J Forensic Sci, May 2009, Vol. 54, No. 3
doi: 10.1111/j.1556-4029.2009.01003.x
Available online at: www.blackwell-synergy.com

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Microscopic Age Estimation from the Anterior Cortex of the Femur in Korean Adults*

ABSTRACT: The purpose of this study was to develop age-predicting equations from the anterior cortex of the femur of Korean adults. Seventy-two femoral samples (44 male and 28 female) were obtained from Korean cadavers and used to develop the equations. The thin sections (<100-μm thick) were prepared by manual grinding; the sections were not decalcified and were stained with Villanueva bone stain reagent. Analysis of covariance showed no significant differences in age-adjusted histomorphological variables between sexes. In stepwise regression analysis, osteon population density, average osteon area, and the most anterior cortical width were selected for an age-predicting equation which produced a high regression correlation (R^2 = 0.789). The average Haversian canal area was not significantly related to age for any specimen.

KEYWORDS: forensic science, forensic anthropology, histomorphometry, age estimation, femur, Korean

Microscopic age estimation of unidentified skeletal remains is accepted as a reliable technique (1) and has been developed from a variety of bones such as the tibia (2–4), fibula (2,4,5), humerus (6,7), ulna (6), radius (4), and rib (8–10). The femur has one advantage in that it is often found in forensic cases and excavations (2), and can be used to provide basic skeletal materials for histomorphometric analysis (6,11,12). Some verification studies have reported that population differences can influence the reliability of histological age-estimation methods (7,13). This incongruity means that the population is one of many variables, in addition to age, that cause differences in bone remodeling and thus can affect bone histology (14,15).

The purpose of this study was to develop age-predicting equations from the anterior cortex of the femur in Korean adults. Sections were prepared by manual grinding and stained with Villanueva bone stain reagent. Histomorphometric analysis was performed after establishing objective measuring fields in the anterior cortex of the femur.

Materials and Methods

Femoral specimens were obtained from 72 cadavers (44 males and 28 females) from the Department of Anatomy of Gachon University, Incheon, and The Catholic University of Korea, Seoul, in Korea. None of the femurs were taken from individuals who had died from a primary bone disease or one during which the subject would have been bedridden for a long time prior to death. The age range of the males was 41–86 years (mean and standard deviation 66.6 ± 12.7 years) and the age range of the females was 35–94 years (70.3 ± 16.1 years; Table 1).

For each specimen, a 5-cm-long segment was cut by saw from the midshaft of the right femur, and the remaining soft tissue and periosteum adhering to the femoral surface were excised carefully. Specimens were processed in 10% formalin and a mixture of chloroform and methanol for several days, and then bleached in 2% H_2O_2 solution for 1 day. The specimens were dehydrated at room temperature, and 1-mm-thick cross-sections were obtained from the femoral segment using a diamond wheel (Isomet® 5000, Buehler Instruments, Lake Bluff, IL) and thin sections (about 100-μm thick) were prepared by manual grinding on graded silicon carbide abrasive papers. Each section was superimposed on a protractor fixed to the vertical axis between the most anterior cortex and the linea aspera, and the margins more than ± 50° were removed using an acrylic cutter. The sections containing the anterior cortex were stained with Villanueva bone stain reagent according to a previously published protocol (16) and mounted on glass slides in the usual manner.

Five subperiosteal areas of each thin section comprising the most anterior point (0°), points ± 10°, and points ± 20° were analyzed microscopically after indicating the points on the glass cover slip with a permanent marker (Fig. 1). Four histomorphological variables—the most anterior cortical width (CW), osteon population density (OPD) and average size of the osteon (OA), and Haversian canal (HA)—were measured using a polarizing microscope (BX-51; Olympus, Tokyo, Japan) with image analysis solutions (Image-pro Plus 4.5.1; Media Cybernetics, Inc., Silver Spring, MD). The combination of 10 × objective and 10 × oculars fitted with 10 × 10 eyepiece reticule provided a grid area of 1.0 mm^2.

The histomorphological variables were averaged over five areas for each individual, and statistical analysis was performed using

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*Presented as a Poster Presentation at the American Academic of Forensic Sciences 60th Annual Meeting in Washington, DC, February 2008.

Received 4 Mar. 2008; and in revised form 12 May 2008; accepted 1 June 2008.

TABLE 1—*Age distribution of the samples.*

Age Range (years)	Pooled Sexes (*n*)	Male (*n*)	Female (*n*)
30–39	1	0	1
40–49	8	6	2
50–59	11	5	6
60–69	20	15	5
70–79	12	10	2
Over 80	20	8	12
Total	72	44	28
Means (years)	68.0	66.6	70.3
Standard deviation	14.2	12.7	16.1

SPSS 13.0 (SPSS, Inc., Chicago, IL). An ANCOVA with Wilks' lambda was performed to test the hypothesis that sex (fixed factor) and age (covariate) have a significant effect on the histomorphological variables (dependent variables). Variance between the sexes was tested for with Levene's test, and all variables satisfied the assumption of equal variances ($p > 0.05$). Finally, standard linear regression (entered model) analysis and multivariate linear regression (stepwise model) analysis were performed to derive the age-predicting equations for Korean adults. In the stepwise regression analysis, after a full model was fitted to the data, a combination of forward selection and backward elimination procedures was employed within the statistical algorithm.

Results

The Villanueva bone staining produced homogenous red staining of the osteoid seams. The HA were stained darkly in the center, the cement lines were orange-red, and the interstitial lamellae appeared unstained or weakly orange. The degree of mineralization density allowed the various histomorphological structures, such as fragmentary osteons, type II osteons, and double-zonal osteons to be distinguished. Combined with polarized light, these gave better structural details to discriminate between them (Fig. 2). Descriptive statistics of all histomorphological variables for the femurs from men and women, pooled and analyzed separately are summarized in Table 2.

Levene's test of equality of error variances indicated that the assumption of equality of variance for each of the histomorphological variables influencing age and sex had not been violated [CW: F (1, 70) = 0.252, $p = 0.617$; OPD: F (1, 70) = 0.422, $p = 0.518$; OA: $F(1, 70) = 1.168$, $p = 0.283$; HA: $F(1, 70) = 1.590$, $p = 0.212$].

Multivariate tests were significant for age [Wilk's lambda = 0.212, $F(4, 66) = 61.286$, $P < 0.01$] and sex [Wilk's lambda = 0.758, $F(4, 66) = 5.272$, $p < 0.01$] indicating that these covariates did account for some of the variance in group membership. For this reason, it was possible to then interpret differences on the individual variables using univariate F tests. Except for CW, ANCOVA showed no significant differences in age-adjusted histomorphological variables between sexes (Table 3), and the data for samples from men and women were combined for further regression analysis.

The histomorphological variables according to age and the related regression results for the sexes combined are summarized in Table 4, which also contains correlation coefficients to compare with other studies. Of the histomorphological variables, OA showed a high regression correlation ($R^2 = 0.668$) with age, followed by

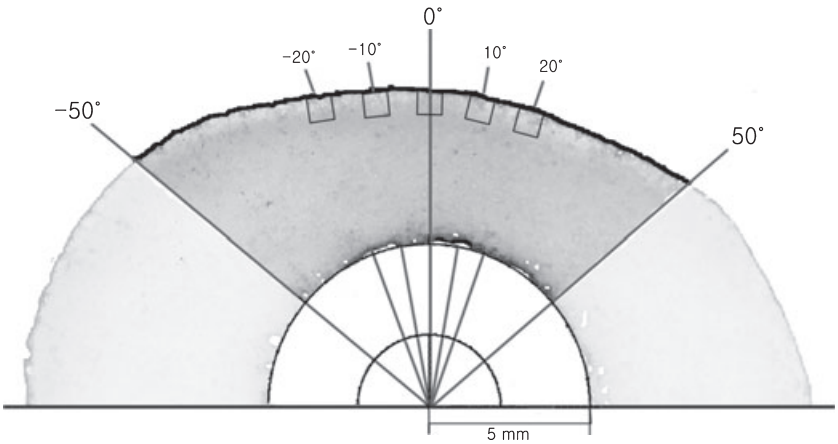

FIG. 1—*Location of five measuring fields (points 0°, ± 10°, and ± 20°).*

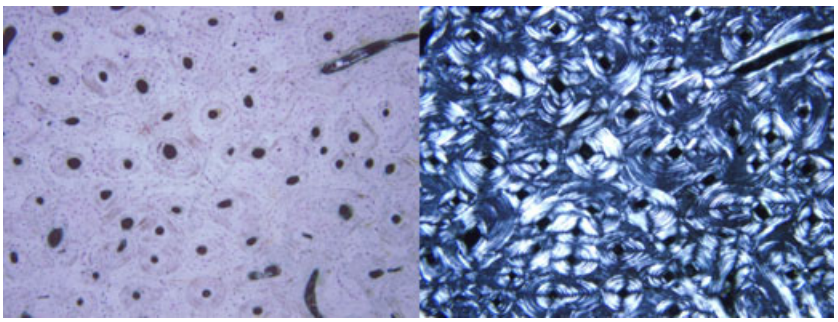

FIG. 2—*Example of a microscopic image of Villanueva bone stain (left) and its field under polarized light (right). 100× magnification from a 69-year-old woman.*

TABLE 2—*Descriptive statistics of histomorphological variables for the pooled and separate sexes (mean ± SD).*

Variable	Pooled Sexes	Males	Females
n	72	44	28
CW (mm)	4.438 ± 1.319	4.836 ± 1.155	3.812 ± 1.335
OPD (#/mm^2)	26.010 ± 6.415	24.887 ± 5.761	27.776 ± 7.075
OA (mm^2)	0.019 ± 0.005	0.019 ± 0.005	0.019 ± 0.005
HA (mm^2)	0.0019 ± 0.0005	0.0020 ± 0.0006	0.0018 ± 0.0004

CW, cortical width; OPD, osteon population density; OA, average size of the osteon; HA, Haversian canal.

TABLE 3—*Results of univariate ANCOVA to test sex differences in age-adjusted histomorphological variables.*

Parameter	*B*	*t*	Significance	Partial η^2
CW*				
Intercept	7.08514	10.681	0.000	0.623
Age	−0.03377	−3.510	0.001	0.151
Sex, female	−0.89943	−3.239	0.002	0.132
OPD*				
Intercept	0.93265	0.428	0.670	0.003
Age	0.35972	11.386	0.000	0.653
Sex, female	1.56008	1.711	0.092	0.041
OA*				
Intercept	0.03695	23.503	0.000	0.889
Age	−0.00027	−11.975	0.000	0.675
Sex, female	0.00079	1.208	0.231	0.021
HA*				
Intercept	0.00262	9.421	0.000	0.563
Age	−0.00001	−2.419	0.018	0.078
Sex, female	−0.00015	−1.259	0.212	0.022

CW, cortical width; OPD, osteon population density; OA, average size of the osteon; HA, Haversian canal.

*The parameter of each dependent variable (Sex, male) was set to zero.

TABLE 4—*Results of linear regression analysis for the relationship between age and histomorphological variables.*

Variable	Intercept	Slope	SEE	Correlation (*r*)	R^2
CW*	87.369	−4.358	13.040	0.406	0.165
OPD*	21.518	1.788	8.373	0.810	0.656
OA*	114.354	−2480.108	8.215	0.818	0.668
HA†	83.993	−8416.586	13.628	0.296	0.087

CW, cortical width; OPD, osteon population density; OA, average size of the osteon; HA, Haversian canal; SEE, standard error of estimate (years).

*$p < 0.001$; $^\dagger p < 0.05$.

TABLE 5—*Age-predicting equations conducted from stepwise regression analysis.*

Equations*	Correlation (*r*)	Multiple R^2	SEE
Age = 1.002 (OPD) − 1467.563 (OA) + 69.365	0.874	0.763	6.994
Age = 0.964 (OPD) − 1363.452 (OA) − 1.805 (CW) + 76.443	0.888	0.789	6.650

CW, cortical width; OPD, osteon population density; OA, average size of the osteon; SEE, standard error of estimate (years).

*Multicollinearity did not exist among the independent variables, and the linear regression model was verified by ANOVA at the 1% level of significance.

OPD ($R^2 = 0.656$). The CW and HA were weakly correlated with age ($R^2 = 0.165$ and 0.087, respectively).

Table 5 presents the age-predicting equations derived from the stepwise regression analysis. In the stepwise method, two histomorphological variables, OPD and OA, were selected (the multiple R^2 and standard error were 0.763 and 6.994, respectively). Three variables, OPD, OA, and CW were then selected as providing a more satisfactory result (the multiple R^2 and standard error were 0.789 and 6.650, respectively).

Discussion

In practical experience, the femur is more easily recognizable and discovered in forensic sites such as hillsides or archeological sites and graves than other skeletal remains. Its midshaft of the diaphysis is anatomically identifiable and survives most conditions of burial or injury better than the epiphysis of bone (2). Thus, the femur may be the most useful skeletal material for microscopic age estimation in anthropological fields.

A number of techniques have been suggested to provide microscopic measuring fields at the femur: four circular visual fields (anterior, posterior, medial, and lateral) by Kerley (2); four square fields on a diagonal axis tangential to the anterior cortex by Ahlqvist and Damsten (11); several fields within a 4 mm diameter through the core by Thompson (6); and five subperiosteal fields at 2.5 mm intervals from the center by Ericksen (12). These studies reported difficulties in the repeatability and constancy for histomorphometric analysis from the viewpoint of providing objective measuring fields. Moreover, in other sites distant from the anterior cortex, there seem to be greater variation in histological structures that do not correlate with age (11). The present study used a modification of the method of Maat et al. (17), and microscopic analysis was performed at the anterior cortex after creating the five measuring fields using a protractor with points 0°, ± 10°, and ± 20°. This produced constant measuring fields for repeatability (Fig. 1).

Morphological age-estimation methods identifying the degree of degeneration from the articulations of bones are inaccurate or inappropriate in skeletons from individuals older than 50 years (18). Microscopic age estimation provides an alternate method to estimate age beyond 50 years (12). The results of the present study seem to satisfy this need, despite the skewness of data in the cadavers from older individuals (Table 1).

ANCOVA revealed no significant differences in age-adjusted histomorphological variables between sexes (Table 3). This is contrary to the observation that sex is an important factor affecting bone remodeling and histomorphometrics (1,19). The age distribution may have been responsible because only a small proportion of bone samples was from females less than 50 years, which would have limited the effects of physiological variables such as menopause. Samples from younger individuals are needed to confirm the existence of sex differences in femoral histomorphometrics.

The CW showed relatively weak correlations in the linear regression model (Table 4). However, CW was selected from the independent variables with a negative slope in the stepwise regression analysis (Table 5). This may evoke an intuitive relationship between the CW and age, but could not be ruled out as a scaling factor that is multiplied times one variable to yield the value of another. One study reported that bone replacement during aging causes a gradual decrease in the percentage of nonremodeled circumferential lamellar bone (17). Therefore, by way of suggestion, careful and attentive application of age-predicting equation including CW should be considered. The HA was not selected from the independent variables in the stepwise regression analysis (Table 4), and this result is consistent with the results of previous studies (6,20,21).

A comparison of other microscopic age-estimation methods relating osteon measurements from the femur is shown in Table 6. Because of different measuring fields and variables, populations,

TABLE 6—*Comparison with other studies relating osteon measurements from the femur.*

Author(s)	Correlation (r)	Multiple R^2	SEE*	Population (n)
Kerley (2)	0.922	–	9.39	American whites and African Americans (67)
Ahlqvist and Damsten (11)	0.965	–	6.71	No ethnic data provided (20)
Thompson (6)	0.862	0.744	7.07	New England whites (116)
Ericksen (12)	–	0.670	10.08	Whites in U.S.A. and a few other populations (328)
Watanabe et al. (21)	–	0.887	6.39	Japanese (98)
Present study	0.888	0.789	6.65	Koreans (72)

SEE, standard error of estimate.

and age distribution of the samples, direct comparison between our data and previous studies is difficult. However, our multiple regression correlation coefficient ($R^2 = 0.789$) and standard error of estimate (SEE = 6.65) were higher than those reported previously. The results of Watanabe et al. (21) showed a high multiple regression correlation ($R^2 = 0.887$) and low SEE of 6.39, which may reflect the use of various staining methods including Villanueva bone stain and microradiograms to demarcate the histological structures more easily in a 100 × field. Forensic practice needs to develop simple and efficient methods that achieve a balance between accuracy and rapidity.

In conclusion, repeatable and constant measurement of this study should provide reliable and consistent results of estimating age at death from the anterior cortex of the femur in Korean adults. In future, there is a need for developing age-predicting equations which includes the data from younger samples and demonstrating the result of differences when using these equations over another population.

Acknowledgments

We thank Sang-Yong Lee and Kwang-Hoon Kim, Institute of Forensic Medicine, School of Medicine, Pusan National University, for providing helpful discussions concerning the histological age estimating from the femur.

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2006-331-E00011).

References

1. Stout SD. The use of histomorphology to estimate age. J Forensic Sci 1988;33:121–5.
2. Kerley ER. The microscopic determination of age in human bone. Am J Phys Anthropol 1965;23:149–63.
3. Thompson DD, Galvin CA. Estimation of age at death by tibial osteon remodeling in an autopsy series. Forensic Sci Int 1983;22:203–11.
4. Stout SD, Stanley SC. Percent osteonal bone versus osteon counts: the variable of choice for estimating age at death. Am J Phys Anthropol 1991;86:515–9.
5. Singh IJ, Gunberg DL. Estimation of age at death in human males from quantitative histology of bone fragments. Am J Phys Anthropol 1970;33:373–81.
6. Thompson DD. The core technique in the determination of age at death of skeletons. J Forensic Sci 1979;24:902–15.
7. Yoshino M, Imaizumi K, Miyasaka S, Seta S. Histological estimation of age at death using microradiographs of humeral compact bone. Forensic Sci Int 1994;64:191–8.
8. Stout SD, Dietze WH, Iscan MY, Loth SR. Estimation of age at death using cortical histomorphometry of the sternal end of the fourth rib. J Forensic Sci 1994;39:778–84.
9. Cho H, Stout SD, Madsen RW, Streeter MA. Population-specific histological age-estimating method: a model for known African-American and European-American skeletal remains. J Forensic Sci 2002;47:12–8.
10. Kim YS, Kim DI, Park DK, Lee JH, Chung NE, Lee WT, et al. Assessment of histomorphological features of the sternal end of the fourth rib for age estimation in Koreans. J Forensic Sci 2007;52:1237–42.
11. Ahlqvist J, Damsten O. A modification of Kerley's method for the microscopic determination of age in human bone. J Forensic Sci 1969;14:205–12.
12. Ericksen MF. Histologic estimation of age at death using the anterior cortex of the femur. Am J Phys Anthropol 1991;84:171–9.
13. Thompson DD, Gunness-Hey M. Bone mineral-osteon analysis of Yupik-Inupiaq skeletons. Am J Phys Anthropol 1981;55:1–7.
14. Aiello LC, Molleson T. Are microscopic ageing techniques more accurate than macroscopic ageing techniques? J Archaeol Sci 1993;20:689–704.
15. Stout SD, Lueck R. Bone remodeling rates and skeletal maturation in three archaeological skeletal populations. Am J Phys Anthropol 1995;98:161–71.
16. Villanueva AR. A bone stain for osteoid seams in fresh, unembedded, mineralized bone. Stain Technol 1974;49:1–8.
17. Maat GJ, Maes A, Aarents MJ, Nagelkerke NJ. Histological age prediction from the femur in a contemporary Dutch sample. The decrease of nonremodeled bone in the anterior cortex. J Forensic Sci 2006;51:230–7.
18. Ubelaker DH. Human skeletal remains, 3rd ed. Washington: Taraxacum, 1999.
19. Martin RB. On the significance of remodeling space and activation rate changes in bone remodeling. Bone 1991;12:391–400.
20. Currey JD. Some effects of ageing in human Haversian systems. J Anat 1964;98:69–75.
21. Watanabe Y, Konishi M, Shimada M, Ohara H, Iwamoto S. Estimation of age from the femur of Japanese cadavers. Forensic Sci Int 1998; 98:55–65.

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