

Article

Age Estimation Through Osteon Histomorphometry: Analysis of Femoral Cross-Sections from Historical Autopsy Samples

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Abstract

Background/Objectives: Age estimation is of fundamental importance in forensic investigations. When traditional methods based on gross bone morphology or morphometric analysis cannot be applied, forensic experts must rely on multidisciplinary approaches. Histomorphometry has consistently proven to be reliable in cases of highly fragmented or incomplete skeletal remains, particularly in older individuals. Building on the foundational study of Amprino and Bairati, this study evaluated the correlations between bone microstructural features in femoral cross-sections and the age and sex of individuals.

Methods: The sample comprised 95 femoral mid-diaphyseal thin sections obtained from autopsy specimens housed at the Institute of Legal Medicine, University of Bari (Italy), representing both male and female individuals aged 18 to 92 years. The numbers and densities of primary, intact secondary, and fragmentary secondary osteons, together with osteon circularity and the mean osteonal area, were measured to investigate age-related variation. Statistical analyses included *t*-tests, Mann–Whitney tests, Spearman’s rank correlation, and General Linear Models (GLMs). **Results:** No significant differences in histomorphometric variables were observed between males and females. However, the number of intact secondary osteons and osteon population density increased with age, while the mean osteonal area and osteon circularity decreased with age. Although some variables displayed significant correlations with age, residual analysis indicated a lack of heterogeneity in variance, which limited the development of a robust predictive model.

Conclusions: The findings highlight both the potential and the limitations of histomorphometry in forensic age estimation. While certain microstructural variables correlate with age, inter-individual variability reduces predictive accuracy. Further research is needed to refine models that account for biological and biomechanical variability, particularly in older adults.

Keywords: age-at-death estimation; compact bone histomorphometry; forensic anthropology; forensic pathology; histomorphometric analysis; histological femoral samples



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1. Introduction

Quantitative bone histology, or histomorphometry, has been used for nearly a century to estimate the age at death, with the first documented study published in 1911 by Balthaz-

ard and Lebrun [1]. In Italy, one of the earliest forensic applications of histomorphometry was carried out by Professor R. Amprino, head of the Medico-Legal Institute of Bari [2]. In 1936, together with Dr. A. Bairati, he tested the method of Balthazard and Lebrun (1911) [1], which relied solely on Haversian canal morphology from the tibial cortical bone. They concluded that this single parameter was too imprecise to reliably estimate the age at death, as error rates were high and results varied considerably depending on the sampling location within the cortex.

Since then, numerous quantitative histomorphometric methods have been developed across different long bones, including the femur, tibia, humerus, ulna, and metacarpals [3]. Several factors have been shown to affect reliability and accuracy, such as sex [4,5], developmental stage (perinatal versus postnatal) [6], population differences [7], biomechanical loading [8,9], sampling location [10,11], sample size and reference collections [12,13], and pathological conditions, such as osteoporosis [14–16]. These findings demonstrate that the bone microstructure must always be interpreted in context and that no single parameter or equation can be universally applied across populations. The interest in developing age estimation techniques based on histomorphometrics stems from the crucial role that accurate age-at-death estimation plays in the forensic practice [3]. Traditional biological profiling methods based on gross bone morphology and morphometric analysis are often insufficient or cannot be performed (e.g., fragmented remains). In such cases, the forensic expert must resort to a more multidisciplinary approach. Histomorphometry has proven particularly valuable in cases involving fragmented or incomplete skeletal remains [17]. The histological determination of age is based on observing age-dependent changes in cortical microstructure, especially remodeling activity [18]. A key distinction must be made between primary and secondary osteons, as they differ in biological origin and forensic relevance. Primary osteons form during initial bone growth when vascular canals become encased by lamellar bone, whereas secondary osteons arise through remodeling, replacing pre-existing tissue. This distinction is critical, as secondary osteons reflect cumulative remodeling events and thus provide stronger correlations with age [4,19]. Variables such as osteon population density (OPD), the number of intact and fragmentary osteons, and the mean area and circularity of osteons have been widely used in age estimation studies, with varying degrees of success [20,21]. Quantifying and analyzing these microstructural features has led to the development of several age-prediction equations. Bone tissue undergoes lifelong remodeling, a coupled process in which osteoclasts resorb bone and osteoblasts deposit new lamellae [22,23]. This cycle, regulated by hormonal regulation, mechanical loading and metabolic demands, progressively replaces primary bone with secondary osteons. Over time, remodeling alters the number, density, and morphology of osteons, making these variables key indicators of age-related change [17,19,24]. This remodeling, therefore, influences variables such as the number of osteons, their density, and their area. Although most scholars have focused on long bones (femora, tibiae, humeri, fibulae, ulnae, and metacarpals), histomorphometric approaches have also been proposed using the axial skeleton (ribs and clavicles) and the mandible [24–31].

One of the main challenges faced by researchers and practitioners lies in determining which histomorphometric variables should be used to develop age prediction equations, and what level of accuracy and reliability each variable is able to provide. Building upon the foundational work of Amprino and Bairati [2], the present study revisits their unique collection of femoral mid-diaphyseal thin sections, housed at the University of Bari. This sample offers a rare opportunity to test modern histomorphometric approaches on historically prepared material. The objectives of this research are threefold: (1) To examine the relationship between histomorphometric variables and both age and sex; (2) To assess the potential of these variables to support a predictive model of age-at-death; and (3) to

evaluate the methodological limitations associated with using a sample heavily weighted toward older individuals. The femoral midshaft was specifically selected because it is a biomechanically loaded region where remodeling is particularly active, making it an ideal site to capture age-related microstructural variation. By integrating this historical collection with updated methods and statistical analyses, this study aims to refine our understanding of how cortical bone remodeling reflects age-at-death and to explore the limitations of applying histomorphometric techniques in forensic practice.

Building directly on Professor R. Amprino's seminal work with femoral mid-diaphyseal thin sections, we revisit his collection to test contemporary, quantitative approaches on historically prepared material. Amprino and Bairati examined 92 femoral diaphyseal sections spanning infancy to advanced age and showed that reliance on Haversian canal diameter alone was insufficient for accurate age estimation; they also observed that measurements vary with cortical sampling depth (periosteal versus endosteal) and that only broad age classes could be distinguished reliably given the methods available at the time. Using adult autopsy sections from the same Bari collection (here restricted to individuals aged 18–92 years and including both sexes), we extend that foundation with explicit variable definitions and statistical modeling aimed at adult age estimation—addressing the sampling and single-parameter limitations identified in the early study.

The choice and interpretability of histomorphometric variables remain central challenges for building age-at-death estimation models: different measures capture distinct aspects of cortical remodeling, and their accuracy and reliability can vary by skeletal element, sampling location, and demographic composition. Accordingly, this study (i) quantifies the relationships between selected femoral cortical microstructural features and age and sex, and (ii) evaluates the extent to which these variables—considered individually and in combination—can support a reliable, transparent model for forensic age estimation in adults. We focus on variables commonly used to characterize secondary osteonal remodeling (e.g., counts and densities of intact and fragmentary osteons) together with mean osteonal area and circularity, as these capture both the accumulation and morphology of remodeling units most closely tied to adult aging processes. This framing clarifies our objectives and the rationale for variable selection in advance of the empirical analyses.

2. Materials and Methods

The sample analyzed in this study consisted of 95 femoral cross-sections with known sex, age and cause of death (see Table 1 for demographic distribution by sex and age, and Appendix A for complete dataset). The subset consisted of autopsy samples housed at the Institute of Medico-Legal Medicine of the University of Bari, Italy. The age-at-death of the individuals ranged between 18 and 92 years, of which 54 were females and 41 were males. The samples had previously been prepared using standard histological protocols described in Amprino et al. [2]. After maceration and cleaning, bones were immersed in a 10% sodium chloride solution to remove residual organic material and to preserve microscopic structures for histological examination. Bone samples were taken from the femoral mid-diaphysis of each of the 95 individuals. However, the exact position around the cortical circumference was not reported in the original article by Amprino et al. [2], which may account for potential micromorphological variation. According to the autopsy records, the study sample consisted of individuals of European ancestry; therefore, the sample was not subdivided into ancestral groups. Cases of exclusion included contamination of the sample and unreadability under the microscope. Although age, sex, and cause of death were known, the study was conducted blind, meaning that demographic data were concealed during analysis to prevent observer bias. After data collection, demographic variables were added to an Excel spreadsheet together with all recorded histomorphometric measurements.

Table 1. Age and sex demographics of the study population. Age is presented as mean $\pm$ standard deviation (SD). N = number of participants in each group. Min = minimum age, Max = maximum age. Male and Female groups are reported separately.

Group	N	Mean Age $\pm$ SD	Min	Max
Total	95	59.3 $\pm$ 20.7	18	92
Male	41	59.0 $\pm$ 19.6	27	92
Female	54	59.6 $\pm$ 21.3	18	92

2.1. Histological Methods

Following a thorough literature review, the following histomorphometric parameters were selected and measured for each individual femur using a Nikon Eclipse 80i light microscope (Nikon Corporation, Tokyo, Japan) fitted with a compatible eyepiece:

Number of Intact Secondary Osteons (On.) is defined by Crowder [32] as the number of secondary osteons with intact Haversian Canals bounded by a scalloped reversal line. If the osteon were connected by a clearly defined Volkmann's canal, then the structures were counted as separate osteons. When two or more structures appeared to share a Haversian canal and/or shared a scalloped reversal line due to the plane of sectioning, then they were counted as one osteon. If, due to the material margin of the histological photomicrograph chosen, $\frac{3}{4}$ of an osteon could be clearly discerned as a Secondary Intact Osteon, then the osteon was included in the count. This parameter was then divided by the Surface area analyzed to have Intact Osteon population density (On./mm²), named as OPD (I).

Fragmentary Secondary Osteons (Fg.On.) is defined by Crowder [32] as the number of secondary osteons with a partially visible Haversian canal that has been breached either by a neighboring osteon or a resorptive bay and secondary osteons with no remnants of a Haversian canal present. Fragments that lacked the Haversian canal but could be identified by concentric lamellar rings and the presence of a defined reversal line with a scalloped (irregular) margin were included. This parameter was then divided by the Surface area analyzed to have Fragmentary Osteon population density (Fg. On./mm²), named as OPD (F).

Primary Osteons (On.Pr.) is defined by Kerley [4], Ericksen [19] and Streeter [21] as primary vascular canals that are formed by the inclusion of small, peripheral blood vessels into the bone by the rapid expansion of the cortex's diameter, which is located on both the endosteal and periosteal surfaces. This parameter was then divided by the Surface area analyzed to have Primary Osteons Population Density (On. Pr./mm²), defined as On.Pr(D).

Osteon Population Density (OPD), defined by Crowder [32], as the total number of intact and secondary osteons by sampled area.

Mean Osteonal Area (On.Ar.) is defined by Crowder [32] and Cummaudo et al. [10] as the average area of bone contained within the cement lines of structurally complete secondary osteons (reversal lines are intact), calculated as the average cross-sectional area of 4 complete osteons per cross-section. Drifting osteons were not included for measurements. The following requirements were followed from Cummaudo et al. [10]:

- Secondary osteon (the Haversian canal area must be smaller than $\frac{1}{4}$ of the osteon area).
- Not in the resorption phase.
- With a well-defined and complete cement line.
- Absence of Volkmann's canals crossing the osteon.
- The ratio between the Haversian canal maximum and minimum diameter must be lower than 2:1.

Osteon Circularity (On.Cr.) is defined by Goliath [33] as the circularity index $[4p(\text{area}/\text{perimeter}^2)]$ that indicates to what extent a measured object is similar in shape

to a true circle. A value of one (1) represents a true circle and values approaching zero (0) represent increasingly elongated shapes. The same osteon inclusion criteria considered for the Mean Osteonal Area variable were applied.

To capture a representative portion of each cross-section, two adjacent areas of the cross-section 1 mm apart (Figure 1) were captured at 40× magnification using the NIS Element D (version 3.1) imaging software connected with the microscope and stitched together using ImageJ SOFTWARE (version 1.8.0) [34]. ImageJ was subsequently used to analyze the femoral cross-section.

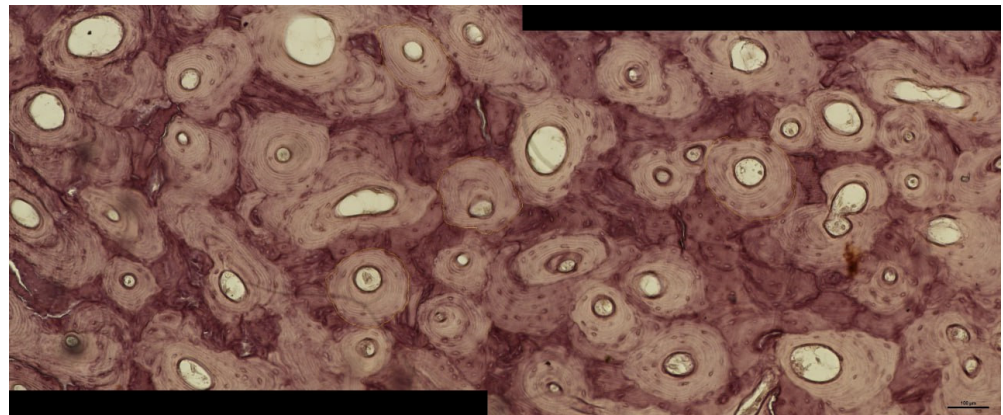


Figure 1. Sample n. 115 showing the osteons chosen to determine On.Cr and On.Ar (highlighted in yellow). Scale at the bottom.

The number of intact (On.) and fragmentary (Fr.On.) secondary osteons and primary (On.Pr.) osteons was manually counted following the inclusion and exclusion criteria aforementioned.

Circularity index (On.Cr.) and Osteon area (On.Ar.) were determined using the area and shape descriptors functions of ImageJ. Three to four osteons with well-defined boundaries were chosen randomly and outlined separately and served the basis for the calculation of osteon circularity (Figure 1). Only structurally complete intact osteons with complete reversal lines and round Haversian canals were measured to avoid measuring osteons represented by tangential cuts. Each analyzed feature was annotated in a separate color-coded layer to preserve the original image, enabling future reanalysis if needed. This allows for the inclusion or exclusion of any variable that was tracked based on the interest of the observer, thus allowing for retrospective reanalysis for different research questions.

2.2. Statistical Methods

The objectives of this study were to determine if any difference between sexes could be discerned in each variable, describe the relationship between age and the cortical bone histomorphometrics the sample, and to determine which variable was more statistically significant in order to be included in an age predicting model. Statistical analyses and the generation of predicting equations were accomplished using RStudio (version 4.4.2) [35]. Mean values for osteon circularity, osteon area, and OPD were calculated for each bone, and these values were employed in subsequent analyses.

Differences between males and females among each variable were established using *t*-Test and Mann–Whitney tests. All variables were assessed for normality, and transformations were applied where necessary to meet normality assumptions. For variables that remained non-normal even after transformation, the Mann–Whitney test was employed as an alternative non-parametric method to compare medians between the two groups.

To establish a correlation with age-at-death and histomorphometric variables, Spearman's Rank correlation coefficient was used, because age data were not normally distributed. Spearman's rank correlation coefficient is a statistical measure of the strength of a monotonic relationship between paired data. In a sample it is denoted by r_s and is by design constrained as follows [36]:

- 0.0–0.19: “very weak”;
- 0.20–0.39: “weak”;
- 0.40–0.59: “moderate”;
- 0.60–0.79: “strong”;
- 0.80–1.0: “very strong”.

Finally, General Linear Modeling (GLM) analysis was used to explore the combined effects of the various potential explanatory variables. All variables were tested for multicollinearity, and those with high correlations ($r > 0.7$ or $r < -0.7$) were excluded to prevent redundancy. Subsequently, a visual inspection of the data was performed to determine whether the relationships between variables and age were linear or non-linear.

3. Results

Descriptive statistics for the sample data are presented in Table 2. The *t*-test results showed no significant sex-related differences for any of the measured variables.

Table 2. Descriptive statistics for the sample data.

	N	Minimum	Maximum	Mean	Std.Dev.
Age (years)	95	18	92	59.263	20.675
On.	95	3	36	17.189	6.317
Fg.On.	95	6	33	17.432	5.727
On.Pr.	95	0	8	1.305	1.936
OPD (osteons/mm ²)	95	6.170×10^{-15}	5.800×10^{-14}	1.786×10^{-14}	6.218×10^{-15}
OPD(I) (osteons/mm ²)	95	1.470×10^{-15}	2.520×10^{-14}	8.846×10^{-15}	3.598×10^{-15}
OPD(F) (osteons/mm ²)	95	3.080×10^{-15}	3.290×10^{-14}	9.012×10^{-15}	3.772×10^{-15}
On.Pr(D) (osteons/mm ²)	95	0.000	3.920×10^{-15}	6.587×10^{-16}	9.787×10^{-16}
On.Ar (mm ²)	95	0	0.099	0.035	0.013
On.Cr.	95	0.788	0.937	0.874	0.035

The correlations between age and histomorphometric variables were found to be very weak and not statistically significant for most variables, as shown in Table 3. However, On., OPD(I), and OPD showed some significant positive correlation with age, while ON Ar. and On.Cr. showed some significant negative correlation. This data shows how the n. of secondary osteons, intact population density and osteon population density increased with age, while osteon size decreased with age.

Because there was not a significant correlation between sex and histomorphometric variables, male and female individuals were combined for the age-predictive models. The results showed high collinearity for the variables shown in Table 4, On.Ar. and On.Cr. did not show high collinearity and were thus retained. Therefore, the selected variables—On., Fg.On., On.Pr., OPD, On.Ar., and On.Cr.—were chosen due to their lack of high correlation with one another, ensuring that each variable contributes unique and independent information to the model.

The analysis of the relationship between age and various osteon-related measurements revealed distinct patterns. On. exhibited a significant cubic relationship with age, with the first two polynomial terms showing strong significance ($p < 0.001$), but the third term was not significant ($p = 0.944$), explaining about 29.5% of the variance in age. In contrast, Fg.On. and On.Pr. displayed weak or non-significant associations with age, as their polynomial terms had *p*-values above 0.05, suggesting no clear pattern. OPD showed a significant

non-linear relationship with age, with both the first and third polynomial terms being significant ($p < 0.05$). On.Ar. also demonstrated a significant cubic relationship with age, with the first and third terms being significant ($p < 0.001$ and $p = 0.013$), while the second term did not show significance. Lastly, On.Cr. exhibited a weak and mostly non-significant relationship with age, with high p -values for the polynomial terms.

Table 3. Spearman rank correlation coefficients between histomorphometric variables and age.

	Correlation's Coefficient	p -Value
Number of Intact Secondary Osteons (On.)	0.335	$p < 0.001$
Number of Fragmentary Secondary Osteons (Fg.On.)	0.047	$p = 0.653$
Number of Primary Osteons (On.Pr.)	−0.055	$p = 0.597$
Osteon Population Density (OPD)	0.294	$p = 0.003$
Intact Osteon Population Density (OPD_I)	0.306	$p = 0.002$
Fragmentary Osteon Population Density (OPD_F)	0.051	$p = 0.626$
Primary Osteon Population Density (On.Pr.D)	−0.051	$p = 0.623$
Mean osteonal Area (On.Ar.)	−0.295	$p = 0.003$
Osteon Circularity (On.Cr.)	−0.216	$p = 0.035$

Table 4. Collinearity test, with high correlation set at >0.7 or <-0.7 .

	r Value	Variable Chosen
On. and OPD(I)	0.845	On.
OPD(F) and OPD	0.851	OPD
OPD(I) and OPD	0.836	OPD
Fg.On. and OPD_F	0.747	Fg.On.
On.Pr and On.Pr_D	0.999	On.Pr.

Variables that exhibited significant non-linear relationships were incorporated into the model accordingly. However, as depicted in Figure 2, the residuals were not homogeneous, violating the assumptions of the model. Consequently, the age-predicting model based on histomorphometric variables could not be successfully established.

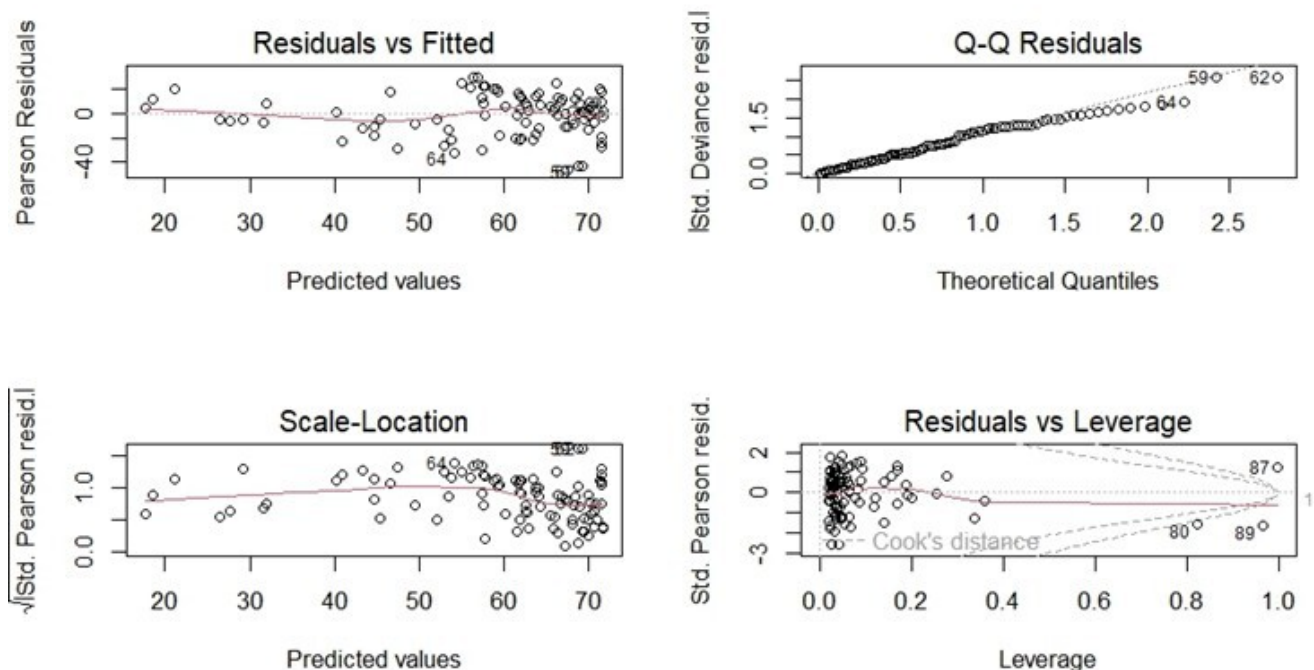


Figure 2. Scatterplot looking at the residuals and homogeneity of the dataset. Each circle shows an observation's residual or diagnostic value, and the red line is a fitted reference or trend for visualizing assumptions across each plot.

4. Discussion

The aim of this study was to develop a histomorphometric method capable of accurately estimating the age at death of the skeletal material from the same sample collection studied by Amprino and Bairati in 1936 [2]. They were among the first medical practitioners in Italy to hypothesize a correlation between osteon morphometry and the cellular degeneration associated with aging. For this purpose, we analyzed the bone microstructure of 94 autopsy thin-section samples from Amprino's histological collection, which is still housed at the Medico Legal Institute of Forensic Medicine of the University of Bari (IT).

4.1. Objective 1a: Correlation Between Sex and Bone Maturation

Differences in cortical bone remodeling rates between postmenopausal women and men of the same age have long been recognized, e.g., [37,38]. Notably, bone remodeling increases in women immediately following the onset of menopause [39]. However, in the study sample presented in this research, no notable difference was observed for each variable between males and females, and the data found in the literature addressing this factor are equivocal. Kerley [4] found no significant sex difference in his sample of 126 lower-limb bones, nor did Stout and colleagues [40–42] in their samples of ribs and clavicles. However, when pooled-sex equations were attempted, it was found that these were less accurate than using sex-specific predicting equations [5,23,43]. When evaluating only OPD values, Gocha [12] found no significant difference in OPD values between males and females, while Crowder and Dominguez [44,45] report significant differences between sexes in their quantification of fragmentary osteons, but no significant differences in intact osteons or combined OPD. They argued that differences between males and females will not be recognized unless the constituent variables of OPD are examined. It has been argued that such discordant results on whether biological sex affects the rate at which remodeling occurs might be the result of the number and age of males and females included in a sample, which will be discussed later in this study. Studies reporting no sex differences often used a sample that was heavily biased towards males; for example, Kerley's [4] sample only included 17 females compared to 43 males. In contrast, the present study included an almost even number of males (41) and females (54), uncharacteristically prevailing towards the females. It has been suggested [46] that perhaps sex-specific equation should take into account the differences in maturation rates and the timing of epiphyseal union between males and females, which could reflect different ages in which the adult cortex is completed by modelling, thus the inability to establish, among the same variable, a difference between males and female histomorphometric. At present, the debate has not settled yet, and future research is needed, perhaps incorporating the different growth rates of the adult cortical bones into sex-specific age estimation models.

4.2. Objective 1b: Correlation Between Age and Bone Maturation

Although no significant differences were found between males and female, some variables such as Number of Intact Secondary Osteons (On.), Osteon Population Density (OPD), Mean osteonal Area (On.Ar.) and Osteon Circularity (On.Cr.) seemed to be significantly correlated with age.

Osteon area (On.Ar.) exhibited a significant, although weak, negative correlation with age, decreasing in size while the bone maturation progressed. This trend has been observed in different bones, such as the humerus [22] and the ribs [11,47], while when femoral samples were examined, the data showed no change in osteon size correlated with age [11,24]. According to Robling and Stout [17], the mechanical loading has been shown to influence bone remodeling dynamics. In fact, also according to Karydi et al. [8], the bone

microarchitecture is affected by imposing mechanical forces, consequently affecting the osteon size, especially when considering a weight-bearing bone such as the femur.

For the study sample, Osteon Circularity (On.Cr.) decreased with age, contrary to most study on the topic, who instead indicate that osteon circularity increases with age [47–49]. Regardless of its strong correlation with age, the average value deviated from the literature, which could be explained by taking into account physical activity and differences in biomechanical stimulation, which can cause intra- and interpopulation variability among the sample itself [8,46]. Imposing biomechanical forces are suggested to influence osteon circularity since more circular shapes offer greater resistance under conditions of high mechanical loading [25]. Being most of the population sample composed of mid- and older individuals from the South of Italy, potentially from the first half of the 20th century, it is likely to assume a type of workload related to farming and other agricultural or manual activities, which greatly differs from the samples analyzed by other researchers. For example, Goliath et al. [48] used a subset of a dissecting room cadaver collection obtained from the Departments of Anatomy of Washington University and the University of Missouri, although of unspecified European ancestry, while Britz et al. [49] used autopsy samples from the Victorian Institute of Forensic Medicine (VIFM) in Melbourne, Australia.

All the Osteon Population Density (OPD) variables displayed significant correlation with age in the femur specimens, showing an increasing pattern, as well as the number of intact secondary osteons (On.), which constitute variable of OPD. With increasing age, secondary osteons undergo remodeling over the lifespan, and their relative density increases with age, meaning that the cortical bone becomes crowded with secondary osteons until an asymptote is reached in osteon counts [14]. Therefore, OPD increases with advancing age until the asymptote is reached. Ericksen [19] reported in his study that the number of secondary osteons/millimeters squared increases with age, a factor they have incorporated into their age-predicting equations, while Richman et al. [50], reported no significant association with age.

To conclude, the lack of correlation between age and Fragmented Osteon Number (On.Fr.) on the femora has also been indicated by Crowder [32]. Bone microarchitecture is indeed greatly affected by the imposing biomechanical forces which reversely affect osteon diameter [9,51]. This phenomenon is even greater in weight-bearing bones such as the femur.

4.3. Objective 2: The Age Prediction Model

The inability to generate a reliable age-prediction model likely resulted from the lack of homogeneity in the sample distribution, especially across age groups. The uneven distribution led to biased predictions, preventing the model from accurately capturing variability across all age categories. This resulted in non-homogeneous variance of error terms, violating the key assumptions of homoscedasticity and making the model unreliable for accurate age prediction.

As previously discussed, methods developed for histological age estimation are limited to the age ranges included in the sample from which they are derived. In addition, no single histological method is applicable to the entire human age span, especially considering the challenges in age estimating sub-adults and elderly individuals [12]. In the former, the pattern for age-associated cortical bone histomorphometry is peculiar, and the remodeling rates are higher and more variable. In the latter, because of the asymptote phenomenon, histological age estimation method has proved to bear low reliability in terms of correctly estimating the age of older individuals. The study sample showed the most represented age range to be the one between 52 and 92 years old, with 63 samples out of 95. The asymptote phenomenon is reached when the cortical bone is totally covered by the Haversian system,

during each remodeling phase a standardized number of secondary osteons is created destroying the underlying layers of microstructures [3,29,52]. As a result, one of the possible explanations as to which the model could not be built using Amprino's study sample is most likely to be appointed in the high prevalence of older individuals, being more likely to be subjected to medical autopsies, compared to the younger demographic. Similar challenges have been reported in other studies. Bantavanou et al. [53] in his study found that their regression equation based on Haversian system density performed poorly beyond 60 years, and Narasaki [54] noted that bone morphology in males was less age dependent than in females. This may be due to estrogen deficiency, which occurs in women after the age of 50 and impacts the remodeling process, ultimately leading to the thinning of cortical bone and a reduction in trabecular bone volume [55]. The reduction in volume size results in a decrease in bone area, which is a fundamental component of the OPD. Consequently, while the cortical area continuously diminishes and the bone becomes full of small osteons that characterize the senile individuals, the OPD increases. It needs to be noted however that OPD values are also heavily affected by factors such as diet, health conditions and social status [17,49]. Ultimately, osteoporosis is a frequent condition that appears mainly on middle and elderly adults and disturbs the remodeling procedure of the bone, creating massive resorption spaces, which, considering the fact that the thin sections used in this study derive from autopsy samples, thus the prevalence of the older demographic, has potentially played a role in disturbing the model [14,15].

For these reasons, histological age estimation methods often prove imprecise and biased in older age groups, explaining the difficulties encountered in developing a robust predictive model in this study.

4.4. Limitations of the Study

One issue that needs to be addressed concerns the collection standards of the histological samples. Although Amprino et al. [2] stated in his study that the thin sections have been collected from femoral mid-diaphysis, the exact sampling site was not specified. Several studies have shown how different portions of different bones result in different sizes of osteons, which is determined by the cortical thickness of each section. Cummaudo et al. [10] showed how in the proximal metaphysis, which in most long bones is characterized by a thinner cortex compared to that of the diaphysis and distal metaphysis, osteons were statistically significantly smaller. The only exception has been observed in the tibia. As expressed by Gocha et al. [46], given that histomorphometric variability in sampling location has been widely studied [11–13,20,56,57] the exact topographic sampling technique should be described and illustrated, which in this case study could only be assumed based on the nature of the sample.

An interesting point has been made by Crowder and Dominguez [44], who argued that for digitally captured images, the lack of depth of fields hinders microscopic structures, while using the fine focus of the light microscope allows for easier and more reliably identifiable reversal lines and lamellar patterns. Due to capacity and time constraints, the samples were analyzed using digitally captured images only, which, in addition to the degradation observed on some samples due to time wear, might have increased the inter-observer error of the author. Moreover, because the two adjacent areas of the cross-section (1 mm apart) were selected by the observer, some degree of sampling bias may be present. However, the consistent sampling protocol applied across all specimens minimizes the potential impact of this limitation on the overall results.

Furthermore, it is also important to acknowledge that, regardless of the negative outcome in constructing a new age-at-death estimation model, such a model—if derived from a collection dating back to the early twentieth century—would have limited applicability to

contemporary populations. Over the past century, substantial changes in nutrition, health, lifestyle, and environmental factors have affected bone physiology and remodeling dynamics. These shifts likely influence histomorphometric variables such as osteon population density, size, and shape. Therefore, even if a statistically valid predictive equation were developed from Amprino's collection, its direct application to modern skeletal assemblages would be questionable without appropriate recalibration using contemporary reference samples. Future research should thus focus on validating or adjusting historical models with modern datasets to ensure their forensic and anthropological relevance.

4.5. Future Research

Histological research within forensic anthropology has traditionally focused on improving existing methods to accurately estimate the age at death, which is understandable considering the applied nature of the discipline. However, over the past decade, some scholars [3] have also focused on understanding bone biology using a variety of technological developments to study bone in new ways. Although the scope of this research was to construct a new age-at-death estimation model—including a substantial number of older individuals, often underrepresented due to histological challenges—the outcome was not what we expected. This posed the basis for a deeper thinking behind the reasons why such model could not be built, and what are the limitations in using such techniques. Future research will focus on implementing the sub-adult age range of the sample, as well as perhaps looking at the different growth rates between females and males, to account for the sex-related variation at which cortical bone is remodelled.

Moreover, most research focused on two main pillars: (1) the use of a linear model to estimate the age at death centered on the assumption that the replacement of primary bone with secondary bone is a continuous process of turnover that occurs at a predictable rate [23,32], and (2) the number of intact and fragmentary secondary osteons per unit of cortical area is considered to be the best indicator of skeletal age at death. For the reasons mentioned above, the current methodologies are inclined to a number of limitations; thus, future research will likely challenge the use of linear models, and a deeper understanding of bone biology and its variability might drive this endeavor. Investigating the influences of extrinsic and intrinsic considerations such as biomechanical stressors, taphonomy, disease, trauma, substance abuse, diet and nutrition, hormones (e.g., vitamin D, estrogen), and asymptotic values [17,24,58], as well as considering the provenance of study samples, might shape future research in a field that has not been fully explored yet.

Furthermore, future studies could benefit from whole-slide imaging (WSI) technology, which enables analysis of entire histological sections rather than preselected regions. This approach minimizes area-dependent variability and allows for more comprehensive bone microstructure assessments [59–61]. Integrating WSI with automated image analysis could enhance reproducibility and promote standardized quantitative evaluations in future research.

5. Conclusions

In summary, this study contributes to understanding the role of histomorphometric variables in estimating the age at death while challenging some widely accepted findings in the literature. By discussing factors affecting reliability—such as sex, population differences, biomechanical stress, sampling techniques, reference standards, and pathological conditions—this research underscores the complexity of histological age estimation. Moreover, although Amprino and Bairati [2] did not attempted to create an age estimation method, they correctly theorized that different age ranges correspond to different osteon morphologies and that even though it might not be possible to get a perfect age estimation

from histological samples, a skilled researcher would be able to distinguish if an osseous fragment belongs to a juvenile, a young adult, an adult or a senile individual. The importance of this statement relies on the fact that, in the forensic practice and especially in cases of highly degraded bone material, the only features that can be successfully determine might only be those regarding species; thus this research, following the correct assumption made by Amprino and Bairati [2], sheds light on the limitations of some studies while highlighting the importance of following best practices when it comes to providing a precise description of an individual.

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Informed Consent Statement: Informed consent was not required for this study, as per Italian national legislation (Legislative Decree 196/2003 as amended by Legislative Decree 101/2018, Article 2-terdecies), since all cases involved deceased individuals and the data were fully anonymized prior to analysis.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed at the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. Breakdown of demographic data.

Sample	Age	Sex	Cause of Death
2	79	F	Metabolic disease
3	79	F	Metabolic disease
6a	75	F	
6b	75	F	
7	27	F	
8	73	M	
9a	72	F	
12a	71	M	
12b	71	M	
13	74	M	
14	71	M	
15	71	M	
16	79	F	
18	81	M	
19	74	M	
20	80	F	
21	47	M	
22	60	M	Metabolic disease
23	52	M	
24	57	M	
25	86	F	
26a	81	F	
26b	81	F	

Table A1. Cont.

Sample	Age	Sex	Cause of Death
27	87	F	
30	86	F	
31	92	F	
32	80	F	
33	76	F	Metabolic disease
34	77	F	
36	77	F	
37	78	M	
38	81	F	
39	79	M	
41	90	F	Metabolic disease
42	78	M	
43	92	F	
44a	71	M	
44b	71	M	
45a	71	M	
48	30	F	Metabolic disease
49	47	M	
50a	69	F	
50b	69	F	
51	56	F	
52	76	F	
53	64	M	
54	60	M	
55	42	F	Metabolic disease
56	44	M	
57	22	F	
58	22	F	
59a	41	M	
61a	41	F	
61b	41	F	
62	41	M	Metabolic disease
63	41	M	Metabolic disease
64	41	M	Metabolic disease
65	19	F	
66	26	F	
67	42	F	Metabolic disease
68	66	M	
69	26	F	
70	52	M	
72	22	F	
73	48	M	
75	27	F	
77	68	F	
78	66	M	Metabolic disease
79	23	F	Metabolic disease
80	67	F	
81	69	F	
84	80	M	
85	27	F	
86a	41	M	
86b	41	M	
87	65	M	
88	65	M	
89	64	M	
90	32	M	
91	32	M	
93	57	F	
95	56	F	
96	81	M	
98	65	F	
99	80	F	
100a	24	F	
101	41	M	
102	34	F	Metabolic disease
103b	24	F	
104	18	F	
105	66	M	
107	69	F	
110	76	F	

Table A1. Cont.

Sample	Age	Sex	Cause of Death
111	65	F	
115	57	F	

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