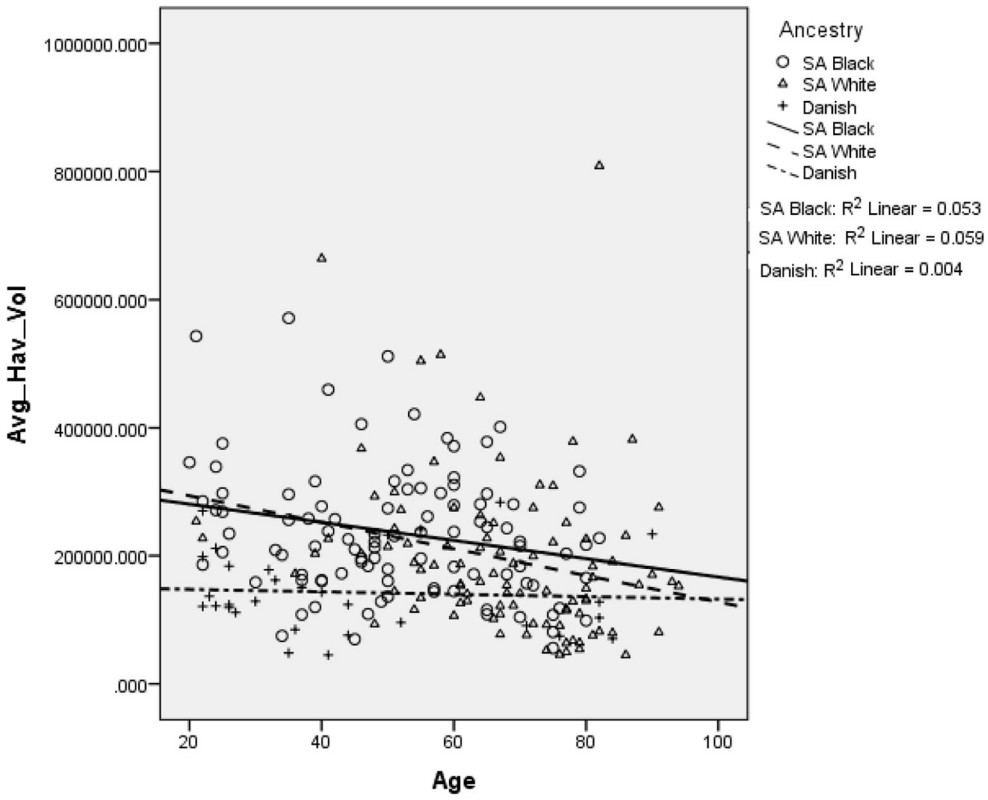

Fig. 7 Correlation between average Haversian canal volume and age

than in black individuals. The results of this study showed that both white/European samples had a higher number of osteons than the black sample, but also that there is a difference between European samples that are geographically unrelated. Whether this is related to climatic conditions and in particular the exposure to ultraviolet light remains to be explored.

Osteon population density has been widely used in age estimation studies as it correlates well with age. It is also the most "user-friendly" variable, as counting may be performed fairly easily. Apart from the advantages of using OPD for age estimation, the results of this study showed that significant differences are present between the study samples, which have implications when it comes to the use of general age estimation regression equations. This suggests that population standards may be necessary for the employment of OPD as a single variable. Alternatively, the reference population (if being applied to more than one ancestral group) for the construction of a regression equation should include a sub-sample of the population group of the individual in question.

Osteon size showed almost no significant differences between the sample for all age categories. Some variation (although not statistically significant) is present in the young and middle-aged adults, but once older age is reached, osteon size is very similar for all samples. Secondary osteon size, which also correlates relatively well with age [45, 52], appears to be a more dependable variable for age estimation, specifically where ancestry is unknown.

Significant differences were seen between the samples for Haversian canal size in younger adults, but as age advances the size of the canals became more uniform between the different groups (refer to Figs. 5, 6 and 7 and Table 4). Haversian canal size has been reported to show a low correlation with age and due to its considerable variation, specifically in the younger ages, should not be used for age estimation as a single factor. As Haversian canals are often irregular and highly inter-connected [53], its shape and size vary much more than that of the external surface of the Haversian system (osteon). The outcome of this study is similar to other studies on age estimation [9, 44, 45] that also found the Haversian canal to show unpredictable changes with age.

Pratte and Pfeiffer [54] tested age prediction equations developed on a sample of European descent and found that it performed poorly when used to predict age-at-death in a South African sample comprising of white, black and coloured individuals. Given these results, as well as evidence for population variation related to bone turn-over rates, density and strength [13, 21, 27] between different population groups, it is expected that population-specific regression formulae for age-at-death estimation provide higher accuracy and precision. However, Pfeiffer et al. [52] reported that equations developed based on an American sample of white, black and ethnicity unknown individuals [50] may be applied to a South African sample of white, black and coloured individuals. It was also found that population-specific standards did not perform

Table 3 Summary of ANCOVA results

Variable	F-ratio	Significance (p value)
Ages 20–39		
	F(2,41)	
Avg_OPD	34.807	0.000**
Avg_Ost_L	4.572	0.016
Avg_Ost_Ar	5.104	0.010
Avg_Ost_Vol	5.785	0.006*
Avg_Hav_L	9.320	0.000**
Avg_Hav_Ar	6.142	0.005*
Avg_Hav_Vol	9.469	0.000**
Ages 40–59		
	F(2,62)	
Avg_OPD	49.656	0.000**
Avg_Ost_L	2.940	0.060
Avg_Ost_Ar	2.814	0.068
Avg_Ost_Vol	2.701	0.075
Avg_Hav_L	5.583	0.006*
Avg_Hav_Ar	3.888	0.026
Avg_Hav_Vol	3.063	0.054
Ages 60+		
	F(2,108)	
Avg_OPD	27.993	0.000**
Avg_Ost_L	0.151	0.860
Avg_Ost_Ar	0.074	0.929
Avg_Ost_Vol	0.018	0.982
Avg_Hav_L	0.823	0.442
Avg_Hav_Ar	1.608	0.205
Avg_Hav_Vol	1.534	0.220

**$p < 0.001$

*$p < 0.01$

better than the "ethnicity unknown" equation. The outcome of this study agrees with Pratte and Pfeiffer [54] that European populations are dissimilar from white South Africans. The white South African population, although having European ancestry, possibly has genetic admixture with other populations. Also, adaptation to the environment may have contributed in variation seen between them and the Danish. It is possible that South African groups and American groups (such as the reference sample by Cho et al. [50] and applied by Pfeiffer et al. [52]) may be more similar to one another, but further investigation is needed to ascertain this possibility.

Although bone loss over time is affected by environmental factors, genetic make-up largely contributes to skeletal variation related to bone metabolism (bone formation and loss). Genetic studies have shown that skeletal growth and density acquired during childhood has a strong genetic foundation, and that the subsequent development and timing of peak bone mass is a result of specific genetic factors [27]. Black individuals tend to reach a higher peak bone mass than white individuals [55, 56], and often present with higher bone mineral density as adults [57, 58], which assist in maintaining higher bone mass throughout life.

On a molecular level, differences between populations have been reported that relate to calcium metabolism [10, 24], sex hormone levels [59], vitamin D levels [60], sun exposure and vitamin D [61] and vitamin D receptor genes [62] and body composition [14, 63, 64]. These studies found a higher calcium retention rate, bone formation rate and oestrogen levels in black individuals. All these factors were linked to a greater bone mass and maintenance in black adult individuals compared to their white counterparts.

Many of the factors, documented to play a role in bone microstructure and variation thereof, are linked to environmental circumstances (e.g. climate), physical activity and

Table 4 Pairwise comparisons (significance; p values) between groups for all variables assessed

	Avg_OPD	Avg_Ost_L	Avg_Ost_Ar	Avg_Ost_Vol	Avg_Hav_L	Avg_Hav_Ar	Avg_Hav_Vol
Young adults (20–39 years)							
SAB/SAW	1.000	0.015	0.010	0.010	1.000	1.000	0.914
SAB/DAN	0.000**	1.000	1.000	1.000	0.000**	0.004*	0.000**
SAW/DAN	0.001**	0.024	0.015	0.005*	0.166	0.337	0.398
Middle aged adults (40–59 years)							
SAB/SAW	0.000**	0.252	0.317	0.397	1.000	1.000	1.000
SAB/DAN	0.000**	0.606	0.544	0.487	0.011	0.038	0.105
SAW/DAN	0.000**	0.080	0.083	0.086	0.005*	0.026	0.051
Older individuals (60+ years)							
SAB/SAW	0.000**	1.000	1.000	1.000	1.000	1.000	1.000
SAB/DAN	0.000**	1.000	1.000	1.000	1.000	1.000	1.000
SAW/DAN	0.001**	1.000	1.000	1.000	1.000	1.000	1.000

**$p < 0.001$

*$p < 0.01$

socio-economic status (e.g. nutrition and health care). The exact influences of everyday life on the health of bone are complex. As bone loss throughout life is multifactorial, all these aspects most likely play a part in the variation we observe in the studied groups. It is clear that variation between black and white, and individuals of shared ancestry but geographically isolated, is associated with various factors. Most of these factors are strongly related to a genetic basis that has been shaped by long-term environmental influences. However, due to a lack of information on general well-being and health on many of the individuals in the study samples, the exact nature of these factors and how it contributed to the variation seen is unclear.

Conclusion

Overall, the highest variation in the studied variables was observed in the younger age groups. The average number of osteons showed significant differences between the three samples across all age categories. The opposite was true for osteon size, with population samples showing similar osteon dimensions over age. Haversian canal size showed variation in the younger age groups, but not in older individuals. As OPD and osteon size correlates well with age, it is recommended that these variables (rather than Haversian canal size) be employed for age estimation using regression analysis.

It is important to consider population differences in terms of OPD before using this variable in age estimation. Osteon size appears to be a more generally applicable variable to employ as it showed the least amount of inter-population variation. In cases where the ancestry is unknown, it is suggested that osteon size, rather than OPD, be used for estimating age.

As there are conflicting views on whether age estimation equations should be generally applied, further studies need to be done on inter-population variation and the use of specific histological age estimation standards.

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Histological age-at-death estimation in white South Africans using stereology.

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Abstract

Various methods are available for estimating age from skeletal remains, amongst them the use of histomorphometry. It is generally argued that age estimation standards are population specific, but this in itself create problems as the reference samples used are often not large enough and/or lack substantial representation of all age cohorts. Traditional age methods have been shown to suffer from problems such as age mimicry. This paper aims at establishing histological age-at-death standards for the white South African population by supplementing the available sample (lacking an adequate number of young adults) with another sample of European descent to avoid over-estimation of age in younger individuals caused by age mimicry. Bone microstructures related to the number of osteons and fragments, osteon size and Haversian canal size that change with advancing age were used for the development of regression formulae. A histomorphometric assessment of the anterior cortex of the femur was done using stereology for the estimation of age-at-death. All sections were analysed using the optical fractionator and nucleator probes. A sample of 94 bone sections (n = 50 male, n = 44 females) of white South African individuals were used. A sample of Danish individuals (n = 14 males, n = 16 females) was combined with the South African sample to create a normal age distribution for the reference sample. Single and multiple regression equations were developed after randomly selecting a hold-out sample (n = 14) for validation. Osteon size (average length, surface area and volume) showed the highest

correlation with age, followed by the number of osteons and fragments per grid area. Haversian canal size showed inconsistent changes with advancing age. Using the regression equations, predicted ages were obtained for the 14 individuals. RMSE values ranged between 14 and 17 years, which we deemed acceptable.

Keywords: femur, age estimation, stereology, linear regression, age mimicry

1. Introduction

Methods for age-at-death estimation in adult skeletal remains are subjected to wide ranges caused by variation in the observed biological structures. Age-related changes in children are more constant, leading to narrow age ranges, but once adulthood is reached, more variation is introduced. The degenerative changes that are often used in adults are influenced by numerous factors, both biological and environmental. The tempo of development (of lack thereof) of specific skeletal features, as well as the pattern in which they emerge, vary considerably between populations and individuals within a population, making the setting of universal age estimation criteria very difficult and often calls for population-specific standards [1]. Of course, many of the problems with existing age estimation techniques are due to small sample sizes, lack of validation and statistical difficulties when combining multiple features.

A major problem in traditional age estimation methods is that of age mimicry, where the estimated age-at-death of an individual tends to mimic the age distribution of the reference sample [2-4]. This phenomenon sets in when the age distribution of the reference sample is statistically skewed. Skeletal collections used as reference samples for the construction of age estimation standards sometimes lack an appropriate number of individuals in certain age cohorts, causing a misrepresentation of the population, which may lead to inaccurate age estimates of an unknown individual.

Biological (rather than chronological) age is observed in the skeleton, which often differs from an individual's age in years (chronological age) as it is affected by the individual's general well-being and health, as well as genetic make-up. Theoretically, a close relationship exists between chronological and skeletal age. This suggests that degenerative changes that take place on either a macroscopic or microscopic level should correlate well with advancing age. However, this relationship is not perfectly linear, which makes it difficult to establish accurate age estimates [1,5]. Current research gives broad explanations on how age and

variability of structures are linked, with no specific reasons as to why certain trends are observed in histological structures with advancing age. Causes for variation are often labelled as "environmental" or "genetic," with in-depth knowledge lacking on the interaction between these two factors.

Although macroscopic methods for age estimation are mostly used, the lack of skeletal elements in some cases makes it nearly impossible to narrow down the estimated age range. Histological techniques provide a valuable alternative in such cases as it requires only one skeletal element (e.g., femur or rib). Studies on histological age estimation have made use of many different skeletal locations, including the occipital bone [6], mandible [7,8], clavicle [9,10], 2nd metacarpal [11], ribs [5,12,13], humerus [14,15], ulna [14], femur [7,16-20], tibia [7,16,21] and fibula [16,22]. Most studies are based on specific populations, except for a few publications combining individuals of different groups into one sample [16,22,23]. Population-specific studies are essential, as it is important to understand the characteristics of histological bone structures with its associated variability and how it differs across diverse groups.

Several characteristics of bone microstructures have been identified to show age-related changes. These included a decrease in osteon and Haversian canals size with age, as well as an increase in osteon number and fragments with age [24,25]. Current approaches in age estimation from histomorphometry are performed on two-dimensional images and are largely based on methods established through earlier studies. Variables widely used include osteon population density (OPD), osteon area (On.Ar) and relative cortical area (Ct.Ar/Tt/Ar). However, inconsistencies exist between studies reporting on these variables. Although most studies agree that OPD increases with age, not all studies report consistent finding regarding osteon area. Some studies reported a negative association between On.Ar and age [5,26], while others failed to note this tendency [10,27,28].

Variation between studies in the selection, definition and measurement of secondary osteons and its associated field of view may be to blame for inconsistencies reported between studies. Such variation makes histological age estimation vulnerable to observer bias and error and leads to extreme difficulty in comparing different methods for the standardization of histological age estimation techniques.

The use of computer-based stereology protocols as a histological tool for assessment offers a way to exclude selection and observer bias. It also allows for standardization of the method

as a set protocol is employed. Stereology is based on systematic random sampling which does not allow for the observer to select a region for analysis, but randomly places a counting grid on the region of interest, creating several counting frames and thus preventing selection bias. Counting rules have been set which should be followed by the observer to exclude observer/method bias [29]. Stereology was chosen as an alternative to traditional two-dimensional methods in this study.

A recent study using stereology [42] dealt with inter-population variation of histological structures used for age-at-death estimation. The results showed a statistically significant difference in the number of osteons between populations, but no difference in osteon size. This suggested that standards using osteon size may be generally applied to individuals of different population groups. The use of population-specific standards for OPD as a single variable for age estimation should thus preferably be used in conjunction with osteon size in cases where the individual's population group differs from that of the reference sample.

The primary objective of this study was to create regression equations for age-at-death estimation in white South Africans. To avoid the effect of age mimicry, the sample was enlarged with a group of the same ancestry but located in a different geographical region. A Danish sample with known ages at death was used for this purpose. Assessment of bone microstructures for the estimation of age through histomorphometry was done using stereology. To test the validity of the regression formulae, a hold-out sample was selected. The actual ages were compared to the estimated ages to assess if the standards produce reliable age estimates. Intra-observer reliability of the method was also tested.

2. Materials and methods

2.1 Sample, slide preparation and stereological analysis

Bone slides used were derived from two study groups. The sample size and age distribution of the two samples is given in Table 1. All bone sections were taken from the anterior cortex of the femur in the region of the mid-shaft opposite the linea aspera. Sections from both samples were unstained.

The white South African sample comprised of 50 males and 44 females (total n = 94) and was selected from the Pretoria Bone Collection [30]. Although the sample is large, it contains very few young adult individuals (n = 4; 20-39 years). The white individuals from the Pretoria Bone collection were most probably of middle socioeconomic status. Cross-

sections with a thickness of 2-4 mm were made just below the midshaft of the anterior cortex of the femur (to not affect bone measurements in future) using an EXAKT 312 pathology saw fitted with a diamond blade (Bone Research Laboratory, WITS). The sections were manually ground by hand until it reached a thickness of around 1 mm according to the Manual for the Preparation of Ground Sections for the Microscopy of Bone Tissue [31]. This method does not ensure a uniform thickness throughout the section. Machine grinding was employed from this point forward to ensure a smooth and uniform surface area. Sections were mounted onto perspex slides measuring 25 x 75 x 1.5 mm and ground to a minimum thickness of 100 µm with the EXAKT 400 CS surface grinder (Bone Research Laboratory, WITS).

The Danish sample comprised of 14 males and 16 females (n = 30). Complete cross-sections from the femoral mid-shaft were made for autopsies performed at the Department of Forensic Pathology, University of Copenhagen, between 1988 and 1990. Sections were prepared by boiling complete sections of the anterior midshaft of the femora (sectioned to be ± 4 cm in length) in water and detergent for a few hours, after which 100 µm slices were cut using a Buehler Isomet low speed diamond water saw. Sections were then mounted on glass slides [32].

Table 1: Sample size and age distribution.

Age cohort	Pooled sexes			Males			Females		
	n	mean age	SD	n	mean age	SD	n	mean age	SD
South African sample									
20-29	2	21.5	0.7	-	-	-	2	21.5	0.7
30-39	2	37.5	2.1	1	36.0	-	1	39.0	-
40-49	6	44.8	3.5	4	43.8	3.9	2	47.0	1.4
50-59	15	54.1	2.8	11	53.7	2.4	4	55.3	3.9
60-69	25	64.6	2.9	11	63.2	3.0	14	65.8	2.4
70-79	24	75.4	2.7	11	74.5	2.3	13	76.2	2.8
80+	20	85.1	4.7	12	85.1	5.5	8	85.0	3.6
TOTAL	**94**	**67.3**	**14.9**	**50**	**66.7**	**14.5**	**44**	**67.9**	**15.5**
Danish sample									
20-29	10	24.2	1.9	5	23.8	2.1	5	24.6	1.9
30-39	6	33.8	2.6	3	34.3	3.8	3	33.3	1.5
40-49	4	42.3	2.1	2	42.0	2.8	2	42.5	2.1
50-59	3	52.3	2.5	1	50.0	-	2	53.5	2.1
60-69	1	67.0	-	-	-	-	1	67.0	-
70-79	2	73.5	3.5	2	73.5	3.5	-	-	-
80+	4	84.5	3.8	1	82.0	-	3	85.3	4.2
TOTAL	**30**	**44.1**	**21.8**	**14**	**41.8**	**20.7**	**16**	**46.1**	**23.2**

For the assessment of histomorphometric variables, stereology was performed. Stereology allows for the assessment of microstructures in an unbiased fashion and has been proven to be more accurate than traditional two-dimensional methods [29]. It is based on random sampling techniques and incorporates a three-dimensional space for the estimation of number, length, surface area and volume [33]. Stereology has previously been employed in the assessment of histomorphometric variables of the femur in black South African individuals [34].

A design-based systematic random sampling protocol using StereoInvestigator software (MicroBrightField, version 11.08.1; 64-bit) was employed. Pilot studies were conducted to obtain an average section thickness (195-250 µm) and to optimise sampling parameters, including counting frame size (1000 x 1000 μm^2) and sampling grid (1250 x 1250 μm^2) sizes, disector height (150-215 µm), guard zones (7.5-10 µm), average number of sampling sites (55) and average coefficient of error (Gundersen, m = 1; 0.12).

Eight variables were observed for both samples and included the following:

1. *Average number of osteons per grid* (Avg_OPD): The total estimated number using number-weighted section thickness for all intact, identifiable Haversian systems divided by the number of counting frames with counts to produce the average number of Haversian systems per grid area in each individual.
2. *Average osteon width* (Avg_Ost_L): The average diameter (µm) of all osteons measured in each individual.
3. *Average Haversian canal width* (Avg_Hav_L): The average diameter (µm) of all Haversian canals measured in each individual.
4. *Average osteon surface area* (Avg_Ost_Ar): The average surface area (μm^2) of all osteons measured in each individual.
5. *Average Haversian canal surface area* (Avg_Hav_Ar): The average surface area (μm^2) of all Haversian canals measured in each individual.
6. *Average osteon volume* (Avg_Ost_Vol): The average volume (μm^3) of all osteons measured in each individual.
7. *Average Haversian volume* (Avg_Hav_Vol): The average volume (μm^3) of all Haversian canals measured in each individual.

8. *Average number of fragments per grid area* (Avg_Frags): The total estimated number of fragments using number-weighted section thickness for all identifiable osteon fragments divided by the number of counting frames with counts to produce the average number of fragments per grid area in each individual.

To estimate the total number of secondary osteons and fragments within a specific region of interest (ROI), the optical fractionator probe [35] was used. One ROI was selected for each individual. Counting of structures was done according to guidelines outlined by Gundersen [36] and Howard and Reed [29], which state that every particle should be marked once with a unique point. Particles/structures lying within the counting frame and/or touching the inclusion lines are counted, whereas particles touching the exclusion lines or lying outside the counting frame are not counted.

To estimate the length, surface area and volume of secondary osteons and Haversian canals, the nucleator probe [37] was used in conjunction with the optical fractionator. Thick sections are required for employment of these probes. An arbitrary point is placed in the middle of the osteon/Haversian canal, from which five radiating lines are drawn to intersect with the cement line/boundary (Figure 1). The software calculates an estimated average length, surface area and volume based on the length of these lines for ROI.

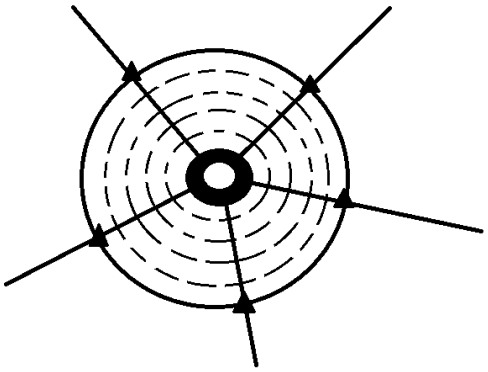

Figure 1. Illustration of five lengths (rays) from an arbitrary point in the centre of the Haversian canal intersecting with the boundary of the osteon. The intersections are marked by the solid triangles (▲).

2.2 Statistical analysis

Upon completion of the stereological analysis, independent-samples t-tests were done to assess differences between males and females of each group for all variables assessed.

Regression analyses [38] were done for single and multiple independent variables vs. age (dependent variable) in a total sample of n = 110 after a hold-out sample (n = 14) of two individuals (one male, one female) per age cohort was selected for validation of the regression standards. Random selection per age/sex cohort was done to include individuals from both population groups (South Africans, n = 10; Danish, n = 4).

Preliminary tests were done to determine if the data are suitable for multiple regression analysis. The assumptions of normality, linearity and multicollinearity were tested to ensure that no violations existed. Also, the sample needs to be large enough (more than 20 entries per variable). Therefore, the pooled-sex sample (n = 110) was used. Cook's distance coefficient was used to detect any outliers, but none was found. Kolmogorov-Smirnov and Shapiro-Wilk tests were non-significant, signifying normality. An acceptable linear relationship between age and the independent variables was demonstrated by a scatterplot (standardized residual vs. standardized predicted value). The assumptions for multiple regression analysis were thus met and the data were found to be suitable for the execution of this method.

However, multicollinearity (when one predictor variable is strongly correlated with another to the extent where redundant information is incorporated into the equation) was detected between osteon length/area/volume and Haversian canal length/area/volume. It is thus essential that only multiple regression equations containing either length, area or volume of a specific object (i.e. osteon and Haversian canal) combined with average osteon/fragment number be used.

Regression formulae were then constructed for all models of single and multiple variables for the estimation of age-at-death that showed a statistically significant change with age for pooled and unpooled sex groups. In order to test if the regression equations produce accurate/reliable age estimates, a single regression equation (Avg_OPD) and multiple regression equation (Avg_OPD, Avg_Frags, Avg_Ost_Vol and Avg_Hav_Vol) was tested using the hold-out sample. The strength of correlation between the actual age and predicted ages was tested and a RMSE was given.

Intra-observer repeatability was tested using Cronbach's alpha and intra-class correlation (ICC) coefficients [39]. Thirty individual sections were re-analysed using the stereological protocol. Cronbach's alpha provides a measure of how closely related two values/scores/groups are to one another. ICC measures the reliability for values/scores for a

specific group. Both these coeffients range between 0 and 1, where 0 represents no relation/resemblance between scores and 1 characterizes perfect relation/reliability.

All statistical analyses were done using SPSS software (version 24) apart from the RMSE values which was calculated in Microsoft Excel.

3. Results

Independent-samples t-tests were done for all variables to detect differences between the sexes of the white South African sample. Males and females differed only in the average number of fragments per grid area ($t(122) = 3.384$; $p < 0.001$), with females having significantly more fragments than males.

The strength of the relationship between age and the variables in the combined sample is given by the correlation coefficient (r) and coefficient of determination (r^2) for pooled and unpooled sexes (Table 2). A positive correlation with age was seen for the average number of osteons (Avg_OPD) and fragments (Avg_Frags) per grid area. All other variables were correlated negatively with progressing age.

Considering pooled data, the average number of osteons ($r = 0.391$), average number of fragments ($r = 0.341$) and osteon size (Avg_Ost_L: $r = -0.456$; Avg_Ost_Ar: $r = -0.455$; Avg_Ost_Vol: $r = -0.446$) showed a moderate correlation with age. Haversian canal size (Avg_Hav_L: $r = -0.118$; Avg_Hav_Ar: $r = -0.080$; Avg_Hav_Vol: $r = -0.046$), however, showed a low correlation with age.

Table 2: Coefficient of correlation (r) and coefficient of determination (r^2) for all single variables (combined dataset, n = 110).

Variable	Correlation coefficient (r)			Coefficient of determination (r^2)		
	Pooled	Male	Female	Pooled	Male	Female
1. Avg_OPD	0.391	0.480	0.307	0.153	0.230	0.094
2. Avg_Frags*	0.341	0.208	0.462	0.116	0.043	0.213
3. Avg_Ost_L	-0.456	-0.507	-0.409	0.208	0.257	0.167
4. Avg_Hav_L	-0.118	-0.129	-0.102	0.014	0.017	0.010
5. Avg_Ost_Ar	-0.455	-0.494	-0.421	0.207	0.244	0.177
6. Avg_Hav_Ar	-0.080	-0.094	-0.057	0.006	0.009	0.003
7. Avg_Ost_Vol	-0.446	-0.473	-0.427	0.199	0.224	0.182
8. Avg_Hav_Vol	-0.046	-0.057	-0.020	0.002	0.003	0.001

* sex-specific difference of $p < 0.001$

A simple linear regression was performed to predict age based on the eight variables selected. Regression analyses of age vs. single variables were done for the pooled and unpooled sex groups. A significant regression was seen for all variables, except for Haversian canal length (Avg_Hav_L), area (Avg_Hav_Ar) and volume (Avg_Hav_Vol). Regression results showing predictable changes with advancing age are summarized in Table 3. The SEE values obtained ranged between 16 and 19 years for all variables analysed.

Table 3. Linear regression analysis of age vs. single histomorphometric variables.

Variable	Correlation coefficient (r)	Coefficient of determination (r^2)	Slope	Intercept	SEE	F Ratio
			Age = (slope x variable) + intercept ± SEE			
Avg_OPD	0.453	0.205	1.308	27.156	17.270	27.896*
Avg_Frags	0.384	0.148	2.005	45.988	17.887	18.694*
Avg_Ost_L	-0.511	0.261	-0.730	139.761	16.658	38.068*
Avg_Ost_Ar	-0.505	0.255	-0.001	99.475	16.718	37.024*
Avg_Ost_Vol	-0.490	0.240	$-3.573e^{-006}$	85.317	16.886	34.147*
Avg_Hav_L	-0.182	0.033	-0.622	80.737	19.051	3.680
Avg_Hav_Ar	-0.127	0.016	-0.002	67.848	19.216	0.187
Avg_Hav_Vol	-0.076	0.006	$-8.413e^{-006}$	64.085	19.317	0.623

* $p < 0.001$

Multiple regression analyses were done to evaluate the relationship between age and a combination of variables. A summary of the results with the associated ANOVA results for four linear combinations of variables is given in Table 4. The correlation coefficients for the models ranged between 0.461 and 0.640, indicating that roughly 46 to 64% of variance in age may be accounted for by the specific combination of variables when estimating age. The combination of Avg_OPD, Avg_Frags, Avg_Ost_Ar and Avg_Hav_Ar had the highest correlation with age on a linear scale when considering the R and R^2 values, of which both indicated an average to strong correlation strength with age.

Table 4. Multiple regression analyses result summary.

Combination of variables	R	R2	Adjusted R2	Regression equation	SEE	F Ratio
1. Intercept Avg_OPD (x_1) Avg_Frags (x_2) Avg_Ost_Vol (x_3) Avg_Hav_Vol (x_4)	0.633	0.401	0.378	$69.645 + 0.469(x_1) + 0.268(x_2) - 5.145e^{-006}(x_3) + 5.686e^{-005}(x_4)$	17.268	17.539*
2. Avg_OPD (x_1) Avg_Frags (x_2) Avg_Ost_Ar (x_3) Avg-Hav_Ar (x_4)	0.640	0.410	0.387	$76.573 + 0.518(x_1) + 0.341(x_2) - 0.001(x_3) + 0.007(x_4)$	15.212	18.208*
3. Avg_OPD (x_1) Avg_Frags (x_2) Avg_Ost_L (x_3) Avg-Hav_L (x_4)	0.632	0.399	0.376	$100.592 + 0.0575(x_1) + 0.395(x_2) - 1.068(x_3) + 1.917(x_4)$	15.097	17.452*
4. Avg_OPD (x_1) Avg_Frags (x_2)	0.461	0.213	0.198	$28.729 + 1.052(x_1) + 0.647(x_2)$	15.227	14.465*

* $p < 0.001$

Descriptive statistics showing the actual mean age compared to the predicted ages for the hold-out sample are summarized in Table 5. The correlation values for actual vs. predicted ages (Figures 2 and 3) for the hold-out sample (n = 14) using the regression equations were r = 0.879 (single variable) and r = 0.586 (multiple variables), with the RMSE values given as 14 and 17 years, respectively.

Table 5. Descriptive statistics of actual and predicted ages of the hold-out sample.

	N	Mean	SD	RMSE
Actual age	14	55.43	20.49	
Predicted age (single variable)	14	57.09	7.29	14
Predicted age (multiple variables)	14	50.33	13.24	17

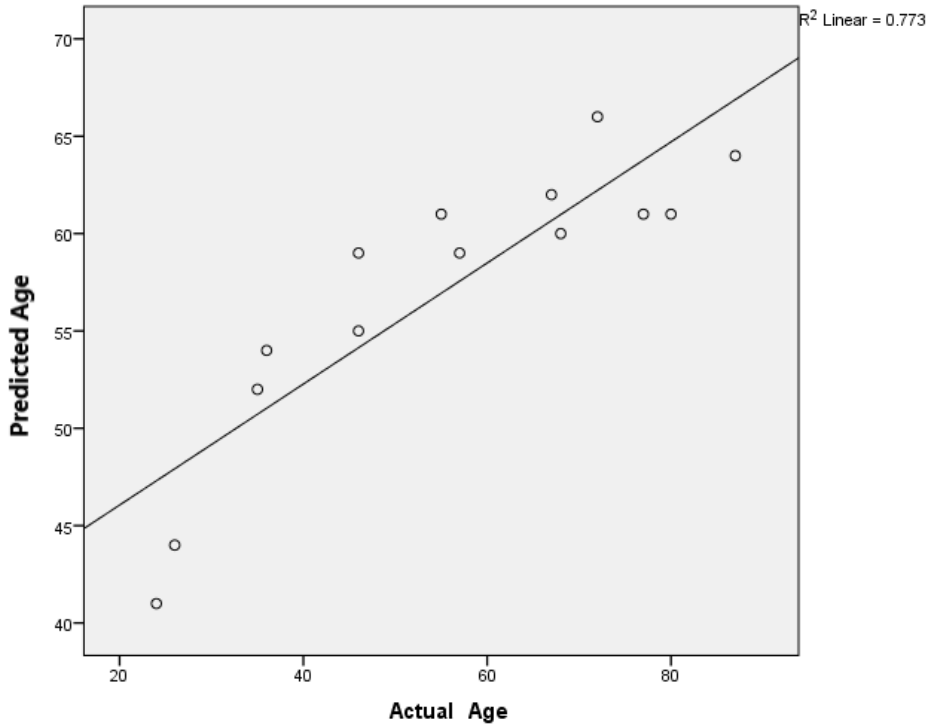

Figure 2. Actual vs. predicted age using single regression (Avg_OPD).

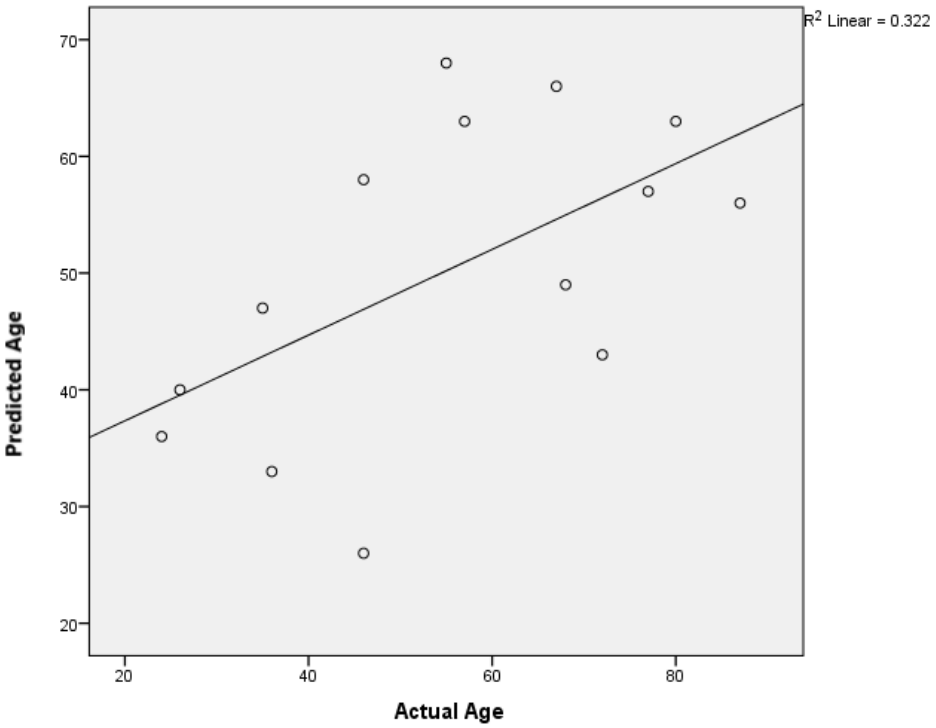

Figure 3. Actual vs predicted age using multiple regression.

Intra-observer repeatability and reliability are presented in Table 6. A high association between repeated measurement for all variables (> 0.80) were seen for Cronbach's alpha and intraclass correlation (ICC) coefficients. The highest repeatability was seen in the average number of osteons per grid area (Avg_OPD), with Haversian canal length (Avg_Hav_L) being the least repeatable. The level of variation between first and second measurements is thus within acceptable limits. Therefore, it can be suggested that the method was repeatable and reliable in this case.

Table 6. Intra-observer repeatability.

Variable	Cronbach's alpha	ICC	95% CI for ICC	
			Lower	Upper
1. Avg_OPD	0.981	0.963	0.908	0.985
2. Avg_Frags	0.973	0.947	0.871	0.979
3. Avg_Ost_L	0.975	0.950	0.879	0.980
4. Avg_Hav_L	0.897	0.814	0.588	0.922
5. Avg_Ost_Ar	0.968	0.939	0.852	0.975
6. Avg_Hav_Ar	0.937	0.881	0.725	0.951
7. Avg_Ost_Vol	0.979	0.960	0.901	0.984
8. Avg_Hav_Vol	0.923	0.857	0.673	0.941

4. Discussion

This study provides histological age-at-death estimation standards using stereology and gives a unique perspective on its application for individuals of shared ancestry (European) but geographically separated from the study sample. Stereology is a time-consuming method, but it was employed for its ability to provide unbiased sampling and analysis.

Although the South African sample is large, the age distribution is slightly skewed towards older individuals, as the collection contains very few young white individuals. This may cause complications such as age mimicry [2-4] but is a factor that cannot be accounted for when working with specific collections. The original sample (white South Africans) in this study had limited representation of young adult individuals. From Table 1 it can be seen that there are only four individuals younger than 40 years of age. This reference sample thus allows for age mimicry to play a role. As age mimicry poses a serious problem in the estimation of age, it was essential in this case to avoid over-estimation of age using regression analysis. Upon adaptation of the original sample by pooling the white South African and Danish samples, the hold-out sample (N = 14) results showed that all individuals could be aged within the SEE range of 14 to 17 years. This suggests that, even though there

may be significant differences between populations, it is possible to pool groups for establishing generally applicable equations. It should be noted that assessment of inter-population variation and construction of population standards must rely on large, normally distributed reference samples. In cases where references samples are skewed in terms of its age distribution, one should compromise on population standards and combine samples for construction of age estimation standards using traditional methods to account for the problem of age mimicry. Thus, statistical problems related to the reference sample outweigh population variation.

In this study, the number of osteons, fragments and osteon size were found to change significantly with age. Haversian canal size, however, showed unpredictable changes with age. Osteon size presented with the highest correlation (Avg_Ost_L: r = -0.456; Avg_Ost_Ar: r = -0.455; Avg_Ost_Vol: r = -0.446), followed by the average number of osteons (r = 0.391) and fragments (r = 0.341) per grid area. Previous studies also reported inconsistent results when assessing Haversian canal size, with large amounts of variation between subjects [20,28,34]. Linear regression analysis results revealed SEE values ranging from 12 to 15 years, which is similar to that of other studies on histological age estimation [20,34,41].

Bone histomorphometry has been established to be useful for age estimation, although it is still unclear how much inter-population variation exist between populations world-wide and if current population standards may be generally applied. Pfeiffer et al. [26] used the Cho et al. [5] methodological approach (based on African-American and European-American groups providing population-specific and ethnicity-unknown regression equations) and applied it to a South African sample from the Western Cape that comprised of white, black and coloured individuals. They found that the equation for the combined group (ethnicity unknown) overall performed better than the population-specific equations and stated that the use of population-specific equations are unnecessary.

The results of this study lean towards agreement with that of Pfeiffer et al. [26] and showed that estimated ages for all individuals of the hold-out sample were in range with the predictive interval/SEE of approximately 12 years. The assessment of histomorphometric variables using stereology have assisted in pointing out that population differences are present. Botha et al. [42] has shown that a statistically significant difference does exist for the average number of osteons (OPD) between the white South African and Danish samples,

with Danish individuals having significantly more osteons per counting area than white South Africans. Although some population differences exist, the correct application of appropriate statistical methods and the validation there of may provide generally applicable standards for age estimation [40].

Therefore, the statistical method employed for estimation of age should also be considered. Although linear regression analysis provides a useful tool for constructing age estimation equations, it has some disadvantages. It is extremely sensitive to outliers, which may cause the regression outcome to be inaccurate. As biological data are subjected to large amount of variation, outliers are often detected and must be removed when employed statistical methods such as linear regression [38]. Linear models by nature tend to over-estimate age in younger individuals, due to the intercept starting at a point above zero, thus already placing an individual at a certain age, without the variable fraction added [26]. Regression analysis is thus limited in its ability to predict age.

Generally applicable methods (as used in the Pfeiffer et al. [26] study) would be ideal, but validation of methods, together with further research on population variation is needed before regression formulae may be used on unspecified skeletal remains. Large reference samples equal in population numbers representing normal age distributions are essential for the development of generally applicable methods.

5. Conclusion

This study showed that OPD, number of fragments and osteon size may be correlated with age to produce significant regression equations. Moderate correlations with age were observed for these variables. Haversian canal size, however, showed a low correlation with age and could not be significantly linked to advancing age.

The regression equations established were shown to be applicable to white South Africans and Danish/Northern European individuals as no individuals from the hold-out sample were severely under- or over-estimated in age. It is, however, recommended that inter-population variation and the use of specific statistical methods are assessed before applying specific equations for the estimation of age-at-death in unknown individuals from geographical and ethnic dissimilar groups.

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Compliance with ethical standards

The authors declare they have no conflict of interest. Ethical approval for the project was obtained through the University of the Witwatersrand. The National Research Foundation provided funding for Authors D Botha and M Steyn.

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PART 3: DISCUSSION, SUMMARY AND CONCLUDING REMARKS

Chapter 8

DISCUSSION AND CONCLUSION

8.1. Introduction

Histology as a discipline is based on the assessment of soft tissue cells or "fresh" material. Due to the absence of fresh cells in skeletal material, the exploration of dry bone histology as a tool for research and analysis became a discipline on its own (De Boer *et al*. 2013). Predominantly employed for age estimation and palaeopathological diagnoses, studies on dry bone histology have delivered a bulk of literature stretching over more than a century, with some papers produced as early as 1891 by Prudden (Stout & Crowder 2012). In the past 50-odd years, the bulk of dry bone studies focused on age estimation, with varying degrees of accuracy reported. Initial studies on histological age estimation reported high accuracies with correlation coefficients around 0.9 (e.g., Kerley 1965; Ahlqvist & Damsten 1969; Singh & Gunberg 1970). With subsequent refinement and revision of the original methods used, later studies revealed somewhat lower accuracies with correlation coefficients ranging from around 0.5 to 0.7 (e.g., Lynnerup *et al*. 2006; Keough *et al*. 2009; this study).

This study focused on the linear relationship between age and secondary osteon histomorphometry (using stereology), its application for age estimation in adult human skeletal remains and the associated inter-population variation of the groups studied. Three distinct populations were assessed, namely black South Africans, white South Africans and Danish individuals. Dry bone samples were taken from the anterior cortex of the femur with the minimum amount of invasion, leaving the bone intact for future use. Bone sections were prepared by grinding bone samples to a minimum thickness of 100µm, as sections grinded thinner than this resulted in unclear histological structures and cracked bone.

The individual relationships of the number of osteons per grid area (Avg_OPD), number of fragments per grid area (Avg_Frags), osteon length (Avg_Ost_L), surface area (Avg_Ost_Ar) and volume (Avg_Ost_Vol), as well as Haversian length (Avg_Hav_L), surface area (Avg_Hav_Ar) and volume (Avg_Hav_Vol) with age were explored. For the first time in a dry bone histological study, stereology was used for its advantage of being an unbiased technique, instead of employing traditional two-dimensional histological methods. Age-predicting equations were constructed for single and multiple

variables with statistically significant results. Furthermore, bone mineral density values obtained from DXA scans were used to investigate the linear relationship between age and bone mineral density. Regression equations for age estimation were constructed using femoral neck density. Changes related to bone histology and bone mineral density over time contribute to our understanding of bony changes with age. As bone comprises not only of its cellular components but also of its non-organic components, it is important to study both histology and bone mineral density. In future, an in-depth assessment of the relationship between bone histology and bone mineral density as it changes over time increase our understanding of how bone ages.

In the first paper published from this study, results from assessing a black South African sample were compared to a previous study performed on the same samle using a different histological method (Keough 2007; Keough *et al*. 2009). This provided information of the use of stereology for this purpose. The second paper dealt with age-at-death estimation from bone mineral density in black and white South African groups. Inter-population variation of the histomorphometric variables used in this study is presented in the third paper. Lastly, validation of the equations set up for white South Africans was done by using a test sample of Danish individuals. A summary of these four papers are given here.

8.2. The use of dry bone in age-at-death estimation (summary of papers)

8.2.1. Histological age estimation in black South Africans using stereology

Chapter 4 introduces the use of stereology in the histomorphometric assessment of bone for age-at-death estimation (Botha *et al*. 2018). A total of 99 bone sections (n = 60 males and n = 39 females) from a black South African sample were used to create regression equations. The original study on this sample (Keough 2007; Keough *et al*. 2009) made use of traditional 2D methods for analysis of 10 histological variables and included the assessment of variables such as the average number of lamellae per osteon, non-Haversian canals and resorption spaces which were excluded in the current study. The results of this study showed a moderate correlation of age with the average number of osteons and osteon size. Haversian canal size, however, showed a low correlation with age. Overall, no statistically significant differences were seen between males and females. Single regression analysis was done for pooled, as well as separate sex groups. However, only pooled-sex multiple regression was done as this statistical method

requires a large sample. The results of this study and that of Keough *et al*. (2009) were similar as both reported the number of osteons to be the most reliable indicator of age. Standard error estimates and correlation values for this variable were also similar between this study and that of Keough *et al*. (2009), suggesting that intrinsic bone factors, rather than the histological method employed, is the limiting factor for achieving narrower age ranges. However, it did show that stereology may be employed for this purpose. The use of stereology also helped to ensure that the results were accurate and precise.

8.2.2. Age estimation using bone mineral density in black and white South Africans

Chapter 5 (Botha *et al*., 2019) deals with the use of bone mineral density in age-at-death estimation. A major advantage of using bone mineral density values for age estimation is that it is not influenced by observer subjectivity but relies on concrete values which make it a reliable and easy method to use. DXA scans were performed for a South African sample of 123 left femora, of which 64 were black (n = 34 males and n = 30 females) and 59 white (n = 29 males and n = 30 females). Independent-samples t-tests revealed a significant difference between black and white groups for total and neck bone mineral density values. Regression equations were constructed for the white and for the total sample, as the black group did not show a significant correlation with age as far as BMD is concerned. Black individuals seem to maintain a more constant bone mineral density over time than white individuals. Bone mineral density values in whites declined more rapidly over time compared to that of black individuals. Overall, the black group showed higher values for femoral neck BMD than the white group, which is similar to what has been reported by previous studies (Daniels *et al*. 1995; Schnitzler *et al*. 1990). Past studies paid little attention to population differences. Our study clearly showed that bone strength and maintenance during one's lifetime differed between black and white study groups, and that age estimation from bone mineral density may be more complex in black than in white individuals.

8.2.3. Variation in histomorphometric variables used for age estimation

Chapter 6 (Botha *et al*., accepted) explores population variation with regard to several histomorphometric features of bone often used for the estimation of age. A sample of 99 black South Africans, 94 white South Africans and 30 Danish individuals were compared by means of ANCOVA. The results indicated that the three groups differed significantly

from one another in terms of the number of osteons per sampling grid, with Danish individuals having the highest number of osteons (thus having the highest bone turnover rate), followed by white South Africans. Black South Africans had the least number of osteons per sampling grid. Previous studies have demonstrated a significant difference between black and white individuals (Weinstein & Bell 1988; Kleerekoper *et al*. 1994), with bone remodelling rates being higher in whites which is consistent with the current results. However, the results of this study also indicated that there is a difference between white groups from various geographical locations. Secondary osteon and Haversian canal size were similar between groups. Osteon size showed a significant correlation with age, whereas Haversian canal size did not show predictable changes with age and was not significantly correlated with age. Overall, the highest amount of variation was observed in the younger ages, with variables becoming more uniform between the groups as age advances. It is recommended that when applying osteon number (OPD) for age estimation, population differences be considered. However, osteon size appears to be similar between different populations and may be more generally applied when estimating age from skeletal remains.

8.2.4. Age mimicry in histological age estimation

Chapter 7 (Botha *et al*., under review) serves as an example of how problems related to the reference sample may affect age estimation accuracy. A reference sample of 94 white South African individuals (n = 50 males and n = 44 females) lacked an adequate number of individuals within the younger adult age cohorts and was thus skewed towards the older ages. The original sample was then combined with a Danish sample of n = 30 to correct for the skewed age distribution. Single and multiple regression equations were then constructed after a hold-out sample was selected containing 14 individuals (one male, one female per age cohort) for which population affinity was not specified. An RMSE value of 15 years was established which was deemed acceptable. These results reiterate that the size and age distribution of the reference sample is extremely important in avoiding problems such as age mimicry.

8.3. General discussion

This study moved beyond the traditional methods for histological age estimation. The use of stereology provides forensic anthropologists with a tool that allows for the unbiased estimation of age, ensuring accurate and precise results. Although stereology has existed for many decades (Bolender 1992; Mouton 2005), stereological assessment of bone for the purpose of age estimation has only surfaced recently (Lynnerup *et al*. 2006; Villa & Lynnerup 2009). Stereology allows for the assessment of any microscopic features of bone previously employed in traditional two-dimensional histological age estimation methods (e.g., osteon surface area and number). In addition to these variables, stereology was used for the estimation of osteon and Haversian canal volume, as volume assessment of histological features of bone has not previously been assessed for the estimation of age from dry bone. Also, researchers may largely benefit from computer-based stereology systems as it provides a number of design-based stereology probes readily available that allows for the assessment of cross-sections (as used in this study) or vertical section orientation, with differentiation between thick or thin sections. By applying different probes, one may potentially evaluate other characteristics of dry bone such as Haversian canal length in vertically orientated sections.

Overall, results of the current study pointed to the fact that there are differences as far as bone remodelling and changes associated with age between populations are concerned. The Danish sample presented with the highest number of osteons per grid area compared to the white and black South African samples for all age cohorts, whereas the black South African sample presented with the least number of osteons. The white South African sample was found to be between the Danish and black South African groups as far as these bony changes are concerned, suggesting that adaptation to the climate/environment and admixture with other African groups may have played a role in the establishment of their bone turnover status. This implies that population-specific standards in age estimation should not be generally applied to individuals belonging to a different group, as doing so may yield inaccurate results. To establish if a specific method may be applied to a different population group, the method should be tested on that population group. Pfeiffer et al. (2016) managed to successfully apply the method outlined by Cho *et al*. (2002) based on an American sample to a test sample from the Western Cape and found that the regression equations established may be generally applied to individuals from South Africa. However, the results of the current study found

that osteon area was similar for all groups, but that caution should be taken when using only OPD as an estimator of age, as bone remodelling rates (OPD vs. age) differed between the population samples.

Furthermore, a decrease in bone mineral density with age was more pronounced in white than black South Africans. These findings point to the black South African sample having a lower rate of bone turnover and loss as compared to white South Africans, thus supporting the results of the histological analysis. In order to better understand bone loss and its associated histological features, one should consider the development and maintenance of the skeleton. A steady decrease in BMD after the establishment of peak bone mass as age advances is considered normal, as bone deteriorates and ages in accordance to the body's physiological processes (Riggs *et al*. 1981, 1982, 1986). Genetic make-up has also been shown to contribute considerably to bone mineral density, seemingly by affecting acquisition of peak bone mass (Brown *et al*. 2005). Due to many factors influencing the rate of bone loss, functional adaptation of bone over time is complex, especially when considering inter-population variability.

The histological and bone mineral density assessment, supported by previous studies (Weinstein & Bell 1988; Schnitzler *et al*. 1990; Kleerekoper *et al*. 1994; Daniels *et al*. 1995; Cho *et al.* 2006) revealed a noteworthy trend, i.e. that bone turnover rates and bone loss over times seem to be higher for European groups than for African groups. Osteoporosis (severe bone loss classified as abnormal) has been reported to be more prevalent in white/European groups than in black/African groups (Hochberg 2007; Cauley 2011). The more pronounced bone loss trend in white South Africans as compared to black South Africans observed in this study may be accounted for by white individuals being more susceptible to developing osteoporosis. Bone remodelling rates have also been reported to be higher for European than for African groups (Schnitzler *et al*. 1990; Bell 1997; Key & Bell 1999; Nelson & Villa 2003). Differences in remodelling rates between groups may be based on a large number of factors causing variability in normal physiology and pathology (Brant & Pearson 1994). For example, a higher bone mass in black American groups have been reported due to the presence of increased levels of parathyroid hormone and vitamin D in the blood, as well as their ability to preserve dietary calcium more efficiently (Slemenda *et al*. 1997; Heaney 2000; Silverberg & Bilezekian 1999). Genetic, physiological and lifestyle factors, as well as

the interaction between these factors may cause variability in bone on a microscopic level between different population groups.

Although the size of the South African samples in this study was large (black: n = 99 and white: n = 94), the sample composition may be responsible for a lack of a stronger correlation between age and the histological variables assessed. As all individuals included form part of skeletal collections of which the remains came from unclaimed bodies (mostly black individuals from lower SES) or donated bodies (from all levels of SES), some individuals assessed may have been affected by disease/conditions that has the potential to alter bone turnover rates. Their general health and nutritional status, as well as levels of physical activity are unknown. It is likely that some of the individuals suffered from chronic disease and reduced physical activity, which would have affected their bone strength/BMD and rate of bone remodelling. Older individuals, particularly those that are indoor-bound, tend to be relatively inactive and often develop vitamin D deficiency due to a lack of sunlight exposure. Studies have revealed that this phenomenon transpires in both black (Harris 2006; Kennel *et al*. 2010) and white (Rassmussen *et al*. 2000) individuals.

It should be kept in mind that circulatory vitamin D ranges for different population groups vary widely depending on ancestry, age, geographical location (i.e. climate and availability of nutrition) and sampling season (Kennel *et al*. 2010). Considering the results of this study, the ANCOVA analysis (Botha *et al.* 2019) revealed a difference in bone turnover rates between white South Africans, black South Africans and Danish individuals. In addition to genetic composition, differences are most likely related to geographical location, i.e. environmental differences between Danish and white South African individuals, with the largest difference being climate. South Africa experiences greater amounts of sunshine all year round in comparison to Denmark, which favours higher levels of vitamin D in the body which promotes calcium absorption and play an essential role in bone formation and remodelling (Holick 2007). Studies on the Danish population have pointed out that vitamin D insufficiency is wide-spread and common especially among children and the elderly (Rassmussen *et al*. 2000; Petersen *et al*. 2016). In contrast to these reports, Vitamin D insufficiency/deficiency is encountered less in African populations due to adequate sunlight exposure (Aufderheide & Rodriques-Martin 1998; Gloth *et al*. 1999) but not completely absent and that individuals with darkly

pigmented skin, and those infected with HIV or *M. tuberculosis* are at risk (Harris 2006; Norval *et al*. 2016). Higher frequencies of insufficient vitamin D levels such as seen in European populations (Holick 2008; Cashman *et al*. 2016) may give rise to accelerated bone remodelling rates to maintain the bone's microstructure, accounting for a higher number of osteons produced. This most likely contributes to explaining why Danish individuals presented with the higher number of osteons per grid area.

Lastly, a general consideration should be made. It is also important to consider the effect of modern-day treatments to either prevent a significant decrease in bone mineral density, or to repair decreased bone mineral density due to a metabolic condition. Hormone replacement therapy has been proven to have a positive effect on bone mass gain in older men (Orwoll 2005) and post-menopausal women (Cauley *et al*. 1995; Villareal *et al*. 2001) and is usually prescribed for individuals that have access to quality healthcare (mostly individuals from a medium to high socioeconomic status). The effect of such treatments should be considered in age estimation using bone mineral density, as an individual subjected to hormone replacement therapy may skeletally appear somewhat younger than others of the same sex and age. It is thus possible that an individual that received hormone replacement therapy on a long-term basis may be aged inaccurately based on population standards.

8.4. Limitations and future recommendations

The age distribution of the white South African sample is the most notable limitation in this study. Unfortunately, this is the result of what is available in skeletal collections in Gauteng, South Africa. Future research should consider expansion of the current sample, specifically within the younger age cohorts by including individuals from other areas in the country. It remains extremely important to consider the reference sample population structure before applying standards to an individual of unknown ancestry/group as this study did point out differences in terms of the number of osteons/osteon population density. It would be interesting to test the standards provided in this study on population groups from other regions or descent and subsequently adapt the regression equations to fit additional groups.

To overcome problems related to populations differences, sample sizes and statistical inferences, it is essential that reference sample sizes be as large as possible and include individuals from all the population groups within the specific geographical area assessed.

The age distribution of the sample must be equally dispersed to avoid skewness and subsequent problems such as age mimicry. Age estimation standards established using such reference sample will yield more accurate results and may then be applied to all individuals form the included ancestral groups. However, care should be taken when applying such standards to individuals outside the designated geographical area.

Future studies should aim at excluding bias by using methods such as stereology, as this would ensure accuracy and precision of results. Employing stereology is, however, a time-consuming process. After bone slide preparation, scanning of the section should be done by an experienced individual before analysis may commence, making it a lengthy process. Also, computerized stereology systems are expensive. However, if such equipment is available, it is much preferred above traditional histological techniques and methods of analysis as it provides one with a selection of probes for assessment.

Furthermore, variation within the sampling location may have contributed to reduced correlation coefficients between age and the independent variables assessed. Skedros *et al*. (2003) reported variation in the number of osteons between the endosteal and periosteal region of the femoral cortex. It would be interesting to re-assess the variables observed in this study using stereology, whilst differentiating between the endo- and periosteal regions to determine which region provides higher correlations with age.

The study showed that BMD decreases with age, whereas the number of osteons increase as age advances. Future directions should include a direct assessment of BMD vs. the average number of osteons per individual to determine if those individuals with abnormally decreased BMD (osteopenia/osteoporosis) have more osteons (higher bone remodelling rates) as compared to individuals with normal BMD in relation to their age. Also, the presence and size of resorption spaces should be taken into consideration, as osteoporotic bone is often weakened by large cavities (Bandela *et al*. 2015), and its influence on age estimation should be investigated.

8.5. Conclusion

In conclusion, the study found that:

1. Stereology is a valid method for the assessment of age-related histological features of bone and that it provides unbiased, accurate and precise results.
2. Regression equations could be constructed for the prediction of age with a SEE of ±13years.
3. The number of osteons and osteon size showed the highest correlation with age. Haversian canal size did not show predictable changes over time and was poorly correlated with age.
4. Inter-population variation indicated that the number of osteons per sampling grid differs between black South African, white South African and Danish individuals.
5. Bone mineral density showed a significant difference between black and white South African groups.
6. Age mimicry can cause highly inaccurate age estimates. In this study, it was demonstrated how a skewed reference sample may be corrected by an alternative sampling strategy.
7. The use of dry bone histology and bone mineral density should not be underestimated in a forensic context. This study showed that histological age estimation using refined methods may be valuable where incomplete skeletal remains are present and provide an alternative to subjective macroscopic methods.

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APPENDICES

UNIVERSITEIT VAN PRETORIA
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Faculty of Health Sciences

06 April 2016

To Whom it May Concern,

Mrs D Botha from the University of the Witwatersrand has been given permission to collect bone samples from skeletal remains from the Pretoria Bone Collection which is housed in the Department of Anatomy, University of Pretoria for her PhD research entitled "*Histological age-at-death estimations in human bone: Assessment of inter-population variation*". The bone samples will be 3mm in thickness, taken 2cm below midshaft on the anterior surface of the femur.

With kindest regards,

GC Krüger, MSc

Museum Assistant

Curator: Pretoria Bone Collection

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Human Research Ethics Committee (Medical)

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Medical School Secretariat: Medical School Room 10M07, 10th Floor. Tel +27 (0)11-717-2700
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Ref: W-CJ-140604-1

04/06/2014

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: School of Anatomical Sciences (Head: Prof T J M Daly).

Project title: research on cadaveric material.

Reason: In terms of Chapter 8, sections 62-64 of the National Health Act No 61 of 2003 donated bodies and their tissues may be used for, among other purposes, health research. Use of such material is subject only to permission from the responsible person in the School of anatomical Sciences – the Head or person designated by the Head.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2014 -06- 04
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy - HREC(Medical) Secretariat : Anisa Keshav, Zanele Ndlovu.

Declaration: Student's contribution to article(s) and agreement of co-authors

I, Deona Botha, student number 331572, declare that this Thesis is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis.

Signature of Student: .....**Date**.....19/02/2019.....

Name of Primary Supervisor: .....Maryna Steyn.....

Signature of Primary Supervisor: .....**Date**.....19/2/19.....

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article by the student as part of his/her Thesis. In cases where the student is not the first author of a published article, the primary supervisor must explain (under comments) by the student is entitled to use the paper for his/her degree purposes.

Article 1: Title: The use of stereological methods in the histomorphometric assessment of bone for age-at-death estimation.

Journal name, year, volume and page numbers: Forensic Science International 2018; 290:353.e1-353.e7.

Authors	Name	Signature	Date
1st author	D Botha		19/02/2019
2nd author	A Bhagwandin		19/02 2019
3rd author	N Lynnerup		19/2-19
4th author	M Steyn		19/2/19

Comments by primary supervisor:

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Article 2: Title: Age estimation using bone mineral density in South Africans.

Journal name, year, volume and page numbers: Forensic Science International (accepted 16 February 2019).

Authors	Name	Signature	Date
1st author	D Botha		19/02/2019

2nd author	N Lynnerup		19/2-19
3rd author	M Steyn		19/2/19

Comments by primary supervisor:

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Article 3: Title: Inter-population variation of histomorphometric variables used in the estimation of age-at-death.

Journal name, year, volume and page numbers: International Journal of Legal Medicine (accepted subject to corrections).

Authors	Name	Signature	Date
1st author	D Botha		19/02/2019
2nd author	N Lynnerup		19/2-19
3rd author	M Steyn		19/2/19

Comments by primary supervisor:

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Article 4: Title: Age mimicry in histological age estimation: an example.

Journal name, year, volume and page numbers: International Journal of Legal Medicine (under review).

Authors	Name	Signature	Date
1st author	D Botha		19/02/2019
2nd author	M Steyn		19/2/19
3rd author	N Lynnerup		19/2-19

Comments by primary supervisor:

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