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TITLE OF THE DOCTORAL THESIS

RADIOLOGICAL APPLICATIONS IN FORENSIC ANTHROPOLOGY.

FRACTURE HEALING AND DATING

S.S.D. MED/43

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***A te Edoardo**,*
a te che dal tuo primo giorno di vita mi hai accompagnato in questo percorso,
a te che hai dato un nuovo senso a tutto,
a te che rendi unico e speciale ogni attimo della mia vita.

***A te Francesco**,*
a te che mi hai sempre sostenuto,
a te che hai sempre capito,
a te che mi hai dato la gioia più grande.

***A te mamma**,*
a te che sei il mio più grande esempio di forza, coraggio e tenacia,
a te che mi hai insegnato prima a rialzarmi che a non cadere,
a te, perchè a te devo la donna che oggi sono.

Vi amo.

ABSTRACT

The accurate dating of bone fractures constitutes a critical component of trauma analysis in forensic anthropology and the research in this field represents to date a challenging opportunity to apply scientific knowledge and methods to real problems of society, including also global humanitarian and human rights issues.

However, the literature review performed in order to describe the state of the art in fracture healing and dating showed that forensic studies are scarce and that the assessment of fractures lacks consensus about both the definition of fracture healing and the duration of the fracture healing process.

Thus, the aim of the present PhD project, including two research lines, was to acquire a better knowledge of the process of bone remodeling both in the living and the dead with regard to the timing of injury as well as to evaluate the applicability of high-resolution radiological techniques for objective dating of the healing phase of the fracture.

The 1st retrospective study, dedicated to the living, was based on digital radiographs from the largest adult living population ever analyzed and was aimed not only at examining time frames for healing of bone fractures but also at investigating the effect of variables, including age, sex, bone type and number of fracures on the timing of healing stages of traumatic skeletal lesions. For these purposes a multivariable model was built, which showed a significant association between the healing stages and the variables analyzed, so that a dynamic nomogram was preliminary proposed to predict a time interval since fracture from digital radiographs.

The 2nd experimental study, dedicated to the dead, was based on dry human bones presenting calluses of different known age in order to preliminary assess the potential of an advanced and non-destructive imaging technology, like micro-computed tomography (micro-CT), in order to obtain a future objective dating of the healing phase of the fracture on post-cranial human bone calluses of known age. The results not only demonstrated the potential utility of micro-CT to obtain a wealth of qualitative details about the microstructure of the callus but also to reach an objective fracture dating, laying promising foundations for further studies on this topic in light of the highlighted existence of a certain trend of some

parameters of trabecular microstructure relative to the age of the callus, including the degree of anisotropy, the connectivity and the trabecular spacing.

SOMMARIO

L'accurata datazione delle fratture ossee costituisce una componente fondamentale dell'analisi traumatologica in antropologia forense e la ricerca in questo campo rappresenta al giorno d'oggi un'opportunità stimolante per applicare le conoscenze e il metodo scientifico a gravi problemi sociali, ivi compresi quelli relativi alla violazione dei diritti umani.

La revisione della letteratura scientifica eseguita al fine di descrivere lo stato dell'arte su tale tematica ha tuttavia evidenziato come gli studi forensi siano scarsi e come manchi un adeguato consenso sia circa la descrizione delle caratteristiche che circa la durata delle diverse fasi di guarigione delle fratture.

Lo scopo del presente progetto di dottorato, comprendente due linee di ricerca, di cui una su vivente e una su cadavere, è pertanto quello di acquisire una migliore conoscenza delle diverse fasi del processo di rimodellamento osseo e della loro durata, anche valutando l'applicabilità di tecniche radiologiche ad alta risoluzione per la datazione oggettiva delle fratture.

Il 1° studio, di tipo retrospettivo e dedicato al vivente, è basato su radiografie digitali appartenenti alla più vasta popolazione vivente adulta mai analizzata ad oggi ed è finalizzato non solo a esaminare i tempi di guarigione delle fratture ossee ma anche a studiare l'effetto di variabili, tra cui età, sesso, tipo di osso e numero di fratture, sulla tempistica delle fasi di guarigione. A tal fine è stato costruito un modello multivariato, che ha mostrato una significativa associazione tra le fasi di guarigione e le variabili analizzate, alla luce del quale è stato quindi proposto un nomogramma dinamico preliminare per datare una frattura.

Il 2° studio, di tipo sperimentale, dedicato al cadavere e basato su calli ossei umani di diversa età nota, è finalizzato alla preliminare valutazione delle potenzialità di una tecnica di imaging avanzata e non distruttiva, come la micro-tomografia computerizzata (micro-TC), per ottenere una datazione oggettiva della fase di guarigione della frattura. I risultati ottenuti non solo hanno dimostrato la potenziale utilità della micro-TC per ottenere una molteplicità di utili dettagli qualitativi sulla microstruttura del callo osseo ma anche per misurarne alcuni parametri, tra cui il grado di anisotropia, la connettività e la spaziatura trabecolare, i quali hanno dimostrato avere un trend definito rispetto all'età del callo, ponendo

quindi promettenti basi per ulteriori studi su questo argomento finalizzati proprio a raggiungere una datazione oggettiva della frattura.

SOMMARIO

1. INTRODUCTION

Forensic Medicine is an interdisciplinary and cross-sectional medical discipline which uses the knowledge of numerous different fields of expertise with the aim of applying justice properly, according to a German definition by Schmidtmann (1905), the last editor of the famous *Handbook of Forensic Medicine* by Johann Ludwig Casper (1857) [1].

Among the different fields of expertise of forensic medicine, **Forensic Anthropology** is considered as an applied subfield of physical anthropology[1] (i.e. one of the four primary subdisciplines of anthropology). This field has evolved and expanded dramatically since the early pioneers first began applying the science and methodology of anatomy and physical anthropology to medico-legal issues.

Stewart's classic text "*Essentials of Forensic Anthropology*" published in 1979 classified the forensic anthropology as "... that branch of physical anthropology which, for forensic purposes, deals with the identification of more or less skeletonized remains known to be, or suspect of being, human" [2] and Christensen A.M. et al. in the recent guide "*Forensic Anthropology: Current Methods and Practice*" defined it as the "application of anthropological method and theory to matters of legal concern, particularly those that relate to the recovery and analysis of the skeleton" [3].

Traditionally, the practice of forensic anthropology has been involved in the study of the dead, focusing on the recovery and analysis of human remains as well as on the identification and detection of signs of trauma. In particular, the summary of estimated biological parameters like sex, ancestry, age, and stature from skeletal material belonging to unknown individuals is referred to as the biological profile, which is compared to missing persons records in an attempt to identify the person to whom the skeletal remains belong [4] while the analysis of skeletal trauma and other alterations may be relevant to the individual's cause and manner of death.

Recently, the scope of forensic anthropology has been expanded to include also issues about the living related to identification [5, 6], age determination [7] and fracture healing timing, so that notions of anthropology and associated disciplines

[1] *Anthropology* = the study of humankind, from the Greek *anthropos* "man" and *logia* "study".

have been applied also to age juvenile perpetrators, to identify bank robbers taped on videosurveillance systems, to establish whether presumed victims of pedopornography are under age [8], to verify the statements of alleged victims about torture or abuse as well as to date fractures from radiographs.

In light of this expansion in the forensic anthropology's applications, in the last few years new researches have been set dealing with trauma interpretation and age estimation not only in the dead but also in the living, with investigation of mass disasters and humanitarian and human rights issues, including immigration, adoption, refugee and asylum seekers, human trafficking and child pornography [7] and for this purpose different methodology approaches from other areas of science have been adapted to address issues in forensic anthropology, including radiology and imaging.

Indeed, the interrelatedness between forensic radiology and forensic anthropology was recognized very early, since X-rays where chiefly used right in the field of anthropology when images of the mummified hand of an Egyptian princess were produced just 1 year after Röntgen's discovery (1896) [9]. Furthermore, the technological innovation of the bio-medicolegal sciences, begun at the end of the 20th century and continuing into the new millennium, has then led to a wide variety of new and more powerful imaging methods with potential utility also for forensic anthropological investigation

Nowadays, as forensic anthropology, also **Forensic Radiology and Imaging,** is considered as a dynamic and rapidly evolving field within the scenario of forensic medicine, which usually comprise the performance, interpretation, and reportage of those radiological examinations and procedures that have to do with the courts and/or the law [10].

Over the years the involvement of both forensic anthropologists and radiologists has become of fundamental importance to identify and describe injuries that could be difficult to objectify and in order to respond to bio-medicolegal issues, such as the identification of unknown persons, the identification of wound vitality, the reconstruction of the chronology of injuries, the mechanism of traumatic injuries, the definition of the specific cause and the dynamic of death, and the determination of the post-mortem interval [9-11].

Therefore, forensic anthropology has attracted extensive research attention just because of the challenging opportunity to apply scientific knowledge and methods, as well as new imaging techniques, to real problems of society, including not only the assessment of foul play in criminal cases but also global humanitarian and human rights issues [4].

Among the applications of forensic anthropology and related research, **trauma analysis** is one of most important contributions that forensic anthropologists make in the medico-legal arena, both in the dead (including on dry bones, where injuries may be preserved for a long time thanks to the resistance of bone tissue to decomposition) and the living.

Through casework and innovative experimental research, forensic anthropologists have learned a great deal about the biomechanics and dynamics of traumatic injury involving bone. Calling upon their experience with both recent and ancient skeletal casework, anthropologists are uniquely positioned to try to differentiate perimortem trauma that may be associated with cause and manner of death (at or about the time of death) from antemortem trauma and postmortem alterations. Assessment of the timing of an alteration demands knowledge not only of the biomechanics of bone fracture but also the skeletal remodeling system, patterns of growth and development and the many variables involved in postmortem alteration [4, 12].

For deceased victims the trauma analysis in forensic casework is crucial not only because it can give important information for the identification of an unknown, decedent especially in the modern scenarios of mass migrations [13-15], but also because, through the accurate dating of bone fractures, it could assist in testing witness statements and contribute to understanding the chronology of events prior to death [16, 17].

On the other hand, detailed information about the timing of injuries given by forensic anthropologist in the living could help to corroborate or contradict the victim's statements about abuse [18-20], maltreatment or torture, which is a delicate and extremely current international problem given the increasing of domestic violence worldwide [21] as well as the increasing influx of asylum seekers to Europe who claim to have been victims of torture and request for assessments for signs of torture [22-25]. In fact, in such cases, living victims often present multiple injuries in different stages of healing sustained through repetitive and prolonged abuse [26, 27], including bone fractures which can bear witness to the incident even years after it occurred [26-28].

However, the assessment of a traumatic event, with particular reference to the estimation of the post-traumatic time interval, could be extremely difficult because of a large amount of variables (e.g. age, health conditions, skeletal location of injury, severity of injury, type of fracture, apposition and mobility of fracture ends), especially when dealing with dry bone.

This project, inserted in the context of the trauma research in forensic anthropology and divided into two distinct parts, is dedicated to the study of fracture dating after bone trauma basing on the tissue healing response after traumatic tissue damage, with the aim of acquiring a better knowledge of the process of bone remodeling both in the living and the dead, which is crucial for a correct analysis of injuries in the skeleton, since it can be very informative with regard to the timing of injury in both living and dead.

In the *first part*, dedicated to the living, it will be presented a multidisciplinary study, including different disciplines like radiology, clinical medicine, biological and forensic anthropology, which has been performed with the aim to examine time frames for healing of bone fractures in a retrospective substantial clinical adult sample and to investigate the effect of variables, including age, sex, bone type and number of fracures on the timing of healing stages of traumatic skeletal lesions using clinical digital radiologic images.

In the *second part*, dedicated to the dead, it will be evaluated the applicability of high resolution radiological technique like micro-computed tomography (μCT) for objective dating of the healing phase of the fracture on dry bones since this topic still needs to be studied in detail and improved by developing new approaches and adding know-how in the interpretation, which could include the use of advanced technologies. Furthermore, as feasibility study to verify the potentials of future applications, also synchrotron radiation micro-CT (SRμCT), which is another non destructive imaging technique with a resolution higher than conventional micro-CT, was considered.

However, before getting to the heart of the project explaining its development in detail, a brief discussion concerning the general topic of bone repair and trauma analysis as well as the state of the art in fracture healing and dating in forensic anthropology, both in living and dead, will be presented.

2. BONE HEALING PROCESS

A fracture is a laceration of bone caused when a force exceeds the ability of the bone to deform [19]. The injured bone tissue, unlike other tissues, exhibits the behaviour of "self-healing" by activating a very complex biological healing process which recapitulates many of the ontological events of embryonic skeletal development (aside from the initial bleeding and inflammatory phases) and it is able to regenerate itself completely as well as to restore the tissue to its original physical and mechanical properties, leading to a final mechanically stable lamellar structure [16, 29].

Bone fracture is one of the more common traumatic injury to humans [29] and it is associated with treatment costs exceeding billions of dollars, societal productivity loss, and individual disability [30-32].

Understanding the complex healing process in detail is crucial not only for orthopedic surgeons to properly create the optimal healing environment for an injured bone [29-30] but also for forensic anthropologists since the accurate dating of bone fractures is a critical and crucial component of trauma analysis in forensic casework, as already reported in the Introduction.

The developmental progression of fracture healing (starting from the early signs of bone healing) has been mostly derived from studies, especially mouse fracture models, focusing on histological tissue changes, cellular and molecular pathways and strength testing [29, 33-35].

Although there is still much to be learned to fully comprehend the pathways of bone regeneration, the overall pathways of both the anatomical and biochemical events have been thoroughly investigated, providing a general understanding of how fracture healing occurs after trauma [35-36], which can be either direct or indirect [16].

The ***direct fracture healing*** refers to the direct attempt to form new Haversian systems through the formation of discrete remodelling units which restores mechanical continuity and this process occurs only in case of anatomic reduction of the fracture fragments reached by internal fixation or decreased interfragmentary strain where there is no formation of bone callus, with no periosteal response but only direct differentiation of osteoblasts from osteoprogenitor cells derived from vascula endothelial and perivascular mesenchymal cells.

The ***indirect healing*** has proven to be the most common process since it is usually involved in case of fractures with no anatomical reduction or rigidly stable condition. In this process a fracture is repaired by the growth of new tissue that develops in and around the site of the fracture and the new tissue produced to bridge the two extremities is called *callus*, whose formation is the result of a combination of two different ossification processes: intramembranous and endochondral. This healing process is conventionally partitioned into different phases, each characterized by a specific set of cellular and molecular events, including the haematoma formation and the early inflammatory phase, the repair phase (i.e. primary soft callus formation-cartilage form and hard callus formation-woven bone) and the late remodeling phase (**Figure 1**) [29, 30, 36-39].

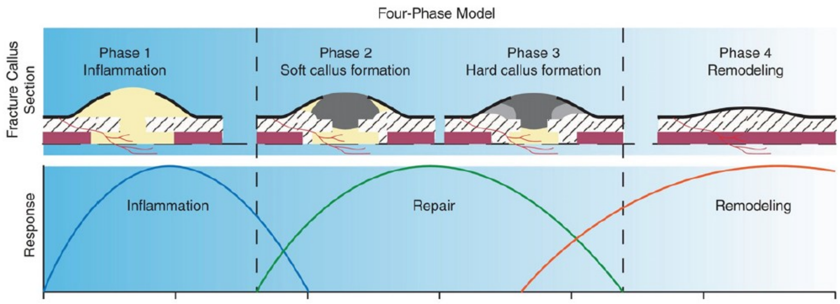

Figure 1. Healing process phases.

The indirect healing starts with an initial anabolic phase, where local tissue volume increases through inflammation. In fact, the first immediate event after a fracture is the haemorrhage from the ruptured bone and the periosteal vessels that are formed within the medullary canal and beneath the periosteum [39], followed by the formation of a hematoma at the fracture site. The hematoma's composition, not yet fully comprehended, is thought to include neutrophils and macrophages that migrate into the fracture gap in addition to the numerous marrow bone cells and erythrocytes who have arrived from the ruptured vessels and damaged marrow spaces. The activated coagulation process follows rapidly in order to form a clot that will act as a haemostatic plug to limit further haemorrhage and then the acute inflammation reaction initiate, with significant increasing of proinflammatory mediators which have chemotactic effects on other inflammatory cells and are critical for bone healing to progress. Then, further aggregation of platelets and angiogenesis take place.

After vascular trauma, the fracture microenvironment becomes locally hypoxic and the necrotic reaction is activated simultaneously, with the osteocytes at the ends of the fracture sites that become deprived of their nutrition and undergo degenerative and/or necrotic changes [39]. Macrophages phagocytize such necrotic areas and facilitate the regeneration stage by releasing growth factors which are responsible for migration, recruitment, and proliferation of mesenchymal stem cells (MSCs) and their differentiation to angioblasts, chondroblasts, fibroblasts, and osteoblasts. Even if the origin of MSCs is still unclear with respect to where they come from and to which are the precise signaling molecules that mediate their homing to the injury site, their recruitment is fundamental for the healing process, since they lead to a chondrogenic phase to form the first organization at the fracture site, consisting of a soft fibrous granulation tissue [40]. In fact, they migrate within at regions juxtaposed to the fracture gap, which are less mechanically stable wherein resident, and start to proliferate and differentiate to chondroblasts and osteoblasts. The endothelial cells, fibroblasts, and osteoblasts participate in filling the fracture gap by the formation of granulation tissue which invades the fracture area only temporarily and houses all the aforementioned mediators. This tissue can be considered a *primary callus*, which is a bone tissue not yet calcified and still slightly soft.

Subsequently, fibroblasts begin to lay down a stroma that helps support vascular ingrowth and during the progressive vascular ingrowth, a collagen matrix is laid down through the activities of skeletal and endothelial cells while osteoid is secreted, leading to the formation of an *intermediate cartilagineous callus*, created precisely on the granulation tissue by osteoblasts that deposit osteoids into the fibrous connective tissue while osteocytes at this step are not included since no new bone is already synthetized [29, 30, 38].

The *periosteal callus* is produced by osteoblasts on the periosteum and it is characterized by a bulging shape that, unlike the previous ones, can be macroscopically identified on dry bone as well as on radiographs and the clinical follow up of injury in the living is based on its evaluation.

The third phase involves the formation and mineralization of the callus and replacement of the mineralized callus with mineralized bone and sculpting of the bone back to its original shape, size, and biomechanical competency via modeling and remodeling. This phase can also be referred to as secondary bone formation and involves converting the irregular woven bone callus into the lamellar bone. This prolonged period is characterized by coupled cycles of osteoblast and osteoclast activity, with osteoclasts that resorb the newly woven bone (hard callus) and osteoblasts that replace this matrix with the lamellar bone in order to reshape the newly formed secondary bone to the structure of the original cortical bone with the final well-oriented bone lamellar tissue (termed "coupled remodelling"") [39]. In the final period of the catabolic phase, also extensive vascular remodelling takes place in which the increased vascular bed regresses and the high vascular flow rate returns to its pre-injury level.

The aforementioned phase model, originated as the result of histological observations of healing fractures in both animal models and human patients and representing histologically the healing process, can be a useful system for describing the basic events that take place but it must bear in mind that, in practical terms, there is rarely such a clear delineation of events, since the different stages often significantly overlap during fracture repair [41].

In fact, although these processes take place consecutively, there is a continuum of changing cell populations and signaling processes within the regenerating tissue [29, 36], so that, to date, it's only possible to describe a general temporal overview of the biological events of fracture healing, as presented in **Figure 1**.

In support of this, it was stated that healing process separation into histological phases is only an abstraction for the purposes of study performed by various researchers who arbitrarily divided healing stages differently or commonly assigned different names to them both from an histological and radiological point of view and that is for this reason that the literature, despite the great amount of articles dealing with bone repair, shows little consistency in terms of stages of bone healing [42].

Moreover, also in the radiological examination, as in the histological analysis, the healing process is generally divided arbitrarily up to six stages, which are based mostly on the features of the fracture line, fracture gap and inner structure of the newborn callus, with a lack of consensus on imaging about the clinical criteria for definition of fracture healing regarding the specific time course as well as a lack of a true gold standard [43] to estimate the post-traumatic survival time so that the reliability and accuracy of post-traumatic survival time estimation on the basis of radiographs remains an “inexact science”.
Therefore, beyond terminology, there is still much to be understood concerning both the exact timetable of changes in bone repair from a histological and radiological point of view and the associated interpretation of time since fracture, and a multitude of factors must also be taken into account in timing evaluation.

3. TRAUMA ANALYSIS IN FORENSIC ANTHROPOLOGY

The word *trauma* refers to the "physical disruption of living tissues by outside forces" and the *trauma analysis* is a major area of interest in forensic anthropology both in the living and the dead, since it can contribute to solve important questions about interpersonal violence, abuse or torture in the living as well as to understand the circumstances of death in cases of burnt, skeletonized, or otherwise decomposed human remains for which a complete autopsy may not be possible [3, 44].

Therefore, in addition to personal identification, the most important issue for forensic anthropologist is just that of correctly identifying and interpreting every single skeletal lesion and trauma.

In particular, the conclusions reached from the analysis of skeletal trauma by the forensic anthropologist typically include the *mechanism* or type of force that caused the trauma (usually categorized as blunt force, high velocity projectile, sharp force, thermal, or some combination of these categories) and the *timing* of the trauma, which consists precisely in ageing fractures for the corroboration or contradiction of the victim's statements about abuse or torture in living as well as in determining when and how a bone injury occurred in relation to death.

The *timing* of the trauma is particularly difficult in case of dry bones where there are no fresh soft tissues which can help to define the injury's vitality and in this case it is necessary to evaluate if it occurred prior to, around time of or after death, that is "antemortem", "perimortem" or "postmortem".

In particular, "*antemortem trauma*" is an alteration[2] having occurred before death and it is characterized by early bone signs of healing or fracture callus while "*perimortem*" is an alteration occurring around the time of death, without any signs of healing but with certain morphological characteristics related to bone elastic response to trauma so that it can't be reliably assigned to either the ante- or postmortem group [45-46]. However, unlike that of the pathologist, the anthropologist's perimortem interval is temporally broad and may span several days to weeks in either direction of time of death.

[2] An *alteration* is any change to the physical properties of a bone.

On the contrary "*postmortem*" alterations are not considered trauma since by definition they no longer disrupt *living* tissue and they occur after death because of environmental and taphonomic processes or human intervention.

The primary evidence of antemortem trauma is osteogenic reaction (or the formation of new bone) since, naturally, such responses will only occur if an individual is still alive and it is usually in the form of fracture healing, which may be evidenced by rounded fracture edges or a fracture callus.

Therefore, indications of antemortem osseous repair are an important arbiter of the antemortem/perimortem boundary and all forensic scientists agree that any evidence of bone remodeling suggests at least minimum survival beyond the occurrence of the injury [12].

However, the use of antemortem or perimortem definitions has severe limitations since it gives no estimation of the length of the time period between the moment the insult occurred and the time of death (the post-traumatic interval) while a more precise definition about the timing of the trauma would be desirable in the forensic scenario, since the estimation of the amount of time that has lapsed from the traumatic event can have substantial implications in legal proceedings, especially in cases of violent death, alleged torture, maltreatment or abuse. In fact, certain antemortem trauma patterns can indicate a particular injury history and can help to corroborate witness testimonies as well as to reconstruct the chain of events leading to eventual death or to establish a sequence when multiple traumata in a single individual are encountered.

4. STATE OF THE ART IN FRACTURE HEALING AND DATING IN FORENSIC ANTHROPOLOGY

The accurate dating of bone fractures, which consist on the post-traumatic survival time estimation after bone trauma, is based on the tissue healing response after traumatic tissue damage [47].

Histological and biomolecular preclinical animal studies (especially mouse fracture models), focusing on histological tissue changes, cellular and molecular pathways and strength testing [16, 29, 33-35, 48-51] allowed the understanding of the general developmental progression of fracture healing (starting from the early signs of bone healing). Moreover, many radiographic studies on the topic of fracture healing have been performed for clinical purposes, mainly for the follow-up of the mechanical stability of fracture repair and mineralization, in order to obtain an objective evaluation of the course of a successful healing.

On the contrary, from a forensic anthropological point of view, little is known to date not only about the precise timing of fracture healing stages in the livings (based on radiographic appearance) and in the dead (based on dry bones) but also about the variables that influence fracture healing, so that the task of ageing bone fractures still remains a tricky issue in the forensic scenario, both in the living and dead.

The present PhD project, inserted in the context of the trauma research in forensic anthropology, is instead dedicated properly to the study of fracture healing and dating, with the aim of acquiring a better knowledge of the process of bone healing from a forensic point of view, given its known and already listed legal implications.

About that, preliminarily to the project development, a literature search was firstly conducted in the electronic databases Pubmed and Web of Science (subsequently updated to October 2020) using a combination of free text protocols, such as "*fracture dating*", "*fracture healing*", "*fracture timing*", "*bone callus*", "*fracture repair*" and "*forensic*" in order to define precisely the state of the art in fracture healing and dating in forensic anthropology, with special reference to the imaging techniques applied for the study of bone calluses, both in living and dead. Further studies were also identified by reviewing the reference lists of the papers that were found in this search and at the same time filters such as full-text and English language were activated.

Moreover they were selected only the studies of fracture healing and dating on both animal and human bone calluses presenting forensic anthropological implications while pre-clinical or clinical studies targeted at the evaluation of bone quality and quantity from a clinical point of view were excluded (i.e. articles concerning the mechanical stimuli needed for a proper fracture healing, the correlation between a specific surgical treatment or pharmacological/endogenous stimulus and fracture healing and the variations in the healing fracture process in case of a specific organic or bone pathology) as well as articles targeted at the macroscopical differentiation between perimortem and post-mortem bone trauma, articles exclusively related to post-mortem bone changes or to mechanisms of skeletal injuries and articles related to the fracture healing process implications in civil tort law.

After the electronic database research process and the review of the reference lists of the papers, more than 180 articles were identified as potentially relevant, among which only 46 papers fulfilled the aforementioned criteria and were analyzed in full-text.

Such selection revealed that, despite the huge need to accurately determine the timing of fracture healing stages in order to ascertain the age when the injuries were sustained with respect to the time of examination in the forensic scenario, the number of articles dealing with fracture healing and dating on human bone calluses from a forensic anthropological point of view is extremely limited (i.e. 46 articles) compared to the total (>180), especially considering that a screening had already been done upstream by including the keyword "*forensic*" in the article search, without which the articles would be more than 2000.

Among the 46 selected articles of forensic interest dealing with the study of fracture healing and dating, 20 are about the living (7 studies in adults, 12 in children and 1 in animals) and 26 about the dead, of which 4 about general considerations, 12 about adults, 8 about children and 2 about animals.

Therefore, in light of the contents of the selected articles, the state of the art in fracture healing and dating in forensic anthropology has been outlined, with special reference to the imaging techniques applied for the study of bone calluses, both in living and dead.

4.1 State of the art of the imaging techniques applied for the forensic anthropological study of the fracture healing and dating in the living

Since fractures cause substantial economic burden to society, both in health-care costs and loss in productivity, most of the existing research about fracture healing (both on animal models and human bones) has been driven with clinical aims, especially by orthopaedics and radiologists, who are interested in the final stages of fracture healing as well as in the investigation about interventions to hasten fracture repair [35, 52-53], as demonstrated by the high number of articles excluded during the literature review.

The clinical assessment of fracture healing and the determination of when a fracture is healed is in fact a mainstay of orthopedic practice since it affects patient management decisions.

In particular, such studies concerned the mechanical properties of bone and the process of bone healing with the specific purpose of identifying the factors which influence bone competence (i.e. bone volume and bone quality) and the best criteria for monitoring the healing response, estimating callus mechanical competence (i.e. strength and stiffness) as well as the best approaches for augmenting fracture repair, both with biophysical or pharmaceutical strategies [54] and for evaluating the weight-bearing capacity of the healed bone [55].

Imaging remains essential for monitoring healing and due to its low cost, wide availability and relatively low radiation profile, radiographic imaging [43, 56] is routinely used for the examination of fractures in clinical medicine so that these radiographs provide a basis for clinical assessment of fractures by displaying the fractured region, callus formation, and bone remodelling.

Among the modalities for fracture healing clinical assessment are also included computed tomography (CT) scanning, dual energy X-ray absorptiometry (DXA), magnetic resonance imaging (MRI), ultrasound imaging (UI) and radiostereometric analysis (RSA), although they are not routinely used [57].

However, to date, despite the technological innovation and the availability of the aforementioned imaging techniques, the precise clinical timing of fracture healing stages in the living continue to remain challenging due to a lack of consensus on imaging and on clinical criteria [35, 43, 47] and, given the lack of a true gold standard to estimate the duration of the fracture healing, the process continues to be based on experience rather than on actual evidence, according to Prosser et al. [18].

The reliability and accuracy of post-traumatic survival time estimation on the basis of imaging continue to be an "inexact science", since the clinical standards one can make use are not always in complete agreement (especially on the intermediate stages of fracture healing), and they don't give further information on what happens beyond the last stage (what every clinician defines as "complete recovery").

Therefore it emerges the presence of a gap in knowledge regarding clinical fracture healing assessment and associated interpretation of time since fracture, which is even more evident in the forensic anthropological setting, as demonstrated by the limited number of studies reported in literature, where almost two thirds of which were performed during the last decades in living children with the aim of trying to establish time frames for fracture healing with forensic purposes [13, 18-19, 33, 56, 58-664] basing on the appearance of radiological features of fracture healing, since radiographic skeletal survey is the primary and commonly technique used for assessing skeletal trauma [56].

Baron et al. also performed a pilot study focused on fracture healing in paediatric population pointing out the forensic relevance of an objective and radiation free approach, consisting of quantitative MRI application, for fracture dating in cases of child abuse and maltreatment but they used living immature rats instead of living children [65].

However, as demonstrated by Drury in 2017 [56] and by Messer et al. in 2020 [66] through the review of the radiographic timelines available for pediatric healing feature, the proposed fracture healing timelines differ significantly among

studies, so that to date their application not only to clinical but also to forensic paediatric cases is challenging and imprecise and they can't even be applied in the adult population since the speed of the healing response generally differs substantially between children of different ages and between children and adults [47, 67] because of the longer period required in the adults for reawakening the osteogenic environment necessary for the induction of all the healing phases [16].
Furthermore, to date, similar studies on dating fractures in adult population are still very rare (3 studies), confirming what was already observed by Malone et al. in 2011 [13] who pointed out how radiographic assessment of adult fracture healing was relatively absent in the forensic literature, highlighting only the study conducted by Hufnagl in 2005 [42] which however consisted of a master's thesis which is not published in any scientific journal.
Subsequently only two research groups of authors investigated the problem of the forensic assessment of post-traumatic time interval in an adult living population. In particular, Baron K. at al. in 2016 [20] performed a pilot study to investigate the applicability of quantitative MRI approach for objective bone fracture dating in adults by systematically investigating time-resolved changes in quantitative MR parameters after a fracture event, providing promising results as a first step towards objective quantitative estimation of the date of a fracture while Tritella et al. [69] demonstrated good foundation for the use of an healing scale for the forensic assessment of the post-traumatic time interval through digital radiographs in an adult population after testing the reliability of using such scale.
Furthermore, it has been demonstrated both in paediatric and adult population that a multitude of inter- and intra-individual variables also affects how bone injury is diagnosed and interpreted, influencing assessment and estimation of timing of injury and understanding fracture-healing processes and their timing [19, 70].
In conclusion, the literature review highlighted the limitation of existing knowledge regarding estimating timing of bone injury in the living, especially in the adult population where only a handful of studies have been performed in the last decades.
Therefore, it emerges the need for further research on fracture healing, especially among adult population, which should be aimed not only at evaluating potential

technological tools for objective fracture dating in order to overcome the current radiographic methods that rely on the expert’s experience, but also at examining the relationship between the healing process and different inter- and intraindividual variables, cited in the paediatric studies as influencing the healing process itself.

4.2 State of the art of the imaging techniques applied for the forensic anthropological study of the fracture healing and dating in the dead

In forensic anthropological practice the request to date skeletal fractures that arrive from autopsies or from human remains is frequent, with particular reference firstly to the distinction between antemortem and perimortem and then to the amount of time elapsed between the time when the fracture was sustained and the time of death.

However, despite the importance of the forensic assessment of the age of bone calluses, the available literature is mainly constituted of clinical studies as already said, while studies for forensic purposes, particularly in looking for better methods for ageing calluses in view of criminalistic applications, still leave much to be desired.

Among the selected articles from the performed literature review, 26 were about fracture healing and dating in the dead, among which most of the article referred to adult population (i.e. 12 studies in adults versus 8 in children), contrary to the living where most of the forensic studies were referred to the pediatric population.

The available studies on fracture healing and dating in the dead are mostly based on dry and skeletal remains, with only few works based on post-mortem skeletal radiography on corpses compared to histopathologic analysis [71-74] in pediatric forensic cases, where the detection and dating of infant fractures plays an important role in the diagnosis of the battered child syndrome [67, 75].

Among the studies based on skeletal remains, Barbian et al. [51] and Maat at al. [76] reported that a minimal period of several days is required for the gross detection of initial osseous evidence of healing response. In fact, on dry bones, unlike in fresh skin, a fracture can only be described as certainly “antemortem” if signs of the healing process such as periosteal new bone deposition and callus formation can be detected, whose macroscopic and radiographic detection require a long time, which is not before 10 to 14 days [12].

Alternatively, when the bone still possesses its elastic properties at the time of the fracture, but no mineralized signs of healing can be observed, the injury can only be classified as "perimortem", meaning that it could have occurred shortly before or after death [8, 44, 77], but this definition leaves open the question of the vitality of the fracture.

The inability to solve the issue of the accurate dating of fractures with traditional anthropological methods has driven forensic researchers over the last decades to test the potential of both histology [17, 67, 75] and new radiological tools [78] for a more precise analysis of the degree of fracture healing, in order to identify vital processes in dry bones, even when macroscopical changes are not visible yet [14, 62, 76].

In particular, Maat et al., after an extensive literature review based only on documented forensic cases and anatomical and pathological text books (since there were no experimental data available from naturally healed fractures in adults), firstly published a detailed healing timetable that linked the elapsed time after bon injury to precisely radiological and histological healing features, testing it both on adults and children [62, 76] while Cattaneo et al. in 2010 [14] firstly experimentally assessed the feasibility of performing histopathological investigations and immunohistochemical staining on human dry bone to verify whether the fracture is vital or not, and, if vital, its time of production (and therefore survival). Takamiya et al. also experimentally demonstrated on animal models that mRNA expression of IL10 would be informative for fracture healing and dating together with macroscopic and histological changes [79].

Subsequently, De Boer et al. compared Maat's timetable and old works based on gross anatomical and radiological observations, focusing on the evaluation of what features can still be detectable and adequate in the assessment of traumatic lesions in dry bone material of unknown origin by using histology and radiology [45] and they demonstrated the complementary use of radiological and histological techniques to differentiate the various healing phases, allowing a more detailed fracture dating. Nevertheless this study didn't prove the validity or reliability of these healing features on known material, so that Cappella et al. tested the accuracy of the approach proposed by De Boer on dry bone fractures with a known post-traumatic survival time [47].

Anyway, in light of the aforementioned studies, it has been accepted that post-mortem histological and immunohistochemical analyses, even on dry bones, are not only essential to ascertain the post-traumatic interval [16], since the gross examination of the bone outer surface does not give a complete picture of the biology of the tissue's reaction [17, 80-81], but also superior to both conventional radiology (whose blurriness does not allow to evaluate the precise evolution of the callus during the healing phases) and CT scanning [47].

Unfortunately, although histological examination allows a precise dating of the early stages of fracture healing, it is a destructive and often labor-intensive process, yielding data that lack a 3D content [54, 75].

Therefore, researchers over the years have also proceeded to study *new high-resolution imaging and non-destructive methods* for a more *accurate and objective analysis of the degree of fracture healing*.

Cappella et al. (2013) demonstrated how cone-beam CT technology was able to visualize different levels of bone callus mineralization, thus representing a helpful and reliable tool to assess the stage of fracture healing [78].

Micro-computed tomography (micro-CT) is another sensitive and high-resolution radiological tool that provides detailed information regarding 3D bone mass distribution and microarchitecture, precise measurements of cortical and trabecular indexes, but also the hydroxyapatite content that relates to bone mineralization status [82]. It is widely used for clinical purposes in animal models [52, 83-91], but only applied to forensic purposes in two studies [46, 92].

These papers, both based on human cranial fractures, pointed out how micro-CT analysis, already useful in forensic investigation [93], can provide accurate information about fracture healing, especially for early healing signs. However, despite the tremendous potential of micro-CT analysis in the field of forensic anthropology, and in particular to fracture dating, no research aimed at studying and dating post-cranial human bone calluses with micro-CT has ever been performed.

Synchrotron radiation micro-CT (SRμCT) has been also applied to understand the bone structural biology [94-95], especially beyond vascular canals [96], but to date no studies on bone calluses for forensic anthropological purposes have been performed.

Finally, Yu et al. very recently published a study, based on animal model, pointing out again how traditional imaging modalities (including conventional radiology and computed tomography) are subjective, time-consuming and have low accuracy, so that they proposed FTIR spectroscopy as a rapid and objective method for analyzing forensic cases in order to distinguish antemortem from peri- and postmortem fractures but similar studies were not performed on human bones [97].

In conclusion, the application of non-destructive 3D imaging technologies to bone healing research is considerable and offers tremendous future potential also in the field of forensic anthropology, with special reference to fracture dating, given the importance of its legal implications.

However, to date no studies aimed at studying and dating post-cranial human bone calluses both with micro-CT and synchrotron radiation micro-CT (SRμCT) have been performed.

Therefore, it emerges the need to evaluate the applicability of such non-destructive 3D imaging technologies for objective dating of the healing phase of the fracture on dry bones since this topic still needs to be improved by developing new approaches and adding know-how in the interpretation.

5. THESIS CHART

BONE TRAUMA ANALYSIS

Participation to ***two research lines***

First research line based on the ***living***

Retrospective time-series study of clinical digital radiologic images of adult patients who sustained a bone fracture and for which information regarding trauma, time elapsed since fracture and following follow-ups were available.

Second research line based on the ***dead***

Experimental study of the inner structure's characteristics of bone calluses collected during autopsies, for which information regarding trauma and time elapsed since trauma in relation to death were available.

5.1 RESEARCH AND AIMS

The review of the forensic literature that was preliminary performed in order to define the state of the art in fracture dating in forensic anthropology revealed a gap in knowledge regarding this topic, with little research being conducted on the healing rates and the associated interpretation of time since fracture for forensic purposes, both in the living and in the dead.

In particular, about the living, the limitation of existing knowledge regarding estimating timing of bone injury was especially highlighted in the adult population, where only a handful of studies were performed in the last decades and the correlations between the healing process and different inter- and intraindividual variables were not yet thoroughly analyzed.

Instead, about the dead, it was highlighted the need for further research to investigate the application of more sensitive and non-destructive radiological tools to perform more accurate analysis on the degree of fracture healing, which is a prerequisite for aging trauma.

Therefore, in light of the aforementioned premises, the entire PhD project, inserted in the context of the trauma research in forensic anthropology, will treat one of the major and complex issues related to trauma analysis that is the *timing of fracture healing*, with special reference to the problem of dating fractures both in the living and the dead through two different but closely interconnetted lines of investigation performed until the end of the PhD in collaboration with the LABANOF Staff, in order to make a contribution to fill the aforementioned gaps.

Specifically, the project is based on *two research lines*, of which:

1. ***one related to the living*** and based on the appearance of specific radiological features during the fracture healing process in a large adult casuistry, after the ethics committee approval, for which information regarding trauma, time elapsed since fracture and following follow-ups were available;
2. ***one related to the dead*** and based on the study of the outer and inner structure's characteristics of the calluses present on soft-tissue free human

bones collected during autopsies, for which information regarding trauma and time elapsed since trauma in relation to death were available.

In this perspective the project was consequently divided in two main sections relative to the two research lines, whose results could help to obtain empirical data useful for fracture dating, which is fundamental in the forensic context for a comprehensive assessment and interpretation of domestic violence and torture in the living, as well as for trauma analysis on skeletal remains in real forensic cases of medico-legal interest.

The purposes of each of the two main research lines is listed below.

1. The *first research line*, dedicated precisely to the *living*, aimed at obtaining data, in terms of time-course, functional for the comprehensive and correct radiological assessment of fractures to be applied potentially in investigations on living who suffered of domestic violence, abuses and tortures and it was possible thanks to the Radiology Unit of the IRCCS Policlinico San Donato in Milan that provided the casuistry (the largest never used before for similar studies, neither in paediatric population) and to the forensic anthropologists from the LABANOF in Milan who participated in the assessment and evaluation of radiological images.

   The main purpose of this research line was to *describe exhaustively the time-course of the radiological appearance of bone fractures at the diverse healing stages in a retrospective substantial clinical adult sample* and to *investigate the effect of variables, including age, sex, bone type and number of fractures in influencing the timing of healing stages of traumatic skeletal lesions*. The observation and description of such data can contribute to build foundations for estimating the post-traumatic time interval of bone fractures more accurately also in adults, allowing forensic experts to interpret correctly traumas for violent events in adults too.
2. The *second research line*, dedicated to the *dead*, aimed at investigating the problem of the interpretation of antemortem injuries and was focused on the evaluation of calluses present on soft-tissue free post-cranial human bones, which are frequently present in the skeletal population since fracture is one of the most common traumas which occurs during life. This research was

possible thanks to the LABANOF staff in Milan who provided the calluses collection and participated in the assessment and evaluation of radiological images and to the team of Elettra Sincrotrone Trieste[3] who provided the bone calluses analysis both with microCT and synchrotron radiation micro-CT.

In particular, the main purpose of this research line was to *preliminary assess the potential of an advanced and non-destructive imaging technology, like micro-computed tomography*, for the analysis of fracture healing and dating, in order to obtain a future objective dating of the healing phase of fractures on dry bones. A preliminary feasibility experiment aimed at assessing the potential of synchrotron radiation micro-computed tomography (SRμCT) for the analysis of fracture healing and dating was also perfomed but only to evaluate if it could provide additional information with respect to micro-computed tomography.

Therefore, in the following paragraphs, after a brief introduction, it will be described the population selected for both research lines, the material and methods and their limits, and the results of each research. Subsequently a general discussion will be performed, in order to analyze how this PhD project has contributed to fill the existent gap of knowledge relating to the healing rates and the associated interpretation of time since fracture for forensic purposes, both in living and dead.

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[3] Elettra Sincrotrone Trieste is a multidisciplinary international research Center of Excellence, specialized in generating high quality synchrotron and free-electron laser light and applying it in materials and life sciences and the collaboration with this Research Center was subordinate to the submission and the acceptance of a proposal (**Proposal n. 20195365**) that was evaluated on the basis of pure scientific merit and potential impact.

6. FIRST RESEARCH LINE: THE PROBLEM OF FRACTURE DATING BASED ON THE RADIOLOGICAL ASSESSMENT OF FRACTURE REPAIR IN AN ADULT LIVING POPULATION

This part of the project is based on two different studies, one of which is going to be submitted to AJR while the second one, subsequently reported as ***Attachment 1****, has already been published, cit: Tritella, S., Obertová, Z., Sconfienza, L.M., Collini, F., Cristini, E., Amadasi, A., Ciprandi, B., Spairani, R., Albano, D., Viero, A., Cappella, A., Cammilli, P., Sardanelli, F. and Cattaneo, C. (2020), Multi-Rater Agreement Using the Adapted Fracture Healing Scale (AFHS) for the Assessment of Tubular Bones on Conventional Radiographs: Preliminary Study*. J Forensic Sci, 65: 2112-2116.*

6.1 Introduction

The estimation of post-traumatic survival time after bone trauma is based on the tissue healing response after traumatic tissue damage [47] and the examination of skeletal injuries can be therefore very informative with regard to the timing of injury in both living and dead victims.

Living victims, in cases of suspected abuse and domestic violence as well as in case of torture, often present multiple injuries in different stages of healing sustained through repetitive and prolonged abuse [26-27], which frequently heal without timely medical attention. Although the severity and types of violence may differ, the most the common injuries in these contexts result from punches, kicks and beatings [26, 98-99], so that the most common type of trauma to the head, face, thorax and the extremities is blunt force trauma, sometimes associated with bone fractures which can bear witness to the incident even years after it occurred [26-28, 100].

However, despite the assessment of fracture healing is a common task for both orthopedic surgeons and radiologists, it remains challenging due to a lack of consensus on imaging and clinical criteria as well as the lack of a true gold standard, especially in the adult living population and this constitute a huge problem in the forensic scenario where the assessment of the estimation of the posttraumatic time interval may provide essential information for the corroboration or contradiction of the victims' statements about maltreatment, abuse or torture, which are delicate and extremely current international problems given the increasing of domestic violence worldwide [21] as well as the increasing influx of asylum seekers to Europe who claim to have been victims of torture and request for assessments for signs of torture [22-25]. Moreover, in order to correctly estimate time frames of healing and to provide a reliable timetable to be used in case of real forensic scenarios, it is also necessary to understand the relationship between the healing process and different inter- and intraindividual variables, which has not yet been thoroughly statistically analyzed in the scientific literature, where it is only stated that a multitude of variables affects how bone injury is diagnosed and interpreted, influencing assessment and estimation of timing of injury and understanding fracture-healing processes and their timing [19].

Thus, the aim of the present multidisciplinary study is to describe exhaustively the time-course of the radiological appearance of bone fractures at the diverse healing stages, not only providing minimal/maximum and peak time frames for each single radiological aspect -related to the adapted fracture healing scale (AFHS)- but also glancing at the effect of variables such as age, sex, skeletal region and number of fractures in influencing the timing of healing stages.

6.2 Material and Methods

Study design and population

This retrospective study included a cohort of adult patients that performed a radiological evaluation for long/tubular bone fracture, between January 1th, 2011 and June, 30th, 2015. Exams were all collected at the Emergency Department (ED) of IRCCS Policlinico San Donato (Milan, Italy).

The entire study was conducted in accordance with the Declaration of Helsinky Ethical Principle and Good Clinical Practices and was approved by the Ethic Committee of the University of Milan (Opinion n. 22/16, approved the 18th of July 2016) that waived the need for informed consent.

All cases were selected and anonymized from the internal picture archiving and communication system (PACS). Inclusion criteria were the presence of at least one fracture in a long/tubular bone (i.e. clavicle, humerus, ulna, radius, metacarpals, hand phalanges, femur, fibula, tibia, metatarsals, foot phalanges), the clear visibility of the fracture line on plain x-rays, accidental fracture history, the known post-traumatic time from injury (which was retrieved from ED medical admission records) and the age > 20 years. Moreover, only patients with at least one radiographic follow-up were included in the study.

Exclusion criteria were the presence of severe radiographic osteopenia, because of its potential impact on bone healing patterns, as well as the presence of pathological fractures.

For each fracture event the patients' age and sex, together with the time interval between injury, the appearance of radiographically detectable healing signs, the duration of these healing features, the anatomical site, the number of fractures (classified as single or multiple) and the timing of radiological follow-up were recorded.

Radiological evaluation

Images evaluation was performed to assess the fracture healing signs, in order to provide a healing score attribution. To this purpose, a specifically adapted fracture healing scale (AFHS) consisting of 7 stages according to Tritella et al. [69] was used, as reported in **Table 1**.

AFHS STAGE	Description
0 - No healing	No healing features; sharp contours of the fracture margin.
1 - Absorption	Absorption of the cortical bone adjacent to the lesion; blunted appearance of the fracture margin.
2 - Periosteal reaction	Start of periosteal reaction= linear elevation and calcification of periosteum in the vicinity or adjacent to the fracture site.
3 - Sclerosis	Increased bone density at the fracture margin.
4 - Callus	Appearance of callus: from the fluffy appearance of early new bone formation to well demarcated callus, which may have margins as dense as the cortical bone.
5 - Bridging	Fracture gap bridged by cortical bone to any extent.
6 - Remodeling	Bone returning to its original shape; from firm bony union, smoothening of contours to complete bone remodeling = bone at fracture site returned to its original shape.

Table 1. The 7 healing stages of the adapted fracture healing scale (AFHS) used in the present study, with detailed description.

This adapted scale was created starting from fourteen bone healing features described in previous studies by Malone [13], Prosser [18] and DeBoer [45], after excluding those healing features considered as rare, difficult to see on plain films, or those patterns of difficult interpretative definition (like "clearly visible periosteal callus", "aggregation of spiculae into woven bone from periphery to center of fracture cleft", "osteoporosis of the cortex", "endosteal callus indistinguishable from the cancellous bone in the marrow cavity", and "pseudoarthrosis").

Image evaluation was performed by two experienced reviewers, a 15 years-expert radiologist and a 10 years-expert anthropologist, both with specific expertise on bone fracture healing dating.

The choice to conduct the assessment by agreement of the two selected reviewers was made in accordance with results provided by a previous publication of the same authors [69] which already addressed an excellent interobserver agreement and concordance between the radiologist and anthropologist included in this study when assessing fractures healing process in several radiographic images.

Both operators were asked to score the healing stage in agreement, by choosing only one of the 7 healing stages as the most representative of the radiographic pattern. Both operators were blinded to the identity of the patient and to the post traumatic time.

Healing feature presence

In order to analyze the time at which the healing features were detected, their combination, their peaks prevalence and their earliest and latest recording, radiological images were arbitrary grouped into 7 different classes, based on the known post-traumatic age of fractures: 1-4 days, 5-10 days, 11-30 days, 31-45 days, 46-80 days, 80-180 days and >180 days. Such classes were created according to the availability of data (with at least 60 radiological images in each time frame) and with the intent to reflect the age estimation process that is commonly done in daily practice: the time frames were narrowed only for the first two weeks, with increasing width thereafter.

Statistical Analysis

The AFHS score assigned to each radiograph was entered into a Microsoft Excel large worksheet and a database including the patient age and sex, the location (i.e. injured bone) and number of fractures (i.e. single or multiple), the time interval between injury and first radiologic examination and the timing of the follow-up in days was created for statistical analysis.

Time since fracture was then modeled as a linear function of covariates. No model selection was performed, and all clinical variables considered as relevant were included. Age effect was included in the model via a restricted cubic spline transformation. A two-way interaction among AFHS, ClusterBones and Gender was also included. Model performance was assessed via R^2 and Slope Index, both adjusted for optimism via bootstrap (10000 runs). Model calibration was evaluated also via bootstrap (10000 runs). Model was presented via a nomogram and a R-shiny app is available at the supplementary material section: https://r-ubesp.dctv.unipd.it/. Analyses were performed using the R System [101] and the rms [102] and DynNom [103] libraries.

Critical considerations about material and methods

This study aims at describing the radiological signs of bone healing that appear after a fracture at diverse time frames on digital images. Although it builds on prior works focusing on the challenging topic of dating long bone fractures, it is unique in some perspectives, including the kind of the selected population. In fact, it represents the first retrospective investigation exclusively dedicated to adults, since the study written by Hufnagl, which is to date the only one based on fracture healing in adults, includes also children and adolescents [42].

Furthermore, among the several radiographic studies focusing on the estimation of the post traumatic time which attempted providing timelines for the fracture healing (as just said mostly related to paediatric populations), this study represents also the largest investigation of this kind in terms of sample typology and numerosity, since to date no other studies have included a fracture sample size and a number of radiographs greater than those analyzed in the present study.

This is confirmed also by a recent review by Messer et al. in 2019 [66] which reported smaller samples (maximum 141 patients, 205 fractures and 707 radiographs-Islam 2000) among the 10 studies concerning radiographic features of pediatric fracture healing (and corresponding timelines) selected and analyzed from the current literature.

On the other hand it needs to be pointed out that in the present study only some of the multiple variables hypothesized to influence the healing time have been analyzed, so that further studies should be performed to evaluate the influence of the multitude of other variables not yet analyzed, including co-morbidities, type of medical treatment (e.g., immobilization), type or severity of fracture, in order to refine the nomogram and make it further suitable in clinical as well as in forensic practice.

Another huge limit of the collected data in this retrospective study consists in the lack of serial radiological follow ups at defined time intervals after fractures and this is the reason why, generally, timelines of fracture healing are so difficult to investigate. In fact, since the obtained radiological images for each fracture are dependent on clinically requirements, their number in terms of follow-up images (varying from two to five for each individuals) and their follow-up time interval (PPT, post traumatic time) result irregular among all the selected fracture events, so that the data represent an unbalanced panel dataset.

Furthermore, critical consideration could be expressed about the lack of precise blood chemistry data about the osteometabolic conditions of the patients included in the study and about the inclusion of fracture treated with means of synthesis in the dataset, given the potential impact of the aforementioned factors on the timing of healing.

Nevertheless, in order to try to minimize the impact of the aforementioned factors on the results of the study, the severe radiographic osteopenia and the pathological fractures were defined among the exclusion criteria for the casuistry selection and also only fractures treated with means of synthesis which determine some motion at the fracture site (i.e. intramedullary nailing, external fixation, internal fixation of comminuted fractures) were included, so that the sample could be considered homogeneous, since all the fractures healed thanks to secondary bone formation, as it happen in case of non operative fracture treatment.

6.3 Results

Starting from an amount of 1016 follow-up digital images deriving from the examinations of 355 fractures found on 244 patients, after the inclusion and exclusion criteria application, a final selection of 661 follow-up digital images deriving from the examinations of 230 fractures found on 167 patients (89 females and 78 males, respectively corresponding to the 53,29% and 46,71% of the total patients) was obtained.

The age of the population individuals ranged between 20 to 97 years old, with an average age of 56 years old (SD ± 20 years old) at the time of the first radiological examination.

Considering age groups, a total of 51 fractures (22.17%) occurred in 34 adults with an age ranging between 20 and 39 years old, 55 fractures (23.91) occurred in 40 adults with ages ranging from 40 to 59 years old and 124 fractures (53.91%) occurred in 93 adults aged > 60 years old. Overall, 220/230 fractures were complete (95,6%), while 10/230 fractures were incomplete (4,4%) (**Table 2**).

Days intervals after injury	Number of image series with a fracture		
	Complete fractures	Incomplete fractures	Total fractures
1-4	103	5	108
5-10	123	4	127
11-30	63	4	67
31-45	121	2	123
46-80	59	5	64
81-180	67	0	67
>180	104	1	105

Table 2. Number of fractures evaluated at each time point.

The sites of the fractures and ages of the patients involved in the study are depicted in **Figure 2**.

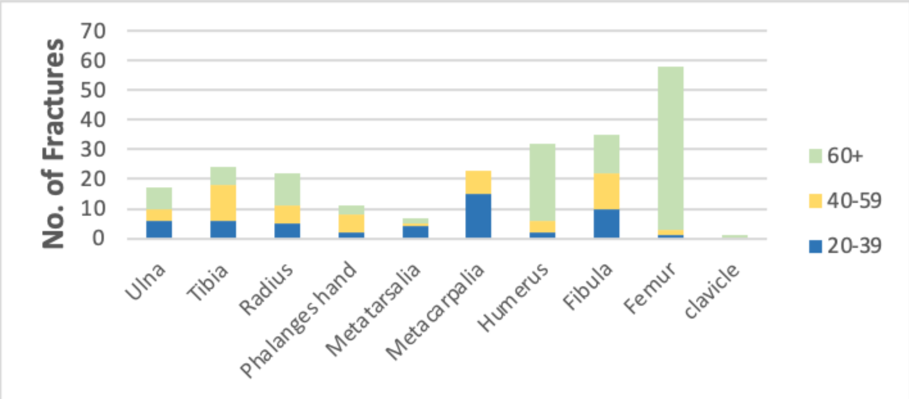


Figure 2. Bar-graph shows the sites of the fractures and ages of the 167 patients in years.

The time since fracture (i.e. post traumatic time) ranged from 1 to 1325 days after trauma, with an average post traumatic time of 88 days (SD ± 157.89).

Based on the known post-traumatic time of fractures (i.e. age fracture), the total number of available radiographs at each post-traumatic time interval class (i.e. 1-4 days, 5-10 days, 11-30 days, 31-45 days, 46-80 days, 80-180 days and <180 day) was calculated. A number of 108 fractures were found in the time interval between 1-4 days, n= 127 in the time interval 5-10 days, n= 123 in the time interval from 31 to 45 days and n= 105 over 180 days, while the radiographs halved in the remaining intervals (n= 67 in the time interval from 11 to 30 days and from 81 to 180, n= 64 in the time interval from 46 to 80 days). Fractures of the femur and fibula were the most represented (19.81% each), followed by metacarpal bones (12.85%)**.**

The 7 bar-graphs reported in **Figure 3** and **4** show the presence of each of the 7 stages of AFHS healing scale according to post-traumatic time interval class.

In particular, the "***No healing-STAGE 0***" is a feature that was seen in images of both upper and lower limb bones at all times, with the except for post traumatic times of over 80 days, with a percentage of presence on radiographic images greater than 70% between 1 and 10 days, followed by a progressive decline at onward times. In fact, the prevalence of STAGE 0 reduced to less than 26% at 11 to 45 days, further declining after 46 days (less than 3% from day 46 to 80). The "***Absorption-STAGE 1***" was first seen at 5 days after trauma (9.52% of radiographic images at times ranging both from 5 to 10 days) in images of both upper and lower limb bones, but this feature completely disappeared in images of upper limb bones after 80 days while it persisted over 180 days since injury (up to 28.57%) on images of lower limb bones. The feature **"*Periosteal reaction-STAGE 2*"** was never seen before 5 days both in upper and lower limb bones images and the preponderant presence of this stage was described at times ranging from 31 to 45 days after trauma in both groups. However, this stage was represented in higher percentages in lower limb fractures (55%), in which however it completely disappeared after 81 days; on the contrary, its complete disappearance in upper limb bones was subsequent to 180 days.

Contrary to the previous features, the "***Sclerosis-STAGE 3***" was never seen before 11 days and 31 days respectively in upper limb and lower limb bones images. In both upper and lower limb bones images its presence progressively increased up at times ranging from 31 to 45 days after trauma (with higher percentages among upper limb bones) and persisted also after 180 days. The "***Callus-STAGE 4***" is a feature that, similarly to stage 3, was observed always after 11 days in upper limb bones images and 31 days in lower limb bones images. However this feature presents a different trend in the two groups: the peak presence on radiographic images of upper limb fracture is earlier (around 31-45 days) lower limb fractures (81-180 days), though it persists after 180 days in both groups. The "***Bridging-STAGE 5***" has a trend similar to what has been observed for the previous stage (callus-stage 4), as it was observed at first at 11 days with a quite early peak presence (46-80 days) on upper limb fractures, while on lower limb fractures it was seen at first at 31 days with a peak presence at 81-180 days. Finally, this stage was noted to persist 180 days after the fractur event in both groups.

Lastly, the feature of "**Remodeling-*STAGE 6***", contrary to all the previous stages, was not observed before 31 days in images of fractures of the upper limb bones and before 11 days in lower limb bones images and presented low prevalence before 180 days, followed by a rapidly increase (peak) after 180 days on images of both upper and lower limb bones.

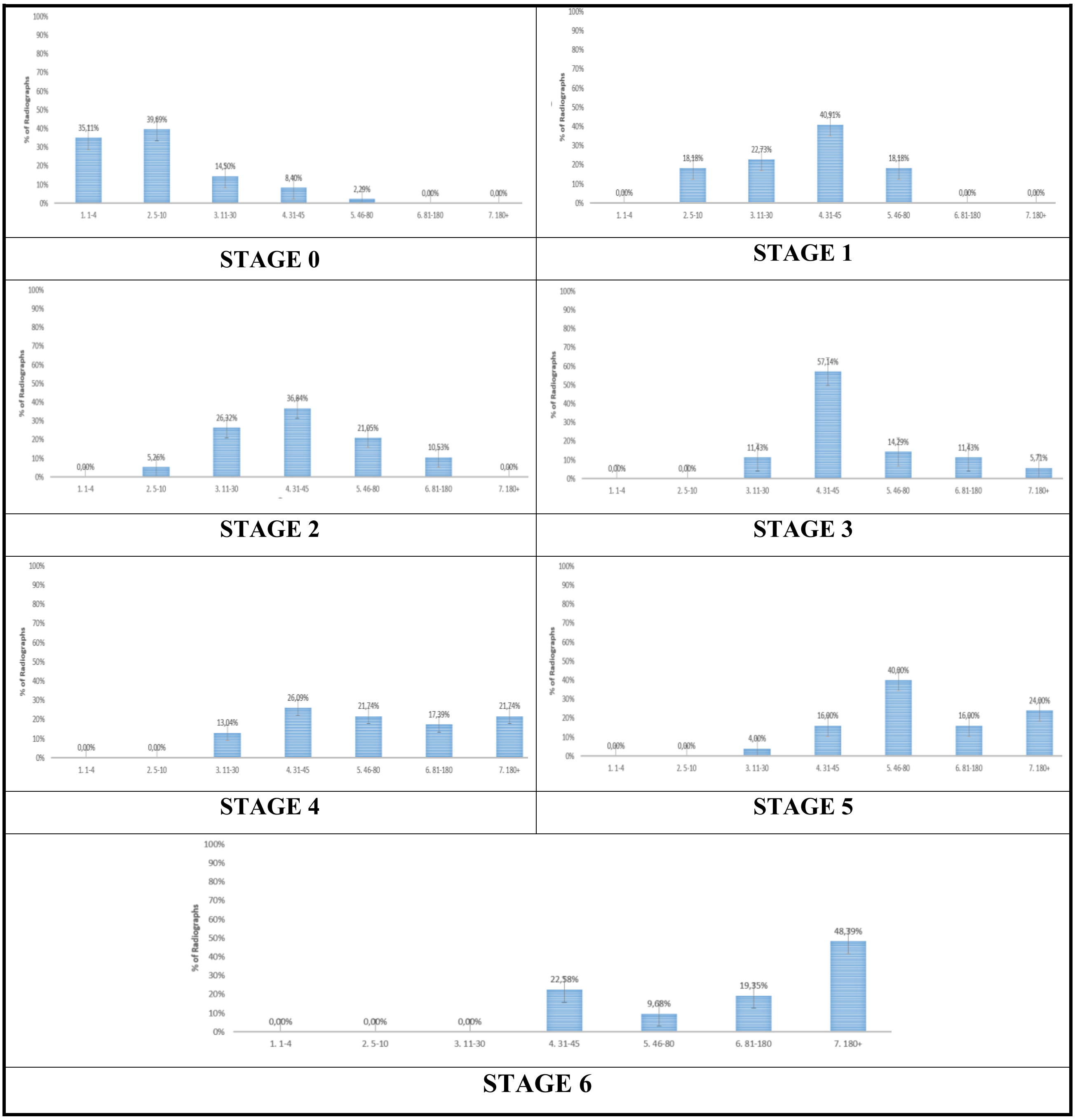


Figure 3. Bar-graphs show the presence of each of the 7 stages of AFHS healing scale at each post-traumatic time interval class (n. of days since fracture) in ***long bone of the upper limb***. Data are split by time bands with 95% Cls (vertical lines). Regarding our casuistry relative to the long bone of the **upper limb**.

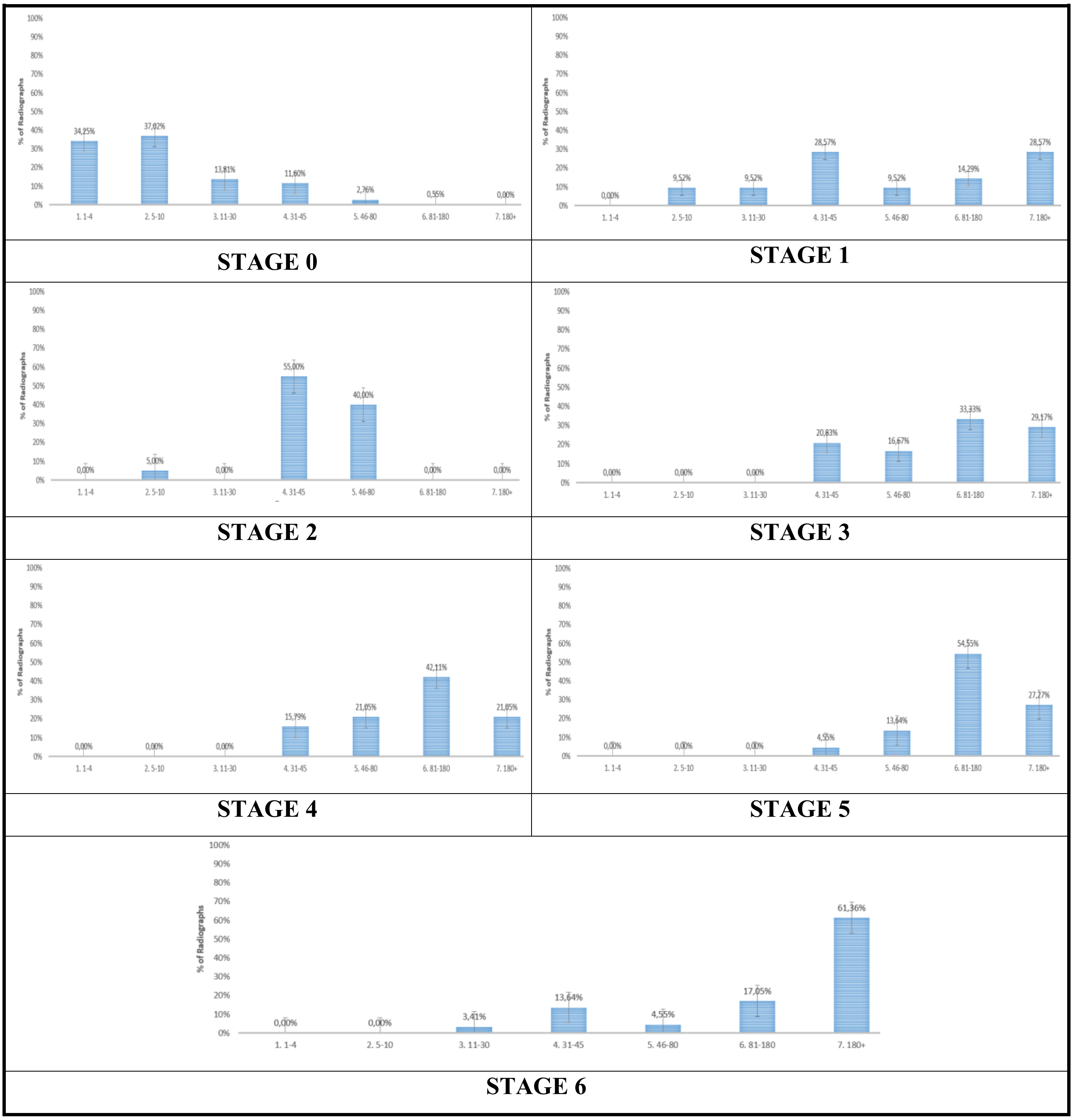
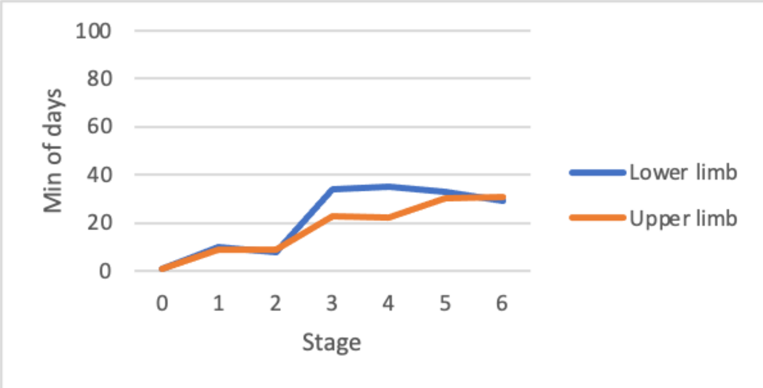


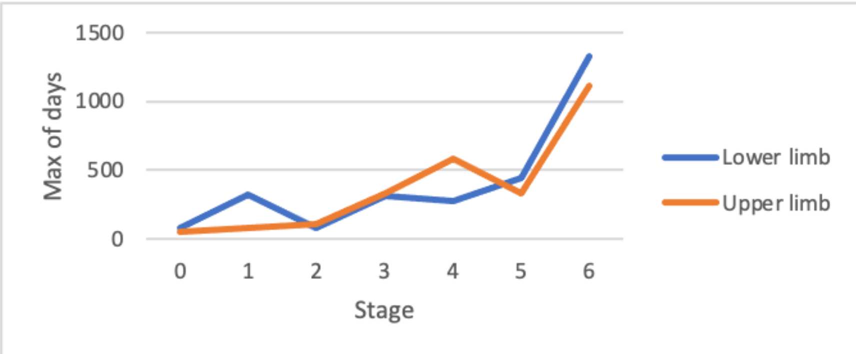
Figure 4. Bar-graphs show the presence of each of the 7 stages of AFHS healing scale at each post-traumatic time interval class (n. of days since fracture) in ***long bones of the lower limb***. Data are split by time bands with 95% Cls (vertical lines). Regarding our casuistry relative to the long bone of the **lower limb**.

The general minimum and maximum post traumatic time concerning each healing feature was calculated separately for the upper and lower limb bones. In particular, the minimum time trends showed little difference between the bones of the two different districts, with upper limb fractures (orange line) showing some intermediate characteristics (as sclerosis, callus and bridging) at earlier times (**Graph 1-part a**).



Graph 1-part a. Graphical trends of the minimum post traumatic time at which the various healing features were observed in lower limb (blue line) and upper limb (orange line) long bones. 0 = no healing, 1 = absorption, 2 = periosteal reaction, 3 = sclerosis, 4 = callus, 5 = bridging, 6 = remodeling.

On the contrary, the maximum time trends showed that some intermediate characteristics (as absorption, sclerosis and bridging) persisted longer in lower limb fractures, while callus and periosteal reaction lasted longer in upper limb bones (**Graph 1-part b**).



Graph 1-part b. Graphical trends of the maximum post traumatic time at which the various healing features were observed in lower limb (blue line) and upper limb (orange line) long bones. 0 = no healing, 1 = absorption, 2 = periosteal reaction, 3 = sclerosis, 4 = callus, 5 = bridging, 6 = remodeling.

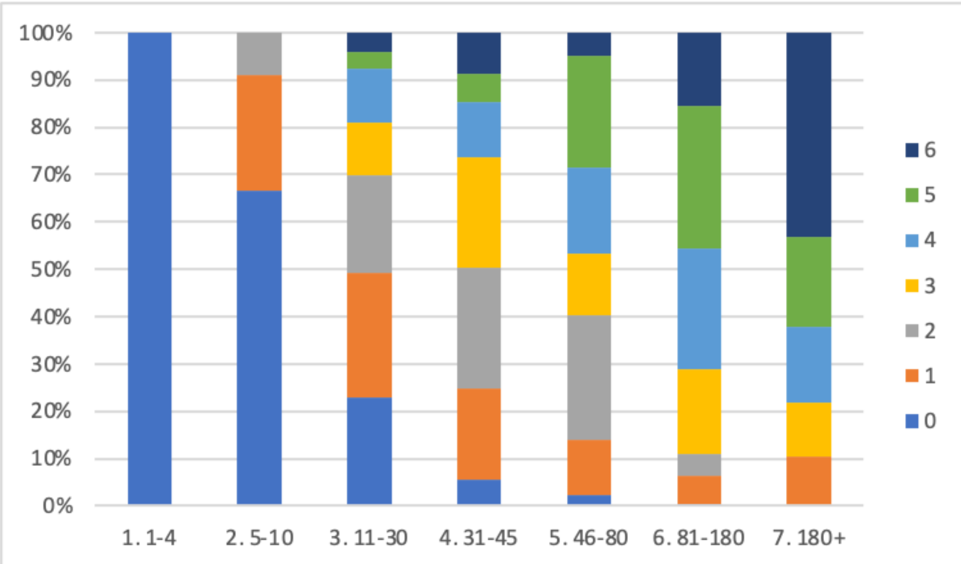
The **Table 3** reports the timescales in which each feature appeared (i.e., the earliest day in which the presence of a feature was described by both reviewers), reached the peak (the period in which the specific healing feature exceeded 35%

of prevalence) and was lastly seen (last day at which the reviewers agreed that the feature was present).

	N. of days since fracture			
Healing Features	First seen	Peak Period	Last seen	**N. of radiographs**
STAGE 0	1	5-10	82	312
STAGE 1	9	31-45	319	43
STAGE 2	8	31-45	105	39
STAGE 3	23	31-45	332	59
STAGE 4	22	46-180	579	42
STAGE 5	30	81-180	445	47
STAGE 6	29	180+	1325	119

Table 3. Days at which each feature of fracture healing was first seen, reached the peak, and was lastly seen on radiological images.

Based on **Table 3**, a histogram was created that represents the various percentage of x-ray exhibiting each healing stage at the diverse posttraumatic time classes (**Graph 2**). The graph highlights that the healing stages from 1 to 6 were simultaneously present at all the posttraumatic time interval classes, although in different percentages, while stage 0 was the only present in first post-traumatic time interval (i.e. 1-4 days after trauma).



Graph 2. Histograms showing the various percentage of images that exhibits each healing stage at the diverse posttraumatic time classes. In general, at intermediate time intervals, almost all features were observed but with different percentages.

Finally, a multivariable model for time-since-fracture was built, as reported in **Table 4**.

Factor	Effect	Lower 0.95	Upper 0.95
Age (74 vs. 39 years)	1.0034	-19.737	21.744
AFHS - 1:0	94.113	27.608	160.62
AFHS - 2:0	36.441	-49.559	122.44
AFHS - 3:0	109.19	50.127	168.25
AFHS - 4:0	91.821	0.4033	183.24
AFHS - 5:0	137.8	70.148	205.44
AFHS - 6:0	355.47	309.3	401.64
ClusterBones (Upper vs. Lower limb)	-2.9861	-41.038	35.065
Gender (Female vs. Male)	1.8423	-34.053	37.738
SingleMultiple (Multiple vs. Single)	40.138	20.191	60.085

Table 4. Multivariable model for time-since-fracture.

The effects were expressed in days and 95% confidence intervals are also presented. AFHS, gender and cluster bones were included via an interaction term and estimates are adjusted to AFHS=0, cluster bones=lower and gender=male. All interaction terms were significant with a p-value <0.001. Goodness of fit of the model R2=0.42 and slope index 0.96.

The model was then represented via a static and dynamic nomogram after calibration, evaluated with 10000 bootstrap runs, as reported respectively in **Figure 5** and **6.**

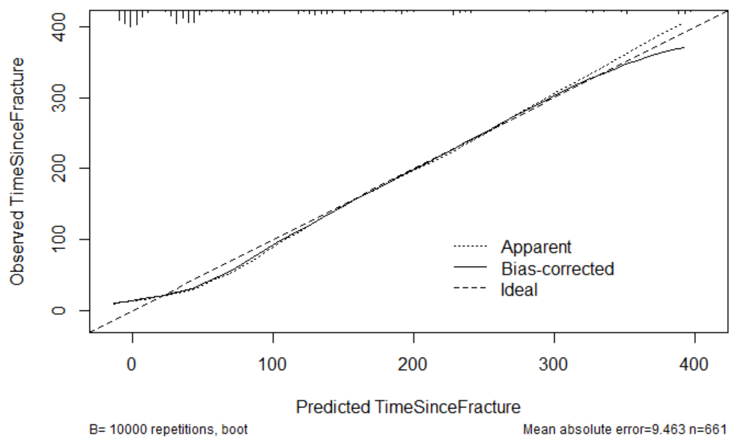


Figure 5. Model calibration, evaluated with 10000 bootstrap runs. For perfect fit, Bias-corrected line (continuous) should overall with plot bisector.

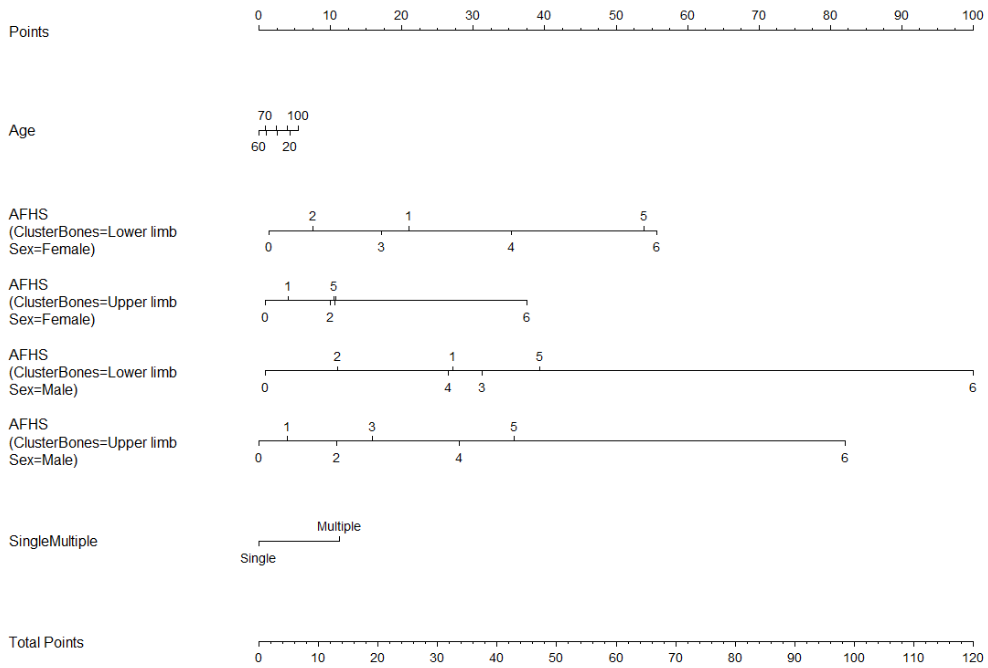


Figure 6. Representation of the model via a static nomogram. Dynamic nomogram is avail at https://r-ubesp.dctv.unipd.it/. Total points are days after fracture. Each patient gets a score according to his/her characteristics: each patient's characteristic gets a point based on the orthogonal projection on the "point" scale. Then, all points are summed up to and evaluated on the "total points" axes.

7. <u>SECOND RESEARCH LINE</u>: THE PROBLEM OF EVALUATING AND DATING DRY BONES CALLUSES

*A paper relative to this part of the project, entitled "**The potential of micro-CT for dating post-cranial bone fractures: a macroscopic, radiographic and microtomography comparison on fractures of known post-traumatic ages**" has been submitted on November 30th to the **International Journal of Legal Medicine** and it is currently under revision.*

7.1 Introduction

In forensic anthropology practice, dating skeletal fractures from autopsies or human skeletal remains is a frequent request. Firstly, the timing of the fracture must be distinguished as "antemortem" (i.e., occurring before death and having time to heal), "perimortem" (i.e., occurring around the time of death, shortly before or after) and "postmortem" (i.e., certainly occurring after death). Secondly, this broad categorization of timing of injury is refined in antemortem (and sometimes perimortem) lesions with the estimation of the amount of time elapsed between the time when the fracture was sustained and the time of death.

However, despite the importance of the forensic assessment of the age of bone calluses, the available literature [43, 35, 52-54, 104] is mainly constituted of clinical studies performed by orthopaedists and radiologists interested in the final stages of fracture healing as well as in the best approaches for improving fracture repair, both with biophysical or pharmaceutical strategies (i.e. mechanical stimuli needed for a proper fracture healing, correlation between a specific surgical treatment or pharmacological/endogenous stimulus and fracture healing, variations in the fracture healing process in case of a specific organic or bone pathology and weight-bearing capacity of the healed bone).

By opposition, studies performed for forensic purposes, particularly looking to improve methods for ageing calluses, are scarce and the traditional anthropological methods are unable to solve the issue of the accurate dating of fractures, especially in the earliest stage of healing when the bone still possesses its elastic properties at the time of the fracture but no mineralized signs of healing can be observed as well as at in the later stage when the outer surface may not give a complete picture of the biology of the inner structure's response [80].

Barbian and Sledzig [51] and Maat [76] reported that a minimal period of several days is required for the gross detection of initial osseous evidence of healing response. In fact, on dry bones, unlike in fresh skin, a fracture can only be described as certainly "antemortem" if signs of the healing process such as periosteal new bone deposition and callus formation can be detected. However, macroscopic and radiographic detection of these features require a long time, and so fractures are classified as "antemortem" after at least 10 to 14 days [12]. Alternatively, when the bone still possesses its elastic properties at the time of the fracture, but no mineralized signs of healing can be observed, the injury can only be classified as "perimortem", meaning that it could have occurred shortly before or after death, but this definition leaves open the question of the vitality of the fracture [8, 44].

The inability to solve the issue of the accurate dating of fractures with traditional anthropological methods has driven forensic researchers over the last decades to test the potential of both histology and new radiological tools [14, 17, 45, 62, 67, 75-76, 79] for a more precise analysis of the degree of fracture healing, in order to identify vital processes in dry bones even when macroscopical changes are not visible yet.

These studies demonstrated that post-mortem histological and immunohistochemical analyses, even on dry bones, are not only essential to ascertain the post-traumatic interval [16], since the gross examination of the bone outer surface does not give a complete picture of the biology of the tissue's reaction [17, 80, 81], but also superior to both plain films (whose blurriness at high magnification does not allow to evaluate the precise evolution of the callus during the healing phases) and CT scanning [16, 47]. Unfortunately, although histological examination possibly allows a more precise dating of the early stages of fracture healing, it is a destructive and often labor-intensive process, yielding data that lack a 3D content [54, 75].

Therefore, research over the years has also proceeded to study new high-resolution imaging and non-destructive methods for a more accurate and objective analysis of the degree of fracture healing, in order to identify vital processes in dry bones even when macroscopical changes are not visible yet [14, 45, 62, 76, 79] as well as to better define the age also of calluses macroscopically and radiologically detectable.

About that, different new radiological tools were applied over the year for the study of fracture healing and compared with conventional radiology.

In particular, Cappella et al. [78] demonstrated how cone-beam CT technology was able to visualize different levels of bone callus mineralization, thus representing a helpful and reliable tool to assess the stage of fracture healing.

Micro-computed tomography (micro-CT) is another sensitive and high-resolution radiological tool that provides detailed information regarding 3D bone mass distribution and microarchitecture, precise measurements of cortical and trabecular indexes, but also the hydroxyapatite content that relates to bone mineralization status [82].

Micro-CT has been widely used for clinical purposes in animal models [52, 83-91], and increasingly adopted in forensic investigations (i.e. for the examination of gunshot entrance and exit wounds, tool marks on bone, bone pathology and post-mortem interval estimation [93]), but it has been applied to forensic anthropology in only two studies [46, 92]. These papers, both based on human cranial fractures, pointed out how micro-CT analysis could provide accurate information about fracture healing, especially for early healing signs.

However, despite the tremendous potential of micro-CT analysis in the field of forensic anthropology, in particular in the setting of fracture dating, no research aimed at studying and dating post-cranial human bone calluses with micro-CT has ever been performed.

Furthermore, despite synchrotron radiation micro-CT (SRμCT) has been applied to understand the bone structural biology [94-95], especially beyond vascular canals [96], to date no studies on human bone calluses have been performed.

The present study aims therefore to preliminarily assess the potential use of micro-CT, an advanced and non-destructive imaging technology for small bone samples, for the qualitative and quantitative analysis of fracture dating, in order to obtain an objective dating of the healing phase on dry bones.

A preliminary feasibility experiment aimed at assessing the potential of synchrotron radiation micro-computed tomography (SRμCT) for the analysis of fractures dating was been perfomed but only to evaluate if it could provide additional information with respect to micro-computed tomography.

7.2 Material and Methods

Nine costal bone fractures of known post-traumatic ages (the time between trauma and death ranging from 8 days to 2 years), were collected from six adult individuals (5 males and 1 female, with ages-at-death ranging between 39 and 55 years) autopsied at the Institute of Legal Medicine of Milan, in accordance with article 41 of the Italian National Police Mortuary Regulation (September 10, 1990; n° 285). Rib fractures were selected in this study because it is one of the anatomical sites where fractures are most frequently found during forensic autopsies.

None of the individuals included in the study was known to have a disease that might have interfered with fracture repair and all of the fractures had a traumatic origin, as inferable from medical records or witness testimonies, due to car accident or aggression. In addition, given the fact that the individuals selected for the study were 5 young adult men and 1 woman in premenopausal age, the possibility of senile osteoporosis is negligible.

During autopsies the flesh was mechanically removed from all of the bone samples which were then macerated in cold water until complete soft tissue removal.

Then, the study was divided into *three parts*, of which the first of qualitative and descriptive type and the second based on quantitative assessment through micro-CT of the inner trabecular microarchitecture of the same 9 bone calluses of known age, while the third was performed using synchrotron radiation micro-computed tomography (SRμCT) only on one of the aforementioned calluses, just for an ultra-preliminary feasibility experiment to speculate future perspectives.

Part I

Each fracture was photographed and studied macroscopically using standard anthropological methods [105]. Imaging studies were then performed using plain

X-Ray conventional radiographic examination (CR) and high resolution radiological micro-CT scans, also to compare the results of both imaging techniques.

Conventional radiological analysis was performed at the Institute of Legal Medicine of Milan using a Poskom PXM-40BT and an X-DR L WiFi with the following technical parameters 50kV and 4mAs and acquired using the Examion® software. Bones were entirely scanned in a single frontal projection, with the sample lying on the side that offered the best visualization of callus area.

For the acquisition of tomographic images, a custom-made system was used. The X-ray source, Hamamatsu L12161-07, was operating at 50 kV and current 400 μA at 20 W (focal spot size 20 μm) with 0.5 mm Al filter. The detector that was used was a Hamamatsu C11701DK-40, 2192x1776 pixels of 120 μm size. The object to source distance was 110 mm and the source to detector distance was 600 mm. The sample was rotating along his axis at a constant speed of 1 ^{o}/s and 1800 projections were acquired over 360^{o} in 6 min. Flat and dark field images were acquired to correct for the detector's pixel variabilities in the active scan area.

The reconstruction was performed using NRecon version 1.7.0.4. by Bruker microCT 2012-2016. The tomographic images were corrected for their misalignment, ring artifacts and beam hardening. The images were saved as 16-bit TIF files of 2192 × 2192 pixels. The reconstruction was done for an isotropic voxel size of 22 μm.

According to bones length, an attempt was made to include the entire sample in the micro-CT scan. When this was not possible, the scan was centered on the region of the bone callus, including as much as possible the remaining normal bone.

The longitudinal assessment of bone callus on CR and micro-CT images was done by a forensic anthropologist with expertise in the imaging of dry bones (main investigator), with the support of a forensic pathologist and a radiologist. According to the macroscopic and radiological appearance, three stages of callus healing were identified [54] as follows.

- *Early stage* characterized by the absence of any clear bone callus. This stage is supposed to indicate the very early phase of fracture repair, corresponding to the time of cartilaginous soft callus deposition (which was not visible on dry specimens).

- *Intermediate stage* corresponding to the formation of woven bone became apparent, due to the progression of endochondral ossification. At this stage, newly formed woven bone is less dense, and mainly located at the level of periosteum and on the cortical margins of fracture line.
- *Late stage* in which the woven bone resulted more solid, with the formation of lamellar ossified callus related to remodeling phase. At this stage, the original cortex may possibly be restored.

A description of each fracture, macroscopically, on CR as well as micro-CT images was provided.

Part II

Micro-CT is well known imaging technique that allows to extract the inner tridimensional microarchitecture of the bone. It permits also to obtain a morphological bone description based on a volumetric data rather than inferring these values from 2D stereological models, as is done when using standard histologic evaluation.

The final goal of the analysis was to find synthetic parameters able to describe the trabecular microarchitecture for objective fracture dating, mixing the anthropological and radiological experience of the participants with more quantitative mathematical models.

For each sample were selected by visual inspection four regions of interest: two in the callus and two far from the fracture borders for adequate comparison of the measured parameters. Because in almost all the cases was impossible to find large homogenous regions for the definition of a REV (Representative Elementary Volume) it was decided to consider for the callus the maximum allowable volume (parallelepipedon) in the selected area and a similar amount for the normal bone. This step could be considered arbitrary, but in cases where other variables can interfere (i.e. fracture typology and location, lifestyle and history of the donors) the trained eye of the expert is the most reliable solution.

The areas containing useful and informative qualitative details about the callus age, also in light of the aforementioned morphological description, were selected by the participants analyzing coronal and sagittal sections (22 µm thickness) obtained from the 3D dataset which revealed features not recognizable on radiographic imaging. The extraction of the trabecular structure was made by hand selecting carefully the regions under investigation.

The process of reconstruction resulted in slices characterized by a histogram with respect to the intensities of the grey levels. Darker areas of the histogram characterized the pores and whiter areas regions of the bone. The first step was the adjustment of the brightness and contrast of the grey level histogram using the automatic adjustment method. Then the various features of the bones were preserved after applying the unsharp filter with radius 5. After this step, the edges of each trabecula were differentiated greatly from the pores in order the segmentation to be accurate and precise. The final step was to segment the bone skeleton from the pore space using the Otsu algorithm. Despite a condition of Otsu's algorithm is the histogram to be a bimodal distribution, in the analyzed tomographic images, a semi bimodal distribution of the histograms allowed the successful application of the algorithm.

All the parameters (anisotropy, connectivity, BV/TV fraction and trabecular thickness and trabecular separation) of the segmented binary images were calculated using the BoneJ plugin in ImageJ.

Part III

Finally, just as a preliminary feasibility experiment carried out just to verify the potentials of future applications, also synchrotron radiation micro-CT (SRµCT) was considered to evaluate if it could provide additional information with respect to micro-computed tomography (micro-CT).

One of the nine post-cranial bone callus samples under study for this thesis, aged 86 days old, was scanned at SYRMEP, the hard X-ray imaging beamline of Elettra, the Italian Synchrotron Radiation Laboratory[4].

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[4] The availability of X-ray sources, like Synchrotrons, with high characteristics of spatial coherence, made it possible the application of the so-called 'Phase Contrast Imaging' (PCI). PCI

Phase contrast micro-CT scans in PB modality were performed with the pink beam microCT setup.
The used X-ray detector was a Hamamatsu CMOS camera C11440-22C with a 2048 x 2048 pixel chip, optically coupled with a 17 µm thick lutetium oxyorthosilicate (LSO) scintillator, allowing for variable pixel size between 0.9 and 5.2 µm.

<u>Critical considerations about material and methods</u>

The first part of the study aims at preliminarily assessing the potential use of micro-CT, an advanced and non-destructive imaging technology, for the qualitative and quantitative analysis of fracture healing and dating. Micro-computed tomography (micro-CT) is a sensitive and high-resolution radiological tool that provides detailed information regarding 3D bone mass distribution and microarchitecture, precise measurements of cortical and trabecular indexes, but also the hydroxyapatite content that relates to bone mineralization status [82].
Although this technique has been widely used for clinical purposes in animal models, and increasingly adopted in forensic investigations, no research aimed at studying and dating post-cranial human bone calluses with micro-CT has ever been performed.
Therefore, this study represents the first investigation dedicated to the study the healing features of post-cranial human bone calluses with micro-CT, from both a qualitative- morphological and quantitative point of view, the latter by means of different histomorphometric parameters.
Given the peculiarity of the inner structure of a single bone callus, which can present simultaneously different healing features in different parts of the same callus, the huge problems when dealing with this kind of analysis consist right in the selection of the most representative trabecular bone volume of interest (REV) within each callus as well as in the test and choice of the histomorphometric

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allows for an improved visualization of details borders, producing the typical 'edge enhancement' effects that brings an improved visualization of regions with different refractive indexes. In case of samples constituted by more components, the application of phase retrieval brings to an improvement in the image contrast and a better identification of the different sample components with respect to the solely absorption contrast.

parameters to be examined, given the lack of previous quantitative studies on this topic. Moreover, the difficulty in this scenario is twofold, since on the one hand the variability is related to the smaller callus homogeneous volumes (compared with the trabecular spacing), due to the different types of fracture, as certain fractures may present with impacted cortical fragments within the callus, frayed opposed margins or they can regularly evolve with restored cortical margins and on the other side the variability may be related to the intrinsic biomechanics of the traumatic and healing process, as in the same callus we can have compression and tension regions.

Considering the maximum allowable volume means losing the fine structure and can introduce some unknown bias, but it is a conservative choice for a preliminary analysis that aims to find synthetic parameters describing a complex structure. The areas of interest were then selected manually, while being aware that it represents a critical limit since this introduce a variability linked to the operator. However, the comparison between the inner characteristics of bone far from the fracture and of the callus is self-consistent since both of the areas analyzed came from the same bone sample.

In relation to the present study, critical consideration could also be expressed about the small sample size, as well as about the lack of homogeneous groups of samples based on fracture type, which are all factors which don't permit any definitive statement but only considerations about any trends and for that reason it was specified in the introduction that it is a preliminary study.

Another limitation of the study could be that the samples were macerated prior to radiological analysis, so that with the removal of all soft tissue, including e.g. granulation tissue and cartilage, a potentially rich source of additional information, was deleted.

Finally, criticism could be made about the fact that micro-CT can analyze only small calluses, no longer than 2-3 cm and no wider than 1.5 cm so that this kind of non-destructive imaging technology, to be really informative, should not be applied to all kind of bones but only to bone calluses which can be fully scanned, in light of their size, without the need to be randomly sectioned. Conversely, big calluses to be analyzed should be sectioned and the section of big calluses could create a huge bias given the heterogeneity of the internal structure of the callus itself.

Therefore, in the present study, given the adequate size for the complete callus scanning and being one of the anatomical sites where fractures are most frequently found during forensic autopsies, only fragments of ribs (including the callus) were selected in order to try to minimize the aforementioned bias.

7.3 Results

Part I

The description of the gross examination of each fracture and of their degree of bone healing on both CR and micro-CT images is reported in **Table 5.**

<table>
<tr><th>Sample</th><th>Post-traumatic age</th><th>Stage of healing</th><th>Macroscopic features</th><th>Radiographic features</th><th>Additional information with micro-CT compared to CR</th></tr>
<tr><td>N1</td><td>8 days</td><td>Early</td><td>Frayed fracture margins with no signs of healing</td><td>Frayed fracture margins with no signs of healing</td><td>Contour of the fracture margins are distinct, whereas they are blurred on x-ray</td></tr>
<tr><td>N5</td><td>28 days</td><td>Intermediate</td><td>Woven bone callus bridging the two ends</td><td>▪ Undefined fracture margins
▪ Marginal sclerosis indicating bone apposition</td><td>▪ Periosteal new bone deposition on the bone cortex
▪ Microstructure of the callus, constituted of woven non-remodeled bone</td></tr>
<tr><td>N4</td><td>42 days</td><td>Intermediate</td><td>Exuberantly bulging lamellar bone callus</td><td>Bulging callus bridging the two ends</td><td rowspan="6">▪ Finer details on the internal microstructure of the callus
▪ Finer outline of the callus and bone ends</td></tr>
<tr><td>N3</td><td>72 days</td><td>Intermediate</td><td>Smooth bulging lamellar bone callus</td><td>Smooth bulging callus</td></tr>
<tr><td>N2a</td><td rowspan="4">86 days</td><td rowspan="4">Intermediate</td><td rowspan="4">Smooth lamellar bone callus, slightly bulging</td><td rowspan="4">Smooth callus with slight sclerosis on the fracture margins</td></tr>
<tr><td>N2b</td></tr>
<tr><td>N2c</td></tr>
<tr><td>N2d</td></tr>
<tr><td>N6</td><td>2 years</td><td>Late</td><td>Remodeled callus, bulging barely visible</td><td>Remodeled callus, bulging barely visible</td><td>Finer appreciation of the microstructure (remodeled lamellar bone) and outline of the callus, unsharp on x-ray</td></tr>
</table>

Table 5. Macroscopic examination, radiographic and micro-CT findings of the 9 fractures.

Micro-CT provided very high-quality images of the callus microstructure, with excellent magnification and definition, thanks to the very small isotropic voxel. Compared to micro-CT, CR suffered from its intrinsic limitation related to the lower spatial resolution, leading to a superimposition of the intermediate healing stages. According to the proposed classification, we found one sample in the early stage (N1, 8 days from the trauma), four samples in the intermediate stage (N5, N4, N3 and N2, with 28, 42, 72 and 86 days from the trauma, respectively) and one sample in the late healing phase (N6, with 2 years from the trauma).

In the youngest fracture (**N1-Figure 7**), micro-CT imaging did not provide additional information, as no sign of bone healing could be found neither on micro-CT nor on the x-ray, suggesting that the fracture was not older than a few days. It is reasonable to think that, dealing with dry bone samples, the soft tissue fraction of the bone callus typically found at this phase (consisting mainly of cartilaginous and granulation tissue) is no longer recognizable, as a consequence of dehydration. No woven bone is clearly visible at this stage.

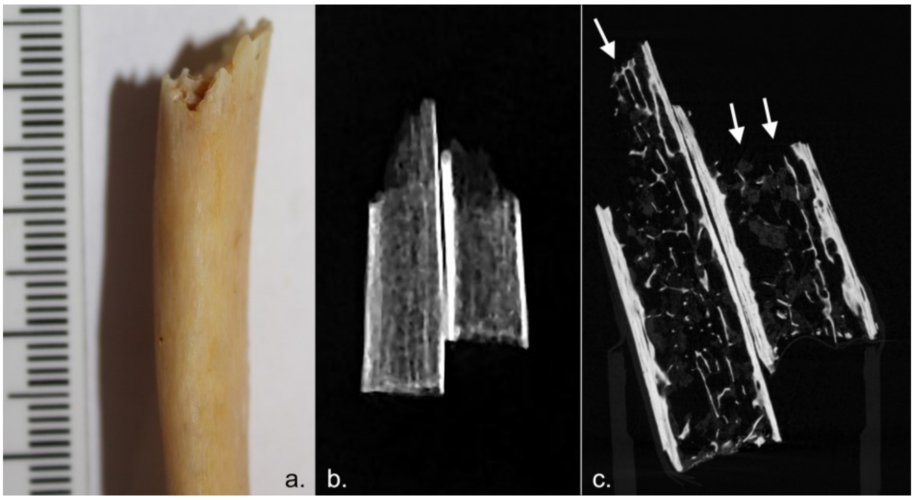


Figure 7. Macroscopic (a), radiographic (b) and micro-CT (c) comparison of **N1-8 days old**. Micro-CT clearly shows no signs of bone healing, with sharp fracture margins next to the bony trabeculae (arrows).

With increasing post-traumatic age, the potential of micro-CT for fracture analysis became undeniable. In those samples at intermediate stage, periosteal new bone deposition in a woven callus could be clearly appreciated on the micro-CT section, while plain X-ray only showed bone sclerosis with vague irregular margins as sign of new bone apposition (**N5-Figure 8**).

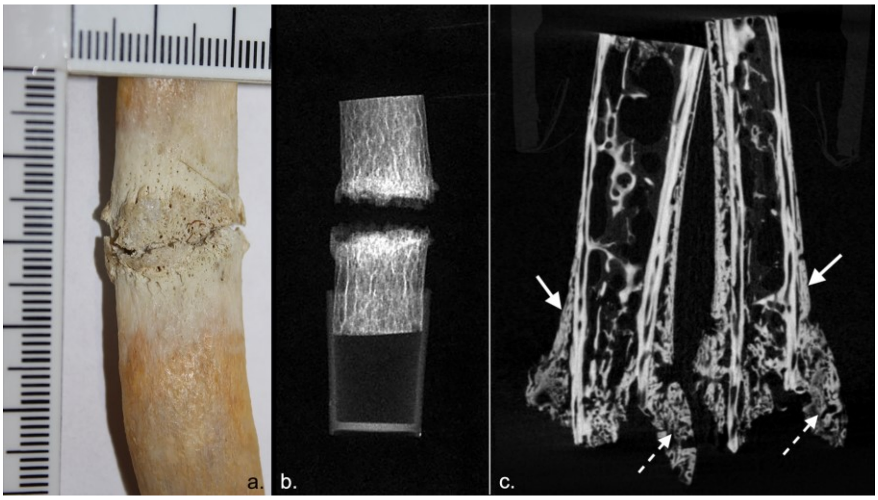


Figure 8. Macroscopic (a), radiographic (b) and micro-CT (c) comparison of **N5-28 days old**. Micro-CT shows the presence of callus with deposition of periosteal bone (arrows) together with woven non-remodeled bone close to fracture line (dashed arrows).

At the same time, micro-CT images offered better detail on those features that are particularly of interest for dating fractures, such as the periosteal callus becoming firmly attached (inseparable) to the bone cortex, which was visible after 6 weeks (**N4-Figure 9**) [45], and the smoothening of the periosteal callus outline, noticeable after 2 months (**N3, N2A, N2B, N2C, N2D – Figures 10-14**) [47].

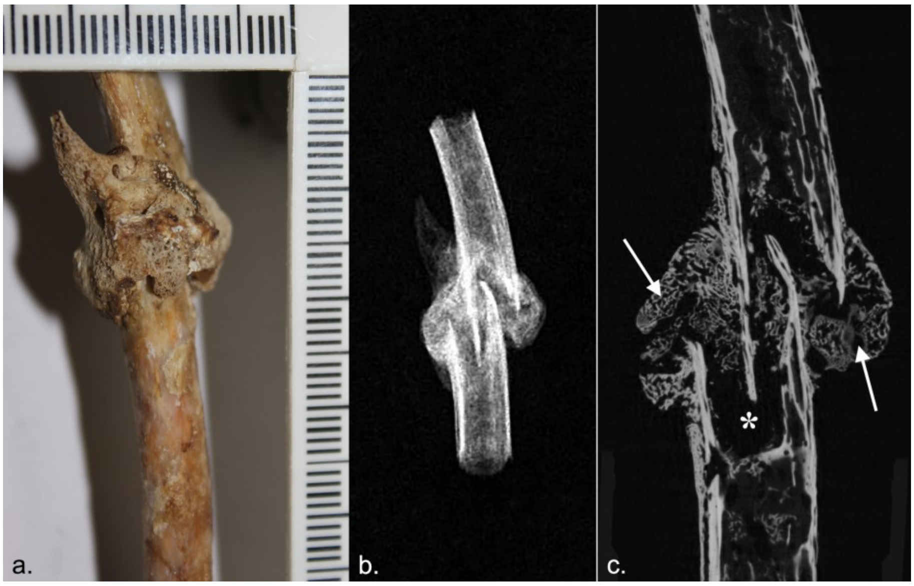


Figure 9. Macroscopic (a), radiographic (b) and micro-CT (c) comparison of **N4-42 days old**. This is a highly impacted fracture and micro-CT shows the partial non-union in the central part (absence of callus, asterisk). An exaggerated bulging callus (arrows) can be seen accumulating at the periphery.

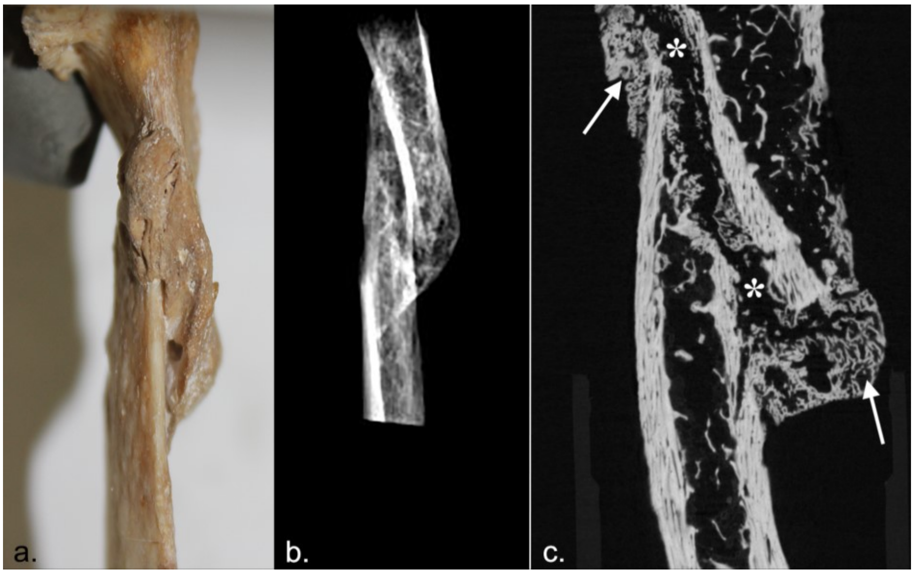


Figure 10. Macroscopic (a), radiographic (b) and micro-CT (c) comparison of **N3-72 days old**. In this displaced fracture, micro-CT highlights the presence of exuberant woven bone formation mainly at the periphery (arrows), with a lack of callus formation in the central part (asterisks).

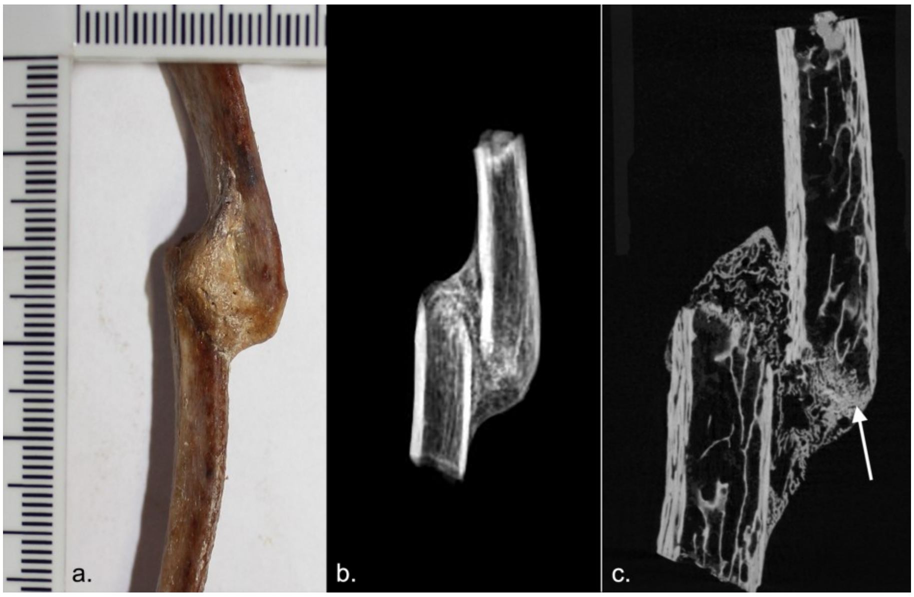


Figure 11. Macroscopic (a), radiographic (b) and micro-CT (c) comparison of **N2A-86 days old.** Micro-CT shows the presence of denser woven bone on one side of the fracture (arrow), suggesting an advanced status of bone healing.

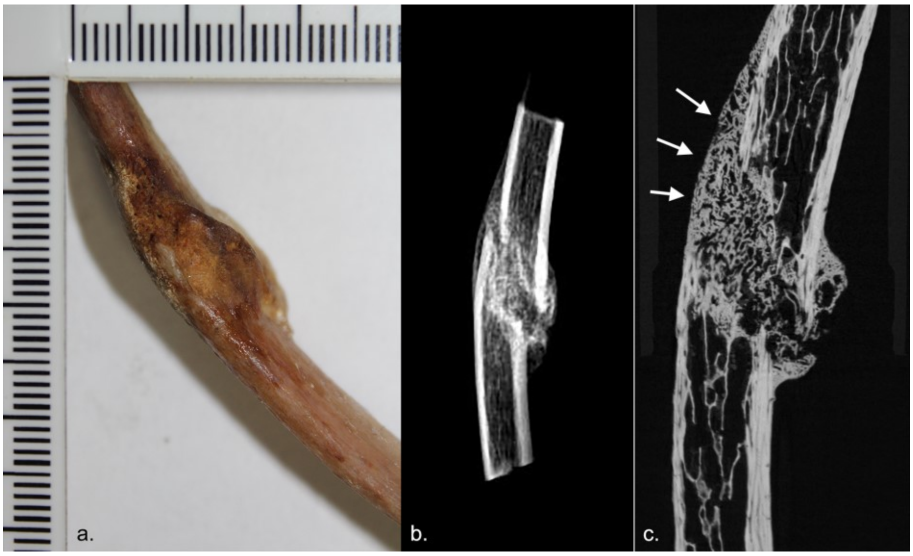


Figure 12. Macroscopic (a), radiographic (b) and micro-CT (c) comparison of **N2B-86 days old**. This compound fracture presents with signs of advanced healing, with lamellar oriented trabeculae and a more continuous and smoother appearance of the cortical regions, especially on the right side (arrows).

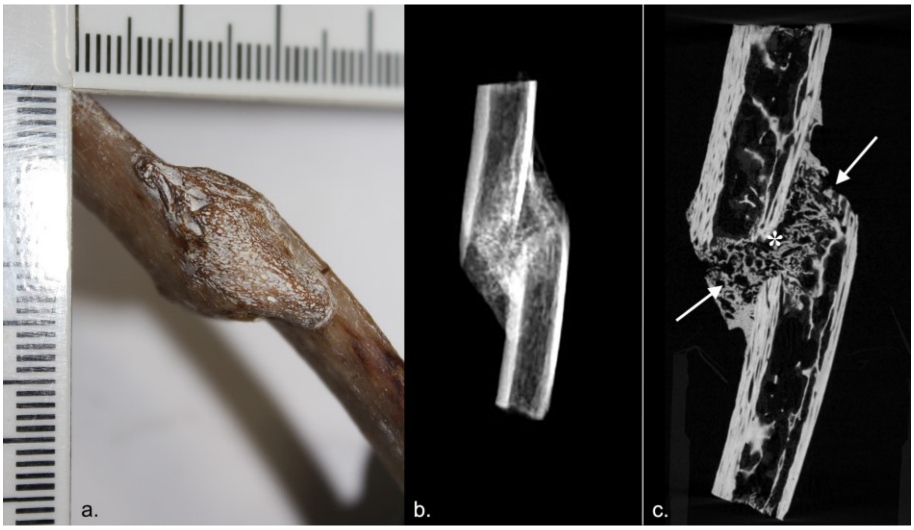


Figure 13. Macroscopic (a), radiographic (b) and micro-CT (c) comparison of **N2C-86 days old fracture**. On the micro-CT, this displaced fracture presents with a callus mainly composed of lamellar and woven bone (arrows), with a residual gap (asterisk).

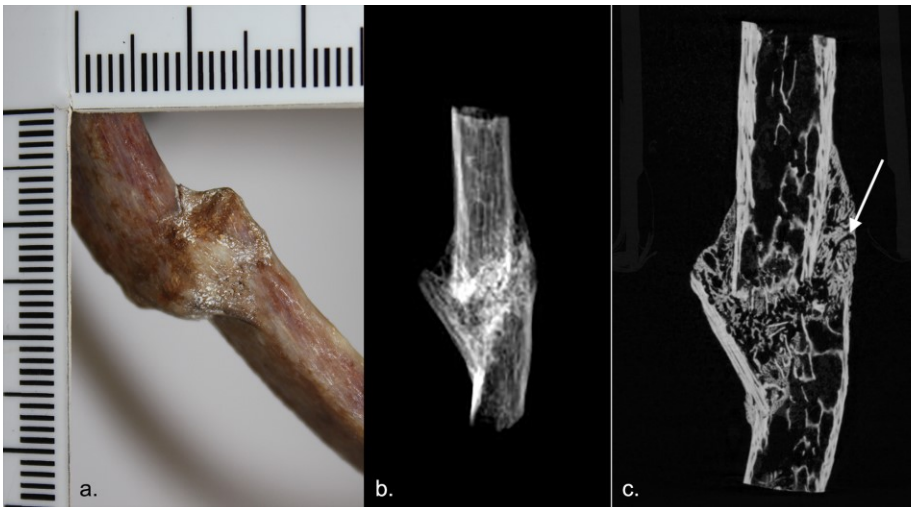


Figure 14. Macroscopic (a), radiographic (b) and micro-CT (c) comparison of **N2D-86 days old fracture**. This impacted but non-displaced fracture presents with dense woven callus and complete bridging on the left side (arrow).

The same features could be just perceived on radiographs, mostly as blurred dense callus, without a clear detail of its microstructure. It should be noted that some characteristics of N2 samples are different from those of the other samples belonging to the intermediate stage. In fact, although the difference in terms of days from the trauma is not too wide (especially compared to sample N3), some calluses of sample N2 show a more advanced state of consolidation. This is the case of sample N2B and N2D, in which the presence of denser woven bone can be appreciated, with lamellar oriented trabeculae and a more continuous and smoother appearance of the cortical regions. A possible explanation is related to the fracture morphology, as N2B and N2D present with a non-displaced fracture line compared to N3, N2A and N2C. It is known that fracture morphology influences the healing process, with displaced fractures requiring longer time and carrying the risk of failure and non-union [106]. This is the case of N3 sample, a full displaced rib fracture; while conventional radiography may show a smooth and complete bulging callus, micro-CT section clearly depicts the presence of woven bone mainly at the periphery, with a lack of callus formation in the central part. Similarly, the N4 sample shows a highly impacted fracture morphology, which possibly led to the formation of exuberantly bulging callus (also macroscopically visible) due to woven bone accumulating at the periphery.

The evaluation of the only later-stage callus (**N6**, presented in **Figure 15**) confirmed that micro-CT provided useful information even when bone remodeling was already radiographically evident. In fact, micro-CT is able to show the fine details of the callus in its most advanced stage of healing, such as the presence of remodeled lamellar bone, the restored outer cortex and medullar trabecular continuity.

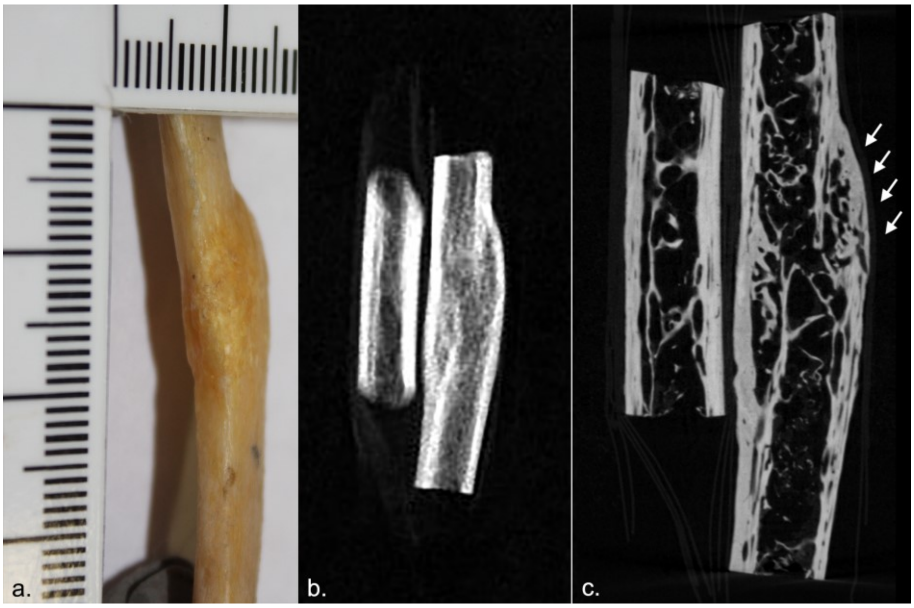


Figure 15. Macroscopic (a), radiographic (b) and micro-CT (c) comparison of **N6-2 years old**. This is a callus in advanced stage of healing; micro-CT shows the presence of remodeled lamellar bone with restored outer cortex (arrows).

Part II

The 3D measurement of the trabecular inner morphology of the bone sample analyzed were performed on the selected areas of interest of each bone (signed with yellow square), both on the callus and on the areas of the bone distant from it (named as healthy bone-HB), as represented in **Figures 16-24**.

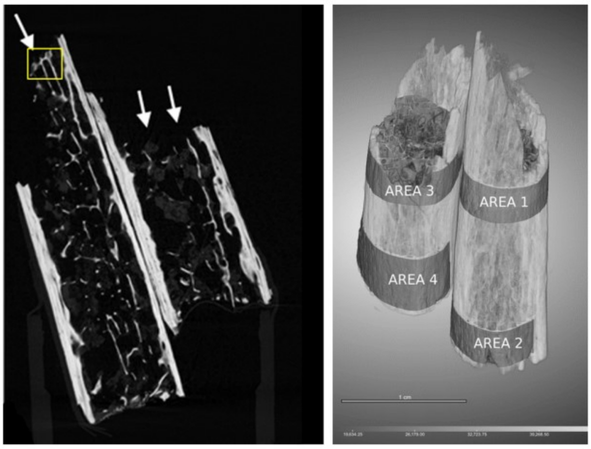


Figure 16. Sample **N1-8 days old.** Area of interest with no signs of bone healing and sharp fracture margins next to the bony trabeculae.

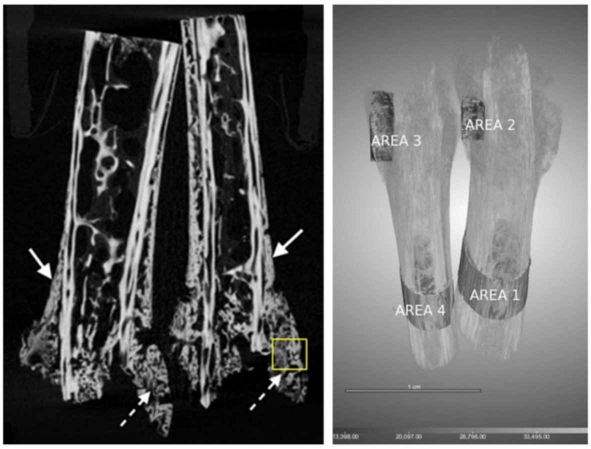


Figure 17. Sample **N5-28 days old**. Area of interest with woven non-remodeled bone close to fracture line.

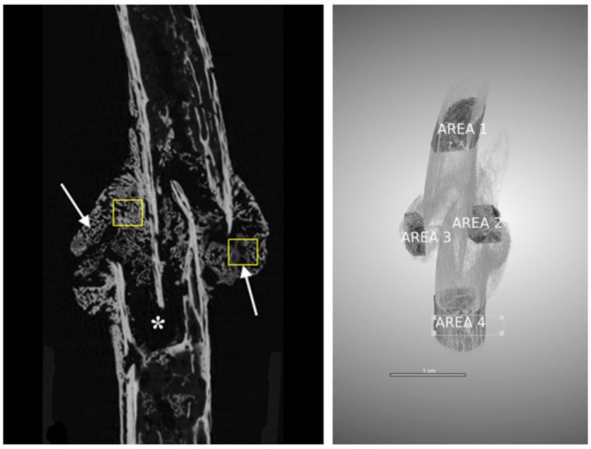


Figure 18. Sample N4 **N4-42 days old**. Areas of interest with exaggerated bulging callus accumulating at the periphery.

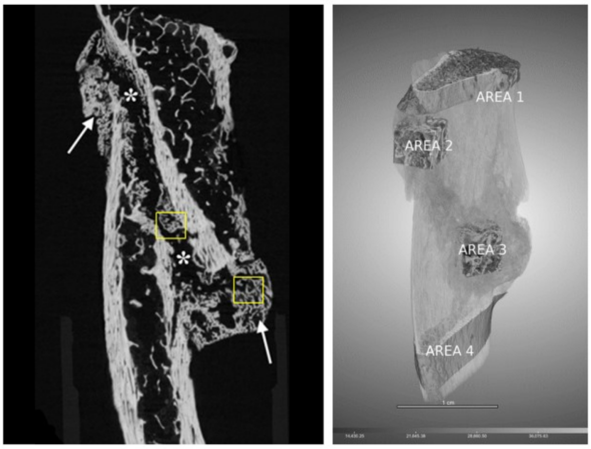


Figure 19. Sample **N3-72 days old**. Areas of interest with exuberant woven bone formation.

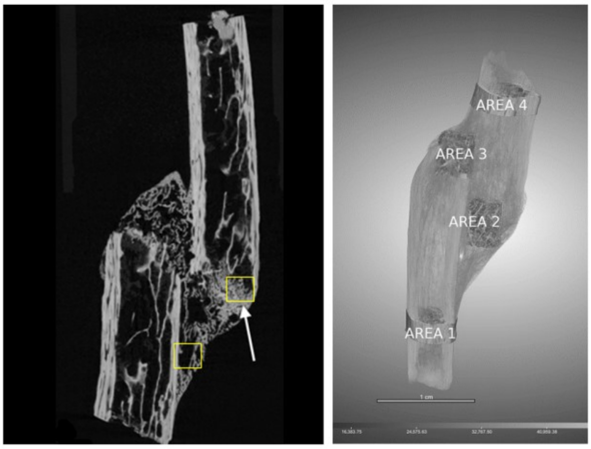


Figure 20. Sample **N2A-86 days old.** Areas of interest with denser woven bone.

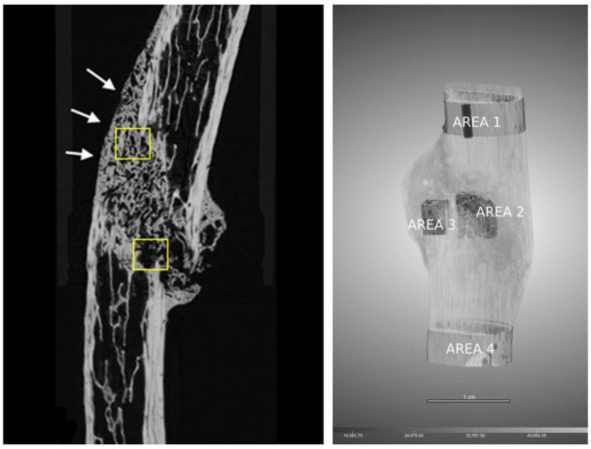


Figure 21. Sample **N2B-86 days old**. Areas of interest with signs of advanced healing, including lamellar oriented trabeculae and a smoother appearance of the cortical regions.

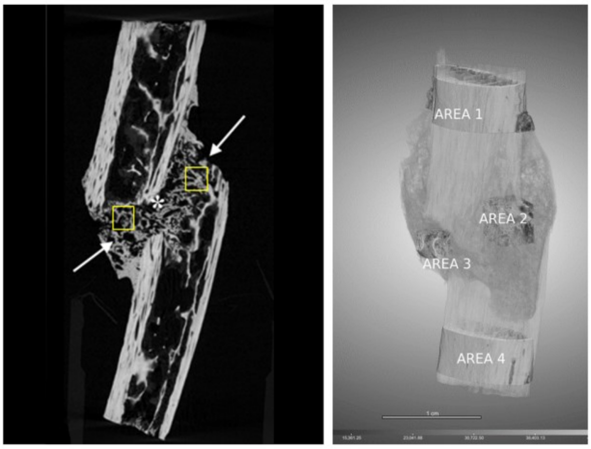


Figure 22. Sample **N2C-86 days old fracture**. Areas of interest with lamellar and woven bone.

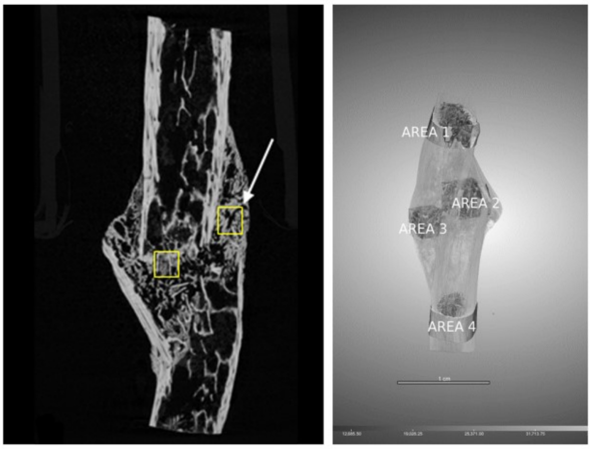


Figure 23. Sample **N2D-86 days old fracture**. Areas of interest with dense woven callus and complete bridging on the left side.

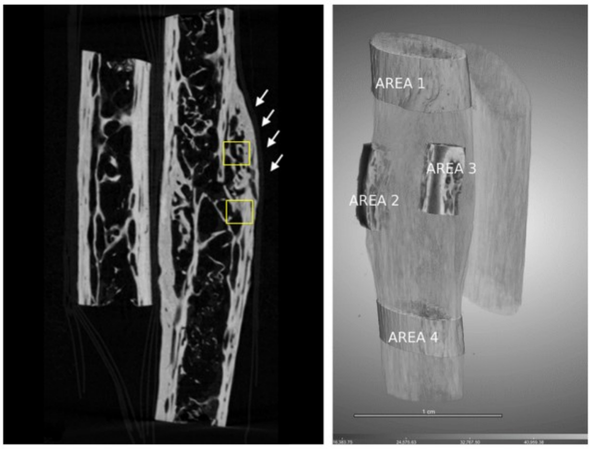


Figure 24. Sample **N6-2 years old**. Areas of interest with remodeled lamellar bone.

Below are then reported the measurements of five histomorphometric parameters performed on the aforementioned selected areas. Such five parameters, whose definition can be found in ***Attachment 2***, are the most commonly used for the study of the 3D bone trabecular microarchitecture with micro-CT [107], including anisotropy, connectivity, BV/TV fraction [%] (bone volume fraction), trabecular thickness [mm] (TbTh) and trabecular separation [mm] (TbSp) (**Table 5**), but they have never been applied in case of bone calluses.

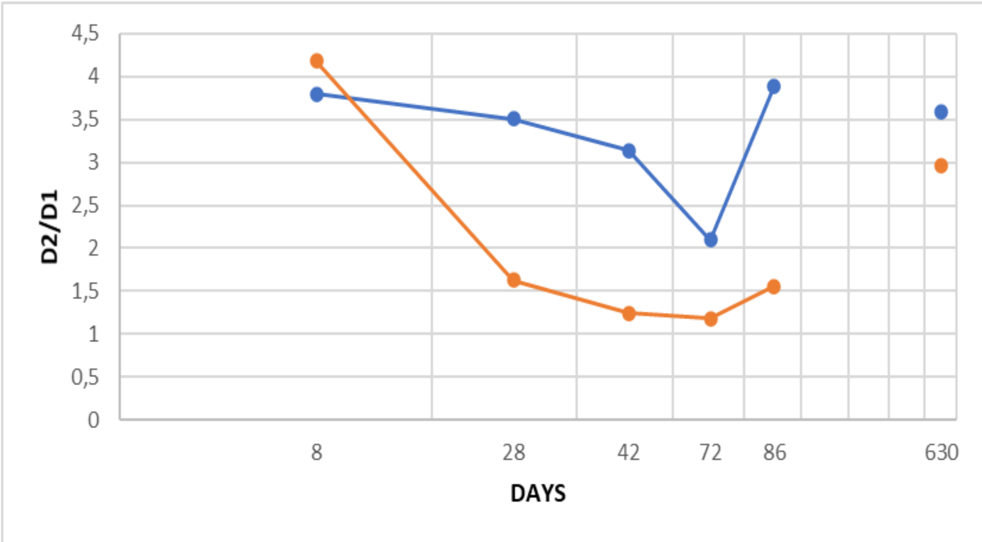
	Anisotropy		**Connectivity**		**BV/TV fraction**		**Mean TbTh**		**Mean TbSp**	
	HB	**CALLUS**	**HB**	**CALLUS**	**HB**	**CALLUS**	**HB**	**CALLUS**	**HB**	**CALLUS**
N1	0.77	0.79	191.69	120.69	0.10	0.10	6.74	6.36	48.09	55.87
N5	0.85	0.43	83.50	1488.38	0.12	0.42	8.03	7.41	51.63	12.64
N4	0.79	0.32	513.06	2519.81	0.12	0.34	7.19	7.22	40.77	17.13
N3	0.74	0.30	109.38	1011.88	0.15	0.38	8.74	10.02	43.15	21.55
N2A	0.80	0.39	44.09	1290.38	0.15	0.44	6.99	8.45	41.22	16.72
N2B	0.83	0.54	44.09	1290.38	0.15	0.44	7.25	10.96	39.24	13.60
N2C	0.80	0.47	44.09	1290.38	0.15	0.44	8.37	9.52	43.99	15.63
N2D	0.81	0.42	44.09	1290.38	0.15	0.44	7.00	8.65	46.28	14.00
N6	0.80	0.73	109.63	169.94	0.13	0.30	6.36	11.08	29.83	29.42

Table 5. Measurements of the histomorphometric parameters commonly used for the study of the 3D bone trabecular microarchitecture with micro-CT

The aforementioned results where then summarized on graphs, in order to evaluate the trend of the histomorphological parameters analyzed both on the callus site and on the same bone far from the fracture.

In particular, about *anisotropy*, which is used to quantify the directionality of trabecular bone and tells whether the trabeculae have a certain orientation, it was noticed that the inner microarchitecture of the callus tends to be anisotropic as the rest of the bone in the early stages of fracture healing and becomes increasingly isotropic during the intermediate stages of healing, to return then anisotropic as it was in the early stages.

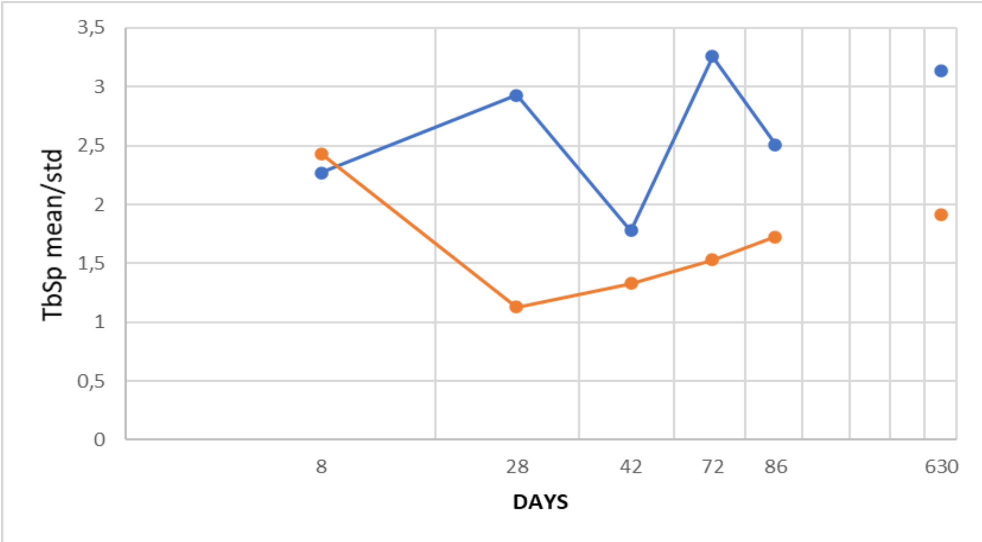
Furthermore, starting from the assumption that the trabecular bone was mainly oriented along the rib longitudinal axis (as demonstrated by the higher degree of anisotropy in the areas of the bone far from the callus), it was also evaluated the ratio between the medium (D2) and the shorter ellipsoid axis (D1) in order to test the degree of anisotropy also in the transverse plane of the rib itself and a more clear trend emerged, as represented in **Graph 3**.



Graph 3. Ratio between the medium (D2) and the shorter ellipsoid axis (D1) in order to test the degree of anisotropy also in the transverse plane of the rib both on the callus (orange line) and on the areas of the bone distant from it (blue line).

Likewise, the *trabecular separation (TbSp)*, represented in the graph as the ratio of the mean value and its standard deviation (**Graph 4**), presented a visible trend. Taking into account that the first point (8 days) refers to a sample that doesn't present any visible callus, we see low values at the beginning of the healing process that are increasing as it is evolving. This behavior is not so clear considering only the TbSp because it represents an average while the standard deviation is connected the spread of the value.

These results show that the callus start growing with a lateral isotropic trabecular fine mesh and during the healing process becomes more oriented with larger spacing.



Graph 4. Ratio of the mean value of the trabecular separation (TbSp) and its standard deviation both on the callus (orange line) and on the areas of the bone distant from it (blue line).

The different aspect of the trabecular microarchitecture of the bone far from the fracture and of the callus can be appreciated, as an example, also through the following 3D rendering, which clearly shows how the distance between the trabeculae was greater in the inner structure of the bone far from the fracture (**Figure 25**) than in the inner structure of the callus (coming from the same bone) at an intermediate stage of healing (**Figure 26**).

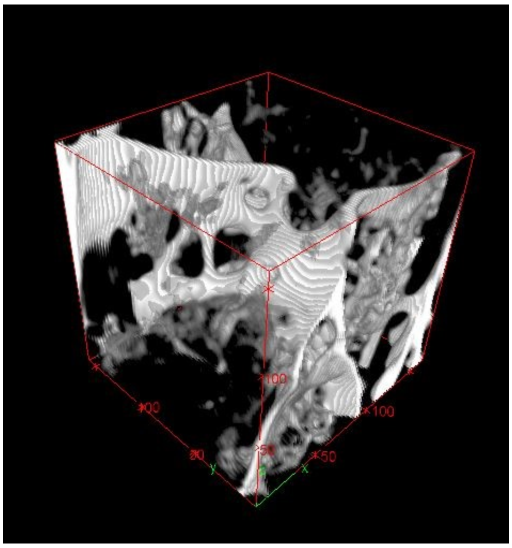


Figure 25. Trabecular microarchitecture of the bone far from the fracture

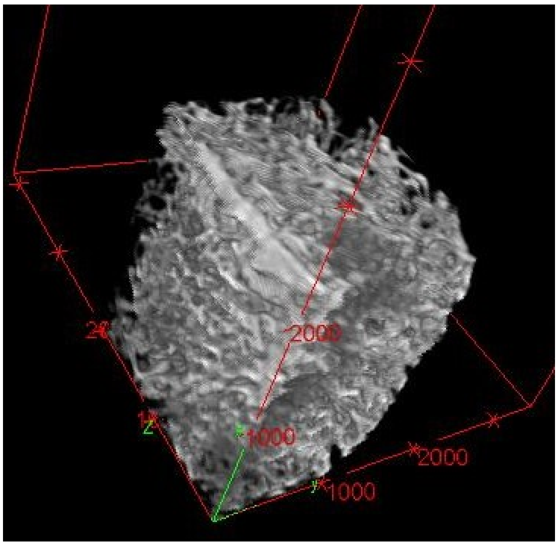
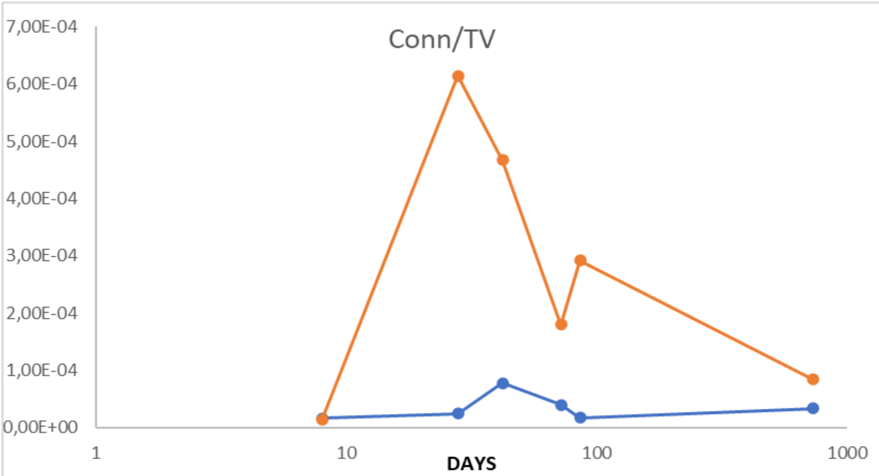


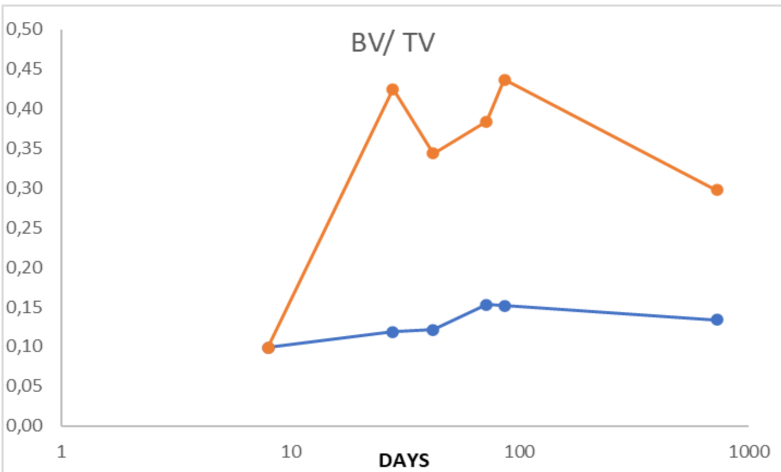
Figure 26. Trabecular microarchitecture of the callus at intermediate stage of healing

Another parameter which showed a trend was the *connectivity*, which is designed to estimate the number of connected structures, i.e. trabeculae in a trabecular network. This parameter is represented in the **Graph 5** as the ratio of the connectivity and the total volume, which present an evident peak during the intermediate stages of the healing process (orange line) while in the later stage presented the same values as in the rest of the bone (blue line).



Graph 5. Ratio of the connectivity and the total volume both on the callus (orange line) and on the areas of the bone distant from it (blue line).

Conversely, the measurement of *trabecular thickness*, that is the average thickness of the trabeculae, did not highlight any trend relative to the age of the callus.
Finally, the *bone volume fraction (BV/TV)* presents a fluctuating trend which seemed to not be completely related to the age of the callus (**Graph 6**).



Graph 6. Bone volume fraction.

Part III

As regards the application of Synchrotron radiation micro-computed tomography (SRµCT), the feasibility experiment allowed to hypothesize that this technique could better distinguish the small differences in density of the different components of the callus, as represented by the three different scales of grey in **figure 27-28.**

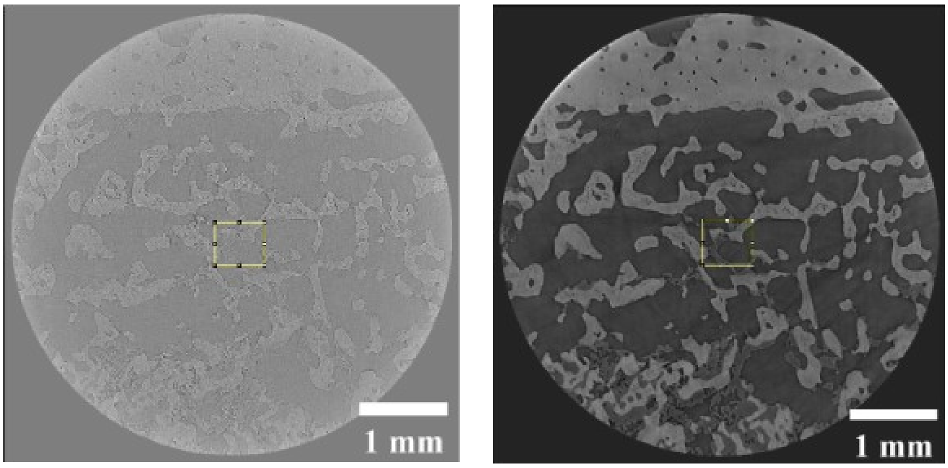


Figure 27. 3D tomography of **N2A-86 days old callus** before (left) and after phase retrieval (right) at 2.5 µm.

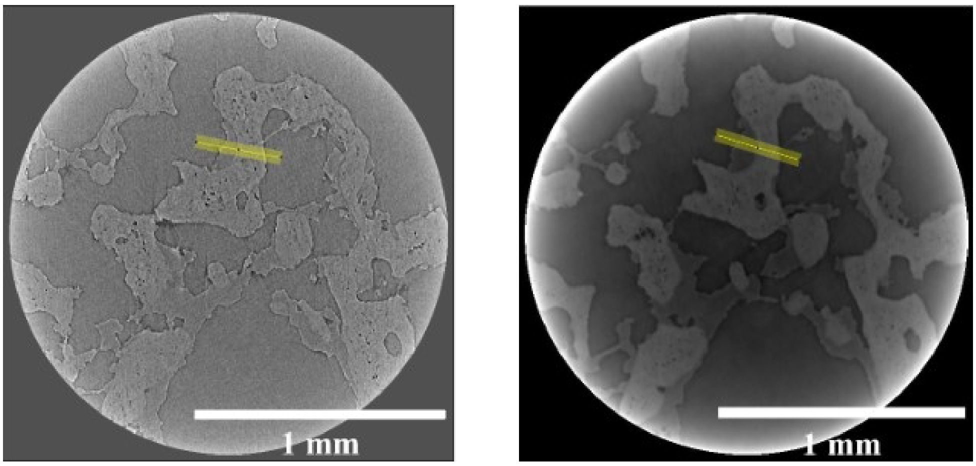


Figure 28. 3D tomography of **N2A-86 days old callus** before (left) and after phase retrieval (right) at 0.9 µm.

8. GENERAL DISCUSSION ABOUT THE PROBLEM OF FRACTURE DATING IN THE LIVING AND THE DEAD

This PhD project, inserted in the context of the trauma research in forensic anthropology and dedicated to the study of fracture dating after bone trauma basing on the tissue healing response after traumatic tissue damage, was developed through two research lines, as summarized in the thesis chart section.

The general aim of the project was to acquire a better knowledge of the process of bone remodeling, which is crucial, as already said, for a correct analysis of injuries in the skeleton, given the substantial implications in legal proceedings, especially in cases regarding maltreatment, abuse or torture in the living.as well as in case of violent death.

On this purpose, a preliminary literature review was performed, which highlighted a general limitation of existing knowledge on this topic in the forensic scenario, both in the living and the dead.

In particular, about the humans living, the main gap lay both in the estimation of bone injury timing, especially in the adult population (where only a handful of studies have been performed in the last decades, among which only one dedicated to radiographic imaging [68]), and in the examination of the relationship between the healing process and different inter- and intraindividual variables, cited in the paediatric studies as influencing the healing process itself. In fact, it was demonstrated both in paediatric and adult population that a multitude of inter- and intra-individual variables could affect how bone injury is diagnosed and interpreted, influencing assessment and estimation of timing of injury and understanding fracture-healing processes and their timing [19, 70] but no statistical analysis has ever been performed to analyze their effective influence in the timing of bone healing process in adults.

Thus, in this scenario, it was performed the present multidisciplinary study, which was based on digital images from the largest living adult population ever studied, with the aim of describing the radiological signs of bone healing appearing after a fracture at diverse time frames in order to obtain data functional, in terms of time-course, for the comprehensive and correct radiological assessment of fractures to be applied potentially in future investigations on living adults who suffered of domestic violence, abuses and tortures.

To achieve that goal, a multivariable model for the estimation of time-since-fracture was created in order to statistically analyze some of the aforementioned variables and the results showed a significant association between the healing stages of AFHS and the variables analyzed, including age, bone segment (i.e. upper or lower limb), gender and number of fractures (i.e. single or multiple). A change in fracture location with increasing age was also demonstrated, with a higher prevalence of metacarpal and fibula fractures among adults aged 20-39 years, tibia and fibula fractures among adults aged 40-59 years old, humerus and femur fractures among adults over 60 years. Therefore, according to Malone's hypothesis [13], this study demonstrated different healing time trends between bones of two different districts (upper and lower limbs). In particular, some intermediate characteristics like sclerosis and bridging appear at earlier times on upper limb bones but persist longer in lower limb bones, while other intermediate features like callus formation appeared at earlier times and persist longer in upper limb bones.

However, although the time associated to the bone healing process in adults depends on many factors and showed variability depending on the individuals and bone type, this study confirms that there is a continuum of radiological healing signs also in the adult population, as previously demonstrated by Prosser et al. [18] in the paediatric population, and that some features follow predictable time trends that can help in dating fractures.

Furthermore, despite the difficulty to provide accurate and precise post traumatic time intervals in light of the multitude of factors playing crucial roles in speeding or in the slowing the time of fracture healing, the proposed dynamic nomogram based on this large investigation, represents an innovative and easy tool to predict a time interval since fracture from digital radiographs, just taking into consideration some of the variables which affect bone healing. The observation and description of such data can therefore contribute to build foundations for estimating the posttraumatic time interval of bone fractures more accurately also in adults, allowing forensic experts to interpreting correctly traumas for violent events in adults too.

In contrast, about the dead, the main gap lay in the need to test new high-resolution imaging and non-destructive methods for a more accurate and objective analysis of the degree of fracture healing.

In fact, to date, conventional radiography is considered the most readily accessible method in most facilities and remains the preferred imaging modality in forensic cases but tend to be observer-dependent and no universally accepted standard exists for the radiographic staging of fractures and calluses [18, 35, 43] while post-mortem histology, despite it is considered the most accurate method for dating fractures and the most precise in the early healing stages even on dry bones, it is destructive, slow to process and requires training for interpretation.

Therefore, over the years, few researchers proceeded to study new high-resolution imaging or non-destructive methods for a more accurate and objective analysis of the degree of post-cranial fracture healing in the forensic scenario while preserving the sample of potential forensic interest. Cappella et al. [78] explored the potential use of Cone-Beam CT (CBCT) for fracture analysis compared with CR, showing that this imaging technique provided unique information on the different degrees of mineralization and a better visualization of the inner and outer callus structure, the fracture line, allowing for an improved differentiation of the different mineralization stages. Steyn et al. [92] and Delabarde et al. [46] demonstrated the use of micro-CT in fracture dating based on human cranial fractures, even in early healing stages when it may be a powerful complement to histological analysis, but no quantitative analysis were performed.

In this scenario, the main aim of the present preliminary study was therefore to test micro-CT imaging technology for the longitudinal evaluation of post-cranial bone healing process because of its high resolution and less destructive characteristics, in light also of the promising results of two qualitative previous forensic studies on cranial fractures [46, 92].

From a “qualitative” point of view, our study results highlighted that micro-CT sections allowed a fine observation of the contour of the fracture margins, deposition of new bone on the cortex, outline of the callus and of the bone ends as well as organization and nature of the microstructure of the callus, providing a wealth of qualitative details that could not be obtained solely by radiographic imaging. The micro-CT images of post-cranial callus samples present in fact a much higher quality, magnification and definition than CR, whose low definition and bidimensionality of images lead to superimposition in the evaluation of the middle healing stages, concurring with Cappella et al. [78]. While micro-CT imaging did not permit the addition of any significant information with respect to CR in the youngest fracture, as previously evidenced with CBCT [78], micro-CT with its higher resolution allowed a clearer observation of the microarchitecture of the older fractures than both CR and CBCT. Indeed, CBCT does not permit the observation of the presence of woven bone nor that of lamellae deposited in an undulated fashion in the initial healing phases and in a more linear one in the late healing phases [78]. Thus, although the results of this preliminary analysis unsurprisingly show that micro-CT imaging can permit a more accurate understanding of the fracture healing process than CR, they also demonstrate a finer analysis of the fracture microarchitecture and healing features than CBCT, suggesting a new line of research for the analysis of fracture healing in forensic anthropology.

Regarding the “quantitative” preliminary analysis performed with micro-CT on the bone calluses, the first of their kind, some considerations can be made from the obtained results. The first thing is that micro-CT highlighted some differences in certain microarchitectural parameters between the callus and the “healthy” bone (HB), as it was arbitrarily defined the bone far from the fracture in the same rib, mainly related to the degree of anisotropy also in the transverse axis of the callus and the spacing between the trabeculae. Anisotropy [108] is a parameter that is used to numerically quantify the trabecular directions in a specific bone segment: from a practical point of view, this parameter tells us if there is a specific orientation of the trabeculae or if they are randomly arranged. In our samples, a clear difference emerged between the degree of anisotropy of the callus and HB in the intermediate stages. In fact, the newly formed callus presented a progressive more isotropic microstructure, i.e. a more anarchic architecture. This finding is in line with the current literature related to the formation of woven bone, which is characterized by collagen fibers (early stage) and bony trabeculae (later stages) irregularly arranged, showing no specific orientation related to cortical bone and vascular spaces [109]. With the progressive callus maturation, this anarchic organization tends to reduce during time, in relation to the formation of lamellar and more resistant bone within the callus which typically show better and more regular orientation.

Similarly, we assisted to a decrease in the values of trabecular separation (TbSp) and to an increase of connectivity in the middle stages of callus formation. This is equivalent, in practical words, to a callus showing less space within the newly formed trabeculae and collagen fibers as well as a higher connection between trabeculae. Again, this is in line with the healing process of bone; in fact, the beginning of callus formation is characterized by a less dense woven bone mixed with cartilaginous and granulation tissue, which is progressively resorbed in favor of denser woven and lamellar bone [54].

Therefore, from such quantitative preliminary micro-CT measurements of the 3D trabecular microstructure of bone calluses from known ages, it emerges the existence of a certain trend of some parameters of trabecular microstructure relative to the age of the callus, including the degree of anisotropy, the connectivity and the trabecular spacing, which could be useful for the future objective fracture dating of the callus.

Certainly, as already said, these are only preliminary considerations, since the small size of the sample does not permit any statistical analysis.

Finally, about synchrotron radiation micro-CT (SRμCT) it can be hypothesized, according to what has been observed about the different image grey levels, that this technique could provide even more detailed information about the microarchitecture of callus than micro-CT, including data about soft and hard callus. Therefore, SRμCT, albeit present only in a few Centers of Excellence all over the world, could represent a promising technique for further studies about fracture healing.

9. CONCLUSIONS

In conclusion, the PhD project provided several useful stepping stones for future research in skeletal trauma analysis, both in the living and the dead.

In particular, when fracture dating concerns *living adults*, it has been demonstrated to be very difficult to provide accurate and precise post traumatic time intervals, and it needs to be taken into consideration the multitude of factors playing crucial roles in speeding or in the slowing the time of fracture healing. Thus, the present study, one of its kind, through the investigation of a large adult population, allowed to build a dynamic nomogram to predict a time interval since fracture from digital radiographs, taking into account the influence of some of the most important variables, like age, bone segment, gender and number of fractures, that were proved to be significantly correlated with healing stage of AFHS. However, given the multitude of other variables hypothesized to influence the healing time in addition to those analyzed in this study, including concomitant pathologies and treatments, further studies are certainly needed to evaluate the influence of these variables in order to refine the nomogram and make it further suitable in forensic clinical practice.

Otherwise, in case of *skeletal remains*, it emerged the need to apply new high-resolution imaging and non-destructive methods for a more accurate and objective analysis of the degree of fracture healing, so that micro-CT was tested for the longitudinal evaluation of post-cranial bone healing process not only because of its high resolution and less destructive characteristics, but also in light of the promising results of two previous forensic studies on cranial fractures.

The comparison with CR demonstrated that micro-CT imaging, for the first time applied to post-cranial fractures, could provide much more detailed information about the fracture microstructure than CR, allowing a more accurate understanding of the fracture healing process. Moreover, these results not only demonstrate the potential utility of micro-CT to obtain a wealth of qualitative details about the microstructure of the callus but also to reach an objective fracture dating through the quantitative measurement of some relevant histomorphometric parameters, laying promising foundations for further studies on this topic.

However, the casuistry should be expanded to carry out an adequate statistical analysis and the sample itself should be homogeneously grouped, but, as it is commonly known, these are ones of the most important intrinsic limits in the forensic research on this topic, since it is difficult to collect a large number of calluses as well as to select homogeneous groups of cadavers, given also the huge interpersonal variability.

Finally, SRμCT, thanks to the application of phase contrast imaging and phase retrieval algorithm could bring to an improvement in the image contrast and a better identification of the different callus components, so that also this technique deserves to be studied in depth with further studies to reach an objective fracture dating.

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Attachment 1

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TECHNICAL NOTE

ANTHROPOLOGY

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Multi-Rater Agreement Using the Adapted Fracture Healing Scale (AFHS) for the Assessment of Tubular Bones on Conventional Radiographs: Preliminary Study*

ABSTRACT: Better understanding of the timing of fracture healing may help in cases of interpersonal violence but also of personal identification. The intra- and inter-rater agreement for the adapted fracture healing scale (AFHS) assessing the post-traumatic time interval on radiographs were tested. This is a preliminary study, providing essential information on method reliability for upcoming studies using the AFHS. Five raters (two radiologists, a forensic pathologist, an orthopedist, and an anthropologist) were presented with a test in three parts consisting of 85 radiographs (from 30 adults) of fractures of tubular bones in different stages of healing purposefully selected from more than 1500 radiographs. The raters were firstly asked to assess 15 features describing fracture healing as present, absent, or not assessable. Thereafter, the raters were asked to choose from the AFHS a single-stage best representing the observed healing pattern. The intra- and inter-rater agreement were assessed using single-rating, absolute agreement, two-way mixed-effects intra-class correlation (ICC) coefficients. The intra-rater ICC of radiologist 1 ranged from 0.80 to 0.94. The radiologists' inter-rater ICC ranged from 0.68 to 0.74, while it ranged from −0.01 to 0.90 for the other raters. The good to excellent ICC among the radiologists and forensic anthropologist provides good foundation for the use of the AFHS in forensic cases of trauma dating. The poor to good results for the other physicians indicate that using the AFHS requires training in skeletal anatomy and radiology.

KEYWORDS: forensic anthropology, fracture, healing assessment, radiology, reliability, inter-rater agreement

The Fracture Dating Study has originated from the need to gather data allowing for comprehensive assessment and interpretation of cases of interpersonal violence, such as child abuse, and institutional torture in the forensic context, with focus on the post-traumatic time interval (PTI). PTI is relevant since the magistrates and judges often query how much time passed between the time when a bone was fractured and either the time of death or the time of examination in survivors. In addition, the assessment of PTI may also be of importance in cases of unidentified bodies. Radiological imaging is crucial in these investigations, particularly since for the living victims it is the only method available for fracture assessment. For postmortem trauma analysis, histological assessment of the post-traumatic time interval is a valuable alongside radiographs (1,2).

Biological literature provides descriptions of the individual stages of bone healing (3–5), but the timing of their appearance is not well known. However, before any association of healing stages to the temporal component can be made, it is important to know whether raters are able to recognize and assign the individual healing stages on radiological images. In regard to fracture dating, the radiological and forensic literature offers various types of assessment scales for bone healing, some of which are clinically driven focusing on features associated with late healing stages (6), while others describe the whole spectrum of the healing process (1,2,7–13).

Several of these studies lack the essential information on the reliability of the given scales (7–10), while some report inter-rater agreement for the individual healing stages as variations of the κ statistic (11–13) or intra-class correlation (ICC) coefficients (2).

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All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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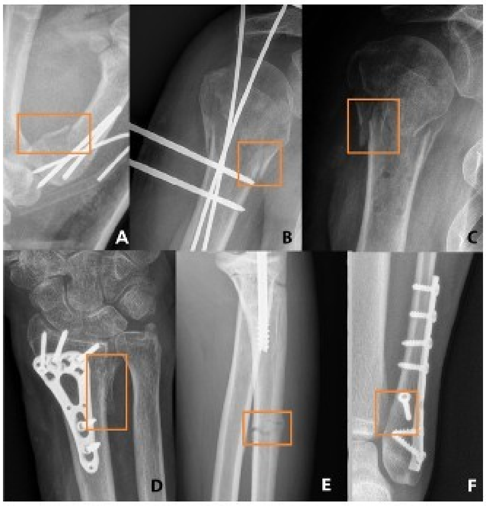

FIG. 1—*Examples of the stages in the adapted fracture healing scale (AFHS): (A) absorption; (B) periosteal reaction; (C) sclerosis; (D) callus; (E) bridging; (F) remodeling.*

The assessment of the reliability of a method is an essential initial step for introducing and subsequently applying any approach where human judgment is required (particularly one intended for forensic purposes); therefore, the aim of this paper is to assess the intra- and inter-rater agreement for the use of a purposefully adapted fracture healing scale (AFHS). The adaptation process for the fracture healing scale was driven by the need to distinguish the early signs of healing (without the need of histological assessment) without including too many stages for a clear and concise differentiation and definition of the stages (Fig. 1).

Materials and Methods

The Fracture Dating Study is a retrospective observational study using series of clinical digital conventional radiographs of fractures of patients, who were admitted to the Emergency Department of the IRCCS Policlinico San Donato, between January 1, 2013, and June 30, 2015, and had at least one follow-up imaging with known date recorded at the same institution. The Study was approved by the relevant Ethics Committees (patients' informed consent was waived). The data collected within the Study include radiographs of fractures of a known date, and demographic and clinical variables, which may affect fracture healing. The main outcome of the study is the fracture "age" in relation to the healing stage, taking into account the effect of demographic and clinical variables on the temporal progression of fracture healing.

Data were collected from the hospital system database. An initial search for the term "fracture" returned 680 individual patients admitted during the given period. After the application of the exclusion criteria (no follow-up, initial access different from the hospital's Emergency Department), the final number of patients decreased to 467 totaling more than 1,500 radiographs. The vast majority of the patients was aged 18 years and older.

TABLE 1—*The adapted fracture healing scale (AFHS).*

Stage (short designation)	Description
0 (no healing)	No healing features; sharp contours of the fracture margin
1 (absorption)	Absorption of cortical bone at the fracture margin; blunted appearance of the margin
2 (periosteal reaction)	Start of periosteal reaction = linear elevation and calcification of periosteum in the vicinity or adjacent to the fracture site
3 (sclerosis)	Increased bone density at the fracture margin
4 (callus)	Appearance of callus; from the fluffy appearance of early new bone formation to well-demarcated callus, which may have margins as dense as the cortical bone
5 (bridging)	Fracture gap bridged by cortical bone to any extent (<50%, >50%)
6 (remodeling)	Bone returning to its original shape; from firm bony union, smoothening of contours to complete bone remodeling = bone at fracture site returned to its original shape

The AFHS (Table 1) used for this study was compiled from several sources (2,9,11). The initial evaluation using the predefined stages of the scale was undertaken by radiologist 1. A test was developed to assess the reliability of the AFHS. The test involved 85 radiographs (from 30 adult patients) of fractures of tubular bones (humerus, radius, ulna, metacarpal bones, femur, tibia, fibula, metatarsal bones) in different stages of healing, also including cases of clinical fixation. The radiographs were purposefully selected from the total pool of images to represent the whole range of healing stages. In addition, ten images were selected to represent nonideal settings (consisting of only one projection, or the fracture being obscured by cast) to resemble real-life scenarios. The option "not assessable" was included for all test questions. The healing process may not be consistent throughout all bone surfaces/margins, so the raters were asked to note the most advanced feature(s).

Part 1 of the test included 15 cases comprising the initial image from the emergency room (which was not evaluated) and two follow-up images from different points in time, both subject to evaluation (30 images to assess, 45 images in total). There were 15 features describing fracture healing, including no healing features, seven features assessing the fracture margin/fracture line/fracture gap, and six features addressing new bone formation adjacent to the fracture site and callus formation, and remodeling (Table 2). Each of these features needed to be evaluated as present, absent, or not assessable. Part 2 included 20 cases consisting of a single radiograph, which needed to be evaluated in the same manner as the series of radiographs in Part 1. For each of the images of Parts 1 and 2, the detailed features noted by the raters were subsequently translated into one of the stages of the AFHS using the principle of the most advanced feature. These were then used for the calculation of intra- and inter-rater agreement. The raters were informed that the order of the healing features was not necessarily related to time. Part 3 included 20 cases of a single radiograph, for which the raters selected only one-stage best representing the healing using the AFHS. Parts 2 and 3 involved only a single radiograph per individual to simulate real-life scenarios, where often only a single radiograph is assessed in forensic cases. For postmortem trauma analysis

TABLE 2—*Features describing fracture healing used in Parts 1 and 2 of the test.*

Healing features
No healing
Fracture margin/fracture line/fracture gap
Absorption of cortical bone at the fracture margin (blunted appearance)
Sclerosis (increased bone density) at the fracture margin
Bridging of < 50% of the fracture gap
Bridging of> 50% of the fracture gap
Start of blurring of the fracture line
Advanced blurring of the fracture line
No evidence of fracture line
New bone formation adjacent to the fracture site/callus formation
Start of periosteal and/or endosteal reaction
Fluffy appearance of early new bone around the fracture site
Periosteal and/or endosteal callus less dense than the cortex
Periosteal and/or endosteal callus of similar density to the cortex
Periosteal and/or endosteal callus more dense than the cortex
Periosteal and/or endosteal callus firmly attached/indistinguishable from adjacent bone tissue
Other
Remodeling (the fracture site returning to its original appearance)

including fracture dating, it is rare that suitable antemortem radiographs are available to help assess the progression of healing. Similarly, for living victims (for instance, suspected torture) it is also rare that imaging other than the queried radiograph taken at the time of victim examination is available for analysis.

Five raters (two radiologists, a forensic anthropologist, a forensic pathologist (legal medicine specialist), and an orthopedist) were presented with detailed written instructions on how to perform the test, which they undertook at their leisure and with no time restrictions. The imaging conditions (i.e., magnification options and software) were comparable among raters. All raters had approximately ten years of professional experience working with radiographs. The raters were chosen in relation to the forensic relevance of the study, including forensic anthropologists and forensic pathologists, but also clinicians who routinely work with radiological images of trauma.

The initial evaluation by radiologist 1 was considered as the optimal assessment, that is, other assessments were compared against it in the ICC calculation. Since there is no golden standard in the assessment of radiographs, the experience of radiologist 1 was set as the standard. Since the initial assessment was not blinded with respect to the time variable due to the evaluation being part of the data collection, the ICC was also calculated based on the blinded second observation by radiologist 1 to assess any potential bias. Moreover, the ICC was computed with the assessments of radiologist 2 as comparative values against other raters (except for radiologist 1) to estimate any potential discrepancies in the evaluation pattern of radiologist 1 with respect to the other raters.

The intra-rater agreement for radiologist 1 was tested on the combined sample of 70 images. Radiologist 1 undertook the second round of observations more than six months after the initial evaluation. The outcome of the intra-rater agreement for radiologist 1 may also be considered as an indication of the test–retest reliability. In addition, there were 15 images, which were repeated in two parts of the test (either in Parts 1 and 2, or Parts 2 and 3, or Parts 1 and 3) to test the intra-rater agreement for each of the raters or the repeatability of the results. Moreover, the values may also represent a measure of internal consistency.

Except for testing the reliability of the AFHS as such, we have also tested whether using multiple images in known order would improve the reliability of the results by comparing the ICC derived from Part 1 to those of Parts 2 and 3 (the assumption was that it would), and whether creating the final stage based on numerous detailed descriptions would improve reliability in comparison with assigning a single stage from a short list of stages by comparing the ICC of Parts 2 and 3 (the assumption was that it would not, since more options would result in more variability and complicate the decision process).

The statistical analysis was performed using Stata 12 (StataCorp, 2011, College Station, TX, USA). The intra- and inter-rater agreement as the measures of reliability were assessed using single-rating, absolute agreement, two-way mixed-effects intra-class correlation coefficients. In general, ICC is high when there is little variation among raters considering the individual stages (or feature descriptions) given to each radiograph.

The ICC values of intra- and inter-rater were categorized following the recommendations of Cicchetti (14): Less than 0.40 was categorized as poor agreement, between 0.40 and 0.59 as fair, between 0.60 and 0.74 as good, and between 0.75 and 1.00 as excellent agreement.

Results

The ICC coefficients for intra- and inter-rater agreement (with 95% CI) are presented in Table 3. The intra-rater ICC of radiologist 1 was classified as excellent, ranging from 0.80 to 0.94. The inter-rater ICC for the radiologists was classified as good, ranging from 0.68 to 0.74. The inter-rater ICC values for the other raters ranged from −0.01 to 0.90 for the three different parts of the test.

One case was purposefully selected to represent the scenario, where none of the features could be assessed due to the presence of a cast obscuring the fracture. All raters assessed this case as "not assessable." This case was not included in the ICC calculations.

The results of the inter-rater agreement for the combined sample derived from comparing the blinded round by radiologist 1 to other raters were as follows: 0.70 (95% CI 0.48–0.83) to radiologist 2, 0.86 (95% CI 0.75–0.93) to forensic anthropologist, 0.51 (95% CI 0.32–0.70) to forensic pathologist, and 0.22 (95% CI −0.03–0.44) to the orthopedist, while the ICC coefficient based on the comparison between radiologist 2 and forensic anthropologist was 0.83 (95% CI 0.69–0.94), between radiologist 2 and forensic pathologist was 0.50 (95% CI 0.32–0.65), and between radiologist 2 and the orthopedist was 0.30 (95% CI 0.08–0.50).

The ICC values representing intra-rater agreement computed on 15 cases repeated in two different parts of the test were 0.93 (95% CI 0.72–0.99) for radiologist 1, 0.98 (95% CI 0.93–1.00) for radiologist 2, 0.97 (95% CI 0.82–1.00) for forensic anthropologist, 0.80 (95% CI 0.29–0.95) for forensic pathologist, and 0.57 (95% CI, 0.06–0.82; 12 cases) for the orthopedist.

Discussion

The aim of this study was to assess the reliability of the AFHS, which has been developed to facilitate the forensic assessment of post-traumatic time interval in both deceased and living individuals. Measuring intra- and inter-rater agreement is essential for determining the reliability of a scientific method. When using a nominal scale, human raters will usually not be in complete agreement. In general, some of the variation in ratings may be due to chance, some due to differences in training or experience among raters but some may be purely due to the scale failing to measure the intended characteristic.

TABLE 3—*Intra- and inter-rater intra-class correlation (ICC) coefficient with 95% confidence interval (CI) for the three parts of the test separately and combined.*

Test (number of cases)	Intra-rater	Inter-rater Radiologists	Inter-rater Forensic Anthropologist	Inter-rater Forensic Pathologist	Inter-rater Orthopedist
	ICC (95% CI)				
Part 1 (30)	0.94 (0.83–0.98)	0.69 (0.38–0.86)	0.90 (0.78–0.96)	0.58 (0.25–0.75)	−0.01 (−0.35 to 0.28)
Part 2 (20)	0.81 (0.44–0.94)	0.68 (0.34–0.85)	0.78 (0.33–0.94)	0.49 (−0.01 to 0.73)	0.24 (−0.22 to 0.64)
Part 3 (20)	0.80 (0.48–0.90)	0.74 (0.40–0.89)	0.80 (0.42–0.94)	0.42 (−0.20 to 0.74)	0.65 (0.39–0.81)
Combined (70)	0.87 (0.78–0.93)	0.70 (0.54–0.80)	0.84 (0.73–0.91)	0.52 (0.28–0.67)	0.24 (0.04–0.41)

In this study, the inter-rater agreement between the radiologists and forensic anthropologist ranged from good to excellent, while the agreement between the radiologists and the other physicians (forensic pathologist and orthopedist) ranged from good to poor. The excellent agreement between the assessments of the radiologists and the forensic anthropologist may be due to the experience of the anthropologist working with conventional radiographs while assessing skeletal development and trauma. The forensic anthropologist was also involved in the development of the AFHS and was therefore well-aware of the specific definitions of the bone changes and stages of healing.

The intra-rater agreement for both radiologists, forensic anthropologist, and forensic pathologist was classified as excellent (confirming the reliability of the AFHS), while the intra-rater agreement of the orthopedist was fair. To cite the participating orthopedist, "Orthopedists use radiographs to visualize patients' complaints, but are not used to assess radiographs without any clinical information."

In previous publications, the inter-rater agreement was reported for a variety of stages with more or less detailed descriptions (2,11–13). de Boer et al. (2) reported the ICC values for 13 features observed during the radiological assessment of fractures. Prosser et al. (11) reported free-marginal multi-rater κ values separately for radiographs showing cases with and without cast, the cast being detrimental for evaluation. Two of the publications specifically mentioned that the raters were radiologists (11,13), while in the other two the profession of the raters is unclear (2,12).

As expected, using a number of different subcategories for a feature such as callus leads to low agreement between raters: For example, in one case, even though the raters agreed on callus being present, the subcategory given ranged from the early-stage "fluffy" callus, through callus being less dense, equally dense, or more dense than the cortical bone up to callus being firmly attached to the cortex (results not shown). When the state of the blurring of the fracture line was reported, a feature, which has been previously used to define certain stages in various fracture healing scales (8,10,12), it was found that, for example, the description "advanced blurring of the fracture line" was almost equally noted for each of the stages of the AFHS (results not shown). This finding suggests that it either posed great difficulty for the raters to assess when the blurring of the fracture line is actually advanced or that advanced blurring of the fracture line occurred at all stages of the healing process (which is unlikely for the early stages). Similarly, Halliday et al. (12) reported difficulties in the assessment of the definition of callus as opposed to presence/absence of callus as well as for the definition of fracture line. The reported κ values for the endosteal callus ranged from poor to good for three pair of observers, who were likely radiologists.

We assumed that using multiple images in known order would improve the reliability of the results, that is, the ICC coefficients will be greater for Part 1 than for Parts 2 and 3 of the test. This was the case for radiologist 1, forensic anthropologist, and forensic pathologist. We also hypothesized that in comparison with assigning a single stage from a small number of clearly distinguished healing stages as was the case in Part 3 of the test, the assignment of the final stage based on detailed descriptions as was the case in Part 2 would reduce the reliability due to the fact that using a multitude of different features and recognizing minute differences between the individual descriptions may complicate the decision process. Our assumption was confirmed by the ICC values for radiologist 2 and especially the orthopedist being higher in Part 3, even though radiologist 1 and forensic anthropologist had similar values in both parts. In summary, it seemed that using the AFHS with a small number of clearly differentiated stages and when possible evaluating a series of images showing the progress of fracture healing in known temporal order benefited the assessment process. Although it may be argued that including a smaller number of stages will result in broad fracture dating estimates, including a greater number of stages has been shown to result in an overall low reliability of the method due to the difficulty to distinguish among the multiple stages.

The agreement between the radiologists was poor when the absorption of bone at the fracture margin was considered (results not shown). This early stage of healing is important from the forensic point of view and is often addressed by forensic pathologists and anthropologists but is rarely if ever assessed by radiologists within the clinical context. Therefore, it is likely that the observations of this stage by radiologist 1 were guided by her awareness of the study's purpose, while radiologist 2 mostly assessed this stage as "no healing" possibly due to the need of using magnification features to correctly assess this stage. In contrast, forensic anthropologist and forensic pathologist showed good agreement with radiologist 1. Similarly, Boer et al. (2) reported poor to fair inter-rater agreement for early stages of fracture healing (smoothening of the lesion margin and absorption of cortical bone adjacent to lesion) when assessed on radiographs in comparison with assessments of histological specimens. Assuming that the technical parameters, such as the image resolution of digital radiographs, were sufficient for the assessment of the initial fracture healing stages, as shown by the good agreement among radiologist 1 and the forensic anthropologist and forensic pathologist, the assessment accuracy may be improved by training of raters less experienced in assessing the early stages of fracture healing.

The limitations of the study may include the fact that the results from the two parts of the test were based on less than 30 individuals (the recommended minimal number for the calculation of ICC), which resulted in broad CIs of the estimates. However, the overall number of individuals and radiographs (85) was sufficient and the different parts of the study provided valuable insight into the different aspects of the rating. Expanding the sample size would prove difficult, since the time required for the completion of the test needed to be restricted due to the workload of the raters.

This study focused solely on the healing patterns in tubular bones of adults. The healing of cranial bones shows a different pattern, lacking the typical callus formation stage. Using the initial, nonblinded assessment as the optimum may be considered questionable, but the excellent intra-observer agreement and almost identical ICC of the inter-rater agreement based on both the nonblinded and blinded assessment by radiologist 1 proved that the choice was appropriate. Moreover, there was little difference in the ICC coefficients between the radiologist and the other physicians when the assessment of radiologist 2 was used as the optimal staging, confirming that the assessment pattern of radiologist 1 did not show any major discrepancies when compared to that of radiologist 2.

The test was undertaken without specific training regarding the AFHS, only using detailed written instructions, to avoid introducing bias by trainers since it has been assumed that the descriptions of the stages are sufficient for practitioners who have got experience with the assessment of fractures on radiographs. Notably, the AFHS has been introduced mainly for forensic purpose. The stages used in the AFHS cover the whole spectrum of fracture healing. Although radiologists are used to assess and report on some of the stages, including callus formation, bridging, or remodeling, the results have shown that a specific training with focus on the less routinely used stages of the AFHS may be needed even for experienced radiologists.

The training in radiological assessment varies between countries, as, for example, in the United States radiology is part of a forensic anthropologist's training. Therefore, the findings would not necessarily apply to forensic anthropologists and other practitioners in the United States and other countries with different training background.

Conclusion

In conclusion, we tested the reliability of using the AFHS intended for the forensic assessment of the post-traumatic time interval on radiographs. The good to excellent ICC values achieved among the radiologists and forensic anthropologist provide good foundation for the use of the AFHS. The poor to good results for the other practitioners indicate that using the AFHS requires advanced training in skeletal anatomy and radiology. Nevertheless, a collaboration of experts from different fields is crucial for a comprehensive assessment of forensic cases of abuse and torture.

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Attachment 2

HISTOMORPHOMETRIC PARAMETERS COMMONLY USED FOR THE STUDY OF THE 3D BONE TRABECULAR MICROARCHITECTURE WITH MICRO-CT [108]

Degree of anisotropy (DA)

It is a measure of how highly oriented substructures are within a volume. Trabecular bone varies its orientation depending on mechanical load and can become anisotropic. This plugin uses the mean intercept length (MIL) method for determining anisotropy. Briefly, a large number of vectors of the same length originating from a random point within the sample are drawn through the sample. When each vector hits a boundary between foreground and background, an intercept is counted for that vector. The mean intercept length on that vector is then the vector length divided by the number of boundary hits. A cloud of points is built up, where each point represents the vector times its mean intercept length. Fitting an ellipsoid to the point cloud, construction of a material anisotropy tensor and subsequent eigendecomposition results in eigenvalues which relate to the lengths of the ellipsoid's axes (as the reciprocal of the semiaxis squared) and eigenvectors giving the orientation of the axes. DA is calculated as 1 - smallest eigenvalue / largest eigenvalue (note that due to the reciprocal square relationship, the longest axis has the smallest eigenvalue). New random points with the same vectors are sampled and DA updated with the new MIL counts until either the minimum number of sampling points is reached or the coefficient of variation of DA falls below a threshold.

Connectivity

The number of connected structures in a network can be determined by calculating the Euler characteristic. Trabecular bone is one such network, and its connectivity density (Conn.D) can be calculated by dividing the connectivity estimate by the volume of the sample. The algorithm in BoneJ's Connectivity uses voxel neighbourhoods to calculate the Euler characteristic of the volume and adjusts this to give the contribution of the volume to the connectivity of the structure it was cut from. An assumption is made that there is only one particle in the foreground; to achieve this, run Purify prior to Connectivity. A warning will be displayed if connectivity is negative, which is an unnatural result.

BV/TV fraction [%] (bone volume fraction)

It is the volume of mineralised bone per unit volume of the sample. Volume Fraction provides two methods for measuring BV/TV: voxel-based and surface mesh-based. In the former, BV/TV is simply the number of foreground (bone) voxels divided by the total number of voxels in the image. In the latter, a surface mesh is generated using the foreground as a template and BV is the volume contained by the surface, while TV is the volume enclosed by a surface wrapped around the total test volume.

Thickness [mm]

The thickness at a point as the diameter of the greatest sphere that fits within the structure and which contains the point. The plugin calculates mean and standard deviation of the trabecular thickness (Tb.Th) and trabecular spacing (Tb.Sp) directly from pixel values in the resulting thickness map. The plugin assumes that trabeculae are the foreground (255) of a binarised image. Processing time is heavily dependent on feature size (in pixels); large features can take a very long time to process. In these cases, consider reducing resolution so that each feature is resolved by fewer, larger pixels.

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