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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 μm^2 ≈ 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

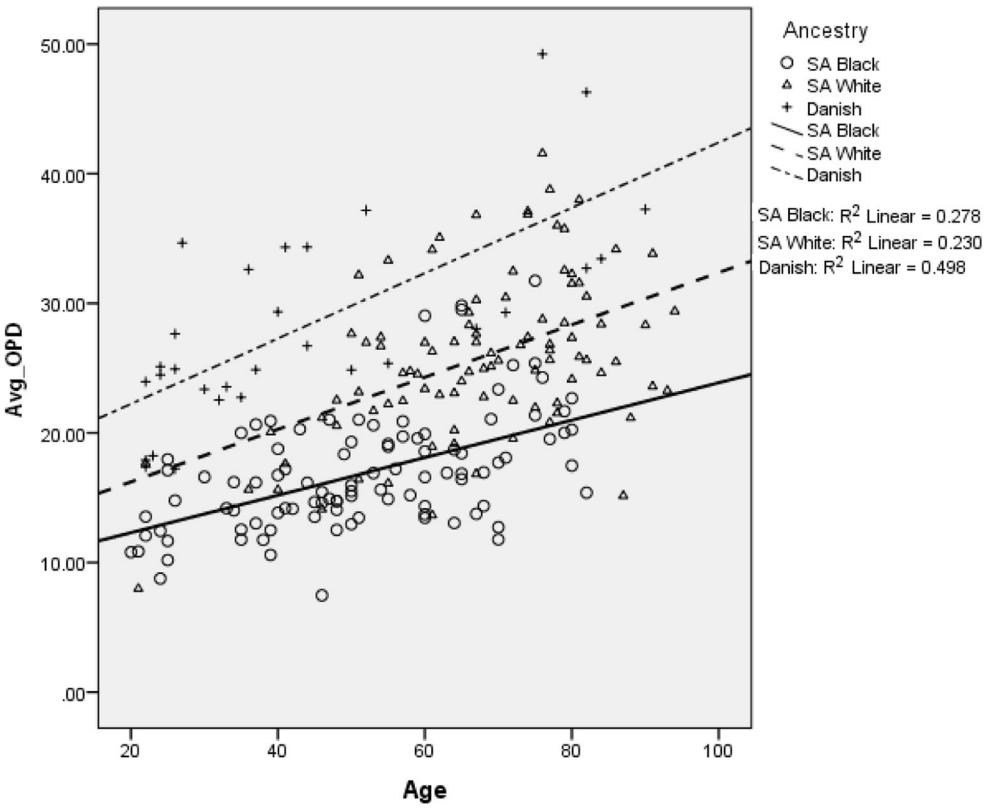

Fig. 1 Regression between average number of osteons per grid area and age

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.

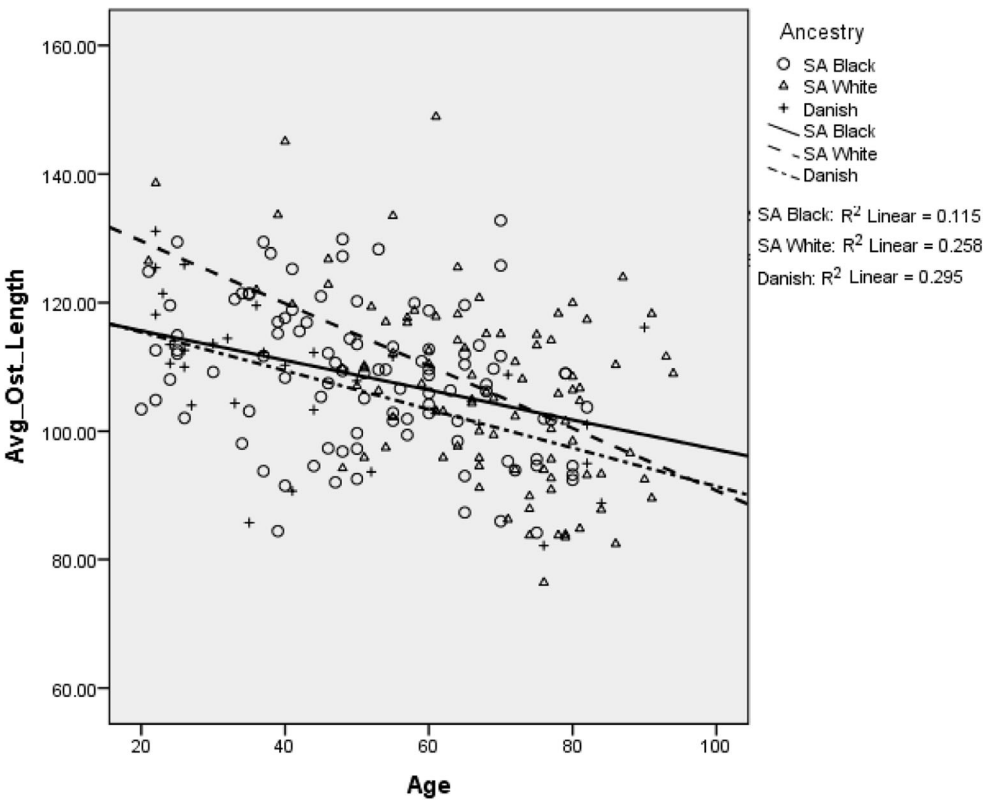

Fig. 2 Regression between average osteon length and age

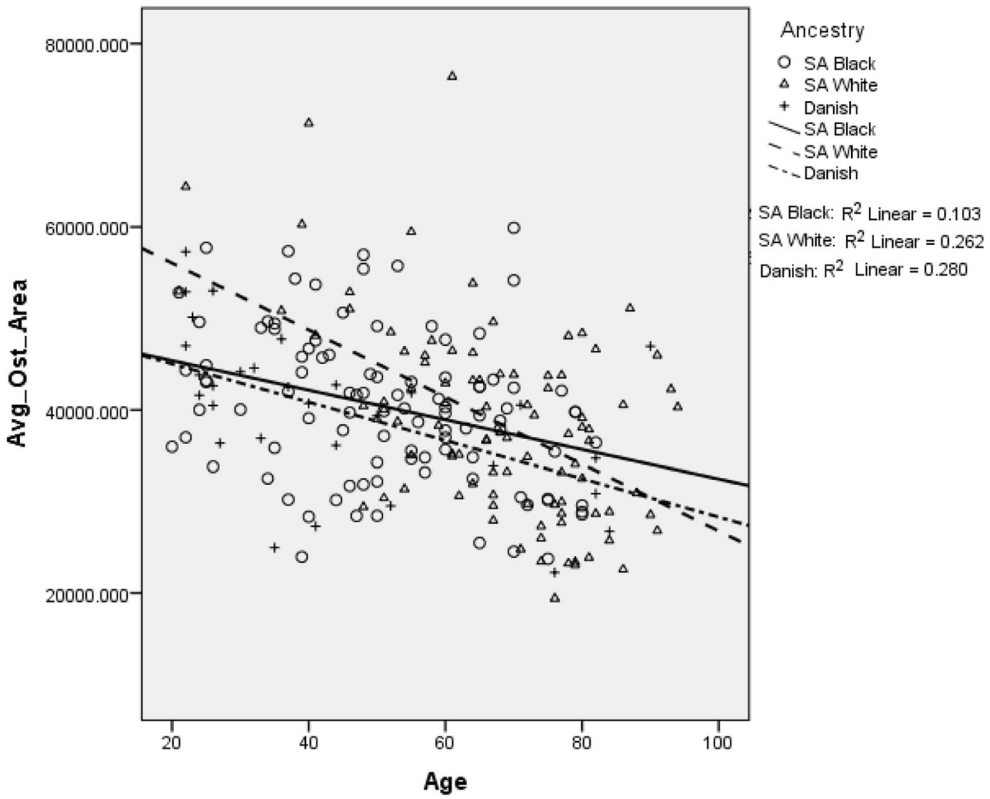

Fig. 3 Regression between average osteon surface area and age

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

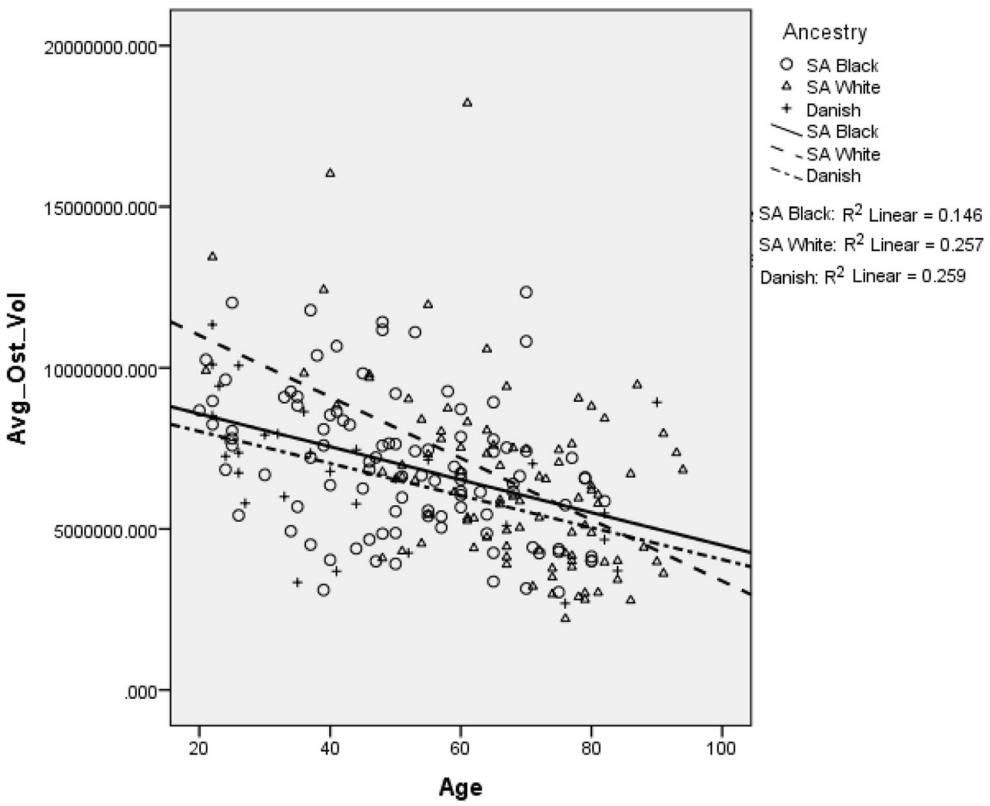

Fig. 4 Regression between average osteon volume and age

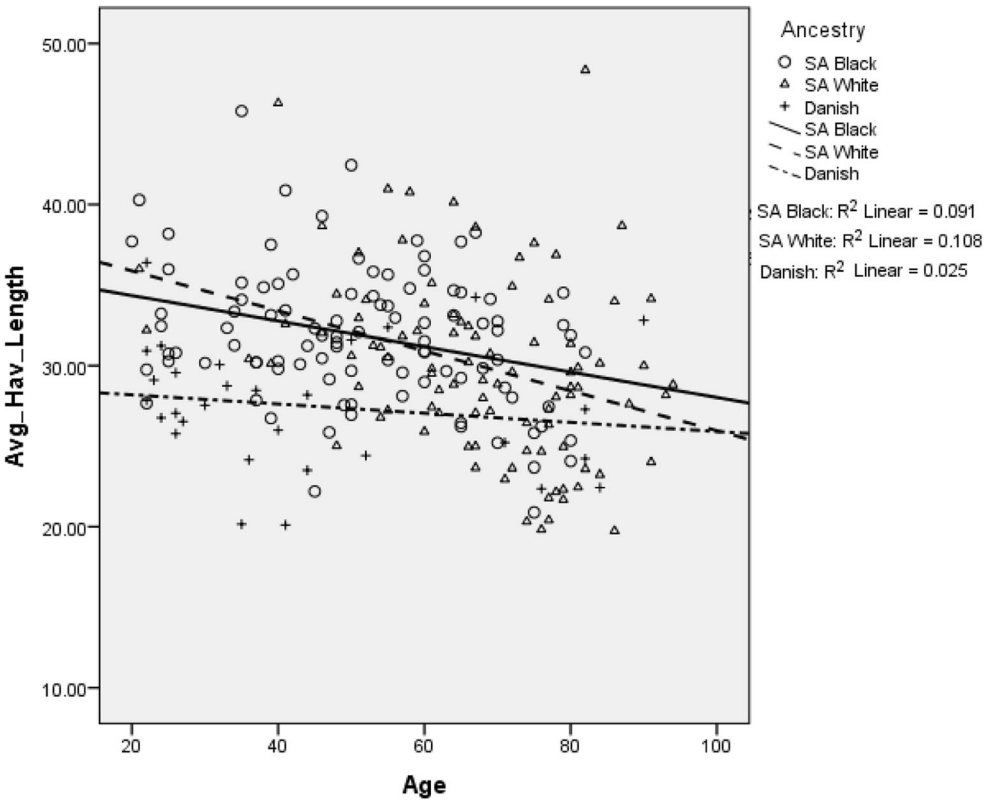

Fig. 5 Regression between average Haversian canal length and age

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

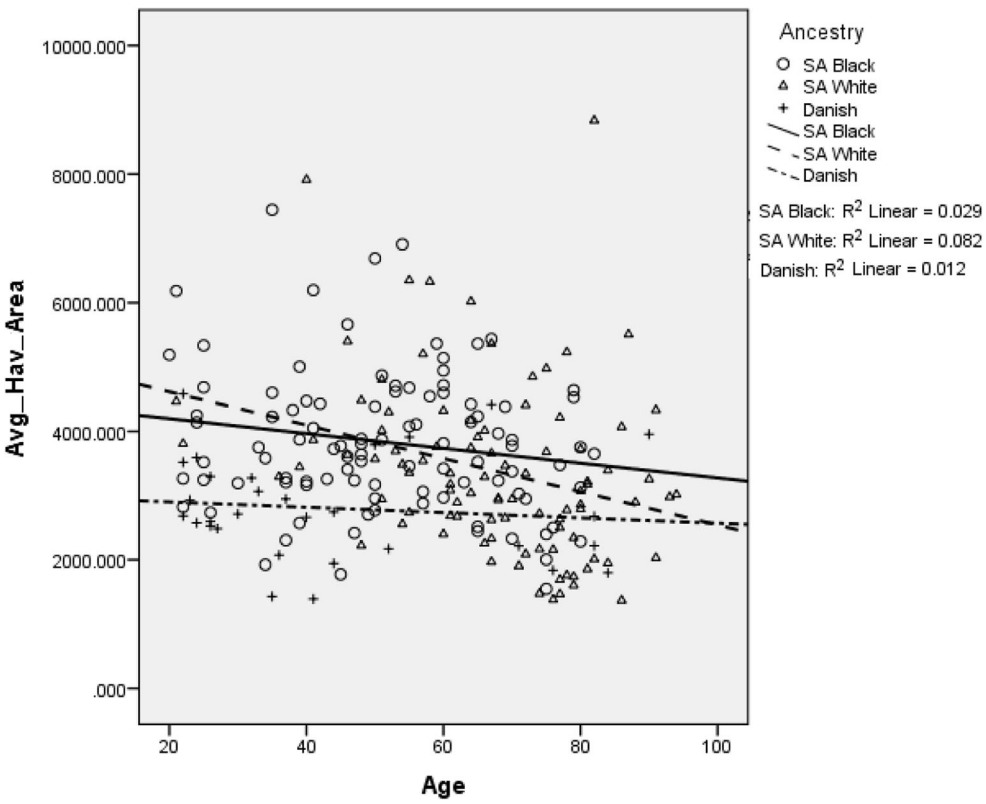

Fig. 6 Regression between average Haversian canal surface area and age

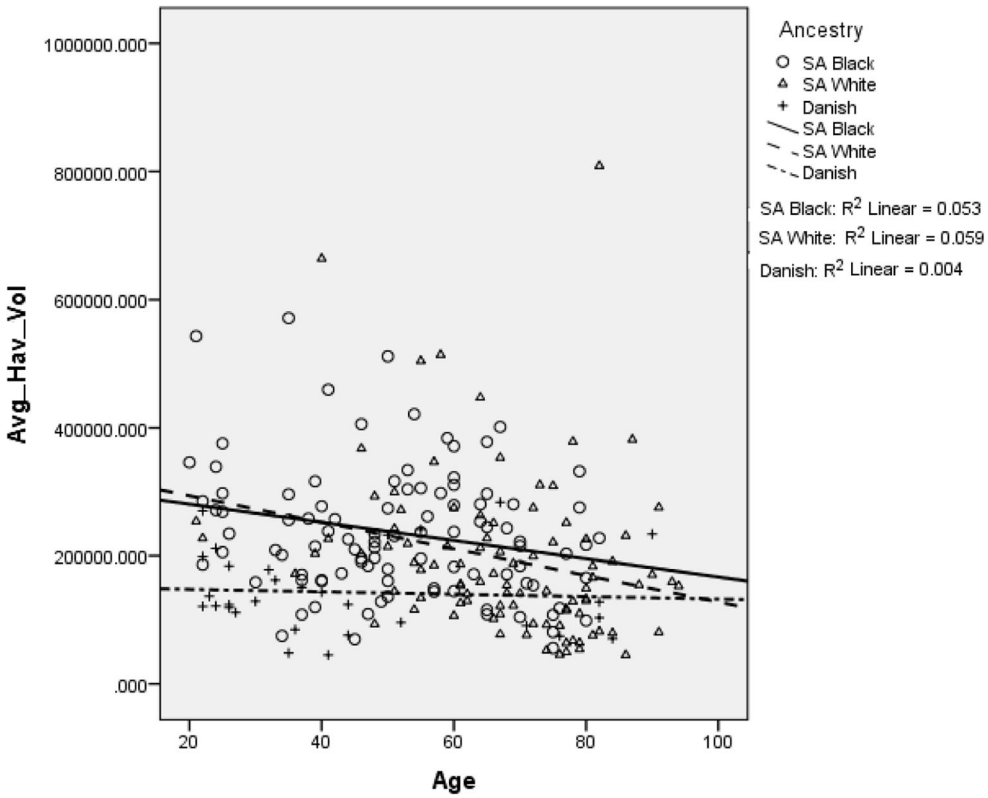

Fig. 7 Correlation between average Haversian canal volume and age

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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