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# Determining 'Age at Death' for Forensic Purposes using Human Bone by a Laboratory-based Biomechanical Analytical Method

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## Abstract

Determination of *age-at-death* (AAD) is an important and frequent requirement in contemporary forensic science and in the reconstruction of past populations and societies from their remains. Its estimation is relatively straightforward and accurate (±3 years) for immature skeletons by using morphological features and reference tables within the context of forensic anthropology. However, after skeletal maturity (>35yrs) estimates become inaccurate, particularly in the legal context. In line with the general migration of all the forensic sciences from reliance upon empirical criteria to those which are more evidence-based, AAD determination should rely more-and-more upon more quantitative methods. We explore here whether well-known changes in the biomechanical properties of bone and the properties of bone matrix, which have been seen to change with age even after skeletal maturity in a traceable manner, can be used to provide a reliable estimate of AAD. This method charts a combination of physical characteristics some of which are measured at a macroscopic level (wet & dry apparent density, porosity, organic/mineral/water fractions, collagen thermal degradation properties, ash content) and others at the microscopic level (Ca/P ratios, osteonal and matrix microhardness, image analysis of sections). This method produced successful age estimates on a cohort of 12 donors of age 53-85 yr (7 male, 5 female), where the age of the individual could be approximated within less than ±1yr. This represents a vastly improved level of accuracy than currently extant age estimation techniques. It also presents: (1) a greater level of reliability and objectivity as the results are not dependent on the experience and expertise of the observer, as is so often the case in forensic skeletal age estimation methods; (2) it is purely laboratory-based analytical technique which can be carried out by someone with technical skills and not the specialised forensic anthropology experience; (3) it can be applied worldwide following stringent laboratory protocols. As such, this technique contributes significantly to improving age estimation and therefore identification methods for forensic and other purposes.

Keywords: age at death, method, estimates, forensics, biomechanical features, bone tissue

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## 1. Introduction

Age at death is one of the main four physical attributes that a Forensic Anthropologist may be called to estimate when attempting to identify unknown skeletal or decomposed human remains, along with an estimation of sex, stature during life and ethnic ancestry. The accurate estimation of age at death in human remains is reliant on recognition of several age-related changes that happen in the skeleton at predictable times during an individual's development. In infants, juveniles and adolescents, these skeletal changes are governed mostly by intrinsic genetic and hormonal factors, which mean that there is little variation between the chronological age at which certain recognisable milestones, such as epiphyseal fusion of long bones, are reached. This makes age at death estimation of sub-adults much easier and more likely to be accurate than in skeletally mature individuals [1].

Several qualitative methods for age at death (AAD) estimation exist, such as the assessment of the eruption of specific deciduous and permanent teeth, or the observation of epiphyseal fusion at different anatomical sites on the skeleton. In juveniles, dental eruption occurs at regular intervals to allow quite accurate (±3 years) age estimation. However, the reliability of age estimation declines with increasing age. Once an individual has reached skeletal maturity (in biomechanical terms this after about 35yrs) the age-related changes that are visible on the skeleton are mainly degenerative and are influenced by a combination of intrinsic and extrinsic factors such as genetics, diet, exercise and activity. This means that it is unlikely for two individuals of the same chronological age to show exactly the same age related skeletal changes. This variation between individuals means that age estimation of adults is notoriously unreliable [2].

After adulthood, age estimation is based on the assessment of degenerative changes to the symphyseal faces of the pubis and the auricular surface of the ilium; as well as progressive fusion and obliteration of the cranial sutures, the ossification of cartilage at the sternal end of the fourth rib, and the degree of femoral cortical remodelling [3]. AAD estimates based on these methods are not usually more accurate than ±10 years, and lead at best to an approximate age range and not an actual age.

There have been several attempts in the past twenty years to improve quantitative methods of AAD estimation, and to introduce methods that do not rely as heavily on the expertise and experience of the investigator, as is often the case with the observational morphometric techniques. These have focussed on the premise that intracortical porosity of bone and bone remodelling increases with age, and assessment of correlation between known chronological age and characteristics of histomorphological bone features, such as primary and secondary osteon numbers/size/maturity level,

trabecular volume and cortical width [4-12]. These methods have been found to be of only limited value as errors for age estimation exceeded ±8 years in over half of the cases in these studies.

The aspartic acid racemisation technique for age estimation [13], first developed in 1979, has been tried and tested in the archaeological and forensic context [14-16]. This method is very laboratory and protocol-dependent [17], and achieves an average accuracy of ±5 years in bone tissue and ±3 years at best in perfectly preserved teeth. An age-dependent accumulation of D-aspartic acid has also been demonstrated in bone osteocalcin [16], which has led to age estimations within ±5 years (at a 95% prediction interval for the data). However, as a forensic technique, aspartic acid racemisation is complex, slow and inherently inaccurate for mature female remains [15].

*Bone tissue and the chronological donor age*

In contrast to previous histomorphometric studies of bone, which are only based in phenomenological changes in the bone cortex, Zioupos et al.[18-22] have engaged in a material characterisation of the various bone phases at the macroscale and the microscale. In an attempt to evaluate the factors affecting the biomechanical properties of bone as function of age (for the benefit of orthopaedic and clinical biomechanics [18]) a number of physical characteristics were measured *in situ*, or in homogenised (bone powder) form. These physical measures were the bone stiffness and strength in relation to its porosity, mineral content, calcium to phosphorus ratios, the dry density [21], the condition of collagen (thermal shrinkage and content in mature x-links) [18], the elasticity of osteonal and interstitial lamellae [22], the numerical and surface-density of the *in vivo* fatigue microcracks [20] and other similar microstructural features. It was observed that meaningful relationships could be established that can predict some of these age related biomechanical bone characteristics as a function of others. The macromechanical Young's Modulus can be predicted [21] from the chronological age, the dry density and the mineral content with an $R^2$=0.98 as shown in figure 1.

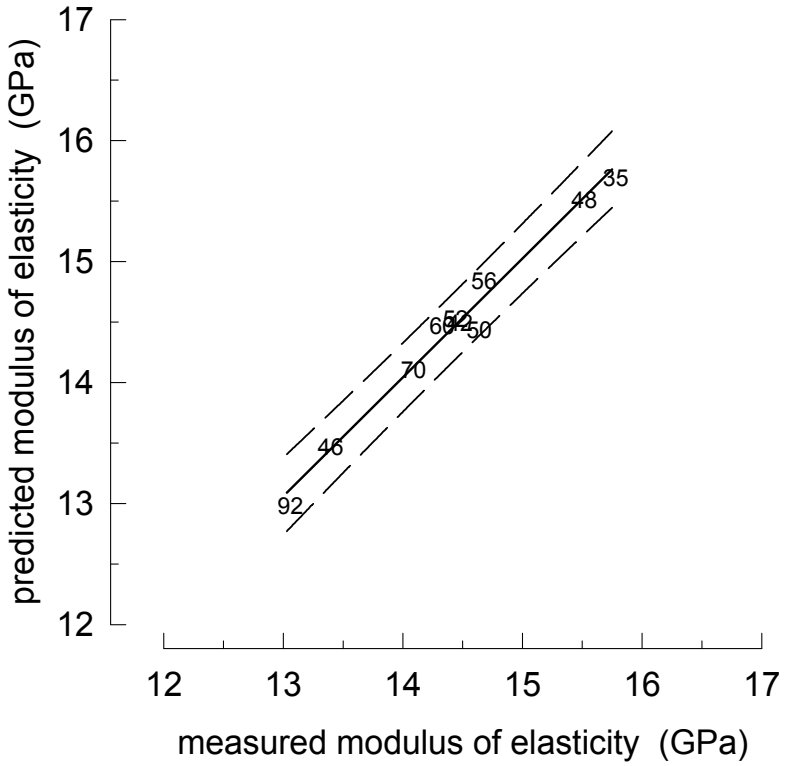


**Figure 1**. Plot of the experimentally measured modulus of elasticity values (from 3-p bending test on bone samples from 10 males donors, age range 35-92 yrs) vs. the predicted values for modulus produced by a predictive relationship which utilises just 3 variables: age, dry density and mineral content [18]. Age itself can explain 48% of the variability in the data. By adding the other two physical characteristics the $R^2$ increases to 0.98. The error in estimating Young's modulus in the worst of cases (50 yrs) is only 1.3% of the actual measured value. Regression line with the 95% prediction interval.

In a similar study [22] the elastic modulus and hardness of secondary osteonal and interstitial bone (Fig. 2), was examined throughout the thickness of the cortex of human femoral bone from 9 male subjects (same cohort 35-95yrs of age) by nano-indentation, which provides both modulus of elasticity and hardness estimates for a material.

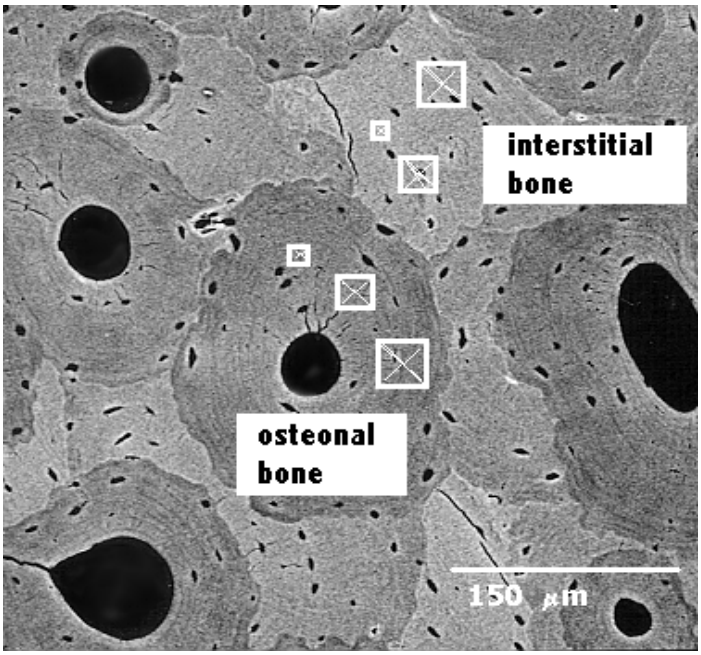


**Figure 2**. Back-scattered scanning electron micrograph produced from a histological section of the mid femur of a 60 yr old donor [19]. Darker areas are those that have been more recently remodelled and contain relatively lower mineral content. 'Older' interstitial tissue areas appear lighter; they are more highly mineralised (higher yield of electrons=higher mineral density). Indentations at three different impression weights were performed in osteonal and interstitial matrix areas [19].

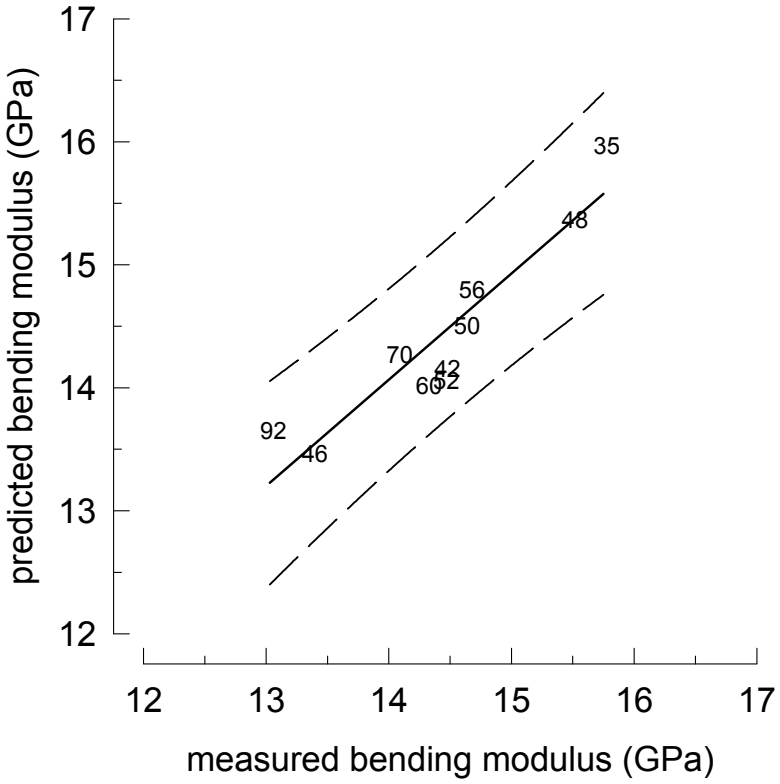


**Figure 3**. An algorithm that combines the mechanical properties of bone at the osteonal and interstitial areas, the relative proportion of these areas and the intracortical porosity can predict the actual experimentally measured elastic modulus of whole bone from these microstructural characteristics with an $R^2$=0.87, which increases to 0.94 if the donor age is added in. Regression line with the 95% prediction interval.

By combining results on the area fraction occupied by secondary osteons, the nanoproperties of these osteons and the intracortical porosity in a 'rule of mixtures' approach, the bending modulus of the whole bone could be predicted with an $R^2$=0.88 as shown in figure 3. If the chronological age of the donor is known and is added in as an extra independent variable, the $R^2$ increased to 0.94. This indicated that 'age' still carried extra non-quantifiable information about the quality of the bone of the donor which is not easily captured by these physical characteristics, but is due to factors such as nutrition, genetics, lifestyle or damage.

From these previous studies it becomes obvious that there may be a number of physical features and microstructural characteristics, which are traceable with the age of the donor and most importantly in cohorts extending beyond the maturity threshold (35yr and older) where most AAD determination methods become inaccurate. The obvious question to ask is: what will happen if one reverses the analysis (i.e. instead of tracking physicochemical or mechanical characteristics as a function of age) so as to estimate age as a function of these bone quality factors?

This paper presents efforts made in the quest for an age estimation technique based on bone matrix features (those produced by material characterisation techniques such as porosity, mineral content, organic fraction, collagen thermal degradation data, osteonal and matrix hardness etc.) to explore whether in this way a more accurate and robust AAD method can be produce.

## 2. Materials and Methods

The bone received from the tissue bank was from 7 males (55-85yr) and 7 females (53-79yr) who had died from causes that did not affect the bone condition and had not been hospitalized for any length of time. The samples were ~3mm thick sections from the mid- femur (Fig.4), which were washed thoroughly with buffered saline solution and kept at -20°C in between tests and during storage. Cylindrical bone disks (pellets) approximately 5mm in diameter were drilled, from the cortical bone area at the anterior part of each specimen to be used in the different tests (Fig. 4). This was achieved by the use of a diamond coring-tool under continuous irrigation with saline solution. Two specimens, both females aged 70 and 56, were found to have too thin and irregular cortices and were used as material for setting up the experiments. Finally a set of 12 (7♂, 5♀) were used for the analysis of this article.

The apparent density ($D_{ap}$), bone matrix (material) density ($D_{mx}$) and the Porosity ($P_{vol}$) of bone were measured in the disk size specimens (diameter 5mm), which were reduced to a pellet of 1.5mm in thickness. The dimensions of each pellet were measured using MITUTOYO Digital Callipers to produce a volume measure ($V_o$). Weights were measured by use of an electronic microbalance (METTER TOLEDO® College B154) either in air, or in submersion using a liquid of known density (distilled water, density ~1 g/cm$^3$). Samples were first weighed submerged ($W_{sub}$) and then in air (wet weight of bone – $W_w$). Between these two operations, samples were placed in a centrifuge (MSE® Mistral 1000) for 3 minutes with a speed of 1,000 rev/s to remove excess amounts of water from their major pores. Samples were placed in the oven for 72 hours at ~38°C and then weighed again in air to produce a dry weight measure, $W_d$. From these values (where $\rho$ is the density of the water solution used):

| | | |
|---|---|---|
| Apparent density, (g/cm$^3$) | $D_{ap} = W_w / V_o$ | (1) |
| Bone matrix, density, (g/cm$^3$) | $D_{mx} = \rho W_w / ( W_w - W_{sub} )$ | (2) |
| Volumetric porosity, | $P_{vol} = [ 1 - ( D_a / D_m )]$ | (3) |
| Dry density, (g/cm$^3$) | $D_{dry} = W_d / V_o$ | (4) |
| Water fraction, | $WF = ( W_w - W_d ) / W_w$ | (5) |

The samples were then demineralised with Ethylenediaminetetraacetic acid (EDTA) 0.5M, pH=7.4 (over two weeks period by changing the solution every two days) and were subsequently dried out over 3 nights at ~38°C, after which the dry demineralised weight $W_{dd}$ was measured to produce the:

| | | |
|---|---|---|
| Organic fraction | $OF = W_{dd} / W_w$ | (6) |
| Mineral fraction | $MF = ( W_d - W_{dd} ) / W_w$ | (7) |

Mineral content was also measured by the more commonly used ashing method. Pellets were weighed wet and dry and they were then placed in the furnace for 20 hours at a temperature of 800°C in porcelain crucibles. The resulting values for the mineral content by ashing were '$Ash_{wet}$' and '$Ash_{dry}$' depending onto whether the ash weight is estimated as a function of the initial wet or dry bone weight respectively.

In addition, porosity ($P_{opt}$) was measured by optical imaging at ×100mag with the use of Image Pro Plus 6.0 (Media Cybernetics Inc, MD 20910, USA) software. Images were converted to grey scale and a mask was applied by using the segmentation command that segregates the areas of interest within the same colour histogram [details in 23]. Once the mask was applied it converts the image into black and white areas of interest. The white areas of the images were calculated and totalled as well as the black areas. The optical porosity data was then obtained by subtracting the white from the black area. Three images were collected per sample to obtain a mean '$P_{opt}$' value.

Microhardness measurements were produced by use of an INDENTEC HWDM-7 instrument to produce Vickers microhardness values (equipped with a square-shaped pyramid diamond tip of $\theta$ =136°). One pellet sample from each donor was dried out as described previously and embedded in epoxy resin (Metprep Kleer-Set Type SSS) with the cross sectional surface facing up (visible histological features). After 72 hours the resin blocks (each containing 3 samples) were metallographically polished to a mirror finish in a METESERV rotary pregrinder, by the use of 400, 800, 1200, 2500 grinding paper and finally on a MasterTex cloth with MICROPOLISH Alum 3B 6OZ. Indentation values were obtained for secondary osteons, and from interstitial lamellae from five locations in each sample identified as North, South, East, West and Centre (where North was towards the periosteal side of the pellet). The indentation loads used in each location

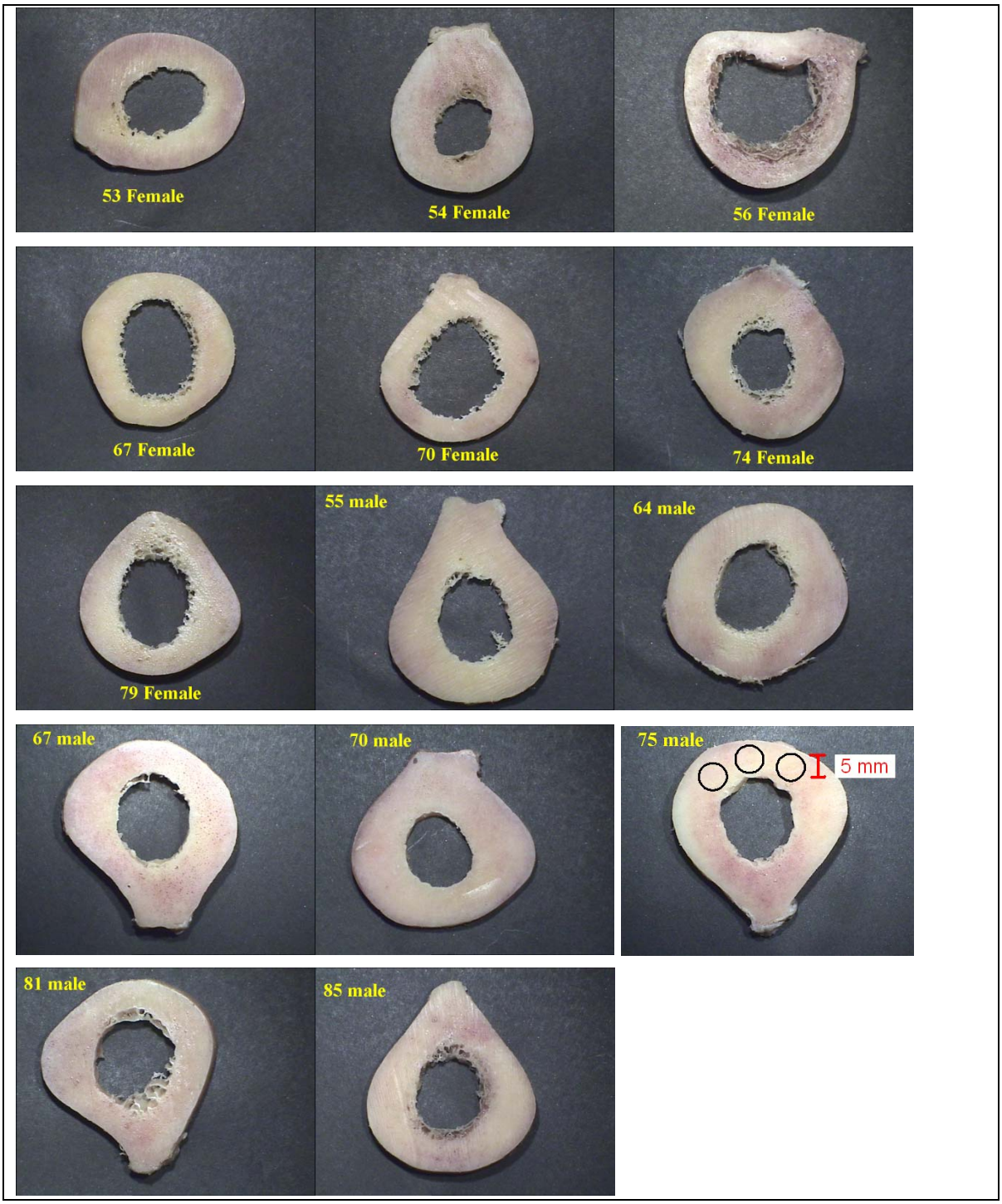


**Figure 4.** Cross-sections from mid-diaphysis of 14 femurs. 'Disk like' specimens were drilled from the anterior side of each cortex for the analysis as shown for the 75 male and were thinned down to 1.5mm thick pellets.

were 10gf, 50gf and 100gf. In total 360 indentations were performed, thirty indents per sample. Different weights were used for selection purposes due to the fact that different weights produce different indentation diagonals, and therefore the ease of measuring under the microscope varies accordingly to weight. The error observed during these processes was found to be approximately ±1-2 Vickers hardness units. During experimentation, great care was undertaken to avoid confounding factors such as, levelling the sample thus allowing the indenter to penetrate in right angles, keeping the loading mechanism free of any vibrations, and discarding any asymmetric or problematic readings.

Nanoindentations were carried out by using a CSM-NHT (system v.3.75, CSM, 2034 Peseux, Switzerland) at 10mN and 100mN max loads and standard loading protocols (load/hold/unload), each indentation lasting 90s (30s in loading/hold/unloading). The nanoindentations were made next to microhardness ones in osteonal and interstitial areas (Fig.5). Five indentations were performed on each site (north/south/east/west/centre) and at 10 and 100mN; first in the

interstitial matrix areas and then in the nearby osteons. This produced 20 nanoindentation readings for each sample and 240 indentations in total. Universal Hardness (UH in MPa) was calculated from load and contact area, and Elastic Modulus values (E in GPa) were produced (assuming a Poisson's ratio value of $\nu$=0.3) in the unloading phase as per the Oliver and Pharr method [24].

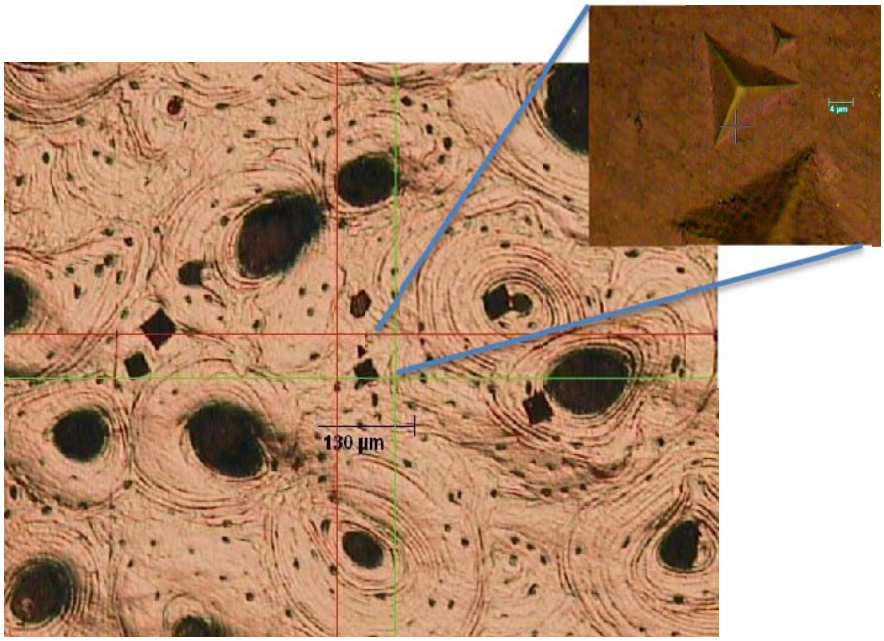


**Figure 5.** Nanoindentations were performed in the same areas as the microhardness ones targeting osteonal and interstitial areas.

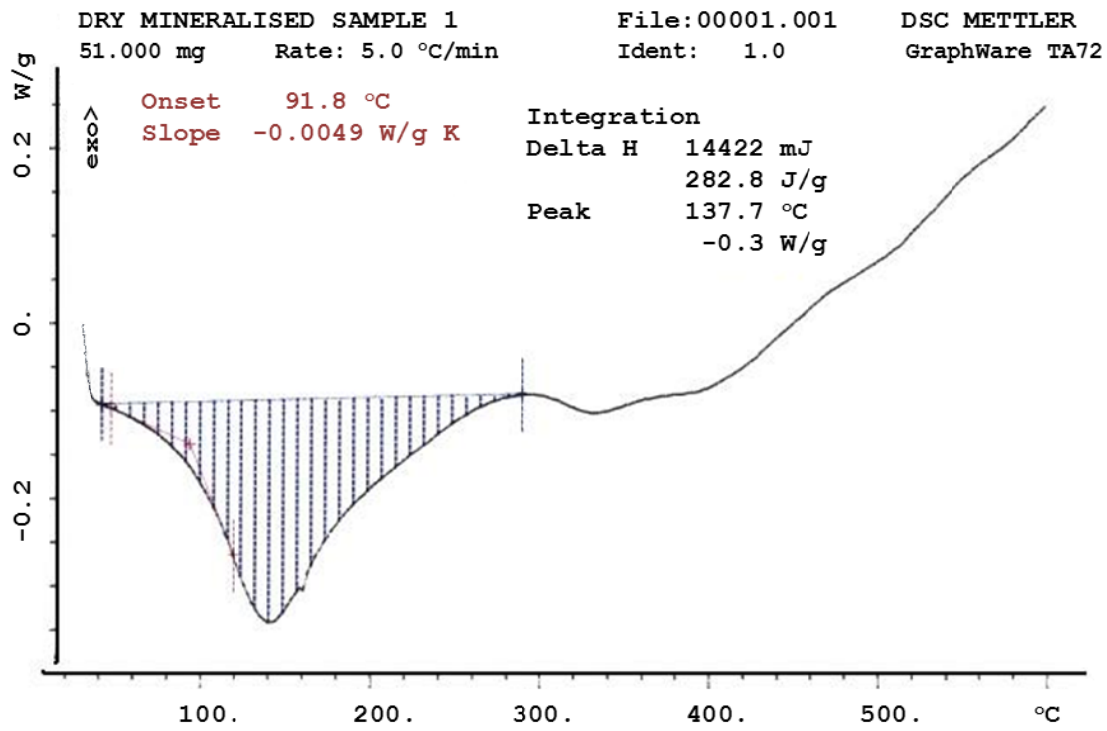


**Figure 6**. A typical DSC printout graph showing the difference in heat flow between the sample and the reference as a function of temperature.

Differential Scanning Calorimetry (DSC) was used to determine the thermodynamic parameters of the denaturation of bone collagen, in the mineralized and demineralized state. It is a standard chemical technique used to characterise compounds that exhibit thermal transitions. DSC has successfully been used to investigate the heat-induced degradation of collagen [25], which is also known to vary with age of the individual. The samples under investigation were initially thinned down manually using grinding paper, under continuous irrigation, to approximately 1.5 mm in height, and left to dry for 5 days. One pellet sample was used in its native state (fully mineralised) and the second one used was demineralised. This second pellet sample provided the demineralised DSC results. The bone samples were then subjected to thermal testing using a Mettler Toledo M3 DSC machine. The temperature inside the central furnace of the machine was raised uniformly at a rate of 5°C per minute from 30°C to 600°C. The reference sample was an identical, empty aluminium crucible. The sample and crucible were weighed after the heating was finished, and the weight of the crucible subtracted from the total, giving the post-testing sample weight. The output graphs from the Mettler DSC machine (Fig. 6) show a clear endotherm, starting at 30°C and peaking at approximately 140°C (for the mineralised samples 'minPeakT'=148°C (SD=4.0); for the demineralised 'demPeakT'= 135°C (SD=10). The mean temperature at onset of the melting phase was found to be 'onsetT' = 98.16°C (SD= 9.65). The point at which the output graph has the steepest gradient represents the temperature at which the greatest rate of change in heat flow between the sample and the reference occurs. The mean value for this was found to be 'maxgradT' = 123.3°C (SD=5.75). However, these two features ('onsetT', 'maxgradT') were not analysed further because they did not provide enough consistency. The mean enthalpy (DeltaH) of the samples was determined by calculating the integral of the curve representing the endotherm (for the mineralised samples 'minDeltaH'=260.4 (SD=33.7); for the demineralised 'demDeltaH'= 133.0 (SD=19.4).

Calcium to Phosphorus ratios were produced by Energy Dispersive Analysis through X-rays (PRINCETON GammaTech IMIX MicroAnalyzer EDAX detector) in an SEM (JEOL JSM-840A) unit. Four calcium to phosphorus ratio values were obtained from each sample. Two values were obtained from secondary osteons and two from interstitial lamellae. The primary data were the percentage values of the normal weights of calcium and phosphorus (WCalcium-m, W Phosphorus -m, WCalcium-o, W Phosphorus -o) and the percentage values of the atomic weights of calcium and phosphorus (Calcium%-m, Phosphorus%-m, Calcium%-o, Phosphorus%-o) in matrix (-m) and osteons (-o). The ratio values were then calculated by dividing the calcium values with those of the phosphorus (WCa/P-m, WCa/P-o, Ca/P%-m, Ca/P%-o). The accelerating voltage used was 10 keV, with a take-off angle of 40°, for the period of 200s in each location measured. The ratio values were calculated automatically from the spectra produced, by the PGT software used by the detector.

### Statistics and Analysis

The level of statistical significance throughout this study is at P=0.05. We are seeking to establish correlations between the various parameters, to observe trends in the data and subsequently to promote multifactorial regressions to predict the known chronological age of the donors from the experimentally measured parameters. The cohort that was finally analysed comprises 12 healthy donors (7♂,5♀). As such it is probably too small to allow meaningful separate analysis for males and females. We will therefore, do the analysis for the mixed cohort. That is not necessarily a drawback as knowledge of sex in forensic remains is not always known, or it cannot be easily determined from just fragments of bone, or sections. It is therefore, advantageous to design a technique that will work regardless of an individual's known sex.

## 3. Results

Table 1 shows the basic statistics for each variable and the correlations with 'Age'. Only the mineral and organic contents (by the EDTA method) showed significant change with age (going down and up respectively) which probably reflects the increased remodelling that exists with donor age (that is the chronological age of the individual, not tissue age). From the material and physicochemical characteristics the microhardness values showed significant correlation throughout. That in essence may show a link to the plasticity characteristics of bone and we know that these are affected by age at both the macroscopic [18,21] and microscopic level [21,22].

**Table 1**. Descriptive statistics of age and the various parameters are shown in groups for histomorphometry, composition, DSC, EDAX, microhardness and nanoindentation.

(*:significant correlation to Age at P=0.05. NS: non-significant)

| Variable | (units) | N | Mean | Median | StDev | P(0.05) |
|---|---|---|---|---|---|---|
| Age | (yrs) | 12 | 68.75 | 69.00 | 10.74 | |
| $D_{ap}$ | ($g/cm^3$) | 12 | 1.884 | 1.864 | 0.0864 | NS |
| $D_{mx}$ | ($g/cm^3$) | 12 | 2.097 | 2.100 | 0.0540 | NS |
| $P_{vol}$ | | 12 | 0.099 | 0.106 | 0.0454 | NS |
| $P_{opt}$ | | 12 | 0.147 | 0.139 | 0.0267 | NS |
| $D_{dry}$ | ($g/cm^3$) | 12 | 1.748 | 1.735 | 0.0802 | NS |

| | | | | | | |
|---|---|---|---|---|---|---|
| MF | | 12 | 0.643 | 0.659 | 0.0378 | * |
| WF | | 12 | 0.066 | 0.065 | 0.0056 | NS |
| OF | | 12 | 0.290 | 0.274 | 0.0399 | * |
| $Ash_{wet}$ | | 12 | 0.579 | 0.580 | 0.0291 | NS |
| $Ash_{dry}$ | | 12 | 0.643 | 0.635 | 0.0239 | NS |
| | | | | | | |
| WCa/P-m | | 12 | 2.19 | 2.24 | 0.109 | NS |
| Ca/P%-m | | 12 | 1.68 | 1.70 | 0.081 | NS |
| WCa/P-o | | 12 | 2.16 | 2.14 | 0.115 | NS |
| Ca/P%-o | | 12 | 1.67 | 1.65 | 0.088 | NS |
| | | | | | | |
| minPeakT | ($^o$C) | 12 | 148.1 | 148.0 | 4.04 | NS |
| minDeltaH | (J/g) | 12 | 260.4 | 253.8 | 33.66 | NS |
| demPeakT | ($^o$C) | 12 | 134.9 | 139.1 | 9.92 | NS |
| demDeltaH | (J/g) | 12 | 132.9 | 129.2 | 19.43 | NS |
| | | | | | | |
| 10gf-o | (kgf/mm²) | 12 | 57.65 | 57.55 | 3.16 | * |
| 10gf-m | (kgf/mm²) | 12 | 65.63 | 65.55 | 3.64 | * |
| 50gf-o | (kgf/mm²) | 12 | 57.85 | 57.70 | 3.50 | * |
| 50gf-m | (kgf/mm²) | 12 | 66.42 | 66.30 | 3.24 | * |
| 100gf-o | (kgf/mm²) | 12 | 58.46 | 58.60 | 3.45 | * |
| 100gf-m | (kgf/mm²) | 12 | 66.83 | 67.20 | 3.32 | * |
| 10gf(o/m) | (kgf/mm²) | 12 | 0.879 | 0.877 | 0.015 | NS |
| | | | | | | |
| E10mN-m | (GPa) | 12 | 22.73 | 22.97 | 1.589 | NS |
| E10mN-o | (GPa) | 12 | 21.95 | 21.83 | 1.431 | NS |
| E100mN-m | (GPa) | 12 | 21.02 | 21.15 | 1.358 | NS |
| E100mN-o | (GPa) | 12 | 20.02 | 20.01 | 1.381 | NS |
| UH10mN-m | (MPa) | 12 | 934.1 | 913.5 | 89.4 | NS |
| UH10mN-o | (MPa) | 12 | 840.3 | 829.1 | 59.8 | NS |
| UH100mN-m | (MPa) | 12 | 770.1 | 770.9 | 38.3 | NS |
| UH100mN-o | (MPa) | 12 | 715.5 | 729.7 | 43.1 | NS |

-o: osteons; -m: matrix; min-: native mineralised tissue; dem-: EDTA demineralised tissue;

The other parameters showed some weak trends with age going up and down as expected (i.e. up for porosity, down for ash content, down for Ca/P ratios etc.), but insignificantly so. When looking separately in male and female data there were also some hints present (Fig. 7) that certain parameters which relate to the remodelling rate (and this rate arguably varies with age) like for instance, the collagen denaturation parameters (peakT and DeltaH) may in fact carry some useful information, which could be further explored through multifactorial regressions.

### *3.1 Stepwise regressions*

The main tool we use here is stepwise regressions performed in Minitab (v.15). These have demonstrated in the past [18,21,22] that the various underlying physicochemical characteristics of bone carry useful information, which can be linked to mechanical characteristics and the chronological age of the donor. These effects are not immediately obvious, as illustrated by the lack of strong correlations in Table-1. However, it has been shown that the various factors interplay and also lie dormant until when combined with another factor produce a relationship of highly significant predictive value. What makes this possible, and a prerequisite for this to happen [21], is that small variations of, for instance, factor A below and above its average value, must be in synchronisation with the related variable (i.e. B) with which it links in a rationalistic manner. A good example is mineral content, bone material stiffness and age. Mineral content changes very little and in fact it may not significantly change with age, but when one examines its variations between individuals it becomes apparent that mineral variations follow similar small and synchronous variations in the modulus of

elasticity. This explains the apparent paradox that although properties A and B may not significantly change with C, they still significantly relate to each other.

It can be argued that modern powerful computer programmes churn out a number of equations with no underlying knowledge of the causal links between the parameters. For that reason we are obliged to apply a 'reasoning' filter by checking whether (1) associations make sense, (2) are expected and (3) go the right way up/down as expected, for instance an increase in mineral content is associated with an increase in stiffness because that is a well-established effect.

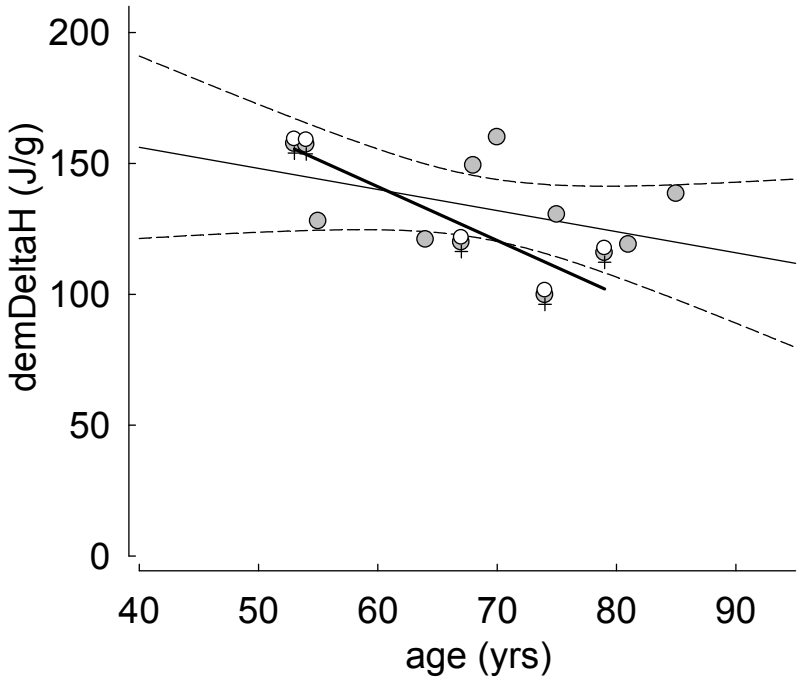


**Figure 7**. Enthalpy values from DSC tests for EDTA-demineralised samples in the mixed cohort (N=12, least squares regression and its 95% confidence interval) and for females (♀ heavier regression line) vs. Age. There is an obvious trend, but while in the mixed cohort this is just below statistical significance level ($R^2$=0.20; P=0.140); in females the effect is much clearer ($R^2$=0.80; P=0.025).

### *3.2 Unrestricted global analysis*

Multifactorial stepwise regressions were attempted with different intents in mind so as to satisfy different interests such as: potential forensic applications, best resources and tests management and/or time management. To start with in an 'unrestricted' case where neither resources nor time is a problem we run first a stepwise regression where the $\alpha$-value to input and withdraw a parameters was set at a=0.10. In this case using the whole set of 32 predictors ($D_{ap}$, $D_{mx}$, $P_{vol}$, $P_{opt}$, $D_{dry}$, MF, WF, OF, $Ash_{wet}$, $Ash_{dry}$, WCa/P-m, Ca/P%-m, Ca/P-o, Ca/P%-o, minPeakT, minDeltaH, demPeakT, demDeltaH, 10gf-o, 10gf-m, 50gf-o, 50gf-m, 100gf-o, 100gf-m, E10mN-m, E10mN-o, E100mN-m, E100mN-o, UH10mN-m, UH10mN-o, UH100mN-m, UH100mN-o) the best performing equations were:

| Equation | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|
| Constant | -114.51 | -86.03 | -54.41 | -45.89 | -24.98 |
| 10gf-m | 2.79 | 2.63 | 2.39 | 2.03 | 2.02 |
| P-Value | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| demDeltaH | | -0.134 | -0.216 | -0.291 | -0.314 |
| P-Value | | 0.012 | 0.001 | 0.000 | 0.000 |
| $P_{vol}$ | | | -53.3 | -45.7 | -49.8 |

| | | | | | |
|---|---|---|---|---|---|
| P-Value | | | 0.014 | 0.005 | 0.002 |
| UH10mN-o | | | | 0.0264 | 0.0219 |
| P-Value | | | | 0.011 | 0.014 |
| demPeakT | | | | | -0.094 |
| P-Value | | | | | 0.064 |
| $R^2$ | 0.894 | 0.949 | 0.977 | 0.991 | 0.995 |
| $R^2_{adj}$ | 0.883 | 0.938 | 0.968 | 0.986 | 0.992 |

The last of these equations reads:

$$Age(yr) = -24.98 + 2.02(10gf\text{-}m) - 0.314(demDeltaH) - 49.8(P_{vol}) + 0.0219(UH10mN\text{-}o) - 0.094(demPeakT) \quad (12)$$

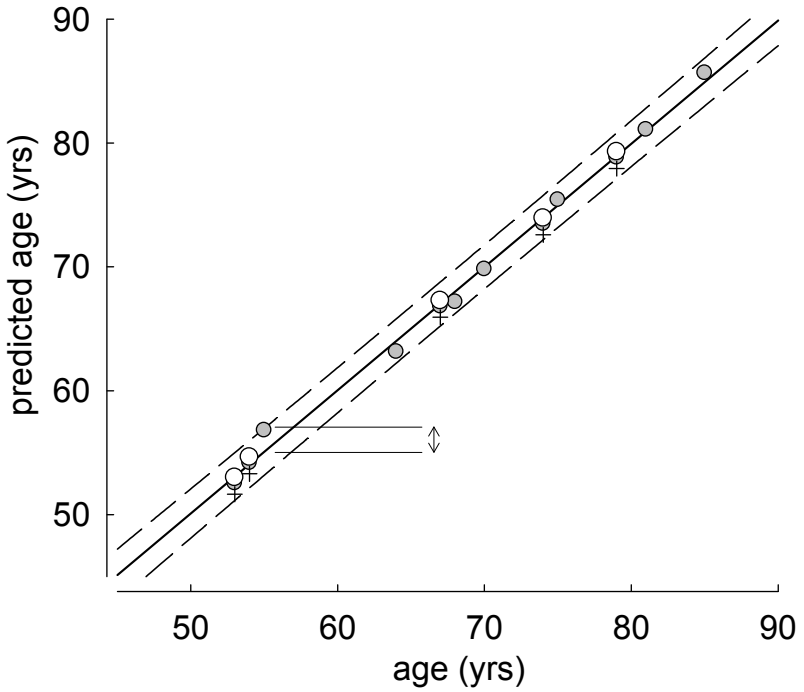


**Figure 8**. Plot of real age vs. predicted age (AAD) from equation 12. The error was on average 0.53±0.48 (SD); in the worst of cases (arrow) was -1.8 yrs equal to a 3.2% of the actual age at death. The $R^2_{adj}$ was 0.992 and the standard error of estimate (SEE) was ±0.95 yrs. The Regression line with the 95% prediction interval is shown.

The $R^2$ adjusted for the degrees of freedom is 0.991 (Fig. 8). The residuals varied between -1.8 and +0.8 years (an error of 3.2% in the worst of cases) a performance as good or better than any alternative AAD method reported in the literature.

Since this analysis is churned out from the statistical package with no imposed preconditions we can apply some 'reasoning' filters for quality control. (1) It would be preferable that no more than 2 parameters are utilised from each test, whereby test is hardness, DSC, EDAX etc. (i.e. no more than 2 microhardness values or no more than 2 nanoindentation values and so forth); this is indeed the case and the programme identified that the cross-correlation of any these 2 parameters (i.e. demPeakT and demDeltaH) is low enough to enter the equation as independent predictors. (2) That all coefficients generated for each equation are themselves significant at P=0.05; entering more parameters does not decrease but rather increases the statistical significance of previous parameters, e.g. the statistical significance of the coefficient of $P_{vol}$ (porosity) increases from P=0.014 to P=0.002 after introducing 2 extra parameters, rather than decrease in value. (3) That the chosen parameters rationalistically link to age and to the age related effects that are already documented in the literature. Indeed (a) two of the five chosen parameters are the micro- and nano-hardness; (b) these were the ones derived at the lowest weights (10 grams force and 10 mN) indicating the well known fact that ageing effects occur at a localised level; (c) two of the five parameters were derived from the DSC tests, which depict

collagen related chemistry (this has been shown to change with age [19,21]); and finally (d) the particular DSc values are those produced from the demineralised samples (rather than the native mineralized one), since demineralisation is expected to enhance the demonstration of this collagen related effect.

### *3.3 Tests and analysis with limited resources*

We further considered two useful permutations in the forensic context, one where resources may be a problem and one where a lab is well resourced but we are seeking a rapid answer within 24 hours. In the first case we run a stepwise regression for parameters that do not require the use of an SEM, a NanoIndenter and a DSC system (the more expensive pieces of equipment). In that case the 16 selected parameters would be: $D_{ap}$, $D_{mx}$, $P_{vol}$, $P_{opt}$, $D_{dry}$, MF, WF, OF, $Ash_{wet}$, $Ash_{dry}$, 10gf-o, 10gf-m, 50gf-o, 50gf-m, 100gf-o, 100gf-m. The resulting best performing equations are:

| Equation | 13 | 14 |
|---|---|---|
| Constant | -114.51 | -95.81 |
| 10gf-m | 2.79 | 4.60 |
| P-Value | 0.000 | 0.001 |
| 100gf-m | | -2.1 |
| P-Value | | 0.094 |
| $R^2$ | 0.894 | 0.924 |
| $R^2_{adj}$ | 0.883 | 0.907 |

$$Age(yr) = - 95.81 + 4.6\ (10gf\text{-}m) - 2.1\ (100gf\text{-}m) \quad (14)$$

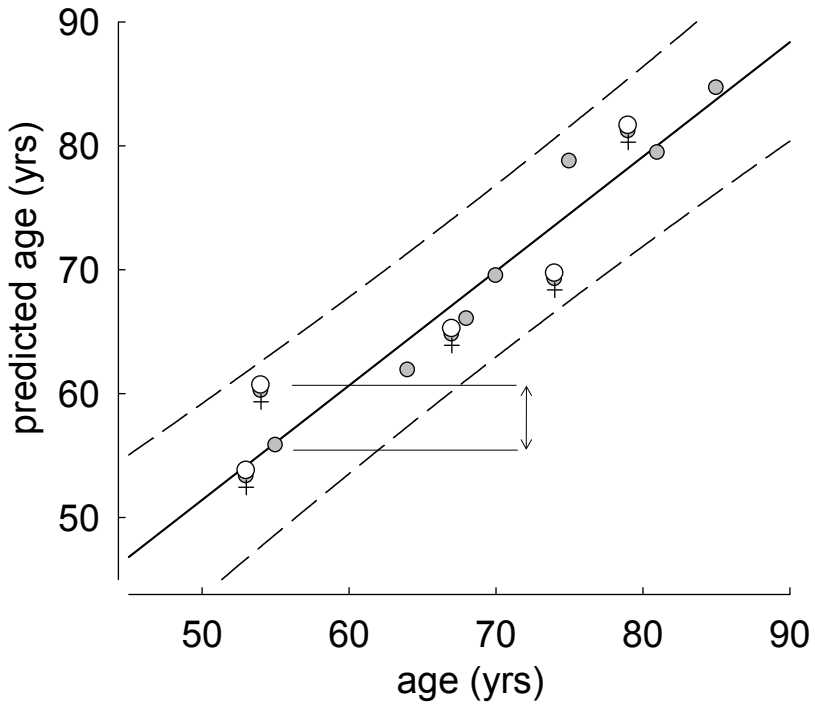


**Figure 9**. Plot of real age vs. predicted age (AAD) from equation 14. The error was on average 2.23±1.85 (SD); in the worst of cases (arrow) was -6.2 yrs equal to a 12% of the actual age at death. The $R^2_{adj}$ was 0.907 and the standard error of estimate (SEE) was ±3.2 yrs. The Regression line with the 95% prediction interval is shown.

The regression results indicate that we can obtain a reasonably good estimate ($R^2_{adj}$=0.907) for AAD by using just two micro-hardness values at 10 and 100 grams force and by targeting the interstitial matrix areas (Fig.9). Again statistics show that matrix areas carry some valuable age related information (not necessarily the osteons) and therefore the

mechanical characteristics of the material are on the whole more important than the histomorphometric features (density, porosity, mineral content etc.).

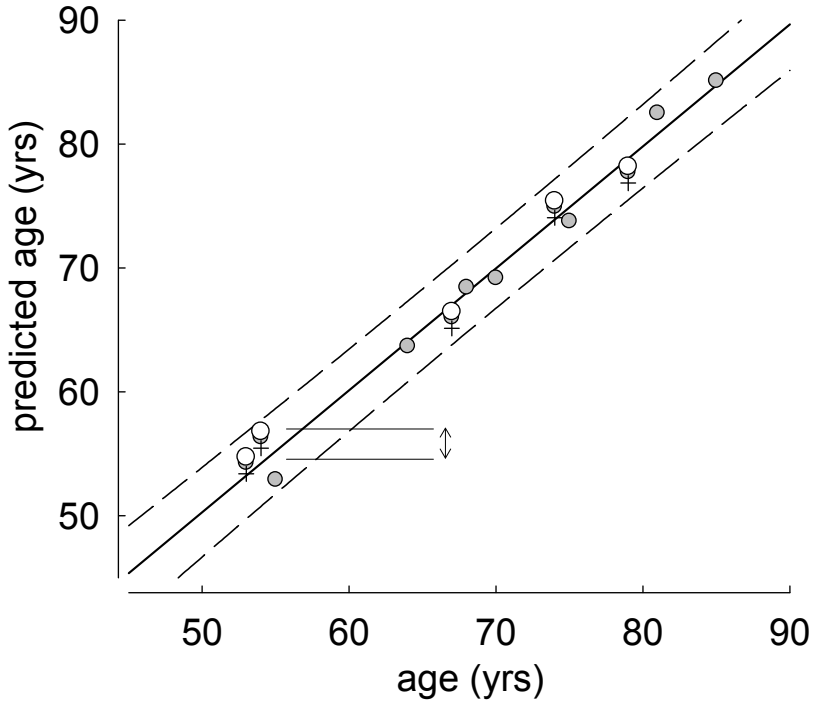


**Figure 10**. Plot of real age vs. predicted age (AAD) from equation 20. The error was on average 1.11±0.67 (SD); in the worst of cases (arrow) was -2.3 yrs equal to a 4.3% of the actual age at death. The $R^2_{adj}$ was 0.976 and the standard error of estimate (SEE) was ±1.6 yrs. The Regression line with the 95% prediction interval is shown.

### *3.4 Tests and analysis that can be completed within 24 hrs*

Next we considered tests that can give a result within 24 hours as for instance in police work where initially a quick even though less accurate answer is often required. This application would require several operators working in parallel. The 27 selected parameters were: $D_{ap}$, $D_{mx}$, $P_{vol}$, $P_{opt}$, $D_{dry}$, $Ash_{wet}$, $Ash_{dry}$, WCa/P-m, Ca/P%-m, Ca/P-o, Ca/P%-o, minPeakT, minDeltaH, 10gf-o, 10gf-m, 50gf-o, 50gf-m, 100gf-o, 100gf-m, E10mN-m, E10mN-o, E100mN-m, E100mN-o, UH10mN-m, UH10mN-o, UH100mN-m, UH100mN-o.

| Equation | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|
| Constant | -114.51 | -95.81 | -96.54 | -69.01 | -73.04 | -163.98 |
| 10gf-m | 2.79 | 4.60 | 3.59 | 2.56 | 1.26 | 1.43 |
| P-Value | 0.000 | 0.001 | 0.009 | 0.029 | 0.060 | 0.007 |
| 100gf-m | | -2.05 | -2.11 | -1.38 | | |
| P-Value | | 0.094 | 0.063 | 0.139 | | |
| 100gf-o | | | 1.21 | 1.75 | 1.85 | 1.91 |
| P-Value | | | 0.105 | 0.018 | 0.018 | 0.002 |
| UH100mN-m | | | | -0.051 | -0.064 | -0.061 |
| P-Value | | | | 0.046 | 0.019 | 0.004 |
| $D_{mx}$ | | | | | | 35.3 |

| P-Value | | | | | | 0.012 |
|---|---|---|---|---|---|---|
| $R^2$ | 0.894 | 0.924 | 0.946 | 0.971 | 0.959 | 0.985 |
| $R^2_{adj}$ | 0.883 | 0.907 | 0.926 | 0.954 | 0.944 | 0.976 |

$$Age(yr) = -163.98 + 1.43(10gf\text{-}m) + 1.91(100gf\text{-}o) - 0.061(UH100mN\text{-}o) + 35.3(D_{mx}) \quad (20)$$

The equations are presented in terms of increasing power or prediction with the $R^2_{adj}$ ranging from 0.883 to 0.976. Equations 16,17,18 contain a combination parameters whereby some coefficients are less significant than P=0.05. Equation 18 is also based on four different hardness values which were produced on matrix and osteons by microindentation and nanoindentation, which is probably not a good practice. Equation 20 is preferred, which contains three hardness values and an independently produced estimate for the material density of bone (Fig.10).

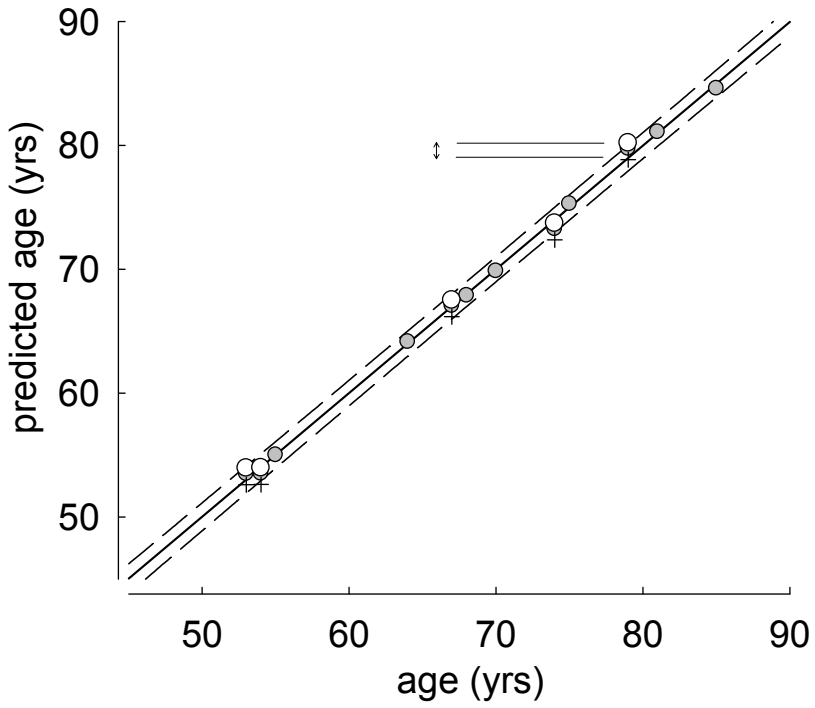


**Figure 11**. Plot of real age vs. predicted age (AAD) from equation 26. The error was on average 0.31±0.26 (SD); in the worst of cases (arrow) was -0.75 yrs equal to a 1.4% of the actual age at death. The $R^2_{adj}$ was 0.997 and the standard error of estimate (SEE) was ±0.6 yrs. The Regression line with the 95% prediction interval is shown.

### *3.5 Selective choice of parameters*

We finally performed a stepwise analysis of regressions by manually selecting a set of eighteen parameters, which we considered most likely to relate to ageing characteristics based on our a priori knowledge of human bone physiology and changes in ontogeny. These were: $P_{vol}$, $P_{opt}$, $D_{ap}$, $D_{mx}$, MF, WF, OF, Ca/P%-m, 10gf-m, 50gf-m, 100gf-m, demPeakT, demDeltaH, 10gf(O/M), UH10mN-m, UH100mN-m, $Ash_{wet}$, $Ash_{dry}$.

| Equation | 21 | 22 | 23 | 24 | 25 | 26 |
|---|---|---|---|---|---|---|
| Constant | -114.51 | -86.03 | -54.41 | -21.74 | -19.83 | -49.31 |
| 10gf-m | 2.792 | 2.629 | 2.395 | 2.294 | 2.232 | 2.246 |
| P-Value | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| demDeltaH | | -0.134 | -0.216 | -0.268 | -0.271 | -0.255 |
| P-Value | | 0.012 | 0.001 | 0.000 | 0.000 | 0.000 |
| $P_{vol}$ | | | -53.3 | -57.5 | -60.6 | -50.1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| P-Value | | | 0.014 | 0.005 | 0.001 | 0.000 |
| demPeakT | | | | -0.138 | -0.156 | -0.089 |
| P-Value | | | | 0.064 | 0.016 | 0.030 |
| $P_{opt}$ | | | | | 35.6 | 42.4 |
| P-Value | | | | | 0.041 | 0.002 |
| UH10mN-m | | | | | | 0.0183 |
| P-Value | | | | | | 0.010 |
| $R^2$ | 0.894 | 0.949 | 0.977 | 0.986 | 0.994 | 0.998 |
| $R^2_{adj}$ | 0.883 | 0.938 | 0.968 | 0.978 | 0.988 | 0.997 |

$$Age(yr) = -49.3 + 2.25(10gf\text{-}m) - 0.255(demDeltaH) - 50.1(P_{vol}) - 0.0892(demPeakT) + 42.4(P_{opt}) + 0.0183(UHmN\text{-}m) \quad (26)$$

Equation 26 turned out to be the most powerful of all produced within this study. It employs parameters, which we know a priori are linked to ageing, and all coefficients produced are significant at P=0.05. The $R^2_{adj}$ is 0.997 and the errors range from -0.75 to +0.73 years (difference between real and predicted AAD), which is ±1.4% of the true age value (Fig.11). This is certainly, and as far as we know, the most successful algorithm for AAD that has ever been reported in the literature.

## 4. Discussion & Conclusions

Estimation of Age at death (AAD) is a notoriously difficult task in mature adult skeletons [26]. It is compounded by the absence of key developmental stages; the variable degree of degeneration of bones between individuals; it can be influenced by lifestyle health and nutrition; and different parts of the skeleton show different traits and rates of change with the chronological age of each individual.

The great majority of the AAD methods are based on gross morphological features of the skeleton and as such they become increasingly inaccurate beyond the age of skeletal maturity (>35 yrs old). They can, in the best of cases, classify the deceased only to within a certain decade of life (i.e. 40-50 yrs old). However, these gross morphology based methods have some advantages. They can be used in situations where the remains have been exposed to harsh environmental conditions or are otherwise physically compromised, and they are not reliant on expensive equipment.

The main technique which utilises physicochemical characteristics of bone and bone like tissues is the amino acid racemization method which is based upon the gradual and temperature depended transformation/racemization of certain biological proteins during the life of an individual. These methods have shown promise and can in the best of cases produce estimate values within ±5yrs of the actual age [13-15,17]. In teeth [27] it produced a 95% CI of ±8.7yrs across the ages and ±6.2yrs for ages less than 35.

Table 2 shows the relative performance of various methods in the literature. As there is no standardised method for expressing the degree of efficiency of the various formulae, there is no better way to compare the various techniques directly.

**Table 2**. Comparison of the performance of various AAD methods in the literature and the present equations 12,14,20,26. SEE: standard error of estimate; $R^2$: coefficient of determination. <Er>: absolute mean error. SDr: standard deviation of the absolute mean error. CI%: confidence interval.

| ref. | tissue | $R^2$ | SEE | <Er> | SDr | CI% |
|---|---|---|---|---|---|---|
| eq-12 | femur | 0.991 | 0.95 | 0.53 | 0.48 | - |
| eq-14 | femur | 0.907 | 3.2 | 2.23 | 1.85 | - |
| eq-20 | femur | 0.976 | 1.6 | 1.11 | 0.67 | - |
| eq-26 | femur | 0.997 | 0.6 | 0.31 | 0.26 | - |
| [9] | femur | 0.574 | 9 | - | - | 68% |
| [11] | pelvis | 0.798 | 6.33 | - | - | - |
| [16] | skull | 0.980 | 2.8 | - | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| [27] | teeth | - | 4.35 | - | - | - |
| [28] | teeth | 0.772 | 8.63 | 6.46 | 5.63 | - |
| [29] | spine | 0.45-0.50 | 11-13 | - | - | - |
| [30] | teeth | 0.35-0.45 | 12-11.2 | - | - | - |
| [31] | pelvis | 0.42-0.50 | - | 8.4-9.3 | 6.4-7.2 | - |
| [32] | teeth | 0.47 | 14.3 | - | - | 65% |
| [33] | teeth | 0.87-0.96 | 7.4-3.9 | - | - | - |
| [34] | skull | - | 18 | - | - | 75% |
| [35] | ribs | 0.55 | 15 | - | - | 68% |
| [36] | teeth | 0.33 | 13.7 | - | - | - |

The present study was organised around a small collection of samples (finally 12 were analysed) on which a maximum number of biomechanics bone analysis techniques were applied. In AAD prediction methods, the collection of samples is usually much larger running in the tens or hundreds. However, this is only required by other methods because the noise contained in the used parameters is such that in order to produce a relationship of statistical significance these large numbers are necessary. In the present study the use of a number of robust measurements, which have proved their worth in bone biomechanics studies, allowed us to produce and demonstrate significant predictive relationships from a small cohort of donors. This in itself is significant. When a study contains 200 participants where this number is needed to obtain a result over the noise, any other blind uncharacterised sample will have to compete against this noise and the result will always bare the same degree of uncertainty. If a good result can be produced by a small random samples of 12 participants it is much more likely that a sample in need of characterisation will find its 'nest' in this cohort easily.

We must emphasize a few other aspects of this study. Firstly, the formulae are produced for sections from the mid-femur. On the one hand, this is a good choice as in cases of dismembered bodies, or bodies where the environment had a damaging effect, this solid compact part of the skeleton is most likely to survive intact [37]. On the other hand, similar relationships have to be produced by other pilot studies for other parts of the skeleton to cover cases where the bone fragments brought for identification are from these other parts of the body. Secondly, further tests may be needed to check the effect of the sampling location on the femur since there are reported variations in bone remodelling at different sites along the long bone (femoral) shaft, and the distribution of histological structures is not uniform at these locations [38]. Thirdly, the ethnicity of our samples: our twelve donors were all Caucasians and although the technique may be useful for samples of any ethnic group, the precise relationship we produce here may be less accurate across populations from outside Europe.

Another limitation of this study is that the method requires considerable resources and time to deliver the best results. Unlike morphology-based methods, this technique demands a laboratory and suitably trained technicians. Morphology-based methods rely more heavily on the expertise, experience and judgment of skilled osteologists, which may mean that a quicker, less expensive result can be obtained. However, in morphology the subjective element which is contained in the analysis performed by the expert osteologist can never be completely ruled out. The current approach even in the absence of some expensive pieces of equipment (SEM, Nano-indenter, DSC) has delivered reasonable estimates (eq-14). Meanwhile, with ample lab resources and technically skilled operators, it can deliver an even better result within 24 hours, should that be necessary for the investigation (eq-20).

Future stages of the validation of the technique will require blind tests by inputting the results from compact bone samples from individuals of similarly known age/race/sex at death into the current formulae. It would also be beneficial to extend this study with a larger sample size, and to have enough samples to allow separation of male and female samples, in order to determine if the accuracy of AAD estimation can be further enhanced with known sex. In conclusion, we suggest that a prediction of AAD based on a combination of biomechanical properties of human bone offers a viable and accurate quantitative alternative to other existing quantitative and qualitative methods used in forensic medicine and archaeo-anthropology.

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