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TECHNICAL NOTE

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Age at Death Estimation in Adults by Computer-Assisted Histomorphometry of Decalcified Femur Cortex

ABSTRACT: In 1965, Kerley pioneered histomorphometry of bone as an aging method. The technique has been modified by several authors, and some have used computer-assisted image analysis. Undecalcified bone sections used in these methods are obtained with a diamond wafer saw or by grinding the sections manually or automatically with abrasive paper. In the present study, we examined the application of histomorphometry to decalcified bone sections, routinely obtained in every pathology lab, from paraffin blocks cut with a standard microtome. This study was divided into two parts: in the first, we tested different decalcifying methods to determine the most appropriate for femoral bone; in the second part, we used computer-assisted histomorphometry to estimate age at death in 29 samples of femoral bone. We measured intact osteon density (N.On), fragmented osteon density (N.On.Fg) and percentage of lamellar bone surface per unit area (Lm.B.Ar) in the cortex of the femoral midshaft, on four or 20 fields per section. We found that 20% nitric acid solution at room temperature proved to be the best decalcifying method, with a mean decalcification duration of 1 week. Fragmented osteon density was found to be the morphometric feature most closely correlated with age, followed by intact osteon density; Lm.B.Ar. did not increase accuracy. The best accuracy (4.1 ± 3.5 years) was obtained for individuals under the age of 70 when measurements of 20 fields were used for the analysis. For all individuals, the inaccuracy was 6.1 ± 6.2 years and 8.1 ± 8 years, with 20 and four fields respectively. The present study shows that decalcification of bone sections can be used for age estimation at death. This procedure is particularly useful in case of mass disaster as it is easily done in any pathology department.

KEYWORDS: forensic science, aging, femur, histology, computer-assisted histomorphometry, physical anthropology

Estimation of age at death is an important concern for forensic pathologists and anthropologists. In addition to macroscopic methods, a number of methods have been developed from bone histology. In 1965, Kerley pioneered histomorphometry of bone as an aging method; his approach is still considered as the histological "gold standard." Kerley's technique consists of the determination of the number of complete secondary osteons, osteon fragments, and non-Haversian canals, as well as the percentage of lamellar bone present in four anatomically defined microscopic fields in the outer portion of the bone cortex of the femur, tibia, and fibula (1). This method has been modified by other authors: Ahlquist and Damsten pooled histological parameters (2), Singh and Gunberg included the number of lamellae per osteon and the diameter of Haversian canals (3), Kerley and Ubelaker revised the regression equations in 1978 (4), Thompson (5), Ericksen (6), and Maat (7) used only the anterior aspect of the femoral section, and Stout and Paine studied the correlation between age and histological structures of ribs and clavicle using entire bone sections (8). Computer-assisted image analysis has been used for both macroscopic and microscopic measurements in femoral samples (9–14).

In most of these studies, undecalcified bone sections were used. Decalcified bone was used in only two studies (2,3). Undecalcified bone sections are usually made using a low speed diamond wafer saw. Frost et al. (15), in 1958, proposed a manual method of grinding bone sections with abrasive paper, an approach recently modified in two studies (16,17). Automatic grinding has also been used. In undecalcified bone sections, photography is challenging because evaluation of the structures under the microscope requires constant refocusing. Some authors have stressed the large intra- and inter-observer variation in histological criteria used in age at death determination based on femoral cortical bone (18,19).

We have investigated (1) the application of histomorphometry to routinely decalcified bone sections from paraffin blocks, cut with a standard microtome, and (2) the potential role for computer-assisted histomorphometry in providing accurate structural identification and measurement.

Materials and Methods

This study was divided into two parts: in the first, we tested various decalcifying procedures to determine the most appropriate for femoral bone; in the second, we used computer-assisted histomorphometry to estimate age at death in 29 samples of femoral bone.

The sample (Table 1) consisted of 59 three-cm thick cross-sections from the femoral mid-shaft taken from 59 bodies autopsied at Lapeyronie Hospital, Montpellier, France. Ethical requirements were fulfilled (in France, the use of tissues from medicolegal cases must be authorized by the regional prosecutor). The individuals had a mean age of 53 ± 19 years and included 44 white males and 15 white females. Femora were excluded if they presented mid-diaphyseal damage, bone pathology, or any endocrine pathology, since these conditions could have interfered with the bone remodeling

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TABLE 1—*Study sample.*

30 Individuals mean age: 53 ± 19 years 11 females, 19 males	Group A (test of ready-to-use decalcifying solutions): 30 femoral sections Group B (test of nitric acid solutions): 30 femoral sections
29 Individuals mean age: 42 ± 15 years 9 females, 20 males Total sample: 59 White individuals	Hystomorphological study: 20 images around the 29 femoral sections

process. After fixation in 10% formalin, each femoral sample was cut into 0.5-cm thick sections. The sections were then cleaned of soft tissues, and fixed again for 24 h in 10% formalin. The samples were then divided into two populations: bones from 30 individuals were used to finalize the decalcification method, while samples from the 29 other individuals were used for histomorphometry.

Decalcification Methods

The bone samples were placed in plastic jars containing one of the decalcifying agents under study (a commercial decalcifying solution or nitric acid), until complete decalcification.

Samples were divided into two groups:

Group A: Samples were decalcified using a commercial decalcifying solution, RDO®, DC3®, available in most pathology labs.

Group B: The remaining samples were decalcified using nitric acid solutions at concentrations of 5%, 15%, 20%, and 30%; formalin was added to the decalcifying agent, according to Bancroft's recommendations (20).

The decalcifying procedure was considered complete when X-rays revealed no residual calcium, and a fine needle could easily penetrate the specimen.

After decalcification, samples were cut into two parts along the sagittal plane to fit in standard cassettes (small plastic boxes used to embed the sample). Sections were embedded in paraffin after dehydration in alcohol and toluene baths. Each paraffin block was cut with a standard microtome. When the block was found to be still too stiff to be cut, it was further decalcified in a 20% nitric acid solution for 20 additional minutes. The obtained 4-μm thick sections were stained with Sirius Red.

In both groups, decalcification was performed at room temperature. The solutions were changed everyday. In addition, some specimens were decalcified at 37°C and 55°C for comparison with the decalcification process at room temperature.

Histomorphometry

Twenty-nine cross-sections from the femoral midshaft were evaluated histomorphometrically. The samples were taken from bodies autopsied at Lapeyronie Hospital, Montpellier, France. Ethical requirements were fulfilled. The mean age of this group was 42 years ± 15, and the median age, 40. The individuals ranged in age from 9 to 96 years.

Three-centimeter thick sections from the femoral mid-shaft were fixed in 10% formalin, and then sawed into 0.5-cm thick sections. The sections were placed again in 10% formalin for 24 h, then cleaned of any remaining soft tissue, and finally decalcified as previously described; all decalcification was performed at room temperature.

After decalcification, samples were cut into two parts along the sagittal plane to fit in standard cassettes. Sections were embedded in paraffin after dehydration in alcohol and toluene baths. Each paraffin block was cut with a standard microtome. When the block was found still too stiff to be cut, it was further decalcified for 20 min in a 20% nitric acid solution. The obtained 4-μm thick sections were stained with Sirius red.

Digitized images of bone sections were made using a digital camera (Kappa DX 30) mounted on a light microscope (Nikon® Eclipse 600) with a X10 objective. All measurements were done in images of 515×650 pixels with calibration factor of 2.22 μm/pixel, representing an area of 1.65 mm^2 of bone tissue. Digitized images were then analyzed using Leica Qwin® software (Leica, UK) running under Windows XP®.

For each of the 29 bone sections, 20 images were analyzed in the sub-periosteal area.

The histological features selected for our study were the following (all abbreviations for histomorphometric features are adapted from Parfitt et al. [21]):

- Intact osteon density (N.On): the number of osteons per unit area. An osteon system was counted as intact provided 80% of its surface could be observed and the Haversian canal was untouched by remodeling;
- Fragmented osteon density (N.On.Fg): the number of fragmented osteons per unit area. A fragmented osteon lacked the Haversian canal or, if present, it had been remodeled by the formation of subsequent generations of osteons;
- Percentage of lamellar bone surface per unit area (Lm.B.Ar).

A digital stylus was used to draw the outline of the area occupied by lamellar bone. Once delimited, the lamellar surface was automatically calculated. After validation of the first step, the operator outlined the complete osteons, and then the fragmented ones. For all steps, the structures which had already been counted were highlighted by a color overlay in order to avoid double counting (Fig. 1*a–d*).

Statistical analysis of the data was performed by the SAS (Statistical Analysis Software) program and consisted of a correlation study between the above descripted features and chronological age.

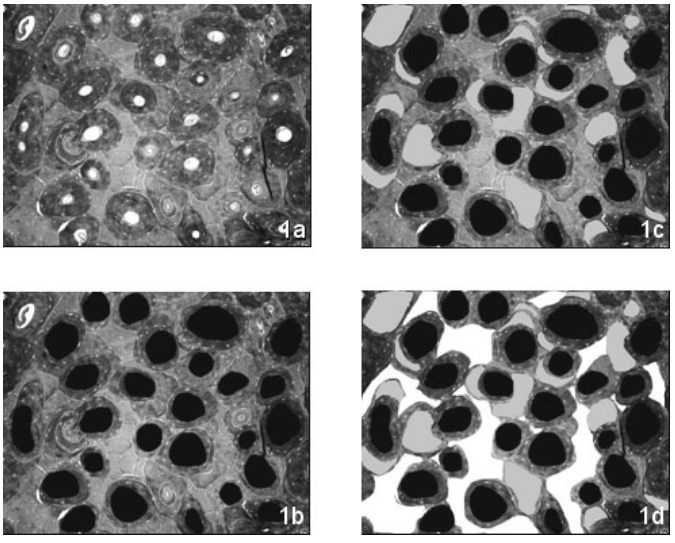

FIG. 1—*1a: Section of decalcified bone. Original magnification: ×10; Stain: Sirius red, 1b: complete osteons highlighted, 1c: fragmented osteons highlighted, 1d: lamellar bone highlighted.*

TABLE 2—*Results and duration of the decalcification.*

Group A	Impossible to cut the blocks after 2 months			
Group B	30%	20%	15%	5%
	Bone sample was completely destroyed after 72 h	Complete decalcification: 8 specimens = 4 days 8 specimens = 6 days 14 specimens = 9 days	Complete decalcification after 15 days	Unfinished decalcification after 2 months

After checking that the distribution of the data was normal, simple linear regression was performed in order to assess correlations between the histomorphometric features and age.

Statistical analysis was performed separately using measurements on four fields as defined by Ahlquist and Damsten (2), and on 20 fields regularly setted around the cross-section and in the outer third of each cross-section.

We analyzed the data using two age groups: all individuals and just those individuals under 70 years of age.

One-dimensional analyses were first applied to confirm the selected age-associated variables (statistical significance, $p < 0.05$). For each variable, the linear regression curve and the correlation coefficient (Pearson product moment correlation coefficient) were computed. Multiple linear regression was then carried out and age predicting equations were proposed.

Inaccuracy and bias were also computed. "Inaccuracy" corresponds to the average absolute error of age estimation for each individual, without reference to over- or under-aging. "Bias" is defined as difference between estimated age and chronological age divided by the number of individuals, thus indicating over- or under-prediction.

Results

Decalcification Method

Group A—Decalcification was complete after 2 months. However, paraffin blocks could not be cut, because of stiffness of connective tissue. High temperatures did not improve the results.

Group B—After 72 h, the samples in the 30% nitric acid solution completely disappeared.

Of the four concentrations, the 20% solution proved to be the most effective, with a mean decalcification duration of 1 week. Among the 30 individual samples, eight were decalcified within 4 days, eight within 6 days, and 14 within 9 days. In some cases, an additional bath in 20% nitric acid solution for 20 min was required.

Samples in the 15% nitric acid solution were decalcified only after 2 weeks of immersion—some were still difficult to cut with the microtome. Samples in the 5% solution were not decalcified after 2 months (Table 2).

Histomorphometry

Figures 2–4 show the linear regression curve corresponding to the analysis of 20 fields per bone section. The number of complete and fragmented osteons increases with age, whereas the percentage of lamellar bone area decreases.

Table 3 shows Pearson product moment correlation coefficients.

Age predicting equations and determination coefficients r^2 ($p < 0.001$) are shown in Table 4.

The equation predictive of age for all individuals was: Age in years = (0.57A) + 21.2 (A = N.On.Fg, $r^2 = 0.75$). For those individuals under 70 years of age, the equation was: Age = (1.8B) + (1.65A) (B = N.On, $r^2 = 0.86$,). In our statistical model, the variable Lm.B.ar was discarded as no additional information to that given by variables N.On and N.On.Fg was provided.

Determination coefficients (r^2) are presented in Table 5, with respect to the two variables retained for the statistical analysis (N.On and N.On.Fg). We found that N.On.Fg is the parameter most closely correlated with age. Nevertheless, taking into account N.On increases the r^2 value. Again, the best results were found for individuals under the age of 70.

We calculated the bias and the global mean (± standard deviation) of the inaccuracy for all individuals (Table 6) and for individuals under 70 years of age (Table 7). The lowest inaccuracy was found for the individuals under 70, when 20 fields were measured (4.1 ± 3.5 years). For all individuals, the inaccuracy was 6.1 ± 6.2 and 8.1 ± 8 years, with 20 and four fields respectively.

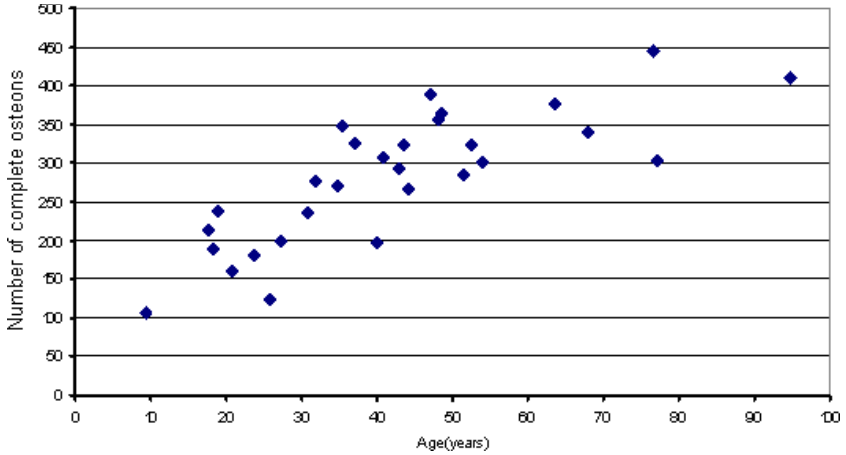

FIG. 2—*Correlation between age and N.On.Ar (each point corresponds to the mean value of the 20 fields for each individual).*

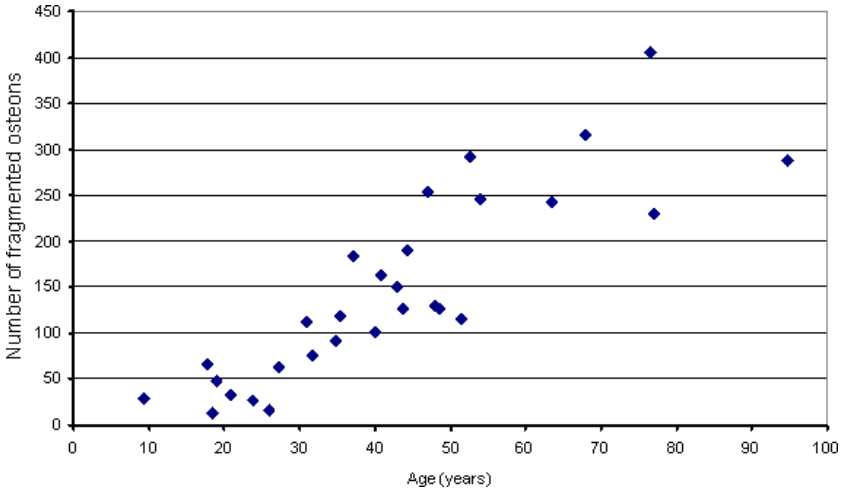

FIG. 3—*Correlation between age and N.On.Fg (each point corresponds to the mean value of the 20 fields for each individual).*

Discussion

The increased use of histological aging methods on human bone reflects their value to forensic scientists. Kerley's pioneering work and Stout and Paine's method represent the "gold standards." Other authors have attempted to reduce the intra- and inter-observer variation in histological criteria used in age at death estimation based on cortical bone (18,19).

In the present study, we elaborated a bone decalcification procedure for those forensic pathologists familiar with decalcified and stained bone sections, which are routinely examined in pathology labs. Decalcification does not require constant supervision, which is helpful in situations involving many, even hundreds, of specimens such as in mass disasters. We also considered the potential role for computer-assisted image analysis of decalcified samples in age estimation.

In surgical pathology practice, bone specimens consist of biopsies—surgically excised lesions—mainly tumors. In these circumstances, commercial decalcification solutions such as RDO® or DC3® are used, because the specimens are small (biopsies) or require gentle decalcification (tumors). Our work demonstrated that these solutions are not appropriate for age-estimation purposes in a forensic context.

The decalcification properties of nitric acid are well known, but required concentrations have not been determined in forensic materials. Of the various concentrations tested in our study, the 20% solution was found to provide the best results. Decalcification was fast (1 week or less), bone structures were not disrupted (Fig. 5a, b), and a standard microtome could be used to cut the samples embedded in paraffin.

It has been suggested that heat can be used to accelerate the decalcifying process. However, our results with 37°C and 55°C temperatures at lower acid concentrations showed that samples could not be cut with a standard microtome regardless of the decalcification duration. On the other hand, at higher acid concentrations, heat destroyed the interstitial collagen matrix or even the whole sample (Fig. 6). Therefore, decalcification should be conducted at room temperature.

Interestingly, our study also showed that, despite X-ray verified complete decalcification, some samples could not be cut with a standard microtome. This was the case with all decalcification methods, whether commercial solutions or the lower nitric acid concentrations, and also with some of the samples decalcified by the 20% solution. This phenomenon was due to the dehydration process: solvent agents like toluene or xylene stiffen the connective tissue. Chloroform has been proposed as an alternative dehydration

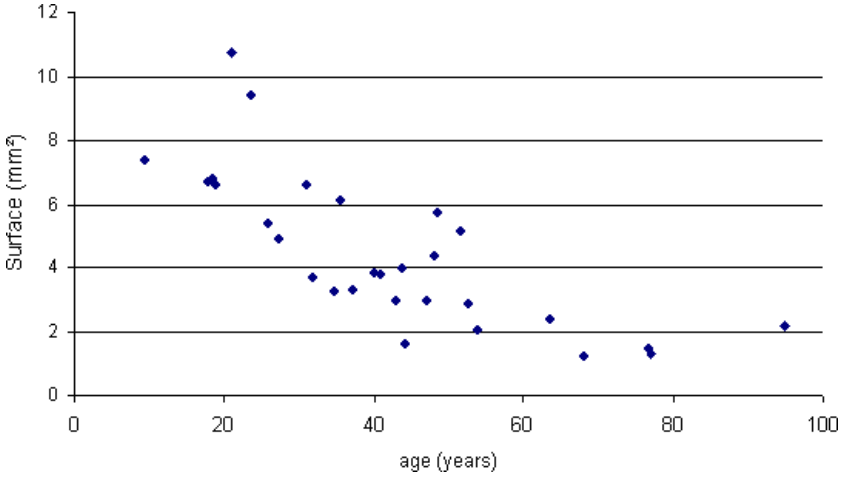

FIG. 4—*Correlation between age and Lm.B.Ar (each point corresponds to the mean value of the 20 fields for each individual).*

TABLE 3—*Correlation coefficient (r), simple linear regression.*

	Correlation Coefficient (r) $p = 0.005$			
	20 Fields		4 Fields	
Parameters	All ages	<70 years	All ages	<70 years
N.On.Fg	0.84	0.87	0.78	0.80
N.On	0.87	0.88	0.82	0.87
Lm.B.Ar	−0.77	−0.78	−0.70	−0.74

TABLE 5—*Determination coefficient (r^2), multiple linear regression.*

	Determination Coefficient (r^2, $p = 0.005$) 20 fields		Determination Coefficient (r^2, $p = 0.005$) 4 fields	
Parameters	All ages	<70 years	All ages	<70 years
N.On.Fg	0.75	0.77	0.68	0.75
N.On	0.71	0.75	0.61	0.64
N.On.Fg + N.On	0.80	0.86	–	–

agent, but for health and safety reasons, it is not available in pathology labs. The problem was easily solved by placing the paraffin blocks in the 20% nitric acid solution for 20 more minutes. This procedure softened the samples, allowing the microtome to cut them into 4-μm thick sections.

The decalcifying procedure was considered complete when a fine needle could easily penetrate the bone, which was not possible in some samples even when X-rays demonstrated that the bone was decalcified. Although X-ray is usually accepted as an accurate method to demonstrate decalcification, our needle test was found more accurate in determining the proper time finishing point for the decalcifying process, avoiding the subsequent stiffening of the sample. In these cases, the samples had to be put back in the nitric acid solution to complete the process. It was not possible in this study to determine the factors involved in this problem.

Our second objective was to determine whether our decalcification method permitted reliable recognition of histological structures. Sirius red stain was used because of its property to selectively stain collagen. By using our method, a narrow clear space is created as a procedural artifact. This structural change separates the osteons from the surrounding lamellar bone and proves helpful in delineating the interface between osteons and lamellar bone, as shown in Fig. 5. Our preliminary results with computer-assisted image analysis demonstrated that these spaces are essential in differentiating the histological structures (22).

Heating has been suggested as an aid to decalcification (20) but in our study, we found that heat produced serious artifactual changes, as shown in Fig. 6. These much larger spaces interfered with the identification of the bone structures.

Finally, the decalcification procedure (room temperature, solution of 20% nitric acid changed each 24 h, estimation of the end of decalcification with a needle) could be an alternative to undecalcified preparation because it could be used easily in all pathology labs and because bone structures could be more easily identifiable.

Regarding age estimation, our results confirmed that the lamellar surface is inversely correlated with age, whereas the number of complete and fragmented osteons increases with age. Each parameter separately, fragmented osteons are the best correlated with age, followed by complete osteons and lamellar surface.

TABLE 4—*Multiple linear regression equations and determination coefficients.*

		Equations Predictive of Age (multiple regression)	r^2 ($p = 0.001$)
All ages	4 fields	Age = (0.77A) + 18.8	0.68
All ages	20 fields	Age = (0.57A) + 21.2	0.75
<70 years	4 fields	Age = (1.9B) + (2A) − 3.2	0.80
<70 years	20 fields	Age = (1.8B) + (1.65A)	0.86

A = N.On.Fg.
B = N.On.

One of the difficulties of the various histological methods is the determination of the number of fields that most accurately reflect bone remodeling (23). Kerley (1) as well as Ahlqvist and Damsten (2) studied four fields in a whole bone section. Ahlqvist and Damsten deliberately avoided the linea aspera since bone remodeling is specific at this site because of the attachments to the muscles of the thigh. In our study, statistical analysis showed that 20 fields should be analyzed.

The multivariate model revealed that the correlation coefficient was not improved (Table 4) when lamellar bone was taken into account. Multivariate analysis revealed that the variable best correlating with age was the number of fragmented osteons. The determination coefficient including the number of fragmented osteons was of 0.75 for 20 fields and all individuals. When the number of complete osteons was also included, the determination coefficient was 0.80. Consequently, complete osteons only added 3% to the

TABLE 6—*Inaccuracy and bias for all individuals.*

	Age Estimation		Bias	
Chronological Age	20 Fields	4 Fields	20 Fields	4 Fields
35.5	41.8	40.8	−6.4	−5.3
40.0	42.4	–	−2.4	–
37.1	49.6	45.7	−12.4	−8.5
31.8	32.8	33.8	−1.0	−2.0
42.9	45.4	50.3	−2.4	−7.4
48.5	48.8	–	−0.3	–
17.8	25.0	26.1	−7.2	−8.3
34.7	34.1	–	0.6	–
76.6	81.4	79.9	−4.9	−3.3
53.9	54.7	–	−0.8	–
40.8	48.0	48.1	−7.2	−7.3
18.9	24.9	23.8	−6.0	−4.9
27.3	23.4	23.0	4.0	4.3
67.9	73.7	70.3	−5.9	−2.5
94.8	65.6	62.2	29.3	32.7
52.6	58.8	63.9	−6.3	−11.4
9.4	16.9	–	−7.6	–
51.5	38.1	34.0	13.4	17.5
47.0	60.0	63.2	−12.9	−16.1
18.4	17.9	17.7	0.5	0.7
20.9	17.0	14.7	4.0	6.2
23.7	19.8	–	3.9	–
48.1	43.7	46.2	4.3	1.8
30.9	35.5	–	−4.5	–
63.5	60.8	–	2.6	–
44.2	45.2	45.4	−1.0	−1.1
77.0	59.0	57.5	18.0	19.5
25.9	19.9	–	6.0	–
43.6	45.0	45.1	−1.4	−1.5

	Estimated Mean Age		Inaccuracy	
Sample Mean Age	20 Fields	4 Fields	20 Fields	4 Fields
42.2 ± 20	42.3 ± 17	44.6 ± 18	6.1 ± 6.2	8.1 ± 8

- = Not applicable.

TABLE 7—*Inaccuracy and bias for individuals under 70 years of age.*

	Age Estimated		Bias	
Chronological Age	20 Fields	4 Fields	20 Fields	4 Fields
35.5	41.0	39.1	−5.5	−3.6
40.0	40.2	-	−0.2	-
37.1	46.6	42.4	−9.5	−5.2
31.8	32.6	32.9	−0.8	−1.1
42.9	43.0	46.4	−0.1	−3.5
48.5	47.8	-	0.8	-
17.8	24.6	25.6	−6.8	−7.9
34.7	33.4	-	1.3	-
53.9	49.8	-	4.1	-
40.8	45.4	44.0	−4.6	−3.2
18.9	25.2	23.6	−6.3	−4.7
27.3	23.0	22.4	4.3	5.0
67.9	66.4	60.4	1.5	7.4
52.6	53.1	54.6	−0.5	−2.0
9.4	16.8	-	−7.5	-
51.5	36.9	32.5	14.6	19.0
47.0	55.7	56.5	−8.7	−9.5
18.4	18.9	18.5	−0.5	−0.1
20.9	17.1	14.9	3.8	6.0
23.7	20.4	-	3.3	-
48.1	42.6	42.6	5.4	5.5
30.9	33.9	-	−2.9	-
63.5	56.6	-	6.9	-
44.2	41.7	41.6	2.5	2.6
25.9	20.5	-	5.3	-
43.6	43.7	42.3	−0.1	1.3

	Estimated Mean Age		Inaccuracy	
Sample Mean Age	20 fields	4 fields	20 fields	4 fields
37.5 ± 15	37.6 ± 14	37. 7 ± 14	4.1 ± 3.5	5.1 ± 4.3

- = Not applicable.

variance. Lynnerup demonstrated that complete osteons could be counted without great inter- and intra-observer variability (19). Taking into account only complete osteons, the determination coefficient was close to that found with fragmented osteons ($r^2 = 0.71$ over 20 fields and for all individuals). For that reason, Stout and Paine (8) proposed to use the total osteon density (i.e., N.On + N.On.Fg). This study confirmed that the combination of several histological parameters yields the best result (23). We also confirmed that age estimation could be improved by excluding the oldest individuals (1,6).

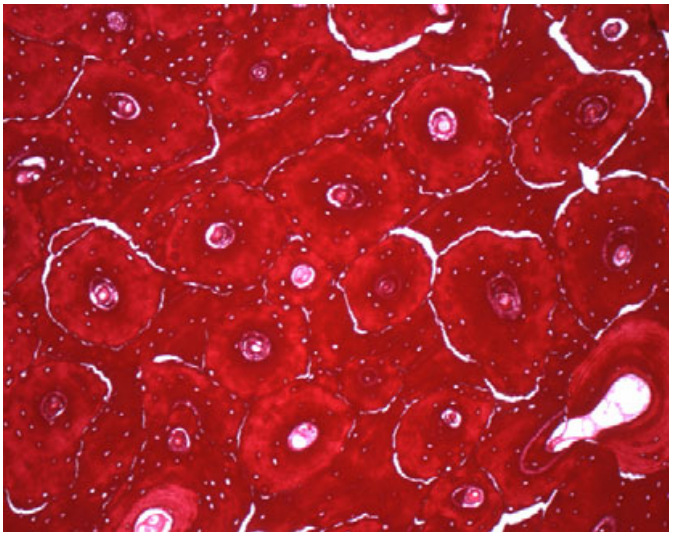

FIG. 5—*Section of decalcified bone. Original magnification: ×10; Stain: Sirius red.*

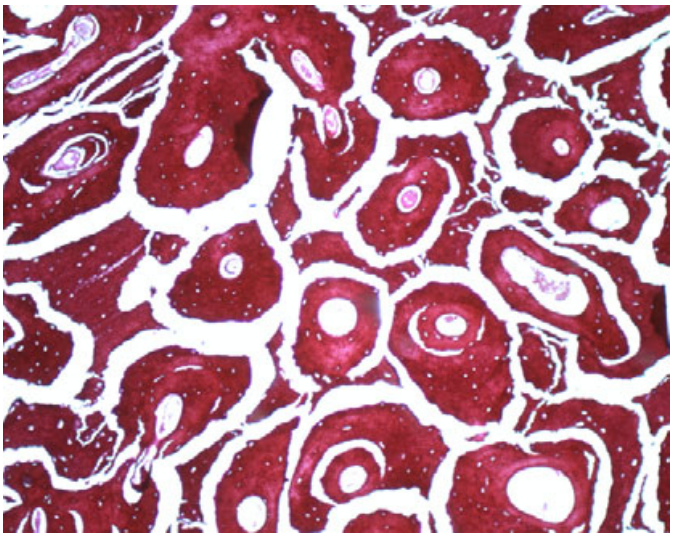

FIG. 6—*Deleterious artifacts caused by high temperature (55°C [131°F]). Large clear spaces separate collagen matrix from osteons. Collagen is disrupted by high temperature. Original magnification: ×10; Stain: Sirius red.*

We chose three of the histological parameters described by Kerley (1). As emphasized by Lynnerup (19), defining the different histological bone structures is a major challenge. In the literature, various descriptive terms may be used for the same histological element which may lead to confusion or major misunderstandings (24). Here, we have used previously published recommended suggestions of terms wherever possible (21).

Several authors have used computer-assisted analysis (9–14). These authors selected features that could be easily identified such as size of the femoral section or bone porosity. These elements did not appear to be strongly correlated with age.

In contrast, we decided to use a semi-automated method, taking advantage of the speed of calculation of the software and the accuracy of interpretation by the analyst.

Potential for Forensic Application

The first application is with standard anthropological cases, either in association with macroscopic methods or when macroscopic techniques are not possible, specifically those in which DNA has been degraded by heat, in which bone elements required for macroscopic methods are absent or damaged or when marked fragmentation has occurred, as after bombings.

The mid-shaft of the femur, used in histomorphometrical age estimation, is particularly robust even when the bone is fragmented. When the more usual macroscopic aging elements are absent or damaged, the femoral mid-shaft will often be available.

The present study shows that decalcification and standard histological processing of bone sections can be used for age estimation at death with good results. Furthermore, the decalcifying procedure can be applied to many bones simultaneously, which can prove convenient in handling mass disasters.

Acknowledgments

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