Correspondence

Commentary on Ohtani S, Matsushima Y, Kobayashi Y, Kishi K. Evaluation of aspartic acid racemization ratios in the human femur for age estimation. J Forensic Sci 1998;43(5):949–953.

Sir:

In a recent paper, Ohtani et al. (1) present results of an "evaluation of aspartic acid racemization ratios in human femur for age estimation."

Since age estimation based on aspartic acid racemization *in dentin* enables very accurate age estimates and has become "the method of choice" for age estimation in adults (2–10), the question of its applicability to bone is an important one of great practical relevance. We have also been interested in the problem (11,12) and believe that we can usefully comment on the findings presented by the authors in the light of our own experiences.

The authors analyzed bone specimens of 39 cadavers. The DL-aspartic acid ratio was determined in three fractions "total amino acid," "acid insoluble collagen" (sic), and "acid soluble peptide" with the following results: In all fractions, the DL-aspartic acid ratios "generally increased with age." However, they were "reduced in aged females, in the acid soluble fraction they were "very unstable" and affected by the prepared bone particle size and conditions of the acid extraction. From their results, the authors conclude: (i) that "for the estimation of age using femoral bone, the use of total amino acid fraction appears to be most effective," (ii) that "in the practical estimation of age using acid-soluble peptide fractions, accurate values may not be obtained unless the size of the powder particles is made uniform," (iii) that "further studies of the reproducibility of this method using various types of bone are necessary," and (iv) that "special care should be taken in applying this method to any remains that might be those of females."

The presented experimental data are generally in agreement with already published results of other authors (11–13), but (for the "total amino acid fraction") three orders of magnitudes faster than the rates using the Arrhenius relationship reported by the corresponding author in another recently published article (14).[1]

Although the authors warn that this new study (1) is only a preliminary study, the conclusions drawn from the data are neither plausible from a statistical and—above all—from a (bio)chemical point of view.

From a Statistical Point of View

The prerequisite for the applicability of a method for age estimation is a close correlation between the measured parameter and age. The correlation coefficients presented by the authors are not very expressive, both because the numbers of samples analyzed are relatively low and the distribution within the given age range (16 to 84 years) is strongly biased towards older individuals, all but 2 being beyond 47 years of age. The relationship between the extent of aspartic acid racemization and age remains unclear. The lack of correspondence in the estimated D/L values at the intercept ($t = 0$ years) between the insoluble and soluble fractions, is too great (for the total data set 0.019 versus 0.052) to be attributed to differences in hydrolysis induced racemization. This implies that a linear increase of the DL ratio cannot be assumed for the whole age range. As already indicated by the two values for younger individuals, the accumulation of D-aspartic acid in the "acid-soluble peptide fraction" obviously is faster in younger individuals, as already described by others (11).

Testing the "applicability" of a method for age estimation in a forensic framework has to include the determination of its accuracy and reproducibility by suitable statistical methods. The authors are to be congratulated for presenting a comprehensive tabulated data set, but unfortunately, the dataset itself does not allow the conclusion that the analysis of total bone protein or of the acid soluble and insoluble bone protein fractions are indeed applicable for age estimation in forensic cases.

From a (Bio)Chemical Point of View

Basic research work on the kinetics of aspartic acid racemization illustrate that the rate of racemization of aspartic acid, (more strictly of "Asx" residues, as it includes a total of seven separate species (15)), is governed by primary, secondary, and higher structure and in particular by the conformational flexibility of the bound residues (15–20). In effect, this means that every residue has its own racemization kinetics. All protein fractions analyzed by the authors are *mixtures of numerous proteins.* The extent of aspartic acid racemization in the analyzed fractions is highly dependent on their protein composition and in the case of the "acid-soluble peptide fraction" particularly influenced: (i) by contamination with unracemized Asx from the collagen helix,[2] and (ii) by loss of highly racemized soluble peptides. The same is true of dentine or dentine protein fractions, but it is dangerous to transfer an approach adopted for dentine to bone because the latter is not metabolically isolated what leads to additional potential problems: The structure and physiological function of bone poses a much greater threat of contamination by blood proteins than does dentine. Many non-collagenous bone proteins degrade during life and several systemic and bone diseases may influence the protein composition of bone (21), so much so that the presence of racemized collagen telopeptides in the blood stream is used to detect turnover (22). The most important reason for the low correlation between the extent of aspartic acid racemization in the analyzed protein fractions and age is the metabolic turnover of bone and metabolic disorders which affect the tissue. Unlike the case of dentine the authors *do not know the protein composition of their samples* nor can they be confident that any changes in composition are due solely to age.

[1]In the latter case (14), it appears that the author has used the wrong units in her calculations (hours instead of seconds).

[2]90% of bone protein is collagen, and most of the Asx residues in this protein are within the triple helix where they are highly conformationally constrained and therefore have an extremely low rate of racemization (15,20).

In the light of the complex biochemistry underlying racemization, we suggest that the authors modify their (albeit preliminary) conclusions as follows: (i) none of the analyzed fractions can be "effective" for age estimation, because the protein composition will vary as a consequence of factors other than age; (ii) the size of the bone particles used for acid extraction may introduce unwanted variation in composition,[3] but standardization of particle size alone cannot circumvent the first conclusion; (iii) it is unlikely that the analysis of "other bones" using the same methods will circumvent the first conclusion; (iv) as identified by the authors, the problem of unreliable results in older female individuals may be related to the higher incidence of metabolic bone disorders.

All problems discussed by the authors can be reduced to one problem; the inconstant, unknown composition of their protein fractions. The solution of that problem has already been presented (12): If we want to use the in vivo racemization of aspartic acid in a complex, non-bradytrophic tissue for age estimation we have to know, what we analyze. We have to determine the extent of aspartic acid racemization in defined, partially purified proteins. We have already shown that this concept works: in purified bone osteocalcin, the correlation between the extent of aspartic acid racemization and age is very close and enables accurate age estimation in both sexes (12). The authors cite our work using bone osteocalcin, but note that the "analysis method is complicated and requires a long time and special apparatus," and therefore conclude that "it is not appropriate for the practical estimation of age." A method for age estimation in a forensic framework requires accuracy and reproducibility.

There is no real alternative between a "complicated," but accurate and reproducible method and a simple, but inaccurate and unreproducible one which cannot solve the questions of forensic practice. We agree with the authors that easily applicable methods should be developed, but theirs is a dangerous step backwards. Instead we have to improve purification strategies for identified fractions such as bone osteocalcin or to identify other permanent bone proteins which can be purified more easily.

With good reason, Houck et al. (24) stated that "although science often hopes to develop "pure" techniques that can be accurately used by novice and master alike, experience is nonetheless the determining feature of any evaluative process. Age determination based on aspartic acid racemization in complex tissues requires specialized and trained laboratories and personnel. This must not be an argument against the selection of such a method. In our era, which is characterized by extremely rapid scientific progress resulting in high specialization of scientists, it is acceptable and *lege artis* to consult specialists—especially if questions with a high legal and social impact for the individual as well as for the community are to be solved as it is the case in age estimation in forensic science.

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[3]The apparent increase in DL ratios observed by the authors in progressively larger size fractions is explained by the partial solubilization of triple helical collagen during grinding or powdering of the sample (23), which in the < 63 μm fraction can be as great as 13% of the total collagen sample. The greater surface area in smaller size fractions, results in more extensive chain scission of the collagen and a higher proportion of newly soluble collagen "contaminating" the soluble fraction thereby lowering the overall DL ratio.

Authors' Response

Sir:

To begin with, we wish to emphasize the following points regarding our report (1): (a) Dentine (also osteocalcin) is the best ma-

terial for age estimation using D/L ratios (2,3). (b) When there is no choice but to use materials other than teeth, one candidate is the femur, since the skull of an unknown cadaver generally has teeth. (c) How reliable is the age estimated from D/L ratios in the femur using our method? (d) The correlation coefficient of the male acid-soluble peptide fraction is 0.969, that of the female acid-soluble peptide fraction is 0.125.

We also wish to stress that our paper is not arguing against the commentators' papers (4,5). Their method is an excellent one, especially in the point that both male and female samples produce extremely high correlation coefficients.

As the commentators noted, we had used the wrong units in our paper (6).

We know very well that samples from young individuals are not suitable. All of the samples used in our study (1) were kindly supplied by a certain hospital. We are trying to make efforts in obtaining specific human samples. We agree with the commentators that a linear increase in the D/L ratio cannot be assumed for the whole age range, only for the older age range.

Osteocalcin is probably the best candidate protein in bones for age estimation using the D/L ratio. However, osteocalcin is synthesized in osteoblasts (7, 8), which are one of the active cell types in the body. The precise function of osteocalcin in bone has not yet been clarified, although its properties are well defined (9). The osteocalcin content of bone varies depending on the type of bone (10), the portion of the bone samples, age, and sex (9). In addition, the osteocalcin content of blood also varies depending on the type of disease (9). Thus, the osteocalcin content of blood is a marker of some bone diseases (11). The possibility of osteocalcin displacement from bone has been suggested (9). All these data suggest that the rate of osteocalcin synthesis is not constant. Thus osteocalcin may not be a permanent protein which constantly ages. Without improvement of the purification strategy, it is not possible for us to test their method, although it is an excellent one.

Use of a single protein itself does not guarantee accuracy and reproducibility. These two parameters depend on different factors. The accuracy for age estimation is indicated by the correlation coefficient, no more than that. Handling samples of unknown mixed proteins does not mean a method is not reproducible. If the profiles of the contents are equal, then the data should be the same. In fact, the correlation coefficient of the male acid-soluble peptide fraction is 0.969, indicating some degree of reproducibility. Of course, we don't think that the protein profiles of all samples are the same. One critically important point for accuracy and reproducibility of age estimation using the D/L ratio is the complete separation of D- and L-aspartic acid by chromatography to obtain accurate values. (Unfortunately, the commentators' papers did not show any chromatography separating the D- and L-forms (4,5,12–14)).

Forensic scientists are required to give answers like "probably 32–years–old, but possibly 31 or 33–years–old." The estimated age is exactly that: an estimate. Thus, nobody can say "He is 32–years–old" even if the correlation coefficient is 0.99999. Again, the most important consideration is how reliable the estimated age is.

This technique is not for use by only a few scientists. There is no facility that can handle all cadavers requiring age estimation from all over the world, and things cannot wait until a good technique is established. It is important to set up standard samples to derive a standard line whenever a cadaver is inspected. In order to do this, more samples are better. This is the reason why we prefer a simple method, even though the obtained correlation coefficient may not be very high.

We believe that only one scientist cannot determine the direction of science, and that wide discussion is necessary, even by a highly established authority. We believe that the direction should be determined by scientific consensus. Ten scientists may have ten different opinions. If all are logically correct, the all should be accepted.

Statistical science tells us that if we obtain a sufficient number of samples at random, even if the overall impression is chaos, then theoretically we can suggest what underlies the chaos. We know very well that our samples are like a chaos and have an uncertain background. However, their method also has a drawback in that it focuses on only one protein, which may magnify or miss some signals. We think it is still too early to conclude that osteocalcin is the best material. Before making a decision, we have to evaluate other non-collagen proteins such as osteonectin, and we need more concrete reasons for using osteocalcin.

As we have said before (15), forensic science has certain aspects similar to diagnostic science. We have to perform age estimation using a well tried method. Therefore, the chosen method is sometimes not the best one in terms of the correlation coefficient (commentators' accuracy).

Finally, we have been considering for a long time that we have to know what we analyze if there is a method we can perform relatively simply, and we eagerly anticipate the identification of the best permanent bone protein (probably osteocalcin) which can be purified easily.

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