Below the *callus* surface: applying paleohistological techniques to understand the biology of bone healing in skeletonized human remains

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Abstract

Objectives: Bone trauma is a common occurrence in human skeletal remains. The macroscopic and imaging scrutiny is the approach most currently used to analyze and describe trauma. Nevertheless, this line of inquiry may be not sufficient to accurately identify the type of traumatic lesion and the associated degree of bone healing. To test the usefulness of histology in the examination of the bone healing biology, an integrative approach that combines gross inspection and microscopy was conducted. **Material and Methods:** Six bone samples belonging to five adult individuals with signs of bone trauma were collected from the Human Identified Skeletal Collection of the Museu Bocage (Lisbon, Portugal). Previous to sampling, the lesions were described according to its location, morphology, and healing status. After sampling, the bone specimens were prepared for plane light and polarized light analysis. **Results:** The histological analysis was pivotal: (1) to differentiate between types of traumatic lesions; (2) to access the post-traumatic interval; and (3) to diagnose other associated pathological conditions. **Conclusion:** The outer surface of a bone lesion may not give a complete picture of the biology of the tissue's response. Accordingly, microscopic analysis is essential to differentiate, characterize, and classify trauma signs.

Key-words

Paleohistopathology, biology of fracture healing, osteomyelitis, age-related osteopenia, Post-traumatic interval, bone remodeling

Introduction

Injury constitutes one of the major causes of death and disability worldwide and a source of health and social concern with regard to prevention and treatment [1]. As nowadays, injury has also played a significant role in past human societies, a role that can be accessed through a careful analysis of the skeletal remains [2]. Bone injury, resulting from interpersonal violence or associated with the hazards of the daily life, is frequently reported in the paleopathological literature as a useful *tool* to decipher past behaviors and health conditions [e.g. 3-14]. The prevalence and distribution of fractures constitute the marker most commonly investigated [e.g. 15-20]. The study of fractures in the skeletonized remains relies on the macroscopic and radiological identification of signs of bone healing [21-23] and allied complications such as bone shortening, deformity, infection, pseudarthrosis, among others [21, 23-25]. As in the clinical context, imaging analysis is considered the best line of inquiry to diagnose and classify fractures [26]. Nevertheless, its application may be conditioned due to the unavailability of equipment or by the presence of diagenetic bone changes that may bias the radiologic interpretation [23, 27]. As a result, the visual inspection of complete or partial callus formation [e.g. 10, 15] and/ or angular deformities [10] constitutes the methodological approach most often used. And what about paleohistology, what information's considering the bone healing can be regained from the application of histological techniques?

The paleohistopathological analysis of skeletal remains offers a glimpse into the products of cellular activity, such as excessive tissue mineralization, signs of abnormal bone resorption, and residual organics associated with past diseases [28-29]. Furthermore, it provides a wide range of information with regard to microstructural changes that bone undergoes during burial, and that can affect disease diagnosis [30]. Wright and Yoder [31] stated that the application of histological methods may be especially important for examining the degrees of bone healing, as well as to identify traces of disease in cases where little bone response occurred prior to death. This assertion is equally shared by Ragsdale and Lehmer [32]: if cells are the effectors of bone changes, understanding their dynamic interaction is a necessary step for the differential diagnosis.

Using two methodological approaches that compare macroscopic and histological observations, this study aims: (1) to describe the macro-and microstructure of the bone callus; (2) to ascertain the biology of bone healing and the post-traumatic survival interval; and (3) to discuss diagnoses by identifying other conditions that might have caused the lesions observed.

Material and methods

Five adult individuals with multiple trauma evidence and housed at the Lisbon Human Identified Skeletal Collection from the Bocage Museum (Portugal) [33] were chosen for analysis. However, only six bone callus on the ribs and long bones were targeted for sampling (table 1). For skeleton number 1196, permission was granted to take two samples. Due to the invasive nature of the histological procedures, bone sampling was adjusted in order to avoid needless damage to the skeletons. For example, when possible the samples were taken close to damaged areas or in bones showing postmortem breakage. Previous to bone sampling, fractures were classified according to their location, stage of healing and/or presence of bone malignment. Only lesions that were healing (presence of woven bone near the fracture edges or surrounding the primary callus) or had a healed appearance (presence of a sclerotic or dense bone) were considered [22]. After sampling, the bone specimens were prepared for histological analysis [34] as the follows: (1) cleaning and dehydration in water and ethyl alcohol (95%), respectively; (2) embedding in epoxy resin; (3) sectioning using a slow speed saw; (4) grinding using graded sandpaper discs, and an abrasive slurry of aluminum oxidate; (5) dehydration in ethyl alcohol (95%), followed by immersion in xylene (vacuum chamber) and cover slipping; and (6) microscopic analysis under transmitted and polarized light. The qualitative description of the callus histomorphology considered: (i) the bone tissue involved: periosteum, cortex, endosteum; (ii) the type of bone response observed: resorption, formation, or both; (iii) the type of new bone produced: woven, lamellar or both; and (iv) the prevalence of osteocyte lacunae.

Results

Macroscopic study of the degree of bone healing

A total of forty-two consolidated and unconsolidated bone calluses were observed in the five adult individuals considered for analysis (table 2). Of these, 35.7% (15/42) were seen in the adult male Sk. 1138 (86 y.o.), 30.9% (13/42) in the adult female Sk. 119 (64 y.o.), and 23.8% (10/42) in the adult female Sk. 1196 (75 y.o.). With the exception of the Sk. 54, all the remaining individual exhibited multiple signs of bone trauma. The majority of the bone callus were recorded on ribs, mainly in those from the lower segment (R7-R10). For example, 15 bone calluses, seven of them unconsolidated, were observed in nine right ribs of the Sk. 1138 individual. This individual also showed a combination of healed and unconsolidated fractures in five ribs. The other bone calluses were observed in the radii (Sk. 1196), tibia (Sk. 54) and fibula (Sk. 198).

Of the six bone elements selected for histological analysis, five presented bone calluses with a consolidated and healed appearance (table 3). That is, the calluses appeared slightly elevated from the bone surface and presented a dense and smooth outer shell. Only the 4th right rib of the Sk. 1138 showed an unconsolidated callus. Depending on the bone element, the callus morphology ranged from a sharp outlook to a mount-shaped or round relief (e.g., Sk. 119, fig. 1A-D). In two individuals (Sk. 54 and Sk. 198), the fracture has introduced slight structural changes in the bone architecture (fig. 2A-D and 3A-C). In the case of the Sk. 198 right fibula, an inefficient stabilization has caused a malignment of the shaft with an overlap of the broken ends and subsequent bone shortening. In addition with some structural changes, a small cloacae (~1mm) with remodeled contours (anterior portion) and a patch of periosteal new bone formation (lateral portion) was observed in the Sk. 54 tibia bone callus. Healed fractures were seen in the ribs and at the distal extremity of the Sk. 1196 radii, causing a slight epiphysis malignment (fig. 4A-B2). In the unhealed rib fracture (Sk. 1138), the broken edges presented an irregular, smooth and polished morphology. Surrounding the affected area, deposits of periosteal new bone were seen detached from the surface No “movable” join-like structure was identified at the fractured ends (fig. 5A-D).

Histomorphology of the bone callus

The histological analysis of the six bone samples showed a well-preserved bone microanatomy with good bone birefringence. Some structural differences were found when the bone calluses were compared (table 4).

Of the six bone samples with an apparently healed callus, only two (Sk. 54 and 1196 – rib sample) presented a truly mature and remodeled microstructure. For example, in the lateral portion of the Sk. 54 right tibia callus, a haphazard arrangement of Haversian systems, interstitial lamellae, and enlarged osteon canals, crisscrossed by Volkmann's canals was observed. Discontinuous rows of intracortical lamellae, which resemble grenzstreifen [see 47-49] were seen separating the inner cortical bone from the secondary compact bone. At some points, erratic resorption lacunae [or sinuous lacunae, see 49] parallel to the intracortical lamellae were also noticed. In the medial portion of the bone callus, a profusion of randomly organized lamellae in different stages of maturation was observed. In this area, a more chaotic structure formed by osteons, Howship's lacunae and remnants of densely packed lamellae were seen. The periosteal microarchitecture ranged from a thin rim of bone to a denser and/or ruffled surface showing, at some points, irregular resorption spaces (fig. 6A-E).

A remodeled callus microstructure characterized by well-defined osteons and interstitial lamellae was also identified in the right rib sample retrieved from the Sk. 1196. In this sample, multiple bays of bone resorption were observed in the endosteal and periosteal surfaces. At some points, larger areas of bone resorption enclosed by thin layers of bone lamellae were seen, in addition with enlarged Haversian canals and numerous osteocyte lacunae (fig. 7A-D). In contrast with the above-mentioned case, the sample retrieved from the right radius of the same Sk. 1196 individual showed a more immature microstructure. For example, an intricate network of trabeculae, some of them with signs of bone resorption, was observed in the posterior side of the bone callus. The typical structure of a mature cortical tissue was recorded as absent from the core of bone callus, as well as from the opposite anterior surface. That is, no clearly-defined osteons, interstitial lamellae, and Haversian canals were observed. Instead, the anterior portion appeared formed by horizons of lamellar bone pinpointed by a high density of osteocyte lacunae, which suggests distinct levels of bone deposition. Irregular lines running alongside the bone lamellae were also seen. Finally, a pattern of disorganized lamellae and immature bone populated by osteocyte lacunae and separated by irregular areas of bone resorption and discrete Haversian canals was seen in the interface between the anterior and the posterior surfaces of the bone callus (fig. 8A-E1). The most striking example of an immature callus microstructure came from the Sk. 198 sample. In spite of the healed macroscopic appearance, the histological study revealed a cortical tissue formed by an intricate system of lamellae, comparable to trabeculae, in which multiple branches and islands of well-preserved lamellae connecting partially digested osteons were observed. A combination of mature lamellae with more immature bone populated by multiple osteocyte lacunae, and large resorption spaces was also noticed. At periosteal level, a rim of lamellae in distinct stages of maturation was seen bordering the outer surface of the bone (fig. 9A-D).

A complete unhealed fracture was observed on the 4th right rib fracture of the adult male Sk. 1138. In this particular case, the most revealing histological feature observed was the presence of an arc-like structure of new bone connected with the underlying cortex by bone pedestals. Intact endosteal and periosteal circumferential lamellae were also visible, as were remnants of blood vessels. In fact, a network formed by rib trabeculae and preserved blood vessels was observed on the endosteal surface. Enlarged Haversian canals and irregular resorption spaces were visible at cortical level (fig. 10A-C1).

With regard to Sk. 119 rib callus, no evidence of cortical bone break was observed. Actually, the cortex exhibited a mature structure composed of numerous rows of osteons and interstitial lamellae. In the upper and lower edges of the pleural surface, a clear separation between the cortical bone and the patches of periosteal new bone was noticed. This level of microstructural organization was clearly distinguishable from the mesh-like pattern of interconnect lamellae and amorphous new bone formation noticed on the central portion of the pleural surface. Moreover, an accumulation of new bone in distinct stages of maturation was also observed. For instance, the deepest layers were formed by densely packed lamellae with small, elongated and erratic osteocyte lacunae, whereas the outermost ones were composed of a random structure with an immature appearance and densely populated by circular osteocyte lacunae (fig. 11A-C1).

Discussion

Apart from the macroscopic and radiologic scrutiny, few paleopathological studies have applied histological techniques to characterize different types of injury lesions [e.g. 38-45] or to understand the timings of post-traumatic healing [e.g. 35-37, 46]. Results of this exploratory investigation showed that gross inspection may be insufficient to describe and characterize trauma lesions or to understand the biology of disease.

Despite the morphology of the outer shell of Sk. 119 rib callus, which resembles a consolidated fracture, the histological study showed an unremodelled architecture compatible with a subperiosteal hematoma, and eventually caused by periosteum detachment, bleeding. The microstructure of hematoma is variable ranging from thin layers that resemble slip-like cover, to relatively short, bulky bone trabeculae with extensive bridging, arc-like formation and/or multiple layers [47-49]. From the present diagnosis, one cannot completely exclude a case of an incomplete micro-fracture. In the case under discussion, the presence of tissues in distinct stages of maturation: immature and more disorganized bone/isodiametric osteocyte lacunae (outer layers) and lamellar bone/flattened osteocyte lacunae (deepest layers) seems to indicate that the bone lesion was undergoing remodeling at the time of the death. Few can be said about the elapsed time after hematoma formation; nevertheless, and during fracture repair, the remodeling of woven bone into longitudinally oriented lamellar is observed occurring 14 to 21 days after injury [36]. Independently of the diagnosis, the presence of nine rib calluses (n=6, right ribs; n=3, left ribs, see table 2) seems to indicate that this female was exposed to chest trauma that caused a minor tissue disruption in the 9th right rib.

With regard to the Sk. 198 right fibula callus, the mesh-like pattern observed seems to mirror the last phases of the reparative stage. The remodeling phase is the longest (it may require 6-9 years in adults) and aims to reestablish the skeletal integrity [50-51]. The reparative phase is characterized by the development of an organized fibrous mass [52] in a process that recapitulates the embryonic intramembranous and endochondral ossifications [53-55]. This soft or fibrous callus will bond the broken ends [24] and guarantee the mechanical stability of the injured area [56]. Other local changes include mineralization [50], degradation of the nonmineralized matrix, and formation of new trabeculae, which compounds the primary bony callus [24]. In the study of bone callus morphogenesis, Gerstenfeld and co-authors [57] showed that during the endochondral process of fracture healing, the cortex and cartilage undergoes resorption, being replaced by an inner supporting network of trabeculae that will stabilize the fracture. Ayoub et al. [58] also observed a characteristic histological picture characterized by islands of newly built bone surrounded by cartilage and interspersed, at some points, by lamellar bone. The reparative stage may last several weeks. In some cases, the bony callus may originate as early as the first week after injury [59]. Analyzing the morphology of mineralized bone calluses, Liu and co-authors [60] noticed that a microstructure composed of poorly organized tissue (woven bone) and well-aligned lamellae develops between the 2-9 weeks of healing.

In contrast with the aforementioned case, the histomorphology of the Sk. 54 bone callus is compatible with a mature and remodeled fracture. The presence of a cloaca and the observation of small patches of intracortical lamellae or grenztreifen confirms that this individual has developed a post-traumatic osteomyelitis. Albeit more common in specific infections characterized by slowly growing new bone formation [i.e. treponematoses, see 47-49, 61], grenztreifen may also occur in cases of nonspecific osteomyelitis [49]. In the case under discussion, the grenztreifen separates the original cortical bone affected by trauma from a secondary inflammatory process imposed by osteomyelitis.

As osteomyelitis, pseudarthrosis is another severe fracture complication. It develops when the broken extremities fail to form a bony union, which may happen, for instance, by the lack of immobilization [62, 63]. The continuing mobility of the affected area may culminate in the formation of a pseudo-joint associated with extensive callus formation [24, 62]. In cases of fracture nonunion, pseudarthrosis may mimic the healing process leading to misdiagnosis [64]. With regard to the unhealed Sk. 1138 rib fracture, the absence of a “false joint” turns a diagnosis of pseudarthrosis improbable. Accordingly, the histological features observed (i.e. periosteal osteogenesis distant from the lesion edges and separable from the cortex and cortical osteoporosis) are more compatible with the reparative stage of the healing process, pointing to a possible post-traumatic survival interval of 7-14 days [see 35]. As mentioned previously, the Sk. 1138 individual exhibited seven unhealed fractures (in a total of 15 bone calluses) distributed through nine right ribs. Although acute chest trauma is a possible explanation for the lesions observed, one cannot put aside the eventual contributions of age and associated bone fragilities. In addition with age and the health condition of the individual at the time of the death, numerous factors such as the type, location, and severity of the fracture; the stability of the fractured ends; or the adequacy of the vascular supply may affect the healing process [24, 52]. Few conclusions can be drawn from the impact of the causes-of-death of the individuals in the duration of the healing process; nevertheless, the age, and possibly the health condition, may have played a role in some of the cases described, namely in the Sk. 1196 calluses healing. The presence of a callus with partially digested trabeculae and unremodeled lamellae seems to suggest the existence of an underlying condition. It seemed that the bone was continuously laid down in a lamellar fashion without being converted into secondary Haversian systems. Some metabolic disorders may reduce the bone turnover [65]. When this happens, there is more time for secondary mineralization to proceed; as a consequence, the bone tissue becomes hypermineralized and more brittle, requiring less energy to microdamage [66]. Aging also diminishes the ability of bone to repair leading to osteopenia and, in severe cases, to osteoporosis [67]. In addition with the presence of fractures (Colle's fracture, vertebrae, hip, and ribs), age-related features of osteoporosis may include increased resorption and/or marked coalescence of resorption spaces, mineralization defects, and microcracks [67 and authors herein]. These changes were noticed both in the radius and in the rib samples. In spite of being difficult to ascertain if the fractures were predisposed by a metabolic disorder, it may be hypothesized that it was affecting the healing process. Through histological analysis, it was also possible to verify that Sk. 1196 suffered multiple traumatic episodes: an early one completed healed at the time of dead (rib fracture); the other was healing when the individual died (radius fracture).

Conclusion

In spite of the reduced number of samples, this study showed that histology is important for several reasons. Firstly, it is essential to accurately identify the stage of fracture healing, a sort of information that cannot be retrieved entirely from gross inspection and radiologic analysis. In fact, some studies consider that radiographic dating of fractures, especially in children, may be uncertain [68-69]. The determination of the post-traumatic survival interval is important not only to understand how past populations coped with injury but also assumes particular relevance in forensic contexts [35]. For most of the sample studied, however, it was difficult to evaluate the impact of factors such as age in the regular progression of the bone healing; a fact that may affect the estimation of the post-traumatic survival interval. Secondly, histology was useful to distinguish between calluses of fracture origin, from others etiologies (e.g. subperiosteal hematoma, Sk. 119 individual). In this scope, it was also important to further corroborate evidence of secondary complications (e.g. osteomyelitis, Sk. 54 individual), showing that histology may complement the differential diagnosis. Thirdly, it revealed that a lack between the macroscopic and the histological morphology of the bone callus may exist. One can infer that the outer-shell is consolidated, but not that the bone callus is completely healed or remodeled. Accordingly, researchers should be cautious when describing bone lesions of traumatic origin. Finally, histology was useful to determine the sequence of traumatic events in the Sk. 1196 individual. Moreover, it revealed a set of microstructural changes compatible with age-related osteopenia and eventually, osteoporosis. One recommends that future studies should consider a similar approach when trauma lesions and the degree of bone healing are under the scope.

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Table 1. List of the bone samples extracted for microscopic analysis from the Identified Human Collection from the Bocage Museum (Lisbon, Portugal).

Sk.	Individual profile			Bone sample		
	Sex	Age at death (yrs)	Cause of death	Bone element	Location	Length (cm)
54	M	24	Pulmonary tuberculosis	Right tibia	Middle diaphysis	1.5
119	F	64	Bronchopneumonia	9th right rib	Sternal end	2.0
198	M	68	Urinary sepsis	Right fibula	Lower third	2.0
1138	M	86	Bronchopneumonia	4th right rib	Sternal end	1.0
1196	F	75	Arteriosclerosis	Right rib	Middle portion	6.0
1196	F	75	Arteriosclerosis	Right radius	Distal portion	1.7

Table 2. Distribution of the evidence of bone trauma in the individuals analyzed by bone element, anatomic location and rate of consolidation.

Sk.	Individual profile		Trauma evidence					Bone sample
	Sex	Age at death (yrs)	Number	Type	Bone element	Anatomic Location	Rate of consolidation	
54	M	24	Single (n=1)	Bone callus	Right tibia	Middle shaft	Consolidated	**Yes**
119	F	64	Multiple (n=13)	Bone callus	Right humerus	Upper portion	Consolidated	No
					Left radius	Lower portion	Consolidated	No
					Right radius	Lower portion	Consolidated	No
					2nd right rib	Sternal end	Consolidated	No
					3rd right rib	Sternal end	Consolidated	No
					7th left rib	Sternal end	Consolidated	No
					8th left rib	Sternal end	Consolidated	No
					8th right rib	Sternal end	Consolidated	No
					9th left rib	Sternal end	Consolidated	No
					9th right rib	Sternal end	Consolidated	**Yes**
					10th right rib	Sternal end	Consolidated	No
					11th right rib	Sternal end	Consolidated	No
					Pelvis	Ischiopubic ramus	Consolidated	No
198	M	68	Multiple (n=3)	Bone callus	Right tibia	Lower portion	Consolidated	No
					Left fibula	Lower portion	Consolidated	No
					Right fibula	Lower portion	Consolidated	**Yes**

1138	M	86	Multiple (n=15)	Bone callus	3rd right rib	Middle portion	Unconsolidated	No
					4th right rib	Middle portion	Unconsolidated	**Yes**
						Sternal end	Consolidated	No
					5th left rib	Middle portion	Unconsolidated	No
					6th right rib	Vertebral end	Consolidated	No
						Middle portion	Unconsolidated	No
					7th right rib	Sternal end	Consolidated	No
						Middle portion	Unconsolidated	No
					8th right rib	Vertebral end	Consolidated	No
						Middle portion	Unconsolidated	No
						Sternal end	Consolidated	No
					9th right rib	Vertebral portion	Consolidated	No
						Sternal end	Unconsolidated	No
					10th right rib	Vertebral portion	Consolidated	No
					11th right rib	Vertebral portion	Consolidated	No
1196	F	75	Multiple (n=10)	Bone callus	Left radius	Distal portion	Consolidated	No
					Right radius	Distal portion	Consolidated	**Yes**
					8th left rib	Vertebral end	Consolidated	No
						Middle portion	Consolidated	No
					9th left rib	Vertebral end	Consolidated	No
						Middle portion	Consolidated	No
					10th left rib	Vertebral end	Consolidated	No
						Middle portion	Consolidated	No
					Unidentified rib fragment	Middle portion	Consolidated	**Yes**
					Right fibula	Distal portion	Consolidated	No

Table 3. Evaluation of the macroscopic features of the bone callus by individual, bone element, location and healing stage.

Sk.	Bone element	Injury location	Macroscopic description of the bone callus features	Healing stage	Complications secondary to bone healing
54	Right tibia	Middle portion of the diaphysis	• Slight elevation of dense bone with an irregular morphology (> medial surface); • Combination of smooth and rough areas; • Postmortem detachment of the bone outer surface (>anterior-superior portion of the bone callus).	Healed fracture	Slight bowing of the tibia diaphysis, with minor changes in the geometry of the affected area. Presence of a small cloaca (~1mm) with round and remodeled contours bellow the callus and on the anterior surface. Presence of a slight patch of periosteal new bone formation in the lateral portion of the bone callus.
119	9th right rib	Sternal portion	• Mount-shaped elevation of dense bone on the visceral surface; • Smooth surface; • Some postmortem changes.	Healed fracture	No angular deformities were identified
198	Right fibula	Lower third of the diaphysis	• Combination of smooth areas of dense bone (lateral portion) with small and irregular deposits of bone with a sharp appearance (medial portion).	Healed fracture	Malignment with overlapping of the fractured ends. Bone shortening.
1138	4th right rib	Sternal portion	• Unhealed complete fracture; • Light colored fracture ends with an irregular, sharp and polished morphology; • Presence of woven bone deposits encircling the injured area (> bony edges).	Unhealed fracture	---
1196	Right rib	Middle portion	• Slight bone elevation; • Smooth appearance; • Small enlargement of the affected area.	Healed fracture	Slight swelling of the affected area
1196	Right radius	Distal portion of the diaphysis	• Possible Colle's fracture; • Slight transverse depression on the anterior surface; • Abnormal amount of bone in the posterior surface (despite the postmortem damage).	Healed fracture	Slight deformation of the distal extremity

Table 4. Evaluation of the histological features of the bone callus by individual, sample, healing stage and type of trauma diagnosis.

Sk.	Bone element	Histological description of the bone callus features	Type of trauma diagnosis	Healing stage
54	Right tibia	*Lateral portion of the bone callus:* • Cortical bone formed by multiple rows of osteons with different sizes and shapes embedded in interstitial lamellae; • Presence of some enlarged Haversian canals, interstitial lamellae and Volkmann's canals crossing multiple Haversian systems; • Presence of a thin rim of periosteal circumferential lamellae enclosing the cortical tissue. *Medial portion of the bone callus:* • Thicker layer of periosteal bone and a more uneven organization of the cortical tissue; • Evidence of osteoclastic activity at the periosteal level; • Presence of dense sheets of lamellae occupying an intracortical position (grenztreifen) and of sinuous lacunae.	Fracture	Healed
119	9th right rib	*Pleural surface:* • Thick deposit of new bone showing a high concentration of osteocyte lacunae and distinct levels of organization: the deepest layer is formed by densely packed lamellae with small erratic osteocyte lacunae. In the core of the periosteal formation, a poor lamellar arrangement accompanied by elongated osteocyte lacunae with their branching canaliculi is seen. The outer shell exhibits a random structure with no lamellar organization and densely populated by circular osteocyte lacunae. *Cortical bone*: • Distinct levels of organization: inner cortex composed of multiple rows of osteons and interstitial lamellae, whereas the outer cortex is formed by individualized sheets of lamellae randomly interconnected by Haversian systems. Inner cortex populated by a large number of osteocyte lacunae.	Subperiosteal hematoma or an incomplete micro-fracture	Healing
198	Right fibula	• Cortical bone composed of an intricate system of lamellae surrounded by a thin rim of bone with a dense appearance; • Presence of partially digested osteons, large resorption spaces, and enlarged Haversian canals; • Presence of lamellae in distinct stages of remodeling and embedded in an unknown blue-grey substance; • Densely packed mature lamellae; • Immature bone with poor alignment of their mineralized collagen fibers and with multiple osteocyte lacunae.	Fracture	Healing, reparative stage
1138	4th right rib	*Pleural surface*: • Presence of an arc-like rim of newly built bone separated from the underlying cortex by resorption spaces; • Presence of enlarged Haversian canals and irregular resorption spaces in the cortex (beneath the periosteal new bone deposition); • Presence of an intricate network formed by trabeculae and preserved blood vessels near the endosteal surface.	Fracture	Healing, reparative stage

1196	Right rib	• Cortical bone formed by a unique row of osteons and interstitial lamellae; • Presence of enlarged Haversian canals and multiple foci of bone resorption (>endosteal surface).	Fracture	Healed
1196	Right radius	*Anterior portion of the bone callus:* • Composed of multiple horizons of lamellae: the outermost layers presented a compact appearance whereas those close to the endosteal surface exhibited a more disorganized structure; • Presence of two incomplete healed micro-fractures running parallel to the periosteal-endosteal surface; • No Haversian systems were observed. *Posterior portion of the bone callus:* • Formed by a bulky network of trabeculae characterized by a haphazard arrangement of their mineralized collagen fibers, as well as by the presence of numerous osteocyte lacunae; • Presence of multiple foci of bone resorption. *Interface between the anterior and the posterior portion of the bone callus:* • Sheets of new bone mixing a rudimentary system of aligned lamellae with a more disorganized appearance (outermost areas); • Presence of numerous primary vascular canals and enlarged resorption spaces and osteocyte lacunae.	Fracture	Healing, remodeling stage

Figure Captions

Fig. 1 A. Sternal portion of the 9th right rib of an adult female (Sk. 119, 64 y.o.) who died of bronchopneumonia. **B.** Detail of the area sampled for analyzes showing a slight round elevation (white arrow). **C.** Rib section collected for histological analysis. **D.** Detail of the bone sample after slide preparation, in which is visible an accumulation of bone on the visceral surface.

Fig. 2 Tibiae from an adult male (Sk. 54, 24 y.o.) who died of pulmonary tuberculosis. **A.** Right tibia showing a consolidated callus on the middle of the diaphysis. Note the presence of slight structural changes of the shaft when compared with the unaffected left tibia. **B:** Detail of the bone callus showing an expanded and smooth surface (medial face). The black star indicates the area sampled for histological analysis. **C:** Magnification of the anterior-lateral portion of the bone callus exhibiting some post-mortem detachment of the outer shell of the callus and a small cloaca below the callus. **D:** Bone sample collected for histological analysis, before (left) and after slide preparation (right).

Fig. 3 A: Right fibula of an adult male (Sk. 198, 68 y.o.) who died of urinary sepsis. **B:** Detail of the bone callus exhibiting a consolidated but irregular morphology. Note the overlapping of the fractured ends. **C:** Magnification of the sample collected for histological analysis, before (left) and after slide preparation (right).

Fig. 4 A: Radii from Sk. 1196, a 75 y.o. female who died of arteriosclerosis showing trauma evidence compatible with a Colle's fracture. **A1:** Detail of the bone callus of the right radius exhibiting an abnormal elevation. Note the severe post-mortem damage (dorsal view). **A2:** Anterior view of the affected radius. **A3:** Magnification of the bone sample collected for histological analysis, before (left) and after (right) slide preparation. The black star indicates the surface sampled for histological analysis. **B:** Right rib from the same individual with a consolidated callus (white arrow). **B1:** Visceral surface of the abovementioned sample highlighting the smooth surface of the callus (white arrow). **C2:** Detail of the sample collected after slide preparation.

Fig. 5 A: Fourth right rib of the adult male (Sk. 1138, 86 y.o.) who died of bronchopneumonia revealing a complete, unconsolidated fracture in the sternal portion. **B:** Magnification of the fractured ends showing a sharp morphology and the presence of a rim of newly built bone (dorsal view). **C:** Visceral surface of the rib showing the fractured edges. **D:** Detail of the previous image showing the new bone foci on the sample (left) and the slide prepared for histological analysis (right).

Fig. 6 A: Microscopic view of the Sk. 54 right tibia sample showing the cortical tissue formed by osteons (white arrow) intersected, at some points by Volkmann's canals (black arrow). The periosteal surface was composed of a thin rim of bone (white star). **B:** Segment formed by osteons, intracortical lamellae (➊), and resorption spaces (➋). **B1:** Detail of the intracortical lamellae (or grenzstreifen) (black arrows) separating rows of osteons (white arrows). **B2:** Another Magnification showing some resorption lacunae (or sinuous lacunae) (white star) in the intracortical lamellae (black arrow) and at the periosteal surface (white star). Note the presence of osteons with enlarged Haversian canal (white arrow). **C:** Segment showing a haphazard cortical microstructure formed by osteons (white arrow), interstitial lamellae (white star) and compact intracortical lamellae (black arrow). **D:** Thick layer of dense bone at the periosteal surface (black stars) connecting the cortical tissue formed by different sized osteons (white arrows). **E:** Another segment revealing erratic osteons (white arrow) intercalated by elongated intracortical lamellae (black arrow. Polarized light, Magnification 40x and 100x.

Fig. 7 A: Micrograph of the right rib sample of the SK. 1196 individual exhibiting the cortical tissue (➊). **A1:** Detail of the previous figure showing a thin row of osteons (white arrows) and interstitial lamellae (white stars). **B:** Another segment revealing bays of bone resorption in the endosteal and periosteal surfaces (black arrows) and some intact cortical osteons (white stars). **C:** Segment showing a larger area of cortical (white star) and endosteal (black arrows) bone resorption. **D:** Illustration of a segment under plane light exhibiting a row of osteons (white stars) with enlarged Haversian canals and numerous osteocyte lacunae. Polarized light, Magnification 40x and 100x.

Fig. 8 A: Micrograph of the posterior portion of the SK. 1196 right radius bone callus showing scattered trabeculae erased by foci of bone resorption (white arrows). **B:** Illustration of the anterior portion of the callus revealing lamellar bone apposition with variable density and organization (white stars). **C:** Another view revealing distinct layers of lamellae (white stars) separated by longitudinal lines (white arrows). **D:** Area showing cortical lamellae - mixed with more immature and disorganized forms of bone (white and black arrows), and spaces of bone resorption (white stars) and discrete vascular canals. **E:** Segment exhibiting a haphazard arrangement of bone lamellae in distinct stages of maturation (white arrows), as well as enlarged resorption spaces (white stars). **E1:** Detail of the previous figure highlighting the orientation of the mineralized collagen fibers and the numerous osteocyte lacunae. Polarized light, Magnification 40x and 100x.

Fig. 9 A: Microscopic view of the Sk. 198 right fibula callus showing the cortical tissue formed by a disorganized net of bone lamellae and remnants of ancient Haversian systems. **A1** and **A2**: Details of the previous image showing large bays of bone resorption being formed after digestion of previous osteons (white arrows). **B:** Another view highlighting numerous areas of bone resorption. **B1:** Magnification of the previous image revealing the large and irregular areas of bone resorption (white arrows). **B2:** Another Magnification showing mature (white arrows) and more recently formed lamellae with osteocyte lacunae (black arrows). **C:** Bone segment combining densely packed lamellae on the bone surface (black arrow) and branches of disorganized lamellae with osteocyte lacunae in the innermost areas of the cortical bone. **D:** Another view showing the outer surface composed of lamellae with a haphazard arrangement and pinpointed by numerous osteocyte lacunae. Polarized light, Magnification 40x and 100x.

Fig. 10 A: Micrograph of the Sk. 1138 rib cortical tissue presenting mature osteons and enlarged Haversian canals (white arrows) and a deposit of new bone with an arc-like microanatomy at periosteal level (white stars). Note the presence of rib trabeculae and preserved blood vessels (black stars). **B:** Another view pinpointing a major area of osteonal bone resorption (white star) and the newly built bone (white stars). **C** and **C1:** Bone segments showing massive foci of osteon resorption (white arrows). Polarized light, Magnification 40x.

Fig. 11 A: Micrograph of the Sk. 119 right rib showing the cortical tissue composed of mature osteons (white arrows) and interstitial lamellae. A clear separation between the periosteal circumferential lamellae and a more disorganized periosteal new bone formation (white star) is visible. **B:** Segment exhibiting several rows of osteons (white arrow) intersected by sheets of lamellar bone with variable density (black arrows). Note the presence of a lumpy deposit of periosteal new bone (white stars). **C:** Periosteal new bone formation exhibiting several degrees of bone organization (black arrows). **C1:** Detail of the previous figure showing the inner layers composed of well-defined lamellae with small and elongated osteocyte lacunae and the outmost layers with a general lack of bone organization and populated by large and rounded osteocyte lacunae. Polarized light, Magnification 40x and 100x.