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Title: Age estimation through histological study of trabecular volume and cortical bone width of the iliac crest.

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The obtained images were digitally processed through a program of image analysis and data were analyzed using the package SPSS statistical. The analysis results showed that the combination of the trabecular volume and cortical width is a predictible variable of the age in those subjects involving no suffering from deterioration of bone and mineral metabolism.

Cover Letter or Cover Page including author details

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Editor-in-Chief

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Dear Sir,
We are pleased to enclose for your consideration a paper entitled "**Age estimation through histological study of trabecular volume and cortical bone width of the iliac crest".**

The paper is signed by three authors: Rafael Fernández Castillo, Douglas H. Ubelaker, Mirjana Djorojevic. No author has any type of financial interest in this manuscript. All authors contributed to the preparation of the manuscript and all have approved the final version.

The undersigned authors hereby transfer, assign, or otherwise convey all copyright ownership to **Science and Justice**. This manuscript has not been submitted or published elsewhere in any form.

Yours sincerely.
Rafael Fernández Castillo

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Abstract

There have been many methods proposed to estimate the age of human bones in forensic medicine or in forensic anthropology, including those assessing histological structures. In this study, age was evaluated through the histomorphometric study of trabecular volume and cortical width of the right iliac crest. A total of 25 samples were obtained from necropsies of judicial cases, ages ranging from 13-58 years, who had died a sudden or violent death. From a total of 25 studied samples, 16 correspond to males and 9 to females. The samples were obtained using Bordier's trocar for bone biopsy, in a period not exceeding 24 hours after death. The samples were examined without decalcification, with 96º alcohol dehydration and embedding in methyl methacrylate, employing a cut with a microtome Reichert of 3 μm thick. The sections were fixed in sheets slide and stained, using toluidine blue, Goldner trichrome and hematoxylin –eosin methods.

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Introduction

Numerous methods of age estimation of the human skeleton from histomorphometric techniques have been described [1,2,3,4].The first important study on histological changes of the bone and its application to calculate the age in adult skeletons, was carried out in 1965 by Kerley[5], who developed a method based on microscopic analysis of long bone cortex.

In that study four cortical components in the most external bone area were determined, evaluated and examined in both sexes independently or in combination. These are: complete secondary osteons, fragmented secondary osteons, laminar circumferential bone and non Haversian channels in the femur, tibia and fibula. Using the profile chart approach, about 88 percent of estimates were within five years of actual age and all estimates were within 10 years. No sex differences were noted. Kerley´s method presents certain disadvantages: it can be used for long bones of the lower extremities only, requiring the entire diaphysis of the bone. Besides, it counts with numerous subjective variables that interfere in the estimation of parameters to be measured, such as the difficulty of distinguishing fragmented and intact osteons and estimating the percentage of laminar bone in a circular field of vision. That is what Ahlqvist and Damsten had revealed, although the modification of Kerley´s method proposed by them gave somewhat lower results [6]. With a method presented at the Annual Congress of the American Academy of Forensic Sciences, Thompson tried to surpass these limitations [7].

On a sample of 116 corpses (64 male and female 52), whose known age ranged between 30 and the 97 years, Thompson obtained a series of 0.4 cm diameter cylinders from the cortical bone of femur, humerus and ulna and evaluated 19 variables in each cylinder.

Microscopic analysis of histological sections was carried out by Morphometric stereological procedures.

19 variables derived from each cylinder were examined by linear regression analysis in order to select a variable or combination of them with a lower standard error and the highest coefficient of determination.

This study resulted in a series of regression equations which showed that the area of cortical bone which contains osteons (osteonal area), is the best individual element to determine the age of skeletal remains.

Since then, many studies have been focused in one way on another on age determination, counting the osteonal areas of femur or ribs [8,9,10] and observing their evolution, but none of them has examined histomorphometric parameters from trabecular bone volume as well as the cortical width of the iliac crest.

The aim of this study is to evaluate the association of age with the trabecular bone volume and the cortical width of the iliac crest.

Methods

Subjects:

A total of 38 samples were obtained from necropsies of judicial cases, ages ranged from 13-58 years, who had died a sudden or violent death. From a total of collected samples, 13 were discarded due to one or more of the following causes: time of death more than 24 hours; insufficient number of samples; error in sample processing; evidence of disease that could alter the bone metabolism. From a total of 25 studied samples, 16 correspond to males and 9 to females.

Methods:

The samples were obtained using Bordier's trocar for bone biopsy of the right iliac crest (Image 1), in a period not exceeding 24 hours after death. From this section, a transiliac cylinder 7mm in diameter and 15 mm long was obtained.

Biopsies were performed without decalcification, with 96º alcohol dehydration and embedding in methyl methacrylate, making the cut with a microtome Reichert of 3 μm thick (Image 2).

The sections were fixed in sheets slide and stained, using toluidine blue, Goldner trichrome and hematoxylin –eosin methods.

The samples were studied under the microscope Leica DMLB and the pictures were taken with CCD Sony adapted to digitizing card.

Once obtained, the bone section images digitalized to 10x 20x 40x and 63x were edited and analyzed, segmenting the area by means of the following functions: transfer image, colour grey, threshold, find object, calibrate and analyze. In addition, the bone surface tissue has been analyzed and discriminated by its gray level (Image 3). The segmentation of total bone area was performed pointing out the contour interactively.

The results of trabecular surface, cortical surface, length and width have been obtained. For the study of morphometric parameters a method devised deliberately with Visilog and Visual Basic has been used.

For segmentation of Trabecular volume (TV), osteoid volume (OSV), osteoid width (WOS) and cortical width a macro with Visilog has been performed.

A program in Visual Basic that processes the data obtained by Visilog has been created. Mathematical formulas based on geometric calculation have been applied to values which Visilog supplies.

The data have been exported to the SPSS statistical package and a descriptive study by age groups, Anova Test, bivariate correlations and multiple regression equations has been done.

All the data were expressed with the mean value ± standard deviation (X ± SD); $p< 0.05$ values were considered statistically significant.

Results

When classifying a study group according to the age, it can be observed that both cortical width and trabecular bone volume show an increase from an early age up to the age of 40, since this age, both variables begin to descend as it can be seen in Table 1 and Table 2.

The trabecular bone volume shows a high correlation index with the age (r:-0.89 $p< 0,01$); on the contrary, the cortical width does not show any correlation index (r: 0,040 p: 0,849).

Multiple linear regression analysis shows that, when considering the age as a dependent variable, there is a coefficient of important determination (R^2: 0,788) with the trabecular volume and cortical width respectively.

In the Tables 3, 4, 5 and 6 it can be clearly seen that the combination of age, trabecular volume and cortical width increase the precision for calculating chronological age, as independent predictive variables, based on their respective coefficients of determination (R^2).

Discussion

The iliac crest is formed by cortical bone and cancellous bone, the latter being the most dependent on changes. With the passage of time, it is possible to observe how the structure of the cancellous bone gradually disintegrates, with loss of bone trabeculae, and finally, thinning of the cortical bone. It has generally been assumed that growth of the iliac crest is dependent on mechanical load factors, which is confirmed by the non-existence of differences between the sexes if it is correlated with body size; as opposed to the differences found in cases of endochondral ossification, such as vertebral bodies, the mineralization of which does not appear to be influenced by the mechanical loads they support

As we can observe in Table 1, the study of age with regard to the quantification of trabecular and cortical bone enables us to confirm the tendency for bone mass to decrease with increasing age, bone mass reaching a peak at approximately 30 to 35 years of age and starting to decline at around the age of 40, its lowest level being reached at about 80 years. These results agree with those of the research of other authors [11,12], using radiological and imaging techniques.

The use of selection criteria for ensuring the normality of the data is essential. Liver pathology, kidney and intestinal diseases that could alter the results achieved, are to be excluded.

As a result of this, the biopsies have been obtained from individuals who died in a sudden or violent manner and whose autopsies have not revealed any functional pathology that could have altered the bone metabolism.

The appearance of autolytic and putrefactive phenomena 24 hours after death[13] is the reason we have excluded the cases with superior time of death.

However, it is possible that quantification of mineralized bone does not suffer any changes although autolysis is present. Hence, its application in Forensic Anthropology is possible.

As stated previously, the place of choice for the bone biopsy has to provide a representative material about the changes which take place in a skeleton as a whole.

To this effect, different locations as spine, iliac crest and femoral neck provide different values for the same histomorphometric parameters, and even different portions of the iliac crest can show important variations that depend on parameter and diagnostics [14,15].

Several factors are invoked in determining the portion of choice to perform a biopsy: ease of access, choice of the area where there is an active bone re-exchange, adequate quantity of trabecular bone, etc [16, 17.]

The place of choice for the bone biopsy needs to contain both cortical bones and spongy bone. Firstly, the presence of the cortical bones allows proper recognition of the medullary cavity, which is used as a reference for many of morphometric parameters. Secondly, the activity within the cortex and the subperiosteal portion can be evaluated and compared with the trabecular bone activity[18].

This work opted to take samples of the right iliac crest, which allow precise selection of those areas that are more suitable for carrying out the measurements.

In the past, and even today, in numerous works in histomorphometry applied to forensic practice or paleoanthropology, the rib has frequently been used as an item of choice to be examined [19,20,21].

The ribs seem to provide a good sample of cortical bone and since the remodelling packets have been oriented to the longitudinal axis of the rib, the sample was obtained by cross-section.

However, due to the lack of trabecular tissue in the rib, as well as the better accessibility of the iliac crest, the latter is the more common place to obtain bone biopsy, which at present supplants the costal biopsy [22].

The sample size also allowed obtaining cylinders of quality, since no samples were taken with chipping or with microfractures that could interfere with the outcome of the research.

The reasons to avoid the decalcification are obvious and numerous; most importantly the conservation of the differences between the calcified bone and non calcified bone to diagnose and measure parameters of interest, in this case the trabecular bone volume and cortical width

Methyl methacrylate has been used for inclusion of the pieces because it allows a slow polymerization without giving off the heat and its hardness is similar to the bone. The only inconvenience to highlight is a preparation time because it is necessary to keep the sample in methylmethacrylate for 5 days at 32ºC approximately, increasing the temperature for 2 degrees every 24 hours, so that the sample can be entirely included in the plastic material and in order to prevent the formation of bubbles that could interfere in the sample processing.

Conclusions

The results show the utility of the bone histomorphometry and determination of the trabecular volume and the cortical width of the iliac crest to be a useful tool for age estimation in Forensic Anthropology.

The regression equation Y= 108,897 + X(-3,079) + Z(1,905), where Y represents the age, X-trabecular volume and Z cortical width value, is highly reliable to infer the chronological age from the examined sample. On the other hand, it would be convenient to expand the sample and carry out more extensive research based on these results.

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Table

Table 1 Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
						Lower Bound	Upper Bound
Cortical width	0-20	6	1,7867	,12832	,05239	1,6520	1,9213
	21-30	8	2,1750	,04175	,01476	2,1401	2,2099
	31-40	3	2,6167	,18230	,10525	2,1638	3,0695
	41-50	6	2,3567	,31379	,12811	2,0274	2,6860
	> 51	2	1,1500	,21213	,15000	-,7559	3,0559
	Total	25	2,0964	,42491	,08498	1,9210	2,2718
Trabecular Volume	0-20	6	29,0417	1,95612	,79858	26,9888	31,0945
	21-30	8	29,5788	2,17619	,76940	27,7594	31,3981
	31-40	3	25,2900	,11533	,06658	25,0035	25,5765
	41-50	6	22,4617	1,13526	,46347	21,2703	23,6530
	> de 51	2	20,0550	2,19910	1,55500	,2969	39,8131
	Total	25	26,4652	3,89158	,77832	24,8588	28,0716

Table 2 Anova

		Sum of Squares	df	Mean Square	F	Sig.
Cortical width	Between Groups	3,635	4	,909	26,025	,000
	Within Groups	,698	20	,035		
	Total	4,333	24			
Trabecular Volume	Between Groups	299,877	4	74,969	23,579	,000
	Within Groups	63,589	20	3,179		
	Total	363,466	24			

Table 3
Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	,888[a]	,788	,769	6,48930

a. Predictors: (Constant), Cortical width, Trabecular Volume

Table 4
Anova

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	3451,558	2	1725,779	40,982	,000[a]
	Residual	926,442	22	42,111		
	Total	4378,000	24			

a. Predictors: (Constant), Cortical width, Trabecular Volume
b. Dependent Variable: Age

Table 5

Coefficients

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	108,897	11,090		9,820	,000
	Trabecular Volume	-3,079	,340	-,887	-9,044	,000
	Cortical width	1,905	3,118	,060	,611	,547

a. Dependent Variable: Age

Table 6

Age= 108,897 + Trabecular Volume x (-3,079) + Cortical Width x 1,905
Y= 108,897 + X(-3,079) + Z(1,905)

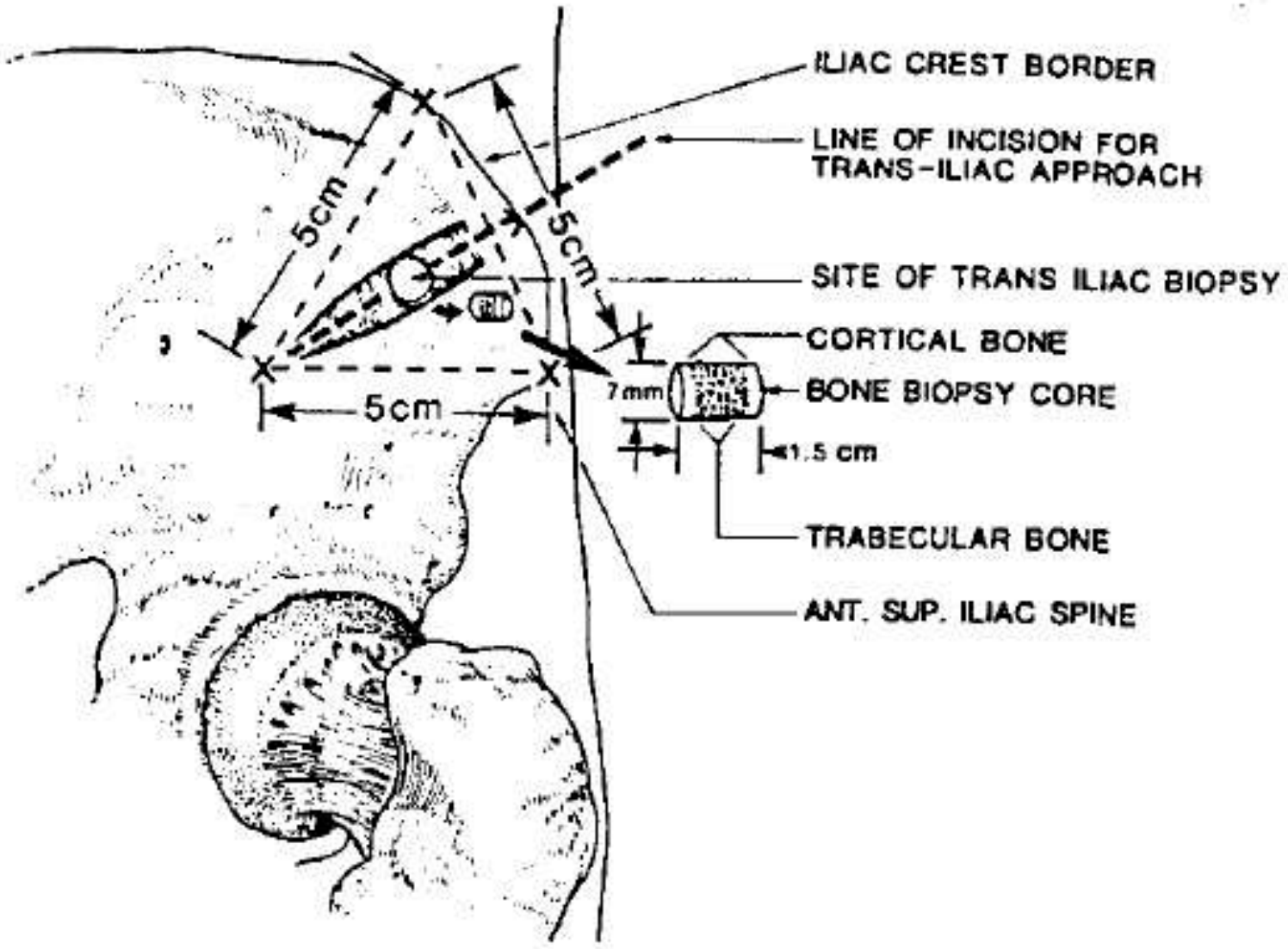
Legend:

Image 1: Detail of two types of iliac biopsy.

Image 2: Bone biopsy specimens embedded in methyl methacrylate

Image 3: Trabeculae segmentation by threshold function by levels of gray.

Figure


ILIAC CREST BORDER
LINE OF INCISION FOR TRANS-ILIAC APPROACH
5cm
5cm
SITE OF TRANS ILIAC BIOPSY
CORTICAL BONE
7mm
BONE BIOPSY CORE
5cm
1.5 cm
TRABECULAR BONE
ANT. SUP. ILIAC SPINE

Figure

Figure