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Estimating the Date of Bone Remains: A Multivariate Study

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ABSTRACT: In previous works we have studied the time of death of bone residuals through the following parameters: total lipids, triglicerides, cholesterol, free fatty acids, total proteins, zinc, iron, manganese, and phosphorus. These elements were quantified in groups of recent bones of 1 and 2 years and of 10, 15, 18, and 20 years postmortem. In this present work we are putting these results under statistical analysis consisting of a stepwise regression. This program selects and introduces in the regression the element that shows the highest correlation with the time of death. In successive steps the partial correlations between the date and the elements not already included in the regression are studied, while keeping the effects of the elements already included fixed. As a result we put forward three formulas in which the time of death appears linked with the parameters that define it best. In the first the time of death of the bones Y is estimated according to the protein X_1.

$$Y = 40.0014 - 7.4275X_1$$

In the second formula the time of death Y, is estimated according to proteins X_1 and triglicerides X_2.

$$Y = 45.5970 - 10.8096X_1 + 0.4104X_2$$

And in the thrid formula the time of death Y is estimated according to proteins X_1, triglicerides X_2, and cholesterol X_3.

$$Y = 52.2032 - 7.8213X_1 + 0.6355X_2 - 3.4930$$

In the three formulas the coefficients of the correlation between the time of death and the variables are improved when the logarithms of the variables are taken, instead of the original measurements.

KEYWORDS: physical anthropology, musculoskeletal system, human identification

Establishing with exactitude the date of bone remains is a frequent problem in forensic science work of great practical importance, since by dating these bone remains, identification is made easier.

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This importance is made evident by the number of professors of legal medicine who have studied this subject since the beginning of the century. We have been left excellent morphological descriptions of bones from different periods in the classical studies of Ref *1*.

Since then other studies have been carried out:

1. Physical studies—specific weight of the bone [*2-3*] is determined.
2. Chemical studies—to show the mineral content of the bone (Tirelli and his ClH test) [*4-5*], as well as the organic content; the presence of lipids was studied by Buerger and Maestre [*6*], and more recently by Berg [*7*]; and the presence of proteins was studied by Beumer [*8*], Bayle et al [*9*], Berg [*7*], and Knight [*10-12*].
3. Histological studies to show the presence of cells in the Havers ion canals [*2-3*].

The present authors have dealt with this subject in depth in previous studies [*13-17*], in which we have determined the relation between the organic material and mineral material in bones of different times since death.

Secondly we have quantified the minerals that are most abundant in the bone (iron, zinc, copper, calcium, magnesium, phosphorus, sodium, and potassium), as well as the principal organic components: proteins, protein fractions, amino acids, total lipids, free fatty acids, cholesterol, and triglycerides.

Statistical treatment, previous to this study, has demonstrated a correlation between the behavior of these elements and the date of the bones studied.

The complexity of the analytical process undertaken made us choose statiscally those elements which showed the highest correlation with the bone date. Those may be the best form of predicting the date by being evaluated together in an equation of multiple regression. This allows the legal medicine laboratory to establish the date of bone remains economically and with maximum reliability.

Material

Our study is based on ten series of human bones:

First series—six different recent bones, from the autopsy room, date zero years.

Second series—the same six bones from the previous series after being kept in the laboratory for six months, date one-half year.

Third series—two bones from the previous series, studied one year later, date one year.

Fourth series—the two previous bones studied two years later, date two years.

Fifth series—five long bones from exhumation of coffins in the soil, date ten years.

Sixth series—5 long bones from exhumation of coffins in the soil, date 15 years.

Seventh series—5 different bones from exhumation of coffins in the soil, date 18 years.

Eighth series—5 metacarpial bones from exhumation of coffins in the soil, date 20 years.

Ninth series—6 long bones, from exhumation of coffins in the soil, date 50 years.

Method

We make a distinction between an analytical method and a statistical method.

Analytical Method

Each bone studied was divided into two samples. The first was mineralized, and in this sample we quantified by atomic absorption, zinc (γ%), iron (γ%), and magnesium (mg/100 mg), and by the Fiske-Soubarow technique, potassium (mg/100 mg). The second sample was divided into two. A Soxhlet extraction of the total lipids by means of another was carried out on the first part, which was reduced to bone dust, from which we quantified triglycerides,

cholesterol, and free fatty acids. The proteins were extracted for 48 h from insulin for the second part and a quantification according to the Lowry process made with the supernatant.

Statistical Method

The statistical method consisted of a stepwise multiple regression. In the first step, the element which shows the highest correlation with the date is introduced into the regression equation. In the following steps the partial correlation with the date and the elements not yet included in the regression are studied while keeping the effects of the elements already included fixed. The elements that show the highest correlation with the date are successively included in the equation. The process terminates when no element reaches the Snedecor F(F-to-enter equal to or higher than 4) [*18*].

At each step the regression of the date is determined on the variables included, together with the multiple correlation coefficient and the standard error of the estimate. Also carried out is a variance analysis of the regression, which is also proved by the Snedecor F(F ratio). A programmed computer was used.

Results

The results of this study are set out in six tables. The first three refer to the regression between the bone date and the other variables in their original units of measurements. The last three (Tables 4, 5, and 6) are a structural replica of the first three, but the variable measurements in the bone (proteins, lipids, and so forth) are not taken in their original units, but in a logarithmic transformation of the units. So, for example, if the simple correlation between the date and the logarithm of the iron content of the bone (Table 4) is $r = 0.1289$, the importance of this transformation on the independent variables will be discussed later.

In Table I the evolution of the correlation coefficients between the date and the other variables are shown at each step of the regression. In Column A the simple correlation coefficients between the date and each of the remaining elements are set out. As 41 bones are studied for 39 degrees of freedom the correlation coefficients of the date with proteins ($r = 0.86$), cholesterol ($r = -0.78$), fatty acids ($r = -0.65$), zinc ($r = -0.63$), total lipids ($r = -0.62$), and triglycerides ($r = -0.54$) are significant at the level of 0.01. As the highest significance is reached by the correlation between the date and the proteins (F-to-enter = 112.8), this is the first variable included in the step-by-step regression. The effects of this inclusion are made evident in Column B where the *partial correlation coefficients* between the date and the other variables are laid out while *keeping the effects of the variable "proteins" fixed*. The sharp drop in the correlation between the date and zinc on the one hand and cholesterol and fatty acids on the other, while the correlation with iron and magnesium is more or less similar, are the effects which particularly stand out. The alteration of the correlation sign between the date and triglycerides will be dealt with later.

Since the partial correlation with this element is the highest (F-to-enter = 16.99), the triglycerides are included in the regression. The effects of this inclusion are shown in Column C, where the partial correlation coefficients between the date and the other variables are set out while keeping the effects of the proteins and the triglycerides fixed. Cholesterol is again significant (F-to-enter = 8.60), which is why it is included in the regression together with proteins and triglycerides. The effects of this inclusion are shown in Column D. The correlation between the date and the other variables are no longer significant and no other element reaches the level of $F = 4$ and as a results are not included in the regression.

Table 2 shows the multiple correlation coefficients between the date and the variables included at each step of the regression and resumes the variance analysis of regression. The variance shown by the regression (which = $100\ R^2$), is 74.31% if only proteins are included, 82.25% if proteins and triglycerides are included, and 85.59% if proteins, triglycerides, and

TABLE 1—*Correlations between the date and the other variables at each step of the multiple regression. Column A is simple correlations between the date and the other variables. Columns* B, C, *and* D *are partial correlations between the date and the other variables keeping proteins* (B)*; proteins and triglicerides* (C)*; and proteins, triglicerides. and cholesterol* (D) *fixed.*

	A		B		C		D	
	r	*F*-to-enter	*r*	*F*-to-enter	*r*	*F*-to-enter	*r*	*F*-to-enter
Iron	−0.0469	0.1	0.0987	0.37	0.0983	0.36	0.0381	0.1
Zinc	0.6267	25.2	0.2297	2.12	0.2565	2.61	0.1468	0.8
Phosphorus	0.0566	0.1	−0.1102	0.47	0.1637	1.02	0.2551	2.5
Magnesium	0.2169	1.9	0.2059	1.68	0.0624	0.15	0.2003	1.5
Lipids	−0.6213	24.5	0.5055	13.04	0.2260	1.99	0.1305	0.6
Cholesterol	−0.7766	59.3	0.0829	0.26	−0.4342	8.60[x]	...	...
Triglycerides	−0.5360	15.7	0.5558	16.99[x]	...	...	...	...
Lipid acids	0.6450	27.8	0.0535	0.11	−0.0359	0.05	−0.1612	1.0
Proteins	−0.8620	112.8	...	...	...	...	...	...

TABLE 2—*Multiple correlation coefficients* (R *and* R^2) *between the date and the variables included at each step of the regression.*

Steps	Variable Included	r	R^2	Increasing R^2	F Ratio
1	proteins	0.8620	0.7431	0.7431	112.8
2	proteins and triglycerides	0.9069	0.8225	0.0794	88.0
3	proteins, triglycerides, and cholesterol	0.9252	0.8559	0.0335	73.3

cholesterol are included. It can be observed how these two last elements contribute only 11.29% to the variance shown by the regression. The highest percentage was explained exclusively by proteins (74.31%). The last column of Table 2 is the F ratio which proves the significance of each step of the regression for the corresponding degrees of freedom.

In Table 3, the equations obtained at each step of the regression are shown. The bone date (y) is estimated from proteins (X_1); from proteins (X_1) and triglycerides (X_2); or from proteins (X_1), triglycerides (X_2), and cholesterol (X_3). This last equation, in accordance with our statistical analysis explains *the highest percentage of variance of the information with the minimum number of variables in the equation.*

The standard errors of the bone date estimation (y) from these 3 are 8.42, 7.09, and 6.47 for each one of them, respectively. The best equation to determine the bone date is the third one, since it offers the lowest error rate in the estimation.

The evidence obtained from previous studies that a curvilinear adjustment may be more convenient than a lineal adjustment have made us carry out a logarithmic transformation of the independent variables. However, we have not done this with the date because it is a variable established as dependent.

Table 4 is interpreted like Table 1. Notice that as the logarithms are taken, the correlation coefficients between the date and the other variables systematically increase, although the same significant coefficients are maintained (for 37 degrees of freedom). Furthermore, the logarithmic transformation reduces the step of the regression to two, in which only proteins ("log. proteins") and triglycerides ("log. triglycerides") are included.

Table 5 brings out this phenomenon. The regression of "log proteins" and "log. triglycerides" in the date explains 95.76% of the variance. But from this variance caused by the regression, 95.27% comes from "log. proteins" while "log. triglycerides" contributes only 0.5%.

The regression equations are set out in Table 6. The standard error of the date estimate using the first equation is 3.49. If we use the second it is 3.34. This is the lowest error of the estimate of all the equations set out. It is therefore this last equation that must be used to predict the bone date with maximum reliability, as will be shown in the discussion.

Discussion

In previous studies we have proposed a complete analytical study of each bone problem on which it was necessary to establish a postmortem date.

However, the diversity of the techniques presented and the complexity of some of them have made us undertake this present statistical study, in which we have chosen those variables that are really responsible for the regression line which express the postmortem aging of the bone.

Protein Behavior

The stepwise regression shows us that the most important variable for finding out the bone date is the quantity of protein present in the bone, to the point where if they are taken in their

TABLE 3—*Regression equations obtained at each step:* y = *date,* X_1 = *proteins (μg/g of bone),* X_2 = *triglicerides (μg/g of bone), and* X_3 = *cholesterol (μg/g of bone).*

$$y = 40.0014 - 7.4275\,X_1$$
$$y = 45.5970 - 10.8096\,X_1 + 0.4104\,X_2$$
$$y = 52.2032 - 7.8213\,X_1 + 0.6355\,X_2 - 3.4930\,X_3$$

TABLE 4—*Correlations between the date and the rest of the variables (after the logarithmic transformation) at each step of the multiple regression. In* A: *simple correlations between the date and the other variables. In* B *and* C *partial correlations between the date and the other variables keeping proteins logarithm* (B) *and proteins logarithm and triglicerides logarithm* (C) *fixed.*

	r	*F*-to-enter	*r*	*F*-to-enter	*r*	*F*-to-enter
Iron	−0.1289	0.63	−0.0309	0.03	−0.0730	0.19
Zinc	0.6996	35.47	−0.0738	0.20	0.0405	0.06
Phosphorus	0.1752	1.17	0.0694	0.17	0.1648	0.98
Magnesium	0.2179	1.84	0.1333	0.65	0.0404	0.06
Lipids	−0.8435	91.22	0.0822	0.25	−0.2552	2.44
Cholesterol	−0.8978	153.71	0.1341	0.66	−0.0855	0.66
Triglycerides	−0.8347	85.00	0.3240	4.22[x]	...	...
Lipid acids	0.7304	42.30	−0.0753	0.21	0.1861	1.26
Proteins	−0.9761	744.95[x]	...	...	...	...

TABLE 5—*Multiple regression coefficients* (R,R^2) *between the date and the variables (previous logarithmic transformation) included at each step of the regression.*

Steps	Variable Included	*r*	R^2	Increasing R^2	*F* Ratio	Degree of Freedom
1	proteins	0.9761	0.9527	0.9527	744.95	1.37
2	proteins and triglycerides	0.9786	0.9576	0.0050	407.01	2.36

TABLE 6—*Regression equations obtained at each step:* y = *date,* X_1 = *proteins, and* X_2 = *triglicerides.*

$$y = 36.7998 - 50.0067 \log X_1$$
$$y = 36.0678 - 56.9910 \log X_1 + 4.6048 \log X_2$$

original measurement (mg/100 mg) they justify 74.31% of the variance in the regression. If the logarithms of this measurement are taken into account, the justified variance becomes 95.27%.

The classical studies of Beumer et al [*9*] had already proposed a study of bone protein, to determine the age of a bone, through an immunological reaction between bone dust and an antihuman serum. This reaction was negative in bones of a certain age. However, this technique could only establish the date within very wide margins which were not very useful from the forensic science point of view. The same applies to more recent studies, but also carried out by semiquantitative methods like those of Berg and Knight [*10–12*].

We propose an extraction of bone dust insulin lasting 48 h in constant movement and a subsequent quantification in the supernatant by the Lowry technique. This is a simple technique which can be carried out in any laboratory dealing in legal medicine or biochemistry.

As we commented in the results, by keeping the proteins fixed and correlating the date and the other variables, there is a sharp drop in the significance of zinc.

The explanation is that zinc is linked to proteins in some way, thus presenting a similar behavior, but in the opposite direction a drop in proteins is parallel to a rise in zinc.

Another phenomenon that is reflected in Column B of Table 1 is a change in the sign of the regression coefficient of triglycerides. The explanation for this is that while proteins fall slowly, triglycerides show a sharper fall. As in any multiple regression equation, the variables are in close interdependence. In the case of our equation the coefficient of the positive regression obtained for triglycerides show that they act as a corrector on the sharp protein fall.

Lipid Behavior

The only elements chosen in the stepwise regression together with proteins are triglycerides and cholesterol.

In Table 2 we show the coefficients of the multiple correlation between the date and the variables included. Proteins justify 74.31% of the variance, 82.25% if triglycerides are included, and 85.59% if cholesterol is included.

However, when the logarithmic transformation is carried out, the regression is reduced to two-step. Cholesterol is rejected, and proteins (95.27%) and triglycerides (0.5%) are sufficient to explain practically all the variance of the regression (95.76%).

This shows that the postmortem aging of bone remains results fundamentally from the loss of the organic material (principally proteins and in a lower quantity lipids) of which it is made.

Classical authors, such as Buerger and Maestre [*6*] had guessed this and tried to show the presence of fats by the submersion of a slice of bone in a solution of copper acetate and osmic acid. Both authors showed that the lipids disappeared from the bone eight or ten years after burial.

By making a Soxhlet extraction of the bone dust in ether we have found lipids even in bones 100 years old. These results coincide with those of Berg [*7*], who used a similar procedure. We insist on the importance of the method with regards to the extraction and subsequent quantification of these bone components.

Estimate of the Bone Date

The estimate of the bone date from the last equation shown, which takes into account the logarithms of the values of the proteins and triglycerides of the bone studied, is a point estimate. That is to say, by applying the formula, we obtain a concrete value for the date. However, we have already stated in the results that this estimate has a standard error of 3.34 years. This means that a more accurate estimate would be possible by using the confidence limits at 10, 5, or 1%.

Taking into account the standard error of the estimate, the confidence interval at 10% is 5 years. The confidence interval at 5% shown an error of 6.5 years, and the confidence interval at 1% is 8.6 years.

Strict methodology induce us to present these confidence intervals which may at first seem very wide. Nevertheless we must not forget that with the formula we obtain a point estimate limited by the confidence intervals. So when we make the estimate of a date, the probability that it will be outside the point estimate decreases as we get further away from the probability.

As a final conclusion of this study, we would like to point out that we have achieved maximum economy of method (quantification of proteins and triglycerides) in determining the date of bone remains and offering at the same time maximum validity in the result.

However, the rest of our studies on the subject which used other techniques such as mineralization and the relation between organic material and mineral material, atomic absorption for quantifying minerals, quantification of aminoacids after protein hydrolysis through autoanalyser, and so forth are interesting because if a laboratory has some of these techniques available and not the ones we propose in our equations, they can be carried out on the bone problem, thereby also offering results which are interesting for the administration of justice.

References

[1] Mueller, B., *Gerichtliche Medizin*, Editorial Verlag, Berlin, 1953.
[2] Aznar, B., "Identificación de restos cadavéricos óseos," *Investigación*. Vol. 210, 1945, pp. 48-50.
[3] Aznar, B. "Identificación de restos cadavéricos óseos," *Investigación*, Vol. 211, 1945a, pp. 79-81.
[4] Tirelli, V., "Considerazioni di Medicine Legale Sulle Ossa Umane," *Archive di Antropologia Criminale*, Vol. 31, 1910, pp. 81-85.
[5] Tirelli, V. "L'osteodiagnossi dell'a eta delle morte," *Archive de Antropologia Criminale*, Vol. 32, 1912, pp. 221-232.
[6] Buerger and Maestre in Lopez Gomez y Gisbert Calabuig, *Tratado de Medicina Legal*, Tomo I, Editorial Saber, Valencia, 1962, p. 439.
[7] Berg, S., "The Determination of Bone Age," *Methods in Forensic Science*, Vol. 2, 1963, pp. 231-252.
[8] Beumer, M., *Lochte Cerichtsarztl Technick*, Editorial Bergmamn, Wiesbade, 1914.
[9] Bayle, Ami, and Rondeau du Noyer, "Estudio de la materia orgánica y del fluor en restos óseos," *Bulletin DLa Societe Chimique de France*, Vol. 5, pp. 1.011-1.014.
[10] Knight, B., "Methods of Dating Skeletal Remains," *Medicine, Science and the Law*, Vol. 9, 1969, pp. 247-252.
[11] Knight, B., "The Dating of Human Bones," *The Criminologist*, Vol. 6, 1971, pp. 19-26.
[12] Knight, B., "Recent Advances in Bone Pathology," *The Police Surgeon*, Vol. 7, 1975, pp. 8-12.
[13] Castellano, M., Villanueva, E., and Lopez Gorge, J., "Etude de l'evolution de la composition proteique des os dans son processus de vieillissement. Sa mise en pratique dans l'estimation de l'âge des restes osseux," in *Actas X° Congres International de L'Académie de Médecine Légale et du Médecine Sociale*, Academie Internationale de Médecine Légale et de Médecine Sociale, Munich, 1976.
[14] Castellano, M., Rodrigo, Ma. D., Villanueva, E., and Luna, A., "Evolution de la composition minerale de l'os avec le vieillissement. L'application á la date de restes osseux," in *Actas X° Congreso de la Acade mia Internacional de Medicina Legal y de Medicina Social*, Academie Internationate de Médecine Légale et de Médecine Sociale, Munich, 1976.
[15] Castellano, M. and Villanueva, E., "Estudio sobre la evolución de los lípiodos en restos óseos en función de la data. I). Lípidos totales," *Zacchia*, Vol. 52, No. 3, Aug.-Sept. 1977.
[16] Castellano, M. and Villanueva, E., "Estudio sobre la evolución de los lípidos en restos óseos en dunción de lat data. II). Colesterol, Triglicéridos y Acidos Grasos Libres," *Zacchia*, Vol. 53, No. 1, Feb.-March 1978.
[17] Castellano, M. and Villanueva, E., "Estudio sobre la evolución de los lípidos en restos óseos en función de la data. III). Análisis de los esteres metílicos de los ácidos grasos libres por cromatografía de gases," *Zacchia*, Vol. 53, No. 3, Aug.-Sept. 1978.
[18] Snedecor, G. W. and Cochran, W. G., *Métodos Estadísticos*, Editorial C.E.C.S.A., México, 1971.

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