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# Using histomorphometry for human and nonhuman distinction: A test of four methods on fresh and archaeological fragmented bones

Lagacé F., Schmitt A., Martrille L., Benassi J., Adalian P.

## ABSTRACT

Positive identification of human remains is the very first step in anthropological analysis, and the task may be particularly difficult in the case of fragmented bones. Histomorphometry methods have been developed to discriminate human from nonhuman bones, based on differences in the size and shape of Haversian systems between the two groups. Those methods all focus on a very specific type of bone, section, and zone. Therefore, the objective of this study was to test the efficiency of four histomorphometric methods on a sample of fragmented bones. The sample is composed of 37 archaeological and fresh specimens, 25 nonhumans (*Bos taurus*, *Equus caballus*, *Sus scrofa*, *Capreolus*, *Canis familiaris*, *Cervus elaphus*, *Ovis*, and *Capra*) and 12 humans (*Homo sapiens*). Eight histomorphometric criteria were collected from all intact osteons visible on each fragment and then inserted into the corresponding discriminate function of each method. The results were compared with the real origin to establish rates of correct classification for each method. The methods of Martiniaková et al. (2006) and Crescimanno and Stout (2012) obtained very low percentages of good classification (32% and 67%). Those of Cattaneo et al. (1999) obtained 94% correct classification, but only after a correction of the units of measurement for Haversian canal area in their formula. The methods of Dominguez and Crowder (2012) obtained an 86% rate for well-classified specimens. Some of the methods tested here contain errors in the original publication that make them unusable in their current state. Plus, it seems that histomorphometric methods developed from specific areas are more difficult to apply to fragments. A reduced number of intact osteons analyzed may partially affect the reliability of the method by being unrepresentative of the entire microstructure. Therefore, this study demonstrates that one should be cautious with the use of histomorphometric methods to distinguish human and nonhuman fragmented bone until further research can refine these methods to achieve greater reliability.

## INTRODUCTION

Forensic anthropologists are frequently confronted with the challenge of identifying fragmented skeletal elements. The identification of the origin of a bone, i.e., human or nonhuman, is a fundamental issue, as it is the very first question that needs to be answered and will radically influence the future of the criminal investigation. Identification of skeletal fragments is often experienced in mass disasters and domestic crimes where it can be mingled, intentionally or not, with animal bones [1]. Hence, identification of each fragment is necessary, and anthropologists seek the fastest, cheapest, and most reliable method. Bone identification is also a major concern in archaeology because it can provide relevant information on burial practice and help researchers understand and interpret the purpose of a structure or a site [2,3]. For zooarchaeologists, distinguishing human bone from other species is the first step before proceeding to further analysis.

Since 1950, many studies have demonstrated notable differences between human and nonhuman bone microstructure [4,5], making histology a common tool used for identification when macroscopic anatomical features are altered or not present [1,6–10]. Two different approaches are currently used for histological determination. First, the qualitative approach is based on the observation of bone organization and characteristics considered specific to human or nonhuman microstructure [1]. Plexiform, or fibrolamellar complex, is a primary organization found in most nonhuman mammals with fast-growing rates [2,3]. Therefore, the presence of plexiform is considered a good indicator of nonhuman bone, although it has been found also in human bone in the case of fracture repairs and juvenile bone [2,3,11]. Other structures, such as osteons banding, have also been considered a good indicator of nonhuman origin [12]. However, recent studies have demonstrated the presence of osteons banding in human bone [2,11,13,14]. Secondary bone, or Haversian bone, is characterized by new bone replacing bone already in place and appears in cortical bone with structures called secondary osteons or Haversian systems [15]. In a majority of mammals and some birds, Haversian bone can coexist with plexiform. In large mammals, the former is mostly found near the endosteal surface, and the latter near the periosteal surface [1,4,16]. Human bone tissue is characterized by either dense or scattered secondary osteons [1,5,17]. However, the presence of Haversian bone alone cannot guarantee a human origin because of its presence in other mammals [15].

The quantitative approach, also referred to as the histomorphometric approach, was developed to differentiate human and nonhuman Haversian bone. Histomorphometric methods assume that the differences of size, shape, and quantity between human and nonhuman Haversian bone structural elements are important enough to distinguish one from the other with discriminant functions analysis [1,15]. Those methods are especially important when there is no qualitative indicator present, like plexiform bone. This can be the case in large mammal bones where subperiosteal surface can be removed by weathering, fire, extreme fragmentation, or other peri/post-mortem alteration, leaving only the endosteal area filled with Haversian bone [1,18]. A review of the available literature provided four histomorphometric methods that were particularly relevant. Their reference sample proposed species likely to be found in forensic and recent archaeological contexts [19]. Those four methods are summarized in Table 1. In 1999, Cattaneo and colleagues published a study that tested histological, immunological, and DNA techniques for human/nonhuman identification. This was the first publication to propose a discriminant function analysis to classify human/nonhuman bones. Their method is based on long bones burnt at 800–1200°C and uses three histomorphometric criteria for the Haversian canal. This method is particularly relevant because it uses a wide range of species (human, horse, cattle, sheep, swine, cat, dog). Martiniaková and colleagues proposed in 2006 a classification method for human and four other species. Their method uses mid-shaft femora section from human, cattle, sheep, swine, and rabbit. Their classification model proposed to use seven histomorphometric measurements based on intact osteons and Haversian canals. Crescimanno and Stout also published a method in 2012 for discriminating human and nonhuman. Their method is based on one histomorphometric criterion: osteon circularity. Their method uses human and three other species (dog, swine, deer) from humerus, femur, and ribs. Dominguez and Crowder published in 2012 a method based on two criteria of intact secondary osteons: area and circularity. Their method is based on three species (human, deer, and dog) from long bone (humerus and femur) as well as flat bone (ribs). They proposed a classification function for the three species, but also a discriminant function for humans/nonhumans.

All these methods succeed at different degrees to correctly identify human/nonhuman bone with correct classification rates from 75% to 100%. They share some common characteristics: they were all elaborated from fresh skeletal elements (only Cattaneo et al.'s method was elaborated from fresh burnt skeletal elements). They are all constructed from the analysis of complete cross-sections and on specific location (mid-shaft for long bones). They

mostly focus on the same specific anatomical regions, like the femur, tibia, or rib. Paradoxically, even if these methods were published to identify bones without any anatomical landmarks, their accuracy on fragments where those parameters are unknown has never been attested [1]. Therefore, the principal aim of this study is to evaluate the reliability of those four histomorphometric methods when they are applied on a sample of fragmented archaeological and fresh bones.

*Table 1: Description of the four histomorphometric methods selected to be tested in this study. Nomenclature was adapted from the original publication to fit the new recommendation in Dempster et al. (2013).*

## MATERIALS AND METHODS

The study sample was composed of 37 different specimens (Table 2). The human subsample consisted of five skeletal elements obtained from a medieval archaeological site (Beaulieu, France) and seven skeletal elements from bodies given to science. Sex and age-at-death was not accessible. These skeletal elements were previously used for unpublished studies; ethical requirements were fulfilled. The nonhuman subsample consisted of 25 skeletal elements from eight different species: cattle (*Bos taurus*), horse (*Equus caballus*), swine (*Sus scrofa*), roe (*Capreolus capreolus*), dog (*Canis lupus familiaris*), deer (*Cervus elaphus*), sheep (*Ovis aries*), and goat (*Capra aegagrus hircus*). The fresh samples were collected from a local butcher, hunter, and an osteological reference collection at the *Maison Méditerranéenne des Sciences de l'Homme*, Aix-en-Provence. The archaeological nonhuman subsample came from Notre-Dame, a medieval site in South France. Most of the specimens were already fragmented. When the specimen was complete (complete cross-section), only a quarter of the section was observed and analyzed, without paying attention to its exact location on the section. Final bone surface observable ranged from 22x11mm to 9x1mm. The goal was to analyze each specimen independently of the others to extract a maximum of information and to mimic a real-life situation where the fragment must be identified.

*Table 2 : Summary of the sample*

The bone preparation technique and mounting on microscope slides all followed the same basic steps described below. All samples were embedded in epoxy resin and put under a vacuum for 30 minutes to remove any air in the resin. Cross-sections were cut with a standard microtome (Leica SP 1600). Thin-section thickness ranged from 60  $\mu\text{m}$  to 150  $\mu\text{m}$ , depending on the quality and resistance of the bone. Thin sections were mounted on a microscope slide with Canada turpentine or geoptic glue (Brot Lab) and a glass cover slip. Slides were observed under a light microscope mounted with a camera (Nikon Eclipse Ci), and images were captured using NSI Element D software. When the bone section was already fragmented, the complete surface of the bone was analyzed.

First, the total bone area was reconstituted with stitching software (Image composite editor). All references of “osteons” refer to secondary osteons (Haversian systems). Primary osteons are not considered in this study. The objective was to identify and capture the maximum number of intact osteons with clear reversal lines on each specimen. Intact osteons were captured with X10 or X40 magnification, according to their size. When an osteon was captured, it was crossed out on the large image to avoid duplicates and to make sure the entire surface was scanned for intact osteon. Histomorphometric analysis was made using ImageJ Version 1.50 software. The calibration of the scale (pixel/ $\mu\text{m}$ ) was set on ImageJ for each magnification (X10 and X40). Each intact osteon and Haversian canal were outlined using a drawing pen tablet (Bamboo Fun Wacom). Only osteons with well-defined reversal lines and Haversian canals with well-defined contours were outlined. Between five and 93 intact osteons with their Haversian canals were traced on each specimen. All the measurements were calculated automatically with ImageJ commands. Eight histomorphometric variables used are presented in Table 3. Those variables were selected following methodology from the four selected methods.

Additionally, measurements were taken by an additional observer on all specimens. Inter-observer error was evaluated using Lin’s concordance correlation coefficient (CCC) to judge the degree of agreement between the two sets of measurements [20]. Statistical analyses were performed on R software, version 3.3.2. The significance level for the test was set at 5%.

In their analysis, Dominguez and Crowder (2012) and Crescimanno and Stout (2012) both excluded osteons with elongated Haversian canals to avoid skewed osteons caused by an

oblique cut of the bone. To reproduce their method precisely, all osteons with maximum Haversian canal diameters more than twice the minimum diameter were excluded from the original data before applying Dominguez and Crowder's discriminant functions. The number of excluded osteons for this method ranged from zero to five osteons per specimen. For Crescimanno and Stout (2012), all osteons with a Haversian canal circularity index less than 0.9 were excluded from the original data before applying their discriminant functions, as recommended in their method. The number of excluded osteons ranged from zero to 28 per specimen for this method.

The average of each variable for all specimens was calculated and inserted into the discriminant function of the four selected methods previously presented and summarized in Table 1. The three discriminant functions proposed by Dominguez and Crowder were tested. The DFAs for this method are not published in the original paper but were provided by personal communication. D1 used On.Ar only, D2 used On.Cr only, and D3 used both variables for classification. Classification of each specimen with each method was computed and then compared with the bone's real origin. Correct classification rates were calculated for the four methods, for human and nonhuman separately, and the two subgroups combined for the total sample.

*Figure 2: Example of a large image (total bone surface) obtained on a fragmented specimen (Equus caballus). This large image was acquired with stitching software (Image Composite Editor) and used as a general view of the bone organization and a reference to make sure all the intact osteons visible had been captured for analysis.*

*Table 3: Description of the eight histomorphometric variables collected. Dempster et al. (2013) [21] nomenclature, adapted from Parfitt et al. (1987) [22] was used.*

## RESULTS

Lin's CCC indicates a very good agreement ( $CCC > 0.95$ ) between the main and the additional observer for all variables except for On.Cr (Table 4). With a CCC of 0.43, the agreement between the measurements of the two observers for On.Cr is considered weak ( $CCC < 0.50$ ) [23].

Descriptive statistics for this study are provided in Table 5, along with the data report in the original papers of the four methods tested. A total of 1,395 intact osteons and their Haversian canals were measured (884 nonhuman and 511 human). The number of intact osteons obtained was highly unequal between each specimen, with a minimum of five and a maximum of 90 (Supplementary material). Since the methods of both Dominguez and Crowder and Crescimanno and Stout exclude skewed osteons from their analysis (see Materials and Method), descriptive data obtained with their respective methodologies are presented separately in Table 6. The total number of intact osteons and canals analyzed with their methodology was respectively 1,365 and 1,056.

Results obtained by the application of the discriminant functions of the four methods tested are presented in Table 7. The assessment of each specimen in detail is presented in Supplementary data 1. For Cattaneo et al.'s discriminant function, all specimens were classified as nonhuman, with a 100% correct classification rate for nonhuman, 0% for human, and 67.57% for the total sample. For Martiniaková et al.'s method, all the specimens were classified as human, with a 0% correct classification rate for nonhuman, 100% for human, and 32.43% for the total sample. With Crescimanno and Stout's discriminant function, 67.57% of the total sample was correctly classified, but only an 8.33% correct classification rate for human and 96% rate for nonhuman were achieved. Three discriminant functions of Dominguez and Crowder were tested. D1 and D2 obtained correct classification rates of 72.97% and 67.57%, respectively, for the total sample. D3 (On.Ar + On.Cr) obtained the most accurate results, with classification rates of 84% correct for nonhuman, 91.67% for human, and 86.49% for the total sample.

*Table 4: Evaluation of the inter-observer error with Lin's Concordance Correlation Coefficients (CCCs)*

*Table 5: Descriptive statistics of this study for all the histomorphometric variables collected. The data reported from the original papers of each method are included in this table for comparison.*

*Table 6: Descriptive statistics after excluding skewed osteons for Dominguez and Crowder (2012) and Crescimanno and Stout (2012) with the methodologies described in their papers.*

*Table 7: Number of correct assessments along with the correct classification rates for nonhuman, human, and total sample separately.*

## DISCUSSION

*Cattaneo et al. (1999)*

Cattaneo et al. (1999) is the first publication to use differences between Haversian systems to produce a discriminant function analysis that classified human and nonhuman bones. The use of Cattaneo et al.'s function on our sample provided unusual results, with all our specimens classified as nonhuman. By looking into the descriptive data more closely, we found that Cattaneo et al.'s study presents uncommonly low results for two area variables (H.Ca.Ar and On.Ar). The mean values of H.Ca.Ar for human and nonhuman samples in our study are respectively  $3,406.46 \mu\text{m}^2$  and  $911.94 \mu\text{m}^2$ , in opposition to  $24.98 \mu\text{m}^2$  and  $7.76 \mu\text{m}^2$  in Cattaneo et al.'s publication (Table 5). This considerable difference between their areas' values and ours may explain why the formula is ineffective. Additionally, our values for areas seem to be more coherent with those obtained in Martiniaková et al. (2006) and Dominguez and Crowder (2012). Comparison with other studies of human histomorphometry also confirmed that our values fall in the normal range for values in  $\mu\text{m}^2$  [24–30]. There is also an inadequacy between the diameters and areas in Cattaneo et al.'s values. The Haversian canal minimum diameter average of  $58.37 \mu\text{m}$  is more than twice the value of the mean Haversian canal area ( $24.98 \mu\text{m}^2$ ). Considering those findings, a possible explanation for this incoherence could be the existence of a typo in Cattaneo et al.'s publication for the unit of measurement of osteons and Haversian canal areas. It would seem as if their results for areas are not expressed in  $\mu\text{m}^2$ .

The idea was to test their discriminant formula with a different unit of measurement. All our values for H.Ca.Ar were divided by 100 to get results in the same range of measurement as in Cattaneo et al. (i.e., an average of 34.06 for human and 9.11 for nonhuman). The discriminant function analysis was again applied on the new values. Correct classification rates with the new values presented a combined successful classification rate of 94.59% (Table 8). Only two specimens were incorrectly identified; both human bones were classified as nonhuman. Those two specimens have respectively 14 and 32 observable intact osteons (Supplementary material). When the unit of measurement for H.Ca.Ar is modified, the method of Cattaneo et al. is the most effective method to discriminate human from nonhuman fragmented bones in our sample. However, without precision from the authors, it is not possible to address with certainty the problem that causes the formula to automatically classify all specimens as nonhuman.

*Table 8: Number of correct assessments along with the correct classification rates for nonhuman, human, and total sample separately for Cattaneo et al.'s method when H.Ca.Ar measurements were divided by 100.*

It is important to note that Cattaneo et al.'s method was built from a sample of burnt bones. The authors mentioned in their publication that there is a significant difference between osteons and canal measurement for burnt and unburnt bones, but they did not give any quantified data to support this statement [6]. There is a great uncertainty around the effect of heat and fire on the bone microstructure, since the few available studies on that matter remain preliminary and their results are contradictory [31–34]. Therefore, with the current literature on the subject, it is difficult to know if a heated/burnt-based method can be used on non-heated/burnt bones, and vice versa.

*Martiniaková et al. (2006)*

Martiniaková et al.'s method proposed a classification function to classify five species, including human. The application of their classification function on our sample did not provide accurate results. Their descriptive data were compared with the one obtained in this study (Table 5). Since no major incoherence was found between our descriptive results, these unusual results led us to examine more closely their classification function. To try to pinpoint the issue, a test with their data set was performed. The mean histomorphometric measurement for the rabbit in their publication (i.e., the species with histomorphometric values that are the most distant from human values) was used and run into their classification. Even with their mean values for the rabbit, the result was still human. Based on those facts alone, it is difficult to say exactly what caused the malfunction of the classification function. It may be only a typo in the numbers of the classification function that led to a systematic error, but without any precision from the authors Martiniaková et al.'s method as proposed in the original paper cannot be considered reliable.

*Crescimanno and Stout (2012)*

In their publication, the authors presented circularity as a discriminant variable for human and nonhuman bones. However, Crescimanno and Stout's method did not provide accurate results. When human and nonhuman samples are taken separately, classification rates were highly unbalanced between the two groups. In their paper, Crescimanno and Stout support that human

bones have less circular osteons and nonhuman bones present more circular osteons [35], and these findings were coherent with those found by Tersigni and colleagues [36]. In our study, we found that humans have a slightly more circular osteonal shape than nonhumans (Figure 3). This observation is consistent with Dominguez and Crowder's method, which supports that humans have more circular osteons than nonhumans [37]. Another study published in 2017 by Keenan and colleagues supports Dominguez and Crowder and our findings [38]. This might partially explain why their method is not effective on our sample.

*Figure 3: A) Boxplot comparing human and nonhuman samples for On.Cr values measured with Crescimanno and Stout's method. B) Boxplot comparing human and nonhuman samples for On.Cr values measured with Dominguez and Crowder's method.*

#### *Dominguez and Crowder (2012)*

Dominguez and Crowder published a method in 2012 that used osteon area and circularity as discriminant variables. Their discriminant function that combined both variables was the most efficient on our sample, with one human specimen classified as nonhuman and four nonhumans classified as humans. When On.Ar was included with On.Cr, the validity of the method increased. Not only did it produce more correct classification, but it also produced more balanced results between human and nonhuman. It seems that On.Ar is more discriminatory than On.Cr for origin determination. This result corroborates what Dominguez and Crowder and Keenan and colleagues found in their studies [37,38]. On.Cr alone might not be the most appropriate criterion for human and nonhuman differentiation. First, even though each study that presented circularity as a discriminant variable found a statistically significant difference between human and nonhuman, there is a considerable overlap of circularity index between the two groups [35,37]. This overlap is also present in our data (Figure 3). Moreover, studies have demonstrated that the lack of standardized methodology to acquire a circularity index can produce a difference in measurement up to 10–20% [38,39]. Even with the same technique, On.Cr measurement is difficult to reproduce, as a result of inter-observer test indicated. Since On.Cr is a very sensitive measurement, even small variations in the outlining process will produce high variations of the index (up to 0.05) [40]. At the same time, the inter-individual variability (mostly between 0.70 and 1) is too small in relation to the measurement error. This is caused by the intrinsic circular shape of osteon. Therefore, it is difficult to attribute the difference observed for On.Cr to a real

species-specific difference, or to other methodological factors. This also partially explains the poor performance of the previous method tested (Crescimanno and Stout, 2012), which used only On.Cr.

Nevertheless, the discriminant function proposed by Dominguez and Crowder, which used both On.Ar and On.Cr, misclassified only five specimens out of 37. From the four nonhumans wrongly classified, two were *Bos taurus* and two *Equus caballus*. This is consistent with the findings of previous studies that found that those species are the ones with histomorphometric values closest to humans [7,41,42]. It seems that the difference between humans and those species for On.Cr and On.Ar is not significant enough to differentiate one from another in every case, especially since those species are not present in the method's sample. This is an important point to discuss. In a real-case scenario where a fragment needs to be identified, there is no way to know beforehand if the specimens are from species that are included in the original sample, even if some can be ruled out with the geo-chronological context. Histomorphometric methods should be inclusive of all potential species in a specific time/geographic frame to maximize their accuracy.

### *Observability and histomorphometry variability*

The effect of diagenetic alteration on the bone microstructure has been attested many times, in particular with microbial attack [43–45]. The degradation process observed in archaeological bones may lead to a partial or complete disappearance of original features (i.e., Oxford Histological Index in Hedges et al., 1995). With Cattaneo et al.'s method (changed units for H.Ca.Ar), the only two human specimens misclassified were from archaeological origin. With only 14 and 32 observable osteons, those two specimens did not have the lowest number in this case, but they were lower than the global mean of 42 osteons/specimen. More generally, the human archaeological sample had less discernable intact osteon than the fresh human sample. This indicates a close relationship between diagenetic alteration and the number of observable osteons, due to the microbial attack concealing and destroying the bone microstructure (Figure 4). For Dominguez and Crowder's method, the four nonhuman specimens misclassified were fresh specimens, with only 4, 15, 17, and 31 intact osteons (Supplementary material). For those specimens, the low number of observable osteons is not due to diagenetic alteration, but to the presence of other structures than dense Haversian bone, i.e., fibrolamellar bone. In this case, the

classification could have been provided with a histomorphological analysis, since fibrolamellar bone is mostly found in nonhumans [10]. This fact supports the idea that qualitative and quantitative approaches must be used together to maximize the accuracy of the classification [1,10].

Several external factors, such as sex and age, can produce variation in bone histomorphometry and must be taken into analysis [1,47]. The relationship between age and bone microstructure is well documented [48,49] and has led to numerous age-at-death estimation methods from histomorphometry in human bone since 1965 [26,50–52]. Aging increases the number of Haversian systems, but it also affects their size and shape [17,24,25]. This is also true in nonhuman bone [53–55]. Sex also seems to influence bone remodeling and histomorphometry [1,56], but its effect is more controversial. Some studies did not find any relationship between sex and histomorphometric variables studied [27,50,57,58]. Among the four methods tested in this study, that of Crescimanno and Stout (2012) is the only one that tested the relationship between sex and histomorphometric variables in the human sample. These authors found no statistically significant difference between male and female [35]. In our sample, sex and age were not accessible. However, identifying nondiagnostic bone fragment implies that those parameters can not be assessed as well. A better understanding of those factors and the normal variation in both human and nonhuman bone is necessary to establish reliable species identification methods. Thus, methods should be based not only on strong discriminative variables for human and nonhuman, but also on variables that are less affected by external factors such as sex or age in both groups.

The number of osteons considered necessary to be representative varies among the relevant publications. Cattaneo et al. proposed the use of only three osteons per specimen [6]. For Crescimanno and Stout, a minimum of 12 osteons may be needed to be accurate [35]. On the other hand, Harsányi, Martiniaková et al., and Dominguez and Crowder recommended using at least 50 intact osteons per specimen [7,41,59]. Some publications on age-at-death estimation in human bone from histomorphometry have also used a minimal number of intact osteons, between 25 and 30 osteons [24,26,60]. It is difficult to say exactly how many osteons are needed to be representative, but it seems that a limited number increases the risk of a misclassification, although this assumption is not systematic. On a small number of osteons, the presence of one outlier measurement can attract the mean in one direction or the other, and cause a misclassification. Factors other than the number of osteons may affect the representativeness. In two studies, Cummaudo and colleagues [61,62] reported the presence of both histomorphological

and histomorphometric variability between different bones of the same individual, in different portions of the same bone, and in different parts of the same section. This variability may affect the effectiveness of histomorphometric methods and lead to wrong conclusions, especially for fragmented specimens [62]. In those cases, the osteons analyzed were limited to a restrained area that may not have been representative of the bone of origin. Thus, the reliability of histomorphometric methods when applied on fragmented bones seems to be affected more by the number of osteons analyzed than the size of the fragment (even if normally more bone surface equals more osteons, but not necessarily). This number can be highly reduced by the presence of diagenetic alteration, and the group of osteons observed might not be representative due to a random effect of histomorphometric variability.

## CONCLUSION

Histomorphometric methods have been developed in recent years with different criteria to discriminate human and nonhuman bones. When a method is constructed, all the parameters regarding the sample can be more constrained than in a real-life situation where methods are blindly applied. The method's sample is narrowed to certain types of bones, mostly long bone and rib, and a certain location, mostly mid-shaft. The entire cross-section is analyzed to reduce bias due to the heterogeneity of the cortex. In a real context where a fragment needs to be identified, those parameters are not available, and they might not be optimal. Additionally, anthropologists must deal with external factors like bone conservation and quality, image quality, and number of exploitable fields. All these elements might undermine the reliability of those methods [61]. In addition, some authors have already expressed some concerns about these methods, since most of them are still preliminary and need validation to be eligible for use in a court of law [10,37]. This research was conducted to shed light on the effectiveness of four available histomorphometric methods to distinguish human from nonhuman bones. Some relevant information from this work should be considered: 1) Some methods might contain errors in the original publication that cause the discriminant function to be inaccurate as currently presented. 2) Since fragmented bones are often encountered in the case of burnt bones, there is a necessity to explore the effect of heat and fire on microstructure with a rigorous methodology before attempting to say whether unburnt-based methods can be applied on burnt bone, and vice versa. 3) Some methods are still preliminary works, and there is still a lack of consensus around the validity of certain criteria

(osteon's shape). 4) Histomorphometric methods that are elaborated from specific areas and bone types may not be suitable on highly fragmented bones due to histomorphometric variability. Putting into perspective the information gathered in this work, it seems clear to us that using such methods in real forensic or archaeological cases must be carried with caution until further clarification. Of four methodologies examined, we demonstrated that Cattaneo et al.'s method (when our unit was changed) was the most efficient with fragmented bone, but the number of intact osteons used in the analysis seems to be an important factor influencing its reliability. A more precise study focusing on the threshold number of osteons required to use this method can help enhance its reliability and justify its use not only in forensic anthropology but also in archaeology and zooarchaeology.

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