

Characterization of bone diagenesis by histology in forensic contexts: a human taphonomic study

Yann Delannoy^{1,2,3,4,5} · Thomas Colard^{2,3,4} · Catherine Cannet⁶ · Vadim Mesli^{2,3} · Valéry Hédouin^{2,3} · Guillaume Penel^{2,4} · Bertrand Ludes^{1,7}

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Abstract The diagenesis of a bone in the postmortem period causes an identifiable deterioration in histology. This degradation is characterized by a collagenous alteration, which can be observed very early. In order to develop a method for determining a postmortem interval for medico-legal use, two ribs collected from six human bodies were studied prospectively over 2 years. Each bone was studied after staining with Sirius red to demonstrate the degradation of collagen as a function of time. This study demonstrated a time-based bone alteration characterized by the architectural degradation of the lamellar bone, without any microbial influence in this postmortem period. The staining was carried out by using Sirius red and correlated this alteration with a collagenic degradation by chemical hydrolysis owing to the affinity of this dye to the amino acids lysine, hydroxylysine, and arginine. Our work asserts that human bone samples that were studied in a

controlled environment and analyzed for 24 months underwent a diagenetic trajectory whose main element was collagen hydrolysis.

Keywords Forensic medicine · Anthropology · Bone diagenesis · Collagen hydrolysis

Introduction

The diagenesis of a bone is influenced by its environment: exogenous material coming from this environment will be incorporated into the bone, and the bone will re-release its endogenous components into the environment [1]. To illustrate this phenomenon of incorporation or release between the bone and its environment, five measures have been recognized in the literature and cover the entire process of diagenesis [1, 2]: collagen loss, crystallinity, changes in porosity, carbonate content, and histological index. The last was established as a result of histological sections that were examined under an optical microscope or by scanning electron microscopy [3–5]. Good histological preservation allows the identification of osteons, interstitial bone, and trabeculae of spongy bone. Poor preservation will show lacunae associated with structural replacements by mineral matter called “hypermineralized nodules,” which eventually disappear to create porosities. This structural alteration is partly related to microbial attacks [3].

The Oxford histological preservation index can be classified according to six stages, numbered from 0 to 5 (Table 1). On thinner sections, another histological preservation index was defined by Garland et al. and adapted by Jans et al. in five identifiable diagenetic modifications [6, 7]:

✉ Yann Delannoy
yann.delannoy@univ-lille2.fr

¹ Institut Médico-Légal de Paris, Université Paris Descartes Sorbonne Cité, 75000 Paris, France

² CHU de LILLE, 59000 Lille, France

³ EA 7367 Forensic Taphonomy Unit - Lille Forensic Institute, Lille University, 59000 Lille, France

⁴ Littoral Côte d’Opale, EA 4490, PMOI, Physiopathologie des Maladies Osseuses inflammatoires, Lille University, 59000 Lille, France

⁵ Institut de Médecine Légale – CHRU de Lille, rue André Verhaeghe, 59037 Lille Cedex, France

⁶ Laboratoire d’Histomorphométrie de Médecine Légale, Faculté de Médecine, 67000 Strasbourg, France

⁷ CNRS UMR 5288, AMIS, Toulouse University, 31000 Toulouse, France

Table 1 Histological index values from Hedges et Millard 1995 [1]

Index	Approximative % of intact bone	Description
0	< 5	No original features identifiable other than Haversian canals
1	< 15	Small areas of well preserved bone present, or some lamellar structure preserved by pattern of destructive foci
2	< 33	Clear lamellate structure preserved between destructive foci
3	> 67	Clear preservation of some osteocyte lacunae
4	> 85	Only minor amounts of destructive foci, otherwise generally well preserved
5	> 95	Very well preserved, virtually indistinguishable from fresh bone

- Microscopic focal destructions (MFD) or tunnels, which are characterized by focal losses of collagen and hydroxyapatite with hypermineralized zones surrounding the tunnels and are described in four categories [3].
- Inclusions corresponding to the presence of exogenous material included in empty bone spaces and mostly related to fungic deposits, sand, and pyrite.
- Infiltrations corresponding to the presence of ferrous exogenous material (Perls Prussian blue staining) and/or altered hydroxyapatite crystals.
- Cracks that colonize the Haversian system (large cracks) or are restricted to the osteons (microcracks). Their size differences are related to their causes: the environment (moisture, temperature) for large cracks and the remodeling associated with chemical organo-mineral alterations for microcracks.
- The intensity of the birefringence, as examined under polarized light on the osteons, which forms an alternation of luminous and dark bands.

On the molecular level, the extracellular matrix represents 92 to 95% of the bone volume. It consists of an organic phase (22%), which is mainly collagenous, a mineral or inorganic phase (69%), and an aqueous fraction (9%) [8–11]. In bone, type I collagen represents 90% of the organic matrix. It consists of three polypeptide chains (α chains) that form a triple helix of 300 nm in length and 1.5 nm in diameter. The triple helix configuration is characterized by the repetition of a Gly-X-Y triplet, where X is usually a proline and Y is a hydroxyproline. Glycine therefore represents one third of the amino acids of collagen, proline approximately 10%, and hydroxylysine with hydroxyproline represent 21%. The other amino acids are predominantly arginine and lysine. Within the endoplasmic reticulum, hydroxylysine and hydroxyproline are formed from polypeptide synthesis by hydroxylation of the amino acids (L-proline and L-lysine) present in the α chains. The hydroxylation of proline contributes to the stability of collagen by inducing the formation of hydrogen bonds. The hydroxylation of lysine allows the stabilization and binding of the molecules in the fibers.

The main hypothesis of this work consisted of an organic degradation of the bone in the postmortem period, in particular a collagenic degradation that would be identifiable both early and prospectively. Once the collagenic degradation was temporally identified, the objective was to demonstrate the mode of organic alteration, including the role of microorganisms (i.e., bacterial attacks) or the role of chemical mechanisms, as described in the literature.

Materials and methods

Materials

To develop a possible method of determining a postmortem interval for medico-legal use, a choice has been made to work on human bone. To comply with ethical standards, the bones were obtained from individuals who had “donated their bodies to science” according to a specific French law, which allows anatomical dissections and research to be performed on human cadavers. Our work was carried out on six human subjects (four men aged 72, 82, 87, and 88 years and two women aged 80 and 92 years) who were free of known bone pathology.

For each, two ribs were sampled on the day of death (1st and 4th right), and the surrounding soft tissues (tendons and muscles) were immediately manually defleshed without any chemical treatment [12]. These ribs were then placed in their different environments. For each subject, the choice of the bones studied was ribs 1 and 4 because they have a recognized medico-legal interest, particularly for determining the age at death [13, 14] and because they have a large surface of exchange with a thin cortex, which confers onto them the characteristic of being subject to diagenesis [15].

These ribs were placed in an outdoor environment (under cover, in clay), and the meteorological data were recorded daily by MétéoFrance © (temperature T_{\max} mean 10.06 ± 3.02 °C and T_{\min} mean 3.98 ± 2.13 °C) (moisture $82.47 \pm 6.86\%$). On the initial day of sampling, every 3 months for a year and punctually at 24 months, the ribs were extracted from their environment. They were sectioned transversely to a thickness

of 5 mm using a diamond disc that is used for histological recutting. Finally, the ribs were replaced in their environments. According to the literature [16], this sampling method, despite re-cuttings, will not result in any long-term diagenetic modification.

Methods

The rib fragments were fixed in 10% neutral buffered formalin (pH 7.2) for 21 days to stabilize the tissue structures. Once they were fixed, the bone fragments were decalcified. The decalcification solution used was Kristensen's fluid, composed of formic acid and sodium formate [17]. This method of decalcification uses a weak acid; hence, it is less aggressive for bone tissue. The decalcification times were 17 h for all rib samples. After decalcification, the fragments were rinsed for 2 h under running tap water, dehydrated according to the scheme applied to the routine organs (cycle of 19 h: ethanol of 50 to 100%, xylene), and finally embedded in paraffin. Numerous protocols for collagen staining exist in histological medical practice. Examples include van Gieson stain, Masson, Mallory, and Heidenhain trichromes. However, Picosirius red (PS) staining using Sirius F3B red (C.I. 35,782) as a dye is the most specific and sensitive of these methods [18]. The Sirius red selectively stains collagens type I and III and is specific to the amino acids lysine and hydroxylysine. The staining is highlighted when it is observed under polarized light. Indeed, Sirius red molecules line up along the collagen fibers and increase their birefringence. The use of this dye on archeological bone remains has recently demonstrated its effectiveness [19]. Thus, 5- μ m sections were cut and stained with

- Hematoxylin and eosin (H&E) to study the general morphology and
- Picosirius red (PS) to demonstrate the collagen fibers observed under polarized light [20].

Slides were examined with a light polarizing microscope (Axioscop A1, Zeiss©) connected to a 2 million pixel 3 CCD camera (Jai200Ge, JAI). The tissue sections were mapped using the Histolab © software (Microvision instruments).

To visualize the microscopic observations, different illuminations were used:

- A classic observation of the tissues under bright-field microscopy,
- An observation under polarized light, which allowed the identification of exogenous materials and the study of the bone microstructure, and
- An observation using the Nomarski Interference Contrast (NIC), which allowed the visualization of bone bioerosion [4, 21].

First, each histological section stained with H&E was mapped under bright-field illumination at $\times 2.5$ magnification. The mapping of large tissue sections, carried out using Histolab © software, gave an excellent overall view of the section. It allowed the accurate localization of any lesion or area of altered bone structure, even when few alterations were present in the tissue even minimal when present on the tissue. Then, each degraded structure was observed under bright-field illumination under polarized light or using NIC at $\times 40$ magnification on the PS staining. This allowed us to better observe the characteristics of collagenic degradation, given the high affinity of the PS to the amino acids lysine, hydroxylysine, and arginine of the collagen.

Results

From these mappings, it was possible to differentiate altered bone areas from unaltered areas. These alterations have been investigated on the osteons and the lamellar bone according to the protocol described by Jans et al. [7].

In undegraded osteons (at 0-month postmortem), the parallel concentric bone lamellae have a continuous appearance and have no architectural alteration. Additionally, the birefringence is visible on the osteons and appears in the form of a cross of Malta (Fig. 1). From 6-month (systematically) to 24-month postmortem, we observe a disorganization of the bone lamellar architecture (Table 2). Up to 12-month postmortem, osteons show an erosion of their most central part, which indicates bone degradation around the Haversian canal (Fig. 2). This degradation appears to be centrifugal, moving from the center to the periphery. At 24-month postmortem, this osteonic degradation continues and is associated with a degradation of the part located in the outermost region of the cortical bone, where the disorganization of the bone lamellar architecture appears. This alteration of the lamellar architecture does not have the characteristics of a lacunary alteration that is linked (by tunnels) to microorganisms. If they are present, these tunnels are usually of larger size and are bordered by hypermineralized zones. A reduction in birefringence is also observed starting at 3-month postmortem, with progressive disappearance that continues until 24 months and confirms collagen degradation.

At the end of 1-year postmortem, there appears to be a marked taphonomic degradation situated in the zone that surrounds the canals of Havers, i.e., the center of the osteons. At 24 months, this degradation continues on the periphery of the osteons and appears in peripheral cortical zone. This degradation is of the collagen type. Indeed, Sirius F3B red is a specific dye of collagen and reacts with the groups lysine, hydroxylysine, and arginine. At 12-month postmortem, it does not show any coloration in the central osteonic zone and shows a decrease in its fixation intensity on the whole degraded osteon (Fig. 3). At

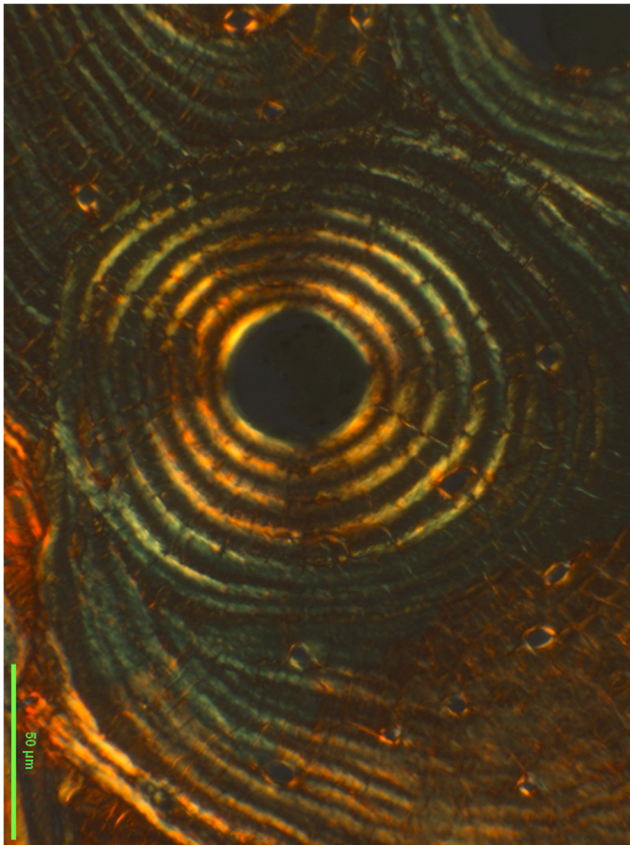


Fig. 1 Undegraded osteons (at 0-month postmortem), the parallel concentric bone lamellae have a continuous appearance and have no architectural alteration. The birefringence appears in the form of a cross of Malta—PS stain under polarized light

24 months, the fixation is also less intense in the external cortical zone. This indicates a disorganization of the collagen fibers. The Sirius F3B red has the property of being fixed parallel to the collagen fibers, which allows an increase in the birefringence of these fibers.

Discussion

Our work, which was carried out over a period of 2 years, made it possible to observe a temporal bone alteration by a microscopic histological study that showed the absence of any microbial attack on this postmortem period. This raises the question of the mechanism involved in the collagenic organic alteration during this period, since this organic alteration (protein or lipid) has been the subject of several histological studies [22, 23], but after longer inhumation times.

Microbial collagen alteration by histomorphology

In our work, the histomorphological study of the samples showed no objective sign of bacterial bone attacks, as identified by the histological preservation index [1]. These signs,

which were originally described by Wedl in 1864 [24], were linked to microorganisms originating from the environment, colonizing bone, and forming tunnels. They were subsequently detailed by Hackett [3] and referred as microscopic focal destructions (MFD). In his microscopy work carried out on 170 human archeological bones (long bones), 113 of the bones showed these tunnels; non-decalcified blocks were included in methyl methacrylate, and 30- μm sections were stained with Masson trichrome and examined under conventional and polarized light. Four individualizable types of these tunnels were highlighted (Figs. 4 and 5):

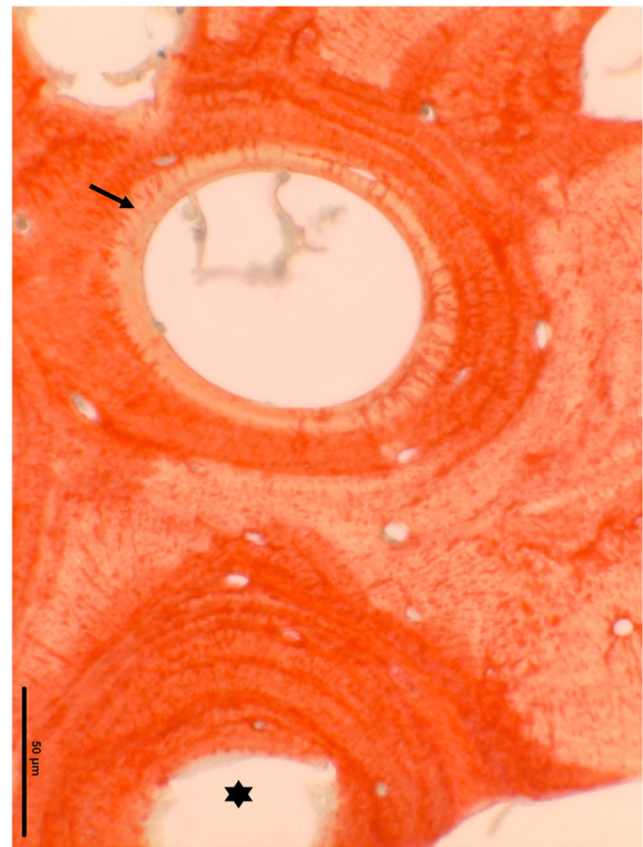
- Wedl tunnels, of relatively uniform diameter, with a range of 5–10 μm . They always pass away from the surfaces of the cortex and the osteon canals on irregular, non-linear paths that are subdivided. Their contours are well delimited; they are mostly empty of contents but occasionally contain spherical bodies that measure approximately half the diameter of the tunnels. They have been attributed to fungic attacks where the spherical bodies sometimes present are spores. Mineral redeposition is absent.
- Linear longitudinal tunnels, measuring from 5 to 10 μm . They are often empty. Expansions are observed in sections in the form of gaps that can measure up to 50 μm .
- Budded tunnels, which run along the osteonic channels. They measure approximately 30 μm and appear irregular in sections. Side shoots are budded off at irregular intervals of approximately 80–90 μm . The contents are streamed and demineralized.
- Lamellate foci, which are rounded and measure from 10 to 250 μm . The expanding lamellate foci are surrounded by rims of retractile bone. Mineral redeposition around these foci occurs frequently.

These tunnels have been described only on tissue areas containing collagen. Thus, these tunnels are not found in dental enamel, unlike dentin [25]. To confirm the role of microorganisms in the genesis of these tunnels, Hackett buried sterilized bones in soil and kept them indoors for 1 year. He showed the presence of fungal deposits but no Wedl tunnels. Only some bone pieces over this 1-year period showed the other three types of tunnels, although this was not systematically observed, which is consistent with our work. The proposed hypothesis was that of bacterial activity of environmental origin. He confirmed this hypothesis by noting that the bones showed a tunnel involvement only on their outermost part and along the Haversian canals. In a study of 233 ancient archeological human bones, Jan et al. showed the presence of these tunnels (or MFDs) of bacterial origin in 74% of their samples [26]. They did not show a gradient of alteration between the external and internal parts of the bone but considered that the bacterial attacks occurred via the natural bone

Table 2 Number of degraded bone areas on a rib section as a function of time (4th rib, for example)

	Rib	Time (months)	Number of degraded osteons	Number of degraded external cortical zone
#1	R4	M0	0	0
		M3	N/A	0
		M6	0	0
		M9	3	0
		M12	5	0
		M24	6	1
#2	R4	M0	0	0
		M3	1	0
		M6	1	0
		M9	4	0
		M12	8	0
		M24	14	2
#3	R4	M0	0	0
		M3	0	0
		M6	1	0
		M9	2	0
		M12	5	0
		M24	10	4
#4	R4	M0	0	0
		M3	0	0
		M6	0	0
		M9	3	0
		M12	8	0
		M24	10	3
#5	R4	M0	0	0
		M3	2	0
		M6	1	0
		M9	5	0
		M12	10	0
		M24	19	5
#6	R4	M0	0	0
		M3	0	0
		M6	2	0
		M9	5	0
		M12	10	0
		M24	15	4

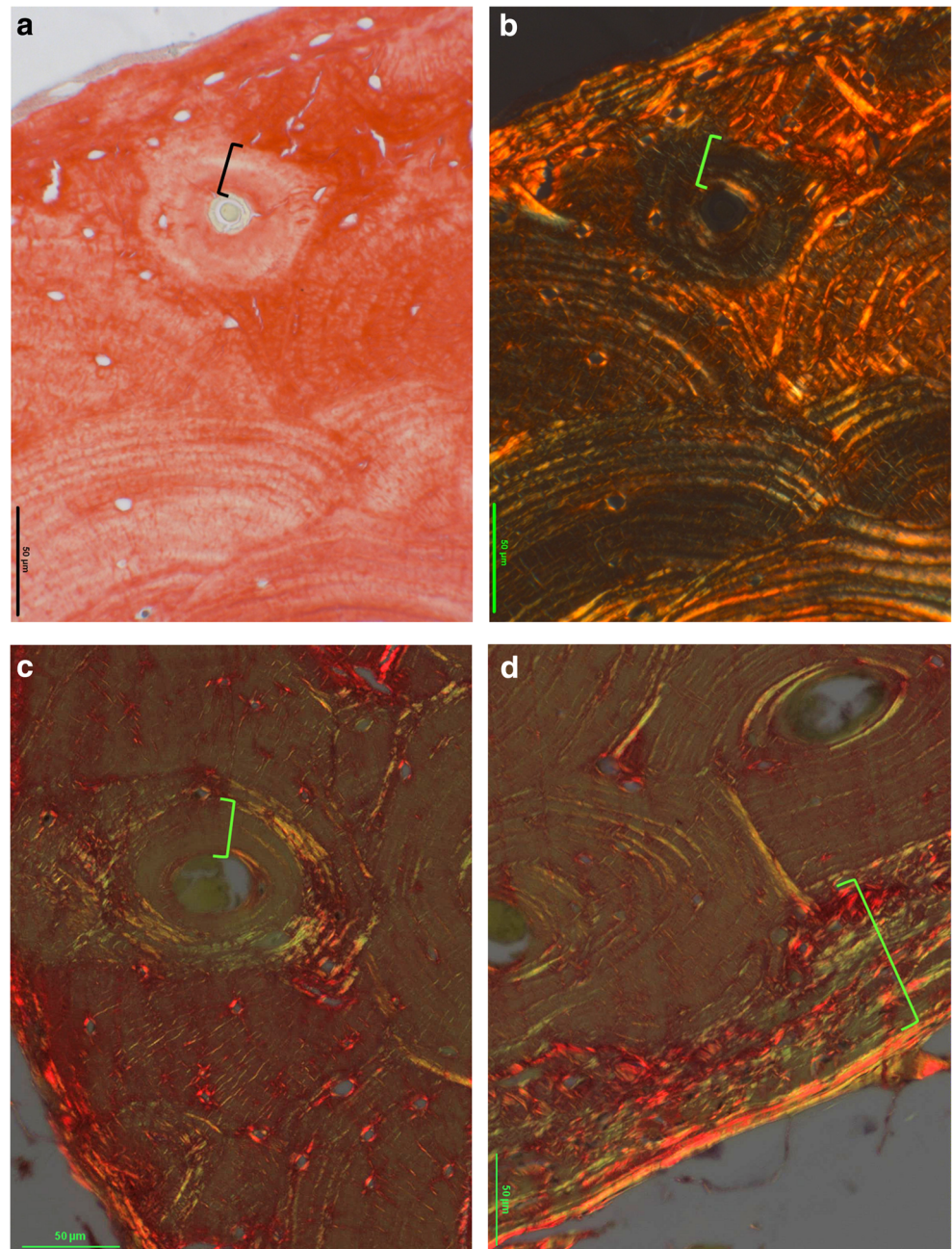
pores and, therefore, mostly via the Haversian canals and the Volkmann's canals. Thus, their observations made it possible to consider that both the endogenous bacteria (which are linked to the putrefactive decomposition) and the exogenous bacteria (which are linked to the environment) could be responsible for these bone alterations. According to their findings, the absence of soft tissue on the bones, which can be linked to extreme temperatures responsible for rapid mummification or to external actions such as dismemberment (via the

**Fig. 2** Microscopic aspect of osteons bioerosion (at 12-month postmortem): loss of Sirius red fixation around the Haversian canal (arrow) vs. osteon without bioerosion (star)—PS stain

action of predatory animals or human manipulation), may occasionally explain the absence of bacterial attack in the absence of endogenous bacteria. These observations were also made in an animal histological study in which Sirius red coloration (highlighting collagen proteins) showed early degradation in periosteal and endosteal areas. This evoked the role of bacteria from soft tissues attached to bone and via decomposing bone marrow [27]. Nevertheless, this question of the action of endogenous bacteria in the creation of bone alterations remains controversial [28]. Some studies have reported bacterial tunnels that only appear after the skeletal stage, which suggests an exogenous bacterial origin [29], whereas others have observed them after a few months post-mortem and evoke an endogenous origin [30, 31]. However, these studies all confirm that although Wedl tunnels are probably related to fungic activity, linear, longitudinal, budded, and lamellar tunnels have a bacterial origin with an enzymatic alteration of collagen via some collagenases [32, 33].

Environmental conditions [12] and the removal of soft parts could partly explain the absence of bacterial attack observed in our work. Indeed, as with any taphonomic study, environmental limitations could represent experimental limits. Further investigations into other environmental conditions are thus necessary in the future. The soil environment and the

Fig. 3 **a** PS stain and **b** under polarized light—marked taphonomic degradation situated in the zone that surrounds the canals of Havers with loss of birefringence (at 12-month postmortem). **c**, **d** PS stain under polarized light—alteration continues at 24-months around the canals of Havers. The fixation is also less intense in the external cortical zone without any fungi or bacterial tunnels



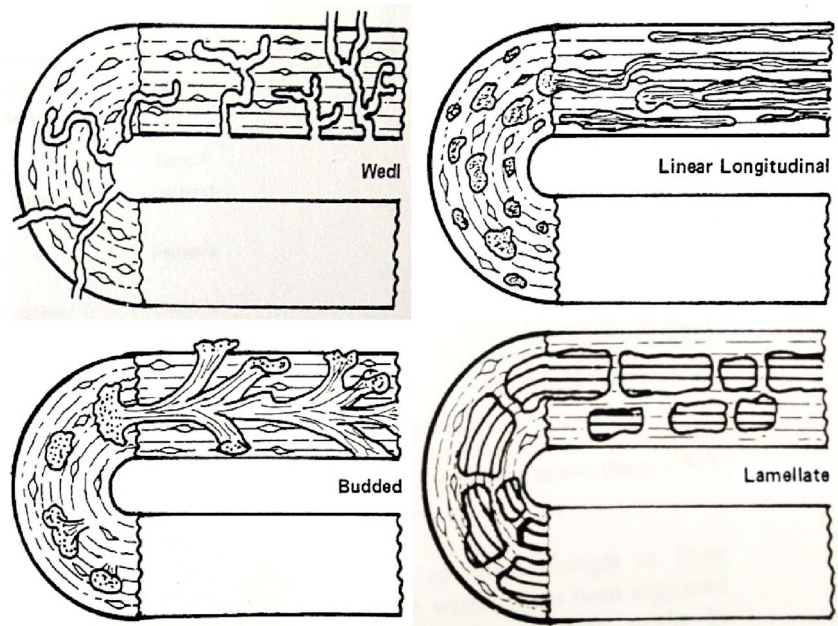
temperature were the most influencing factors in our protocol. Therefore, the absence of bacterial colonization of our samples could be related to (1) a low temperature (T_{\max} mean 10.06 °C; T_{\min} mean 3.98 °C), since at a shallow depth of burial (less than 1 ft), temperature fluctuations are comparable to those occurring on the soil surface [34] and (2) the soil condition (clay) which tends to retain moisture [12, 35]. Indeed, humidity, like low temperature, tends to preserve the remains [36]. A body buried in a shallow grave will thus tend to undergo a process of early mummification, especially in the winter at low temperatures since these will delay or even halt the putrefactive process. This is what we have observed in this

work. Moreover, without protective soft tissues around the bones (our bones were completely defleshed), the desiccation begins to occur immediately after death and limits the bacterial putrefaction period.

Non-bacterial architectural histological modifications

In addition to the classically described Oxford histological preservation index, where these tunnels are individualized into sections [1], another histological preservation index was defined on thin sections by Garland et al. and then adapted by Jans et al. in identifiable diagenetic modifications [6, 7].

Fig. 4 Four individualizable types of bone tunnels. Adapted from Hackett 1981 [3]



Because our histomorphological work did not reveal tunnels suggestive of bacterial alterations, other diagenetic bone changes measurable according to the studies of Garland and Jans were explored to identify the mechanisms involved in the bone degradation that we observed. Thus, two of these histological modifications were sought in our work: one was qualitative, corresponding to the modification of the intensity of birefringence, and the other was quantitative, by accounting for architectural lesions (microcracks).

When bone sections are examined under polarized light, a birefringence pattern can be identified as an alternation of bright bands and dark bands. For the lamellar architecture of

the osteons, this pattern is expressed as a cross of Malta. The intensity of the birefringence is dependent on not only the amount and orientation of the collagen fibers but also the thickness of the section studied. A reduction in this intensity (or even an absence) can be interpreted as being related to the deterioration of collagen [37]. In our work, this birefringence was visible on the first samples at 0-month postmortem; it was reduced from 3 months to completely disappear at 12 months and therefore occurred a fortiori at 24 months. This disappearance of the birefringence thus confirms a degradation of the collagen on this “short” postmortem interval, without any sign of bacterial alteration of the bone.

The quantification of microcracks is most often established based on a structural unit that is formed by an osteon. In our work, we counted the altered osteons and showed that starting at 6 months, degradations were present systematically on each sample and were quantitatively more numerous at 9 months, then at 12 months, and finally at 24 months. Although we did not visually observe these microcracks, the bone lamellar architectural disorganization was particularly evident on our samples around Haversian canals up to 12-month postmortem, and finally, a disorganization of the external cortical zone was associated with this observation. This result confirms the data from the literature on prospective bone studies over short postmortem periods. Peretzschner’s study showed that no microcrack was experimentally produced on the interstitial bone [38] and that microcracks can occur sufficiently long periods of time to undergo microscopic observation. It seems to be the case in our work because the microcracks would explain the loss of the lamellar architecture that we observed. In the literature, these microcracks are attributed [7] to the organo-mineral alterations of the bone and, more particularly, to the swelling that is caused

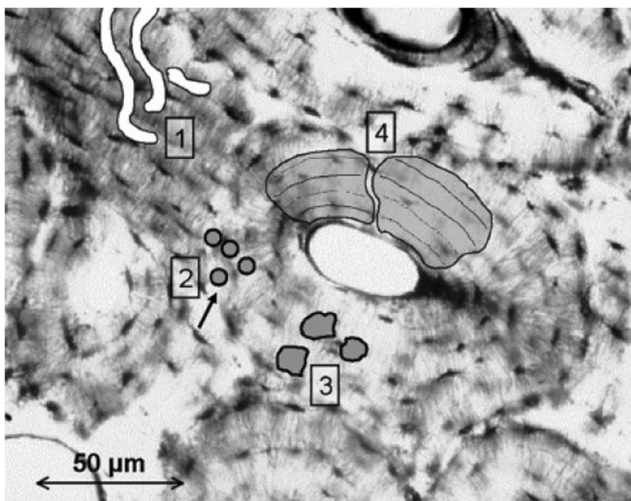


Fig. 5 Four individualizable types of bone tunnels: 1 (Wedl tunneling), 2 (linear longitudinal tunneling), 3 (budded tunneling), and 4 (lamellate tunneling). From Jans et al. 2004 [24]

by the gelatinization of collagen (collagenic alteration responsible for an increase in its solubility) [38].

The staining that was done using Sirius red (no fixation on these altered zones) allowed us to attribute this alteration to a possible collagen degradation of the chemical hydrolysis type due to the affinity of this dye to the amino acids lysine, hydroxylysine, and arginine. The hypothesis that the microcracks that are responsible for the loss of the lamellar bone architecture are related to an organic degradation of chemical type is therefore probable.

Collagen alteration by chemical hydrolysis

The study of the depolymerization of non-mineralized collagen by chemical hydrolysis is well known in medicine, particularly through works on the polymers and material that are used to treat skin, muscle, or vascular diseases [39]. This hydrolysis is also present in mineralized collagen in bone. The triple helix configuration of collagen is characterized by the repetition of a Gly-X-Y triplet. There are very few intra-chain hydrogen bonds, but the numerous interchain hydrogen bonds allow the stabilization of collagen. In bone, mineralized collagen can therefore be altered via hydrolytic cleavage, depending on the temperature and pH of the environment, in three steps [40]: Step 1: hydrolytic cleavage of the intra-chain peptide bonds, step 2: cleavage of interchain hydrogen bonds, and step 3: dissolution of the fragmented chains.

The environment plays an important role in this chemical hydrolysis [41]. The pH of the soil, whether very acid or very basic, will accelerate this chemical modification. Collins et al. estimated that a pH between 2 and 7 significantly increased the mechanism. Similarly, a pH from 7 to 10 increased the hydrolytic phenomenon by a factor of 10 [40]. In our work, the environment was neutral with a pH very close to 7, which explains why the hydrolysis, although present, was demonstrated only after several months. In contrast, by experimentally increasing the pH of an environment to 14 over 5–6 h [38], microcracks appear and can be considered evidence of this collagenous hydrolysis. A dry environment is also a limiting factor for hydrolysis. Indeed, bone dehydration causes an increase in hydrophobic interactions by reinforcing the intra-chain bonds of collagen [40]. This can also be explained by the fact that arginine and lysine, which are specific reaction sites with Sirius red, are two of the most hydrophilic amino acids of collagen. Thus, the fragmented chains (step 3) that contain them will rapidly be dissolved. The early bone desiccation observed under our environmental conditions [12] can therefore explain why only very gradual chemical hydrolysis was observed.

These limiting parameters of the environment probably did not fully inhibit the creation of microcracks, even if the microcracks were not visible microscopically. The disorganization of the Haversian system both around the circumference of the osteons and at the periphery of the cortical bone is an

argument evoking such an alteration. Their identification by marked and extensive bone ruptures is therefore a long-term phenomenon that can take several years or even decades, as noted in the literature [38].

Conclusion

Our work makes it possible to assert that human bone samples that were studied in a controlled environment and analyzed for 24 months underwent a diagenetic trajectory evolving at the second stage that has been defined in the literature [26, 42–44], the so-called accelerated collagen hydrolysis, in which the classically described Oxford histological index remains high [1]. These elements indicate that the organic degradation observed in our study was not caused by bacteria but was instead due to chemical hydrolysis. This result represents one of the approaches to be pursued in future research on bones on a “forensic” timescale.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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