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INTERPRETATION OF TRAUMA AND TAPHONOMY IN A MODERN KNOWN SKELETAL POPULATION: IMPLICATIONS FOR FORENSIC ANTHROPOLOGY

Ph.D Thesis

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To my family

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ABBREVIATION:

ASL	Azienda Sanitaria locale (Healt Local Unit)
BMU	Basic Multicellular Unit
BFT	Blunt Force Trauma
CBCT	Cone Beam CT
DPR	Presidential Decree of the Italian Republic
DR	Digital Radiology
DXA	Dual-energy X-ray absorptiometry
DJD	Degenerative Joint Disease
FA	Forensic Anthropology
Gly-A	Glicoforin A
Hb	Haemoglobin
H&E	Hematossilin and Eosin staining
ISTAT	Istituto nazionale di statistica (National Statistics Istitute)
LABANOF	Laboratory of Forensic Anthropology and Odontology
PMI	Post Mortem Interval
PTI	Post-Trauma Interval
RBC	Red Blood Cells
SEM	Scanning Electron Microscopy

LIST OF PAPERS:

Papers published/accepted/submitted in several International Journals and included in the thesis as a part of methodologies and results:

***Cappella A**, Amadasi A, Gaudio D, Gibelli D,Borgonovo S, Di Giancamillo M, Cattaneo C;*
The application of Cone-Beam CT in the aging of bone calluses: A new perspective?
International J Legal Medicine, 2013; 127(6):1139-44. doi: 10.1007/s00414-013-0824-*9.*

***Cappella A**, Castoldi E, Sforza C, Cattaneo C*
AN OSTEOLOGICAL REVISITATION OF AUTOPSIES: COMPARING ANTHROPOLOGICAL FINDINGS ON EXHUMED SKELETONS TO THEIR RESPECTIVE AUTOPSY REPORTS ON SEVEN CASES.
Forensic Science International. DOI 10.1016/j.forsciint.2014.09.003

***Cappella A**, Amadasi A, Castoldi E, Mazzarelli D, Gaudio, Cattaneo C*;
The difficult task of assessing perimortem and postmortem fractures on the skeleton: a blind test on 210 fractures of known origin.
Journal of Forensic Sciences 2014 Jul 3. doi: 10.1111/1556-4029.12539

***Cappella A**, Stefanelli S, Caccianiga M, Rizzi A, Bertoglio B, Sforza C, Cattaneo C.*
Blood or spores? A cautionary note on interpreting cellular debris on human skeletal remains.
International J of Legal Medicine. Accepted

***Cappella A**, Gibelli D, Muccino E, Scarpulla V, Cerutti E, Caruso V, Sguazza E, Mazzarelli D, Cattaneo C.*
THE COMPARATIVE PERFORMANCE OF PMI ESTIMATION IN SKELETAL REMAINS BY THREE METHODS (C-14, LUMINOL TEST AND OHI): ANALYSIS IN 20 CASES.
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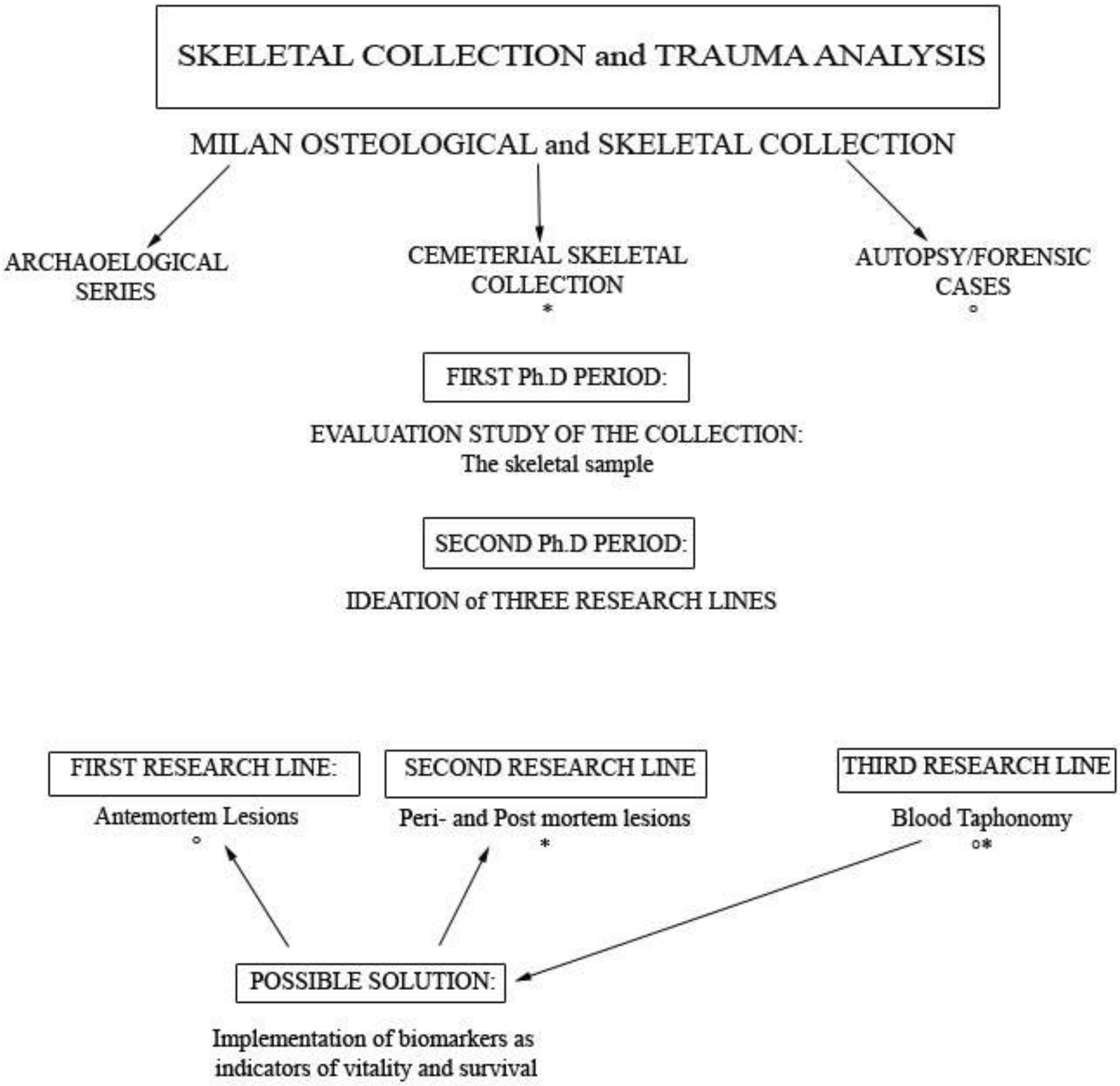
ABSTRACT

In Forensic Anthropology, trauma analysis is essential for a correct understanding of the cause and the modality of death as well as for the interpretation of previous traumatic events which occurred in life. Both have important implications in criminal and humanitarian scenarios: to demonstrate whether a crime was committed, and to ascertain torture and maltreatment. Nevertheless, the assessment of a traumatic event, whether antemortem, perimortem or potmortem, is extremely challenging and often limited and altered by multiple variables, namely taphonomy. Few are the validated scientific methods which can help anthropologists in the interpretation of skeletal trauma, and which are often limited by the lack of known skeletal material on which to conduct research in order to acquire data, to validate methods and to study and create theoretical trauma and taphonomy models.

Among the many known documented skeletal collections already presented officially to the international scientific community very few are equipped also with known/control data on trauma, and cause and modality of death. The Milan cemeterial Skeletal Collection, which is only part of the more consistent Milan Osteological Skeletal Collection housed at LABANOF (University of Milan) is one of the latter, and its presence has offered the chance to conduct research on the difficult field of trauma and taphonomy included in the Ph.D project.

The first ever anthropological study of the skeletal sample of the Milan skeletal collection here presented, has been the starting point which permitted us to highlight the numerous diagnostic difficulties concerning trauma analysis and has provided the material on which to conduct the applied research for analysing the state of the art currently used by the discipline. As a consequence of the many limits arising from the first two research lines (on the diagnosis of antemortem lesions as well as the identification of perimortem injury), which appeared not satisfactory enough for a correct interpretation of skeletal lesions, the research has focused on the novel field of blood taphonomy in order to provide knowledge for the use of cell components and biomarkers as indicators of vitality and survival on skeletal trauma which represents more reliable future possible alternative solutions.

THESIS CHART



Chapter 1

1.1.
INTRODUCTION

Forensic Anthropology (FA) is a sub-discipline of physical anthropology that traditionally deals with the study of human skeletal remains for legal purposes: the term “forensic” refers to sciences applied to legal and criminal subjects. In fact, as a sub-discipline, forensic anthropology represents "the application of physical anthropology to the forensic context" which offers a valid contribution, often complementary, to the medico-legal investigations and practices. For instance, scenes of crime in which the findings involve partially or completely skeletonised human remains demand more specific knowledge in osteology and anthropology, and often forensic pathologists might not have a proper training to retrieve and deal with this topic. On the other hand, forensic anthropologists investigate fully or semi-skeletonised modern human remains by using scientific techniques developed in the field of physical anthropology as well as of forensic sciences, and their main objective consists in assisting a positive identification of the decedent, and, if possible, assessing skeletal trauma, fundamental for the diagnosis of cause and manner of death.

For a correct understanding of the cause and the modality of death, the analysis of trauma is essential, especially on bone, where the lesions are preserved for a long time, thanks to the resistance of bone tissue to decomposition. The analysis of trauma should include both determining the time when the injury occurred with respect to death (antemortem, perimortem or postmortem) and the modality of production of trauma (gunshot injury, blast force, sharp force or blunt trauma). In this scenario, the forensic anthropologists can play a crucial role in the investigation of skeletal remains, especially regarding trauma analysis; thanks to their specific knowledge on bone trauma analysis, they can identify skeletal traumatic signs of great importance, especially in a court of law where the interpretation of that trauma, and all the events related to it, could have tremendous implications in terms of legal consequences. The trauma analysis permits one to detect possible skeletal lesions, and to interpret the mechanism of production of the injury from which the diagnosis of the cause of death could derive. It therefore appears clear how such an analysis offers important evidence that can demonstrate whether a crime was committed.

The assessment of a traumatic event could be of extreme difficulty; it is, in fact, often limited by multiple variables that play a key role in altering or hiding fundamental evidence, specifically the effects derived from taphonomy. Forensic anthropologists, in fact, do not have access to a number of biological markers that forensic pathologists have in the case of analysis of fresh cadavers. The diagnosis of bone lesions that consists usually in distinguishing between antemortem, perimortem and postmortem is based uniquely on macroscopic and morphologic parameters. However, both the interferences of taphonomy and the lack of features specific of vitality and early bone healing reactions make it even harder to reach a correct and successful interpretation of a traumatic event in skeletal remains. This is true also when additional information regarding trauma is required. In fact, discriminating trauma that occurred at or immediately prior to the time of death (which can provide also important evidence about cause and manner of death) from that occurring after is provided by the morphology of lesions; however not always is it possible to differentiate this from post-mortem damage to fresh or nearly fresh bone. Thus, the vital reaction of bleeding or bone remodelling with healing could be the only sign for demonstrating that a traumatic event preceded death; but at the moment no detailed information about their persistence and appearance during decomposition is available in terms of time and detectability. Many are the questions about the chance to determine with more precision the timing of injury especially regarding life's traumatic events or trauma around death. In this perspective, it is important not only to distinguish between peri and post-mortem lesions, which sometimes reveals itself to be very hard (especially in case of blunt trauma and fractures) but also interpret the perimortem lesions with respect to vitality and time elapsed since trauma. If any traces of blood or biomarkers of early healing reactions are found in dry bone tissue in skeletal lesions could they be used as a tool or markers for giving information about the vitality of that lesion?

Although anthropology has developed enormously in the last few decades, especially in the extrapolation of demographic data from the analysis of skeletal remains, the more difficult topics such as injury interpretation and taphonomy have not achieved the same success, in part due to the absence of material in which these characteristics are known and where to conduct research in order to build models and deduce knowledge to apply in the field.

This research project is based strictly on the anthropological study of part of the Milan skeletal collection, which is unique and of worldwide significance thanks to its own characteristics (a contemporary and very consistent population) and to the existence of information concerning both demographics and data related to trauma and cause/manner of death. The study of part of the collection, here presented for the first time, is only partial, but it has made it possible to investigate in depth the issues related to the analysis of trauma as well as taphonomy in order to interpret correctly skeletal injury in human remains.

The analysis of skeletal trauma is a topic in which the forensic anthropologist can greatly contribute to the reconstruction of the circumstances of death; nevertheless this field still needs to be proven in detail and improve its own knowledge by developing new approaches and adding know-how in the interpretation, which could involve biomarkers and the use of advanced technologies for their detection.

However, before approaching at the main issues of the present project, a brief discussion concerning the general theme of skeletal collections and trauma in forensic anthropology is necessary.

1.2.
SKELETAL COLLECTIONS AND SCIENTIFIC RESEARCH

The existence of skeletal collections has always provided a great contribution to science through the development of basic information in various disciplines; their importance for anthropology, medicine, anatomy and biology (human variability) is well documented (1-5).

The acquisition of useful knowledge from a scientific point of view is the reason behind the creation of such collections, whose assembly often includes complete and partial skeletons, as well as anatomical parts derived from cadavers (6-8). The presence of university and museum skeletal collections is therefore a valuable material for anthropology and forensic science in the study of trauma, disease, or simply of biological variation; it is for this reason that it is necessary to disclose the availability of such collections as well as all results derived from research conducted on them.

Among the many known documented skeletal collections from the scientific academies - whose existence is known due to the numerous scientific publications that have made their reputation - the collections most mentioned are the Hamann-Todd Osteological Collection housed at the Department of Physical Anthropology, in the Cleveland Museum of Natural History (http://www. cmnh.org), the "Terry skeletal collection" housed in Washington, DC at the Department of Anthropology in the National Museum of Natural History (2), the "William and Bass donated skeletal Forensic Collection", just to name some of the most famous. Nevertheless many other collections exist and some are still under study or in development (1-5, 9-10) but their presence is not known often because the lack of their international scientific dissemination.

Most of the referenced osteological collections date back to the late 18th, 19th century; nevertheless the most common standard technical methods were developed in the past decades on them, like on the Hamman-Todd Collection of "Cleveland Museum of Natural History", the "Terry and Hungtinton Collection" of the Department of Anthropology at the National Museum of Natural History, and the "Dart Collection" of the Department of Anatomical Sciences at the University of the Witwatersrand (Johannesburg, South Africa).

All the above mentioned collections were assembled in the nineteenth century or during the first half of the twentieth century (1-2, 7, 11).

Nowadays, other important referenced collections known are listed below among the most known:

“Robert J. Terry Anatomical Skeletal Collection”, Washington, DC, USA:
This collection, housed at the Department of Anthropology in the National Museum of Natural History, includes 1728 individuals (461 white males, 546 black males, 323 white females, 392 black females, 5 Asian males, and 1 individual of unknown origin) with dates of birth ranging from 1822 to 1943 and ages at death ranging from 16 to 102 years. Demographic information includes age, sex, ancestry, cause of death, and pathological conditions. Anthropometric measurements, cadaver photographs, dental charts, death masks, and hair samples are available for some individuals. It is considered as one of the most important skeletal collection in the word and over the time many aging methods were examined on the skeletons derived from this specific collection and so considerations on sex, metric and non-metric variants as demonstrated by the numerous publications (12-20).

“Hamann-Todd Osteological Collection”, Cleveland, Ohio, USA:
The collection is housed at the Department of Physical Anthropology, in the Cleveland Museum of Natural History and it includes over 3000 skeletons. The composition and the documentation is still unknown despite the numerous methods and publication based on its sample (21-24),

”William M. Bass Donated Skeletal Collection”, Knoxville, Tennessee, USA:
assembled at the Department of Anthropology, University of Tennessee, Knoxville and it contains around 900 adult individuals along with some infant and fetal remains and cremains. Demographic data is available for most individuals and includes age, sex, ancestry, cause of death, and body mass (25-26),

“Raymond A. Dart Collection of Human Skeletons”, Johannesburg, South Africa:
It is a modern collection of South African individuals of both European and African ancestry housed at the School of Anatomical Sciences, University of Witwatersrand . The total number of individuals and demographics of the collection are unknown despite the numerous publications reporting results of new methods starndardized on its skeletons (27-29).

“Cape Town Documented Skeletal Collection”, Cape Town, South Africa:
The collection is derived from dissection cadavers used by the Department of Anatomy and Cell Biology, University of Cape Town (where it is also housed), between 1980 and 1996 and is of unknown size. Documentation includes sex, ancestry, and age (29).

“George Huntington Collection”, Washington, DC, USA:
This collection comprised of over 3600 individuals with dates of death from 1892 to 1920. Age, sex, nationality, and cause of death are known. All individuals are either European immigrants or New York City residents. Many skeletons are incomplete due to their origin as dissection cadavers. As the “Terry Collection”, it is housed at the Department of Anthropology, National Museum of Natural History

”Coimbra Identified Skeletons Collection”, Coimbra, Portugal:
Composed of 505 identified skeletons. Dates of birth range from 1826 to 1922, and dates of death range from 1904 to 1938. The collection is housed at the Department of Anthropology, University of Coimbra (30-32),

”Luís Lopes Collection (Lisbon Collection)”, Lisbon, Portugal:
housed at the Department of Zoology and Anthropology, National Museum of Natural History (Bocage Museum) in Lisbon; it consists of 1,692 identified skeletons from modern cemeteries in Lisbon, Portugal. Dates of birth range from 1805 to 1972 and dates of death range from 1880 to 1975. Only 699 are currently documented and available for study. Demographic data available includes age at death, date and cause of death, place of birth, occupation, and place of residence. 92 of the individuals are subadults, while the majority of the individuals were age 40 or older at time of death. (9),

“Christ Church Spitalfields Collection”, London, England:
housed at the Natural History Museum of London (“British Museum”) and it incudes 968 individuals excavated from the crypt at Christ Church, Spitalfields, dating from 1729 to 1859 AD. Information about the individuals in the collection stems from coffin plates associated with them (33-35),

”Maxwell Museum Prehistoric Native American Collections”: Albuquerque, New Mexico, USA housed at Maxwell Museum of Anthropology, University of New Mexico; It includes a large number of prehistoric Native American remains collected from archaeological sites in New Mexico and surrounding areas. All the remains are registered in accordance with NAGPRA, and some repatriations have been completed. The majority of the currently housed remains were excavated before 1960. The Collection also includes 257 individuals as of February 2008, although only 207 of those individuals are positively identified, and all but one of the individuals were residents of New Mexico. Ages at death range from fetal to over 80 years. The amount of documentation varies depending on the source of the remains, but typically includes age, sex, and ancestry (8),

“University of Athens Human Skeletal Reference Collection”, Athens, Greece:
The collection is housed at the Department of Animal and Human Physiology, University of Athens and contains 225 modern skeletons from the mid to late 20th century from cemeteries around Athens, Greece. Documentation is available for almost all individuals and includes age, sex, occupation, and cause of death (3),

“J.C.B. Grant Collection”, Toronto, Ontario, Canada:
The collection consists of 202 individuals who died and were received by the Department of Anthropology, University of Toronto, between 1928 and the early 1950s. Most of the individuals are male and aged 40 years or older. Many were transients, migrant workers, or recent immigrants at time of death. All but one are of European ancestry. Demographic information consists of sex, age at death, and cause of death (36).

“Sassari Collection” Sardinia, Italy:
Part of this collection is the Frassetto collections, which is housed at the Museum of Anthropology, University of Bologna. In general the collection contains complete skeletons from 606 individuals who died in the early 20th century and were buried in local cemeteries. Sex, age, and date of death are available for most individuals, and date of birth and occupation are available only for some, but more details are not documented (37)

“Scheuer Juvenile Skeletal Collection”, Dundee, Scotland:
It is a collection of over 100 juvenile skeletal remains from archaeological, anatomical, and forensic sources, housed at the Centre for Anatomy and Human Identification, University of Dundee. Not all skeletons are complete or fully documented.

“Weisbach Collection”, Vienna, Austria:
It is a medico-historical collection from the end of the 19th Century hosted in the Federal Museum for Pathological Anatomy and contains a large collection of osseous material and clearly genetics disorders amongst the adult skeletal specimens (38). It is an important research resource despite it is not widely known and documented.

“Francis J. Rainer Human Osteological Collection”, Bucharest, Romania:
It is one of the largest collections of identified skeletons, named after its founder Rainer, housed at the Anthropology Institute of the Romanian Academy in Bucharest. Its origin dates back to the early 1900s, continuing until the 1950s, with a main core originating from the 1930-1940 period. It consists of approximately 6800 well-preserved human skulls (which 3585 have been inventoried and belonged to 2123 adult males, 1194 adult females and 214 children) and only half have known identity. Also post-cranial specimens are available but not yet documented (39).

“Romulo Lambre Collection”, La Plata, Argentina:
The Lambre Collection is housed in the School of Medical Sciences of the National University of La Plata and it consists of skeletal remains ceded by the Municipal Cemetery of La Plata. The collection has more than four hundred skeletons, with information on age, sex, nationality, date and cause of death (40).

The collections mentioned above are just some of the most famous osteological reference collections; a complete list, which also includes other worldwide collections, is reported in a recent publication (11).
During the last five decades, many methods for determining sex and estimating age were developed using samples from the skeletal populations mentioned above. In particular, the vast majority of methods used for forensic anthropological analysis on human skeletal remains of judicial cases, are mainly based on reference samples from both the Terry and Hamman-Todd Collection (11). As a result, there has been a global trend of growth of the

scientific research in forensic anthropology for the validation of methods and techniques adapted to the specific characteristics of local populations, which meet the existence of biological variability. This was achieved through the creation of local collections of samples and reference (39-46).

The characteristics of a reference skeletal collection so-called 'ideal' according to Usher *et al.* (11) are three: known age at death for all skeletons; adequate representation of the variability of a population in relation to the different socio-economic status, race and health status; and good presentation of all ages and both sexes. This is especially true with reference to the study of biological anthropology on the ancient populations of the past that takes advantage of the anthropological studies of skeletal reference populations known from a demographic point of view, but belonging to much more recent times.

In this scenario, the question of limits and problems derived from the use of specific skeletal collections as reference for all populations is still a currently open debate in anthropology (8,11-12, 47); the core issue consists in the validity of using such skeletal collections as an unambiguous reference for comparative study of various aspects of biology population of the past. The differences in variability between different populations in this respect are already recognized, however, they can neither be identified nor quantified specifically for each sample or skeleton (8).

These issues have been the focus of a great research in fields such as paleodemography, paleo- and bioarchaeology, born as a result of a number of methodological criticisms raised by various authors (48-52), however the same debate about the use of reference collections as a model for the development of anthropological methods applied also to the forensic field is not highlighted equally despite the resulting implications might be different and far more serious. If, on the one hand the implementation of skeletal collections belonging to more recent times (on the eighteenth, nineteenth and twentieth centuries) and used as a reference for skeletal biology and variability of past populations is widely questioned, their use as a reference to the contemporary population does not have the same dispute.

The key issues arise from various distortions in the estimation of fundamental demographic parameters for the construction of a biological profile, which must be as close as possible to that of the individual to which the remains belong to (age, sex, ancestry, stature, specific characteristics). These issues are very relevant to the planning of the research in the field of forensic anthropology, which must inevitably rely on local contemporary skeletal reference collections. Thus, when the research focuses not only on the variability but also on the most difficult forensic topics of related-cause of trauma- possibly significant for modality and cause of death- there is almost a total absence of reference skeletal collections in this regard, so we come to the urgent need to assemble skeletal populations that could be useful as a control also for a field of study as complex as trauma analysis.

In this scenario, the Milan skeletal and osteological collection housed at the University of Milan, still in progress of assemblage and study, has not been yet officially presented in detail to the academic world; despite its limited fame and short life span this collection represents one of the largest worldwide collections as regards both the population size and diversity, as well as the presence of either various trauma models and violent cause of death. Its importance lies in all associated documentation where demographics, clinical data and causes of death are reported. In this sense, the collection has all the advantages deriving from the study of a contemporary population, for both the demographic and trauma analysis purposes, and so it represents to all effects the typical local reference collection on which to conduct scientific research in the forensic field.

1.3.
TRAUMA ANALYSIS IN FORENSIC ANTHROPOLOGY

The term “injury” or “wound” can be best defined as “damage to any part of the body (tissue and organs) caused by the application of a mechanical insult“. An injury or a wound occurs only when the intensity of the applied force exceeds the tissue capability to absorb or adapt to that specific stress. The effective damage depends on the nature of the tissue as well as the type of the mechanical insult (53).

As a concern, even in anthropology as in pathology, a “trauma” is commonly referred to as a physical injury or wound to the living bone tissue caused by an external source, whether intentional or accidental.

In case of investigation on skeletal remains, burned corpses and badly preserved cadavers, one of the most important issue for forensic anthropologists it is to identify and to interpret correctly every single skeletal lesion and trauma. In every case, in fact, an accurate skeletal trauma analysis is always necessary in order to provide key clues/evidence in the determination and manner of death, which of course can be determined at the end only by specialists like forensic pathologists (54).

In trauma analysis, the association of a skeletal injury to the time of death is the first consideration to be made; for every single lesion (no matter the typology) it is always necessary to evaluate if it occurred prior to, around the time of, or after death (55). This last account is true and applicable for all types of lesion, whether they are fractures, cut or sharp force marks, blunt force injuries or gunshot wounds. Then, a detailed analysis is indispensable whenever bone traumatic injuries are found and distinguished from environmental and taphonomic alterations (56), which consists in evaluating the position, the morphology and the significant characteristics of all injuries, and whenever possible in trying to interpret the nature of a trauma and the probable weapons involved in the modality of its production (57). The resulting pattern of trauma will contribute to the determination of cause of death and the interpretation of events around death in the medico-legal setting (58). To sum up, the analysis of trauma carried out by the forensic anthropologist consists in three distinct steps:

- The identification of the skeletal defects and establishment of the timing of injury;
- The determination of trauma patterns and the following interpretation of its modality of production;
- The linking of the trauma patterns to the anatomical context and the comprehension of the sequence of events that could have produced the observed defects (59).

As mentioned above, the identification of skeletal lesions and their establishment of the timing of injury is the starting point for any evidence of trauma found in the skeleton and so it is for this present study. To clarify this concept, it must be said that the timing of injury could be defined more specifically as "determining when the bone injury occurred in relation to death"; this is a very critical issue for various aspects that will be explained in some of the next paragraphs, in general, injuries to bone can be defined as "*antemortem*", if they occur during life, "*perimortem*" if they are formed at or near the time of death or as "*postmortem*" if they are produced after death. The diagnosis of a lesion in this sense is based commonly on the gross macroscopic appearance that will be discussed in detail in the next paragraphs as are all the considerations, limits and problems involved in the determination of such evidence

Fig 1.1: Example of antemortem lesion (left), perimortem fracture (center) and post-mortem fracture (right) in long bones.

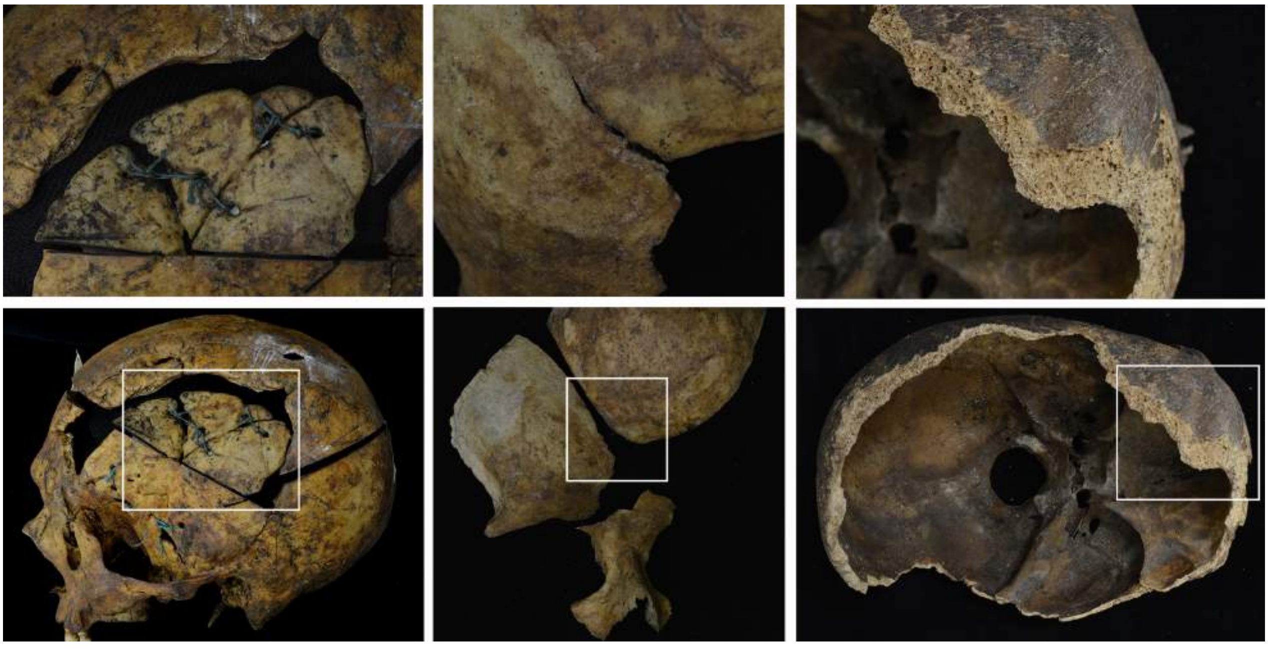


Fig 1.2: Example of antemortem lesion (left), perimortem fracture (center) and post-mortem fracture (right) in the skull.

1.3.1.
ANTEMORTEM TRAUMA

The analysis of trauma with respect to time is only established in reference to the time of death: the lesions defined as "*antemortem*" have all occurred before death. The possibility to define when an antemortem trauma event occurred could reveal precious information in the identification process of skeletal remains belonging to unknown individuals, but also in the trauma interpretation as in cases of maltreatment, abuses and tortures that a person was subjected to during his life. Once a lesion has been designated as "*antemortem*", because of the evidence for bone remodelling, no further temporal information are determined in a detailed way in case of dry bones; nevertheless a well-understood series of events normally ensues (60-61), and some can be recognizable in skeletonised remains through gross macroscopic appearance. In fact, bone is a living material that is habitually exposed to mechanical environments, which challenges its structural integrity. During life, when such integrity is disrupted (as in cases of fractures) the bone tissue (being a living tissue) can repair itself, no matter what is the cause of fracture or the typology of damages. In this perspective, new osseous tissue can be formed where there is a damage or disruption thanks to the osseous tissue regeneration power of bone that is strongly activated during the healing process. (62). Once a bone is broken, strength and stability have to be restored quickly and correctly for giving it the same property it had before and to allow it to continue functioning properly. Bone healing is a natural process only partially understood according to Hendrix (63), from both a pathological and physiological point of view. In fact, it has been studied at a clinical, radiographic, molecular or histological level. It is a very complex process in which many cells and events are involved and in which the consequent result is the reconstitution of the injured tissue and the recovery of the original form, functions and physiology.

1.3.1.1.THE HEALING PROCESS AND CALLUS FORMATION

Histologically, the healing process consists of a coordination of multi-steps where the coordinated participation of different cells is involved in: immigration, differentiation and proliferation of platelets, inflammatory cells, angioblasts, chondroblasts, endothelial cells, fibroblasts and osteoblasts. All these cells participate actively by synthesizing and realising bioactive constituents of bone tissue components (growth factors, collagen and so forth). Among these types of cells, osteoblasts are considered to be the cells responsible for the synthesis and mineralization of the bone matrix (64-66). At the same time also a resorption activity is performed by osteoclasts, which eliminate the necrotic and dead tissue around the injury site.

In histological terms fracture healing could be divided into two different healing steps: direct fracture healing defined as “primary” and indirect fracture healing named also “secondary”. In most cases the events of the healing process have always been elucidated by using experimental fracture healing in animal models (mostly rats) (67) or by using clinical radiological follow up analysis in human patients (63, 68).

Direct fracture healing refers to a direct attempt to form new Haversian systems through the formation of discrete remodelling units (‘cutting cones’) which restores mechanical continuity; this typology of process occurs only in case of anatomic reduction of the fracture fragments reached by internal fixation or decreased interfragmentary strain (69). In this process there is no formation of bone callus, in fact little or no periosteal response is present, but only direct differentiation of osteoblasts form osteoprogenitor cells derived from vascular endothelial and perivascular mesenchymal cells (69-70).

Nevertheless, the indirect fracture healing represents to all effects the most common process observable in case of fracture healing. In this process a fracture is repaired by the growth of new tissue that develops in and around the site of the fracture The new tissue, which sooner or later unites the two extremities by forming a bridge, is called callus. In this case, in fact, the process involves the formation of a callus as a result of a combination of two different ossification processes: intramembranous and endochondral (70). In this typology of healing the periosteal activity takes place in a key role: here reside osteoprogenitor and undifferentiated mesenchymal cells that migrate in the fracture site where intramembranous ossification has started to form new bone directly, without firstly forming cartilage. The final result is the formation of a callus, histologically called “hard callus” and in this typology of healing, also the marrow bone gives its contribution at the very early stages, when endothelial cells differentiate into polymorphic cells which express osteoblastic phenotype (71).

The recruitment, proliferation, and differentiation of the migrant undifferentiated mesenchymal cells are governed during the endochondral ossification; a process in which the new cartilage tissue just formed becomes calcified and then replaced by new bone. This type of fracture healing, is contributed to from both the adjacent to the fracture periosteum and the surrounded external soft tissues, which all provide an early bridging callus, histologically characterized as ‘soft callus’, that stabilizes temporarily the fracture fragments (67).

This healing process for most of the authors (62, 66-67) consists of six identifiable stages:

1. an initial stage of haematoma formation and inflammation,

2. subsequent angiogenesis and formation of cartilage
3. cartilage calcification,
4. cartilage removal,
5. bone formation
6. bone remodelling

Although, according to Hendrix (63) that reported the healing process as a process separated only arbitrarily into stages, the literature shows little consistency in terms of stages of bone healing.

A great amount of literature deals with bone repair, for instance, over the past decades they have named the callus in different ways, which can confuse the reader. Actually, to simplify what happens in the bone repair process, one should just know that only one callus develops and is remodelled as it grows as any other bone structure (72).

Whenever, there is an agreement on the events which occurred in a bone fracture, before the healing and remodelling process: blood pools into the fracture area due to the disruption of vessels where it coagulates and forms a hematoma and a blood cot (73-74); osteoblasts proliferate and form new bone as a consequence of the disruption of osteogenic tissue lining the bone (endosteum and periosteum) and finally necrotic and dead bone derived by the separation of bone cells from the vascular system (osteocytes have not longer a proper nutritional connection) is resorbed. This latter event does not necessary take place immediately after fracturing but can be included into the subsequent healing process (73). After fracturing occurs, some osteoprogenitor cells differentiate into fibroblasts and other supporting cells, which form the first organization at the fracture site: a soft fibrous granulation tissue. This tissue will be formed at either end of the fracture segments and extend into the blood clot. It is formed by the activity of a group of cells: osteocytes at this step are not included because no new bone is already synthetized. Instead, osteoblasts start to product new bone, osteoclasts resorb some necrotic tissue, progenitors cell continue to generate new osteoblasts, macrophages eat away the hematoma, fibroblasts extend their activity in producing intercellular material. In addition, new blood vessels come into the fracture line from the surrounding soft tissue (63, 74). Then, a formation of a primary callus across the fracture represents the next stage; when it becomes complete it will give great stability, even if it is composed by woven immature bone. The primary callus, in fact, is a bone tissue not yet calcified and still slightly soft. At this stage it is possible to recognize different parts of the callus based on its position and origins: the intermediate or sealing calluses, which lie at the two fracture ends; the endosteal callus which is the part of the callus that units the open marrow spaces; and the periosteal callus that is the most visible because it bridges the two end fragments around the outside of the fracture.

There are no structural distinctions between these three types of calluses (which are distinguished only as a concept of the process) but the only difference is linked to their origin. The intermediate callus is created on the granulation tissue by osteoblasts that deposit the osteoids into the fibrous connective tissue, where they become ossified, and where their orientation follow the fibrous matrix one. The endosteum callus is formed into the fracture line from osteoblasts derived from endosteum. The periosteal callus is produced by osteoblasts on the periosteum and its particularity is to have often a bulging shape; this latter is visible macroscopically in skeletal remains (or bone samples) and more identifiable than the other two on radiographic images, on which is based actually the clinical follow up of injury (63, 74).

Once a primary bone callus is created, the remodelling stage will take place; the replacement of woven bone with a more organized lamellar bone operated by basic multicellular unit (BMU) will remodel the primary bone into a callus referred to as “secondary”, a more stable callus. The BMU are units that remodel the tissues in all stages of the process after fracturing: calcified cartilage is first resorbed and then substituted by woven bone then woven bone is removed and replaced with new lamellar bone. The cells produced in BMU are osteoclasts that remove a packet of preexisting tissue and then osteoblasts that replace the removal of osteoclasts with a packet of newly bone. The remodelling stage by BMU always follows precise activity in a specific sequence: activation, resorption, formation which require 3-4 months for building a “secondary” callus since its start (74). The BMU are to all effects the ones responsible for the modelling stage, which means conversion of calcified cartilage to woven bone and of woven bone to lamellar bone; the resultant mature callus will be sooner subjected to a remodelling stage that permits a final healing.

These phases are in general those that represent histologically the healing process even when different researchers rarely describe the process exactly as one or the other (63). In fact, as mentioned before, many researchers name or divide the stages differently and so the exact time associated to it (73-78); nevertheless an agreement in the general process, especially in the first stages could be summarized as reported above.

The process has been rather investigated in radiology for clinical studies (mostly in the orthopaedic field) in the in vivo follow-up of the mechanical stability of fracture repair and mineralization as an objective evaluation of the course of a successful healing. Also in radiological examination, as in the histological analysis the healing process stages are generally divided arbitrarily. As reported in detail by Hufnagl (68) some researchers (63, 79) have presented in agreement the best description and outlines of what stages of bone healing are observable in radiographs: up to six stages of bone healing have been described based mostly on the features of the fracture line, fracture gap, and inner structure of the newborn callus, using criteria such as the presence of bridging or obscuration of the fracture line (lucent, sclerotic, or invisible), fracture margin (that can be sharp, blurred, or invisible), visible bridging activity (starting, partial, or complete), and the deposition of new tissue inside the callus and its mineralization.

1.3.1.2. THE STATE OF THE ART IN DATING FRACTURES IN FORENSIC ANTHROPOLOGY

While histological and biomolecular research in animal models for the understanding of bone healing and radiographic assessment of fracture healing for orthopedic clinical examinations has been carried out, similar applications in the forensic field are relatively absent and few are reported in literature. If on one hand the healing process is well understood in forensic pathology (80), especially concerning soft tissue lesions, the timing of specific response remains still unexplored directly on dry and skeletal remains. In addition defining post traumatic interval becomes a really difficult task on both the bone calluses and the evidence of healing when visible on skeletal remains. Bone tissue response also follows a strict time-dependent sequence that can vary depending on multiple variables like type of lesion, location, bone type involved, age, health status (74, 77, 81-83). In literature some works (84-86) report the minimal period required for initial osseous evidence of healing responses on skeletal remains, especially based on the gross appearance. Other important studies focused on histological fracture dating of fresh and dry bone tissue (86) reporting a detailed healing phases and time table for natural fracture healing based on documented forensic cases and an extensive pathological literature on fracture healing (86-92). The approach has been proven in both adult and child individuals (85-86). In addition, recent research (93-94) has focused on the evaluation of what features can be still detectable and adequate in the assessment of traumatic lesions in dry bone material by using histology and radiology; while the results have shown the chance to still find many features useful in the estimation of post-traumatic time interval of fractures and amputations, they were based only on archaeological material /callus or fractures of unknown origin) with the intent to prove the chance to use such features in dating fractures and not to prove their validity or reliability on known materials.

1.3.2.
PERIMORTEM VS POSTMORTEM TRAUMA

Distinguishing between postmortem and perimortem lesions in skeletal remains is one of the major problems in the forensic field. While there is the chance to exclude a lesion as antemortem because of the lack of features such as the osteogenic reaction or signs of healing, it is much less easy to define signs as perimortem, although of great importance for the interpretation of the cause and modality of death. There are many factors influencing the success in the identification of a fracture (such as perimortem or postmortem) that must be taken into account if a forensic evaluation of trauma is required: the different taphonomic and environmental conditions, the time elapsed since death (PMI), the type of bone tissue attacked by the injury and the variety of postmortem events which might determine post-mortem changes on the remains. The taphonomy and postmortem variables are therefore the main problem to be taken into consideration if the anthropologist approaches the analysis of trauma on the remains (55).

However, perimortem injuries, postmortem changes and taphonomic alterations (these latter can naturally appear during the decomposition process) may be observable and potentially distinguished (95).

The term "postmortem trauma" refers to a bone lesion occurring after the death of an individual; typical post-mortem lesions are those caused by environmental factors, such as carnivore tooth marks, surface erosions, sun bleaching, weathering etc, and by accidental events produced by weight, human activity, transport and fortuitous trauma (96).

The term "perimortem lesion" in forensic anthropology, means a lesion occurring shortly before or shortly after death; any injury directly associated with the manner of death or the handling of the remains is considered a perimortem injury (55). Nevertheless, according to Wieberg and Wescott (98), the term "perimortem" seems to be ineffective in a forensic context since it refers to a temporal period instead of a physical condition (fresh or dry). Few authors discuss how long perimortem fracture characteristics persist into the post-mortem interval (PMI) or the cause of their changes. Therefore it is unclear what exactly constitutes "near death" in terms of skeletal modification.

The morphology of the fracture is the most indicative factor in determining whether a lesion is perimortem or postmortem based on the distinction between fresh or dry bone, depending on the moisture content of the bone tissue (99-106). A "green fracture", typical of fresh bone tissue, has characteristics which differ greatly from those exhibited by a fracture typically formed on dried bone: these differences allow us to evaluate when a fracture was produced in relation with the time of death.

The fracture characteristics, in fact, are closely related to the moisture content and the quantity of collagen matrix (which gives flexibility to the bone tissue); at or around the time of death the bone tissue is characterized by the same features observable also in living bone tissue (with the same properties of strength, elasticity and flexibility). Once the bone tissue begins to lose its original components, such as collagen matrix and content of water or humidity (because it goes towards the decomposition), its own properties will change radically and invariably. At this time bone tissue becomes gradually "dry ", a type of bone tissue in where it is noted a significant loss of flexibility and resistance to subsequent stress (101, 104).

Depending on the bone quality just mentioned, there will be inevitably a different response to the stress applied which means differences on the fracture features; fractures in bones definable as "fresh" (with a high content of liquids and matrix as well as fat, and collagen) will have undoubtedly a morphological appearance dictated by high moisture content, on the contrary, the morphology belonging to fractures that occurred in "dry" bone will exhibit dissimilar characteristics due to the loss of the moisture. The differences observable in lesions occurring at different times in relation with death may therefore be quite significant especially if the bone properties in issues differ so greatly, because the answers to the same insult will appear extremely divergent. However, there will also be a phase in which the properties of tissue will present characteristics intermediate between "green" and "dry" bone tissue, thus causing intermediate responses that will result definitely more difficult to interpret.

What is important in determining changes in tissue properties is not only the passing of time since death, but also all of those cumulative effects resulting from the process of decomposition, taphonomic events and environmental factors that occur over the time (55, 98, 106, 102, 107).

Hence it is impossible to specify an exact time in which the bone properties differ little from those in dry or fresh bone; at such postmortem time the liquid content and matrix preservation in the bone will be therefore not so high (as in the fresh bone) and neither will it be completely lost (as it happens in dry bones), and this confers intermediate properties to the bone tissue and various responses to the stresses that will occur.

In this scenario, one must figure out how to deal with a correct trauma analysis on skeletal remains in which it is fundamental to distinguish between perimortem or postmortem lesions

(108-109). In these terms the diagnosis of “perimortem *vs* postmortem” needs to be based on reliable criteria for achieving such a result. For this reason it is necessary to consider all the limitations and unsolved questions around this important issue and to clarify the current state of knowledge, to develop or improve approaches still inadequate and to designate possible future research needed to solve the debate. (55, 97, 99,103).

1.3.2.1. THE STATE OF THE ART IN INTERPRETING PERI- AND POST MORTEM TRAUMA:

The analysis of trauma found in skeletal remains is very important to reconstruct accurately the circumstances around and after death. The characteristics of a fracture depend entirely on type of bone response to an insult, as already mentioned. The latter is based on bone proprieties that are subjected to changes due to decomposition process, passing of time and also divers environmental factors, at which the skeletal remains could be exposed to (107).

The perimortem lesions occurring when the bone tissue is still "fresh" and able to absorb stresses due to its high moisture content and original presence of tissue components as in the same living tissue. The high moisture content and richness in the bone matrix components determine high elasticity and plasticity, which ensure better tolerance even in cases of great stress and strain before the failure. This is very typical of living bone tissue, bone tissue around the time of death or in the very early stages of the decomposition process, unless some particular taphonomic factors are involved (96-107).

As soon as the organic matrix begins to degrade after death and the moisture content starts to decrease, the consequence will be the decrease in elasticity of the bone tissue, which becomes progressively more "dry". In this sense, the response to an insult will determine models of fracture and deformation completely different to those produced in "fresh" bone. "Dry" bone, in fact, is characterized by little moisture content and organic matrix that cause more rigidity and brittleness (101-103) as well as the loss of the typical viscoelastic properties of the original tissue. In presence of these new properties, a fracture can be produced even as a result of much less stress and will be characterized by a set of fracture attributes different than those shown by a fracture occurring in a fresh bone.

Several authors have claimed the chance to distinguish easily between peri- and post-mortem fractures on the basis of their specific macro-morphological characteristics, which are in their opinion closely correlated with bone quality and properties (102, 106, 108-109). They state that the proper assessment of trauma appears totally dependent on the characteristics of the fracture, such as: the fracture morphology, the outline of fracture, the angle and tactile roughness of the fracture margins (96-98,102, 105), and last but not least, the colour of the margins fractured (110-112). The features of the fracture are defined similarly by most of forensic anthropologists; regardless of their differentiation within the two typologies of fracture (post and perimortem), few studies have focused on the problem of validity and reliability of such common criteria when they are applied to human skeletal remains. This verification is limited by the lack of human remains where different kinds of fractures are known. In addition, not much is known about potential modification of original lesions with relation to time or as a consequence of environmental factors.

In general, the perimortem nature of an injury is sometimes indicated by the breaking pattern, which is more complex than the breaking pattern of a dry bone; concentric, circular, and spiral fractures are usually typical of green fractures (fractures occurring in living or defleshed bone). Nevertheless, some authors have described such patterns even for dry bones (97) in which typical fracture characteristics include a different production of several small fragments, brittle flaking or shattering, and surface cracking (113). In fact, Ubelaker and Adams (97) demonstrated the presence of butterfly or spiral patterns also in bones where the fracture occurred 1 year after death. In addition, other fracture-related features of perimortem fractures are bone tear, break-away notch and plastic deformation with also the adhering of small fractures fragments adjacent to the fracture site or impact (102).

The fracture angle and fracture colour are other additional features analysed and considered according to Wieberg (98) as the main characteristics in the determination of post-mortem trauma: dry bones usually show irregular to jagged fracture edges, which are often lighter in colour than the darkened adjacent bone (97). On dry bone the fractures also exhibit breakage nearly at right angle to their long axes with almost flat ends (seen the perpendicular, parallel or diagonal breakage). On the contrary, smooth, often bevelled, sharp fracture edges with sporadic sharp projections are associated with perimortem trauma and so are the smooth fracture surface and obtuse or acute fracture angles. In this latter case, these morphological aspects indicate that the injury was inflicted when the bone still had elastic properties, which could be in life or just after death (102). Furthermore, according to many authors (97-98, 114-117) green fractures are characterized by smooth fractures edges usually of the same colour as the rest of adjacent bone; thus colour staining can be present due to decomposition fluids, blood, soil, dirty water, leaf stains and organic matter. In this sense, if the colour is homogenous for both fracture margins and the external bone layer, the fractures could be considered to be perimortem since both have been remaining exposed for the same time to staining materials. On the contrary, the difference in colour of post-mortem trauma is connected to the fact that the fracture has occurred a long time after death and so the fracture surface has a different colour (lighter) than the adjacent external layer because exposed probably when the decomposition process has already finished.

However, the coloration of fracture edges should be used cautionary given its very variable nature, which depends on the depositional environment. This caution was also suggested by several authors (98, 102-103, 117-118) who have reported an equalization of colour between fractures margins and external cortical bone over time.

To explain the differences between peri and postmortem trauma a brief sum is presented in the following table.

FRACTURE FEATURES	FRESH BONE CHARACTERISTICS	DRY BONE CHARACTERISTICS
Outline	Radial pattern circling diaphysis	Perpendicular/horizontal fracture surface
Colour surface	Homogeneous colour with external bone	Heterogeneous colour with external bone
Surface	Smooth	Rough
Fracture angle	Obtuse and acute angles	Right angles
Other	Loading point present	Loading point absent
Other	Fracture front never crosscut epiphyseal ends	Fracture front can crosscut epiphyseal ends

Table 1: Morphological comparison between fresh and dry bone features according with Johnson (101), and Wieberg (98)

However, despite the numerous publications on the subject, the criteria for distinguishing between perimortem and postmortem lesions are based on observations of fractures involving long bones or elements characterized by cortical tissue. When, on the contrary, we consider different types of bone, as elements rich in spongy tissue or bones where the cortical tissue is not present as the main constituent, it should take into account specific criteria not yet investigated thoroughly. In addition, the criteria for the assessment of a fracture should also consider the taphonomic contexts in which the bone elements have been subjected: different contexts could modify differently the bone tissue properties and as a result produce diversified responses to the same post-mortem stress, perhaps not easily interpretable. A typical example is the burial within a closed system, such as inhumation in a coffin, where wet conditions and putrefied fluids derived from decomposition could determine a greater and longer persistence of moisture content, and so for the original bone elastic properties. The main features of fractures, in fact, depend on the moisture and bone component content; the speed of bone tissue drying is dependent on several factors and could influence the characteristics of postmortem trauma. As a result, in wet environments - such as in an inhumation in a coffin- the moisture may be retained for a longer time and in such conditions that it could produce changes that mimic perimortem bone damages. (98, 102, 107, 117). This last problem needs to be investigated through an anthropological study conducted on skeletal remains derived from inhumation or similar conditions.

1.3.3.

BLOOD TAPHONOMY: POTENTIAL BIOMARKER OF VITALITY IN PERIMORTEM LESIONS

Once a bone lesion is interpreted as perimortem, it becomes essential to understand if there is any evidence of vitality and how long the lesion has survived before death. This issue is common also in the interpretation of antemortem injury in dry bones where there are not yet visible events of bone remodeling and healing reaction macro- or microscopically (in the very early stages of repair). Similarly to what has been studied on both skin and tissue wounds (119-129), one of the most ambitious goals of anthropology is to diagnose the vital reaction in a bone lesion (130), which coincides with verifying if the perimortem injury has been inflicted in life, and consequently, for how long time that person survived after the occurrence of the lesion and before dying.

In general, the diagnosis of the vitality of a wound still remains an unsolved problem also in forensic pathology examinations on certain points (120, 123, 126). The origin of a wound can be either post-mortem or vital on the basis of both macroscopic and microscopic analysis; in many cases a correct diagnosis is based on the more sensitive and specific markers of vitality that have always been investigated in the past decades (124, 130). The aim of the research on this specific topic has been always the better understanding of the acute inflammatory reaction as well as the evidence of haemorrhagic infiltration (131). This is based specifically on an easy common concept adaptable for all living tissues (or almost all), which is the existence of a "vital reaction" to any insult (121). It is in this respect that as there is a vital reaction described such as an "acute inflammatory reaction" for soft tissue, so there must be a vital reaction following the haemorrhage even in bone tissue once a lesion has occurred on a living individual. The inflammatory reaction -that ensues, as a response of trauma- is a complex mechanism consisting in the restoration of its natural function through tissue repair after the removal of damages on it. This process is still not well and properly understood despite the fact that it includes many processes that involve cellular activity, molecular inductions, hemodynamic and vascular changes, among others. Before all the events, which occur in this reaction, the first manifestation is the bleeding, the blood extravasations in surrounding tissue then the consequent platelet aggregation and their production of coagulation factors as well as the activation of the metabolism of prostaglandin and complement. As reported by many authors (80, 123, 126, 132) the process of healing in cutaneous tissue starts in presence of an injury that causes the loss of integrity and the consequent disruption of blood vessels (and extravasations of blood constituents) (80). As they report, three main phases in wound healing (inflammation, tissue formation and tissue remodeling) exist similarly to those described for bone fracture healing (60-77); once a trauma causes the loss of integrity and the blood extravasations, the consequent formation of a blood clot will re-establish haemostasis and provide a provisional extracellular matrix for cell migration (80). Platelets permit the haemostatic coagulation and also secrete several mediators, which activate macrophages and fibroblasts (80, 119-129). All these cells, the complement, the mediators and molecules produced and involved at this stage are essential for the recruitment of inflammatory leucocytes, granulocytes and neutrophils to the site of injury.

Clearly, all the aforementioned do not occur in non living tissue and so there is no evidence of them in postmortem tissue damage.

In fresh skin, in fact, the red-purplish coloration (hemorrhagic extravasations) of a cut or bruise reveals its vitality, and therefore antemortem formation (to distinguish it from the similar colour of livor mortis), whereas the change in coloration, from a macroscopic perspective (from blue to brown to yellow, etc) reveals the time of survival. In more difficult cases (eg, when hemorrhagic infiltration is not so evident or when a more accurate time of survival is needed), microscopic analysis has to be performed in order to provide a more meticulous diagnosis (80, 121, 130).

Similarly for what has been described in literature, during a fracture, cortical bone, periosteal tissue, and surrounding soft tissues are ruptured, destroying the blood vessel and consequently causing tissue bleeding (132). Thus, both blood and bone marrow cells, such as immune cells, erythrocytes, and stem cells ingress at the injury site. This process leads to local tissue hypoxia and an inflammatory response, which is a result of migration of inflammatory cells, leucocytes, and macrophages into the fracture gap, triggering the formation of granulation tissue (72-78, 133-134).

In this scenario, it appears clear that the detection of blood extravasations, erythrocytes or bone marrow cells into the gap fracture, haematoma, and inflammatory reaction could be very important in both term of vitality and survival at death. Cattaneo *et al.* (130) are the only authors that have investigated this specific topic on dry and macerated bone. But before giving any kind of interpretation in case of the presence of evidence of vital reaction on a perimortem fracture found in skeletal remains (such as blood extravasations, erythrocytes, haematoma among the other blood and inflammatory cells) one must focus on the taphonomy of all these markers in order to have basic knowledge and the understanding of their meaning in this issue. Only once the comprehension of persistence, modifications in relation with time and taphonomy of all these markers in controlled conditions is well understood it will be possible to comment these specific biomarkers in a perimortem bone lesion and their meaning in terms of t vitality and survival time.

For understanding the real potential of the search for blood biomarkers or their own molecular products it is necessary to explore firstly blood taphonomy; in particular how cells degrade during decomposition process and how long they can persist into bone tissue. Only once these fundamental points are clarified the next step would be that to use this knowledge for understanding a possible key role in the interpretation of vitality even in perimortem skeletal injuries. The presence and identification of erythrocytes as a marker of blood extravasations or the recognition of a haematoma in perimortem lesions, even when the decomposition has already started since some time, could be a proof of vitality and eventually revolutionize the interpretation of lesions in the anthropological trauma analysis. As long as the research does not focus on taphonomic changes of erythrocytes among the other blood cells, as well as the chance to reveal their persistence over time and in the bone tissue exposed to diverse decomposition contexts it will never be possible to recognize and identify such biomarkers and consequently their presence as a sign of the vitality.

Once again, taphonomy shows its importance and involvement in the forensic analysis, which must be taken into consideration before advancing any forensic anthropological interpretation. The morphological changes due only to the process of blood degradation itself will become a fundamental piece of information for investigating at a higher taphonomic level, which is the alteration of blood, the timing and persistence of it within the bone tissue in controlled conditions (i.e. in absence of vital injuries and in known decomposition conditions). In addition, the differences of all the above findings but in relation to particular taphonomic conditions can give important clue in this field. In this perspective, the research needs to describe the normal process of decomposition of blood in general, and, specifically, of blood within the bone tissue in standard or particular conditions (in terms of time and context) that will be critical to investigate in more detail what differences are related to the presence of a vital bone lesion which has occurred shortly before death.

Chapter 2

2.1.
OUTLINE OF THESIS

The thesis will explore three major issues related to the analysis of trauma, all three overlapping each other: the problem of dating and interpretation of bone calluses or antemortem injuries; the problem related to the distinction between post-mortem and perimortem lesions, which is necessary for the subsequent interpretation of the modality and cause of death; and, last but not least, the issue regarding the more specific diagnosis of vitality of perimortem injuries, which needs to pass first through the study of the taphonomy of biomarkers potentially used for this, such as blood cells (namely erythrocytes). The three main issues addressed in this work have been investigated thanks to the presence of known samples, for which the information concerning trauma and time elapsed since trauma in relation with death, or vice versa, are available.

This opportunity was implemented mainly thanks to the presence and realization of a major osteological collection of global significance hosted at LABANOF (Laboratory of Anthropology and Forensic Odontology, Department of Biomedical Sciences for Health, University of Milan), namely the Milan Osteological Collection.

Besides the samples derived directly from cadavers (collected in the past 15 years according to Mortuary Police Regulations and Judicial Policy), the Milan Cemetery skeletal collection was the main source of materials in this project, without which the realization of such a study would not have been possible (always according to Police Mortuary Regulation art. 43). This is true not only for the availability of sample material on which to conduct some of the research here presented, but as a source of anthropological knowledge and problems encountered during the analysis of the skeletal remains, in which both the inhumation and exhumation processes as well as time and taphonomic factors have irreversibly altered the bone and some of the traumatic original features.

Through the anthropological study of a small part of this collection, it was possible to start a detailed anthropological analysis useful not only for demographics, disease interpetation and identification purposes, as summarized briefly in the activities section carried out in parallel to the three main topics of the project research, but for an in depth study of the analysis of trauma on skeletonized remains. Hence, this thesis develops into separate sections: the first intends to illustrate the details of the part of collection up to now studied with the purpose of highlighting the issues and the need to investigate the topics covered. The second part will investigate in depth the problem related to the outcomes of the dating of calluses and antemortem lesions obtained by applying methods already in use for trauma analysis interpretation in skeletal remains as well as for the diagnosis of clinical cases. The following part will provide an insight into the difficulty of interpreting an injury or fracture in perimortem skeletal remains where a number of postmortem and taphonomic factors sometimes turn out to be enemies of correct trauma interpretation. Finally, once a lesion is interpreted as perimortem, if possible, the biggest challenge still remains open, which is to define if the injury has been produced before death, and perhaps is related to the same cause of death; the vitality of a lesion represents a topic not yet solved in forensic anthropology. This important issue needs to find solutions through the tracing and identification of markers useful in this direction: a perimortem injury could therefore be an antemortem lesion which has occurred in a very limited time prior to death and in which either osteological reactions or healing process are not detectable macro- or microscopically. In this sense, as for skin/soft tissue wounds, the search for markers that indicate haemorrhage or inflammatory reaction on bone lesions may prove to be innovative and fundamental in the diagnosis of vitality. The comprehension of behaviour of blood, its tracing or the identification of bleeding, beyond the meaning that they might acquire when they are identified on perimortem bone lesions, must go through the understanding of the taphonomy of blood as well as its deterioration due to both the decomposition and the taphonomic factors to which it is subjected, particularly within the bone. Only once it is fully understood how the blood presents itself in the decomposition process and how it behaves in the later stages will it be possible to investigate the importance of its potential use in the diagnosis of vitality.

All of the above issues will therefore be divided into different chapters: the first (Chapter 3) is the presentation of the methods and results derived from the anthropological study of part of the Milan Cemetery skeletal collection, through which it was possible to select cases to be submitted to the specific research on trauma. The second (Chapter 4) is the study of antemortem trauma, based on the work of two different scientific projects (a paper published and one in submission); the works concerned the search for valid criteria and techniques used to determine the PTI as precisely as possible and also to emphasize the many limits still not solved. The third (Chapter 5) presents the study of the interpretation of peri- and post-mortem lesions based on two studies conducted in control cases selected from the collection, the results of which have already been published in the form of two papers. Finally, the fourth research (Chapter 6) includes an experimental part on the taphonomic study of blood, namely erythrocytes, specifically aimed at the understanding of how they change during the time when evaluated as single cells, but also within the bone tissue during the decomposition process; this was addressed for clarifying their potential as biomarkers and thus their possible key role in the interpretation of the vitality of perimortem injury. At the end, the last chapter (Chapter 7) will show the research work conducted on the skeletal collection in parallel to the main issues addressed during this PhD project; the results highlight again the importance for research in the field of anthropology of having at our own disposal a skeletal collection such as the "Milan Cemetery collection".

2.2.
RESEARCH AND AIMS

The entire research project aims at exposing the results of different investigations performed during the doctorate at the Institute of Legal Medicine, Laboratory of Forensic Anthropology and Odontology (Laboratorio di Antropologia ed Odontologia Forense – LABANOF) in order to disseminate important information about several forensic anthropological issues. The laboratory provided both materials and equipments (explained in the next chapters) required for the implementation of the applied research as well as the experimental investigations.
Specifically, the project is based on several research projects that focus on the complex issue of trauma analysis and taphonomy in skeletal remains through the study of a consistent part of the "Milano osteological and skeletal collection", without which the realization of the project would not have been possible.
In this perspective the project is divided in two main sections: the first consists of presenting data regarding the new Milan skeletal Collection and thus the first results related to the anthropological analysis conducted on about 200 skeletons and concerning demography, pathology and taphonomy. This part was fundamental and preliminary to the following section, the main object of the thesis, since the collection had never been studied before and it was necessary to verify its potential on a significant sample.
The second section, more specifically, concerns the research and results relative to the main problems regarding the interpretation of trauma in skeletal remains.
In particular, the second section is based on three major research lines which are all dedicated to the specific topic of trauma analysis. The results presented in this thesis could help in disclosing the main issues and possible solutions which have to be considered when trauma analysis is performed in real forensic cases of medico-legal interest.
The purposes of each of the three main research lines, which function as a baseline, are presented below:

1. The first study aims at investigating the problem of the interpretation of antemortem injury and focuses primarily on the evaluation of bone calluses, which constitute a very frequent pathological feature in the skeletal population.
The main purpose of this research line is to verify methods of properly assessing and estimating the post-trauma interval in known bone callus samples through radiological and

microscopic analysis in order to verify how features and criteria described extensively in literature are valid or limited for this purpose.

2. The second research is based on investigating the validity of the macroscopic morphological criteria that are commonly accepted and used for interpreting trauma in skeletal remains. More specifically, the research focused on two different assessments: firstly how the bone lesions, that are related to the cause and manner of death, which were observed at autopsy in fresh cadavers, are still identifiable in buried skeletal remains (after the long time elapsed since inhumation, and in presence of well known taphonomic conditions); secondly if the lesions that occur after death (certainly caused by postmortem events or as result of taphonomic factors) can appear misleading for a correct interpretation.
The conclusive objective to all effects is the applicability and efficiency of that knowledge acquired until now by forensic anthropology to the diagnosis of perimortem lesions and to the distinction between peri- and postmortem trauma, with the intent to verify its real validity as well as limitations and problems.

3. The third research line has focused on the investigation of the taphonomy of blood cells, namely erythrocytes, in order to prove their key role in the interpretation of the vitality even on skeletal lesions. The research investigated firstly the persistence and changes of isolated erythrocytes and possible mistakes with similar structures of different nature, such as pollen or spores. Secondly, the enquiry was extended to how blood components change in intact bone tissue in controlled decomposition conditions, and for how long the erythrocytes can still be traceable with the passing of time or when several lab experimental conditions are considered, with the intent to acquire useful information on decomposition of blood in non injured bone and to use this knowledge on the diagnosis of vitality in bone lesions.

Chapter 3

3.1.
INTRODUCTION

The PhD project started with the study of a part of the new cemetery skeletal collection, included in the "Milan osteological skeletal collection" housed at LABANOF (University of Milan), acquired at the begin of 2012. Among the many skeletal series comprised in the collection, the Milan skeletal collection is the new one whose assembly started only since 2012, in concomitance with the beginning of this Ph.D project. While the rest of the Milan osteological and skeletal collection, which includes archaeological as well as forensic/autopsy series, was already cleaned, prepared, studied and analysed in depth from an anthropological point of view, the contemporary and reference skeletal collection was completely novel and needed to be study and assessed generally in its potential for giving information on demographics and trauma. Its great dimension (over 1300 known skeletons) and importance as a skeletal collection required a preliminary anthropological study: a small skeletal sample (but consistent) was hence the first candidate ever to be tested and approached, whose study was the starting point of the entire thesis.

An initial study of a selected skeletal sample, in fact, is always necessary when a new collection is created; it permits one to have a preliminary idea of the type of material concerning conservation, taphonomy, demography, pathology, and trauma as well as the diagnostic and critical problems related with the anthropological analysis. Only once the study of a new collection has started will it be possible to have an idea on the importance and quality of a skeletal collection, as well as its potential for scientific research.

As said above, this chapter will describe the Milan Osteological and skeletal collection, which is the main material on which the research included in this project was conducted, as well as the results concerning this first anthropological endeavour.

The Milan Skeletal Collection: history, composition and first analysis of a part of the modern documented cemetery skeletal collection housed at the University of Milan, Italy

3.1.1. THE MILAN REFERENCE OSTEOLOGICAL AND SKELETAL COLLECTION

The Milan osteological and skeletal collection, housed at Labanof, offers a valuable series of osteological remains and skeletons of various types. Specifically, the collection includes a wide range of bone samples (from autopsy), several historical skeletal populations (derived from various archaeological sites and necropoles of different historical periods, located across Italy), a series of skulls and jaws from the early 1900 (donated by the Psychiatric Hospital of Mombello), a series of complete skeletons and skeletal parts both belonging to forensic cases, and a consistent reference contemporary skeletal collection derived from the Cemeteries of Milan.

The autopsy osteological series consist of bone calluses (of different types and at different stages of healing), several examples of trauma (gunshot injury, sharp lesions, cut injury, blunt force trauma, fractures), some samples of pathological nature, and osteological elements useful as diagnostic traits for age estimation (such as the pubic symphysis, 4th ribs and monoradicular teeth) or for the personal identification process.

The historical skeletal collection counts approximately 3000 individuals deriving either from various Italian geographic areas (mostly from Milan) and different historical periods; this specific collection provides an important source of historical and scientific information such as demographics, pathological conditions, and health status as well as occupational stress. An example is the population which was found in a mass grave in Via Sabotino (Milan): here individuals who died from *Yersinia Pestis* were buried during the plague epidemic of the XVIIth century (anthropological studies have confirmed from a scientific point of view what is reported in historical documents, such as described by Alessandro Manzoni in "*I Promessi Sposi*"); but many other different populations represent a precious resource of research in this field.

The cranioteca from the Psychiatric Hospital of Mombello consists of almost 100 complete skulls belonging to individuals interned for mental health reasons; once again the collection includes some very interesting samples, which demonstrate biological variability as well as the presence of diseases such as hydrocephalus, microcephaly, macrocephaly, and others more.

The Autopsy Osteological Series is composed by many osteological specimens of various nature, as diverse trauma examples, bone calluses at different stages of healing, several pathological exemplars, numerous articular surfaces of known age and many other useful bone elements, including all related known data. This series is a valuable source of comparison for real forensic cases and for research purposes.

Finally, the Forensic Skeletal Collection includes over 40 partial or complete skeletal remains from forensic cases that have been analysed in Labanof since its birth and never claimed. This series includes complete and partial skeletal remains belonging to unclaimed identified individuals as well as to individuals still unidentified.

Last but not least, in the last three years the Labanof has created the Milan Cemetey Skeletal Collection, assembled thanks to an agreement between the University of Milan, Milan City Council and the "Cimitero Maggiore" in Milan.

This latter is the collection on which the Ph.D. thesis was started: its massive size and novelty has required a first preliminary investigation on a more limited skeletal sample, whose results will be described in the next paragraphs.

All is in accordance to Police Mortuary Regulations and the Italian Criminal Code.

3.1.2. THE CEMETERIAL SKELETAL COLLECTION

The cemetery collection began in January 2012 with the acquisition of the first 265 individuals belonging to unclaimed exhumed skeletons who died between 1990 and 1991. Since then this collection has seen a rapid increase in the number of skeletons and with them also the gathering of important data, both of demographic and clinical/autopsy nature. Over the past three years its expansion has reached a substantial number of complete or almost complete skeletons, reaching more than 1300 specimens, most of which come from the "Cimitero Maggiore" of Milan, but some also from the cemetery of Lambrate (Milan).

The skeletal remains included in the collection mentioned above belong to individuals who died between 1990-1998, (a very small part of the collection, in contrast, is constituted by individuals who died between 1962-1980), all characterized by 15 years of burial in wood coffins and subsequent exhumation performed similarly by cemeterial workers. The presence of known demographic information such as age, gender, ethnicity, and date of death, proves invaluable for its use in function of the validation of anthropological methods, which is of importance for creating an identikit (key stage in the study of human remains of judicial relevance). In addition, this skeletal population, unlike the more worldwide skeletal collections, is composed only of remains belonging to individuals who lived in the modern times, a fact that confers greater value to the collection even in the forensic field. In fact, the methods commonly used in anthropology, derived from the studies of the main known reference populations, which correspond as mentioned before to populations that lived in the previous centuries. For these reasons, the Milan skeletal collection well reflect the characteristics of an ideal population on which to test and validate methodologies useful for forensic anthropology.

The availability of demographics and its modernity make the collection a useful scientific resource; however, its usefulness is not limited only to demography, but is extended to a much more ambitious aims of the discipline such as the study of taphonomy and trauma. This, in fact, is made possible thanks to the presence of detailed autopsy reports (albeit just for a limited number of individuals for which the autopsy had been performed at the Institute of Legal Medicine of Milan prior to the inhumation) as well as of ISTAT death certificates (death certificate of the National Statistical Institute) for all individuals, whose collection is still under development.

Currently, the collection includes 153 skeletons belonging to new-born and children (which age is ranged between 0-3 years) and over 1200 adult individuals; the demography of part of the population already stored and archived is shown in Table 3.1, and includes 46% of males and 54% of females. Demographic data on the rest of the population is being collected and needs to be verified, but it will be soon disclosed.

Age (years)	Males		Females		Total	
	n	%	n	%	n	%
0-3	93	8,6%	60	5,4%	153	14%
3-10	0	/	0	/	0	/
11-20	1	0,05%	1	0,05%	2	0,1%
21-30	5	0,45%	2	0,15%	7	0,6%
31-40	6	0,55%	6	0,55%	12	1,1%
41-50	11	0,95%	4	0,35%	15	1,3%
51-60	29	2,7%	21	1,9%	50	4,6%
61-70	74	6,8%	68	6,3%	142	13,1%
71-80	138	12,7%	142	14%	280	26,7%
81-90	112	10,25%	206	18.95%	318	29,2%
91-100	17	1,45%	85	7,65%	102	9,1%
>100	0	/	3	0,3%	3	0,3%
Total	486	44,8%	598	55,2%	1.084	100%

Tab 3.1: Demography of part of the Milan Cemeterial skeletal Collection: percentages of males and females and their distribution for each age class.

3.1.2.1. THE USE OF HUMAN SKELETAL REMAINS FOR SCIENTIFIC PURPOSES IN ITALY:

Over the past five years, Labanof in close collaboration with the city of Milan and the "Cimitero Maggiore" (the main cemetery of Milan) has concluded an agreement for the creation and the assemblage of a new and contemporary cemetery skeletal collection and the gathering of all related documentation (ISTAT certificates in which the causes of death and pathological conditions are reported, autopsy reports, and demographics)- this final point in collaboration with ASL, Azienda Sanitaria Locale, Milano. The acquisition of the entire skeletal collection was carried out in full accordance with the rules of the national police mortuary regulation in which art. 43 (D.P.R. n. 258/1990) gives to the cemetery the permission of assigning skeletal remains to Universities for educational purposes and studies.

Specifically, Article 43 of the Presidential Decree of the Italian Republic (DPR) n. 285 of September 10th, 1990 provides:

Release of bone to Universities and Hospitals:

1. The coordinator of the local health unit, upon written request by the directors of the autopsy rooms, may authorize the release to the University of the bones deposited in the municipal cemetery.
2. The bones, which are listed on a regular delivery and acceptance record, are taken into commission by the director of the mortuary room, and will have purposes as education as well as scientific study.
3. In all other circumstances it is prohibited to remove bones from cemeteries.
4. The trade of human bones is banned.

3.1.2.2. DEMOGRAPHIC DATA AND AUTOPSY INFORMATION ASSEMBLING PROCESS:

The skeletal population here presented, includes more than 1300 individuals (150 infants / children and 1200 adults) of both gender and of different classes of age (Fig. 3.1). Because of the large size of the collection, the assemblage is still in progress, and a consistent number of individuals are expected within the next two years. All remains, once skeletonised and exhumed, were stored in metal boxes equipped with an identifier plate in which name, date of birth, and date of death are recorded; in addition, each metal box (containing a unique individual) is also provided with a cemetery form where, besides the personal data, information regarding the burial and the exhumation (modality, date and place) are also reported. The demographic information of each individual, which is sensitive data according to law, were treated in agreement with the regulations and with prior authorization from the National Data Protection Supervisor.

Fig 3.1: Demography of the skeletal remains in the Milan Cemeterial Skeletal Collecti**on.**

All individuals were stored and archived after gathering the data of interest (gender, age, date of death and manner of burial) and then every individual was made anonymous through the acquisition of a progressive number. Among the various data at our own disposal, it is important to highlight the availability of the national death certificates, in Italy named ISTAT certificates. The certificates mentioned above are became an obligation under the national law (which has come into force in 1990) in order to ascertain the cause of death for each deceased. Since 1990 the investigation into the deaths and causes of death is a survey that detects total health information and demo-social data for all deaths occurring in Italy. On ISTAT certificates the doctor who ascertains death must, among other information, indicate the morbid sequence that causes the death as well as any other relevant disease states. Apart from a small group of individuals, who have died before 1990, there was the opportunity to retrieve ISTAT certificates for any deceased who died after 1990, which corresponds to a high percentage of the skeletal population. In these terms, the death certificates clearly offer the chance to gain important information about the causes of death and the presence of important pathological states; thus they provide valuable information that function as a control for the diagnosis of diseases which is, in anthropology, solely based on the presence of pathological signs on bones, often nonspecific.

A more important source of information, similarly to the ISTAT certificates, are the autopsy reports kept at the Institute of Legal Medicine of Milan. The autopsy reports, now collected, are available only for some individuals subject to autopsy prior burial, most of which have died of a violent death among other causes. In the autopsy reports a variety of information about the individual, such as height, weight, health status are described as well as a detailed description of the external examination and the analysis conducted on the organs. Finally, cause and manner of death are reported in detail, and so are the injuries, which have involved the soft tissue and skeletal elements. It seems obvious that, similar information acts as a valuable source for interpreting the same trauma in two different decomposition stages: they were observed and seen before at autopsy, and then analysed in the exhumed skeletal remains 20 years after autopsy and inhumation. This last approach is of great interest especially in cases of violent death, in which some lesions interesting the skeletal apparatus have been described at autopsy.

3.1.2.3. FIRST ANTHROPOLOGICAL STUDY OF THE MILAN SKELETAL COLLECTION

Unlike the anthropological study and results of the archaeological and forensic/autopsy series included in the Milan osteological and skeletal collection, the anthropological study of the new cemetery skeletal collection, whose birth dates back just to 2012, has taken place only in conjunction with the start of this PhD project. This project therefore begun through the study of a skeletal sample consisting of skeletons from which the first data concerning the whole skeletal collection was obtained. A sample of approximately 200 skeletons is a representative sample of the entire cemetery collection for many anthropological aspects such as: the degree of conservation and taphonomic profile, the diagnosis and estimation of biological characteristics belonging to the individual's profile (sex, age, stature), pathological and trauma profile, etc. Similar anthropological data and results were unavailable on the skeletal population at the moment of the start of the Ph.D. project, and the study of the skeletal sample is equivalent to the first ever anthropological approach, which was necessary for the understanding of population characteristics as well as of the diagnostic and methodological issues related to the type of material. The study of this sample, as a consequence has permitted firstly to shed light on the type of skeletal material, and then to select the samples (from the entire Milan osteological and skeletal collection) on which to conduct more specifically the research directed on the issues more strictly related to this thesis. The description of this first doctorate phase is explained in the next paragraph and it corresponds to the anthropological study of what turned out to be the starting point of this project.

3.2. MATERIALS and METHODS

3.2.1. SKELETAL SAMPLE FROM THE "MILAN SKELETAL COLLECTION"

The study sample of the present project consists of a copious number of adult skeletons derived from the collection mentioned above, which was studied in detail from the anthropological point of view. It includes about 200 skeletons that were selected in part randomly from the entire collection, and in part by reason of their well-known cause of death and of the availability of autopsy reports, fundamental for conducting the research lines clarified in Chapter 2. Demographic data concerning gender and age distribution of the skeletal sample are reported in Fig. 3.2; elderly individuals whose age is over 60 years old represent most of the skeletal sample in both genders (>80% of the skeletal sample).

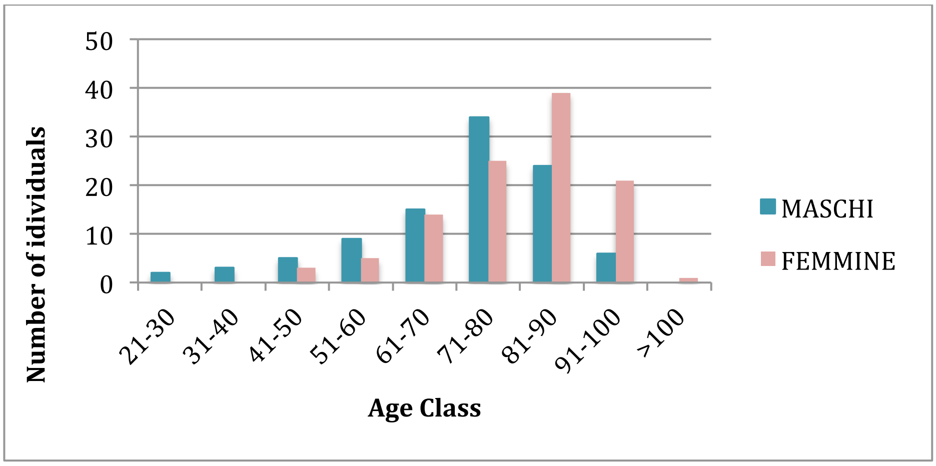


Fig 3.2: Age at death distribution of the male and female skeletons included in the skeletal sample.

All individuals included in the study sample have been exposed to the same conditions: buried in similar wooden coffins in the same cemetery ("Cimitero Maggiore" in Milan), interred between 1990 and 1991, exhumed and re-buried twice in 15 years after inhumation and then stored separately in metal boxes which have been moved and kept in the ossuary for 5 further years. The skeletons were recovered by cemetery workers without any forensic anthropological attendance; the lack of any anthropologist's supervision has unfortunately caused the loss of some skeletal elements of some individuals, mainly hand, vertebrae and ribs. In addition to the many taphonomic alterations and the eventual perimortem lesions, further post-mortem fractures or damages occurred at this stage, which have made the trauma analysis more difficult.

3.2.2. PREPARATION PROCESS FOR ANTHROPOLOGICAL ANALYSIS

Preparing a skeleton for study is a considerably long process: before carrying out the cleaning of any skeleton for the consequent anthropological examination a specific procedure was devised for describing the taphonomic profile. As a matter of fact, all, both biological and inorganic elements (hair, soft tissue remains, botanic and entomological material, textile fibres or clothes, fungi and moulds, buttons, dental plates, etc.) found attached on bones or into the metal box were categorised, described and reported in a taphonomic sheet, documented by photography, and then collected for further research. Once the taphonomic profile was documented for each individual, the cleaning up of all bone elements/fragments was performed by using cold water, small brushes in order to remove soft tissue remains, soil, dust or organic traces, all often attached to the external surface. This passage was conducted in the most delicate way possible in order to make the recognition of both specific and unspecific osteological signs achievable, which are of fundamental importance for pathological diagnosis as well as interpretation of injury.

3.2.3. ANTHROPOLOGICAL ANALYSIS:

After all skeletons were washed and dried, a detailed general anthropological analysis was performed, which consisted in reporting several characteristics in terms of:

-Inventory of the skeletal parts
-Description of survival, integrity and completeness for each bone element
-Description of metric and non-metrics variants
-Dental profile

Then, for all skeletons the anthropological analysis focused on the building of the biological profile, consisting in the age estimation, sex determination, ancestry diagnosis, and stature estimation. Only once the anthropological-demographical details were obtained, the anthropological profile was completed by adding results from the trauma analysis as well as the pathological considerations.

Both the anthropological and taphonomic sheets were compiled for all of the 200 skeletons included in the sample; in addition a photographic reportage was done, in particular on the trauma, details and pathological signs of greater interest.

Finally, the anthropological together with demographic data were collected on a database in which the general information from the skeleton was reported in order to compare results on the first 200 modern individuals with the known data about each individual.

3.2.3.1. Sex determination:

The determination of sex is based upon observations of morphological features known to differ between the genders and measurements of dimorphic dimensions (proximal humeral, femoral and radius epiphyses).

For all individuals sex was determined using first the dimorphic morphological features of the pelvis and cranium, and then also postcranial metric data were evaluated in case of necessity.

Pelvic traits are considered to be the most reliable sex determinants because of the special function of the female pelvis in childbirth (136, 137); in particular, if the pubic bone was well preserved, the method of Phenice (138) was preferable among all the others because of its high reliability, but all traits have been evaluated when possible. The cranial morphology has been also considered despite its reputation of being less accurate than the sex determination based on the pelvic bone (139), in particular if the evaluation on pelvic bones could not be accomplished because of the taphonomic conditions (a frequent case).

The morphological skeletal criteria used for the sex determination are listed in detail below:

Cranial indicators:

- angulus mandibulae
- arcus superciliaris
- crista supramastoidea
- glabella
- inclinatio frontalis
- mandibula-general appearance
- margo supraorbitalis
- mentum mandibulae
- os zygomaticum
- planum nuchale
- processus mastoideus
- protuberantia occipitalis externa
- tubera frontalia
- tubera parietalia

Pelvic indicators:

- angulus (arcus) pubis
- arc composé
- crista iliaca
- incisura ischiatica major
- ischiopubic portion
- Phenice triade
- sulcus praeauricularis
- foramen obturatum

3.2.3.2. Age estimation:

Accurate age estimation of human skeletal remains is crucial when skeletal local populations are studied, especially in terms of demographic analyses which is essential for building a correct biological profile particularly when personal identification is required in forensic skeletal cases. The methods of ageing skeletons are based on three main biological criteria: stages of skeletal maturation, changes to dentition (both for the estimation in subadults and adults), and morphological changes at joints where movement is either limited or non existent (e.g. cranial suture closure, pubic symphysis, auricular surface of the ilium). Since the human growth undergoes a progressive development of bones and teeth, sub-adults and young adults can in general be aged more precisely than older individuals. Subsequent changes in the adult skeleton are often degenerative and therefore not so well correlated with chronological age (137).

In this study age at death was estimated using multiple morphological indicators that have been used for standardizing several anthropological aging methods, which are widely reported in literature. Once the age estimation was performed, the results were compared with the known age, available for all individuals, in order to prove which methods are the best in terms of accuracy and precision, even in elderly individuals (basically the majority of the population). For each skeleton the age estimation was based on all aging methods stated below, but the chance to apply the methods depended on the taphonomic conditions and preservation as well as on the availability of the diagnostic morphological traits taken into account by the relative method. Despite some aging methods were applied to the sample when possible, the results were not considered in this project since they are already contemplated as obsolete and scarcely accurate methods if applied to elderly individuals (such as cranial sutures).

- estimating adult age from cranial suture closures (140)
- estimating adult age from the Pubic Symphyseal Surface (141)
- estimating adult age from the Auricular Surface of the Ilium (142)
- estimating adult age from the palatal suture closures (143)
- estimating adult age from the 4th Esternal rib end (144-145)
- estimating adult age from Combination of Auricular Surface and Os Coxae (146)

The age estimation for all individuals was based on the application of the methods stated above then the results were reported in each anthropological sheet. The data analysis of this specific topic, and so the relative problems and limits in relation with older ages and taphonomic conservation of bone elements, is well discussed and reported in one of the research works inserted in Chapter 7.

3.2.3.3. Stature Estimation:

Over the past century, researchers have designed numerous techniques to estimate adult living stature from various complete skeletal elements (e.g. 147-153), as well as from fragmentary remains (154-156). Most of the techniques are based on the correlation between adult living stature and maximum length of long bones. Despite the numerous long bones that can help in estimating stature, the most reliable results were obtained by employing lower limb bones, particularly the femora as reported by Trotter et al. (150).

In the present study, the approaches involving regression equations by Trotter & Gleser (149-150) were applied to determine the stature of the examined individuals. Nonetheless, all the possible post-cranial and cranial measurements were taken for all individuals in order to facilitate further scientific research and data analysis regarding metric aspects and stature estimation (but also metric dimorphism differences between males and females) in this specific local population.

3.2.3.4. Trauma analysis and pathological diagnosis:

Indicators of skeletal and dental health as well as trauma can reveal information about various aspects of living conditions in individuals and populations.

In the present study, for every skeleton the degenerative diseases were reported as well as any other particular pathological condition (among the infectious, metabolic, cancerous, and traumatic diseases). The diagnosis in these terms was based on the type of signs (bone tissue formation, bone tissue loss, periostites, etc), location on the skeleton and location on each bone element; in case of doubt, only some consideration or the suspicious of a certain pathological condition was reported. The main source of help in identifying pathology has been Ortner & Putschar (95), and eventually some specific publications for particular cases.

The general signs that have been searched for are: cribra orbitalia, porotic hyperostosis, hyperostosis frontalis, non-specific infections, specific infections, trauma, degenerative joint

disease, tumors, button osteoma, pathological dental states (e.g. caries, periapical and periodontal disease, antemortem tooth loss), and many other aspecific pathological features.
In addition, for all individuals who had died due to a traumatic/violent death (selected and included in the skeletal sample for the PhD project) or those in which perimortem skeletal lesions were found, an in-depth trauma analysis was carried out: traumatic lesions were classified by type (e.g., peri-, post-, antemortem), morphology, location on the skeleton, location on each bone, and if possible by modality of production, according with the literature stated in Chapter 1, in the paragraph 1.3.1.2. "*perimortem vs post-mortem*". Then for many cases the results obtained were compared with the data and descriptions reported during the autopsy. In these latter cases, the work has allowed detailed investigation into the complex issue of interpreting perimortem trauma on inhumed skeletal remains, in which taphonomy often causes damages and misinterpretation; the resulting research and findings have been already published in the form of two separate papers which will be shown and explained in Chapter 5.

3.3. RESULTS

At present, the skeletal sample from the collection is composed of about 200 skeletons (206 identified skeletons) with reliable demographic information obtained from the cemetery and ASL of Milan, and for most, also documented life and death data (death certificates and autopsy reports).
In the first 206 individuals subjected to anthropological analysis the following comparison between anthropological results and, both demographic data and death information (or autopsy reports) was performed for a consistent number of individuals, from which some very interesting findings concerning demography, pathology, and taphonomy were highlighted. General data concerning percentage of males and females, age class distribution, are reported in Tables 3.3 and 3.4; while the percentage of the several detectable pathological conditions and trauma are reported in Table 3.5 and Fig 3.3.
All 206 skeletons result as complete or almost complete, and only in few cases were several important parts missing or completely destroyed. The integrity of the present elements is quite good and the fracturing often concerned cancellous bones. Postmortem and taphonomic changes are revealed to be a feature quite common in every single skeleton and follow a similar pattern in all the skeletons of the study sample; part of the results about state of preservation and the percentage of new post-mortem fractures will be explained in some of

the research lines; one is included in Chapter 5 (concerning trauma analysis) and the other in Chapter 7 (concerning age and Post Mortem estimation), and both remark on the problem of taphonomy in the interpretation of trauma and in the survival and use of the articular surfaces in aging or chemical and proteomic compounds in PMI evaluation. Nevertheless, if on one hand the overall preservation of the remains is reported as good enough for sex determination, it is on the other hand not always possible to perform an age estimation. Although several sex indicators can survive and allow for sex determination (even when the pubis is not recoverable or is badly preserved) there is variation in the degree in preservation of the bone surfaces, which means differences in the preservation of the morphological traits that are considered in cases of aging methods. Some surfaces, in fact, appeared eroded, due to the specific taphonomic condition, namely decomposition on a closed system, as the coffin in which the remains can deteriorate differently and faster than skeletal remains buried directly in soil. In addition, some of the teeth and other skeletal elements (mostly hand bones, but also ribs or small parts that are useful for estimating age-at-death) fell out from the coffin probably during exhumation or during their moving into the new metal boxes, and could not be retrieved at the moment of the study. Both the lack and the poor preservation of parts used for several aging methods have stressed the difficulties in estimating anthropological age on this specific population. This result led to a specific research concerning the problem of validity in aging methods and their applicability to inhumed skeletons (the research will be discussed in detail in Chapter 7). Despite some problems encountered in the age estimation, in general the anthropological analysis was possible in all skeletons and has allowed the extrapolation of important anthropological data concerning the individual biological profile with respect to both demography and pathology. For instance, concerning stature, estimation was possible even in the worst conserved skeletons, where at least a single long bone was perfectly preserved. The long bones have demonstrated to be the most preserved elements and in each skeleton some upper or lower long bones have survived well beyond 15 years of inhumation, better than spongy-rich bone elements. Finally, metric and non-metric variants could be assessed only on the skeletal materials that appeared non deteriorated; the double facet of the calcaneous and atlas have revealed to be the most frequent variants in this local population, and so are some additional foramina and supranumerary ossicula in the skull.

	Males		Females		Total	
Age classes	**N**	**%**	**N**	**%**	**N**	**%**
21-30	**2**	**1%**	**0**	**/**	**2**	**1%**
31-40	**3**	**1,5%**	**0**	**/**	**3**	**1,5%**
41-50	**5**	**2,4%**	**3**	**1,5%**	**8**	**3,9%**
51-60	**9**	**4,4%**	**5**	**2,4%**	**14**	**6,8%**
61-70	**15**	**7,3%**	**14**	**6,8%**	**29**	**14,1%**
71-80	**34**	**16,5%**	**25**	**12,2%**	**59**	**18,7%**
81-90	**24**	**11,6%**	**39**	**18,9%**	**63**	**30,5%**
91-100	**6**	**2,9%**	**21**	**10,2%**	**27**	**13,1%**
>100	**0**	**/**	**1**	**0,5%**	**1**	**3,4%**
TOT	**98**	**47,5%**	**108**	**52,5%**	**206**	**100%**

Tab 3.2: age and sex distribution on the 206 skeletal remains included on the skeletal sample

Regarding the combined age and sex distribution of the skeletal sample (a total of 206 individuals included), the female average age-at-death exceeds the male average (respectively 82 against 77 years-at-death). Age-at-death resulted more uniformly represented in the male cohort, although there is an absence of individuals older than 100 years (as observed in Table 3.2). Conversely, the female cohort includes adults in the 100 year old category, but a relative absence of individuals in the young, middle and mature adult groups (21–30, 31-40 years) is noteworthy (Table 3.2). Table 3.4 provides descriptive statistics for these data.

Descriptive statistics (*Age in years*)	Females(*n=108*)	Males (*n=98*)	Total (*n=206*)
Median age	77	82	79
Mean age	72,63	80,65	76,78
Standard Deviation	11,53	15,20	13,98
Minimum age	48	28	28
Maximum age	101	97	101

Tab.3.3: Descriptive statistic for the skeletal sample.

Comparing demographic distribution of the 206 individuals studied with the distribution of the entire collection there appears to be a tendency, very similar to the skeletal sample, to represent the entire skeletal population (sub-adult individuals a part).

The similarity of demography between the skeletal sample and the contemporary population confirms the suitability of the skeletal sample for such studies.

The most significant results concerning trauma and/or pathological signs are shown in Table 3.4 and Figure 3.3.

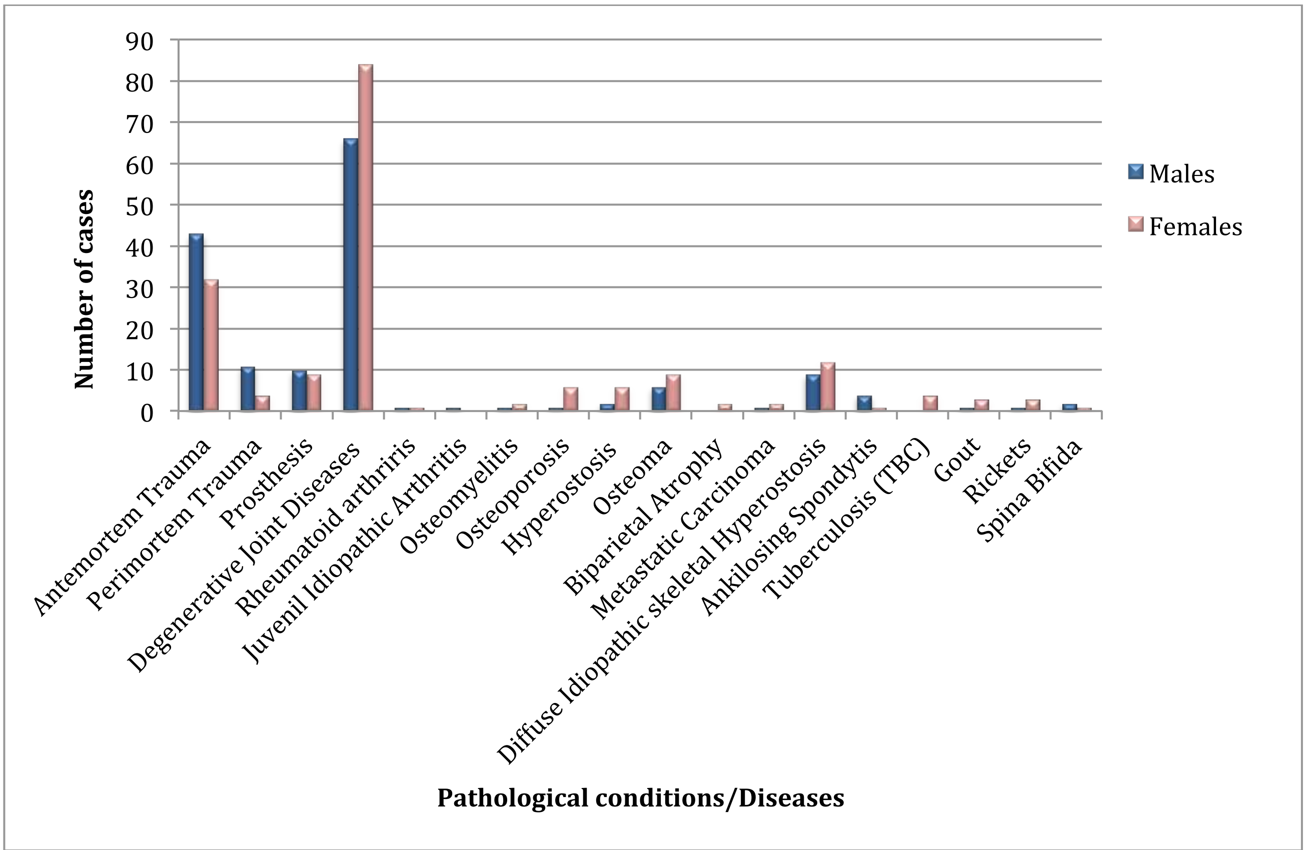


Fig. 3.3: Pathological conditions distribution in males and females of the skeletal samples

The most suggestive pathological condition is Degenerative Joint Disease (DJD) that was observed in the majority of the sample. This specific term refers to the many conditions related to a degenerative joint status like osteoarthritis or osteoarthrosis, whose interpretation has been based only on the presence of any joint alterations observable in the articular surfaces (formation of marginal osteophytes, pitting of the joint surface, alteration of the joint contour, and/or presence of subchondral cysts). A 75% (33% of males and 42% of females) of the skeletal population exhibits some degenerative changes (at least moderate) in more than one-two skeletal parts. In many cases the DJD is very advanced, in particular in the vertebral column where it is often observed with the formation of many huge osteophyte bridges between adjacent vertebrae and the eburnation as well as porosity and Schmorl's nodules. As with DJD, another important pathological aspect found to be very frequent as a pathological condition is the presence of at least one antemortem trauma (37% of the total sample), which is often combined with the presence of a surgical treatment or orthopaedic device (in 9,5% of the total sample); among bones in which orthopaedic devices are implanted, the most commonly affected bone is the left femur (2.5% of males, 3.2% of females out of the total sample).

% Percentage	Pathological conditions/ Disease typology
75%	*Degenerative Joint Diseases*
37%	*Antemortem Trauma*
10,50%	*Diffuse Idiopathic skeletal Hyperostosis (DISH)*
9,50%	*Prosthesis*
7,50%	*Osteoma*
7,50%	*Perimortem Trauma*
4%	*Hyperostosis*
3,50%	*Osteoporosis*
2,50%	*Ankilosing Spondytis*
2%	*Tuberculosis (TBC)*
2%	*Gout*
2%	*Rickets*
1,50%	*Osteomyelitis*
1,50%	*Spina Bifida*
1%	*Biparietal Atrophy*
1%	*Metastatic Carcinoma*
1%	*Rheumatoid arthriris*
1%	*Amputation*
0,50%	*Juvenil Idiopathic Arthritis*

Tab 3.4: Percentages of the different diseases in the total amount of skeletons of the skeletal sample. The pathological conditions order follows the tendency in the population (the Degenerative Joint Diseases, as expected, are the most common).

As antemortem trauma, bone calluses (healed lesions) are frequently observed, especially in the male population (54.4%, versus 29.5% of females): the most affected bones are ribs (13.9% of males, 8.4% of females), followed by tibia and fibula (5.1% of males, 1.1% of females), and scapula (3.8% of males, 1.1% of females).

Some important findings also concern the presence of perimortem trauma, which is more frequent in males than in females (respectively in the 5,5% and in the 2%). The high number of skeletons with at least perimortem lesions (15 skeletons out of about 200 regardless of the type of trauma) required a more detailed investigation; in these terms the availability of autopsy reports, if any, has provided control information for the trauma analysis conducted in those skeletons belonging to cadavers subjected to autopsy prior to inhumation. This has permitted an opportunity to carry out an interesting study about survival of perimortem lesions (often lethal) on inhumed human remains exposed for 20 years to taphonomic factors, in relation to the type of trauma, number of bone lesions reported at autopsy and type of the bone affected by the trauma. As a matter of fact, some skeletons belonging to individuals who died of a traumatic death, and for which the autopsy records were collected from the autopsy archive in the Institute of Legal Medicine of Milan, were selected and used for conducting the research line on the subject of the interpretation of perimortem trauma and on the distinction between perimortem vs post-mortem lesions.

Interestingly, many other pathological conditions are present in small percentages and resulted in being less usual with respect of the more significant pathological conditions mentioned above. As expected, among the many pathological signs, some derive from tumor diseases and are represented by a percentage a little under 10% of the sample, such as osteoma and metastasis. On the contrary, despite the very small number of cases, some skeletons unexpectedly exhibit the presence of more typical of the past (as Tuberculosis and Rickets), while others show several particular conditions more typical of the contemporary population, such as the aforementioned tumor diseases but also osteoporosis and gout. In many other cases, the presence of general periostitis and/or in lack of any other noteworthy signs, does not permit us to specify certain pathological conditions and so only unspecific osseous signs were reported. It is necessary to state that all the diagnoses are based on the gross morphological appearance, and in just a few cases more specific analyses were conducted (e.g. radiological and stereomicroscopic analysis); this is to say that the results can only just give an idea about the general pathological conditions on the population; nevertheless further analysis as well as future research based on the comparison between the pathological control data (still in progress of collection) and the pathological findings obtained by the anthropological analysis needs to be carried out.

3.4. DISCUSSION:

The organisation of the Milan Osteological Skeletal Collection began only in 1995 after the birth of the Laboratory of Forensic Anthropology and Odontology (LABANOF). Over the years the laboratory started to gather several unclaimed skeletal remains from forensic cases as well as osteological specimens from cadavers removed for both forensic and anthropological assessment. After many years some important archaeological/historical skeletons from numerous ancient sites have been added to the pre-existing collection, and only a few years ago (in the early 2012) the gathering of numerous identified skeletons from modern cemeteries has started: the Milan Cemetery Skeletal Collection, as presented here.

Its existence has only just begun and so has its anthropological study, but in this short time, it is expected that the development of the Milan Cemetery Skeletal Collection Project will help deepen the knowledge of skeletal features of the contemporary population of Milan, with a look also at the accuracy of results concerning sex, age and pathological conditions, which are of most importance in local forensic investigations. The study of the initial skeletal sample from this collection aims to describe the composition and first anthropological results (demography, taphonomy and pathology) of this important European Skeletal Collection. It corresponds only to a descriptive analysis of a small sample from the collection and intends to highlight the real distinction between a documented collection and a representative sample (and also of the many problems arising). Obviously the results of this study cannot be generalized to every skeleton of the whole skeletal series since they are still insufficient to produce stringent guidelines for research design. However, as every skeletal collection whether modern and documented, historical, cemeterial or archaeological in origin, the Milan skeletal collection will be biased in its own unique way according to the sampling or methods used. This is to say that what has been shown in this study, from all points of view (taphonomy, demography, pathology and trauma), is just a first representation; nevertheless it could illustrate quite well the rest of the collection and in all probability the problems and limits related to diagnosis of age, pathology, and trauma remarked by this research as aspects correlated highly with the specific taphonomy of inhumation but also with forensic cases. The last account is proven in part by the comparison between the model life tables of both the skeletal sample and collection; in fact, it reveals a high similarity for what concerns the demography and mortality of modern populations, which allows for the ability to presume also a similarity in the presence of several pathologies and traumas (as was found as in the skeletal sample). Being a contemporary population whose skeletons belong mainly to old individuals (about 80% of individual are older than 60) the resulting pathological situation reflects fairly well what would have been expected for a contemporary population. The most significant pathologies, in terms of frequency, are the Degenerative Joint Disease which is not surprising in an older population as such. Thus, also some very common important diseases found nowadays in the contemporary living population (also in young and mature individuals) like metastatic tumors were found, even if in lower percentages. It has to be specified that such a pathological condition may not be easily detectable in inhumed skeletal remains where taphonomy could have caused changes and deterioration on a bone tissue which had already been attacked by the tumors; but this hypothesis needs certainly to be confirmed in future research based on the availability of data concerning the living pathological state of the individuals reported in the death certificates in order to control and verify these findings.

Nevertheless, the results deriving from the anthropological analysis have shown interesting data concerning demography as well as pathology: the chance to build a correct biological profile for each individual depends on the degree of preservation of the skeletal remains, and not always can be performed. If, on one hand, for each individual several characteristics can be easily detectable and provide important information for the identikit, on the other hand the indicators for age estimation, such as the many articular surfaces, have revealed to be much more subjected to both taphonomic changes and deterioration and even when the diagnostic traits are well preserved they do not always allow for a correct or a valid age estimation, due to the many limitations of aging methods in giving a suitable and accurate age range for old individuals. This specific issue was investigated in depth in the research that will be presented in Chapter 7.
Results about pathological and trauma analysis reveal important suggestions: trauma is a very common characteristic, even in a contemporary skeletal population, and as such needs to be interpreted as accurately as possible. Antemortem lesions are the most represented (37%) but also lethal trauma was demonstrated to be present in a fair amount of skeletons (7,5%) as shown in Table 3.5.

Trauma	Males	Females	Total
Antemortem Trauma	21,50%	15,50%	37%
Perimortem Trauma	5,50%	2%	7,50%

Tab.3.5: Percentages of either antemortem and perimortem trauma found in the skeletal sample. Differences in the percentage between females and males are also shown.

Certainly, the high probability of detecting such features as evidence of a traumatic event during life and/or of a traumatic death (in the skeletal sample it has shown a total percentage of 44,5%) pushes the research into further investigations: what additional information could be given as concerns timing and type of trauma, how can the interpretation of trauma help anthropology in the study of skeletal remains, how can taphonomy determine changes in bone tissue that creates difficulties in distinguishing between peri and postmortem, and then which kind of pre-existing trauma can still be interpreted after 20 years of inhumation and are the resultant interpretations valid, helpful and specific in terms of trauma analysis? And again, can any more precise information be given by anthropology regarding trauma around death in the case of lack of any bone signs or reaction and could there be biological markers that can facilitate this task?

Specifically, in the case of antemortem trauma it has been possible to highlight only those cases in which a clear osteological reaction was present, and the description is limited to the mere distinction between healed or healing lesion; no additional information about the traumatic event is attainable at this moment. In addition, one must also consider the possibility that among all injuries observed, some have been described as perimortem because of the absence of bone reaction- typical of the bone healing processes- which begins to appear from around 10-15 days (if eventually the reaction signs have survived to all taphonomic surrounding environment), although they could have occurred hours/days before death (therefore not necessarily related to death) and so they should be considered more appropriately as antemortem injuries (at the earliest stages of healing).

Similarly to what has been said above, the lesions defined as perimortem were determined as such on the basis of specific morphological characteristics which survived to time and to taphonomic conditions. These lesions could provide more precise information on the mode and type of production, but there is still the chance that in inhumed skeletal remains some of them may not have been recognized due to the taphonomic transformations that masked their clearly perimortem morphologic characteristics.

All this is to say that the percentages relating to the presence of trauma in this population sample could be misleading in some aspects, which arise from the limits of the material as well as from the limits of the anthropological diagnosis.

In conclusion, the results obtained from the anthropological study of this sample allow us to illustrate interesting anthropological aspects that concern demography, pathology and morbidity of this skeletal population; in addition they highlight the need to investigate further the issues related to trauma analysis correlated to taphonomy, which have turned out to be a tough foe for any diagnosis in question and which are the mean topic on which the next research lines are based on.

Chapter 4

4.1
FIRST RESEARCH LINE: THE PROBLEM OF DATING BONE CALLUSES

The high frequency of bone calluses and antemortem lesions emerging from the anthroplogical analysis of the study sample highlighted the need to clarify to all effects the limitations still present in dating calluses. This part of the study thus aims at verifying the possibility of elucidating knowledge and tools available to the anthropologists for dating the antemortem injuries in dry bones through the application of the more advanced techniques used in clinical follow-up, histology, radiology and anthropology as well. Despite the extensive experience on this topic from all points of view, even when signs of bone remodeling or calluses are visible, more definite information on PTI (post-traumatic interval) can result very difficult to obtain, and this issue still remains little explored. Given the need to provide more accurate answers concerning the antemortem trauma, often found in victims of torture or in case of maltreatment and abuse, and so to better interpret the traumatic events which occurred in the victim's life, one of the issues addressed in this PhD study was to verify the potential of new technologies already in use by other disciplines.

In order to define more precisely the post trauma interval of an antemortem injury found on skeletal remains, it is therefore necessary to further investigate techniques commonly used in the clinical-pathological field with the objective to establish osteological parameters useful when applied directly on skeletal remains or dry bones.

This first part of the study is based on two different works that will be illustrated on the next two paragraphs; they take into account different aspects, both possible thanks to the availability of bone calluses with a known PTI.

The first study has been published in the International Journal of Legal Medicine: here results obtained by using Cone-beam (advanced CT) on several callus samples are reported in order to prove the potential of such advanced technology.

The second study was possible thanks to an international partnership between different University Departments: Labanof (Department of Biomedical Science for Health, University of Milan), Radiology Unit of Scientific Institute (IRCCS) Policlinico San Donato (University of Milan School of Medicine, Department of Medical and Surgical Sciences) and Laboratory of Pathology of Academish Centrum Medisch of Amsterdam. The study will be presented only partially since some results are still in elaboration.

4.1.1.

PART I:

APPLICATION OF A ADVANCED TECHNOLOGY FOR DATING BONE CALLUSES

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TECHNICAL NOTE

The application of cone-beam CT in the aging of bone calluses: a new perspective?

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Abstract In the forensic and anthropological fields, the assessment of the age of a bone callus can be crucial for a correct analysis of injuries in the skeleton. To our knowledge, the studies which have focused on this topic are mainly clinical and still leave much to be desired for forensic purposes, particularly in looking for better methods for aging calluses in view of criminalistic applications. This study aims at evaluating the aid cone-beam CT can give in the investigation of the inner structure of fractures and calluses, thus acquiring a better knowledge of the process of bone remodeling. A total of 13 fractures (three without callus formation and ten with visible callus) of known age from cadavers were subjected to radiological investigations with digital radiography (DR) (conventional radiography) and cone-beam CT with the major aim of investigating the differences between DR and tomographic images when studying the inner and outer structures of bone healing. Results showed how with cone-beam CT the structure of the callus is clearly visible with higher specificity and definition and much more information on mineralization in different sections and planes. These results could lay the foundation for new perspectives on bone callus evaluation and aging with cone-beam CT, a user-friendly and skillful technique which in some instances can also be used extensively on the living (e.g., in cases of child abuse) with reduced exposition to radiation.

Keywords Forensic science · Forensic pathology · Forensic anthropology · Fracture repair · Bone callus · Fracture healing · CBCT

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Introduction

The analysis of skeletal trauma is a crucial task in forensic anthropology. The age of a bone fracture which occurred during life is usually reflected in an active or well-established callus. Needless to say, the age of a callus is crucial both for identification purposes as well as for reconstructing the history of violent trauma suffered by a victim. As concerns identification, it is for example of extreme importance to assess whether a bone callus of an unidentified decedent is 2 or 10 years old: this in fact may be crucial in searching among hospitals for possible victims of accidents who correspond to a specific biological profile. Aging calluses is also crucial when assessing if the person was tortured or beaten weeks or months before death, for example during imprisonment.

This however may not be a simple task, especially when dealing with dry bone. Most research in the area of the timing of bone reparation and of callus formation has focused on clinical studies (mostly in the orthopedic field), both with classic radiological techniques and digital radiography [1–3], as well as with more advanced technologies like computed tomography or magnetic resonance imaging [4–10]. Radiological investigations of fracture healing are routinely used also in the forensic field for the assessment of child abuse [11–15] and more generally for aging fractures and calluses. The same issues have been investigated with sporadic histological and biochemical studies on humans and animals [16, 17]. The in vivo follow-up of the mechanical stability of fracture repair and mineralization due to production of trabecular bone within the fracture gap is a necessary tool for an objective evaluation of the course of a successful healing. But once a certain stage of healing has been achieved, not many further observations exist.

Up to six stages of bone healing have been described in radiology [18, 19]. This staging, based mostly on the features of the fracture line, fracture gap, and inner structure of the newborn callus, has several limitations: firstly, the entire process is basically evaluated with a combination of subjective assessments. This is performed by a visual and subjective evaluation of radiographs, using criteria such as the presence of bridging or obscuration of the fracture line (lucent, sclerotic, or invisible), fracture margin (that can be sharp, blurred, or invisible), visible bridging activity (starting, partial, or complete), and the deposition of new tissue inside the callus and its mineralization. It is clear that a grading of the fracture repair and callus from an objective and quantitative point of view can hardly be performed with current radiological approaches. Moreover, from an osteological point of view, it is difficult to pinpoint where and at what different stages different parts of a callus are. Some anthropological studies have marginally investigated the morphological appearance of bone remodeling in the skeleton [20, 21] with a rough evaluation of features like osteoblastic/osteoclastic activity and line of demarcation to the surrounding bone. Some other studies have concerned macroscopic analysis in cases of child abuse [12, 22]. However, the general blurriness of radiological images in vivo does not allow us to evaluate the particular evolution of the calcified tissues during healing.

The present study therefore wishes to investigate if the use of more sensitive and specific radiological tools can help us gain knowledge in this field and lead the way towards a more accurate analysis of the degree of fracture healing, which is a necessary prerequisite for aging trauma. In particular, we wish to explore the potential of cone-beam CT technology with a pilot study in the analysis of calluses/-fractures of known age, aimed at visualizing different levels of bone callus mineralization.

Materials and methods

Bone calluses

Thirteen bone fractures (with or without evident formation of bone callus) of known age were taken according to Police Mortuary Regulation from eight individuals upon autopsy (Table 1). Samples were taken during autopsies and the flesh was mechanically removed. In three cases (cases 1, 2, and 3), calluses were too young for mineralization to be present, whereas in the other ten samples, the presence of the callus was clearly visible: the more regular and smooth, the older the lesion. Each lesion was subjected to radiological investigation with digital radiography (DR) and cone-beam CT (CBCT), in order to evaluate any difference in DR and CT images and any additional information this new technology can provide in the analysis of bone fractures. The evaluation of the bone healing degree by DR was based on the description and outlines of the following six stages [18, 19]: stage 1 is designated as "fracture event" and the radiographic characteristic is the absence of visible bone healing; stage 2 is defined as "granulation" with signs of initial visible healing such as the widening of the fracture gap due to resorption, blurring of the fragment edges, and appearance of faintly mineralized buds of callus ("cloudy," "fluffy," or "immature callus"); stage 3 is the "mature callus" that appears radiopaque and is indicated by new bone growth of similar density as normal bone and the clear demarcation of the fracture line is still clearly delineated; stage 4 shows partial bridging and represents the moment when the callus is connected across the fracture gap in some points; and stage 5 classifies the fracture as clinical union because the mature callus has nearly bridged the fracture completely and the fracture line remains faint and characterized by almost "complete bridging" and almost "complete obscuration." Stage 6 represents the complete bridging and complete eradication of a fracture line that is no longer visible on the X-ray.

DR investigation

Digital radiographs were performed in an anteroposterior projection. DRs were performed with a triple-phase X-ray tube with fixed plant and rotating anode (power 72 kV, inherent filtration 3.5 mm of Al) with a double localized spot (1.2 × 1.2 and 2 × 2 mm) and a distance of 100 cm from fire to film. The tool has a CR Agfa Compact® system, put together with the same radiological tube.

Cone-beam CT

Samples were subjected to radiological investigation with cone-beam CT, using the Newtom 5G Cone Beam CT QR System (Verona, Italy) with high resolution, thanks to the small isotropic voxels. The medium time of reconstruction of the image is 45 s. The acquisition of the images is reached with a rotation of 360° around the samples.

DR and cone-beam images were then evaluated in blind by an expert in the field of radiology, and every callus was

Table 1 Differentiation in stages with DR and further detectable features with cone-beam CT

Number	Site of fracture	Presence of callus	Age of the fracture	DR stage	Additional information with cone-beam CT
1	Distal radius	No	5 days	1	No additional information if compared with DR
2	Rib	No	10 days	1	No additional information if compared with DR
3	Cranial vault	No	18 days	1	•Fracture more easily detectable
					•Fracture course clearly detectable in the cortical and spongy bone
4	Supraspinous fossa of the scapula	Yes	20 days	4	•Detection of fracture line (with DR, it is almost undetectable)
					•Outer outline of the callus ("blurred" in DR images)
					•Inner structure of the callus ("blurred" in DR images)
5a	Rib	Yes	28 days	3	•Detection of the inner line of fracture ("blurred" and incomplete in DR images)
					•Evaluation of the outer outline of the callus: continuous on one side and patchy on the other (not assessable with DR)
					•Different deposition of mineralized tissue inside the callus (with DR, no differences in the gray scale are detectable)
5b	Rib	Yes	28 days	4	•Detection of the course of the fracture line (not assessable in 2D images with DR)
					•Outer outline of the callus ("blurred" in DR images)
					•Organization and disposition of different layers inside the callus (not assessable with DR)
					•Different deposition of mineralized tissue inside the callus (with DR, no differences in the gray scale are detectable)
5c	Rib	Yes	28 days	4	•Detection of the course of the fracture line (not assessable in 2D images with DR)
					•Outer outline of the callus ("blurred" in DR images)
					•Organization and disposition of different layers inside the callus (not assessable with DR)
					•Different deposition of mineralized tissue inside the callus (with DR, no differences in the gray scale are detectable)
5d	Rib	Yes	28 days	3	•Detection of the fracture line (not assessable with DR)
					•Outer outline of the callus: patchy on both sides (with DR, this feature cannot be assessed)
					•Inner organization in layers and different deposition of mineralized tissue (uniform gray aspect in DR)
6a	Rib	Yes	2 years	6	•Degree of mineralization of the surface of the callus (not assessable with DR)
					•Outer outline of the callus ("blurred" in DR images)
					•Smoothness of the surface of the callus (not assessable with DR)
					•Inner organization of the different layers of the callus (not assessable with DR)
6b	Rib	Yes	2 years	6	•Degree of mineralization of the surface of the callus (not assessable with DR)
					•Outer outline of the callus ("blurred" in DR images)
					•Smoothness of the surface of the callus (not assessable with DR)
					•Inner organization of the different layers of the callus (not assessable with DR)
6c	Rib	Yes	2 years	6	•Degree of mineralization of the surface of the callus (not assessable with DR)
					•Outer outline of the callus ("blurred" in DR images)
					•Smoothness of the surface of the callus (not assessable with DR)
					•Inner organization of the different layers of the callus (not assessable with DR)
7	Sternotomy	Yes	2 years	6	•Detection of the course of the fracture line (not assessable in 2D images with DR)
					•Detection of different stages of mineralization around the fracture line and inside the callus (not assessable with DR)
8	Femural head and acetabulum	Yes	14 years	6	•Different deposition of mineralized tissue ("blurred" in DR images)
					•Organization of the different layers of the callus (not assessable with DR)

scored according to the six stages of bone healing stated by Hendrix [18] and Toal and Mitchell [19] and previously described. These standards were used in the evaluation of DR images; for the CBCT images, a case by case description was provided since the previous classification was difficult to apply in a comparative manner.

Results

Table 1 shows the results of the classification of the DR images in six stages and the comparison between DR and cone-beam images. Regarding DR, the increase in the stage evaluation roughly follows the increase in the age of the fracture: the older the fracture, the higher the stage, up to the last stage (sixth) of fracture healing. Beyond this threshold, when the bone callus unites the two margins of the fracture and is easily detectable, both with macroscopic and radiological analyses, DR images show similar patterns. Conventional radiography, because of the lack of definition and magnification, led to a superimposition in the evaluation of the middle stages of fracture healing and to a sort of "smoothing out" of any lesion into stage 6 when it was beyond a certain age; CT images, instead, allowed the observer to gain a more precise and defined knowledge of the inner and outer structures of the callus, thus recognizing different kinds and stages of mineralization (Figs. 1, 2, and 3), a clear identification of the fracture line (when still present and detectable), and a vision of the inner organization of the callus in 3D (Fig. 4). In detail, in very "young" fractures, CT images do not add important information to radiographs (fracture numbers 1 and 2), especially when the fracture line is the only feature one can detect in the radiological images: in these cases, CBCT gave in fact no additional information. On the other hand, things change when the fracture is older: the greater the age of the fracture, the more information CT images added in detecting features that can help in the evaluation of the process of fracture healing. The great potential of CBCT is proven by the additional information listed in the last column of Table 1: the enormous amount of information that such tomographic images can provide to the observer is evident. The greater the stage of fracture healing, the more useful and informative features increase on the radiological images: fracture lines become more visible, with the outer and inner layers of the callus become clearer for example. Moreover, different and more subtle levels of bone mineralization become assessable and distinguishable, as well as the inner morphologic organization. Many of these features were clearly not visible with more conventional methods even to the trained eye of the radiologist. To sum up, cone-beam CT imaging gave us a clearer image of the inner and outer structures of the callus, fracture line, inner organization of the different layers of the callus, and different stages of mineralization.

Discussion

The task of aging bone fractures in skeletal remains is still a crucial and tricky issue in the forensic field both in the case of the living and of cadavers. One only has to think of the hundreds of cases every year of child maltreatment in which aging a fracture correctly may make the difference in diagnosing child abuse and thus contribute to ensuring the child's safety, or of war crime scenarios where properly establishing the age of a bone callus on a skeleton may reveal torture during imprisonment. At the moment, particularly on dry bone, very few studies have investigated the morphological features of bone remodeling [20–22] beyond standard radiology, giving only a rough evaluation mostly on single cases. Moreover, the clinical standards one can make use of are not always in complete agreement (especially on the intermediate stages of fracture healing), and they do not give further information on what happens beyond the last stage (what every clinician defines as "complete recovery") [1–10, 18, 19]. On one side, this radiological study demonstrated the problems a trained observer has to face when evaluating fracture healing with conventional radiography: the low definition and bidimensionality of the images lead to a superimposition of some stages, thus leading to observer-dependent observations, without a clear and common concordance, most of all in the intermediate stages like stage 3, stage 4, or stage 5. Concerning all fractures falling into stage 6, the DR images were essentially similar in every case. The major aim of the study was, however, to test for the first time the skills of CBCT in the analysis of the structure of bone calluses on dry bone, a feature every anthropologist has to cope with quite frequently. CBCT was chosen because it is considered as the most valuable tool in the

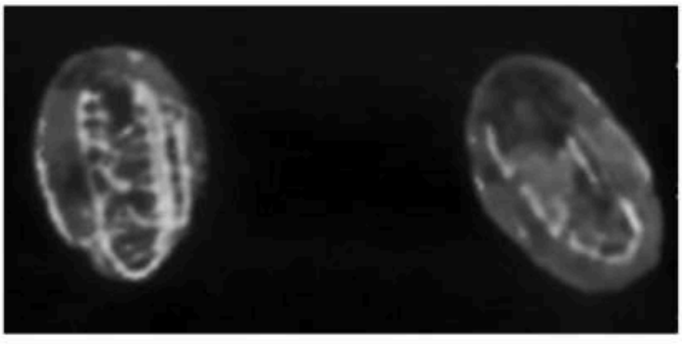

Fig. 1 Different features in the inner structure of calluses with the same age (28 days) in cases 5c (*left*) and 5d (*right*). On the *left*: high mineralization and regular outline of the callus; on the *right*: poor mineralization and disorganized structure of the callus

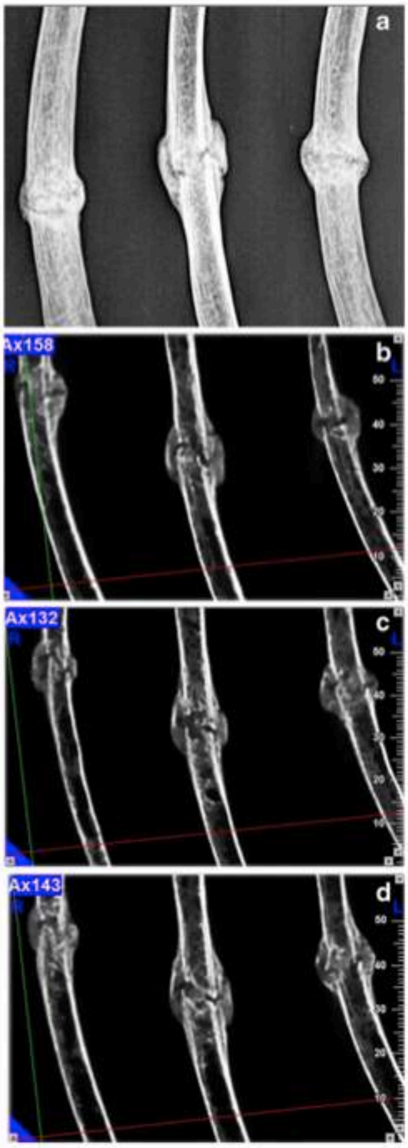

Fig. 2 Comparison of DR (**a**) and cone-beam CT images (**b**–**d**) of three coeval calluses (age 28 days). The callus evaluation resulted to be more precise with CBCT due to its high resolution and its possibility to give subsequent slices on different planes, providing more information on the inner structure of the callus. The rib on the right (**a**) shows a stage 4 healing (the fracture hidden by new bone formation) in the DR image; the same rib view in CBCT images (**b**–**d**) clearly shows more detail

evaluation of hard tissues for in vivo evaluations (particularly bone and teeth of the maxillofacial area). Firstly, in the earliest stages of fracture healing, when the presence of initial callus formation is macroscopically and radiologically (with DR) undetectable, CBCT seemed to bring no significant advantages, since any sign of early changes in the fracture pattern seemed to be undetectable. On the other hand, CBCT provided much more information concerning the different stages of mineralization of specific areas of the callus and other possibly useful features such as the different disposition of bone and cartilage inside the fracture gap, the sequence of new formation, the inner and outer structures, and the characteristics of bone remodeling after the callus has been formed and strengthened. The skills of this kind of radiological investigation are clearly visible in the two cases where coeval fractures on

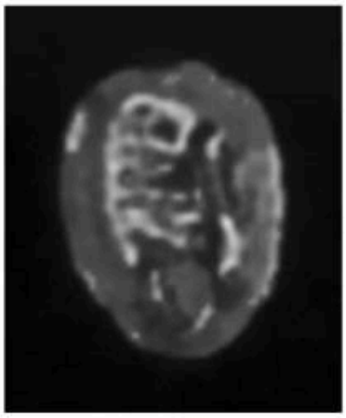

Fig. 3 Inner organization of the callus (age 28 days) and different stages of mineralization. Cone-beam CT image (case 5b)

Fig. 4 Old callus (2 years) in case 6b, cone-beam CT image. The inner part of the fracture line is still detectable, while the outer outline of the callus is smooth and continuous

different ribs were investigated: on one hand, the stages in which the bone calluses fell with DR were substantially similar because of similar features of the radiological images; on the other hand, the high definition of the CT images led to a precise view of the inner structure of the callus, with different dispositions of the layers inside the callus, several degrees of mineralization, different features of the fracture line, and characteristics of the surface of the callus. This clear aspect of the inner structure in coeval calluses can be at first sight misleading: if the stage assessed with DR is the same for every callus, the different features of the inner structure with CBCT can lead to a different stage evaluation. However, the results of this study have to be read as a key element for the future: the clearer features of the structures or the pattern of the fracture line can provide an easier definition of the stages of bone healing. To our knowledge, a radiological technique like cone-beam CT can be a helpful and valuable tool by which to begin a novel classification of bone healing.

The interest in radiological techniques has been rapidly developing in the forensic field [23–26] and in our opinion cone-beam CTs can be particularly helpful: sensitivity is frequently higher than the standard CT technology and the sample does not need to be as small as in the case of micro CT and pQ CTs. Furthermore, the skills of a radiological technique like cone-beam CT must be taken in serious consideration in another crucial forensic field like that of child abuse: one only has to think of the low amount of radiation and the quick acquisition time of the images (CBCT usually needs seconds when a traditional CT needs minutes), provided the lesion is in small parts of the body (like upper or lower limbs) which will fit into the gantry.

Such a technique could thus open up the possibility of making a concrete progress in this field, aiming at correctly evaluating injuries and fracture healing processes. Therefore, cone-beam CT, already known in the forensic field for gunshot and sharp force trauma, can be a helpful and reliable tool for the final purpose of a clear and defined view of bone fractures and calluses, thus trying to make the stages of fracture healing more clear and to create a new classification of bone remodeling.

References

1. Aro H, Eerola E, Aho AJ (1985) Determination of callus quantity in 4-week-old fractures of the rat tibia. J Orthop Res 3(1):101–108
2. Eastaugh-Waring SJ, Joslin CC, Hardy JR, Cunningham JL (2009) Quantification of fracture healing from radiographs using the maximum callus index. Clin Orthop Relat Res 467(8):1986–1991
3. Lujan TJ, Madey SM, Fitzpatrick DC et al (2010) A computational technique to measure fracture callus in radiographs. J Biomech 43 (4):792–795
4. Augat P, Merk J, Genant HK, Claes L (1997) Quantitative assessment of experimental fracture repair by peripheral computed tomography. Calcif Tissue Int 60(2):194–199
5. Den Boer FC, Brarem JA, Patka P et al (1998) Quantification of fracture healing with three-dimensional computed tomography. Arch Orthop Trauma Surg 117(6–7):345–350
6. Lynch JA, Grigoryan M, Fierlinger A et al (2004) Measurement of changes in trabecular bone at fracture sites using X-ray CT and automated image registration and processing. L Orthop Res 22 (2):362–367
7. Schmidhammer R, Zandieh S, Mittermayr R et al (2006) Assessment of bone union/nonunion in an experimental model using microcomputed technology. J Trauma 61(1):199–205
8. Nyman JS, Munoz S, Jadhav S et al (2009) Quantitative measures of femoral fracture repair in rats derived by micro-computed tomography. J Biomech 42(7):891–897
9. Morgan EF, Mason ZD, Chien KB et al (2009) Micro-computed tomography assessment of fracture healing: relationships among callus structure, composition, and mechanical function. Bone 44(2):335–344
10. Hayward LN, de Bakker CM, Lusie H et al (2012) MRT letter: contrast-enhanced computed tomographic imaging of soft callus formation in fracture healing. Microsc Res Tech 75(1):7–14
11. Islam O, Soboleski D, Symons S et al (2000) Development and duration of radiographic signs of bone healing in children. AJR Am J Roentgenol 175(1):75–78
12. Klotzbach H, Delling G, Richter E et al (2003) Post-mortem diagnosis and age estimation of infants' fractures. Int J Legal Med 117(2):82–89
13. Cattaneo C, Marinelli E, Di Giancamillo A et al (2006) Sensitivity of autopsy and radiological examination in detecting bone fractures in an animal model: implications for the assessment of fatal child physical abuse. Forensic Sci Int 164(2–3):131–137
14. Halliday KE, Broderick NJ, Somers JM, Hawkes R (2011) Dating fracture in infants. Clin Radiol 66(11):1049–1054
15. Malone CA, Sauer NJ, Fenton TW (2008) A radiographic assessment of pediatric fracture healing and time since injury. J Forensic Sci 56(5):1123–1130
16. Schindeler A, McDonald MM, Bokko P, Little DG (2008) Bone remodeling during fracture repair: the cellular picture. Semin Cell Dev Biol 19(5):459–466
17. Vetter A, Epari DR, Seidel R et al (2010) Temporal tissue patterns in bone healing of sheep. J Orthop Res 28(11):1440–1447
18. Hendrix RW (2002) Fracture healing. In: Rogers LF (ed) Radiology of skeletal trauma, vol 1, 3rd edn. Churchill Livingstone, Philadelphia
19. Toal RL, Mitchell SK (2002) Fracture healing and complications. In: Thrall DE (ed) Textbook of veterinary diagnostic radiology, 4th edn. Saunders, Philadelphia
20. Barbian LT, Sledzik PS (2008) Healing following cranial trauma. J Forensic Sci 53(2):263–268
21. Wieberg DA, Wescott DJ (2008) Estimating the timing of long bone fractures: correlation between the postmortem interval, bone moisture content, and blunt force trauma fracture characteristics. J Forensic Sci 53(5):1028–1034
22. Walker PL, Cook DC, Lambert PM (1997) Skeletal evidence for child abuse: a physical anthropological perspective. J Forensic Sci 42(2):196–207
23. Yang F, Jacobs R, Willems G (2006) Dental age estimation through volume matching of teeth imaged by cone-beam CT. Forensic Sci Int 159(1):78–83
24. von See C, Bormann KH, Schumann P et al (2009) Forensic imaging of projectiles using cone-beam computed tomography. Forensic Sci Int 190(1–3):38–41
25. Lee WJ, Wilkinson CM, Hwang HS (2012) An accuracy assessment of forensic computerized facial reconstruction employing cone-beam computed tomography from live subjects. J Forensic Sci 57(2):318–327
26. Murphy M, Drage N, Carabott R, Adams C (2012) Accuracy and reliability of cone beam computed tomography of the jaws for comparative forensic identification: a preliminary study. J Forensic Sci 57(4):964–968

4.1.2.
PART II:
APPLICATION OF HISTOLOGY AND RADIOLOGY ON KNOWN BONE CALLUSES

How valid are microscopic and radiological features for assessing Post Trauma Interval on bone calluses?

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4.1.2.1. INTRODUCTION:

Anthropologists are used to conducting various analyses on skeletal remains for the search of skeletal defects as well as trauma; both can give fundamental information with regard to the biological profile (essential for a potential personal identification) as well as interpretation of the events surrounding death (as in case of maltreatment, torture or survival at accidental or traumatic events). One of the first concerns, in fact, is to develop studies in the forensic field that may test the techniques and the knowledge acquired up to now and usually derived from clinical medicine, physiology and pathology in order to designate some useful interpretative models which can be applicable also to real forensic cases.

The analysis of antemortem lesions, specifically regarding the determination of the post trauma interval (PTI) in bone calluses, has become in the past few years an issue that urgently needs to find solutions; the specific information gained from the evaluation of a bone callus, in fact, can be valuable for personal identification purposes (a callus represents a unique and personal characteristic) as well as for the interpretation of abuse or a traumatic event prior to death. Regardless of the fact that an antemortem lesion in skeletal remains is easily detectable when there is a presence of bone reaction/healing (from a macroscopic and radiological point of view), the anthropologists are more often called to give more precise details about the time elapsed since trauma, and so are the pathologists.

If the recognition of a lesion as antemortem is deemed to be quite easy, the identification of the very early signs of healing or the definition of the PTI in the later stages of the healing process (especially when a lesion became healed) on the other hand is considered more problematic and not yet amply studied on dry bones. (84-86, 93-94)

Once a fracture has occurred during life, the bone tissue response follows a strict time-dependent sequence that is well known and widely explained in literature by several authors (60-83) who have reported results especially based on the histology (60, 62-67, 69-70), but also with clinical evaluation by radiology widely investigated on the living (63, 68, 87-88).

Despite the much information on the healing process from all viewpoints, the problem still remains unsolved when one needs to evaluate a precise post trauma interval on a dry bone callus.

A recent study has focused on the age evaluation of dry human bone calluses with a known age by using the new advanced CT technologies (cone-beam CT) which has revealed the advantages of highlighting inner features otherwise not visible by using conventional radiology (RD) (157). If on the one hand advanced technology assures the ability to see in more detail the inner characteristics of a bone callus, on the other hand it does not solve the problem of interpreting these features in relation to the healing process in terms of the time elapsed since trauma. Other studies in literature have reported results derived from the estimation of PMI using histology and conventional radiology on dry bone: Matt et al. (85-86). The authors have suggested a time-table reporting the elapsed time after bone tissue injury based on radiographic and histological healing features and also have pointed out what features can be seen on a dry bone callus allowing for an evaluation.

Other studies have focused on the application of the features suggested by Matt on dry bone calluses and perimortem lesions in order to prove what features are best visible and what are more objective. The results have shown that a considerable amount of healing features are still reliably detectable on dry bone tissue, and that, with significant inter-observer agreement, a reasonable estimation of the minimal and maximal time elapsed after an injury can still be made (93). The study in this perspective was conducted with a non-known sample (only classified as healed, healing or perimortem lesions), a fact that has not permitted to verify the validity and precision of such methods when applied to dry bones. Both, the lack of materials and the difficulty of collecting control samples are, in fact, some of the reasons behind the small amount of literature on such a topic in anthropology; but the subject needs to attempt solutions and methods need to be improved.

It is clear that grading the fracture repair and callus from an objective and quantitative point of view can hardly be performed with current radiological approaches. Moreover, from an osteological point of view, it is difficult to pinpoint where and at what different stages different parts of the callus are. Nevertheless, DR and histology could reveal important features that help in this perspective; but their validity in calluses/fractures of known age has to be proven or eventually improved.

In this view, the present study draws attention to this specific problem with the intent to investigate the tangible validity of microscopic parameters described by Matt and De Boer (85-86, 93-94) observable in dry human bone samples. The histologic analysis conducted in calluses with different known ages has permitted the observation of the potential ability in defining the PTI with attention to precision, accuracy and limits. In addition, the study provides an opportunity to microscopically observe any differences or similarities correlated with both, the typology of bone tissue (rib and skull), and the various stage of the healing process in the same bone tissue type.

4.1.2.2. MATERIALS and METHODS:

4.1.2.2.1. Bone callus specimens:

Nine bone fractures (with or without evident formation of bone callus) of several known PTI were examined from eight individuals who underwent autopsy according to Police Mortuary Regulation (Table 4.1.2.1. shows general information about the study sample). The samples were defleshed mechanically and by maceration in cold water. Only the antemortem lesions from presumably healthy adult individuals were included in this study in order to avoid possible bias due to pathological conditions. In two cases (cases 1, and 6), the stages of the healing process resulted too early to be detected macroscopically nor mineralization or osteogenic reaction was present at death; whereas in the other seven samples, the presence of the callus was macroscopically visible: the more regular and smooth, the older the lesion. The bone specimens selected for the study consist in only two different bone typologies: 5 rib calluses and 4 skull calluses (Figure 4.1.2.1.), in order to limit the variability factors already amply present.

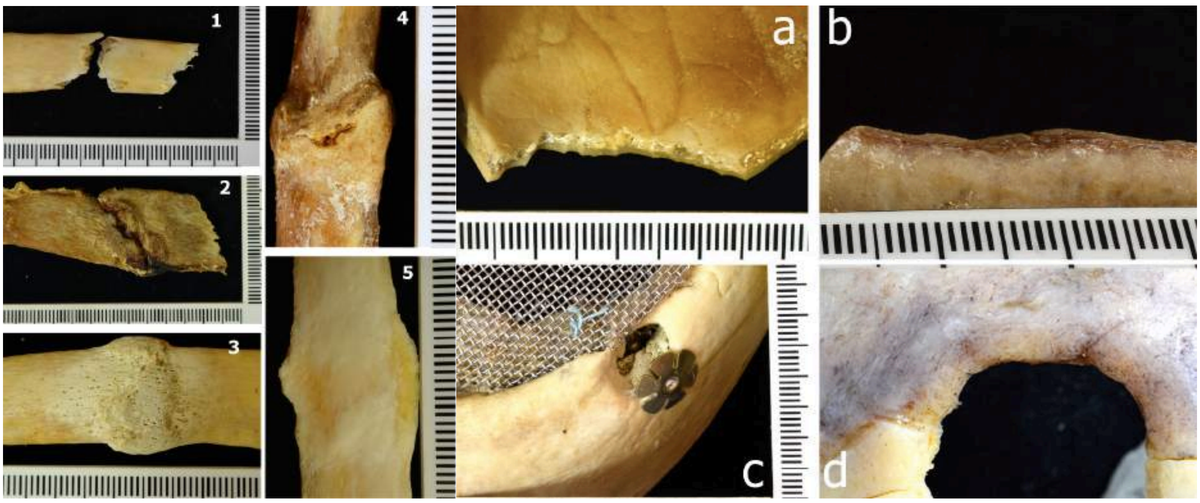

Fig 4.1.2.1.: Photographs of the 9 samples corresponding in rib calluses and skull antemortem lesions of different ages with divers macroscopic appearance: 1) sample 1, 2) sample 2, 3) sample 3, 4) sample 4, 5) sample 5, a) sample 6, b) sample 7, c) sample 8 and d) sample 9.

The samples were selected based on the different known ages in order to cover a large time interval and the various phases of healing (data about each callus are summarized in Table 4.1.2.1.), which range between the very early stages of bone healing (few days since trauma) to the final remodelling stages of the healed fractures (several years after the trauma).

SAMPLE NUMBER	***BONE TYPOLOGY***	***AGE AT DEATH SINCE TRAUMA***	***ADDITIONAL DATA***
1	Rib	9 days	No surgery
2	Rib	20 days	No surgery
3	Rib	28 days	No surgery
4	Rib	90 days	No surgery
5	Rib	2 years	No surgery
6	Skull	17 days	Cranial surgical lesion
7	Skull	90 days	Cranial natural healed lesion
8	Skull	1,5 year	Cranial surgical lesion
9	Skull	15 years	Cranial healed lesion

Table 4.1.2.1.: Bone samples and related data in terms of age of trauma and typology of bone**.**

4.1.2.2.2. Methods:

All samples were photographed and studied in their gross anatomy. Subsequently, conventional radiography as well as Computed Tomagraphy were accomplished prior to the destruction of samples for performing histology. Conventional radiographs were performed in an antero-posterior projection. Once the radiological imaging was performed, the samples were prepared for histology following the protocol standardized for making un-decalcified dry bone sections for microscopic analysis (93). The so-called 'thick slices', were excised from the centre of each bone sample (not subjected to the decalcification process) perpendicularly to the longitudinal plane for rib bodies and perpendicularly to the fracture direction for the skull samples by using a band saw. From each thick slice at least two thin sections were produced: one thin section remained unstained, the other was stained with haematoxylin to enhance the visibility of some tissue architectural aspects with a minor adaptation according to the method of De Boer et al. (93). All sections were microscopically investigated using bright and polarized light. The evaluation of the degree of the bone healing by both microscopic and radiological analysis was based on the description reported in the timetable suggested by De Boer (93) which derived from the combination of features described in literature by Maat (86) and Barbar (158).

Then, the post-trauma interval assessment was obtained for each antemortem bone lesion, in order to verify validity and precision of this method.

Category of lesion	***Healing features***	***Time interval***
Common	Frayed bone lamellae at the lesion margins*	Before 48 hours
Common	Absorption of the cortical bone adjacent to the lesion°	After 4-7 days
Common	First Howship's lacunae at the lesion margins*	After 4-7 days
Common	Smoothening of the lesion margin °*	After 4-7 days
Common	Start of endosteal and periosteal osteogenesis separable from cortex *°	After 7 days
Common	Periosteal osteogenesis at distance from the fracture site	After 7 days
Common	Clearly visible endosteal callus formation *°	After 10-12days
Common	Aggregation of spiculae into woven bone *°	After 12-20 days
Common	Primary bone tissue deposition*	After 12-20 days
Common	Osteoporosis of the cortex*°	After 12 days
Common	Margin of the lesion appears more sclerotic°	After 12-20 days
Common	Start of the transition primary woven bone into secondary lamellar bone*	After 14 days
Common	Cortical 'cutting and closing cones' orientated towards the lesion*	After 14-21 days
Common	Fields of calcified cartilage at sites of callus formation	After 14 days
Common	Clearly visible periosteal callus*°	After 15 days
Common	Endosteal callus becomes indistinguishable from the cancellous bone in the marrow cavity*°	After 17 days
Common	Periosteal callus becomes firmly attached (inseparable) to the cortex *°	After 6 weeks
Specific for fractures	First scattered bone tissue spiculae between the lesions ends*°	After 4-7 days
Specific for fractures	Union by bridging of the cortical bone discontinuity*°	After 21-28 days
Specific for fractures	Smoothening of the callus outline*	After 2-3 months
Specific for fractures	After inadequate immobilization: Pseudoarthrosis development*°	After 6-9 months
Specific for features	After adequate immobilization: quiescent appearance indicating subsided healing*°	After 1-2 years
Specific for amputations	Visibility of cut marks on the amputation surface°	Less than 13 days
Specific for amputations	Start of 'capping' of the medullary cavity°	After 'not many weeks'
Specific for amputations	Complete capping of the medullary cavity°	After 'several months'

Table 4.1.2.2.: Timetable of features of healing process (*Features visible by plain radiographic analysis; °Features visible by histological analysis) reported by Maat et al. (86)

The microscopic observation was carried out on each sample to search for the characteristics described in the timetable mentioned above (Table 4.1.2.2.); then for all samples the PTI was assigned and obtained from the various minimum times corresponding to each features placed. The final PTI was then derived by the extrapolation of a common interval resulting from all the time intervals related with the microscopic characteristics observed. Thus, some images were captured by optical microscopy.

Secondly, the search for features visible only through radiological analysis, as specified by Matt *et all*. (86), was conducted on the radiologic images for each bone callus, in order to add or to implement information with those obtained from histological analysis.

4.1.2.3. RESULTS:

Microscopic observation of both thin undecalcified sections (stained by hematoxylin and not stained) obtained from each known rib callus has allowed for the detection of important features in estimating the PTI; each characteristic observed through microscopy corresponds to a specific stage of the healing process and at different related times that were assessed for all samples as shown in Table 4.1.2.3; for each sample a diagram was also reported representing the estimate of the PTI obtained from the microscopic analysis (Fig. 4.1.2.2-4.1.2.5.).

Sample	*Age of the trauma*	*Estimated PTI*
1	9 days	7<x<12 days
2	20 days	14<x<28 days
3	28 days	21<x<42 days
4	90 days	x>42 days
5	2 years	x>1 year

Table 4.1.2.3: Resultant PTI from the microscopic observation for rib lesions

The microscopic observation of sample 1 (which showed macroscopically neither osteological reaction or callus) revealed the absence of both, frayed bone lamellae and callus formation (Fig. 4.1.2.6.), which means that the lesion survived respectively at least more than 48 hours but less than 12 days. In addition the start of endosteal and periosteal osteogenesis at distance from the fracture site, which usually appears after 7 days, and the absorption of the cortical bone adjacent to fracture site together with both the smoothening of the lesions margins and the presence of first Howship's lacunae at the fracture site, have allowed to restrict the interval ranged between 7 and 12 days (Fig. 4.1.2.2.).

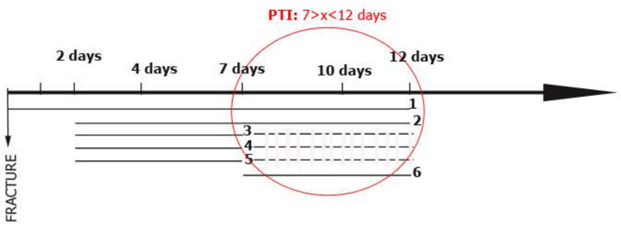

Fig 4.1.2.2.: PTI estimation for sample 1 based on the microscopic analysis

Contrary to what was reported for sample 1, sample 2 had demonstrated microscopically the start of both periosteal and endosteal callus formation (which respectively appear after 7 and 10-12 days). In addition, despite the callus formation formed by just woven bone as observable in Figure 8 (which emerge after 14 days), and the presence of cortical "cutting and closing cones" orientated towards the lesion as observable in Figure 7 (the formation of which starts 14-21 days after the fracture), no union between the two ends was detectable, a fact that allowed to evaluate a maximum age of 21-28 days (Fig. 4.1.2.3).

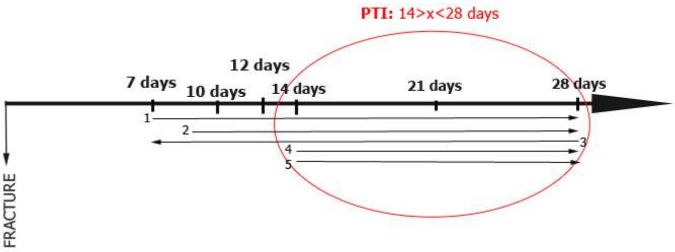

Fig.4.1.2.3: PTI assessment for sample 2 based on the microscopic evaluation

Sample 3 shown some primary bone tissue deposition and a clearly well formed visible endosteal callus formation (Fig. 4.1.2.9.), both features emerging after the 10th days since trauma. Since the periosteal callus was not yet firmly attached, the callus revealed to be younger than 42 days, but the start of union by bridging of the cortical bone discontinuity (Fig.4.1.2.9.) is a characteristic that has allowed for an increase in the minimum of time from 10 days to 21 days (Fig. 4.1.2.4.).

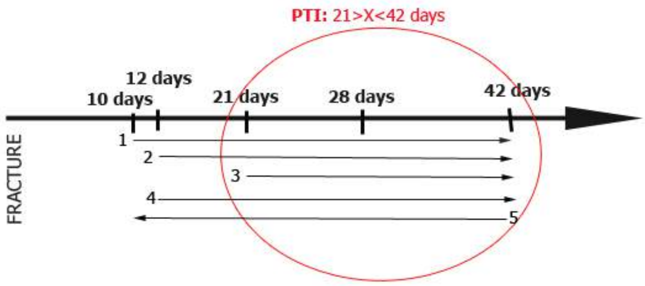

Fig.4.1.2.4.: PTI assessment for sample 3 based on the microscopic evaluation

With regard to samples 4 and 5, the microscopic evaluation permitted only to give a minimum time; in sample 4 the periosteal callus showed to be firmly attached (Fig 4.1.2.8, 4.1.2.9) which means an age superior to 42 days, but because of the lack of any quiescence (Fig 4.1.2.7) no additional time information could be given (Fig. 4.1.2.5). Similarly, for sample 5 it was possible to assign only a minimum age of at least 1 year (the smoothening of periosteal callus outline and the quiescent appearance) since no other features were observable (Fig. 4.1.2.10).

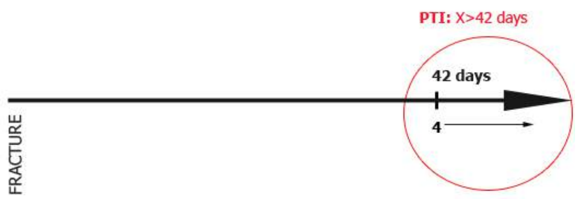

Fig 4.1.2.5.: PTI assessment for sample 4 based on the microscopic evaluation

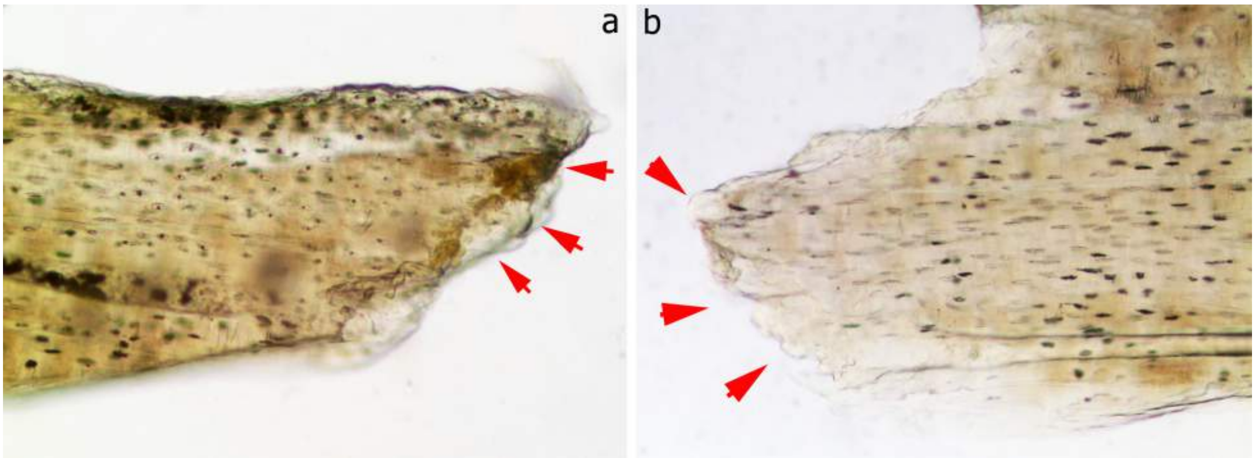

Fig 4.1.2.6.: The two ends of the fracture site in sample 1 do not have any frayed lamellae margins (a, b), which show some initial smoothening process (9 days). Magnification is 100X for both a) and b).

Fig 4.1.2.7.: One cortical 'cutting and closing cones' orientated towards the lesion in sample 2 (Magnification of 100X). The red arrows indicate the mark left by osteoclast during the removal of necrotic bone, as a sort of Howship's lacunae.

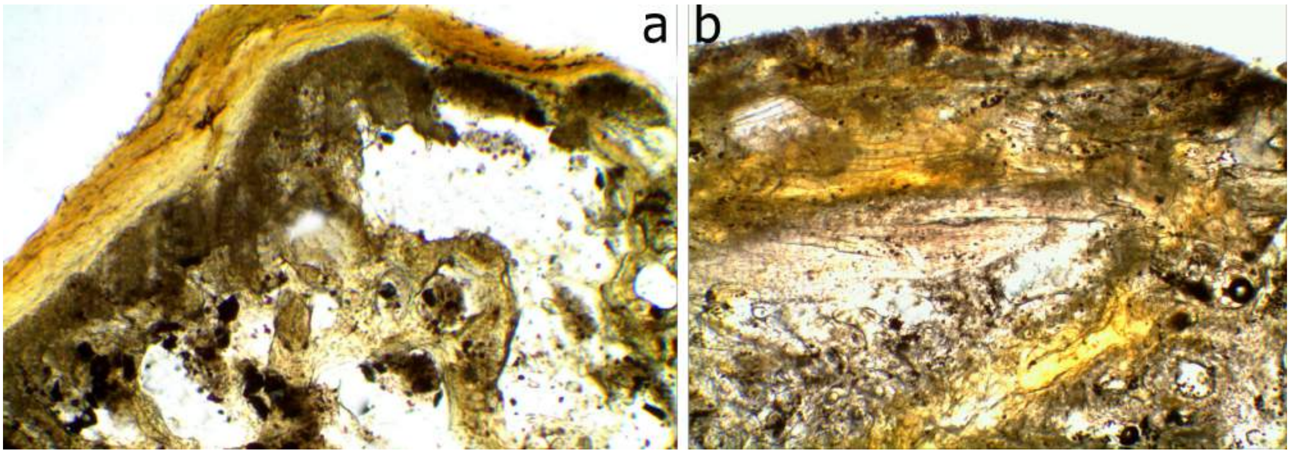

Fig 4.1.2.8.: Two examples of the periosteal callus: a) starting of a clear periosteal callus with some primary woven bone and cartilage, but still separated from the cortex bone in sample 2 (20 days); b) periosteal callus well formed with transition of woven bone into secondary lamellar bone, already firmly attached to the cortex bone in sample 4 (90 days). Magnification is 40X for both a) and b).

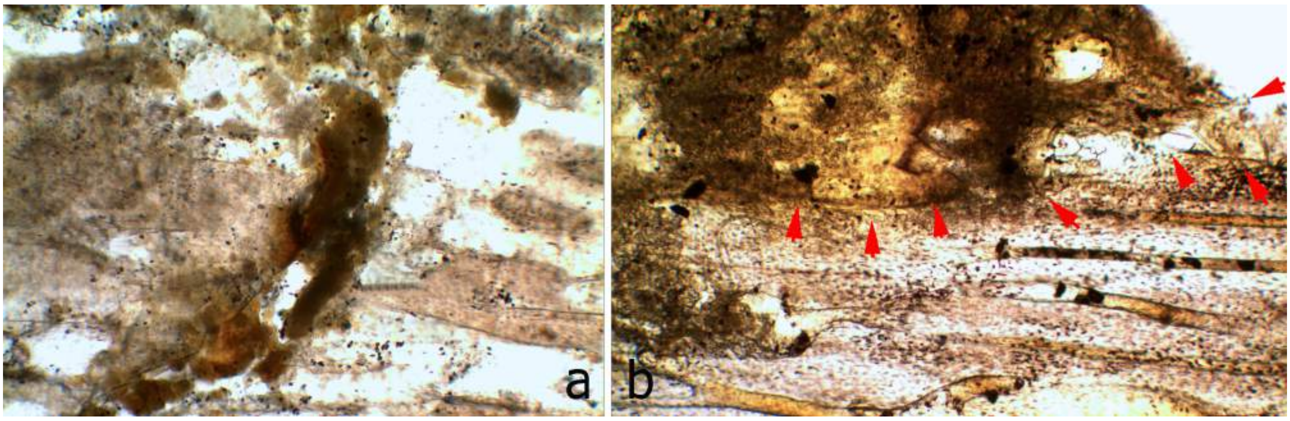

Fig 4.1.2.9.: Sample 3 (a), Magnification of 100X) and Sample 4 (Magnification 40x): in a) some union points by bridging of the cortical bone discontinuity, still present in the rest of the fracture site (28 days). b) The clear and mature periosteal callus (well formed) is firmly attached to the cortex bone in sample 4 (90 days) and the endosteal callus becomes almost indistinguishable from the cancellous bone in the marrow cavity.

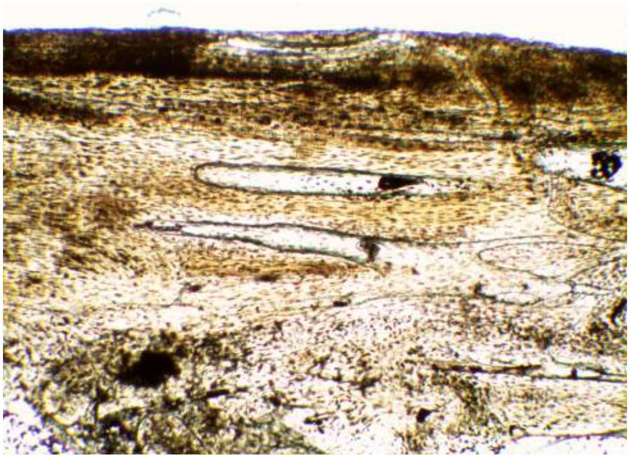

Fig 4.1.2.10: Sample 5 (2 years), magnification of 40X. The periosteal callus is firmly attached as sample 4 but with smoothening outline. The quiescent appearance indicating subsided healing and none additional characteristic gives a time interval more detailed than that given by the quiescence (> 1 year).

With regard to the radiological appearance, the analysis confirmed results from histology, and no more detailed information was added to that derived from the microscopic appearance. Nevertheless, the comparison between calluses of different age revealed evident differences in the degree of mineralization (namely the opalescence visible in the several radiographic images shown in Figure 4.1.2.11) but no more specifics on time could be assessed.

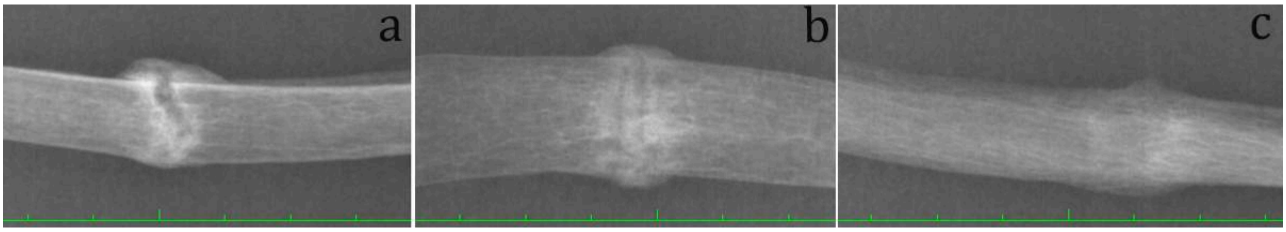

Fig 4.1.2.11.: Sample 3 (a), 4 (b) and 5 (c) in radiographic images. The periosteal callus is observable in all the samples, but in sample 5 is well visible a smoother outline; in addition, the endosteal callus is more mature and has demonstrated to be similar to the adjacent bone tissue. On the other hand, in sample 3 (a) the fracture line is still very visible and only a start of bridging is observable, while in sample 4 (b) the union between the two margin ends is almost complete. All radiographic features confirm the microscopic evaluation performed in the thin undecalcified sections and do not give any additional information, mineralization of the callus excluded.

Analogously, results on skull lesions are reported in Table 4.1.2.4. The microscopic description for skull samples resulted in much more difficulty because of the lack of many features that were not detectable. In sample 6 for instance, few frayed bone lamellae were still visible at the margins that resulted as not yet well smooth and just few isolated Howship's lacunae were found in the vicinity of the lesion site. Since the presence of Howship's lacunae was very limited, the PTI chosen for the lesion was over 48 hours but no further precision could be given. Sample 7 has shown some peculiar features better visible on rib calluses, such as the deposition of new woven bone and abundance of Howship's lacunae. In this case, the start of a sort of 'capping', similar to what happens in the healing process on long bone after the amputation, permitted the ability to assert that the callus is older than several weeks, but even in this case a no more precise post-trauma interval was given. Similarly, sample 8 shown a sort of a 'capping of diploe' which resulted as almost complete, demonstrating in this case a healing process started several months before but no specific amount of months could be specified.

Finally, sample 4 disclosed some microscopic features similar to those observed in sample 3, but the presence of some quiescence permitted the ability to assign at least an age older than 1 year.

Sample	*Age of the trauma*	*Estimated PTI*
6	17 days	x>48 hours
7	90 days	x>several weeks
8	1,5 year	x< several months
9	15 years	x>1 year

Table 4.1.2.4: Resulting PTI from the microscopic observation for skull lesions and calluses

The radiological analysis (Fig 4.1.2.12), as for rib samples, did not add any additional information with regards to the PTI. Thus, many problems arose from the search for features related to the healing process but just a few features specific for amputation were detectable.

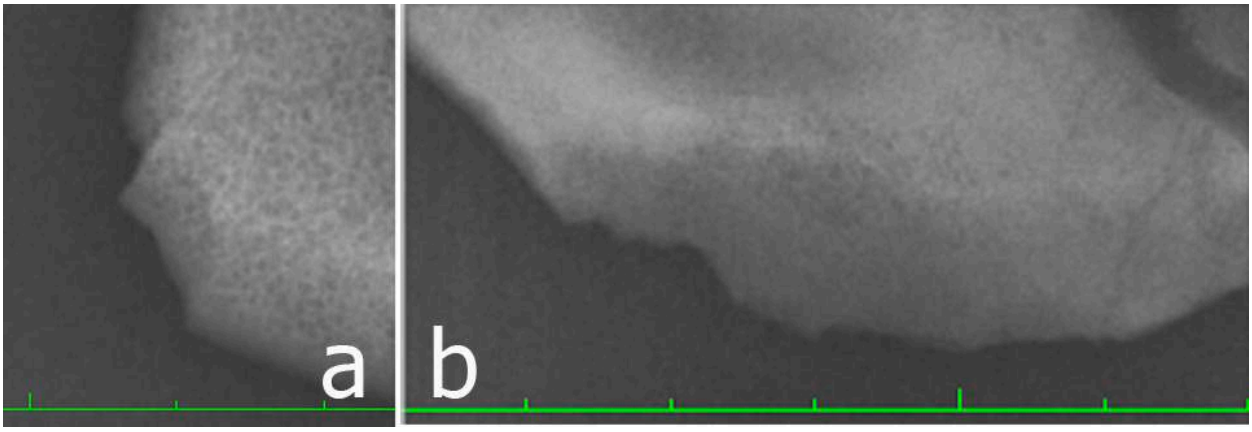

Fig 4.1.2.12.: Sample 6 (a) and 7 (b) in radiographic images. No mineralization is visible and so are other additional features detectable by radiology. In this case the radiologic analysis do not give any details for the evaluation.

4.1.2.4, DISCUSSION:

Aging antemortem bone fractures and calluses in skeletal still remains an extremely difficult and crucial issue; despite the capability of anthropology to declare an antemortem lesion as healed or/and healing based on gross macroscopic appearance, often the forensic anthropologist is called to give more accurate and precise information with respect to time elapsed since trauma (85-86, 93-94, 157-160). At the moment on dry bones very few studies have investigated the microscopic appearance (84-86, 93-94) and features of bone remodelling by histology (158, 86, 93-94); thus the studies often have only focused on the detectability of such microscopic features in order to reveal the histological characterization of bone healing processes at different stages and the chance to observe the specific microscopic aspects related to time directly on thin bone tissue sections. In addition, there is some ambiguous knowledge derived from experimental studies on animal models or from the evaluation of single human cases (85, 94), or unknown bone samples (93-94).

The major intent of this study was, however, to test for the first time, the potentiality of knowledge in this specific forensic issue through the application of microscopic and radiologic osteological parameters described in literature by Matt et al (86) and summed up in a useful timetable suggested for anthropological purposes. In addition, the study also intended to investigate the differences in aging the antemortem lesions according to the bone type considered, namely rib or cranial surgical lesions. The Post-trauma interval estimated by either the radiologic and histologic analysis revealed to be concordant, nevertheless histology has shown greater capacity to detect more features useful for the evaluation, as in the lesions at the early stages of healing (sample 1 and 6). In fact, some peculiar characteristics, like the smoothening of frayed bone lamellae as well as the abundant presence of the first Howship's lacunae, have shown to be important and valid early markers that can give a minimum time of few days since trauma accordingly to literature (74-77, 85-86, 93); similarly, at the same stages, but only in sample 1, the lack of features of the periosteal callus formation together with the beginning of removal of bone tissue from the fracture site or the start of endosteal and periosteal callus formation activity at a distance from the fracture site, are suitable microscopic parameters that have to be considered for giving a maximum time in the PTI evaluation. In case of early stages of healing (of a few days) radiologic images did not give any additional information, resulting in such a way as an unnecessary analysis. The same could not be said for the older lesions, for which as the time elapsed since trauma, increases in the PTI result becomes less precise. The difficulty of dating older antemortem lesions was already demonstrated by the previous study (157) in which the analysis concerned the skill of CT Cone-Beam in revealing important details that, however, demonstrated their inability in giving more specified temporal information for the healed calluses evaluated as stage 6 (the final radiologic grade) according to the classifications commonly accepted by the scientific community (63, 74, 77, 79, 82,). This last account was also proven in skull lesion, in which not surprisingly the only features observable by microscopy were those specific for amputation (except for sample 6) and in this case the bone tissue behaved differently from what happens in rib fractures, a fact that could hypothetically be due to the presence of osteotomy or cranial surgery instead of to bone typology, also reported in a recent study (94). In general the evaluation of PTI was correct and fair for all samples and for both the bone typology; of course the precision of time intervals seems higher in the younger antemortem lesions and can be affirmed as not satisfactory in all cases. Despite the many difficulties in giving a fair estimation of minimal and maximal time lapsed since trauma for healed lesions or older calluses, the radiologic analysis, as shown in Fig. 4.1.2.11, has pointed out an important aspect that still needs to be investigated in detail: the quantity and localization of the mineralization of the inner endosteal callus could be potentially a good parameter for assessing more satisfactory the PTI, but this specific point still needs to be considered especially by using certain technologies able to detect and quantify this particular feature. Thus, as testified by recent publications (93-94, 157), in forensic anthropology the assessment of aging the antemortem lesions with the assignment of a valid PTI has improved a lot if compared to diagnosis such as "healed" or "healing", but still remains inadequate and needs further adjustments especially for the more advance stages according to Cappella et al (157). In addition, some further investigations are recommended for the search of markers that can estimate with more precision the PTI also in the very early stages of the bone healing process; the future goal would be that of giving a more restricted time interval with the intent to evaluate the lesions also in terms of both vitality and of survival with regards to death, which means recognizing the lesions that have occurred just a little time prior to death as antemortem despite the absence of any bone reaction.

Chapter 5

5.1.
SECOND RESEARCH LINE: THE PROBLEM OF INTERPRETING PERIMORTEM LESIONS

The presence of a skeletal collection similar to that described in Chapter 3 made it possible to conduct an in depth study on the evaluation of the interpretation of perimortem trauma, in particular on the common criteria used to distinguish between peri- and postmortem lesions (criteria already widely described in paragraph 4 of the introduction). A similar study aims at detecting limits still present in this specific issue, at least in detail for buried skeletal remains, and problems in interpreting trauma potentially related to the cause and modality of death. In this case the availability of skeletal remains with known perimortem and postmortem trauma, as well as the causes of death, allowed us to conduct two different studies, both concerning taphonomy's role in altering or in transforming perimortem lesions correlated with death. The interference of environmental taphonomic factors, in fact, may contribute to altering pre-existing lesions and in creating new skeletal damages and fractures, an aspect that make it harder for anthropologists to assess trauma.

Despite the many publications which report results on the differentiation between peri. and post-mortem trauma based on macro-morphological parameters like differences in colour, angle, morphology of fracture margins and in the fracture outline, there are very few works in which these criteria have been subjected to an examination with the intent to observe the real applicability and effectiveness. This fact is due particularly to the lack of known osteological material on which to conduct similar assessments.

The presence of the Milan skeletal collection made it possible to conduct an evaluation of the criteria used for the diagnosis of perimortem lesions and its limits. This research line has developed in two consecutive steps, both of which performed on several skeletal remains with the presence of both known perimortem trauma and postmortem changes; all cases were selected from the skeletal sample analysed and described in Chapter 3. The results have been already published in forensic international journals in the form of two different papers.

The first work, published in "Forensic Science International" focused on a macro-morphologic trauma analysis performed on several skeletons for which the cause of violent death is known and in the autopsy reports the lesions correlated with death and concerning both soft tissue (skin and organs) and hard tissue (skeletal apparatus) are described in detail. The analysis was conducted in order to verify if and how the diverse type of trauma can survive after 15 years of inhumation since death. In this study the comparison between autopsy findings (the description of letal lesions found in the cadavers) and the anthropological findings obtained from the analysis performed on the same individual 20 years later (skeletonized remains) show important results concerning the persistence of some specific lesions, which can still lead at one correct interpretation of the trauma. Nevertheless, this study highlights also the role of taphonomy in making difficult the interpretation of trauma by hiding important perimortem features and adding a consistent number of new postmortem alteration/fractures which often alter pre-existing lesions, as for what concerns blunt force trauma. In particular, this research permitted to notice that gunshot and sharp force lesions do not change considerably and remain highly specific for the assessment of the correlated trauma when analysed, even though similar lesions cause by taphonomic events can be found and may result easily misinterpreted at anthropological analysis. Contrary, blunt force trauma resulted to be much more altered by taphonomy and so an additional project was required to investigate in more details this aspect.

The second study, in fact, concentrated on the most challenging lesions whose assessment in the first research was extremely difficult, namely fractures produced by blunt force trauma. The aim in this study was to verify the validity and reliability of macro-morphologic parameter on a consistent number of fractures (over 200) of known origin from skeletons selected from the skeletal samples. This research was carried out with the intent of highlighting the limits of these common criteria, which proved to be unsatisfactory and difficult to be applied to cancellous bone. The application of these criteria to over 200 fractures of known origin performed by two expert anthropologists was a partial failure and proved there to be very few objective assessments depending on the type of bone tissue as shown by the results from the intra-error evaluation.

The following two works presented in this Chapter highlight again the difficulty and problems that lie behind the so-called "fracture timing", mainly for fractures that occur on spongy bone which display a thin cortical surface; the structural differences between long bones and no long bones (such as pelvis bone, vertebrae, ribs) may play a crucial role in exhibiting specific morphologic features in case of injury as well as taphonomic effects. Considering that the distinction between perimortem and post-mortem fracture is based solely on macro-morphologic parameters and that these last are mainly established on characteristics observed in cortical tissue of long bone, it appears evident the need to developed new models and parameters in future research.

5.2.1.

PART I:

THE INTERPRETATION OF PERIMORTEM LESIONS IN SKELETAL REMAINS AFTER 20 YEARS FROM INHUMATION: WHAT ANTHROPOLOGY CAN STILL SAY AMONG THE DIFFICULTIES DUE TO TAPHONOMY

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Forensic Anthropology Population Data

An osteological revisitation of autopsies: Comparing anthropological findings on exhumed skeletons to their respective autopsy reports in seven cases

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ABSTRACT

Forensic anthropologists and pathologists are more and more requested to answer questions on bone trauma. However limitations still exist concerning the proper interpretation of bone fractures and bone lesions in general. Access to known skeletal populations which derive from cadavers (victims of violent deaths) who underwent autopsy and whose autopsy reports are available are obvious sources of information on what happens to bone trauma when subjected to taphonomic variables, such as burial, decomposition, postmortem chemical and mechanical insults; such skeletal collections are still however quite rare. This study presents the results of the comparative analysis between the autopsy findings on seven cadavers (six of which victims of blunt, sharp or gunshot wounds) and those of the anthropological assessment performed 20 years later on the exhumed dry bones (part of the Milano skeletal collection). The investigation allowed us to verify how perimortem sharp, blunt and gunshot lesions appear after a long inhumation period, whether they are still recognizable, and how many lesions are no longer detectable or were not detectable at all compared to the autopsy report. It also underlines the importance of creating skeletal collections with known information on cause of death and trauma.

1. Introduction

Contrary to what happens when dealing with a well preserved cadaver, data available on skeletal material rarely allow the precise reconstruction of a lethal event, although signs of trauma may persist in bone [1,2] and strongly suggest foul play. In fact, examination of a skeleton differs widely from examination of a well preserved cadaver, essentially because of the presence of soft tissues in the latter which obviously provide much more information on cause of death, usually lost with decomposition of a corpse.

Many studies have investigated different types of trauma involving bone with the intent of establishing patterns of all types of lesions, though none truly verify how and how many signs of trauma show up on bone after natural decomposition and taphonomic insults [3–18].

The aim of this work was to provide through seven cases a rare illustration of how skeletal lesions appear after burial and decomposition compared to their appearance on the fresh cadaver. The novelty of such material lies in the fact that of these seven skeletons autopsy reports and photographs were available, describing the soft and hard tissue lesions found on the well preserved cadaver. The bodies were then buried and exhumed after 15 years, once skeletonized – providing a unique chance of verifying the persistence of bone trauma after natural decomposition and a comparative analysis of signs found at autopsy vs those found upon skeletal analysis.

This study was possible thanks to Italian Police Mortuary Regulations, which allows skeletal remains of known individuals to be studied if the remains have not been claimed by relatives. This material provides insight concerning the changes a body, and in

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particular its skeleton, undergo in 15 years of inhumation. Several skeletal collections have already been used for anthropological studies (e.g. Robert J. Terry Anatomical Skeletal Collection, George Huntington Collection, Collection of Identified Human skeletons housed at the Bocage Museum, William M. Bass Donated Skeletal Collection, Dart Collection of Human Skeletons) [19–21]. These collections however have ante mortem information mainly on sex, age, and time of death, but few have known causes of death, particularly violent deaths, and especially autopsy reports available. If in fact for some skeletal collections the cause of death of individuals is known, they almost exclusively include individuals who have died of natural death and rarely of violent deaths.

The Laboratorio di Antropologia e Odontologia Forense (LABANOF), situated at the Dipartimento di Scienze Biomediche della Salute (University of Milan), houses numerous skeletal and osteological collections accumulated since 1995, which have become the Milano Osteological Collection. The various collections consist in complete and partial skeletons derived from archeological contexts, mass graves, and modern cemeteries but also identified and unidentified skeletons (complete and incomplete) from forensic cases.

In particular, the lab houses a well-documented skeletal collection, coming from the Cimitero "Maggiore" of Milan, which offers over 1300 complete skeletons, unclaimed, belonging to individuals who died between 1990 and 1998 and who were buried for 15 years prior to exhumation. For each skeleton all relevant demographic information (resulting from death certificates) are available as well as, for a smaller group of skeletal remains, data on health issues and autopsy reports. The cause of death for many is traumatic, which makes the collection an interesting model also for trauma analysis.

Furthermore, the state of preservation is similar since all individuals were buried in similar wooden coffins, in the same cemeterial plot and exhumed 15 years later with an excavator.

In this perspective, this study aims to compare these seven cases what the pathologist saw at the autopsy and what the anthropologist found 15 years later on the same individual with the intent of observing the survival of signs of trauma on bone after several taphonomic variables were involved.

2. Material and methods

2.1. Seven unique cases

The sample of this study consists of seven individuals with complete autopsy case files involving well preserved cadavers of individuals who died from different causes in 1990–1991, and the consequent anthropological analysis performed 20 years later on the exhumed skeletonized remains. The collection, still under construction, at the moment involves a much larger number of skeletons (over 1300), and the study of the skeletons and acquisition of autopsy files and ante mortem clinical data are still underway; the authors wish to focus on the first seven most complete and interesting cases so far encountered which had been subjected to autopsy, in order to demonstrate the unique comparison between autopsy and anthropological data.

For each skeleton of the seven selected cases a detailed autopsy was available; the seven autopsies were performed by different forensic pathologists between 1990 and 1991 on well-preserved cadavers at the Institute of Legal Medicine of Milano. Each autopsy file contains essential data: demographic information (age at death, sex, and possible pathology), death certificate, external body examination, photographs or sketches, and autopsy findings. The exhumations were carried out after 15 years as mentioned previously and the remains moved separately to metal boxes in an ossuary where they remained for the following 5 years. It is necessary to specify that the exhumation was conducted by cemetery workers with no anthropological support and was performed with a small excavator and shovels; this may have caused additional postmortem fractures: thus the handling of remains, together with the mechanical damage they might have been exposed to, could inevitably have provoked some damage to bone elements. The exhumation consists of the use of excavators to reach the buried coffin which is already frequently open because it has collapsed. Then the remains are manually moved to a container, dried, put into closed metal boxes and transported to the University. It appears clear, once more, how some postmortem damage can occur in each of these phases. However it is important to stress the fact that all skeletons have the same taphonomic history and might show similar postmortem features and patterns with respect to environmental events.

The gathering and study of the seven skeletons, and in general of the skeletal collection, was performed according to article n. 43 of the mortuary police D.P.R. n. 28 (September 10, 1990), which authorizes studies on human remains not claimed by relatives.

The selected cases consist of individuals who have died of different violent causes and include diverse types of trauma: gunshot (2 examples), sharp force (1 example) and blunt force trauma (3 similar examples of traffic accident) (summarized in Table 1). In addition, a case of natural death (lack of traumatic lesions) was included in which surely there was no trauma found upon autopsy but only taphonomic fractures.

The study was performed by first cleaning the skeleton and verifying sex, age, ancestry and pathology, then trauma analysis was carried out blindly (before consulting the details of autopsy reports) by a trained forensic anthropologist on the seven individuals considered. For trauma analysis, the anthropologist was asked to describe and classify all bone trauma observed on each skeleton; all alterations were differentiated as perimortem and postmortem, and then transferred to a chart with specific color codes for perimortem and postmortem trauma. Then they were asked to differentiate between blunt force, sharp force and gunshot wound according to morphology.

For the differentiation of perimortem from postmortem alterations the anthropologist used multiple features typical of patterns of lesions of dry and wet bones according to the criteria suggested in the anthropological field by many authors [22–25]. Mainly, the evaluation was based on macroscopic fracture

Table 1
General data of each of the seven cases.

Case number	Sex	Age	Event	Cause of death
1	Female	81	Natural death	Purulent pneumonia
2	Female	79	Traffic accident	Multiple skeletal and visceral lesions
3	Female	83	Traffic accident	Multiple skeletal and visceral lesions
4	Male	60	Gunshot injury	Abdominal lesions
5	Male	76	Sharp force injury	Multiple stab lesions
6	Male	28	Gunshot injury	Cranial and cerebral lesions
7	Male	78	Traffic accident	Multiple skeletal and visceral lesions

morphology (fracture margin morphology, fracture angle, outline and surface appearance) and on color variation according to what has been recently reported by Moraitis et al. [23] and Weiberg and Wescott [22]. Perimortem damage refers to an injury occurring at or around the time of death. Because of the viscoelastic properties of fresh bone, a perimortem lesion is recognized on the basis of evidence of the biomechanical response of fresh bone, which gives traits specifically reported and noted in some recent publications [22,23,25]. In this sense features used by the anthropologist in this study that indicate perimortem trauma on the 7 skeletons include, according to the above mentioned literature: fracture outline, fracture surface morphology and the fracture angle, and color (homogenous or heterogeneous with external cortical bone).

For each case we then reported in a set of forms for trauma analysis both the superficial (external examination) and deep findings and the bone fractures described at autopsy. Finally, the comparison was performed for each case presented, which permitted us to verify as scope of the study the presence of lesions seen both at autopsy and upon anthropological examination, the presence of lesions seen only at autopsy and not clearly classifiable as perimortem fractures after exhumation as well as the presence of apparently perimortem lesions seen only upon anthropological analysis after 15 years of inhumation, and not at autopsy, summarized in Table 2.

3. Results

3.1. Case 1

Female, 81 years old, found in her own home. Estimated PMI (postmortem interval): less than 24 h. No previous pathological conditions mentioned. Cause of death: purulent pneumonia. No soft tissue or hard tissue traumatic lesions were detected at autopsy.

The anthropological analysis conducted on the skeletal remains showed the absence of perimortem lesions. Nevertheless, 32 fractures classifiable by the criteria mentioned in the introduction as postmortem were detected; examination both at the autopsy and on the dry skeleton show the absence of any trauma at or around death.

3.2. Case 2

Female, 79 years old, who died in hospital a few hours after being run over by a car. No previous pathological conditions were mentioned. Cause of death: multiple skeletal and visceral lesions.

The external examination at autopsy mentioned several different soft tissue lesions and fractures (Fig. 1). The lesions reported were described mainly as "*superficial abrasions*" and as

Table 2
Data regarding the number of lesions (external and skeletal) as described in the autopsy reports and according to the anthropological analysis after 20 years.

Case number	Event of death	Abrasions and lesions seen at the cadaver external examination	Osteological findings at autopsy	Perimortem fractures found at anthropological analysis	Postmortem fractures/alterations
1	Natural	0	0	0	32
2	Traffic accident	7	17	7	79
3	Traffic accident	3	29+ cranial and pelvis fracture complexes	19	62
4	Gunshot injury	1	0	0	61
5	Sharp force injury	30	4	4	55
6	Gunshot injury	1	2+ cranial fracture complex	2+ fracture complex	23
7	Traffic accident	12	7+ multiple rib fractures	15+ 48 rib fractures	47

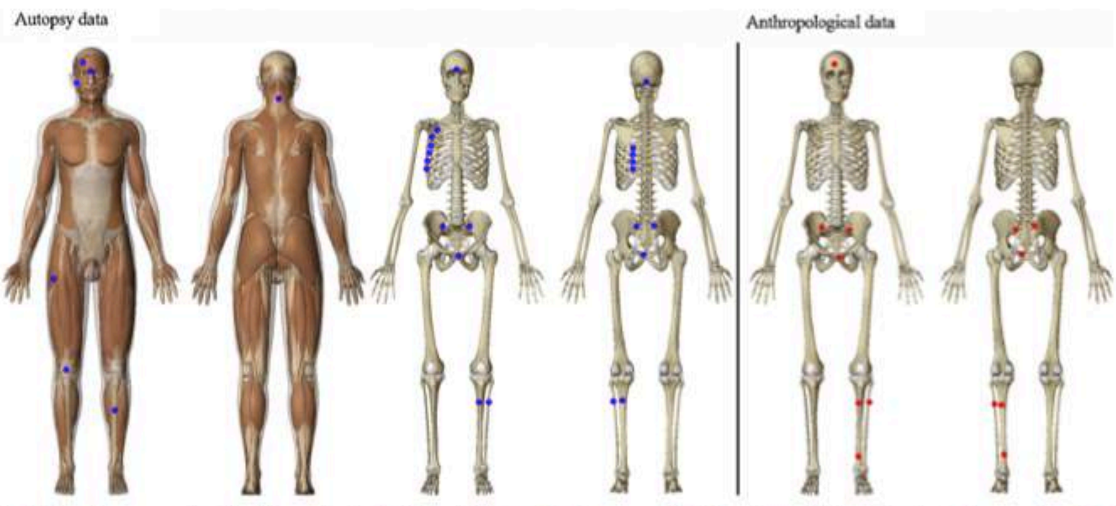

Fig. 1. Schematic representation of the lesions noticed on the body and skeleton of Case 2 at autopsy (left body and central skeleton, blue dots), and upon anthropological examination after 20 years (right, red dots).

"*fractures with abnormal mobility*" (e.g. in the nasal bones and in the left leg), for a total amount of 17 bone fractures found at autopsy after more thoroughly investigating specific anatomical regions. Some discrepancies can be noticed between the anthropological and autopsy examinations, since the analysis of the skeletal remains counted only seven bone fractures which according to classical criteria could be considered perimortem. These fractures showed macroscopic morphological features classical of wet bone. Perimortem lesions of the pelvis and left leg were easily identifiable, for a total of five fractures which corresponded to the ones described at autopsy. In particular, for the fractures on the tibia it was still possible to see butterfly fractures and thus recognize the area of compression and the direction of force. Thus the analysis highlighted the evidence of a massive trauma involving pelvis, right ilium and both auricular surfaces. Some fractures seen and described at autopsy could not be clearly classified as perimortem at the anthropological analysis, both for the taphonomic transformations encountered (e.g. the nasal bones, Fig. 2a, and ribs), and the severe taphonomic destruction of the skeletal regions involved (e.g. cervical vertebrae and ribs). Furthermore, a fracture with typical perimortem characters, located at the distal third of the left tibia, whose appearance was characterized by smooth surfaces, V-shaped outline and homogenous color with the external cortical bone (Fig. 2b), and another one at the frontal bone (which appears to be perimortem for the presence of a smooth surface and acute angle in a radiating fracture regardless of the heterogenous color of the external cortical surface) were observed upon anthropological investigation, and had not been noticed at autopsy. Finally, a total of 79 postmortem fractures showing classical features related to dry/degraded bone (characteristics very much similar to those of the fractures seen on Case 1) were noticed upon anthropological examination.

3.3. Case 3

Female, 83 years old, who died as a result of a traffic accident (run over by a truck) a few minutes before death. No previous pathological conditions mentioned. Cause of death: multiple skeletal and visceral lesions.

Upon autopsy a head injury in the temporal area and an underlying bone fracture was found, as well as an abrasion in the right trochanteric area and a bruise affecting the right leg. The autopsy findings also listed 28 rib fractures, plus multiple cranial fractures, a complex pelvic fracture, composed of a total of three fractures, and a fracture of the sternal extremity of the left clavicle (Fig. 3).

The anthropological findings highlighted the presence of 19 bone lesions classifiable as perimortem according to criteria stated above (Fig. 3). These results indicate that many fractures seen at autopsy are no longer visible: e.g. the fracture on the clavicle and many of those affecting ribs, due to their bad state of preservation. Additional fractures which could qualify as perimortem in some areas according to the above mentioned standards (e.g. right ilium; Fig. 4) could indeed be recognized even after the 15-year inhumation, despite being missed at autopsy. The comparison between autopsy and anthropological findings showed a good correspondence, although the number of fractures visible decreased from the first analysis to the second one mainly due to taphonomy.

Once again, numerous postmortem fractures (62) were found in addition to those diagnosed as perimortem on the skeleton.

3.4. Case 4

Male, 60 years old. Suffered from kidney failure. Cause of death: septic shock, after surviving 56 days following an abdominal gunshot injury.

The only evidence of the abdominal gunshot injury is reported at autopsy on the soft tissue (cadaver body on left, Fig. 5). No lesions on bone tissue were specified in the autopsy report, stating that "*backbone, rib cage, pelvis and bones of the limbs are unscathed*".

According to the anthropological analysis the only lesions involving bone tissue in this case derived from taphonomic factors and no evidence of gunshot injury was noted. It is important to specify that, as in the previous cases, a total of 61 clearly postmortem fractures were recorded.

Altogether, no evident differences are noticeable between autopsy and anthropological analysis, since neither revealed any bone lesion though death was by gunshot.

3.5. Case 5

Male, 76 years old, found dead in the street after having been robbed and stabbed by a drug-addict a few hours before. Estimated PMI: less than 6 h. No previous pathological conditions mentioned. Cause of death: multiple stab wounds.

In the autopsy report a total of 30 cut marks and stab wounds were counted, each of which was photographed and accurately described. Only 4 are however described as reaching the

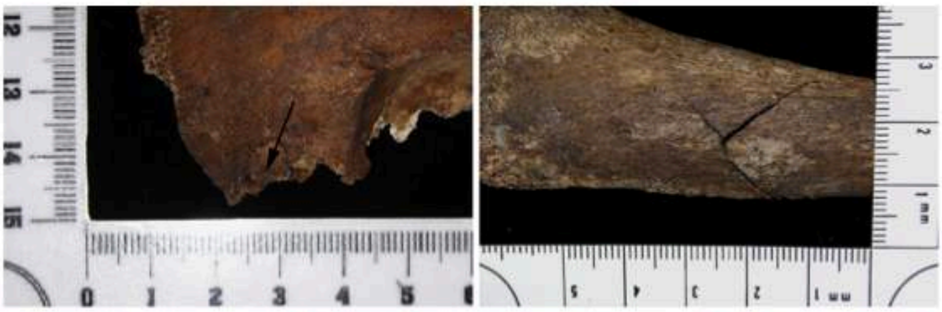

Fig. 2. (a) The black arrow shows an example of how a fracture produced at the time of death and thus in theory perimortem (on the nasal bones), whose presence was observed at autopsy and described in detail in the autopsy report, was no longer detectable at anthropological analysis due to taphonomy. In this case taphonomical events led to the "acquisition" of postmortem characteristics on the fracture (e.g. a whitish margin laterally), which do not allow for the clear recognition of the original morphological features (Case 2). (b) An example of a perimortem fracture (distal end of the left tibia): the type of bone (compact bone) and the good preservation of original perimortem indicators have permitted the correct identification of a perimortem fracture upon anthropological analysis although it was not mentioned in the autopsy report (Case 2).

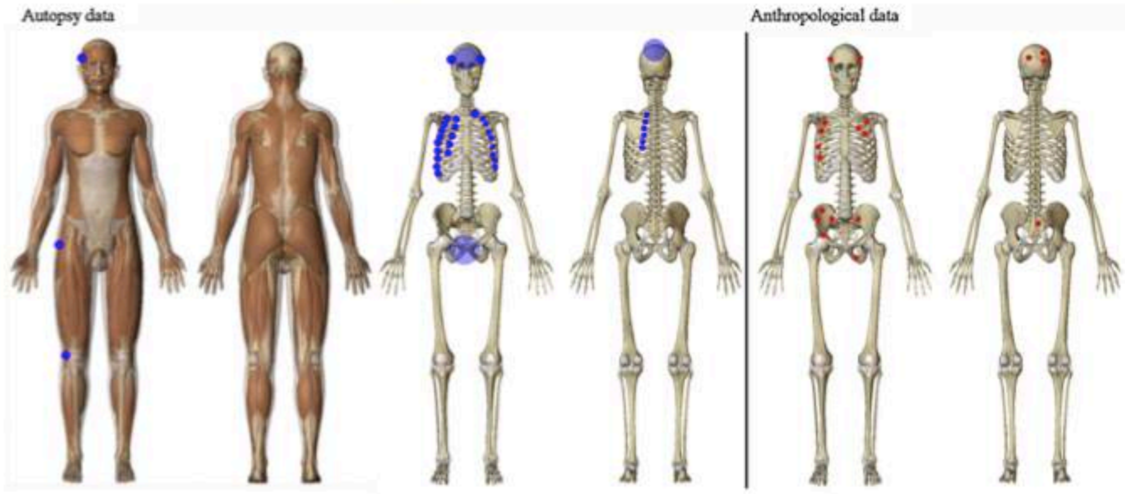

Fig. 3. Schematic representation of the lesions noticed on the body and skeleton of Case 3 at autopsy (left), and upon anthropological examination after 20 years (right).

Fig. 4. The black arrow indicates a fracture which satisfies perimortem standards, of the right ilium (Case 3). The anthropologist has given a perimortem assessment thanks to the survival of perimortem features according to Weiberg and Wescott like the color of margins, the "green" aspect of a part of the fracture and the smooth aspect of fractures edges.

underlying bone tissue: 2 in the left inferior margin of corpus sterni and 2 in the 8th and 10th right ribs (Fig. 6).

The anthropological findings on the skeletal remains consist of 4 sharp force injuries, 2 of these correspond to what was reported at autopsy (the sternal lesions). Nevertheless, whereas in the left inferior corpus sterni the 2 stab wounds (with a slightly triangular shape of the bony discontinuation) seen at autopsy were still visible even 20 years after (Fig. 7), the state of preservation of the ribs did not allow investigation of the other lesions mentioned at autopsy. The 2 additional sharp force wounds (cut marks,) observed on bone are placed posteriorly: one in the right scapular spine and another situated in the left inferior articular facet of the

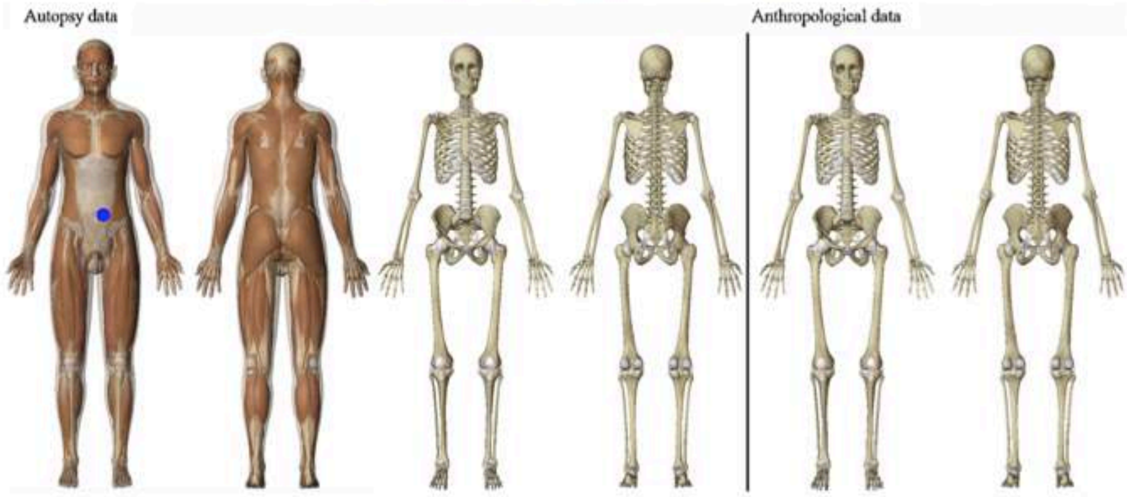

Fig. 5. Schematic representation of the lesions noticed on the body and skeleton of Case 4 at autopsy (left), and upon anthropological examination after 20 years circa from death (right).

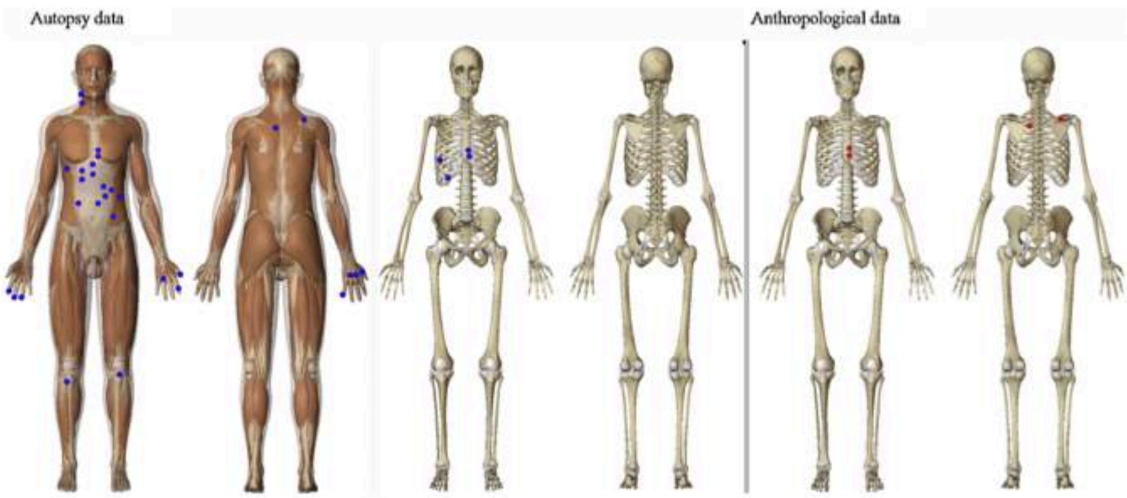

Fig. 6. Schematic representation of the lesions noticed on the body and skeleton of Case 5 at autopsy (left), and at anthropological examination after 20 years from death (right).

third thoracic vertebra (Fig. 7). These lesions were not described in the autopsy report, which reveals lack of sensitivity of radiological and/or autoptic procedures. The anthropological analysis also detected 55 fractures showing classical postmortem characteristics.

3.6. Case 6

Male, 28 years old, found dead in his apartment after committing suicide by gunshot to the head. Estimated PMI: less than 8 h. Cause of death: cranial and cerebral lesions.

The autopsy reported a "*round firearm lesion approximately at the center of the right temporal squamous portion, [...], 0.7 cm in diameter, and with internal bevelling*" (Fig. 8). In the left posterior parietal region, a "*Y-shaped fracture [...] is visible ... the cranial base is crossed by numerous fracture lines in the anterior and medial cranial fossa*". These fractures were caused by a retained bullet (which did not exit the skull completely), which had impacted the inner cranial table upon exiting without penetrating it.

A perfect concordance was found at the anthropological analysis (Fig. 10) which showed the same fracture pattern (Figs. 8 and 9). The skeletal material suffered very little taphonomic alterations, which permitted us to find the same lesions (a perfect round lesion with internal beveling, which testifies the entry wound, and a "Y-shaped fracture") on bones even 15 years after the autopsy. Several (23) postmortem fractures were visible on the skeletal remains.

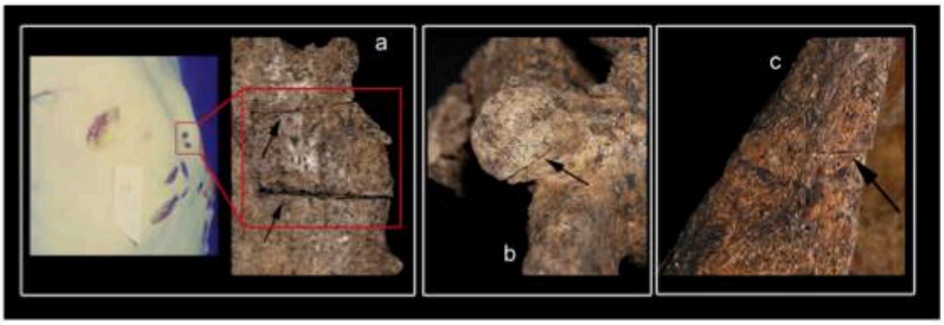

Fig. 7. Lesion found at the anthropological analysis in Case 5: (a) 2 stab wounds on the left inferior side of corpus sterni (concordant with what was reported and seen at autopsy as shown in the picture of the cadaver on the left). Cut marks on the articular facet of the third thoracic vertebra (b), and on the right scapular spine (c).

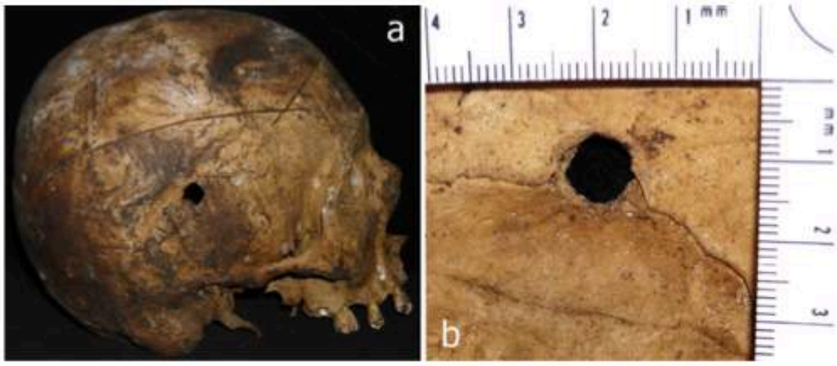

Fig. 8. Crania of Case 6: gunshot entry wound on the right temporal bone (a). A detail of the internal side of the gunshot entry showing evident beveling (b).

3.7. Case 7

Male, 78 years old, who died as a result of a traffic accident (run over by a tram). No previous pathological conditions mentioned. Cause of death: multiple skeletal and visceral lesions.

The external cadaver examination reported 12 lesions, mainly excoriations (Fig. 11), and the autopsy outlined a total of 7 bone fractures (namely one in the cranial base, 1 in the right os coxae and another in the left one, 1 in the right and 1 in the left femur, 1 in the spinous process of the 5th thoracic vertebra, and 1 in the left tibia) and also multiple rib fractures not fully described.

The analysis performed by the anthropologists allowed for the detection of all 7 fractures described in the autopsy report; furthermore a great number (48) of rib fractures was seen and described in depth (Fig. 11) although postmortem modifications had severely altered some fracture margins making them of dubious interpretation. Furthermore, the analysis of the skeletal remains highlighted the presence of 6 fractures with clear perimortem characters, not reported at autopsy: 4 located in the lower limbs (mostly butterfly type with very smooth surfaces and color similar to the external cortical bone and acute and obtuse angles), 1 in the left ischial spine, 1 on the 6th thoracic vertebra, and 1 in the left and in the right scapula (Figs. 11 and 12).

Additional fractures (47 fractures) showed characteristics that were compatible with postmortem trauma.

4. Discussion

For the forensic anthropologist, the interpretation of trauma is extremely difficult due to the recurring pitfalls in finding or recognizing the nature of bone injuries. In general, in cases of well preserved cadavers the autopsy can easily reveal trauma, thanks also to the presence of soft tissues. In those cases, however, in which the corpses are badly preserved or skeletonized, it may be impossible to find or correctly interpret bone lesions.

The accurate interpretation of cause and manner of death in general is one of the most important and difficult tasks especially in the case of skeletal remains. First of all one has to determine whether a fracture is peri- or postmortem (a task full of pitfalls); then, if the lesion is perimortem, one must verify its nature and how it was produced in order to aid the pathologist in the reconstruction of such lethal events.

A traumatic event on bones is commonly classified as a result of sharp force, gunshot or blunt force [4,5,26]. Each of these forces generates unique skeletal signs that are usually readily identifiable in unmodified remains. However, exposure to time and taphonomic factors can significantly blur evidence. Buried skeletal elements can typically exhibit fragmentation and fracturing limiting the interpretation of perimortem trauma. Recently, some research has attempted to find variables in order to distinguish taphonomically induced trauma [15,16,22–27], and a few studies

Fig. 9. The black arrow shows a lesion caused by a bullet on the left temporal bone of Case 6, with very little taphonomic modification.

have been conducted to determine specifically how bone changes over time, and consequently the fracture morphology [22–25]. Nevertheless, more specific investigations are necessary to formulate criteria by which to accurately differentiate between perimortem fractures (traumatically induced) and taphonomic-related fractures.

Numerous authors have described experimental studies of different patterns of injury, their frequency and severity, how advanced imaging can help in interpreting them (SEM, pQ TAC, CT-Cone Beam) and their relation with natural taphonomic factors. However, these studies have been mainly conducted on animal bones (mostly pig) or relate to a few forensic cases, frequently with little information [27,28]. Other studies have been performed on exhumed corpses with the intent of investigating the correlation between the time a corpse has remained buried and the findings upon exhumation, showing the maximum time a pathological finding might still be detectable in relation to the degree of decay, and demonstrating that exhumation performed a long time after death can still reveal important morphological detail [29,30]. Nevertheless, no investigations were ever performed which could allow a comparison between autopsy examination and anthropological analysis on the same individual, thus checking for incongruity and/or changes.

The comparison between autopsy and anthropological data conducted in this study highlights the difficulties in forensic anthropology. The study calls attention to several issues: (1) sometimes a lesion which is clearly visible in soft tissues, cannot be detected on the underlying bone because it did not reach the bone; (2) sometimes an original lesion on bone can be modified by time and taphonomic factors thus becoming perhaps detectable but no longer identifiable as perimortem; (3) at times autopsy does not reveal all fractures, particularly the smaller ones, which can be seen upon anthropological analysis.

It must be stressed that the anthropological analysis proved to be fundamental in acquiring crucial data: in fact, in this series of seven cases, there was a perfect correlation between what was seen at autopsy and at the anthropological examination in three cases (in the case of natural death (Case 1) and abdominal gunshot (Case 4), the absence of perimortem bone lesions; and in presence of a cranial gunshot (Case 6), two particular lesions to the head). In four cases out of seven the anthropological analysis showed the presence of perimortem lesions not detected at autopsy, particularly in the case of sharp force trauma. Finally, only in one case were there no bone lesions despite a traumatic cause of death (abdominal gunshot wound). From an anthropological point of view, the determination of trauma could be ascertained in five of the seven (71%) cases studied because of the presence of lesions which could clearly be associated with perimortem trauma, regardless of taphonomic deterioration. On the other hand, autopsy reports proved a violent death for six cases (Cases 2–7) among those studied (Table 2) and in one case no skeletal trauma was visible. Cases 1 and 4 presented at autopsy showed respectively no lesions and a mortal abdominal firearm lesion affecting only the soft tissue. There can be a lack of skeletal signs both where there was no actual mechanical trauma at the time of death (Case 1) or where lethal lesions were present but did not involve the skeleton (Case 4). Though not many studies have been

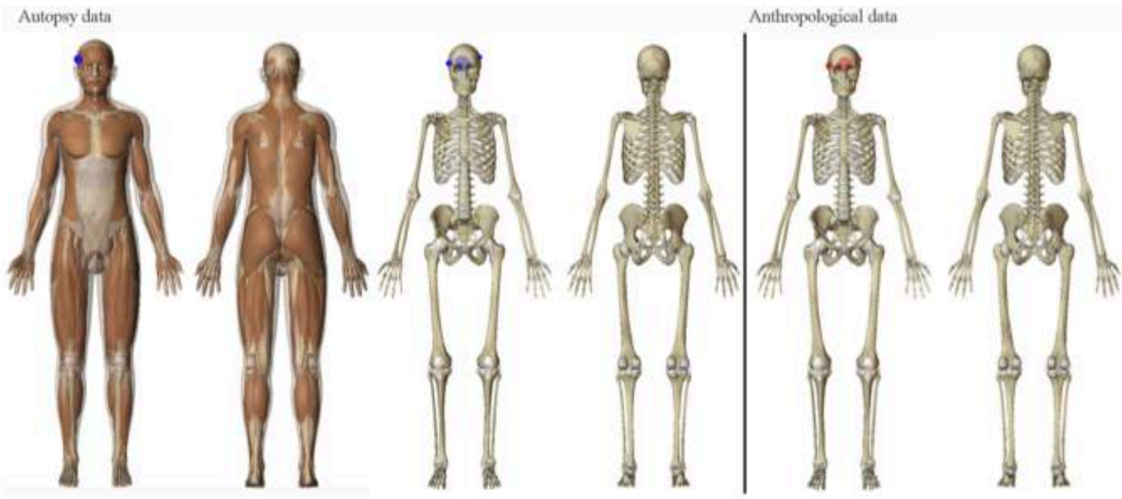

Fig. 10. Schematic representation of the lesions noticed on the body and skeleton of Case 6 upon autopsy (left), and at anthropological examination (right).

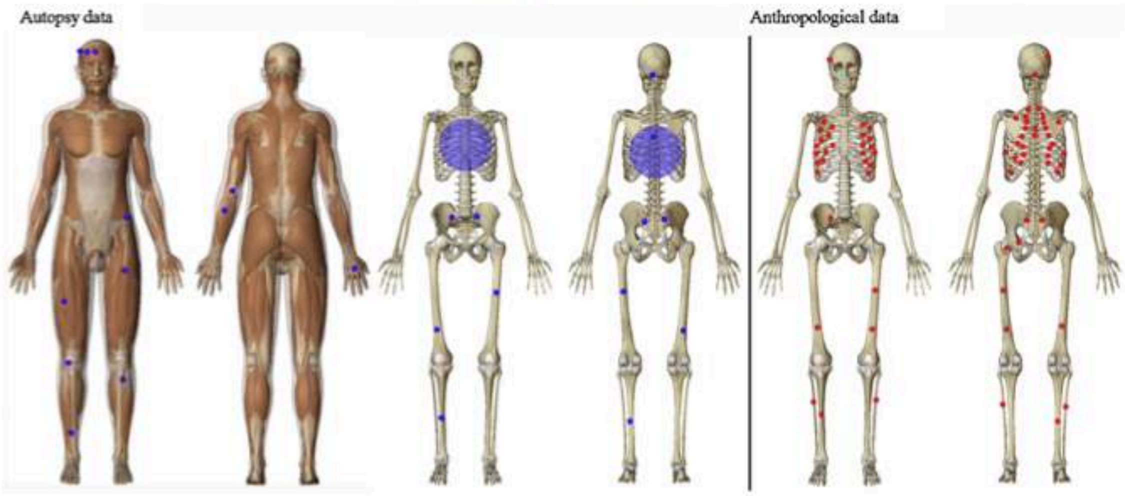

Fig. 11. Schematic representation of the lesions noticed on the body and skeleton of Case 7 at autopsy (left), and at anthropological examination (right).

Fig. 12. (a–c) Case 7. (a) Example of a fracture of the fibula with clear perimortem characteristics not described at autopsy; (b) Example of a fracture of the rib seen at autopsy and showing perimortem traits upon osteological analysis 20 years from death; (c) fracture of the femur seen at the autopsy and evaluated as perimortem at the present day anthropological analysis. (d) Case 2. An example of fractures on the right pubic symphysis and superior ramus of the pubis, which are known to have been present at death (because described in the autopsy report) but could no longer be identified as such at the anthropological analysis 20 years later because of taphonomical effects. In this figure some of the types of consistencies and inconsistencies detected between the autopsy report and the anthropological analysis on the skeleton performed 20 years later are illustrated.

performed on the relative frequencies, it is commonly know that many undamaged skeletons may belong to victims of gunshot, blunt force injury and sharp lesions [31–35].

On the other hand, stab or cut marks (Case 5, Fig. 7) and gunshot injuries (Case 6, Fig. 8) are quickly and easily identifiable even after almost 15 years of inhumation and do not seem to change significantly regardless of taphonomy. The same cannot be said for lesions derived from blunt injury, which are in different cases highly modified by time and taphonomic factors, making it often impossible to identify them correctly. The results showed in fact the impossibility sometime to determine whether a lesion was perimortem or postmortem. Some perimortem fractures acquire in fact postmortem characteristics due to the multitude of taphonomic variables that can remove important perimortem indicators by adding new postmortem fractures, and warping as well as abrasion can round off the fracture edges and modify color, outline or other important features of the fracture (Fig. 11). The final result could be a complete acquisition of postmortem characteristics, which totally modify the original appearance and do not permit one to diagnose that lesion as being perimortem. This can be a very frequent problem in similar taphonomic contexts where a list of considerable factors may affect preexisting lesions: exhumation, handling and transportation, fracturing and warping caused by sediment pressure, lid and burial collapse, and finally the alteration provoked by taphonomic factors like soil, decomposition fluids, humidity and a wet environment. This particularly concerns fractures of bone elements rich in spongy tissue like ribs, pelvic bones, and vertebrae [36].

The present study has shown the importance of such known skeletal populations for trauma analysis. In the present study on seven cases, at least 14 blunt lesions described at autopsy could not be identified on the skeleton 20 years later. The availability of such collections provides the instruments that allow the forensic anthropologists to better understand the limits of his or her field. On the other hand, the analysis of the skeletal remains emphasized the competence of the forensic anthropological practice in providing additional information concerning the presence and nature of bone trauma that the autopsy did not reveal.

This study has however not thoroughly entered the thorny issue of the distinction between peri- and postmortem trauma concerning blunt force injury in this population, which has already been approached elsewhere [36].

5. Conclusions

In conclusion, much information can still be obtained from the analysis of skeletal remains which have undergone decades of taphonomic insults, particularly as concerns sharp force and gunshot trauma, although blunt force trauma interpretation may be severely hindered by postmortem factors.

This study has thus proven the extreme relevance of skeletal populations with known cause of death and autopsy reports, for a comparative assessment between data from the well preserved cadaver and from the skeleton years later, which may shed light on which aspects of bone trauma interpretation are more at risk or are best preserved in human remains.

Acknowledgments

We thank the City of Milan and the Cimitero Maggiore: without their assistance this study would not have been possible.

References

[1] N.J. Sauer, The timing of injuries and manner of death: distinguishing among antemortem, perimortem and postmortem trauma, in: K.J. Reichs (Ed.), Forensic Osteology: Advances in the Identification of Human Skeletal Remains, second ed., C.C. Thomas, Springfield, 1998, pp. 321–332.
[2] P. Andrews, Y. Fernàndez-Jalvo, How to approach perimortem injury and other modifications, Methods Mol. Biol. 915 (2012) 191–225.
[3] D. Semeraro, N. Passalacqua, S. Symes, T. Gilson, Patterns of trauma induced by motorboat and ferry propellers as illustrated by three known cases from Rhode Island, J. Forensic Sci. 57 (2012) 1625–1629, http://dx.doi.org/10.1111/j.1556-4029.2012.02177.x.
[4] H.E. Berryman, O.C. Smith, S.A. Symes, Diameter of cranial gunshot wounds as function of bullet caliber, J. Forensic Sci. 40 (1995) 751–754.
[5] H.E. Berryman, S.A. Symes, Recognizing gunshot and blunt cranial trauma through fracture interpretation, in: K.J. Reichs (Ed.), Forensic Osteology: Advances in the Identification of Human Remains, second ed., C.C. Thomas, Springfield, 1998, pp. 333–352.
[6] M.M. Houck, Skeletal trauma and the individualization of knife marks in bones, in: K.J. Reichs (Ed.), Forensic Osteology: Advances in the Identification of Human Remains, second ed., C.C. Thomas, Springfield, 1998, pp. 410–424.
[7] V.J.M. Di Vaio, Gunshot Wounds, CRC Press, Boca Raton, 1999.
[8] A.J. Schwoeble, D.L. Exline, Current Methods in Forensic Gunshot Residue Analysis, first ed., CRC Press, Boca Raton, 2000.
[9] E.J. Bartelink, J.M. Wiersema, R.S. Demaree, Quantitative analysis of sharp-force trauma: an application of scanning electron microscopy in forensic anthropology, J. Forensic Sci. 46 (2001) 1288–1293.
[10] R. Ferllini, Macroscopic and microscopic analysis of knife stab wounds on fleshed and clothed ribs, J. Forensic Sci. 57 (2012) 683–690.
[11] D.H. Ubelaker, Hyoid fracture and strangulation, J. Forensic Sci. 37 (1992) 1216–1222.
[12] P. Betz, W. Eisenmenger, Frequency of throat-skeleton fractures in hanging, Am. J. Forensic Med. Pathol. 17 (1996) 191–193.
[13] M.W. Spence, M.J. Shkrum, A. Ariss, J. Regan, Cranio-cervical injuries in judicial hangings: an anthropological analysis of six cases, Am. J. Forensic Med. Pathol. 20 (1999) 309–322.
[14] B.K. Tucker, D.L. Hutchinson, M.F.G. Gilliland, T.M. Charles, H.J. Daniel, L.D. Wolfe, Microscopic characteristics of hacking trauma, J. Forensic Sci. 46 (2000) 234–240.
[15] L.P. Karr, A.K. Outram, Bone degradation and environment: understanding, assessing and conducting archaeological experiments using modern animal bones, Int. J. Osteoarchaeol. (2012), http://dx.doi.org/10.1002/oa.2275.
[16] L.P. Karr, A.K. Outram, Tracking changes in bone fracture morphology over time: environment, taphonomy and the archaeological record, J. Archaeol. Sci. 39 (2012) 555–559, http://dx.doi.org/10.1016/j.jas.2011.10.016.
[17] B. Wheatley, Perimortem or postmortem bone fractures? An experimental study of fracture patterns in deer femora, J. Forensic Sci. 53 (2008) 69–72, http://dx.doi.org/10.1111/j.1556-4029.2008.00593.x.
[18] A. Stirland, Patterns of trauma in a unique medieval Parish cemetery, Int. J. Osteoarchaeol. 6 (1996) 92–100.
[19] D.R. Hunt, J. Albanese, History and demographic composition of the Robert J. Terry anatomical collection, Am. J. Phys. Anthropol. 127 (2005) 406–417.
[20] H.F.V. Cardoso, Brief communication: the collection of identified human skeletons housed at the Bocage Museum (National Museum of Natural History), Lisbon, Portugal, Am. J. Phys. Anthropol. 129 (2006) 173–176.
[21] M.R. Dayal, A.D.T. Kegley, G. Strkalj, M.A. Bidmos, K.L. Kuykendall, The history and composition of the Raymond A. Dart Collection of Human Skeletons at University of the Witwatersrand, Johannesburg, South Africa, Am. J. Phys. Anthropol. 140 (2009) 324–335.
[22] D.A.M. Wieberg, D.J. Wescott, Estimating the timing of long bone fractures: correlation between the postmortem interval, bone moisture content, and blunt force trauma characteristics, J. Forensic Sci. 53 (2008) 1028, http://dx.doi.org/10.1111/j.1556-4029.2008.00801.x, 1034.
[23] K. Moraitis, C. Spiliopoulou, Identification and differential diagnosis of perimortem blunt force trauma in tubular long bones, Forensic Sci. Med. Pathol. 2 (4) (2006) 221–229.
[24] D.H. Ubelaker, B.J. Adams, Differentiation of perimortem and postmortem trauma using taphonomic indicators, J. Forensic Sci. 40 (1995) 509–512.
[25] S. Symes, E. L'abbé, K.E. Stull, M. Lacroix, J.T. Pokines, Taphonomy and the timing of bone fractures in trauma analysis, in: J.T. Pokines, S.A. Symes (Eds.), Manual of Forensic Taphonomy, first ed., CRC Press, 2014, pp. 341–365.
[26] B. Knight, Exhumation, in: B. Knight (Ed.), Forensic Pathology, second ed., Arnold, London, 1996, pp. 38–40.
[27] S.E. Calce, T.L. Rogers, Taphonomic changes to blunt force trauma: a preliminary study, J. Forensic Sci. 52 (2007) 519–527, http://dx.doi.org/10.1111/j.1556-4029.2007.00405.x.
[28] C. Wynnyckyj, L. Wise-Milestone, S. Omelon, Z. Wang, M. Grynpas, Fracture surface analysis to understand the failure mechanisms of collagen degraded bone, J. Bone Miner. Metab. 29 (2011) 359–368, http://dx.doi.org/10.1007/s00774-010-0233-9.
[29] D. Breitmeier, U. Graefe-Kirci, K. Albrecht, M. Weber, H.D. Tröger, W.J. Kleemann, Evaluation of the correlation between time corpses spent in in-ground graves and finding at exhumation, Forensic Sci. Int. 154 (2005) 218–223.
[30] B. Karger, G. Lorin de la Grandmaison, T. Bajanowski, B. Brinkmann, Analysis of 155 consecutive forensic exhumations with emphasis on undetected homicides, Int. J. Legal Med. 118 (2004) 90–94.
[31] B.R. Sharma, P.S. Virendar, D. Harish, Neck structure injuries in hanging – comparing retrospective and prospective studies, Med. Sci. Law 45 (2005) 321–330.
[32] S. Jayaprakash, K. Sreekumari, Pattern of injuries to neck structures in hanging – an autopsy study, Am. J. Forensic Med. Pathol. (2012)[Epub ahead of print].
[33] R. Clément, J.P. Guay, A. Sauvageau, Fracture of the neck structures in suicidal hangings: a retrospective study on contributing variables, Forensic Sci. Int. 207 (2010) 122–126.
[34] A. Banasr, G.L. De LaGrandmaison, M. Durigon, Frequency of bone/cartilage lesions in stab and incised wounds fatalities, Forensic Sci. Int. 131 (2003) 131–133.
[35] G.L. De LaGrandmaison, M. Durigon, Frequency of bone lesions: an inadequate criterion for gunshot wound diagnosis in skeletal remains, J. Forensic Sci. 46 (2001) 593–595.
[36] A. Cappella, A. Amadasi, E. Castoldi, D. Mazzarelli, D. Gaudio, C. Cattaneo, The difficult task of assessing perimortem and postmortem fractures on the skeleton: a blind test on 210 fractures of known origin, J. Forensic Sci. (2014), http://dx.doi.org/10.1111/1556-4029.12539[Epub ahead of print].

5.2.2.

PART II:

THE INTERPRETATION OF FRACTURES IN BURIED SKELETAL REMAINS: PERIMORTEM *vs* POSTMORTEM

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TECHNICAL NOTE

ANTHROPOLOGY

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The Difficult Task of Assessing Perimortem and Postmortem Fractures on the Skeleton: A Blind Text on 210 Fractures of Known Origin*

ABSTRACT: The distinction between perimortem and postmortem fractures is an important challenge for forensic anthropology. Such a crucial task is presently based on macro-morphological criteria widely accepted in the scientific community. However, several limits affect these parameters which have not yet been investigated thoroughly. This study aims at highlighting the pitfalls and errors in evaluating perimortem or postmortem fractures. Two trained forensic anthropologists were asked to classify 210 fractures of known origin in four skeletons (three victims of blunt force trauma and one natural death) as perimortem, postmortem, or dubious, twice in 6 months in order to assess intraobserver error also. Results show large errors, ranging from 14.8 to 37% for perimortem fractures and from 5.5 to 14.8% for postmortem ones; more than 80% of errors concerned trabecular bone. This supports the need for more objective and reliable criteria for a correct assessment of peri- and postmortem bone fractures.

KEYWORDS: forensic science, forensic anthropology, blunt force trauma, perimortem, postmortem, bone fracture, taphonomy

In the field of forensic anthropology, the importance of correctly identifying bone injury as perimortem and its correlation with the events around the time of death are crucial (1–4). At the moment, several reports focus on bone trauma (5–9), but very few experimentally approach the issue of the distinction between perimortem versus postmortem lesions (10–15). The distinction between lesions which occurred immediately prior to or around death and those after death still represents a difficult task. With well-preserved cadavers, a pathologist can rely upon several features of soft tissues (like hemorrhaging), but for the osteologist, the challenge is more difficult. Anthropology is limited to a diagnosis of "perimortality" which means at or around death or "postmortality", that is, after skeletonization or when the bone tissue has lost its elastic component. Aside from the vital reaction of bleeding or bone remodeling with fracture healing, there are no further clues that a traumatic event occurred prior to death (12). Thus, anthropologists can only rely upon macroscopic and morphological features to distinguish between perimortality and postmortality. Nevertheless, several authors have already stressed the relative weakness of such observer-dependent methods in forensic cases (12–14,16,17). In this perspective, pilot studies were performed to identify more reliable criteria, based on signs of hemorrhaging on fracture edges (18) or red blood cell modifications following decomposition (19,20), as well as on the micro-morphology of fracture margins (15). The correct evaluation of bone lesions is at the moment strictly dependent on features such as fracture pattern, fracture angle, tactile roughness of the fracture margins/outline (8,10,13), or features such as the presence of collagen and elastic fibers or the color of the fractured margins (5,10,14). A few studies (7,8,10,13,14,16,17,21–23) have shown that environmental factors (weathering, plants, soil, fire, and so on) can hinder the characteristic features of bone injuries. Taphonomic processes can indeed change bone-making forensically relevant features totally illegible (8). Typical characteristics of blunt force trauma (such as radiating, concentric, or hinge fractures) may be disguised by the effects of environmental stress (21). Ubelaker (16,17) illustrated that weathering cracks can resemble blunt force trauma, showing that bone macroscopic changes in aquatic environments can severely change and hinder important indicators of perimortem trauma by rounding off the fractured edges (12,21). Wieberg and Wescott (14) underlined the unreliability of color because it can change over time. Furthermore, Ubelaker and Adams (10) noticed the formation of butterfly factures, typically considered to be distinctive of perimortem injuries, up to 1 year after death as well. However, the actual reliability of these parameters has never been tested on a large scale and on known material.

Hence, the possibility to study a skeletal collection deriving from cadavers which underwent autopsy 20 years before, with clear information concerning the cause of death and the possible presence of antemortem injuries, provides an important tool for this kind of investigation. A description of the macro-morphology of bone injuries from a skeletal collection and forensic cases was conducted by Moraitis et al. (24), but the study was merely descriptive and no differential analysis was performed.

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We therefore had two forensic anthropologists undergo a discriminative test of perimortem versus postmortem injuries on skeletons, which had been buried for 20 years and then exhumed. The availability of autopsy reports provided the positive control, revealing the presence or absence of perimortem injuries and their precise location, thus giving an answer to questions such as: How many times will a postmortem or taphonomical fracture be wrongly assessed as a perimortem fracture and vice versa? and Which bones are the trickiest?

Materials and Methods

The material of this study consisted in a total of 210 fractures identified on 4 skeletons. These are part of a collection of 250 modern skeletons from the Cimitero Maggiore in Milan, made available for the study according to article 43 (September 10, 1990, n.285) of Italian Mortuary Police Regulation. All individuals were subjected to the same conditions: buried in the same cemetery (Cimitero Maggiore in Milano) between 1990 and 1991, and exhumed and reburied twice in 15 years, by means of heavy vehicles (and consequently without any regard to the intact preservation of the bodies) until complete skeletonization. (Postmortem trauma had thus been certainly inflicted at exhumation on the dry bones.) Finally, they were moved (in 2006) to an ossuary for 5 years and stored in metallic boxes.

The individuals subjected to the blind test were selected according to cause of death: Autopsy reports, with detailed descriptions of soft and hard tissue lesions, and death certificates were in fact available and functioned as a control for the anthropological data. The autopsies had been thoroughly performed 20 years before. Three of these subjects died of traumatic deaths in traffic accidents, and the fourth (natural death) was selected as a negative control. The autopsy reports described the presence and location of the antemortem injuries (bone fractures and hemorrhaging), which were then identified on the three individuals who had died of traumatic death.

The presence and position of the bone fractures was documented at the moment of the autopsy by visual and palpatory assessment and by dissection of soft tissues. X-rays were not performed on all cadavers: This may have in theory hindered the detection of very small fractures. It is, however, less likely (though not impossible) for a fracture to have been produced *in vivo* without corresponding soft tissue evidence of at least some hemorrhaging, which would have induced the pathologist to look for a bone fracture.

This allowed us to know in most cases which bone fractures were already present at autopsy (and therefore upon skeletonization were to be considered perimortem fractures) and those which were caused by postmortem events (when there was any doubt on a fracture not having been perceived upon autopsy, it was removed from the test).

For all the four cases, the number and site of bone fractures detected at autopsy in 1991 were recorded as well as those known to have been certainly produced postmortem (because not present at autopsy). Two hundred and ten fractures were thus selected (all the perimortem and some of the postmortem) and submitted to the "blind" observers (Table 1). Ribs were all excluded from the study because bones were too fragmented and ruined by taphonomical factors, so the exact identification of the ribs themselves and the positioning of the fractures was impossible. The position of bone fractures was documented on recording sheets, and two experienced forensic anthropologists (observer A and B, respectively, with 10 and 4 years of experience in the field) were asked to score all lesions as perimortem, postmortem, or uncertain. The analysis was made separately and without any assistance and was then repeated for a second time after 6 months (time 2), by the same observers and on the same lesions. Observers were both unaware of the results of their own assessment at time 1 and of the assessments of the other observer during the test. Results were then evaluated by comparing the scores to the real perimortem or postmortem nature of the fracture, as described in the autopsy reports. Thus, the general success rate, the intra- and interobserver agreement, and the bones with highest and lowest success rates were evaluated.

TABLE 1—*Data concerning the 4 skeletons in analysis.*

Case	Cause of Death	Known Perimortem Fractures	Known Postmortem Fractures
1	Pedestrian hit by truck	8	36
2	Pedestrian hit by tram	11	51
3	Pedestrian hit by car	8	50
4	Congestive cardiac failure	0	46
Total		27	183

Results

A summary of the results is given in Table 2.

The results of the osteological analysis show the higher success rate for both observers in the correct identification of postmortem fractures, with percentages for correct identification on average of 77.3%; for perimortem fractures, correct assessments fall to a percentage of 69.4%, with partial differences between observers A and B (61.1% and 75.9%, respectively) and between the two tests (time 1 and time 2) of the same observer. Similarly, incorrect interpretations for perimortem fractures are higher (22.2%) than for postmortem fractures (10.4%). The number of dubious fractures considerably increases at time 2 (after 6 months), especially for observer A. Correct postmortem evaluations do not show peculiar differences between the two tests of observer B, whereas they decrease significantly from the first to the second time for observer A.

As previously stated, the assessments on perimortem lesions were incorrect in 22.2% of cases. Of this percentage, 83.3% concerned fractures on trabecular tissue, whereas the remaining 16.7% regarded cortical bone. On the other hand, the error rate on postmortem fractures was 10.4%: Of this portion, 70% concerned spongy bone and 30% cortical bone. Finally, the overall

TABLE 2—*Average values of the blind test.*

Evaluation	Observer	Average T1, %	Average T2, %
Perimortem correct	Observer A	63	59.3
	Observer B	81.5	70.4
Postmortem correct	Observer A	83.6	56.2
	Observer B	84.1	85.2
Perimortem wrong*	Observer A	37	14.8
	Observer B	18.5	18.5
Postmortem wrong†	Observer A	14.8	9.8
	Observer B	11.5	5.5
Dubious on perimortem	Observer A	/	25.9
	Observer B	/	11.1
Dubious on postmortem	Observer A	1.6	34
	Observer B	4.4	9.3

*Perimortem detected as postmortem.

†Postmortem detected as perimortem.

TABLE 3—*Error in the evaluation of trabecular and cortical bone.*

	Trabecular and Flat Bone, %	Cortical Long Bone, %
Errors on perimortem fractures	83.3	16.7
Errors on postmortem fractures	84.1	15.9
Dubious	81	19

amount of assessments stated as dubious by the observers (on both perimortem and postmortem fractures) was 11.9%. These were mostly observed on trabecular bone (Table 3).

Discussion

The difficulty in evaluating perimortem blunt force trauma (BFT) has been already highlighted and documented by several authors (8,13,14,17). It usually relies upon macro-morphological features of the fracture like the breakage pattern or the type of margin. A correct distinction between perimortem or postmortem fractures is, however, not always easy to perform. In addition, taphonomic factors can make the task even tougher because they can severely alter the morphological features of bones. The application of the commonly used and widely accepted morphological parameters seems to be sometimes insufficient. This blind test performed by trained anthropologists has revealed several issues: (i) the effective difficulty and a quantified error in the correct assessment of peri- and postmortem lesions; (ii) which skeletal districts mostly hinder anthropological assessment; and (iii) how the evaluation may be observer dependent.

The analysis of the results shows the higher complexity in the identification of perimortem bone fractures for both the observers, if compared with postmortem ones: Perimortem lesions have in fact lower success rates and the higher amount of incorrect and dubious interpretations. This may be related to the superimposition of taphonomic factors on perimortem fractures, which can severely alter the common morphological features of "fresh" fractures, already widely described in the forensic literature. Perimortem lesions may undergo environmental change, making them unrecognizable or imperceptible (8). The erosion and the environmental modifications can severely alter perimortem features of the fractured margins, making them more similar to postmortem fractures. The highest number of mistakes (on both perimortem and postmortem bone fractures) was noticed when the observer had to evaluate trabecular bone (mostly bones of the pelvis). In fact, some skeletal fractures occurred on bones with a high percentage of spongy bone and were not recognized as perimortem in all tests, by both observers, even if their perimortal origin was confirmed by the autopsy reports (Fig. 1).

The correct identification of postmortem bone lesions showed fewer problems. Nevertheless, a small percentage of postmortem fractures was assessed as perimortem (7.65% on the average). Of these, 70% were once again located on trabecular bones. This may be due to the fact that very few parameters exist for trabecular bones, where the thin cortical layer may not provide sufficient characters of "elasticity" and therefore perimortality. Furthermore, both the trabecular structure and the fine cortical portion are easily modified by taphonomic factors, which can strongly alter crucial information (Figs 2 and 3). Even if these factors can severely prevent a correct assessment, in long bones, perimortem fractures are frequently more easily identified, probably because the presence of the thick cortical layer leads to a better preservation of the fracture features (Fig. 4). Nevertheless,

FIG. 1—*Examples of a perimortem fracture not recognized at blind test: right auricular surface of the sacrum (Case 3).*

FIG. 2—*Postmortem fracture on the right ischio-pubic ramus (Case 3).*

FIG. 3—*Postmortem fracture on the distal epiphysis of the left radius (Case 4).*

FIG. 4—*Perimortem fracture on the proximal third of the left tibia (Case 3).*

some of the well-known criteria for the distinction between peri- or postmortem, even on long bones, are not so reliable when they are subjected to taphonomical alteration.

Finally, there is a clear increase for both observers (31.4% for observer A and 5.7% for observer B) in "dubious" assessments at Test 2. The time lapse may have made them more hesitant or insecure when facing this differential diagnosis. Being subjected for a second time to the same test, totally unaware of their previous scores, may have led to greater uncertainty in an already difficult assessment.

In conclusion, the blind test once again points out the relative unreliability of the commonly used morphological criteria in the correct diagnosis of perimortem and postmortem fractures. Whereas they can usually provide valuable information in the study of long cortical bone, the lack of data concerning the fractures in trabecular and flat bones still represents a big obstacle toward a correct diagnosis. Further analysis should be carried out, perhaps even with radiological assessment of the cadavers before autopsy, to extend control populations and to find more reliable criteria that can provide valuable help in the anthropological assessment of bone fractures.

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References

1. Cunha E, Cattaneo C. Forensic anthropology and forensic pathology: the state of the art. In: Schmitt A, Cunha E, Pinheiro J, editors. Forensic anthropology and medicine: complementary sciences from recovery to cause of death. Totowa, NJ: Humana Press Inc, 2007;18–54.
2. Cattaneo C. Forensic anthropology: developments of a classical discipline in the new millennium. Forensic Sci Int 2007;165:185–93.
3. Sauer NJ, Berryman HE, Symes SA. Trauma analysis. In: Reichs KJ, Bass WM, editors. Forensic osteology: advances in identification of human remains, 2nd edn, Vol. 4. Springfield, IL: Charles C: Thomas Publisher, 1998;319–32.
4. Dirkmaat DC, Cabo LL, Ousley SD, Symes SA. New perspectives in forensic anthropology. Am J Phys Anthropol 2008;51:33–52.
5. Maples WR. Trauma analysis by the forensic anthropologist. In: Reichs KJ, Bass WM, editors. Forensic osteology: advances in identification of human remains, 2nd edn. Springfield, IL: Charles C Thomas, 1986;218–28.
6. Berryman HE, Symes SA. Recognizing gunshot and blunt cranial trauma through fracture interpretation. In: Reichs KJ, Bass WM, editors. Forensic osteology: advances in identification of human remains, 2nd edn. Springfield, IL: Charles C. Thomas, 1998;333–52.
7. Herrmann NP, Bennett JL. The differentiation of traumatic and heat-related fractures in burned bone. J Forensic Sci 1999;44(3):461–9.
8. Calce SE, Rogers TL. Taphonomic changes to blunt force trauma: a preliminary study. J Forensic Sci 2007;52(3):519–27.
9. Semeraro D, Passalacqua N, Symes S, Gilson T. Patterns of trauma induced by motorboat and ferry propellers as illustrated by three known cases from Rhode Island. J Forensic Sci 2012;57(6):1625–9.
10. Ubelaker DH, Adams BJ. Differentiation of perimortem and postmortem trauma using taphonomic indicators. J Forensic Sci 1995;40:509–12.
11. Sauer NJ. The timing of injuries and manner of death: distinguishing among antemortem, perimortem and postmortem trauma. In: Reichs KJ, Bass WM, editors. Forensic osteology: advances in identification of human remains, 2nd edn. Springfield, IL: Charles C. Thomas, 1998;321–32.
12. Sorg MH, Haglund WD. Advancing forensic taphonomy: purpose, theory, and process. In: Haglund WD, Sorg MH, editors. Advances in forensic taphonomy. New York, NY: CRC Press, 2002;3–29.
13. Wheatley B. Perimortem or postmortem bone fractures? An experimental study of fracture patterns in deer femora. J Forensic Sci 2008;53:69–72.
14. Wieberg DAM, Wescott DJ. Estimating the timing of long bone fractures: correlation between the postmortem interval, bone moisture content, and blunt force trauma characteristics. J Forensic Sci 2008;53(5):1028–34.
15. Pechnikovà M, Porta D, Cattaneo C. Distinguishing between perimortem and postmortem fractures: are osteons of any help? Int J Legal Med 2011;125:591–5.
16. Ubelaker DH. Perimortem and postmortem modification of human bone. Lessons from forensic anthropology. Antropologie 1991;29:171–4.
17. Ubelaker DH. Taphonomic applications in forensic anthropology. In: Haglund WD, Sorg MH, editors. Forensic taphonomy: the postmortem fate of human remains. Boca Raton, FL: CRC Press, 1997;77–90.
18. Cattaneo C, Andreola S, Marinelli E, Poppa P, Porta D, Grandi M. The detection of microscopic markers of hemorrhaging and wound age on dry bone. A pilot study. Am J Forensic Med Pathol 2010;31:22–6.
19. Laiho K, Pentilla A. Autolytic changes in blood cells and other tissue cells of human cadavers. II. Morphological studies. Forensic Sci Int 1981;17(2):121–32.
20. Bardale R, Dixit PG. Evaluation of morphological changes in blood cells of human cadaver for the estimation of postmortem interval. Med Leg Update Int J 2007;7(2):35–9.
21. Nawrocki SP. Taphonomic processes in historic cemeteries. In: Grauer AN, editor. Bodies of evidence: reconstructing history through skeletal analysis. New York, NY: Wiley-Liss, 1995;49–66.
22. Karr LP, Outram AK. Bone degradation and environment: understanding, assessing and conducting archeological experiments using modern animal bones. Int J Osteoarcheol 2012. doi:10.1002/oa.2275.
23. Karr LP, Outram AK. Tracking changes in bone fracture morphology over time environment, taphonomy and the archeological record. J Archeol Sci 2012;39:555–9.
24. Moraitis K, Eliopulos C, Spiliopoulou C. Fracture characteristics of perimortem trauma in skeletal material. Internet J Biol Anthropol 2009;3(2):1–8.

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Chapter 6

6.1.
THIRD RESEARCH LINE: TAPHONOMY OF BLOOD

The research lines reported in the previous chapters, demonstrated the need to focus on the search for further fundamental parameters and markers in the interpretation of skeletal lesions, different from those already present and used by the discipline. The macroscopic and morphological criteria proved sometimes to be unsuccessful, particularly in light of the interpretation of blunt injury: the effects of taphonomy demonstrated once more to be crucial in hiding perimortem signs of key importance in the trauma analysis on skeletal remains. In addition, with regard to the more precise interpretation of healing or healed bone lesions, the considered parameters showed not to be satisfactory, despite the application of different techniques, sometimes innovative ones. It is therefore clear that there is a need to investigate some aspects of different nature, such as the presence of specific biomarkers for vital reactions, like blood extravasations, haematomas, and inflammatory or osteogenic reactions, all events subsequent to the moment of production of a vital lesion and necessary for the activation of the bone healing process. The presence of such molecules, cells or cellular mediators ensures the possibility of evaluating the bone lesions with a higher level of certainty: not only a greater validity in the diagnosis of a lesion as perimortem, but also the ability to ascertain the presence of vitality of the lesion, and then also the time survival to it. This results in interpreting the injury not only as perimortem, but also as vital, defining the moment of production of that lesion in a more detailed manner in relation with time of death . A similar goal is remarkably ambitious, and its potential application is still entirely to be ascertained and to be investigated on all fronts.

This research line primarily focused on a specific aspect of the above mentioned issue: the detectability of blood cells, namely red blood cells, with respect to taphonomy. The research does not aim to solve the question on the diagnosis of vitality of a perimortem lesion, but intends to comprehend the taphonomy (degradation process) of erythrocyte over time, and within the bone tissue in decomposition. The goal is therefore to acquire important information about taphonomy of blood in order to clarify the chance of tracking down these components in skeletonized remains, regardless of the meaning and the role that such bio-markers, if found in a perimortem bone lesion, can assume.

The study of both, the blood components and the methodologies used for their search, could offer in future an interesting contribution in the diagnosis of perimortem lesions, thus representing a possible field to be explored thoroughly.

The presence of cells related to blood, particularly erythrocytes, in fact could be a promising solution for the interpretation of a lesion, especially with respect to time of death. In these terms, a similar approach would not only ensure the potentiality of a reliable diagnosis (the diagnosis between perimortem and postmortem lesions), but also an attempt to interpret the vitality of that lesion (i.e. if produced in life and in that moment before death), similar to what is done with cutaneous wounds.

In this perspective, the study on the decomposition of the blood components in bone tissue investigates the problems concerning the possible detectability of such cells, which is clearly linked to the taphonomic processes of this tissue; that is to say that time, the decomposition process and the related taphonomic events are all factors that complicate the diagnosis of lesions. This reinforces the importance of specific studies that intend to clarify the taphonomy of these markers, a step necessary for the validation of their use as markers in such terms. The taphonomy of blood, for example, is still one subject which has not been adequately studied and never faced thoroughly from a microscopic point of view. For approaching correctly the diagnosis of perimortem lesions through the search of potential markers such as those reported above and by using several techniques (for example by microscopy, histology and immunohistochemistry) it is necessary firstly to observe how these components decompose, appear and persist when considered as isolated cells in decomposing blood; subsequently it is necessary to verify the way blood components decompose in the bone tissue with respect to time and to extrinsic factors to the bone tissue (environmental taphonomic factors or factor related with the processes of decomposition of a corpse). Only once this aspect is fully understood (purely tied to the decomposition and taphonomy of bone tissue) the meaning of the presence or absence of erythrocytes (and/or of any their related alteration) will be clarified as a matter of the vitality of a lesion; without this step, the detection of such cells otherwise results as an instrument with no interpretation. This chapter therefore describes an in depth study on the taphonomy of blood through microscopy, histology and immunoistochemistry; specifically the study has focused on erythrocytes, both as cells isolated from decomposing blood, and as components within the decomposing bone tissue.

In particular, the research includes two specific works: the first has focused on the study of the persistence and the morphological changes of isolated erythrocytes and possible mistakes in their identification through the scanning electron microscopy investigation (SEM); the second work investigated a more complex taphonomic level corresponding to the detectability, persistence and changes of blood components within the bone tissue in relation to the process of decomposition and to the process of skeletonization. The latter is based on the microscopic evaluation performed thanks to both histological and immunohistochemical techniques (through the use of specific antibodies for blood antigens, such as glycophorin A) conducted on decomposing bone fragments monitored at different times since death (different stages of decomposition) and in various taphonomic contexts, with the intent to observe the taphonomy of blood cells relating only to normal conditions of the bone tissue decomposition. The main goal is represented by the acquisition of important knowledge potentially applicable, in a subsequent phase, to the legibility and interpretation of perimortem bone lesions. This is only a preliminary study, nevertheless it aims to offer interesting findings and a good starting point for future research; it reveals some basic information about how blood cells preserve in decomposed bone tissue and how their presence / absence could become a potential tool for diagnosis of vitality and survival in perimortem injuries.

6.1.1.

PART I:

How red blood cells decompose in cadaveric blood:

Distinguishing these cells from similar structures of different origin through Scanning Electron Microscopy (SEM)

The following article was recently accepted in:

"*International Journal of Legal Medicine*"

Blood or spores? A cautionary note on interpreting cellular debris on human skeletal remains

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6.1.1.1. ABSTRACT

The identification of red blood cells on both skeletal human remains and decomposed corpses is of remarkable importance in forensic sciences, irrespective of its diagnostic value; their presence is often perplexing and difficult to interpret especially when in the context of decomposition and taphonomical variables. Some clinical research has focused on the morphological changes of red blood cells over time by Scanning Electron Microscopy (SEM), but no research has investigated whether botanical structures can be confused for red blood cells. Since some literature has recently presumed the detection of erythrocyte-like cells on skeletal remains (even ancient) as surely erythrocytes and most have never taken into consideration the chance of an origin different from blood, such as botanical, the present study aims at verifying the possibility of confusion between erythrocytes and botanical cells by applying SEM analysis and at highlighting the pitfalls in this particular issue through a test submitted to pathologists and natural scientists, asked to discriminate between red blood cells and different vegetal structures (60 images obtained by SEM analysis).

The results showed that although there are diagnostic features useful in identifying red blood cells from botanical structures, some spores resulted very similar to decaying red blood cells, which calls for attention and great caution when studying decomposed human remains.

6.1.1.2. INTRODUCTION

Forensic pathology and anthropology must frequently deal with blood residues on cadavers, bone or simply stains, for a series of reasons which go from the need to verify the nature of debris on clothing to the proper interpretation of soft tissue residues or what may look like blood clots or cells [93, 98, 130]. In anthropology, the reasons for wanting to identify cellular elements of blood, in particular red blood cells, are many (both in the anthropological and forensic scenario); regardless of the reason behind the interest proper morphological identification of blood may be crucial but also full of pitfalls, especially on decomposed

material [Simmons], where contamination from the environment itself from botanical, zoological and geological elements, is likely to occur.
In literature, several publications have reported evidence of red blood cells and erythrocyte-like structures on ancient skeletal remains [161-166]; it seems however that red blood cells are too often and too easily detected without proper controls, particularly as concerns taphonomical degeneration and other elements which may mimic these structures.
Very few articles have in fact dealt with the degradation of the elements of the hemopoietic system: Penttilä and Lahio [167] demonstrated through a Scanning Electron Microscopy study how white cells, red cells and platelets, isolated from the blood of cadavers, alter their shape and dimension after death and in relation to the passage of time. Focusing on erythrocytes, the authors showed the changing pattern from a vital biconcave shape to a spiculed sphere and eventually to a smooth sphere. Other studies have been carried out for medical and surgical purposes, but only in relation to how long blood cells can be stored without damage and alteration of shape and function [168-174].
But how often can the morphology and ultrastructure “trick” pathologists and anthropologists into believing they are looking at a red blood cell when in fact they are looking at environmental debris? Botanical contamination in particular can be a serious problem since very little information exists on the potential of some botanical structures, such as pollen grains or spores, in mimicking decomposed elements of the hemopoietic system. The authors have verified this problem in several forensic cases and therefore wished to perform the following study.
The aim of this study was to investigate the degradation process of erythrocytes using Scanning Electron Microscopy and, with the same technique, the different morphology of the most common plant spores and other botanical structures, which can simulate decomposing red blood cells. This work would enable the identification of botanical structures which might mimic red blood cell appearance and the actual presence of erythrocytes on different types of tissues as skin or bone.

6.1.1.3. MATERIALS AND METHODS

6.1.1.3.1. Blood Samples and preparation of red blood cell (RBC)

Blood was obtained from well preserved cadavers (6 individuals of same age and sex, no pathological conditions or blood diseases reported) during autopsy and according to Italian Mortuary Police Regulation procedures, and then left in Falcon tubes with no heparin or sodium citrate used as anticoagulant. All the cadavers selected, from whom the blood was extracted for the study, were fresh (with an estimated PMI inferior to 24 hours).

For all the 6 different blood samples the observation was carried out specifically on erythrocytes, isolated from the rest of blood components, and consisted in the evaluation of their morphological modifications (due to both the passage of time and the process of deterioration) and the time at which the cells started to be no longer visible. The daily extraction of a small amount of blood from each of the 6 samples, in which the RBC separation was performed, permitted us to monitor their morphological transformation every day from T0 (day 0) to the day at which the RBC appeared to be deteriorated and no longer observable. Therefore, every day the separation of red blood cells was performed from the whole blood of each sample by repeated centrifugal sedimentation (500 µl of cadaver blood at 2500 rpm for 10 minutes), removing the supernatant (the remaining blood components) and washing up the solid pellet with physiological solution (®Eurospital) in agreement with literature [175-176].

6.1.1.3.2. Fixation and dehydration of RBC

Previous to RBC separation, cells were examined in whole blood by light microscopy to assure their presence and state of preservation; following the RBC separation the cells were then prepared for SEM analysis which required the involvement of fixation and dehydration. Gluteraldehyde (2.5% diluted with Na-Cacodylate buffer 0.1M pH 7.4 and distilled water) was used as a fixative, as it is considered to be optimal in keeping unaltered the morphological shape of erythrocytes. Fixation was carried out overnight at 4°C; then dehydration in ethanol was performed by passing the cells through increasing concentrations of alcohol followed by propylene oxide. [177-180].

6.1.1.3.3.Botanical samples

Plant samples, spores and pollen Plant material was selected with the intention of observing the most common and ambiguous species: we collected spores from Fungi, Bryophytes, Pteridophytes and pollen grains of an Angiosperm, deriving either from cadaver or natural sources (Tab. 6.1.1.1). The Genus of Fungi collected from cadavers had an uncertain determination, but this did not affect our results, since our main focus was the appearance of spores. We decided to focus more on spores over pollen due to the greater probability of "misinterpretation" as summarized in table 1. As with blood samples, the spores and pollen grains were prepared for the SEM procedure through washing steps with distilled water or through the rubbing of sporangia. For *Tortula* sp., *Bryum* sp., *Asplenium trichomanes*, *Asplenium ruta-muraria* and *Dryopteris* sp. spores we used a carbon conductive tape as support for the observation by SEM. The fixation and dehydration steps involved the same protocols followed for red blood cells, consistent with literature in this field [181-182].

KINGDOM	DIVISION	FAMILY	GENUS	SPECIES	SOURCE	LOCATION
Fungi	Ascomycota	Trichocomaceae	*Aspergillus*	Sp.	Cadaver	Milan
			Penicillium	Sp.	Cadaver	Milan
		Morchellaceae	*Morchella*	Sp.	Natural	Bergamo
		Saccharomycetaceae	*Saccharomyces*	*S. cerevisiae* (Meyen ex E.C.Hansen)	Natural	Milan
	Zygomycota	Mucoraceae	*Mucor*	*M. racemosus*	Cadaver	Milan
			Rhizopus	Sp.	Cadaver	Milan
	Basidiomycota	Strophariaceae	*Psylocibe*	Sp.	Natural	Milan
			Psylocibe	Sp.	Natural	Milan
			Psylocibe	Sp.	Natural	Milan
			Psylocibe	Sp.	Natural	Milan
Plantae	Bryophyta	Pottiaceae	*Tortula*	Sp.	Natural	Milan
			Bryum	Sp.	Natural	Milan
	Pteridophyta	Equisetaceae	*Equisetum*	*arvense*	Natural	Milan
		Aspleniaceae	*Asplenium*	*A. ruta-muraria* L.	Natural	Como
			Asplenium	*trichomanes* L.	Natural	Como
		Aspidiaceae	*Dryopteris*	Sp.	Natural	Milan
	Magnoliophyta	Ericaceae	*Camellia*	*C. japonica* L.	Natural	Como

Table 6.1.1.1: List of Species and botanical structures considered in this study

6.1.1.3.4. SEM Analysis

The Scanning Electron Microscopy analysis was conducted by using a Cambridge Stereoscan 360 with electron gun, vacuum pump, and image acquisition software (Oxford Link Pentafet, Oxford, UK). Once the preparation of RBC and vegetal cells was completed, a small drop of the cell suspension was allowed to spread on a glass slide where desiccation takes place almost instantaneously in room air. Before examination by SEM the cells must be coated with Gold-Palladium for creating a fine layer of metal and making the surface conductive.

The evaluation by SEM focused on shape and dimension of every RBC and spores/pollen samples analyzed, and also the survey of any specific and peculiar characteristics of every single cell, which could function as diagnostic features. In addition metrical analysis was also performed in order to evaluate the general data for dimensional characteristics.

6.1.1.3.5. Blind test

Sixty photos were acquired during the study, showing red blood cells isolated or in groups at different times of decomposition and all types of botanical structures chosen for the test. These were then presented to 6 experienced forensic pathologists, well acquainted with red blood cell examination particularly in scenarios where the presence of blood and haemorrhaging needs to be proven, and 6 natural scientists with a strong background in botany, in a blind study in which they were asked, for each photo, to recognize the cells (isolated or in group), choosing from 7 options: “erythrocytes”, “spores”, “pollen”, “other (botanical origin)”, “other (animal origin)”, “other (general)” and “uncertain”. The pathologists and natural scientists were chosen because of their strong experience (10 to 30 years of academic and research activities) respectively in the forensic and botanical field; they also represent the professional figures who most frequently have to deal with the interpretation of such structures in cases where they are found on human remains. Numbers and percentages of correct and incorrect answers were then calculated, along with the “uncertain” answers.

6.1.1.4.RESULTS

6.1.1.4.1. Erythrocytes

The decomposition of erythrocytes revealed the presence of different morphological appearances and sizes in relation to the passage of time. Precisely, our analysis showed 5 steps of degradation: the discocyte (fig. 6.1.1.1.-red circle), with its peculiar biconcavity; irregularly contoured discs (fig.6.1.1.1.-yellow circle), when the cell starts forming bumpy expansions on the membrane; flat and bumpy cells (fig.1-blue circle), when expansions are more visible and the cell loses its biconcavity; the echinocyte (fig.6.1.1.1-green circle), when the cell swells and shows a great number of evenly distributed spicules; the spherocyte (fig.6.1.1.1-pink circles), when the cell loses its spicules and appears as a smooth sphere.

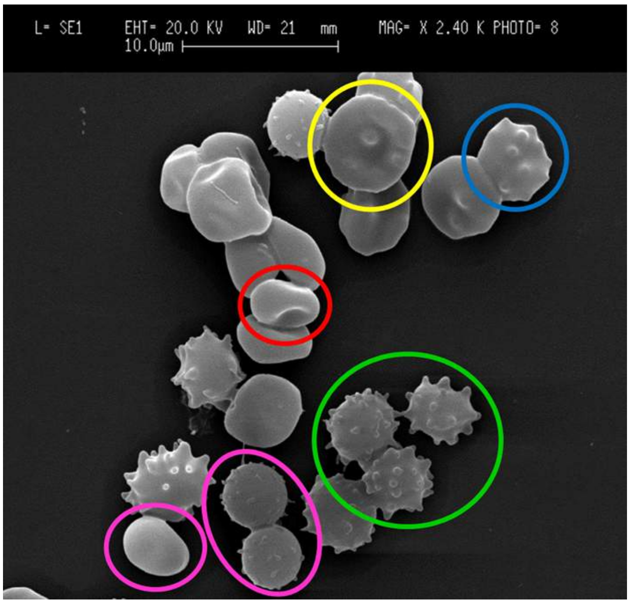


Fig. 6.1.1.1,: Discocyte (red), irregularly contoured disc (yellow), flat and bumpy cell (blue), echinocyte (green), spherocyte (pink).

These peculiar morphologies denote a range of time within death of an individual and the complete disappearance of cells: discocytes are already modified at T0, first day of analysis, and are detectable up to 3 days later (fig. 6.1.1.2.a). Echinocytes and spherocytes come into view as isolated cells already at T0 and T1, to become more numerous and the most common morphology at T2 and T3: they also modify their behavior, with an increasing agglutination as time passes by (fig. fig. 6.1.1.2b-c).

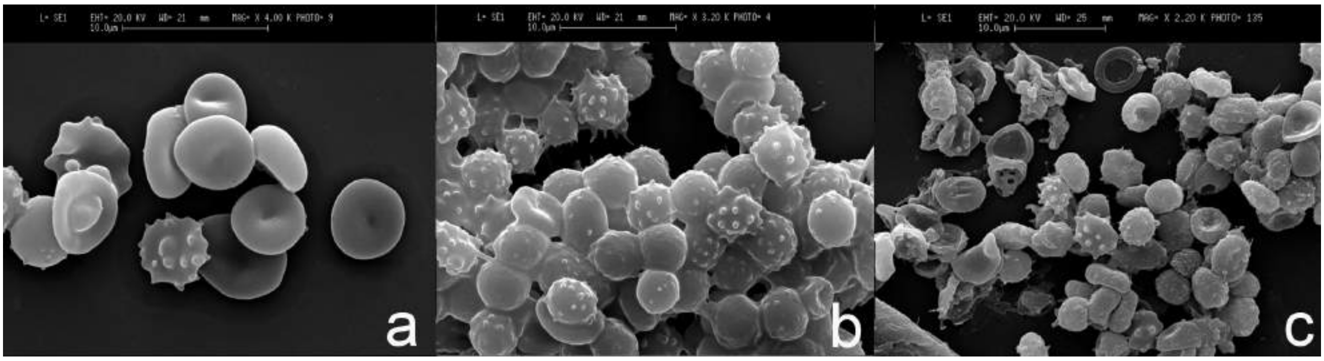


Fig. 6.1.1.2.: Discocytes are the most common morphology at T0 and T1 (a); at T2 most of cells are already spiculed and tend to stick together (b); erythrocytes appearance at T3 (c).

After 4 to 5 days, almost all erythrocytes had already shifted to the spherocyte morphology (fig. 6.1.1.3.a) and it was difficult to isolate single cells since they appeared as an agglutinated mass (fig. 6.1.1.3.b) until it was no longer possible to distinguish any cell, as from T6 onward (fig. 6.1.1.3.c).

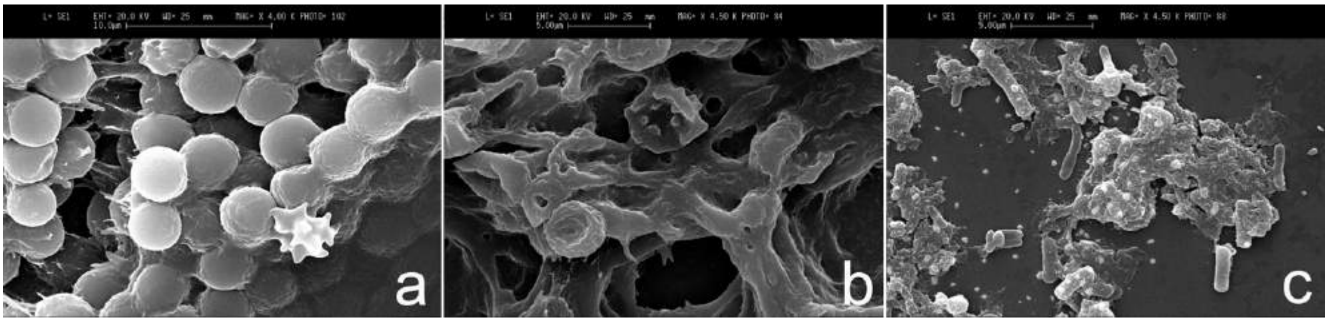


Fig. 6.1.1.3.: almost all cells have shifted to the spherocyte morphology after 5 days (a) and appear clumped together (b); no cell was still visible after 6 and 7 days (c).

At the same time, a thick coat of debris developed which made cells stick together, partly covering them: it derived most probably from the deterioration of cells and their content. Besides the morphological changes, a dimensional difference was revealed: discocytes measured 6 to 8 μm in diameter, while spherocytes 4 to 6 μm in diameter (fig. 6.1.1.4.).
This change is due to the formation of spicules at the expense of the outer membrane of cells.

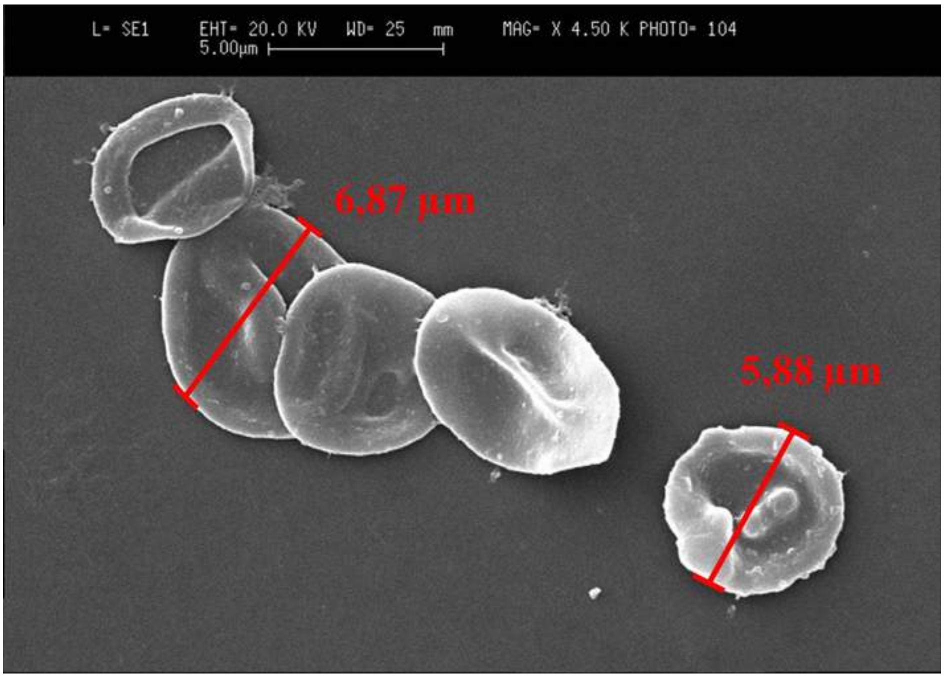


Fig. 6.1.1.4.: measurements conducted on cells in order to identify changes in dimension.

6.1.1.4.2. Botanical samples

Plant samples showed various spore morphologies according to different kingdoms and species. Our intention to compare the appearance of erythrocytes and spores resulted in some features that can help with the distinction.

Regarding the discocyte morphology, spores of *Aspergillus*, *Penicillium*, *Saccharomyces* and *Psilocybe* can be misleading, because they show a clear concavity, very similar to that of red blood cells (fig.6.1.1.5.).

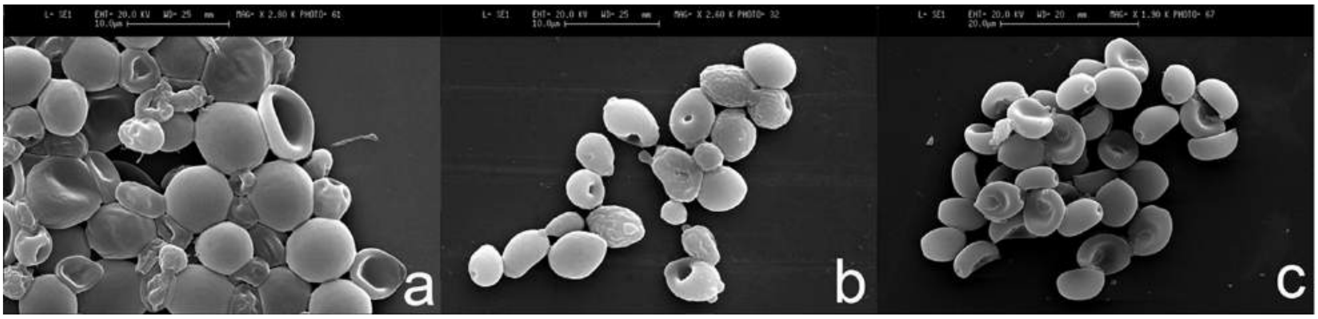


Fig. 6.1.1.5.: one image of *Aspergillus* spores (a). Spores of *Saccharomyces cerevisiae* (b) and of *Psilocybe sp.* (c), both presenting a cell concavity.

Also spores of *Drypoteris* sp. and *Asplenium ruta-muraria* were characterized by a pronounced depression, but it is probably due to an incomplete dehydration of cells and resulting deflation of spores (fig. 6.1.1.6.a-b). Other spores recalled the morphology of echinocytes, such as *Rhizopus* spores (fig. 6.1.1.6.c), and spherocytes, such as *Aspergillus*, *Penicillium* and *Equisetum arvense* spores.

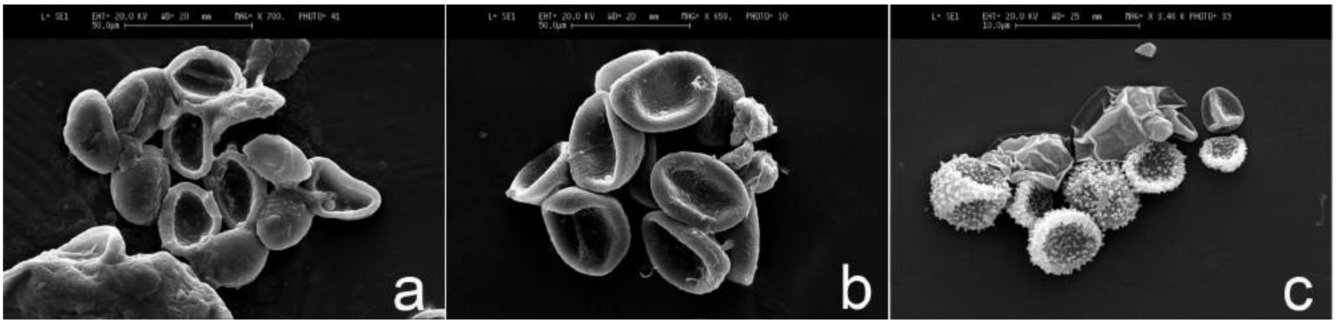


Fig. 6.1.1.6.: a group of *A. ruta muraria* spores (a) and of *Dryopteris* sp. spores (b) and *Rhizopus* spores with tiny spicules on the surface of cells (c).

Other samples analyzed with SEM resulted in clear images of spores, but their appearances were too distant from those of decomposing erythrocytes in order to make them "dangerous" for forensic interpretation (fig. 6.1.1.7.).

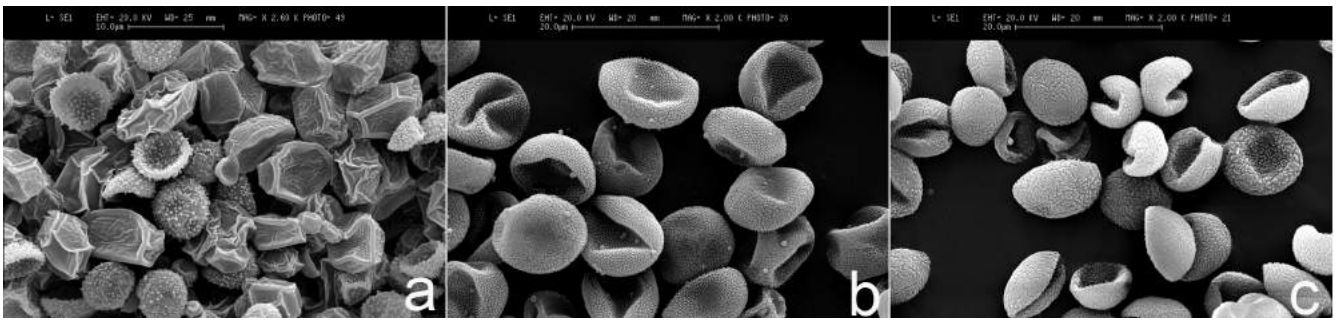
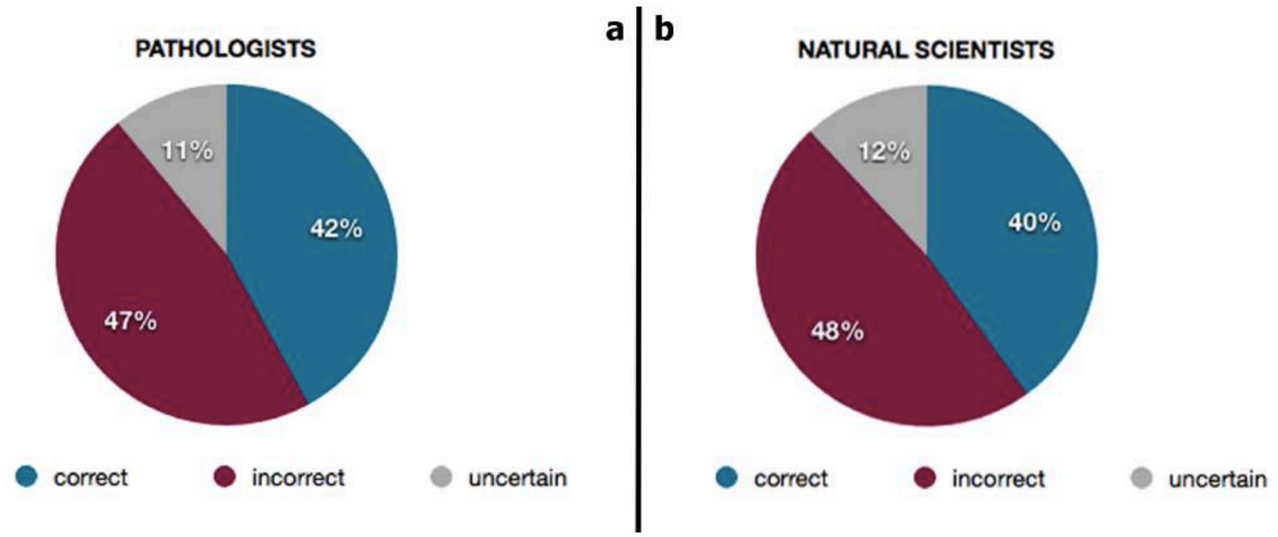


Fig. 6.1.1.7.: spores with a wrinkled outer membrane probably belong to the genus *Mucor* (online on www.cbs.knaw.nl) (a); *Bryum* spores (b) and *Tortula* spores (c).

6.1.1.4.3. Blind test

From the results of our test it emerged that both categories of specialists had similar difficulties with the distinction of structures; despite the knowledge and specific competence in their respective fields of study, the greatest number of mistakes fell on the same pictures, in particular spiculed and spherical red blood cells and spores of Fungi and less known species of plants, some of them similar in appearance to erythrocytes. Percentages of answers were calculated (graph 6.1.1.1.), showing a comparable rate of mistakes: this is mainly associated with the higher number of photos showing altered erythrocytes and spores of species which are not commonly studied, therefore harder to recognize.



Graph. 6.1.1.1.: Results from the blind test**.**

6.1.1.5. DISCUSSION

One of the greatest concerns in forensic science is presenting evidence in court. Experts who deal with the effects of contamination and taphonomy should be aware of the dangers of misinterpreting environmental debris as belonging to human tissue, blood for example. This research was carried out to investigate the potential misunderstanding between human erythrocytes and botanical material and to elicit diagnostic criteria.

This study showed interesting findings on morphological changes of erythrocytes in relation to their decomposition, confirming in part what was already reported in literature (167, 169-170). In addition, important information was gained also concerning the persistence of erythrocytes as definite cells: in fact it was demonstrated that, even if red blood cells were morphologically transformed, they were observable in blood left to decompose up to 6 days, after which only debris was observable (Table 6.1.1.2.). This is supposed to be due to the decomposition process and not to the many lab procedures the cells were subjected to (isolation, fixation and dehydration); in fact the same protocol was strictly followed at each experimental time but the numerous morphological shapes were seen in sequence after some time. Moreover, the procedure followed for blood sample preparation is based on the standard practices used in diagnostics of blood transfusion and diseases (169-170), where it guarantees red blood cell preservation. Furthermore, previous literature has described the same red blood cell morphological alterations as related to decomposition: this we think strengthens the hypothesis that such changes are due to taphonomy. Nevertheless possible changes in morphology due to the lab procedures also may be taken into consideration in future studies.

BLOOD SAMPLE NUMBER	TIME AT WHICH RBC ARE STILL IDENTIFIABLE
1	10
2	8
3	4
4	4
5	6
6	8

Table 6.1.1.2.: Time at which the erythrocytes of each blood sample were still visible at light microscopy and SEM analysis

For what concerns the comparison between the morphology of erythrocytes and botanical structures, the results have showed that while only few specific spores and pollens have a peculiar morphology and are easily discernable from all the forms of red blood cells, on the contrary most spores of various species appear very similar to blood cells and are difficult to distinguish. The typical biconcavity of red blood cells has comparable morphologies to those of *Aspergillus, Penicillium. Saccharomyces, Psilocybe* and *Dryopteris*. Some fungal spores showed short spicules, a second element that could mislead towards echinocytes.

Although the appearance of spores and erythrocytes in some cases seems to be hard to discriminate, one fundamental parameter is the dimension of cells, usually larger in spores rather than in erythrocytes (fig. 6.1.1.8.).

Spores, in addition, are characterized by diagnostic features, which erythrocytes do not possess, enabling the discrimination: fungal spores usually have a chain setting, resulting from a conidiophore arrangement, while erythrocytes are organized in rouleaux (fig. 6.1.1.9.).

A second important element is the interspore bridge left from meiotic division, continuous with the outer layer of the spore wall, which is impossible to observe in erythrocytes. The germinating pore is another peculiar feature of spores, like the one visible on *Psilocybe* spores and on pollen grains of *Camellia*, as described in Table 6.1.1.3., which summarizes the general findings with respect to the different types of cells and elements of discrimination.

DIAGNOSIS				
Elements		***General shape***	***Dimension***	***Peculiar features***
Erythrocytes				
	Discocytes	Biconcave, disc-shaped and smooth surface	6-8 μm	Arrangement in rouleaux
	Echinocytes	Spherical, with 20-30 elongated spicules	5-6 μm	When immature, appear like flat cells with bumpy surface
	Spherocytes	Spherical, with smooth surface	4-6 μm	Spicules are thick with rounded tips Agglutinated when reaching the last stage of degradation
Spores				
	Fungi	Smooth, rough or spined outer membrane. Spherical, elliptical or nut-shaped	Between 2 and >100 μm	Arrangement in chains. Germinating pore and hilar appendage for some Basidiomycota
	Plants	From essentially smooth to papillose, highly ridged, spinode or reticulate. Spherical or elliptical shape.	Between 5 and 200 μm Usually ranging from 12 to 50 μm	Interspore bridge. Laesure of monolete and trilete spores
Pollen				
	Angiosperms	Spherical, ovoid, triangular shape. Reticulation, spines, granules, papillae ornamentation	From 2 μm to more than 200 μm Usually ranging from 20 and 30 μm	Colpi and pores on the surface. Thick pollen tube during germination Sometimes two laterally-placed bladders (sacca)
	Gymnosperms			

Table 6.1.1.3.: Summary of features and strict characteristics found on erythrocytes, spores and pollen grains and useful in differential diagnosis.

This short study has shown how there may be dangers in the interpretation of degraded cellular elements but at the same time the possibility of distinguishing erythrocytes, even when degraded, from other structures using SEM. However, extremely qualified personnel needs to be used. In fact, the blind test conducted on six pathologists and six natural scientists revealed the uncertainties still surrounding this topic: despite being experts in their respective fields, both categories showed some difficulties in recognizing altered erythrocytes from spores and Fungi.

This means that the knowledge on how red blood cells degrade and how reproductive spores of some species might appear needs to be deepened and divulged both in the forensic and the biological fields. It is clear that with the combination of the knowledge of forensic scientists and naturalists this type of analysis can be ameliorated. However forensic scientists should always be aware of the crucial effects taphonomy may have even at the microscopic level.

6.1.2.

PART II:

How blood components decompose in bone tissue:

a study on the detectability of such biomarkers in the perspective of micro-taphonomy

The taphonomy of blood components in decomposing bone and its relevance to forensic anthropology

Cappella A, Bertoglio B, Maderna E, Di Giancamillo A, Domeneghini C, Andreola S, Cattaneo C

6.1.2.1. ABSTRACT

The microscopic detection of blood components in dry bone (which could be a potential diagnostic tool in forensic science, especially for trauma analysis and post-mortem interval estimation) has unfortunately not been thoroughly investigated. In literature, no study seriously takes into account variations and persistence of blood components within the bone tissue during the decomposition process, particularly at the early stages of decomposition and in different taphonomic conditions.

The aim of this research was thus to investigate, using histological (Hematoxylin-Eosin, H&E) and immunohistochemical (Glycophorin A, GlycoA) analyses, 32 parietal bone samples collected from eight individuals at different post-mortem intervals (PMI): (i)< 24 hours, (ii) 48-72 hours, (iii) 15 years and (iiii) 400 years. Three fresh bone samples were subjected to a decomposition process in air, monitored every week for 2 months (for a total of 8 fragments per sample), another one of these underwent laboratory processing (maceration, boiling and freezing); four bone samples were analysed in conditions of putrefaction and skeletonization (dry bone). To identify possible changes caused by the experimental conditions, a control sample was employed.

In order to evaluate the influence of time and taphonomy on survival and persistence of blood components, the presence of red blood cells (RBC) in every bone tissue portion (within Haversian and Volkmann canals, canals localized in bone trabeculae and marrow spaces) was examined.

The immunohistochemical investigation for GlycoA showed the presence of red blood cells under the form of erythrocyte debris or fragments unidentifiable from a morphological and structural point of view using only H&E staining. Results showed that well-defined erythrocytes can be seen only in the control sample, while after a week of decomposition, these structures can be seen only by using GlycoA immunohistochemical staining. No difference was detected in persistence of blood components in the first two months of air exposure with respect to the control fragment (i.e. more than 50% of canals and marrow spaces of each section showing RBC fragments). Similar results were visible on real decomposed cases.

Unlike the decomposed samples, the RBC detection varied after treatment of the tissue; particularly, compared to the control, a reduction (not more than 30% of canals and marrow spaces showing RBCs) together with a more drastic morphological alteration were highlighted in boiled and macerated samples, while preserved RBC structures and percentages close to the control were identified in the frozen one. Despite the longer PMI in modern skeletal samples (dry bone with a PMI of 15 years) it was still possible to detect, only by GlycoA investigation, erythrocytes in 10% of canals and marrow spaces, while a total loss of RBC fragments was observed in the sample taken from an ancient skeleton (PMI: 400 years).

In conclusion, this study highlights the usefulness of immunohistochemical detection of GlycoA in RBC investigation and gives an idea of the persistence of erythrocytes in different taphonomic and PMI conditions, in contrast to results reported by some authors in literature. Another important result concerns the detection of discrete quantities of red blood cells in dry bone with a PMI of 15 years which opens the way to the possible use of RBCs in trauma interpretation.

6.1.2.2. INTRODUCTION

After death, the body is exposed to different weathering and environmental conditions that affect the progression of post-mortem modification, but also the appearance and structure of body, tissues, cells and biomolecules. In literature many works show how body changes and appears at the different decomposition stages (fresh, bloated, decay, postdecay and skeletal) and environments (183-186), thus several authors have dealt with how bone varies in the different taphonomic conditions. Bleaching and small irregular defects for example are the result of water activity while whitened areas are associated with sun exposure and damages with regular, attenuated and thin edges are correlated with mechanical erosion (187). Also to be considered is the activity of scavengers, that inevitably leave marks of their passage on bones (gnawing, chewing and crushing) (187), and the effects of microbial invasion. On the latter many studies have focused on the characterization of the microbial attack and how it looks at the microscopic level (188-190). Another important aspect is how the organic and inorganic components of bone tissue react and change over time after death; Dent et al (191) show how protein degradation and loss of mineral hydroxyapatite take place and emphasize the link between bone preservation and burial environment.

Unlike the macroscopic and microscopic changes on bone tissue caused by environmental factors, scavengers and microorganisms, little is known about bone tissue and its components in relation with decomposition, post-mortem period, different states of preservation and taphonomic factors.

Bone tissue is characterized by a protein structure (mainly collagen), stiffened by a mineral constituent (hydroxyapatite), and other organic components (e.g. mucopolysaccharides and glycoproteins) (192-194). Like every tissue in the body, bone tissue is also characterized by vascularization and therefore includes all cells and typical components of the blood: erythrocytes, leukocytes, platelets, albumin, fibrinogen and other plasma proteins. These components are ubiquitous in bone tissue, specifically they can be observed at the periosteum (within the vessels located here), into Haversian and Volkmann canals located in both lamellar bone (transverse or oblique canals that connect the meaning functional bone units, the so-called osteons), and trabecular bone, in vessels placed into bone marrow spaces where hematopoiesis takes place and blood cells are present at various stages of maturation.

All of these components may be useful for understanding microscopic changes occurring within bone tissue during the decomposition process and due to different environmental conditions in which a body decomposes (water, high or low temperature, humidity, air). In this regard it would be interesting to know what variations take place (persistence and morphological changes of blood components) and in which specific part/structures of the tissue. Once some data about the persistence of blood components in the bone tissue of a decomposed body has been discovered one can consider differences due to decomposition (early and advanced) in different contexts, in order to comprehend knowledge in a field still in evolution such as microtaphonomy or for future research on histological markers useful in the interpretation of taphonomy and in the next level of trauma analysis.

In literature the histological knowledge about bone tissue blood and marrow components in bone tissue are limited to diagnostic histopathology or paleopathology (195-203).

In the forensic field Penttilä and Laiho (168) have investigated the morphological changes of blood components (such as erythrocytes, white blood cells and platelets) in cadavers blood at different post-mortal intervals; their results have highlighted modifications in shape and size over time and a persistence of 10 days maximum. Differently, other studies have focused on morphological changes of blood cells over time with the intent of dating bloodstains and supplying possible information useful in estimation of time since death (204-207). Finally, attention was also given to possible mistakes in differential diagnosis between erythrocytes and spores and pollens (208). But the observation of changes of blood components within bone tissue in regard to Post-Mortem Interval (PMI) and decomposition process has not yet been fully explored: for how long well-preserved blood components are observable in bone tissue of cadavers and still detectable over time, in which area of bone tissue are mostly protected or unaltered, and again what information can be gained from their persistence in relation with different environmental conditions, all questions that still remain a great field to be investigated. Despite the lack of precise information about the points aforementioned, in literature more often some authors report the presence of erythrocytes or structures like-erythrocytes in ancient skeletal remains (163-164, 167, 208-210), but these findings are not yet demonstrated by the specific techniques that science owns. Thus there is a necessity to gather as much information as possible on what can be still observed in decomposing bone tissue, with particular focus on what changes can be highlighted in the early and advanced stages of the decomposition or in the different taphonomic conditions to which a body is exposed. Such information could help forensic anthropology in interpreting taphonomy in skelelal remains and eventually the evidence of bleeding in trauma, an aspect this last that has never been investigated in this perspective.

Therefore the study aims to probe the changes and persistence of blood components (with special attention to erythrocytes) in bone tissue in relation to decomposition process over time and taphonomic conditions by using scientific techniques such as histology (Hematoxylin-Eosin staining, H&E) and immunohistochemistry (Glycophorin A, Glyco A). The observation includes a histological assessment conducted on bone samples from both well and badly preserved cadavers (early decomposition) as well as from modern and ancient skeletonized remains in order to observe how blood components appear, if they are noticeable, and for how long their persistence can be still detected in such conditions. The present study may contribute to clarify the use of some valid techniques as an aid to the understanding of taphonomy of microscopic bone structures and acquiring new important information useful in taphonomic and in forensic field.

6.1.2.3. MATERIALS AND METHODS

6.1.2.3.1. Bone Samples:

The study was conducted on a total of 32 samples of parietal bone fragments, which had been previously sampled from forensic cases from 8 cadavers (with no blood diseases or bone pathological conditions reported) at the Institute of Legal Medicine of Milan. The cadavers were characterized by different states of decomposition (early and advanced): the diverse PMI as summarized in Table 6.1.2.1.

sample n°	Decomposition degree	PMI
1	fresh cadaver	< 24 hrs
2	fresh cadaver	< 24 hrs
3	fresh cadaver	< 24 hrs
4	fresh cadaver	< 24 hrs
5	putref cadaver	48<hrs<72
6	Skeleton	20 yrs
7	Skeleton	20 yrs
8	Skeleton	400 yrs

Table 6.1.2.1.. Samples and related state of decomposition and PMI

The parietal bone fragments from the three well preserved cadavers (samples 1, 2 and 3, all with a PMI <24 hours) were exposed to decomposition in air at standard environmental conditions (constant humidity as well as temperature), and then monitored weekly for the two following months (for a total of 8 fragments per sample). Unlike the previous, sample 4 (derived from a well preserved cadaver with a PMI< 24 hours) was divided in fragments subjected to diverse lab procedures in order to simulate some taphonomic contexts, consisting in boiling (for 2 hours), maceration (immersion in cold water for 1 month) and freezing (-4°C for a month). From every sample mentioned above, a small portion was used as a control for the evaluation of all the experiments and so was not subjected to any experimental conditions. In addition, parietal fragments were taken also from human remains in which both the decomposition skeletonized process occurred in real conditions, in order to compare results obtained from real conditions and experimental setting. The real conditions consisted in a cadaver at the early stages of putrefaction (sample 5, with a PMI ranging between 2 and4 days) and in skeletonised remains of different PMI (sample 6, 7 and 8). In particular samples 6-7 were obtained from 2 well-preserved modern skeletons (from "Milano Skeletal Collection", a modern referenced skeletal population), while sample 8 was taken from a badly conserved ancient skeleton (1600-1630 AD); as summarized in Table 6.1.2.2.

	Time of air decomposition (weeks)							
Sample n°	*T0 (ctrl)*	*T1*	*T2*	*T3*	*T4*	*T5*	*T6*	*T7*
1	< 24hrs	1 w	2 ws	3 ws	4 ws	5 ws	6 ws	7 ws
2	< 24hrs	1 w	2 ws	3 ws	4 ws	5 ws	6 ws	7 ws
3	< 24hrs	1 w	2 ws	3 ws	4 ws	5 ws	6 ws	7 ws
	Laboratory treatment							
Sample n°	*1*		*2*			*3*		*4*
4	ctrl		freezing			boiling		macerated

Table 6.1.2.2. Experimental conditions for sample 1, 2, 3 and 4. While sample 1, 2 and 3 were subjected to decomposition in air, sample 4 was subjected to laboratory treatment for simulating different taphonomic conditions.

6.1.2.3.2. Histological and Immunoistochemical Analysis

The same protocol was used for preparing all bone fragments for both histological and immunohistochemical analysis, consisting in fixation (in 10% Formalin for 24 hours) and decalcification (in DECAL solution, 14% idrochloric acid, Histo-Line Laboratories, Milan). The decalcification was performed at room temperature for a time dependent on size and thickness of the fragment (from 4 hours for ancient bone fragment to 2 days for fresh bone samples). Once the optimal flexibility was achieved, washing up under running tap water was performed for 24 hours before proceeding with dehydration and with the embedding into paraffin. From each samples so treated some 5-microns sections were obtained and subsequently stained with Hematoxylin and Eosin for the histological analysis as well as immunohistochemical staining for the identification of Glycophorin A (antigen of erythrocyte membrane) for the immunohistochemical analysis. The latter was carried out using a monoclonal antibody (antihuman Glycophorin A, clone JC159, Dako-Dakopatts, Denmark) diluted 1:400 by a Dako Autostainers as according to the literature (130, 211).

6.1.2.3.3. Microscopic Evaluation

Finally, the microscopic observation was performed by the optical microscope (LEICA DMLB) at several magnifications (4x, 10x, 20x, 40x, 63x) with 10x eyepieces). By means of a photographic system (Eurekam 3.0, DV-3000) photographic surveys related to the most significant areas were captured.

The evaluation of changes and persistence of the blood components was based on the presence/absence and variation of red blood cells in the different portions of the bone tissue and in all structures containing blood: within Haversian and Volkmann canals, canals present in bone trabeculae of the diploe and vessels located in the marrow spaces as well as periosteum. A semi-quantitative assessment was also possible by calculating the percentage of canals and bone marrow spaces containing red blood cells. For all samples both the histological and immunoistochemical evaluation was performed in order to confirm the results.

When the histology gave no chance to identify with certainty well-defined cells but only a strongly eosinophil accumulation (referred as hematic components by the authors, as confirmed by immunohistochemical analysis) the term "non-specific accumulations" was used.

6.1.2.4. RESULTS

Histological analysis and immunohistochemistry (respectively obtained by using EE staining and glycophorin A-antibody) made it to observe in detail the blood components into every single bone tissue structure: specifically in the Haversian and Volkmann canals, in canals and vessels localized in bone trabeculae, and in bone marrow spaces.

Our first objective was to analyse how the features of red blood cells in bone tissue modify in the monitored samples at the early stages of decomposition in air (for a maximum of 2 months): the evaluation concerned the quantity in respect with the total canals, vessels and marrow spaces, and the recognition of single/groups of well definite red blood cells before their decomposition and disappearance. The same assessment was conducted also on all samples in which the decomposition process has already started a few days before (sample 5) or the skeletonization had occurred over a period of years or century (sample 6, 7, 8). Finally, the potential differences in the persistence of blood components depending on taphonomic factors were investigated: the samples were subjected to those experimental treatments that are usually practiced in the laboratory and then examined as any other samples.

Results from EE staining show the possibility to recognize well definite red blood cells only in control samples (1, 2, 3 at T0 and 4 not treated) as well as in sample 5 (fragment taken from putrefied cadaver with PMI between 48-72 hours). In other words, red blood cells were no longer observable as cells after a week of decomposition in air; from this time onward solely non-specific accumulations were noticeable by using EE staining (Figure 6.1.2.1.).

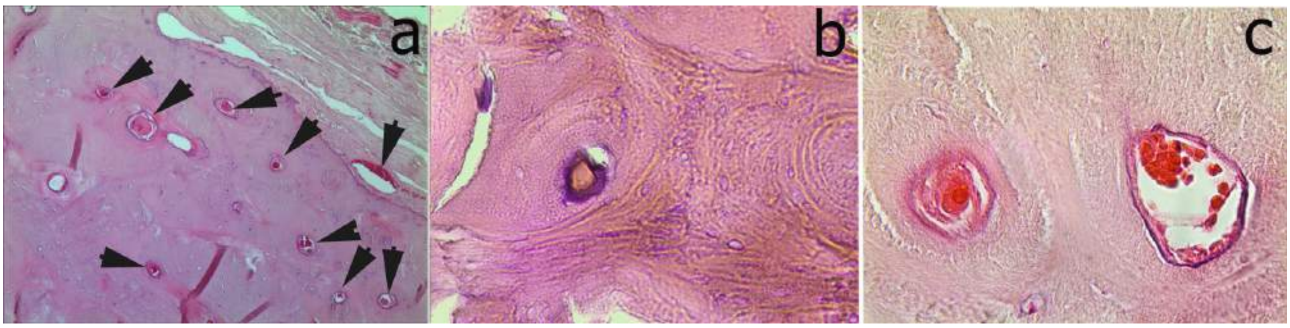


Fig 6.1.2.1.: a) Sample 2 at T2 shows numerous Haversian canals still full of blood components (10X magnification); in this case histological analysis permits only to highlight non-specific accumulation as that shown in b) at a greater magnification (40X) in contrast with the well definite erythrocytes visible at To shown in c) observed at 40X magnification.

On the contrary, the Immunohistochemical analysis adopted for the search of glycophorin A (a specific membrane antigen of erythrocytes) allowed to detect the presence of red blood cells even when degraded (no cells well definite but only hematic debris), if any, otherwise not detectable by the histological examination; thus, this analysis permitted to confirm the haematic nature of most non-specific accumulations located into canals and vessels stained by H&E (Figure 6.1.2.2.).

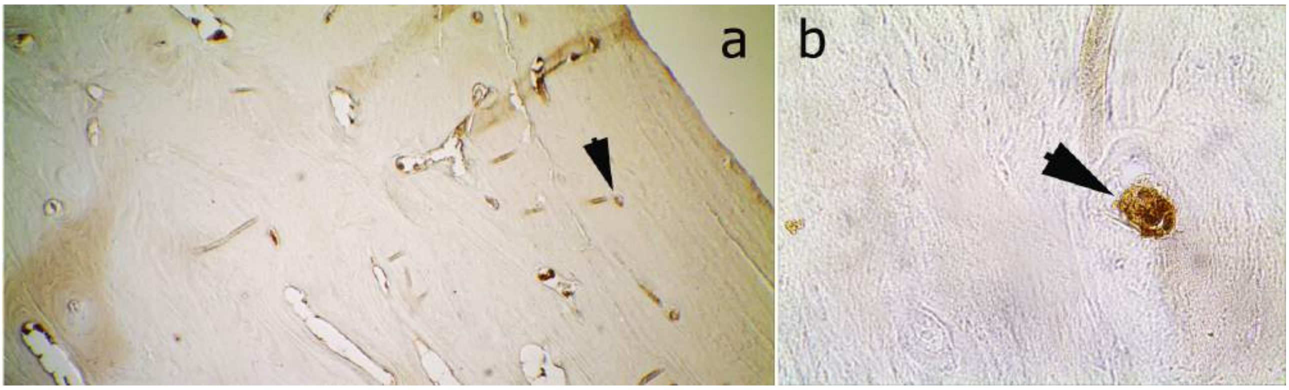


Fig 6.1.2.2.: a) Sample 2 at T3 shows numerous Haversian canals still full of haematic components (4X magnification); in this case immunoistochemical analysis permit to proved the presence of the erythrocytes in the non-specific accumulation: in fact the dark brown coloration means that into canals and vessel is located surely the antigen Glic A, proving in this way the haematic origin also for those non-specific accumulation highlighted by he histological analysis. In b) A Haversian canals of sample 3 at T4 at greater magnification (40X).

Regarding the persistence of blood components (assessed as the percentage of bone tissue structures containing RBC) (Figure 6.1.2.3.), no great changes in terms of percentage were observed concerning the passing of time in the first 2 month of decomposition in air; the percentages obtained by the evaluation in control samples are similar to those found in sample at all experimental time and also after about two months of decomposition in air (Fig 6.1.2.3.). The differences found are more concerning the abundance of coloration: if at very early time like T0, T1 and T2 the canals are completely full of components, at the more advanced time the same structures shown a lighter staining and few space is occupied by the components (Fig 6.1.2.3.).

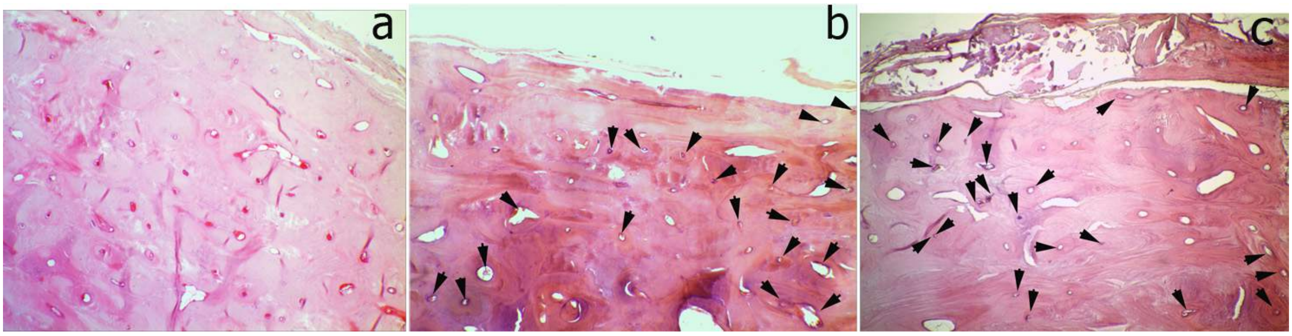


Fig 6.1.2.3.: a) Sample 1 at T1 shows numerous Haversian canals still completely full of blood components, stained by H&E (general view at 4X magnification). In this case the canals does not need to be indicated by arrows as they are very well observable even without any help; in b) a total view (4X magnification) of sample 1 at T6: despite the bone fragment has decomposed for about 1 month and half, Haversian canals still have blood components inside in a surprisingly high percentage out of the total of these structures, and so is for sample 1 at T7 (one week later) shown at 4x of magnification in c). In both, b) and c) the non specific accumulation still located in the canals are indicated by black arrows in order to help the observer in their identification.

As regard quantity of structures among the total in which the components are stained, H&E revealed percentages between 60% and 80%, also confirmed by Glyco A that demonstrated percentage a slightly higher but very alike. As a matter of fact, the immunohistochemistry in general showed higher values compared to the common histological analysis, revealing in this sense not only the presence of entire cells but also the RBC fragments in samples at a more advance stages not detectable by H&E (percentages around 80% in cases where H&E revealed just 60%).

Similar results were found also in real putrefaction (sample 5, PMI 48-72 hours), in which well-defined cellular elements as well as abundant non-specific accumulations are represented by percentages greater than 60% in both histological and immunohistochemical analysis. Unlike the previous, the chance to detect RBC in skeletonised human remains samples characterized by a higher post-mortem interval (15 years 6, 7) was possible only by using the monoclonal antibody Glycophorin A (H&E did not allow to observe neither well definite blood components and non-specific accumulation, but only debris and contamination by botanic structures); while the modern skeletal remains disclosed surprisingly RBC residues in more than 10% of canals and marrow spaces (Figure 6.1.2.4.), the ancient skeleton (sample 8) shows completely lack of any cellular debris, as confirmed by Glyco A stained sections.

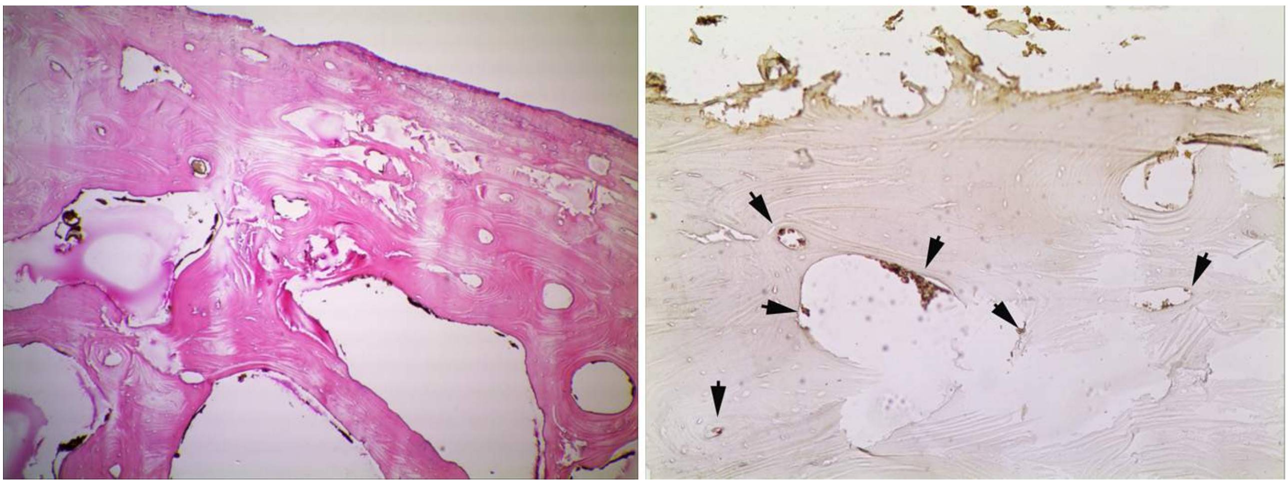


Fig 6.1.2.4.: a) Sample 6, derived from cemeterial skeletal remains (PMI of 15 years) stained by H&E (left image at 4X Magnification) and by Glyco A staining (right image at 10X Magnification). Only immunoistochemical analysis allows to observe the presence of blood components residues (indicated by black arrows in the right image). The histological analysis, in fact, shows only the presence of some brown structures, which can resemble erythrocytes in terms of morphology and size, but since they are not stained by Eosin as the components in all the other samples (which staining includes non specific accumulation and definite blood components) their origin is presumably botanical or some other contamination.

Concerning results from the evaluation of samples subjected to the lab treatments relevant details have arisen: both analysis revealed a great similarity between control and the frozen sample which has displayed both erythrocytes and non-specific accumulations in consistent percentages (>50%), similar to what seen in the control (60%). If on one hand the presence of blood components is maintaining quite constant in the frozen sample (Figure 6.1.2.5), on the other hand the reduction of blood components (values close and under 30%) has been emphasized in the boiled and macerated sample. In this last case solely the immunohistochemical investigation stressed the RBC presence in canals and marrow spaces.

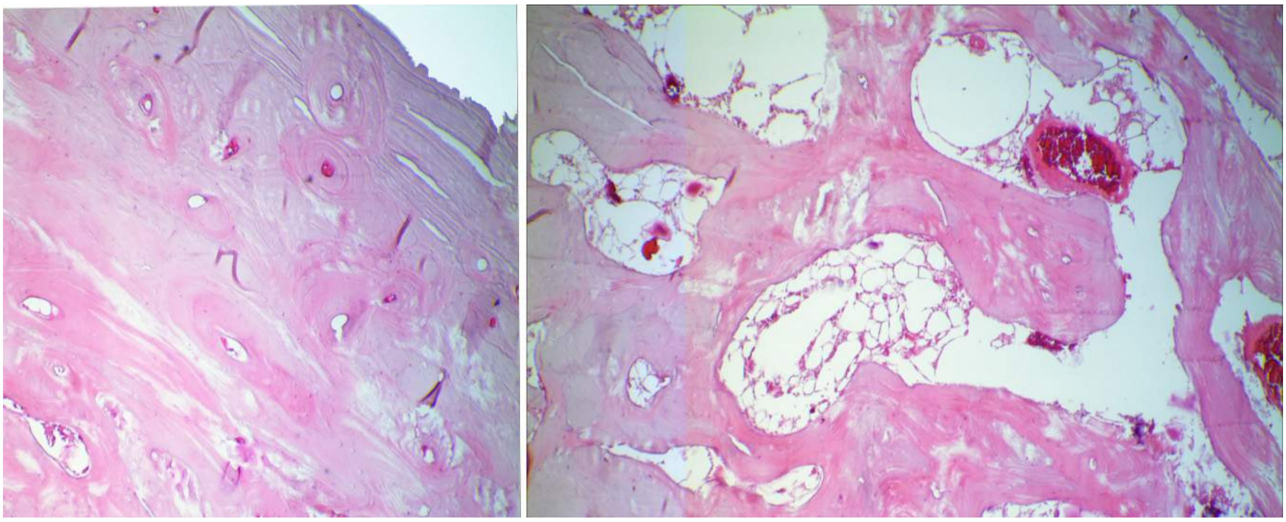


Fig 6.1.2.5. Sample 4-2 (freezing treatment) stained by H&E: At left 40X of Magnification, at right 100X Magnification. The persistence and presence of erythrocites is well localized anywhere in cortical bone into Heversian Canals and into marrow spaces. The treatment seem to maintain constant the level of components demonstrating to be a conservative lab procedure.

6.1.2.5. DISCUSSION:

Given the lack of knowledge about the decomposition and taphonomic effects on blood components in the bone tissue, the present study has focused on the acquisition of such information through histology and immunohistochemistry. Decalcified sections of parietal bone fragments, characterized by different states of decomposition and various post-mortem intervals were analysed with the intent to observe differences due to various steps of decomposition. An evaluation on the presence/absence of erythrocytes or their residues was considered in all canals and marrow spaces of the sections and so the attendant percentage. The immunohistochemical technique, which uses antibodies for Glycophorin A (a specific erythrocyte membrane protein) has allowed the authors to obtain important information about the chance of tracing the presence of cellular debris or remains from blood decomposition. In particular, this technique make it possible to highlighting what is not noticeable in the sections stained by H&E: the so-called non-specific accumulations were proved to be (or not) certainly of RBC origin. Obviously, they may contain however other cellular and haematic debris, which have to be still ascertained with further specific immunoistochemical investigations.

Results from both analysis have demonstrated that cells appear as well defined only if the period of time is shorter than 1 week (in control sample and in sample 5), a fact that proves the influence of time on the preservation of morphology as reported in a recent publication (208). If the morphological preservation of RBC is quite dependent on time, since the very early stages of decomposition, on the contrary the persistence of their debris survive longer than expected: the percentage of bone tissue structures in which such cell remains are still detectable, especially by immunohistochemistry, are constant in the first 2 months of decomposition in air. The confirmation of these results comes also from the assessments conducted on samples taken from a cadaver in putrefaction (PMI less than a week), representative of a real decomposition (in a decomposed body), in which biconcave red blood cells are still well identifiable and are still observed in considerable percentages of vessels and canals.

Surprisingly RBCs are still traceable in modern skeletal remains with a PMI of 20 years (even if in very low quantity). In this last, in fact, despite the long time elapsed since death (20 years), a little percentage of canals and marrow spaces (10%) still contains RBCs, clearly only discernable by immunohistochemistry; meanwhile a total loss of the same was observed in samples with PMI in order of centuries (sample 8). This last account, revealed results in contrast with findings from some recent publications, where it is often reported the identification of red blood cells even in ancient remains and through different techniques (162, 164, 166, 209-210, 212). Despite the present study has not investigated conditions equal to those reported in some publications, such as fossilization or mummification, the authors have observed great difficulties in recognizing well defined cells after a week of decomposition, and in most samples this was provided just because of the implementation of immunohistochemistry, otherwise it would have never been possible. The crucial point in this sense is obviously the technique used to demonstrate the presence of red blood cells, which has to be based not only on morphology but also on more satisfactory requirements. In fact the structure of botanical nature sometimes reveals to be very tricky and may mimic the presence of erythrocytes as reported by a recent work (208), which show the similarity between red blood cells and spores/pollen as well as providing important information about the survival and morphological modification of erythrocytes *in vitro*. In this sense the suggestion is caution before hazarding any wrong conclusions which need to be proved by always carrying out further analysis on sample.

Interesting are also the results obtained from samples subjected to maceration, boiling and freezing: the normal lab practices that are normally required for cleaning up bones (maceration and boiling) which have demonstrated to remove most of cell components of the tissue, even though some minimal quantity of debris can survive in tissue, which are in this case detectable only by immunohistochemistry.

Unlike boiling and maceration, the conservative procedure, such as freezing, revealed as expected good propriety of conservation of cellular components, not only in terms of presence and quantity but even in terms of preserving morphology. Moreover in the frozen sample there is the presence of some cracks caused by the thermal shock, which can cause also hypothetically migration of non-specific accumulations or debris; in this way the histological examination could be a little misleading for the study of distribution of such components and tissue integrity but freezing is proved to be a suitable procedure for histological and immunohistochemical analysis since it preserves both bone tissue and its components adequately.

In general the study was based on experimental conditions, (control samples and samples from real conditions - putrefied cadaver and skeletal remains - a part) which simulate a process of decomposition not easily comparable to what happens in the decomposition of a body (as in a closed system like a cadaver): in the first case, it is the external lamina to be directly in contact with the surrounding environment, in the second, however, it concerns the most superficial tissues that cover and protect the underlying bone tissue at the early stages of decomposition. This fact could give rise to some variation in the detection of blood components and their survival in decomposed or decomposing bone tissue, nevertheless the potentiality of a technique such as the immunohistochemistry in proving their presence and in being an optimal analysis for the detection of blood components residues, even if in traces does not change. This study highlights the usefulness of immunohistochemical detection of GlycoA in RBC investigation and gives an idea of the persistence of erythrocytes in different taphonomic and PMI conditions, often in contrast with results reported by some authors in literature, as mentioned above. The presence of RBC, as definite cells or as cellular residues, can be a possible marker not only in relation with PMI, taphonomic conditions and decomposition, but also as key cells in the bleeding or interpretation of trauma in bone; clearly this study is only a first approach to blood taphonomy and future researches need to be conducted in order to acquire knowledge in this field. Nonetheless, an important fact has emerged from this study which is the great capacity of the immunohistochemistry in searching for specific biomarkers in both fresh and dry bone tissue; the use of biomarkers such as RBC through immunohistochemistry could be crucial for forensic purposes such as the interpretation of PMI (analogous use of Luminol) or of trauma (for the first stages of healing or for the interpretation of vital lesions), all topics that still need to be investigated and clarified.

Chapter 7

7.1. SECONDARY RESEARCH LINES

The previous chapters focused on the study of trauma and on how taphonomy proves to make difficult the interpretation of lesions. As a matter of fact, when skeletal remains are retrieved, information about the individual's life and its biological profile are extrapolated only from bone elements, and so are all signs related to possible trauma, if any.

As the interpretation of antemortem injuries (based on the presence of bone reactions that indicate the healing process) and perimortem lesions (based solely on morphological pattern), the construction of the biological profile of an individual is determined on the basis of the observation of morphologic characteristics and variations belonging to specific skeletal traits. In the latter case, the determination of sex, the estimation of age and stature, as well as the presence of any pathological signs that lead to a diagnosis of a pathological state, are extremely important for defining the identikit in the forensic field. Through the analysis of the skeletal sample studied in this PhD project it has been possible to highlight how taphonomy determines not only alterations on possible injuries pre-existing - misleading in the interpretation of trauma - but also possible changes on the skeletal traits fundamental in the diagnosis of age of individuals. In addition some research was also conducted on the problem of evaluating the Post Mortem Interval, often related to the persistence of indicators of modernity, which are slightly correlated with the persistence of the biomarkers investigated in Chapter 6.

This introduces the problem of taphonomy in relation to the estimation of age but also of PMI, remarking once again that the postmortem insults together with the taphonomic effects due to burial into coffin cause damage and irreversible alterations to bone tissue, critical to the determination of the biological profile and to the evaluation of PMI on inhumed skeletal remains. Once again taphonomy becomes a protagonist of important anthropological issues and creeps up as an absolute rival in the application of methods and criteria commonly used in forensic practice.

This chapter illustrates three sideline analyses carried out during the PhD project in parallel to the study of the skeletal sample and to the research lines conducted on the more specific issues of the trauma analysis, highlighting once again how taphonomy plays a central role for all matters discussed. This is to say that taphonomy must be taken into account in all anthropological practices, starting from the building of the biological profile, through the identification of disease states up to the more difficult task of interpreting injury. Taphonomy represents a problem for all activities and practices related to forensic anthropological which reinforces the need for new research and methodologies, perhaps alternative approaches, which differ from the more common morphologic parameters too often subject to taphonomic transformations, need to be investigated.

7.1.1.
The issue of age estimation in a modern skeletal population: are current aging methods satisfactory for the elderly?

Cappella A, Cummaudo M, Arrigoni E, Sforza C, Cattaneo C

7.1.1.1. ABSTRACT

The skeletal age estimation of individuals is one of the main tasks in forensic anthropology. From 1920 to date, several methods have been developed, each with its limits, mean error and age ranges in which it proved to be more reliable. The main idea behind the age assessment in adults is related to the analysis of the physiological degeneration of particular skeletal structures with age. The main issues with these procedures are due to the fact that they have not been tested on different populations and in different taphonomic contexts and that they tend to underestimate the age of older individuals. The methods currently used by anthropologists have been in fact standardized on archaeological and historical collections that may not completely reflect the characteristics of a modern population, especially from a demographic point of view. In addition to this the increased life expectancy at birth has pointed out the need for further research on the estimation of age ranges in skeletal remains belonging to elderly individuals.

In the present study the following methods were taken into account: Suchey-Brooks (symphysis pubis), Lovejoy (auricular surface of the ileum), Iscan (fourth rib's cartilaginous end), Rougé-Maillart (acetabulum combined with auricular surface) and Beauthier (palatine sutures).

The purpose of this study was to test the applicability and the reliability of these methods on a contemporary population of skeletal remains of 145 elderly individuals of known sex and age (ranging between 50 and 98 years) and exhumed 20 years after their burial. Although the skeletal remains were generally in good conditions, some skeletal sites showed a lower survival due to taphonomic influences and consequently it was not always possible to test all the methods on the entire population. The results show that the methods with the highest percentage of applicability were Lovejoy (89,6%) and Rougé-Maillart (81,3%), followed by Suchey-Brooks(59,3%).Those with the lowest Beauthier(26,2%) and Iscan(22,7%).

As regards the age estimation accuracy, Lovejoy (81,5%) showed the best results in terms of the correct identification of the age ranges in which the chronological age of the individuals was included. These percentages are reduced to 68,6% with Suchey-Brooks(2σ), to 66% with Beauthier, 54,5% with Iscan, and 19,8% with Suchey-Brooks(1σ). Rougé-Maillart method,

although it has not been standardized yet, showed the best potential in age estimation of elderly individuals. The main limit of this method was due to the fact that the age ranges are too small (9 years). Therefore, at the moment, the probability of associating an individual to the wrong age class is too high. The first step to improve the method could be the redefinition of the age classes in wider ranges without neglecting the need for further testing on a wider sample.

This research has shown how for older adults the study of both acetabulum and auricular surface may be more reliable for aging. This is also in accordance with the fact that auricular surface and the acetabulum are the areas more frequently surviving taphonomic insult.

7.1.1.2.INTRODUCTION

Age estimation of skeletal remains is one of the main challenges for forensic anthropologist when trying to create a biological profile of unknown individuals.

More and more, experts are asked to deal with the retrieval, recovery and study of badly decomposed human remains, also due to the growing rate of crime and clandestine immigration (213).

As pointed out by Ritz-Timme (214), methods for age estimation must meet the following criteria: (1) they must have been presented to the scientific community through peer-reviewed publication; (2) their accuracy must be tested using valid statistical procedures and described by clearly defined terms; and (3) the method must be accurate enough for routine forensic application.

Current methods to assess age-at-death can be divided into those that are based on the growing skeleton and dentition, and those based on the degenerative changes of the skeleton. Age estimation is more difficult in adult than in sub-adults because the skeletal and dental development is already complete. In both cases it should be taken into account that forensic anthropologists can try to achieve the physiological age, which might be quite different from chronological age (215).

The most popular methods based on the examination of the teeth are cementum annulation by Kagerer and Grupe (216), the Lamendin method (217-218), Logan and Kronfeld (219), and Schour and Massler charts (220-221), with revision by Anderson et al. (222) and Ubelaker (223); as regards the skeletal examination, diaphyseal length from long bone measurements (224-225), cranial suture obliteration as indicated by Meindl and Lovejoy (226), Masset (227), Nemeskeri et al. (228) and Baker (229), pubic symphysis evaluation by Todd (230-231) and Suchey–Brooks (141, 232), chondral articular surface of IVth rib analysis by Iscan, Loth and Wright (144-145, 233), ilium auricular surface observation by Lovejoy et al. (142), microscopic analysis of bone structure and osteon counts by the Kerley method (234), improved in the Kerley and Ubelaker revision (235) and revisited in the Ahlqvist and Damsten method (236), Stout and Paine (237).

Although the large amount of methods that have been developed, the main problem of forensic anthropology, as in many other forensic disciplines, is the lack of consensus or uniformity of procedures and methods used (215). Some guidelines have been suggested for forensic practices (214-215) but at present there is not an agreement on which method rely directly on a precise age range (238-240) or which methods can be combined (241-244). Similarly when evaluating the possibility to combine methods (multifactorial approach) it should be taken into accounts that their formulation is based on specific local reference samples (245). Therefore the variability between the different populations requires to assess whether the distribution of standards elaborated from a specific reference sample is the same for an individual not included in that reference group (238).

Moreover, although there is no doubt that skeletal changes have some relationship with age, the so called "senescence" is characterized by "*a complex set of ongoing interactions (genes-culture-environment)*" (246]; therefore the relationship between chronological age and skeletal age indicators is neither constant nor linear nor uniform across populations (247).

For this reason, data about the applicability of aging methods to samples from various populations and knowledge of population variation in aging processes are fundamental to successful adult age estimation and still only few studies (242,248-250) have evaluated population differences in the accuracy of aging methods. Similarly, these methods usually don't take into account the problematic of both taphonomy and postmortem events, which may alter the preservation of skeletal remains and so of the diagnostic bone elements concerned.

Age estimation, in fact, leans on the skeletal elements available for analysis; some bones are constitutionally more resilient than other to taphonomic insults, resulting in different levels of preservation (239).

If these methods have proved to be effective when dealing with individual ranging from subadults to mature adults, on the other hand age estimation is definitely more challenging on elderly individuals, whose age is often underestimated (214-215, 239).

This limit of the current aging techniques should not be underrated because it is plain for all to see that in the last decades life expectancy at birth in developed countries is steadily increasing, therefore the possibility for the forensic anthropologists to deal with the finding of human remains belonging to elderly individuals is raising (251).

As proof of that, the Special Commissioner for Missing Persons of Italian government reported that in Italy, the number of missing adults since January 1st 1974 to June 30st 2013 was 15385 of which 1595 over 65 years old (252). The development or the validation, to all intents and purposes, of age estimation methods capable to estimate the age with precision and accuracy even in elderly individuals is becoming so a problem to be solved urgently.

The present study aims to test the applicability and the reliability of the current age estimation methods on a contemporary population of skeletal remains (145 elderly individuals of known sex and age derived from Milan skeletal Collection) with the intent to verify their potential use in this specific issue.

7.1.1.3.MATERIALS AND METHODS

The study sample comprises 145 individuals (66 males and 79 females) of known sex and age at death from the "Milano" skeletal collection, which consists of more than 1300 skeletons. The skeletal remains has been exhumed 15 years after their inhumation in a coffin and they belong to individuals with a mean age of 73,3 years old (ranging between 28 and 103 years old). As shown in Graph.1, most of them are over 60 and the most representative age classes are 71-80 and 81-90 years old (62% of the sample).

The following methods were applied to each skeleton (depending on the availability of their relative skeletal traits) by one single experienced observer: the Suchey–Brooks (SB) method for the pubic symphysis (141), the Lovejoy method for the auricular surface of the ilium (142), the Işcan (IC) method for the fourth ribs (144-145), the Rougé-Maillart method for the acetabulum combined with the auricular surface (146) and Beauthier method for the obliteration of palatine sutures (143).

Since the Rougé-Maillart method has not been standardized yet, the data acquired from the study were considered separately. As indicated by the authors, the method provides seven categories of overall score corresponding to eight age groups and the probabilities of belonging to an age group depending on the overall score were calculated using the Bayesian method.

At the same time the applicability of each method was tested on the entire sample to assess how the 20 years burial in coffin affected the preservation of the various skeletal diagnostic portions.

The collected data were subsequently analyzed to evaluate, for each method, the percentage of correct identification of the age ranges in which the individual's actual age at death is included.

7.1.1.4.RESULTS

Table 1 shows the applicability of each method on the total sample. Overall, hip represents the trait more frequently survived to taphonomic insults. The palatine process and consequently its method based on the palatine suture obliteration was applicable in 26,2% of the sample; the Işcan method for fourth ribs was applicable in just 21% of the sample.

METHOD	% OF APPLICABILITY
Lovejoy	89,6%
Rougé-Maillart	81,3%
Suchey-Brooks	59,3%
Beauthier	26,2%
Iscan	22,7%

TABLE 7.1.1.1.: Percentage of applicability of each method based on the survival of their relative skeletal sites.

As regards the percentage of correct identification of the age ranges in which the individual's real age at death is included (Table 7.1.1.2.) the Lovejoy was the method with the highest success rate (81,5%) followed by Suchey-Brooks 2σ (68,6%) and Beauthier (66%). The methods that showed the least percentage of correct identification were Iscan (54,5%) and Suchey-Brooks 1σ (19,8%).

METHOD	% "SUCCESS RATE"
Lovejoy	81,5%
Suchey-Brooks 1σ	19,8%
Suchey-Brooks 2σ	68,6%
Beauthier	66%
Iscan	54,5%

TABLE 7.1.1.2:Percentage of correct identification of the age ranges in which the individual's age at death is included

As regards Rougé-Maillart method (146) for the acetabulum and the auricular surface, the collected data were processed through the Bayesian method to obtain the conditional probabilities of each age category, for each overall score category (Tables 7.1.1.3. and 7.1.1.4.).

Overall score category	Score	Number	Average (years)	Median (years)	Minimum (years)	Maximum (years)
I	7-10	0	/	/	/	/
II	11-14	1	28	28	28	28
III	15-18	0	/	/	/	/
IV	19-22	7	48,14	48	40	61
V	23-26	22	66,59	68,5	50	81
VI	27-30	67	80,27	80	67	97
VII	31-32	21	89	88	82	101

TABLE 7.1.1.3:Rougé-Maillart method: score category and related descriptive statistic.

7.1.1.5. DISCUSSION

The aim of this study was to assess whether the current methods of skeletal age estimation were satisfactory when dealing with elderly individuals.

The first result concerned the applicability of each method based on the survival of the skeletal traits for which they are developed. Every individuals were characterized by the same taphonomic profile therefore the collected data regarding the survival of the skeletal traits are of particular interest since they are representative of a contemporary cemeterial population. Each individual was buried in coffin in the same cemetery and exhumed after 20 years. In general, the skeletal remains appeared to be in good condition of preservation (Fig 7.1.1.1.), although some skeletal parts survived better than others (Fig. 7.1.1.2).

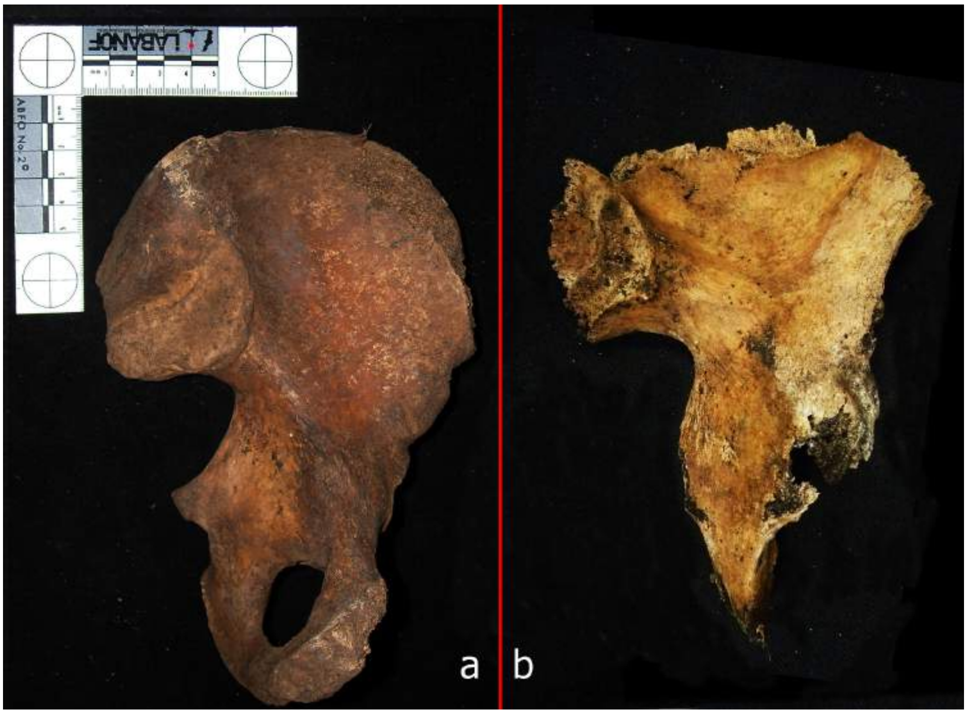


Figure 7.1.1.2.: Two os pubis showing different degree of preservation: (a) advanced degree of deterioration; (b) very good preserved.

In detail, the hip showed the better survival ensuring the possibility to study the auricular surface of the ilium in 89,6% of the cases and both the auricular surface and the acetabulum in 81,3% of the cases. Different responses to taphonomic insults regarded pubic symphysis, surviving in just 59,3% of the sample. Worse results concern the maxillary 26,2%) and the fourth rib (22,7%).

Although the importance of the fourth rib for the study of elderly individuals as suggested by Cunha et al. (215), it is evident that the strong deterioration of the ribs resulted in the impossibility of the application of Iscan for a percentage of 77% of the individuals.

As concerns the performance of each single method, when applicable, the situation is more complex. Assuming that 60% of the sample consists of individual between 70 and 90 years old, results showed in table 2 assume a different value. In fact, although with Lovejoy method it was possible to correctly assess an age range for 81,5% of the individuals, it has to be taken into account that the last phase of the method provide a generic age estimation of ">60 years". Similarly, Iscan method provides, with its last phase an age estimation corresponding to ">65" for males and ">70" for females.

Though this methods allow to successfully discriminate between over and under 60 years old individuals, this can represent a limit considering that life expectancy at birth is steadily increasing and therefore the possibility to deal with human remains of unknown individuals whose age at death is over 70 or 80 years old is raising considerably in the last decades.

As regards Suchey-Brooks, probably the most used aging method (253), the present study confirms what Baccino et al. (254) already pointed about its unreliability for individuals over 40 years of age. This method provides for each phase, two age intervals: 66 and 95% confidence. With the 66% confidence interval it was possible to provide correctly the age estimation in just 19,8% of the cases. This is due to the fact that the last phase give and age interval of 49-73 years for males and 48-72 for females. Referring to the 95% confidence interval the percentage raises to 68,6% but it provides large intervals (34-86 years for males, 42-87 years for females).

Concerning Beauthier method, the survival of palatine sutures in less than 30% of the cases made the method difficult to apply and the results regarding success rate pointed out its tendency to underestimate the age of individuals over 80 years of age. Nonetheless it proved to be useful with individuals ranging between 60 and 80 years of age. The main limit seems to be related to the last phase whose score is unlikely to be reached even by very elderly individuals.

Finally, Rougé-Maillart method, which combine the auricular surface of the ilium with the acetabulum, showed a good potential when dealing with individuals over 60 years of age.
With this method the authors stated that "it *is possible to categorize individuals over 60, and distinguishing individuals with a high probability of being over 70 as well as over 80*". Data obtained with this study confirmed this assumption.
In details, according to Table 7.1.1.4.:
-with an overall score of VII all the individuals were over 74 years of age, 85,7% were over 84;
-with an overall score of VI all the individuals were over 64 years of age;
-with an overall score of V 95,3% of the individuals had an age ranging between 55 and 84 years;
Since approximately 86% of individuals on which it was possible to apply the method were aged between 65 and 101, lower overall scores were not statistically relevant and therefore they have not been taken into account.

Probability	**OVERALL SCORE CATEGORY**						
Age groups (years)	I	II	III	IV	V	VI	VII
1 (15 - 24)	0,000	0,000	0,000	0,000	0,000	0,000	0,000
2 (25 - 34)	0,000	1,000	0,000	0,000	0,000	0,000	0,000
3 (35 - 44)	0,000	0,000	0,000	0,428	0,000	0,000	0,000
4 (45 - 54)	0,000	0,000	0,000	0,428	0,045	0,000	0,000
5 (55 - 64)	0,000	0,000	0,000	0,143	0,348	0,000	0,000
6 (65 - 74)	0,000	0,000	0,000	0,000	0,391	0,224	0,000
7 (75 - 84)	0,000	0,000	0,000	0,000	0,174	0,463	0,142
8 >84	0,000	0,000	0,000	0,000	0,000	0,313	0,857

Table 7.1.1.4: Rougé-Maillart method: probability of belonging to an age group according to the score category

Unlike the other discussed methods, Rougé-Maillart showed a greater suitability to discriminate between individuals over and under 84 years of age. Nonetheless, at present, it does not seem possible to provide a defined age range for each overall score since there is a considerable overlapping between the age classes (Table 7.1.1.4.). Further research may be useful to narrow down this issue.

7.1.1.6. CONCLUSION

Results of this study highlight two important points: firstly, the survival of diagnostic morphological traits in skeletal remains is the first concern that forensic anthropologists have to consider since taphonomy reveals to limit the application of the aging methods; secondly when it is possible to apply several aging methods to skeletal remains that have survived well to taphonomic factors, one has to take into account the chance that those skeletal remains belong to an elderly person and so results from the numerous aging methods could seem not satisfactory. Results indicate that the most common methods of skeletal age estimation are not satisfactory when dealing with elderly individuals. Some tend to underestimate old individuals (Suchey Brooks, Beauthier) (141, 143), some provide inaccurate age ranges (Lovejoy's ">60", Iscan's ">65" and ">70") (142-145). The method that showed the best potential was Rougé-Maillart (146) regarding the combination of the auricular surface of the ilium and the acetabulum. Since the method is not standardized yet further research is needed in order for it to be used for forensic purposes but nevertheless it allowed to successfully discriminate between over and under 74 and 84 years old individuals.

Tests on wider sample and a revaluation of the scores of each criteria on which the method has been developed may be useful to reduce the overlapping between the age classes provided.

All in all this method is a good starting-point in the study of skeletal age estimation of the elderly in accordance with the results indicating the auricular surface and the acetabulum as the areas more frequently surviving to taphonomic insults.

7.1.2.

The following study has been submitted recently to the

"*International Journal of Legal Medicine*"

THE COMPARATIVE PERFORMANCE OF PMI ESTIMATION IN SKELETAL REMAINS BY THREE METHODS (C-14, LUMINOL TEST AND OHI): ANALYSIS OF 20 CASES

A. Cappella, D. Gibelli, E. Muccino, V. Scarpulla, E. Cerutti, V. Caruso, E. Sguazza, D. Mazzarelli, C. Cattaneo

7.1.2.1 ABSTRACT

When estimating PMI (Post Mortem Interval) in forensic anthropology, the only method able to give an unambiguous result is the analysis of C14, although the procedure is expensive. Other methods, such as Luminol tests and histological analysis, can be performed as preliminary investigations, and may allow the operators to gain a preliminary indication concerning PMI, but they lack scientific verification, although luminol testing has been somewhat more accredited in the past few years. Such methods in fact may provide some help as they are inexpensive and can give a fast response, especially in the phase of preliminary investigations.

In this study, 20 court cases of human skeletonised remains were dated by the 14-C method. For two cases results were chronologically set after the 1950s; for one case, the analysis was not possible technically. The remaining 17 cases showed an archaeological or historical collocation. The same bone samples were also screened with histological examination and with the Luminol test. Results showed that only 4 cases gave a positivity to Luminol and a high OHI index at the same time: among these, 2 cases were dated as recent by the radiocarbon analysis. Thus only two false positive results were given by the combination of these methods and no false negatives.

Thus, the combination of two qualitative methods (Luminol test and microscopic analysis) may represent a promising solution to cases where many fragments need to be quickly tested.

7.1.2.2. INTRODUCTION

When skeletonised human remains or single bone elements are recovered in suspicious contexts, one of the first concerns is to distinguish whether or not the material is historical or recent, in which case it would merit juridical attention. Determining the post-mortem interval as accurately as possible can represent a challenge especially if the resources are limited.

Morphological observation is sometimes one of the criteria erroneously used in distinguishing if the remains date back decades or centuries; this alone may not be sufficient because characteristics like the gross aspect, odour and tissue preservation are usually subjected to numerous environmental factors (temperature, body size, location of the body, insect and animal activity) that have an influence on the decomposition process and in most cases allow for a correct PMI evaluation (255). Sometimes even skeletal remains that have always lain in earth sites can be affected by numerous environmental factors; in fact rapid decomposition can occur, determining a quick transformation which makes the remains look more antique than they actually are; on the contrary an optimal situation can preserve the remains very well for decades making their appearance deceptive. Thus, despite the simple distinction between recent with forensic relevance or historical can reveal many difficulties, forensic anthropologists are requested to express their considerations according to Daubert criteria, and so they have to substantiate their assertions with scientifically tested methods (99). The problems of scientific validation and applicability of some dating methods, and the need to give answers with regard to admissibility of scientific evidence in the court room have pressed both the anthropologist and pathologist to employ the most accurate and analytical methods consisting in those in which the PMI assessment is not affected by environmental variability (255, 256-257), and the results are consistently supported by scientific data.

Regardless of methodological limitations, PMI estimation usually begins with the macroscopic examination of skeletal remains followed by the microscopic examination of a bone section (188-189, 258-259); both have shown lack of any degree of confidence but have revealed some important information especially concerning changes of the histomorphological layout caused by microbial attack. The significance of the microscopic analysis, with regard to the degree of preservation of the tissue, can offer a preliminary assessment to those biomolecular tests which require the presence and the good preservation of biomolecules (genetics and isotope analysis).

Concerning the techniques that are used for the PMI estimation, several studies are reported in literature. Some of these studies speak of a comparison of multiple methodologies (259-265), which are otherwise singularly focused on in other studies, and that can be schematically divided in:

- Luminol methods (266-268)
- Radiocarbon methods (255,269-273)
- Other chemical and physical methods: macroscopic UV-fluorescence (274), chemical methods (256-257, 275-277) and the use of various radionuclides (278-282)

- Microscopic methods (258,283)

Table n.7.1.2.1. shows the most important articles concerning techniques for PMI estimation on bone.

When facing forensic cases, a set of problems arise regarding costs, time, possibility of using some methods with respect to others, the state of preservation and deterioration of the bones, the size and number of pieces, their provenance and so forth.

The aim of the present case report is to reveal the issues arising from the estimation of PMI in some forensic cases of retrieval of bones, whose provenance can be extremely different. In particular, this study shows how in the forensic field it is necessary to follow different steps depending on the characteristics of the case (provenance and state of preservation of human remains) and on resources available (cost and time).

Three methods were chosen for the evaluation of the PMI in the present cases: first, the radiocarbon analysis, because at the moment it is the most widely and conventional used dating technique to distinguish modern from non modern samples, also from a judicial point of view; then, the Luminol test, because it is a fast, inexpensive method; it is also easy to perform and its value has been attested in literature (265,267). Finally, an original attempt to assess bone microscopic alteration by histological techniques was made, in order to strengthen the information provided by the Luminol test concerning the "recent origin" of bones. The destruction of the osteological tissue is easily visible in thin sections and has been extensively investigated for a better understanding of the diagenetic process and preservation of the tissue in the archaeological field (258, 190-284); Hackett (189) in particular described four different types of microscopic focal destruction (MFD), referring to either tunnels or borings (a histomorphologic change determined by microbes).With the intent to better understand the extent of bioerosion in archaeological bones, Hedges (285) created the Oxford Histology Index (OHI) to evaluate histological destruction based on the estimation of the amount of unaltered bone and of that affected by microbial attack and bioerosion, ranging from a score 5 (< 5% of bone tissue is affected, and the morphohistology of the tissue appears identical to that of fresh bones) to a score of 0 (no original histological features visible).

Regarding the present study, the comparison of different methods (histological, biochemical and radio-chemical ones) applied to the same cases and of the same method applied to different cases can offer a broad point of view illustrating a set of problems as well as the assets of each method.

MULTIPLE METHODS AND "GUIDELINES"		
Berg, 1963	[260]	Various methods are mentioned. Optical morphological procedures are considered superior to chemical-physical methods.
Knight and Lauder, 1967	[262]	Nitrogen content, positive benzidine reaction and the presence of more than 7 amino-acids at chromatography can correlate with the PMI.
Knight and Lauder, 1969	[261]	Good correlation between time and: nitrogen content, amino-acids content and typology, UV-fluorescence, benzidine reaction, gel diffusion test.
Facchini and Pettener, 1977	[264]	Benzidine reaction, ultra-violet fluorescence, specific gravity and supersonic conductivity can mark a clear difference between samples of the three first centuries and the ones belonging to more ancient periods examined.
Castellano et al., 1984	[263]	Three regression equations are proposed to quantify correlation between PMI and the levels of proteins, triglycerides and cholesterol.
Yoshino et al., 1991	[259]	Microscopic findings on bone and UV-fluorescence have correlation with time since death.
Ramsthaler et al, 2011	[165]	A negative result (for Luminol) or a weak result (for UV-fluorescence) is more meaningful to exclude forensic relevance than a positive result (for Luminol) or a strong result (for UV-fluorescence) admitting forensic relevance.
CHEMILUMINESCENCE		
Introna et al., 1999	[266]	Possible relationship between the PMI and luminol chemiluminescence in powdered bone.
Ramsthaler et al., 2009	[267]	Luminol test is not valid as a single method, but it could help in PMI assessing.
Creamer and Buck, 2009	[268]	Historical bones produced a demonstrably weaker reaction than those of medico-legal interest.
RADIOCARBON		
Taylor et al., 1989	[255]	14-C method can be use for human bone samples for forensic purposes
Ubelaker et al., 2006	[270]	Bomb radiocarbon analysis may be useful to set bones and teeth on the pre-1963 or the post-1963 side of the modern bomb-curve.
Ubelaker and Parra, 2011	[272]	Trabecular bone radiocarbon values are closer to the tropospheric values at the date of death than the values from cortical bone.
UV-FLUORESCENCE		
Hoke et al., 2013	[274]	UV fluorescence alone is inappropriate to discriminate bones with forensic relevance.
CHEMICAL METHODS		
Cook, 1968	[257]	Chemical dating of bone has to be done on a statistical basis and considering the environmental soil conditions.
McLaughlin and Lednev, 2011	[275]	Great potential of Raman spectroscopy for estimating the burial duration of bone for forensic purposes.
Howes et al., 2012	[276]	The potentiality of infrared Spectroscopy to evaluate the modification associated with bone aging in different soil environments is stressed.
Patonai et al., 2013	[277]	Statistically significant changes in the Cristallinity index and in the Carbonate-Phosphate index measured by FTIR were observed between forensic and archaeological bones
RADIONUCLIDE		
MacLaughlin-Black et al, 1992	[278]	Usefulness of 90Sr to identify the moment of death in a period that is chronologically set after or before the 90Sr level peak, in the 1960s.
Swift, 1998	[279]	A proposal of measuring the levels of 210Po and 210Pb to evaluate the PMI. Limitations are diagenesis, time and cost.
Neis et al., 1999	[280]	Useful results of 90Sr to determine the PMI, but more investigations should be done to determine the cut-off year of 90Sr activity.
Swift et al., 2001	[281]	Correlation between various radionuclides content and the PMI. Larger studies should be done to confirm the results and to provide a calibration.
Schrag et al., 2012	[282]	The combined measure of 90Sr and 210Po increases the reliability of PMI value. Trabecular bone is subject to 90Sr and 210Po diagenesis
MICROSCOPIC METHODS		
Yoshino, 1991	[283]	Good estimations of the bone date by using microradiography, electron microscopy and UV-fluorescence by microscopic spectrophotometry.

Table 7.1.2.1: The table summarizes various articles concerning methods for PMI estimation on bone.

7.1.2.3.MATERIAL AND METHODS

A total of 20 cases of human skeletal remains are presented, for which the main problem was dating i.e. determining the forensic or historical relevance.

In all samples, the radiocarbon analysis was conducted as the starting point; thus the other analyses (Luminol test and histological analysis-OHI index) were performed in order to compare results and to observe problems and contributions that some popular methods can add in the forensic practice.

7.1.2.3.1. Cases

Radiocarbon tests, Luminol tests and histological analyses were conducted on different anthropological cases examined in the last 3 years. The casework consisted of skeletal findings found in different places (open air, buried, indoors) and in different taphonomic contexts (mixed remains inside metal boxes, complete buried skeletons, scattered remains, bones inside bags...). In general, the circumstances of the discovery was unusual, accidental or based on the deposition of witnesses. The characteristics of all cases (type of preservation, anatomical part and entirety of body, context and city of discovery) are summarized in Table n.7.1.2.2. and the macroscopic features of some are illustrated in Figure 7.1.2.1. and 7.1.2.2.

The difficulty of these cases consisted in the lack of any evidence such as artefacts (historical or modern such as clothes and personal objects), greasiness and odour which could not rule out forensic relevance. On the other hand, the presence of suspicious circumstances, together with the need to exclude or confirm a forensic interest with a certain probability, induced the specialists to approach the cases from a forensic point of view.

Figure 7.1.2.1: The macroscopic appearance of some examples of skulls (cases n. 2, 3, 4, 5, 16 and 18)

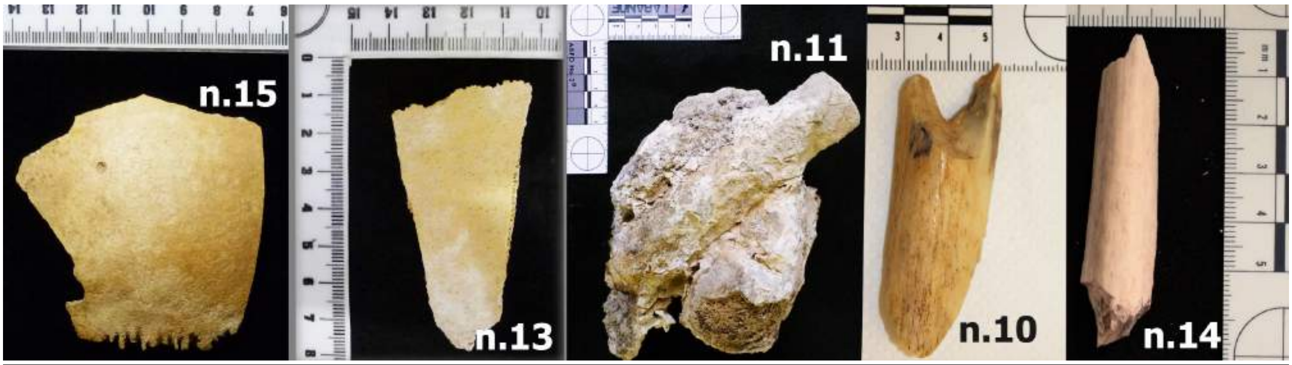


Figure 7.1.2.2: The macroscopic appearance of some examples of bone fragments (cases n. 10, 11, 13, 14 and 15)

7.1.2.3.2.Microscopical Observation

Undecalcified compact bones and/or cranial vault fragments (depending on which type of bone was available) were used for making cross-sections (with no fixation or dehydration). The undecalcified sections were prepared with a ground wheel to create a 150 μm thick section for light microscopic observation (Optical Microscope Leica, Leica Microsystems Wetzlar GmbH).

The histological analysis conducted on the thin section considered different features: presence or absence of tunneling and bioerosion referring the peripheral and mid-zone area or the entire tissue cross-section, type of MFD (classified in the four different types as reported in literature by Hackett (189)) when possible and Oxford Histological Index (OHI) described by Hedges (285) as the amount of bone not affected and that affected by tunnelling. In particular, for this study an OHI score of 4 and 5 was considered as “high”; an OHI score equal to or lower than 3 was considered “low”.

7.1.2.3.3. Luminol Test

The bone samples were pulverized and then a quantity of 50 mg was placed into an Eppendorf tube containing the Luminol solution (prepared following the protocol reported in literature and according to Weber (286)) where hydrogen peroxide was added). The entire protocol was performed in a dark room so that the chemiluminescence was observed in the best possible way. For each sample the intensity of chemiluminescence was assessed, as negative (-), weakly positive (+), positive (++) and strongly positive (+++), according to Ramsthaler et al. (265). Two positive controls (a diluted blood solution and powered bone from a femur with a PMI of 1 month) and a negative control (powered chalk) also were prepared and tested.

CASE WORK	*MATERIAL RECOVERED*	*CITY*	*CONTEXT and CIRCUMSTANCES*	*TYPE of PRESERVATION*
1	Almost complete skeleton (60%)	Piacenza	Scattered superficially in a wooded area	Outdoor (open air)
2	Cranium	Milano	In a bag left outside a church	Unknown
3	Cranium	Vicenza	Left superficially in a cave in the woods	Outdoor/water (open air)
4	Several bones	Locri	Interred in a crypt of a monastery	Outdoor (open air)
5	Complete skeleton (over 90%)	Lecco	Buried under the floor of a restaurant	Burial
6	Humerus	Milano	Found on the surface of a field	Outdoor (open air)
7	Complete skeleton (over 90%)	Milano	Scattered superficially on the sides of a carriageway	Outdoor (open air)
8	Several bone fragments	Francofonte	Buried bones	Burial
9	Complete skeleton (over 90%)	Teramo	Interred beneath the ground near a pigsty	Burial
10	Small diaphyseal fragment	Roma	In metal boxes containing several bone fragments and also soil in the crypt of a church	Burial/indoor
11	Femora embedded in mineral	Roma	In a crypt of a church	Indoor
12	Almost complete skeleton	Roma	Skeletonized in a wooden coffin situated in the crypt of a church	Burial
13	Fragmented Cranium	Roma	In metal boxes containing several bone fragments and also soil in a crypt of a church	Burial/indoor
14	Small fibular fragment	Roma	In metal boxes containing several bone fragments and also soil in a crypt of a church	Burial/Indoor
15	Cranium	Roma	In metal boxes containing several bone fragments and also soil in a crypt of a church	Burial/Indoor
16	Cranium	Roma	In metal boxes containing several bone fragments and also soil in a crypt of a church	Burial/Indoor
17	Mandibula	Roma	Buried in the ground of a church	Burial
18	Cranium	Roma	In a crypt	Indoor/water
19	Cranium	Roma	In a bag outside a church	Unknown
20	Fragmented long bones	Rimini	In a plastic box outside a restaurant	Burial/Indoor

Table 7.1.2.2.: The table contains information on cases and describes the characteristics regarding the type of material, provenance and context of the discovery and preservation.

7.1.2.3.4.Radiocarbon Analysis

The C14 examination of samples were performed in two different laboratories: CEDAD (Centro di Datazione e Diagnostica) of the University of Salento, in Italy; BETA ANALYTIC RADIOCARBON DATING LABORATORY in Florida. A small fragment (from 4 to 10 gr) was cut from each sample, and sent to one of these two laboratories where it was analysed using accelerator mass spectrometry (AMS) on collagen extracted from bone tissue; the procedures for pre-treatment and extraction of collagen for radiocarbon dating were performed according to the specific standard laboratory protocols for the analysis of bone samples (272, 287-291).

7.1.2.4. RESULTS

In Table n.7.1.2.3. all results concerning the 3 analyses conducted on all samples are summarized: the presence of tunneling and the respective OHI for the microscopical observation, the presence of chemiluminescence for the Luminol test and C14 analysis.

The radiocarbon analysis established a successful result for 19 cases out 20; in case 11 the analysis was not able to give any response. Only 2 cases (6 and 7) among the 19 cases resulted of forensic relevance because dated as post 1950; all the other cases were considered archaeological and historical samples.

The microscopic analysis (Figure 7.1.2.3. and 7.1.2.4. show a microscopic section of some cases) showed for only 9 among the 20 (cases 1, 3, 6, 7, 12, 13, 14, 17 and 19) with an intact or almost intact tissue and unperceivable focal destruction by microorganisms except for rare and isolated areas (with an OHI = 4 or 5): according to radiocarbon dating, only two cases (6 and 7) were of forensic interest dated post 1950 while for the other cases the 14C analysis pointed out a non-modern origin. On the other hand, a total of 8 cases (4,5,8,9,15,16,18 and 20) showed an OHI=0 which means a tissue completely degraded, where numerous small cavities were the only evidence of an original osteonic structure. The radiocarbon analysis clearly indicated that all these 9 samples are non-modern, with an age surely greater than 200 years (before 1800 A.D.) and of no forensic concern; in particular case 5, 8, 9, and 18 showed an age of about 1000 years or more by 14-C.

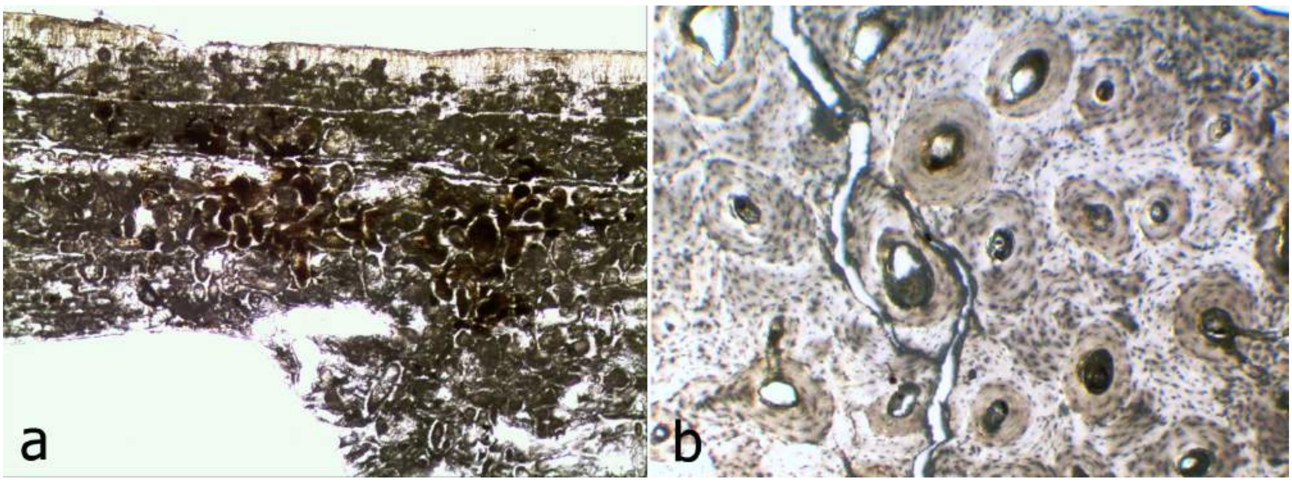


Figure 7.1.2.3.: An example of microscopic appearance of two samples dated by radiocarbon: while case 2 (historical according to the radiocarbon analysis) shows an OHI=2 with intensive destruction with tunnelling in the mid-zone and a good preservation in the external layer (peripheral area) case 6 (dated as post 1950 at C14 analysis) has an intact tissue with very rare focal destruction by microrganism and so shows an OHI=0.

CASES	*TYPE of BONE*	*TUNNELING*	*OHI*	*LUMINOLTEST*	*C14ANALYSIS*
CASE 1	Cortical	Peripheral	4	-	*1810-1870 a.D*
CASE 2	Cranial	In the mid-zone	2	-	*1800-1860 a.D.*
CASE 3	Cranial	In all tissue	4	-	*1850-1910 a.D.*
CASE 4	Cortical	In all tissue	0	-	*1760-1820 a.D.*
CASE 5	Cortical	In all tissue	0	-	*420-480 a.D.*
CASE 6	Cortical	Rare	5	+	*Post 1950*
CASE 7	Cortical	Rare	5	+	*Post 1950*
CASE 8	Cortical	In all tissue	0	+	*2350-2020 b.C.*
CASE 9	Cortical	In all tissue	0	-	*1020-1080 a.D.*
CASE 10	Cortical	In all tissue	2	-	*1840-1900 a.D.*
CASE 11	Cortical	In all tissue	2	+	*Not possible*
CASE 12	Cortical	In all tissue	4	-	*1760-1820 a.D.*
CASE 13	Cranial	In all tissue	4	++	*1690-1750 a.D.*
CASE 14	Cortical	Rare	5	++	*1740-1800 a.D.*
CASE 15	Cranial	In all tissue	0	+	*1500-1560 a.D.*
CASE 16	Cranial	In all tissue	0	+	*1600-1660 a.D.*
CASE 17	Mandible	Peripheral	4	-	*1770-1830 a.D.*
CASE 18	Cranial	In all tissue	0	++	*950-1010 a.D.*
CASE 19	Cranial	Peripheral	4	-	*Pre-1950*
CASE 20	Cortical	In all tissue	0	-	*1470 -1680 a.D*

Table 7.1.2.3.: The results of microscopic observations (OHI scoring), Luminol test (presence of positivity) and C14 analysis (estimated period) are summarized for each case.

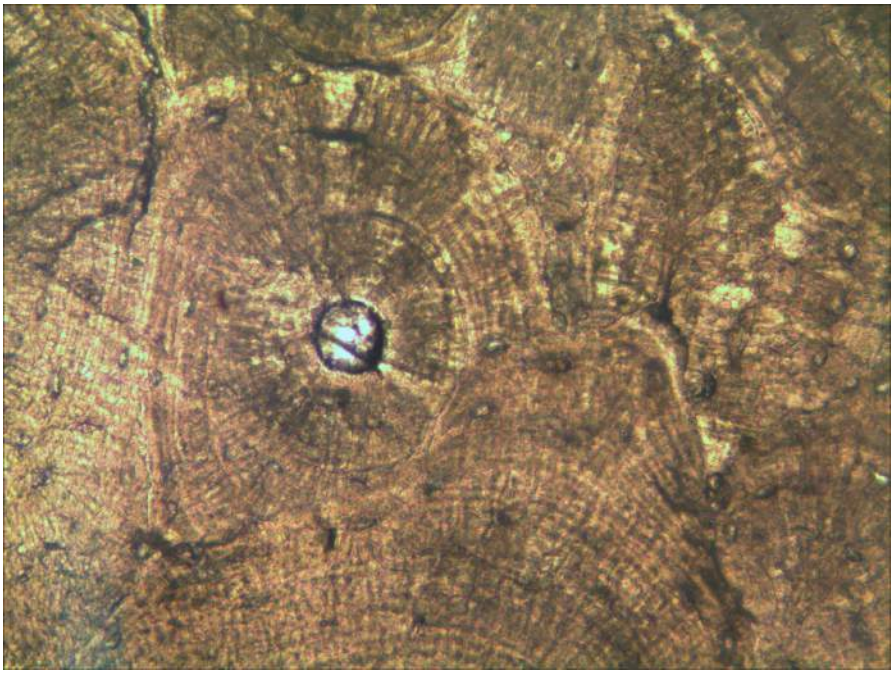


Figure 7.1.2.4.: Microscopic view of case 11, which still remains undated.

Unlike the cases cited above, other 3 cases (cases 2, 10 and 11) revealed some intermediate tissue characteristics with an OHI ranging between 2 and 4 in which the original tissue was still visible, but the destruction operated by the tunneling processes had already started, heavily altering the entire tissue; dating by 14-C, ranged between 1700 and 1900 A.D., and indicated a historical origin testifying a non-modern interest.

The only exception was case 11, that showed a partial destruction of tissue by tunneling and an OHI=2 but no results from the radiocarbon analysis since carbon could not be extracted.

The absolute absence of tunneling was not observed in any case, not even in those which resulted of forensic relevance. Additionally, the identification of tunneling was possible only in rare cases coinciding with those with a greater OHI and limited tunneling.

For what concerns the Luminol tests the positive control (blood sample) shows a very strong chemiluminscence (+++), while the negative control (chalk powder) developed no chemiluminescence. None of the samples demonstrated a chemiluminescence similar to the intensity developed by positive controls. However, 9 cases among 20 (6,7,8,11,13,14,15,16 and 18) showed a chemiluminescence of different intensity and perdurable; case 13, 14 and 18 resulted the most positive despite the radiocarbon analysis indicating a non-forensic interest (dated before 1800 a.D.). The only 2 cases considered as modern samples (cases 6 and 7) in the array at the 14C analysis produced a weak chemiluminescence as well as some archaeological samples (8, 11, 15 and 16). All the other cases (1,2,3,4,5,9,10,12,17,19 and 20) did not show any chemiluminescence proving in this way to be negative samples (with no hemoglobin content) as the chalk powder.

In addition, the positivity at the Luminol test was not limited only to the cases (6,7 and 14) that showed a limited alteration by tunneling and an elevated degree of OHI, but also to 4 cases (8,15,16,18) which resulted completely altered by this process with an OHI of 0 degrees.

7.1.2.5. DISCUSSION AND CONCLUSION

Determining the PMI in human skeletal remains has always represented a difficult challenge especially when rapid dating for possible forensic relevance is required in order to verify whether the remains are of forensic interest. Recognising forensic significance of a bone can require the application of expensive and time-consuming methods; the results given by some methods can sometimes not be validated in court because of their lack of assessment. In literature some studies [265-268, 283] have shown the possibility to easily screen potential modern bones in order to distinguish those which are historical from modern ones.

In this casework report a total of 20 court cases of human skeletal remains were studied. Regardless of costs and time, radiocarbon analysis (bomb curve) was performed. We however wished to comparatively test the efficiency and applicability of two popular tests, the Luminol test and histological analysis (OHI index).

The reasoning behind this choice is similar to the reasoning performed by Hoke et al. (292): in their study, the authors used UV-fluorescence and histological examination to screen which bones are well preserved and not altered by diagenetic processes so as to successfully perform biomolecular analysis (collagen and DNA). Thus, in a similar and experimental perspective, in the present study the combination of Luminol testing and microscopic observation was used to search for an alternative screening method for modern bone.

Among the 20 samples analyzed, only 2 proved to be of forensic interest and therefore subjected to further anthropological investigations aimed at identifying the individual and the events that occurred. Most cases (17 cases) were found to be archaeological or historical and therefore of non-judicial interest and only one case still remained unsolved from a point of view of the PMI, due to the type and deterioration of bone tissue (probably modified by taphonomic factors which had determined the loss and substitution of mineral constituents making the tissue similar to ancient fossils) that did not allow for determination of the carbon content.

Considering that among 20 samples only 10% was found to be recent, it is evident that a valid screening protocol would have been be useful and necessary to reduce the greater costs and long waiting times linked to C-14 testing.

The results obtained by testing with Luminol and OHI showed the difficulty in defining recent bone; cases dated as post 1950 by radiocarbon analysis (case 6 and 7) resulted weakly positive to the Luminol test and well preserved upon histological analysis with an OHI=5.

On the other hand, the samples determined as archaeological or historical by C14 analysis did not give univocal results. Some cases were characterized by negative results for both the Luminol and the microscopic analysis (cases number 4, 5, 9, 20) or, in alternative, by a discrepancy between the results given by the two types of tests: sometimes there was a strong positivity to the Luminol test (equal or higher than in recent bones), with an ancient histomorphologic pattern (partial or total invasion by tunneling, extensive deterioration of the structure, with OHI score equal or lower than 3), as in cases 2, 8, 10, 11, 15, 16 and 18; otherwise, the Luminol test was negative and the microscopic analysis revealed a high OHI score, as in cases 1, 3, 12, 17 and 19. Surprisingly, archaeological or historical cases did not always show ancient characteristics in at least one of the two techniques. In fact, modern features were detected upon both Luminol and microscopic observation in few cases, i.e. in the 2 forensic cases (6 and 7): cases 13 and 14 demonstrated a perfectly well preserved tissue, with minimal trace of microbial attack, an OHI of respectively 4/5 and 5 and a strong positivity at the Luminol test (++), simulating to all intents and purposes the appearance of modern bone. Probably, the false positivity in these 2 cases may be interpreted according to Ramsthaler et al. (265): the action of environmental and burial factors, together with diagenetic processes between the skeletal remains and the soil may give the chemiluminescence also in absence of haemoglobin.

In the same article of Ramsthaler et al (265) reported that about 30% of ancient bones (PMI>100 years) showed a positivity to Luminol testing. A slight increased percentage (41.1%, corresponding to 7 cases of ancient bones positive for Luminol, over a total of 17 ancient cases for radiocarbon analysis) was observed in the present study, considering only the Luminol screening. In this sense, the differences in rates of false positives with respect to previous studies may be due to diversity of study design, cohort grouping and definition of terms (such as what is "recent" and what is not). Analogous results are registered for the OHI score: 7 cases (41.1%) of ancient bone are characterized by a high OHI score. If the contribution of microscopic analysis and Luminol testing are combined, the percentage decreases (about 11.7%, corresponding to the 2 ancient cases which are positive for Luminol and show a high OHI score, with respect to a total of 17 cases proven ancient upon radiocarbon analysis), stressing the positive effect of the combination of the Luminol test and the microscopic analysis in the screening of bones. In fact, this is another focal point of the study: the combination of two qualitative methods can permit a decrease in the number of false positive cases. In view of a future practical application of this combined screening, the decrease of the number of false positive cases results in the decrease of the bone samples showing modern features and thus suitable for radiocarbon analysis, in a cost-effective and time-effective logic. These results are summarized in Table n.7.1.2.4. and n.7.1.2.5.

LUMINOL TEST ONLY		*OHI SCORE ONLY*	
(+)	(-)	(+)	(-)
→FALSE POSITIVE	→TRUE NEGATIVE	→FALSE POSITIVE	→TRUE NEGATIVE
7/17= **41.1%**	10/17=**58.9%**	7/17= **41.1%**	10/17=**58.9%**

Table 7.1.2.4:The results of Luminol testing performed on the ancient cases (17 cases): 7 cases (41.1%) showed a positivity to the test (corresponding to false-positive cases), while 10 cases (58.9%) resulted negative (true negative cases). Analogous results are shown for the use of the OHI score.

COMBINATION OF LUMINOL TEST AND OHI SCORE	
(+) and (+) →FALSE POSITIVE 2/17= **11.7%**	(+) and (-) (-) and (+)(-) and (-) → TRUE NEGATIVE 15/17=**88.3%**

Table 7.1.2.5: The results of combined Luminol tests and microscopic observation (through OHI score), again performed on the above mentioned ancient cases (17 cases). For what concerns the OHI, a score of 4 or 5 was considered a parameter of positivity, while a score equal or lower than 3 as negative. Only 2 cases (11.7%) showed a positivity to both the methods (resulting as false-positive cases), while 15 cases (88.3%) revealed a negative result in at least one of the two methods (true-negative cases). The luminol test in combination with microscopic observation decreased the percentage of the false-positive cases and indeed showed a greater potential in selecting few ancient bone samples.

Thus, the cases that showed positivity with the Luminol analysis and a high OHI score were 4 (cases 6,7,13 and 14): 2 of them were classified by 14-C analysis as forensic cases; the other 2 cases were dated by 14-C as historical. For one sample, the 14-C method was not able to give a result. Finally, for the remaining fifteen samples, there was a discrepancy between the Luminol and the histomorphological observation or, alternatively, both the analyses showed results consistent with ancient features: all these 15 samples were chronologically set before 1950 by the radiocarbon method, demonstrating that a negative result for at least one of the 2 qualitative methods can be considered as an interesting criterion for excluding a modern origin.

When facing the problem of PMI estimation in forensic anthropology, much attention should be paid to the presence of certain features of modernity in both the histological analyses (high OHI and lack of extensive tunneling) and the Luminol test (positivity for chemiluminescence, intensity and persistence): these features may be one of the criteria for selecting those bones on which to perform a further confirmatory and scientifically validated analysis, such as the radiocarbon test, which can definitely confirm or exclude a PMI of forensic relevance. On these bone elements it would make sense to perform tests such as radiocarbon with reference to the modern bomb-curve, which would eventually permit one to define their PMI with reference to 1963 (270).

The radiocarbon method still remains the "gold-standard" technique to estimate the post-mortem interval, though it is not always capable of giving a result. Yet, its expensive cost and its complexity (only few laboratories are technically equipped to perform this type of analysis) could take advantage of a preventive screening of the bones to be tested, in order to choose which elements are worth being tested. Concerning the costs of radiocarbon analysis, these are within a three-figure range and are much higher than those for luminol and microscopic techniques.

Therefore, there is a need for the realization of a screening protocol. The present study sets a valid starting point for this purpose: in particular, as previously mentioned, the combined detection of modern features with the Luminol test and microscopic observation seems to be promising: in fact this pattern of results may be effective in identifying suspected modern bones and, at the same time, in pointing out most of the archaeological ones. Furthermore, the implications on the overall costs could be very encouraging. Indeed, in the present study, only 4 cases were positive to the Luminol test and OHI score, with two true positive cases and only two false positive cases. Nevertheless, these results are based on a very small sample size (n=20) which represents a series of cases where only two samples were proven to be modern by radiocarbon. The sample size certainly limits their value, and for this reason the combination of Luminol testing and OHI score as a screening method that meets the criteria proposed by Daubert cannot be verified. However it seems an important combination of variables which, if further tested, may in the future be an interesting manner of sorting recent samples when the material is too numerous for envisaging C14 dating of all bones.

A screening protocol which includes both chemiluminescence and histological markers may be a very useful tool for judicial purposes, with beneficial effects on the overall costs and on the required time but it needs to be further investigated in order to reach an acceptable level of reliability.

7.1.3.

How valid is the Luminol test in detecting modern skeletal bones? A test on several bone tissue types: a caveat for PMI interpretation

7.1.3.1. ABSTRACT

Forensic pathologists and anthropologists often have to deal with human remains that come into a lab without any information about the burial context. Determining the postmortem interval (PMI) is a difficult challenge that can be faced, in a preliminary way, by conducting the luminol test. Many authors have already analysed luminol-based methods and, thanks to these studies, it is now known that the luminol alone is not a reliable way to determine the PMI of human skeletal remains. Nevertheless it continues to be used in labs as a rapid and cheap method useful in orienting technicians in further studies, such as isotope dating. During the study presented here, we analysed ten individuals, with a recent PMI, taking specimens from three different sites, for a total of sixty samples, in order to investigate differences in responsiveness in the same individual. Particularly we took samples from the cranial theca, from the midshaft of a long bone, and from the spongiosa of a lumbar vertebra. Sampling and analysis were repeated after two months by the same operators and it was possible to notice that, where a variation was registered, the three regions gave a different response. It was also observed that there was a trend in negative results from some of the samples that were positive in the first analysis.

7.1.3.2. INTRODUCTION

The determination of PMI of human skeletal remains is one of the most important challenge that an anthropologist has to face with. This analysis plays a key role in the forensic field since there will be further investigations only if the bones are proved to be modern (post 1950, but variable depending on the national law). It is clear that the main risk in the estimation of the PMI consists in denying to the skeletal remains the due consideration by judicial authorities. Many times PMI determination is very challenging if no supported by circumstances that can help in giving additional information, as for instance skeletal remains that are found in the open. At the same time the anthropologists are also well aware that other different conditions such as burial and deposition highly influence the bone decomposition, hence their macroscopic and microscopic appearance but also tissue components and biomolecules. (293). The PMI analysis is based on various criteria which are all considered as predictive of the origin of bones: morphologic and microscopic observation always need to be reinforced by chemical analysis whose results can assess the PMI with a greater scientific admissibility also in court. There are, for this purpose, several methods based on physico-chemical analysis aim in helping anthropologists in determining the time since death (260-262, 264). Among these many methods, the golden standard still remains the radiocarbon analysis whose scientific validation is demonstrated by the huge number of publication in literature (255, 269-273, 290). Among the chemical methods used in forensic field one of the most performed is Luminol test, which is based on luminol chemiluminescence reaction as a tool for the detection of haemoglobin and its derivatives, also in traces, in bones of medico-legal interest. In literature, several studies report the Luminol test as a promising technique albeit with some limitations, for the assessment of skeletal material that may be potentially of medico-legal interest (268). Its high sensitivity permits technicians to reveal blood and blood-stains even when they are no longer visible: in skeletal remains it has been demonstrated that even low haemoglobin concentrations (between 1:100000 to 1:5000000) can develop a detectable chemiluminescence reaction [8]. Unlike other analogous methods, the one based on luminol reaction produces as in response to it, a bluish-white glow, which is easy to read and interpret. As reported by Introna et al. (266), the CL's intensity increased with decreasing PMI: very modern bones showed a clear CL, while ancient samples characterized by a greater PMI show a slight CL or no CL in case of PMI older than 80 years. Despite the great capacity of Luminol in detecting even traces of blood in bones, the same authors have also reported a significant number of false negative results (33%), which may derive from modern samples. A recent study performed by Ramsthaler et al. (265, 267) has evaluated the Luminol method analytically (266). Interesting finding were observed consisting in the presence of luminescence also in historical samples with PMI greater than 80 years (7,5% of false positives) and the absence of a positive response to Luminol in recent sample, confirming the results showed by Introna. Nevertheless when the percentage of false negative are considered, some discrepancies are shown between the two studies: Ramsthaler reported a percentage about 15% of false negatives, less than that observed by Introna. The unequal rates of false positive and false negative results between the studies mentioned above may be- at least in part- due to different study designs, cohort grouping, and definition of terms (such as what is “recent” and what is not), a fact that should be considered in general, but that highlights an important aspect correlated with the evaluation of PMI in skeletal remains which is the little possibility to rely solely on the luminescence as a predictor of a PMI of forensic interest. An other important aspect is linked to the typology of bone considered by the study: only cortical tissue (from femur) has been investigated until now, but what about bone elements characterized by trabecular and cancellous tissue? This question leads also to an other important matter that has not been yet investigated which is the possible variability that may be highlighted if different bone elements from the same individual are considered when Luminol test is performed. Knowledge about the persistence of blood components or of blood traces in respect to bone tissue typology is an aspect that needs to be explored; Creamer et al. (268) have tested Luminol in different bone elements belonging to the same skeletal individual finding no variability between the elements; in this study only humerus and femurs belonging to the same individual were considered and no results about variability between trabecular, diploe and cortical tissue have been conducted yet.

In order to examine the validity of Luminol test in modern skeletal samples and to verify the possible differences in response to it, the authors have investigated Luminol results on different bone elements of several skeletal remains with a same PMI (20 years). The study has focused specifically on differences that may be highlighted when Luminol test is performed in various skeletal remains with a comparable taphonomic profile and PMI; in addition also several regions (skull, femur, vertebrae) were considered, in order to verify variability in responses. Indeed it could be interesting to observe how different types of bone of an individual can respond to luminol test. Finally, the analysis was conducted twice by the same operators, with also the intent to observe the repeatability of the method after two months. Perhaps this approach may reveal new considerations never noticed before, but able to make the Luminol test more reliable. However, it must be specified that this method is easily subjected to interference (294-295) and this is why it will be always only a preliminary method of relative value.

7.1.3.3. MATERIAL AND METHODS

The sample includes bones from 10 skeletons selected from the "Milan Skeletal Collection" housed at LABANOF (Laboratory of forensic anthropology and odontology, University of Milan). All skeletons are characterized by the same profile PMI and taphonomic conditions: the individuals were buried in 1991 in a wooden coffin and then exhumed after 15 years. One of the criteria in which the selection was based consisted in an objective acceptable, macroscopic preservation of bones and depending on this the skull, the femur and a lumbar vertebrae were chosen for a total of 30 bone elements.

The specimens (small fragments of 10x10 mm) were taken from the 30 selected bone elements using a multipurpose minitool (Dremel) and then powdered with a grinder. The sampling was repeated after 2 months for the second luminol test and the fragments in this case were removed from the site where the first sampling was performed. Both, the sampling and the test were repeated after two months by the same operators. At the end a total of 20 bone powder samples from each region were obtained and so the analysis was conducted on 60 samples (weight of 50mg) stored in an Eppendorf tube, of which 30 analysed at the first analysis, and the other 30 tested in the second routine. The Luminol solution (Sigma-Aldrich) was prepared according with Weber et al. (286) then the test was conducted in a darkened room. After luminol was adding to the bone powder, the tube was gently shaken for few seconds and the response immediately recorded. The intensity of the response was graded in five categories listed in Tab 7.1.3.1.

Values	*Meaning*
-	No Chemiluminescence
+	Very faint CL, barely visible on the naked eye but persistent
++	Clear but weak CL
+++	Clear CL
++++	Intense CL

Table 7.1.3.1.: Luminescence grades

7.1.3.4. RESULTS

As expected the tests gave back a prevalence of positive outcomes and about 26% negative results, between the first and the second analysis. Overall results are listed with percentage values in Tab 7.1.3.2. The negative results refer to all effect to false negative.

Values	*n. samples*	*%*
-	16	26.6
+	5	8.3
++	21	35
+++	9	15
++++	9	15
Tot.	60	100.00%

Table 7.1.3.2.: Overall results

It is interesting to notice that it is the second analysis that shows the most presence of negative results. Among the total percentage of false negative, the 25.0% belonged to the first routine while the 74.9% to the second. Total negative results are listed by region in Tab

7.1.3.3., and the negative outcomes in the first and in the second analysis concerning bone typology are listed respectively in Tabs 7.1.3.4. and 7.1.3.5.

Bone Type	*(-)*	*%(-)*	*%(Tot)*
Cranial	8	50.0	13.3
Diaphysis	3	18.7	5.0
Spongiosa	5	31.2	8.3
Tot.	16	100.0%	26.6%

Table 7.1.3.3: Negative results by bone region

Bone Type	*(-)*	*%(-)*	*%(Tot)*
Cranial	2	12.5	3.3
Diaphysis	-	-	-
Spongiosa	2	12.5	3.3
Tot.	4	25.0%	6.6%

Table 7.1.3.4: Negative results by bone region to the first analysis

Bone Type	*(-)*	*%(-)*	*%(Tot)*
Cranial	6	37.5	10.0
Diaphysis	3	18.7	5.0
Spongiosa	3	18.7	5.0
Tot.	12	74.9%	20.0%

Table 7.1.3.5: Negative results by bone region to the second analysis

Furthermore it is noticeable that during the first performance of the test no diaphysis have given negative results and among the two routines, it seemed that this bone region was the most reliable, giving back only 18.7% of total negative results out of the total negative outcomes, corresponding to the 5% of false negative in the total amount. Nevertheless other regions, particularly cranial, showed a stronger CL than those released by diaphysis during the first analysis as observable in Tab 7.1.3.6, even though its positivity was not confirmed by the second test conducted.

Despite some region gave a response that switched from positive to negative between the two analyses, no negative result became positive at the second examination. Moreover most of the samples that kept a positive value in the two observations, showed a trend in reducing intensity or a visible decrease in CL. Viewing all the samples as a total of 30 specimens examined twice, about 46.6% of the samples changed from a positive to a less positive or to a negative result.

7.1.3.5. DISCUSSION and CONCLUSION

The experience reported in this paper in general shows that Luminol test is not a reliable method in estimating correctly the PMI and in revealing the modern origin of skeletal remains. The high number of false negative (about 26%), little different from those reported in other publications (265-267) suggests that this method lacks of validity in reveal bones as recent: despite its very sensitiveness in detecting hemoglobin traces, the absence of such molecules in skeletal remains does not corresponds necessarily to an ancient origin. In fact, as demonstrated by this study, in some bones this component was not more noticeable, although the PMI was relatively restricted to 20 years.

An important finding that has also to be considered is that the bone district on which the luminol test is performed may affects both, reliability and repeatability of the response. The study has in fact highlighted that the skull is the bone element more unreliable in giving appropriate results, in other words it is a skeletal district to be avoided during sampling. On the contrary, long bones and vertebrae appear to be more reliable as bone in which performed such analysis. In addition, it is evident that the integrity of the bone at the site where the sampling is performed plays a key role in obtaining reliable results, and that the bone elements in which hemoglobin more persists is the vertebrae if compared with the others. In the light of these observations, it is desirable to adopt protocols based on the sampling of only the most integral parts of bone and as little as possible exposed to the outside environment like femur (as suggested in literature) (265-268) and vertebrae.

Results on the repeatability of the tests show that the second observation always confirms what was seen in the first only about the absence of chemiluminescence. On the contrary, some samples that showed chemiluminescence at the first assessment became negative in the second. The type of bone that showed more problems in the reproducibility of the test was once again the skull (40% of discrepancy between the results of the two analyses), followed by the femoral shaft (30%). The spongy tissue is instead revealed to be more reliable in the reproducibility of the test, confirming almost all the results obtained in the first test. These results highlight that there is loss of protein molecules (such hemoglobinic residues) in bone fragments that are damaged and exposed directly even for short periods.

Finally, differences about the intensity of chemiluminescence were observed: as a matter of fact the chemiluminescence seems to be variable when the bone tissue typologies are considered, but the tendency is to stay steady or decrease in intensity (in about 23% of the 60 samples) if the chemiluminescence is observable present also in the second observation as shown in Table 7.1.3.6.

In conclusion, the present study highlights the unreliability of Luminol test as a method in the evaluation of PMI: the high percentage of false negative results when the test is conducted on different skeletal regions does not guarantee a satisfactory evaluation for bones of modern origin. Indeed, if used as a single method, the risk of incurring in the absence of Hb content even if modern specimen, becomes too high with the more serious implication to exclude a medico-legal interest instead due. In this perspective, it appears clear that the distribution of Hb and its breakdown rate is not constant throughout the skeleton, but varies considerably depending on the tissue typology. If the diagenesis and the general environmental variables are usually indicated as major factors of possible complicating and of variability (268), from this study a fact very clear emerge and highlights some distinct considerations: the Hb content is different in similar skeletons characterized by the same taphonomic and diagenetic processes and so its variability could be due also from other factors non yet elucidated. In addition, it appears clear that the absence of Hb traces is not a criteria on which the distinction between historical and modern bones can be solely based on. Additional examinations are always necessary when the evaluation of PMI is required for skeletal materials, and luminol test, despite its widely use in forensic practice, has demonstrated too many limits in detecting modern skeletal remains; this test needs surely to be combined with other methods capable of detecting additional modern features for becoming a screening test in order to correctly distinguish between archaeological and forensic human skeletal remains.

Individual	Sample	Result	Sample	Result	Bone Typology Cranial	Diaphysis	Spongiosa
1	1	-	31	-	*		
	2	+++	32	+		*	
	3	-	33	-			*
2	4	-	34	-	*		
	5	++	35	++		*	
	6	-	36	-			*
3	7	++	37	++	*		
	8	++	38	++		*	
	9	++++	39	++++			*
4	10	++++	40	+	*		
	11	+++	41	+++		*	
	12	+++	42	++++			*
5	13	++++	43	+	*		
	14	++	44	-		*	
	15	++++	45	++			*
6	16	++	46	-	*		
	17	+++	47	-		*	
	18	++	48	+			*
7	19	++++	49	-	*		
	20	++	50	++		*	
	21	++	51	++			*
8	22	++	52	-	*		
	23	++	53	++		*	
	24	++	54	-			*
9	25	++++	55	++++	*		
	26	++	56	+++		*	
	27	+++	57	+++			*
10	28	++++	58	-	*		
	29	++	59	-		*	
	30	++	60	+			*
Tot.					20	20	20

Table 6: Samples list and results. Samples 31-60 correspond to the sampling conducted after two months and so is Luminol test.

Chapter 8

8.
DISCUSSION

Forensic anthropologists often deal with trauma analysis in order to discover evidence which can be correlated with cause and manner of death. Trauma analysis needs a thorough methodology; once, in fact, the timing of traumatic events is determined and classified according to literature as antemortem, perimortem or post-mortem (53-57, 97, 99, 108, 106, 296-297), some further specific details can help the investigation in understanding circumstances and all events which have occurred before and after death. However the great contribution that anthropology can give is often limited by the few validations of anthropological methodologies (based on descriptive approaches), and by the taphonomic processes which may affect the remains and lead to an inaccurate interpretation of trauma (297-298). Trauma analysis and taphonomy together represent complex issues for forensic anthropology, which have been taken into account as main subjects in this PhD dissertation. The project started with the study of a consistent skeletal sample from the "Milan skeletal collection", the first overall anthropological study of such a collection. The study of the skeletal samples in fact highlighted several problems which may arise when anthropologists deal with skeletal remains such as exhumed and buried skeletons. One of the most difficult problems proved to be the analysis of trauma, whose diagnosis is not only essential, but also problematic in such materials, especially when taphonomic effects limit the chance to interpret correctly the common morphologic parameters used by Forensic Anthropologists (FA). Recent applications have appeared satisfactory for interpreting correctly lesions found in skeletal remains, and so different approaches are needed.

The first two research lines in fact verified the efficiency of different methodologies used by FA in trauma analysis, while the third research line faced the more experimental subject of taphonomy of cellular components as a common response and possible aid for both the previous research lines. Knowledge acquired by this later research line could be helpful in building models necessary for the interpretation of antemortem lesions in which no osteogenic reactions are visible, as well as in the interpretation of vitality in perimortem lesions as a potential solution also in the distinction between peri- and postmortem lesions.

8.1.
FIRST RESEARCH LINE:
ANTEMORTEM TRAUMA ANALYSIS

8.1.1. INTRODUCTION

The first research line focused specifically on the diagnosis of antemortem trauma.

The high number of antemortem trauma found in the skeletal sample (described in Chapter 3), which was studied for the first time, stressed the importance of the analysis of antemortem trauma, in both healing or healed lesions. The importance of these lesions lies in the role which they could play in the personal identification of unknown skeletal remains, and in demonstrating evidence of violence, maltreatment and torture which the individual has been subjected to in his life (86, 93-94). One of the focal points in the interpretation of antemortem injuries consists of the estimation of time elapsed between the trauma producing the injury and the death of the individual (PTI) (93-94), which is the mean topic of this first study. The diagnosis of antemortem lesions revealed to be challenging and many problems arose from it.

In order to perform a research project focusing specifically and analytically on the diagnosis of antemortem trauma the first concern was to collect known skeletal material, which is not easily available. Since for calluses and antemortem fractures found in the skeletal collection there were actually no specific antemortem data concerning their time since trauma, this specific research line was conducted on several known bone calluses and antemortem fractures derived from both the autopsy series (collected during the Ph.D course according to mortuary regulations) and the forensic collection included in the Milan osteological and skeletal collection, housed at LABANOF; for all these samples data concerning time of survival of the individuals before death are known and therefore also the time of fractures and callus formation. The availability of known materials is an advantage which permits us to verify the validity and limits of methodologies and techniques in evaluating the time of a lesion since trauma; at the same time, the material also permitted us to analyse the practical problems arising from the diagnosis of antemortem lesions. This approach appears rather novel and innovative for several aspects: despite the many studies which have investigated the healing process time for its application in dating fractures (63, 68, 79, 88-89, 91-92, 160, 299), at the moment very few studies investigated the same issue directly on dry bones (84-86, 93-94) and they are often based on single cases (99, 86, 159) or samples without specific antemortem information (93-94).

A total of 24 known antemortem fractures with different time periods since trauma (with and without evident callus formation) were therefore collected and used for conducting the two researches, each of which tested the usefulness and limits of several techniques in evaluating antemortem lesions, and both elucidated what specific diagnostic problems affect such a procedure and need to be solved. In both studies a detailed analysis was conducted by applying different methods in order to test the morphologic criteria (84, 86) and the radiological classification (63, 79) commonly used in determining the time since trauma of an antemortem lesion. The first study also evaluated the aid which can derive from cone-beam CT application (a new advanced technology) in the investigation of the inner structure of fractures and calluses; this technique has never been tested before in the evaluation of antemortem fractures but its potential is well known to the scientific community, and also in forensic sciences (68, 85-89, 157, 160). In the second study the research was extended by applying the radiological analysis together with the histological examination, to several known antemortem lesions in dry/macerated bones in order to test the dating method recently proposed by De Boer et al (93), thus providing analytic and critical information concerning a possible additional application in this topic. Before reaching the conclusions of this research line some considerations on results obtained from both the studies should be made.

8.1.1.1. PART I: APPLICATION OF NEW TECHNOLOGIES FOR DATING BONE CALLUSES

The first study included in the first research line showed important results concerning the use of the new advanced technologies such as cone-beam CT (CBCT) in detecting features of bone remodelling, otherwise not visible by standard conventional radiology. The innovative aspect in this study is represented by the application of a new technology (cone-beam CT), whose application has rapidly developed in these years in the forensic field, and its usefulness for several purposes in forensic anthropology had been already highlighted (300-304). Nevertheless, it has never been tested in order to evaluate bone calluses for assessing their time since trauma. The features that cone-beam may detect in the inner structure of a callus (as proven in part I of Chapter 4) could be very important for a correct evaluation of the Post Trauma Interval, namely the time elapsed between trauma and death. However, in dry bone some limits in dating antemortem lesions still remain, especially when the earliest stages of healing or old healed lesions are considered. If in the earliest stages of fracture healing, when the presence of initial callus formation is macroscopically and radiologically (by DR) undetectable, CBCT seemed to bring no significant advantages and alternative techniques have to be considered as the histological analysis proposed by Maat et al. (86). On the other hand, CBCT provided much more information concerning the different stages of mineralization of specific areas of the callus, in particular if the latter one is well formed. In addition, CBCT also proved to be capable of investigating other possible useful features: the different disposition of bone and cartilage inside the fracture gap, the sequence of new bone formation, the inner and outer structures, and the characteristics of bone remodelling after the callus has been formed and strengthened. For instance, all the characteristics mentioned above are related to the inner structure of the callus which are detected well by CBCT, but not by the DR (standard radiology). The interpretation of such characteristics can help in assessing the age of the fracture more accurately than the other radiological characteristics. Thus, the degree of mineralization could be considered as a key element in the future, especially for lesions in which the callus is already well formed: the clearer features of the structures or the pattern of the fracture line can provide an easier definition of the stages of bone healing by creating a novel more detailed classification of bone healing.

This study pointed out the difficulties still present in this specific topic; although the presence of a bone callus is a common feature in a modern population, as demonstrated by results from the study of the skeletal sample reported in Chapter 3 (a frequency of 37% of individual with at least one antemortem lesion), the diagnosis of antemortem lesions still proves to be very challenging and not always possible for what concerns their time since trauma. The many radiological classifications reported in literature (63, 79) seemed not to be so accurate in dry bone when lesions at the earliest stages are considered, even if advanced technologies are employed. This was also proven by Steyn et al. (94) in a recent case report where the application of advanced technologies, such as micro-CT scanning, was unable to identify some healing features only assessable with light microscopy. For what concerns well-formed bone calluses this advanced technique demonstrated a great potentiality, and to be more useful in observing additional features not visible by DR. Thus, although the study has also demonstrated that the level of bone callus mineralization is well assessed by CBCT, this specific feature has not yet been considered in the radiological classification as a criterion for dating calluses and thus up to now it cannot allow us to reach a better interpretation. Further investigations are needed in order to provide useful key elements, such as the level of callus mineralization for healed lesions, as well as microscopic features (visible by histology) and biomarkers (detectable with immunoistochemestry or biomolecular techniques) for the interpretation of early healing fractures, a field where radiology, either DR and CBCT, does not provide any additional information for the posttraumatic survival time.

8.1.1.2. PART II: APPLICATION OF HISTOLOGY AND RADIOLOGY ON KNOWN BONE CALLUSES

The results from the first study highlighted the greater contribution that cone-beam CT can give for the final purpose of a clear and defined view of bone fractures and calluses. Nevertheless some pitfalls in the diagnosis of antemortem lesions still remain and along with the need to find a solution, in particular for dating early healing fractures. An alternative methodology that can aid in the interpretation of antemortem data with respect to PTI is the histological analysis. This has never been performed until now in human known antemortem lesions with the intent to verify its validity and helpfulness in dating fractures in FA; some recent studies (93-94) propose the use of a method based on the timetable suggested by Matt (86), which is the result of a careful review of all data reported in literature concerning this issue (amply explained in Chapter 1). But the suggested method has not yet been validated and tested on known material and only unknown archaeological/historical samples were examined, without proving its effective reliability. For all these reasons, this second research was conducted with the intent to verify the potentiality of histology together with radiology (DR) in detecting microscopic features that could be more informative and accurate in the interpretation of posttraumatic survival time. Both, histological and radiological analyses were performed on several known calluses (as samples used in the first study, the material derived from the autopsy series as well as forensic collection) of different age and of different typology of bone tissue (rib and skull).

From this study some interesting facts emerged: some osteogenic signs can be observed only microscopically according to literature (93-94, 157) and they allow us to narrow down the post trauma interval obtained from a macroscopic and radiological observation, which would be otherwise too inaccurate. This fact can improve the deficiency of cone-beam, or other advanced radiological techniques, in detecting features at the earliest stages of healing, as revealed by the previous study. However, defining the age of very young antemortem lesions, whose posttraumatic survival time ranges between few hours to 10 days, is certainly a difficult task and its realization is currently not achievable; perhaps by using immunohistochemical and molecular techniques some biomarkers could be detected and may help in giving additional information also for young lesions such as haemorrhaging traces that were noticed with immunohistochemistry in a preliminary study reported by Cattaneo et al.

(130). However this latter chance is still remote, unless there will be a specific investigation on the taphonomy of such biomarkers which aims at verifying the effective survival of such components in dry bone and decomposing samples. In parallel, in healed lesions (the older ones), both the radiological and histological analyses showed the inability to provide more precise information about the time elapsed between the trauma and death, a more diagnostic problem already shown by the study reported above (paragraph 8.1.1.1). as a consequences calluses of different ages, whose age differs even of years, could present a very similar aspect and the resultant PTI assigned will be the same. For more accuracy, the creation of a novel classification based on the degree of mineralization in the inner structure of the callus is needed, which can be tested through the use of methodologies able to quantify its rate, such as the dual-energy X-ray absorptiometry (DXA). Unless the discipline does not investigate this specific topic, the determination of age in healed lesions will be rather inaccurate, providing only imprecise information. A little digression should also be done for what concerns the variability of the healing process, which depends on the characteristics of the individual (such as the age and the health status), but also on both the type of fracture and bone tissue typology (74, 77, 81-82). These are all aspects that should be considered and investigated more in detail; this second study, in fact, shows that both the type of wound and bone typology on which the fracture is produced determine differences in the healing response and in the assessments of the PTI, that sometimes may be discordant also for contemporary calluses (157). In other words, the healing of a rib fracture can differ greatly from the healing of a surgical skull injury, especially if the latter determined the removal of bone fragments, thus causing a reparative behaviour much more similar to that resulting from an amputation conducted on long bones, a fact that was hypothesized also by Steyn (94). As a consequence evaluating the PTI in antemortem cranial injuries deriving from surgery will require different parameters from those used in clinical settings for the follow-up of the healing, which are based solely on radiological characteristics established on healing processes of long bones (63, 79, 68). As concerns the PTl assessment resulting from the histological analysis, cranial surgical lesions require more specific parameters than those described in the literature (86, 93), and perhaps more similar to those described as specific for lesions from amputations (158-159). Similarly, the type of bone tissue involved in the healing process can determine some variation on the time trend of the healing: contemporary fractures occurring in different types of bone tissue may require different timings for starting healing and/or for becoming healed. For instance, the rib bone tissue could reveal a healing time trend that varies greatly in comparison with that proposed in the literature for long bone (cortical bone tissue), being the latter substantially based on the observation of healing on long bones in human and animal models; this is another aspect that needs to be verified. Therefore it seems clear that there is a need for testing methods and techniques used in this research in a larger and more varied known sample, not only according to the time of the lesion, but also the bone tissue type considered (spongy bone, long bone, trabecular and cancellous bone).

8.1.2 CONCLUSIONS OF THE FIRST RESEARCH LINE

A focal point in the interpretation of antemortem injuries consists in the estimation of time elapsed between the trauma which has produced the injury and the death of the individual (PTI), an issue which has been more and more investigated in the last years (68, 85-86, 93-94, 160). A detailed analysis was conducted in order to evaluating the criteria used in determining the age of an antemortem lesion by applying different methods. Both radiological (standard and advanced) and histological techniques permit us to highlight a good potential in the presentation of greatly informative details. However, in dry bone samples, the diagnosis of PTI seems to be not entirely satisfactory. This is due in part to the difficulty of interpreting details little investigated, such as the levels of mineralization within the bone callus (in case of a healed lesion), or never investigated, such as the presence of mediators, cells and molecular products (collagen, metalloproteinases, etc) which are involved in the very early stages of the healing process (72, 74, 77, 80-81) as widely described in paragraph 1.3.1 and 1.3.3 of Chapter 3; in other words, no research has been conducted for investigating markers specific of bone tissue healing, a part from rare examples(130).

The work presented and conducted for this research line showed that for healed calluses or healing lesions which are rather young, in which there are no visible signs of bone reactions macro- or microscopically, the evaluation of an accurate PTI is extremely difficult and requires additional parameters, different from those used in anthropology, in radiology and in clinical assessment. Actually, the lesions in which no osteogenic reactions are found, neither radiologically nor microscopically, are lesions which will be diagnosed as perimortem without adding any information about the posttraumatic survival time. The problem with assessing these lesions is that they are not unequivocally antemortem lesions; at least some different indicators should be considered such as biomarkers. Molecular methods and immunohistochemistry for instance may be good alternative solutions, which could implement the search for specific biomarkers as indicators of the earlier stages of healing or eventually for vital reaction as addressed in some research conducted on skin lesions (119, 121, 123-129) and macerated bone fractures (130). In these terms, the search for components related with the earliest stages of healing together with the presence of a hematoma and blood extravasation, as well as of the cascade of inflammatory mediators, if performable even on skeletal remains, would demonstrate the vitality and survival of a lesion, whose diagnosis, in anthropology, is currently limited to the mere distinction of a lesion which occurred close to death, e.g. perimortem, based solely on the lack of indicators or osteogenic reactions. The latter one proved to be a common problem in the diagnosis of both antemortem and perimortem lesions and in these perspectives, it would be interesting to investigate the usefulness of these biomarkers, if they persist in dry bone. However at the moment no data about their detectability and persistence in dry bones or skeletal remains are available, and research in this specific experimental field still needs to be performed.

In conclusion, the studies conducted on a limited number of known antemortem fractures and healed/healing lesions demonstrated the crucial developments in anthropology in the last decades, thanks to the application of advanced methodologies and techniques. Despite good capacities in supporting a diagnostic assessment of the lesions, no longer limited to the mere distinction between healed and healing lesions, the issue of dating antemortem injuries still reveals a considerable degree of inaccuracy, especially for what concerns healed lesions (where the callus is well formed) or lesions in which the bone healing reactions cannot yet be observed macro or microscopically, an issue which overlaps with the problems deriving from the interpretation of vitality and of survival time of the perimortem lesions, which has been thoroughly investigated in the second research line (Chapter 5).

8.2
SECOND RESEARCH LINE
PERIMORTEM TRAUMA ANALYSIS

8.2.1. INTRODUCTION

The second research line focused specifically on the diagnosis of perimortem trauma and on the difficulty of distinguishing between perimortem and postmortem lesions. The study of over 200 skeletons from the Milan skeletal collection (described in Chapter 3) permitted us to underline which diagnostic problems are behind the trauma analysis in exhumed and buried skeletal remains, simulating the practical approaches that forensic anthropologists perform in case of investigations conducted in skeletal remains of legal interest. Being the first study ever conducted on this important reference skeletal collection, through the study of the skeletal samples it was possible to highlight some interesting complications regarding the diagnosis of perimortem lesions in skeletons belonging to individuals who died of traumatic death, whose correct interpretation is often limited by the appearance of taphonomic transformations and new post-mortem fractures accordingly with literature reported in paragraph 1.3.2. of Chapter 1. After having observed some problems concerning the diagnosis of perimortem lesions, among all the cases of the skeletal sample studied, some cases of traumatic death were selected for conducting this second research line (Chapter 5). Thanks to the selection of the most interesting cases of traumatic death, and to the collection of autopsy reports related to the same cases that have functioned as their control data, thus determining the known origin for all the lesions present, the second research line of this Ph.D project was carried out in order to investigate the limits and the problems related to the interpretation of perimortem trauma in buried skeletal remains with an approach never experimented before.

In trauma analysis the only tool anthropologists can adopt for the diagnosis of a lesion is the lesion pattern based solely on macro-morphologic parameters (98, 102-103, 106, 108-109), which however proved to be not fully satisfactory (97, 99, 103, 106-107) as demonstrated also by results deriving from the two studies concerning this specific topic (Chapter 5). In this case, the chance to compare autopsy data with those extrapolated by anthropological analysis permitted us to be critical on what has been in use until now, which are the aspects of research which need to be renewed. In these perspectives, the two studies highlighted important considerations concerning what happens to a bone lesion with the passing of time, which has a huge relevance for the analysis of trauma and in the consequent determination of

cause and modality of death, if the correlation between the trauma and the cause of death is achievable. The availability of control data, such as autopsy reports, provided the information regarding the errors which lie behind the diagnosis in trauma analysis and offered a concrete awareness concerning the limits: a singular event, since it is a rare investgation performed on human bones of known origin (102-103). Most studies have been in fact performed on animal models and archaeological bones (98, 105, 111-112, 117) and rarely there is a chance in FA to test methodologies in known materials derived from real conditions, as in this research.
The first study, in fact, allowed us to compare autopsy and anthropological findings focusing mainly on the investigation of the potentiality of anthropology in performing a correct trauma analysis on skeletal remains inhumed for 15 years. Thus, the investigation paid particular attention to the verification of : i) if and how the lesions correlated with death survive in time in burial conditions, and ii) if their anthropological analysis can lead to a correct interpretation of the trauma. The second study faced most specifically the diagnostic problems arising from the first study, most of which concern the diagnosis of fractures produced by Blunt Force Trauma (BFT), namely their distinction from post-mortem fractures according to what is reported in literature (97, 99, 106).
The two studies conducted in this research line are taken into account separately before approaching the general conclusion of this second research area.

8.2.1.1 PART I: THE INTERPRETATION OF PERIMORTEM LESIONS IN SKELETAL REMAINS AFTER 20 YEARS FROM INHUMATION

The first study of this second research line focused on the analysis of lesions correlated with the modality and cause of death. A traumatic event on bones is commonly classified as a result of sharp force, gunshot or blunt force (53, 55-56, 114, 296). Each of these forces generates unique skeletal signs that are usually readily identifiable in unmodified remains. But perimortem lesions, usually recognized by a green bone response which occurs when the bone is still elastic, i.e. fresh bone (97-98, 102-103, 105-106, 114,), are often difficult to identify, since the exposure to time and taphonomic factors can significantly blur evidence. The difficulty in verifying the presence of perimortem lesions has never been tested on inhumed skeletal remains where lesions correlated with death are known since reported at autopsy. Such characteristics gave the chance to test the criteria described in literature (reported in paragraph 1.3.2.2. of Chapter 1) on such known skeletal materials, and to investigate analytically this topic. Buried skeletal elements, in fact, can typically exhibit fragmentation and fracturing limiting the interpretation of perimortem trauma, as proven by research conducted for determining specifically how bone changes over time, and consequently the fracture morphology (97-98, 102-103, 106). Nevertheless, no investigations were ever performed which could allow us to make a comparison between autopsy examination and anthropological analysis on the same individual, thus checking for incongruity and/or changes.

This study focused on the diagnostic problems deriving from the analysis of perimortem lesions found in 7 skeletal remains selected from the skeletal sample. The comparison between autopsy and anthropological data conducted in this valuable little sample highlighted the several diagnostic difficulties in interpreting perimortem lesions; in particular the study called attention to the chance that an original bone lesion correlated with the cause of death can be modified by time and taphonomic factors thus becoming perhaps detectable but no longer identifiable as perimortem, according to Calce *et al.* (107). Although the anthropological analysis proved to be fundamental in acquiring crucial data in the interpretation of modality and cause of death, some problem related to the diagnosis of lesions were underlined. Particularly, if on one hand stab or cut marks and gunshot injuries are quickly and easily identifiable even after almost 15 years of inhumation and do not seem to change significantly regardless of taphonomy, on the other hand the same cannot be said for lesions derived from blunt injury, which appeared to be in many cases highly modified by time and taphonomic factors, making it often impossible to identify them correctly. The results showed in fact the impossibility sometime to determine whether a lesion is perimortem or postmortem since often some perimortem fractures acquire postmortem characteristics due to the multitude of taphonomic variables that can remove important perimortem indicators. The final result could be a complete acquisition of postmortem features, which totally modify the original appearance and do not permit one to diagnose the lesion as being perimortem and eventually correlated with death. The results faced the specific issues in diagnosing fracture deriving from BFT as peri or postmortem, a problem that was then elaborated more specifically in the second study whose results are discussed in the next paragraph.

This study proved the extreme relevance of skeletal populations with known cause of death and autopsy reports, for a comparative assessment between data from the well preserved cadaver and from the skeleton years later, which may shed light on which aspects of bone trauma interpretation are more at risk or are best preserved in human remains.

8.2.1.2 PART II: DISTINCTION BETWEEN PERI AND POSTMORTEM LESIONS IN FRACTURES OF KNOWN ORIGIN

Much information can still be obtained from the analysis of buried skeletal remains which have undergone decades of taphonomic insults, particularly as concerns sharp force and gunshot trauma, although blunt force trauma interpretation may be severely hindered by postmortem factors. The difficulties in the diagnosis of perimortem lesions, as the results derived from the first study have emphasised, are due mostly to the superimposition of taphonomic damages and to the occurrence of new post-mortem fractures to the pre-existent lesions correlated with the cause and modality of death, and thus the most problematic interpretation correspondS to the diagnosis of fractures derived from BFT. These are the motivations for conducting this second part, which focused the interest on the analysis of fractures of known origin with the intent to show limits of macro-morphological parameters used in FA, specifically in the distinction between peri and post-mortem fractures.

The implication of assessing wrongly a fracture is a risk that anthropology must avoid since if perimortem lesions are not diagnosed correctly, due to their misidentification through the common morphologic criteria, the resulting lack of evidence of perimortem trauma will not permit the interpretation of lethal events and traumas. For this reason the second study was fundamental in verifying what was found in the first study; the research was conducted on a large sample of fractures (210) of known origin (both peri- and post-mortem), which were selected from the skeletal sample derived from the cemeterial skeletal collection, with the intent to verify the validity of morphologic parameters - widely accepted in the scientific community – in the evaluation of perimortem and postmortem lesions in buried/inhumed skeletal remains.

Since the evaluation of a fracture usually relies only upon its macro-morphological pattern (96, 98, 102-103; 105-107, 114, 296), and up to now no new criteria have been found or investigated in order to solve this issue, the present study considered solely such parameters at this point of the research. However the study clearly displayed the limits of such an approach: there is, in fact, a higher complexity in the identification of perimortem fractures when compared with postmortem ones since the common morphological features of “fresh” fractures (which occurred in a bone with tissue proprieties similar to those belonging to the living bone tissue) (97, 101, 103, 106) are severely altered by taphonomy, which in turn causes changes, making the original fracture unrecognizable or imperceptible (107, 305). The final result is that a pre-existent fracture linked to a lethal event will become more similar to a post-mortem fracture, and at the end will be erroneously assessed as highlighted by result for the previous study (paragraph 5.1.2.). This fact was amply demonstrated also by the high number of mistakes that the operators made in the assessment of the 210 fractures: the errors regarded both peri and post-mortem lesions, but most of mistakes concern mainly trabecular bones, in which the evaluation is barely possible since no parameters exist for trabecular and spongy bones. In literature only parameters based on characteristics observed in long bone are available as criteria for distinguished between peri- and post-mortem (98, 102, 105).

Results remarked how some of the well-known criteria for the distinction between peri- or post-mortem, particularly on trabecular bones but even on long bones, are not so reliable on buried skeletal remains because of the taphonomic alterations. The relative unreliability of the morphological parameters pointed out by this study leads us to conclude that for the correct anthropological assessment of a fracture found in inhumed skeletal remains there is the necessity to find more reliable criteria able to provide a more valuable help, and to solve problems and limits. Perhaps, some more detailed experimental approaches might be carried out in order to verify the possibility to search for more specific indicators with respect to the morphological characteristics: for instance biomarkers, unlike the morphological parameters, could give information concerning the presence of a vital reaction like bleeding and blood extravasation; the latter one could be one of the solutions in the diagnosis of lesions that show ambiguous morphological characteristics. If signs of haemorrhage or haemorrhagic infiltration are detectable even on dry bones or skeletal injury, these will permit a more accurate diagnosis of perimortality by acquiring also the more valuable meaning of vitality of a perimortem lesion. This represents a common focal point with the previous research line: vitality or posttraumatic survival time in skeletal lesions are characterized both by the absence of any osteological signs micro-, macro-, and radiologically observable, and in every case the only definition that FA can give eventually is "perimortality". It appears clear as future researches in this specific field are essential in order to provide further criteria able to give a diagnosis of perimortality with a certain degree of precision and accuracy greater with respect to the current anthropological approaches tested in this second research line.

8.2.2 CONCLUSION OF THE SECOND RESEARCH LINE

The presence of skeletons belonging to individuals who have died by known traumatic and violent death (whose lesions were fully described at autopsy prior to the 15 years of inhumation) and whose taphonomic profile is known (in terms of PMI and modality of deposition) allowed us to conduct this second research line, innovative for several aspects noted in the previous paragraphs.

Both the studies included in this research line were performed thanks to the availability of known materials/skeletons deriving from the cemeterial skeletal sample from the collection described in Chapter 3. The results demonstrated that anthropology has a crucial role in identifying trauma that has occurred close to death, even after 20 years since inhumation (as remarked by the first study). In inhumed skeletal remains, even if uncompleted or deteriorated by taphonomy, it is still possible to find important signs of trauma, which are essential clues for the interpretation of cause and modality of death. The results in fact showed the great contribution that anthropology can give in trauma analysis, despite the many new postmortem fractures, taphonomic erosions and transformations that are disclosed in skeletons who were in such taphonomic environmental conditions for a long time. Nevertheless, some critical points arose from this research: if the signs derived from a gunshot injury as well as marks/cuts from sharp force trauma - occurring in the skeletal apparatus - appear still somewhat well definite regardless of the passing of time together with the acquisition of some little taphonomic changes, on the other hand the signs from BFT are not quite clear and survive well only if long bones are involved (sometimes not even in such elements). Such types of signs are not always assessed correctly, even if the presence of additional fractures (due to the same lethal event) located in other skeletal districts has allowed us to interpret appropriately the typology of trauma and so the correlated cause of death. This means that results derived from the analysis of BFT cases highlighted the problem of interpreting ambiguous fractures, especially those occurring in spongy and flat bones, in which all the parameters described in literature (96, 98, 102-103, 108-109, 296) and used by FA for the distinction between peri- and postmortem seem to fail. Starting from results of the first study, which in part confirm what has been reported in literature for cemeterial skeletal remains (102-103) the research line then focused in detail on the problem of interpreting the fractures in a consistent number of lesions of known origin from the known inhumed skeletal remains from the Collection. Two important aspects were observed in this second study: the modification of pre-existing perimortem lesions as a result of the taphonomic effects as highlighted also by previous research conducted in animal bones (105, 107), and the emergence of new lesions of dubious interpretation during the decomposition/skeletonization process (296-297). In fact taphonomic effects can ruin the pre-existent fracture pattern once the fracture is exposed to the environment after decomposition; at this point the fracture characteristics described as typical of fractures occurring in fresh bone - especially for lesions concerning the easily perishable bones with a very thin cortical surface (as ribs, pelvis, vertebrae processes, epiphyses) - are altered, a fact which renders the lesion no longer detectable as a consequence of a perimortem injury. Thus, the presence of intermediate characteristics in some fractures, that means some typical characteristics of fresh bones (perimortem fractures) and other distinctive of dry bones (post-mortem fractures), determine an ambiguous appearance, hence the impossibility to assess the origin of that fracture (297). As a consequence, the multiple features considered for distinguishing between post-mortem and perimortem fractures are reliable only when the assessment is performed on long bones –if possible- disclosing a clear fracture pattern of easy interpretation. Since the parameters commonly used in anthropology are essentially based on morphological characteristics of dubious utility in some circumstances, it appears clear that there is a need for relying on different criteria like biological markers, which could be a successful answer. In fact, when lesions are correctly assessed as perimortem, there is a problem which still remains unsolved: which lesions can be related to the cause of death (in case of traumatic one) and what lesions are differently related only to the bone proprieties depending on the high moisture content of the bone tissue instead of being correlated with death. This point could be solved only through the search for haemorrhagic traces and blood infiltration which, if found, will give great help not just in the distinction of the fracture as perimortem, but also in the interpretation of its vitality and perhaps of its correlation with a traumatic event related with the cause and modality of death. As a matter of fact, the trauma analysis must distinguish the lesions correlated with death from those considered as perimortem only because they occurred in a sort of "fresh" bone in which the moisture content is still high. Since the distinction of perimortem lesions from postmortem fractures seems to be inaccurate as the results have demonstrated, the use of additional indicators like biological markers could be fundamental.

This is the most focal point: if some signs of vitality are found in lesions which seem perimortem, these could be used in the correct diagnosis of the lesion as perimortem, and in its correlation to a traumatic event that occurred just prior to death (perhaps with a high significance for the interpretation of the cause of death), or, in case of absence, in the exclusion that the fracture is implicated in the death, although its characteristics are typical of fresh bone, similarly to existing studies on cutaneous lesions in forensic pathology (80, 123-129). One may hope to reach similar results also in skeletal lesion and this may open new perspectives and novel research fields in FA, to which we may apply knowledge and techniques derived from biology and science.

The search for biomarkers indicating vital reaction in perimortem lesions is an ambitious goal and could solve many diagnostic problems in the interpretation of perimortem lesions in trauma analysis on skeletal remains. This is still a field not yet investigated on dry bone, and before approaching it in this term it is essential to understand the taphonomy of blood in general before to clarify its role in the diagnosis of perimortem lesions and its vitality.

8.3.
THIRD RESEARCH LINE:
TAPHONOMY OF BLOOD

8.3.1. INTRODUCTION

This third research line represents the more typically experimental research conducted in this Ph.D project in order to solve the diagnostic problems stressed by the first and second research lines: all results obtained from the previous examinations in fact highlighted through testing the current methodologies adopted by FA, such as the diagnosis of both antemortem and perimortem trauma, how these are problematic and require new more accurate methodologies, able to overcome the limits amply reported in the previous paragraphs (8.1 and 8.2).

The use of biomarkers, as mentioned briefly, might be a possible aid in the diagnosis of both antemortem and perimortem lesions, whose diagnostic problems sometimes are even overlapping. In fact, either lethal perimortem lesions or lesions surviving for a few hours/days do not exhibit any signs of osteogenic reactions, (macro/microscopically and radiologically), and currently there are no chances or tools to ascertain vitality and survival in a lesion. Both immune/histochemical and biomolecular techniques are useful applications of biology to the search for biomarkers and proteins and their identification; such an experimental approach has never been performed in detail on human skeletal remains or applied as a support in the diagnosis of skeletal lesions. Only a pilot study in literature focused on the usefulness of some histological markers for the dating of traumatic lesions, but the experiment was conducted on fresh macerated bone and only few preliminary results were obtained, without any confirmation by further studies (130). But the great contribution which may derive from such applications is still unknown, thus currently no knowledge is available at this regard. The detection of specific biomarkers such as the blood components and the inflammatory mediators/proteins with advance techniques could give information concerning vital reaction in a lesion, first and foremost in terms of timing of survival: haemorrhage, blood extravasation and infiltration, haematoma formation and haemostatic clots are all signs which could help the distinction of a perimortem from a post-mortem one, especially with reference to the concept of vitality. The earliest healing signals, as those involved in the reactive phase of healing after the occurrence of a fracture, may call attention to the importance of their implication in the diagnosis of antemortem lesions by indicating more precisely the survival time of the lesion, as well as the search for the bleeding signs. In addition of course the diagnosis of both antemortem and perimortem lesions, whose limits have been analysed in the first two research lines (respectively Chapter 4 and 5), are two topics overlapping in such a perspective and both could benefit from a more accurate interpretation, in which finally the term of "perimortem lesion/fracture" could be considered more precisely as a vital lesions which has not survived or survived for little time, analogously to the "*modus operandi*" of forensic pathology for cutaneous lesions (80, 123-129). Although many studies in literature have clarified the haemorrhage phases, the coagulation cascade and the healing process, from a physiological and timing point of view (as reported respectively in paragraph 1.3.3 and 1.3.1.), none has never exploited and verified such knowledge in bone lesions of decomposed corpses and skeletal remains. Regardless of the meaning of these biomarkers in skeletal lesions, an experimental study such as this requires an understanding of their decomposition process in bone tissue first, in order to prove if such components are still detectable in skeletal remains. Taphonomy in these perspectives is a field which consists essentially in providing information in order that cell and molecular components can be used as biomarkers of vitality and survival in skeletal lesions, but currently no research has ever been conducted on the subject of micro-taphonomy or taphonomy of biological markers. This is the reason behind the choice to conduct an experimental project finalized at comprehending taphonomy of blood, and at the acquisition of basic information on this topic. Clearly taphonomy of all components correlated with vital and healing reactions needs to be explained, but since the many problems especially arose from the diagnosis of perimortem lesions, this research represents only a preliminary study which will be extended also to other blood and inflammatory components in the future. This research in fact was limited only to the observation of how blood cells, namely erythrocytes, decompose in general as isolated cells and in specific as cells within the bone tissue, a step necessary before approaching future research on the detection of such components in skeletal lesions as potential markers of vital haemorrhage.

This third research line therefore focused on some aspects of taphonomy of blood, whose understanding was possible thanks to two different studies (Chapter 6); the first concerned the decomposition of red blood cells that were monitored as isolated cells at different times of decomposition by the use of a technique like the Scanning Electron Microscopy (SEM) in order to discover and identify all possible morphological changes acquired by erythrocytes during decomposition, and thus to facilitate their correct identification, avoiding possible mistakes that may derive from the presence of rather similar botanical structures (Paragraph

6.1.1.). The second study observed changes of blood components at light microscopy, namely within the decomposing bone tissue monitored at several times of decomposition, but also in putrefied cadavers and skeletal remains, as well as in some simulated taphonomic conditions (paragraph 6.1.2.); the aim of this second study was the detailed observation of changes and persistence of these blood cell components over time and in many sham and real decomposition conditions by using histological and immunoistochemical analysis, in order to provide knowledge concerning how these cell components decompose in normal conditions, but also on their chances of being detected even in skeletal remains, regardless of the presence or absence of a fracture at this stage, a factor that will need to be investigated in future research.

Before approaching a general conclusion, some considerations on the two studies carried out in this research lines are due.

8.3.1.1 PART I: THE DECOMPOSTION OF RED BLOOD CELLS

This first experimental study included in the third research line of the Ph.D project showed important finding regarding the decomposition of red blood cells and the potential towards their recognition with respect to botanical structures that may mimic their presence in debris. This first experimental study included in the third research line of the Ph.D project showed important findings regarding the decomposition of red blood cells and the potentiality of their recognition, if compared with botanical structures (like spores and pollen) that might mimic their presence in the decomposition debris. A study like this represents the starting point for clarifying the blood taphonomy: the investigations in such a field require several preliminary steps to prove that the use of biomarkers can be useful in skeletal lesions as indicators of vitality and survival. The first step, in fact, is to verify if RBC are identifiable when decomposing (at least by scanning electron microscopy). This study provided data about the transformations, concerning morphology and sizes that erythrocytes gain during their decomposition. In addition, important information was gained also concerning the persistence of erythrocytes as definite cells: in fact it was demonstrated that, even if red blood cells were morphologically transformed, they were observable in blood left to decompose up to 6 days, after which only debris was observable. Once discovered all the erythrocytic changes in relation with the decomposition process and their persistence, as was done in this study, one has all data that will help in the identification of these cell components whenever present in the debris of decomposed and skeletal materials.

Since some literature presumes that the detection of erythrocyte-like cells on skeletal remains (even ancient) as surely erythrocytes (163, 168, 209-210) and most have never taken into consideration the chance of an origin different from blood (167), such as botanical, the research also considered the possible confusion between erythrocytes and botanical cells by applying SEM analysis in order to highlight the differences and similarity between both.

The results showed that although there are diagnostic features useful in identifying red blood cells from botanical structures, some spores resulted very similar to decaying red blood cells, which calls for attention and great caution when their presence is assumed in skeletal remains and in particular in skeletal lesions.

In addition, in spite of scanning electron microscopy revealing the variations concerning erythrocytes when decomposing and the possibility to distinguish erythrocytes, even when degraded, from other structures, more focal points arose from this research which still needed to be analysed, like - what happens to these cells into the decomposing bone tissue and how can they be visualised through light microscopy?, -if these cells are still present and how in skeletal remains, and finally -if erythrocytes no longer recognizable by morphology because of the deterioration, could be still detectable by using more sensitive techniques like immunoistochemistry. The need to find answers to the questions stressed above pushed the research towards a more complex level, which is the investigation on the decomposition of blood components within the decomposing bone tissue, a subject faced in the second study of this third research line.

8.3.1.2 PART II: THE DECOMPOSITION OF BLOOD COMPONENTS IN DECOMPOSING BONE TISSUE

Blood taphonomy is a matter that has never been studied before, and so, as highlighted in the first study, many are the questions and the aspects around this topic. The first study showed only some partial results about decomposition of red blood cells, but also other aspects not yet investigated (stressed in the conclusion of the previous paragraph), need to be considered and so were examined in this second research (Paragraph 6.1.2.). Once one has verified the morphological changes and the persistence of erythrocytes in the decomposing blood, the next step required an investigation on the decomposition and persistence of blood cell components within the bone tissue in fresh cadavers, in decomposed bodies and in skeletonised remains.

The study aimed to evaluate the influence of time and taphonomy on survival and persistence of blood components in bone tissue (parietal bone fragments collected from cadavers at different PMI) during the decomposition process, and thus the presence of red blood cells (RBC) in every bone tissue portion (within Haversian and Volkmann canals, canals localized in bone trabeculae and marrow spaces) was examined using histological (Hematoxylin-Eosin, H&E) and immunohistochemical (Glycophorin A, GlycoA). The choice to perform also the immunoistochemical analysis is due to its great sensitivity that has been already demonstrated in some macerated bone by a recent study where the authors needed to investigate the potentiality of this technique in highlighting presence of haemorrhaging (130). In addition, it being that erythrocytes cells deteriorate morphologically after 7 days, according to what is reported in the previous studies, this study requires also the application of techniques more specific than histology. The results from this study showed that well-defined erythrocytes can be seen only in the first week of decomposition (confirming results from the previous study), while after a week, these structures can be confirmed with certainty only using GlycoA immunohistochemical staining, while with histological analysis they can be defined only as an eosinophilic non specific accumulation. The immunohistochemical investigation for GlycoA, in fact, showed the presence of red blood cells under the form of erythrocyte debris or fragments unidentifiable from the morphological and structural point of view using only H&E staining, providing interesting results on the capacity of detecting such components in decomposed and skeletonized material. The sensitivity of immunoistochemical analysis proved to be useful and essential in verifying the presence of blood residues otherwise not detectable by the histologic/microscopic observation. In fact, even if the morphological alterations are now well know thanks to results from the first study of this research line (paragraph 6.1.1.), the second study has shown the problems in identifying these cells within the bone tissue already after a week since death, and also highlighted the impossibility of recognizing their presence only on the basis of morphological features. The study of how blood decomposes within a bone tissue appears to be fundamental for the acquisition of knowledge which could be applicable to some important issues: first, the detection of a vital reaction within a wound, and then the persistence of blood components in the bone tissue might be related to changes in the properties of the bone tissue of a "fresh" bone that is becoming "dry", i.e. in the explanation of the biodynamic bone models widely used in the diagnosis of perimortem and postmortem lesions, which have demonstrated sometimes to fail as reported in the second study of the second research line (Chapter 5). Another important result concerns the detection of discrete quantities of red blood cells even in dry bone with a PMI of 15 years which opens the way to the possible use of RBCs in trauma interpretation even in skeletal remains. This means that since this study has demonstrated that RBC are still detectable in skeletal remains with a PMI of 15 years by using sensitive techniques, even the presence of markers that indicate signs of bleeding, of haematoma formation or of a platelet clot, not yet specifically studied, could be perceivable; if such components are detectable in skeletal lesions also longer after death -in a rather advanced decomposition process – this will ensure a diagnosis of lesions far more accurate, and surely more effective than the diagnosis relied solely on morphological parameters, e.g. the common definition of "perimortem lesion".

8.3.2 CONCLUSION TO THE THIRD RESEARCH LINE

The results from the first two research lines highlighted the numerous problems concerning trauma analysis in skeletal remains. Since morphological criteria and the practical methodologies currently used by FA in the diagnosis of both, antemortem and perimortem trauma were tested in known skeletal materials from the Milan osteological and skeletal collection, and proved to be non satisfactory for interpreting correctly and adequately traumatic signs in inhumed skeletal remains, alternative approaches are mandatory. A scientific and valid aid could be provided by the use of biomarkers and cell components which might demonstrate the vitality and survival in a skeletal lesion, a common problem that overlaps in between perimortem and antemortem lesions. However such approach needs first to investigate the taphonomy of biological components in the decomposition process, namely their persistence and detectability in skeletal remains. Actually, no knowledge is available concerning taphonomy of biological markers such as blood components, and so the Ph.D project moved its research towards this specific field, in order to start a general comprehension of this subject, essential for a future use of biomarkers in the diagnosis of trauma in skeletal remains.

The experimental research directed on the taphonomy of blood components, corresponding to this third line, demonstrated surprising and promising results which open the way to possible usefulness of biomarkers in the interpretation of trauma in skeletal remains.

This third research line has permitted us to collect some very helpful information about taphonomy of blood components and has remarked the possibility to detect blood residues even in skeletal remains with PMI of 15 years (derived from the cemeterial skeletal collection) through the use of sensitive techniques such as immunohistochemistry. These results will be helpful if applied in further research that will focus on differences that may arise in the examination of skeletal perimortem and post-mortem fractures: the presence of such cell components in a perimortem lesion may mean that there has been a vital reaction, and so the trauma occurred when the individual was still alive and not already dead. The existence of such cell components in skeletal lesions, depending on the origin of the fracture, which means whether it occurred in life or after death, can have a key role in the interpretation of trauma and could improve the trauma analysis in skeletal remains.

Of course this research is still preliminary, nevertheless it shows very promising results that need to be extended at the most complex levels that have not yet been developed: for instance the detection of blood markers linked with certainty to a vital reactions resulting from a trauma occurred in life. Future studies will be aimed at tracing biomarkers in vital bone lesions also when considering decomposing bodies, in order to verify the potential in interpreting the vitality in lesions found also in skeletal remains.

It appears clear how taphonomic aspects of the bone tissue components can provide important clues in the interpretation of presence/absence of important biomarkers for vitality (119, 124, 130); knowledge of such components normally present within a vital wound conducts to a more certain diagnosis in terms of vitality and survival (124-130), but also the understanding of how and when these components normally decompose, are all aspects that need to be learnt in order to provide explanations for the classic issues still unsolved.

In conclusion, this research is not enough for clarifying all the outstanding issues, but provided important preliminary information on the process of decomposition of blood elements, specifically erythrocytes, within the bone tissue under normal decomposing conditions. ; the research in fact has not yet considered differences of their detectability due to extrinsic factors such as the presence of a vital lesion. Only starting from good basic knowledge of blood component taphonomy and of taphonomic models related to the decomposing bone tissue at different stages it will be possible to find out how to read trauma in bones.

8.4
DISCUSSION AND CONCLUSION

This thesis was developed on several study levels whose complexity is schematised and summarized at the beginning, in the "Thesis Chart" section. The project began with the anthropological study of a consistent part of the "Milan skeletal collection" for the first time ever, a step which allowed us to have an idea on the type of skeletal material in several respects (conservation degree, taphonomy profile, demography, presence of trauma and pathology), but also to analyse the problems that the discipline faces during the analysis of skeletal remains.

The anthropological analysis of the study sample has encountered major problems in the analysis of trauma, both for the antemortem diagnosis of lesions and the interpretation of injury perimortem. The main problem related to the diagnosis of antemortem lesions lies in the impossibility to provide an accurate estimate of the time elapsed between trauma and death of the individual, a fundamental aspect for personal identification of the individual to whom skeletal remains belongi to, and thus for the collection and information on traumatic events in life suffered by the subject (fundamental implication in cases of torture, maltreatment and child abuse). As evidence of the importance of a similar diagnosis, there is the large number of bone calluses (37%) found in the skeletal sample analyzed (described in Chapter 3), which -being a contemporary and local population- could represent well also the probability of finding similar lesions in skeletal remains of medico-legal interest.

In contrast, the main problem concerning the interpretation of perimortem injury lies in the difficulty of detecting the presence of similar lesions in conditions such as those belonging to buried remains, which are subject to numerous taphonomic effects and events post-mortem/ depositional. In this scenario, the appearance of taphonomic transformations as well as of new postmortem fractures makes it difficult to individualise pre-existing injuries related to mortal traumatic events, as shown by the analysis of the concerned skeletal sample together with the substantial number of postmortem lesions found in it (described in Chapter 3). But the diagnosis of perimortem lesions requires a high degree of certainty, especially for lesions considered ambiguous, where there is the necessity to find the presence of alternative indicators that may allow for a more specific diagnosis.

In this scenario, given the difficulties which emerged in the study of 200 skeletal individuals included in the study sample (Chapter 3), the PhD project was then developed on two specific researches, one concerning the diagnostic approach to antemortem injuries, which is the first research line (Chapter 4), the other focused on the diagnostic approach to perimortem lesions, e.g. the second line of research (Chapter 5). Both studies were extended because of the presence of known material on which to test and apply the diagnostic methods used in FA. The analysis of antemortem injury was conducted on known calluses and fractures obtained from the "Milan osteological and skeletal collection" (specifically from autopsy and forensic series); diversely, the analysis of perimortem lesions was performed on skeletal material - equipped of control autopsy information - selected from the study sample of the skeletal cemetery collection.

From the two observational studies (Chapter 4 and 5), one implemented by the use of techniques and technologies (such as radiology and histology applied to the analysis of antemortem trauma, Chapter 4), the other limited by the application of observational criteria (such as the macro morphological parameters tested for the analysis of perimortem trauma, Chapter 5), some important questions (addressed in the previous paragraph: 8.1. and 8.2.) emerged, which require alternative solutions with respect to the methodologies currently in use by FA. The PhD project also highlighted the premises of a solution, consisting in the possible use of bio-molecular methods, widely used also by science and forensic pathology. The premise of conducting experimental research on biomarkers may prove successful also on skeletal lesions, similarly to what is performed by forensic pathology in the interpretation of skin lesions. In this regard, the last year of PhD course intended to investigate more specifically this aspect: the use of biomarkers for the interpretation of trauma in skeletal remains. Detecting biomarkers as specific indicators of vitality and survival to an injury could enable us to solve the common problems encountered in the interpretation of both antemortem and perimortem injury due in particular to the absence of perceptible bone signs, macro-, micro- and radiographically. Ultimately, the detection of biomarkers would lead to a more precise diagnosis of antemortem lesions surviving trauma also a very short time, which are currently defined only as perimortem, considering the absence of bone remodelling, which do not confer it an antemortem origin. But also the diagnosis of perimortem injuries could benefit of such an application: their distinction from postmortem lesions is often difficult, sometimes impossible, unless the meaning of vitality is considered, which could assume a more reliable key value in this perspective with respect to the morphological patterns of lesions commonly used in FA. Given the lack of scientific research in the use of cellular biomarkers as indicators of vitality and survival time in skeletal lesions, the Ph.D. project focused finally on the taphonomy of blood (Chapter 6). Basic research in this area is

fundamental; at the moment no data is available on the persistence and traceability of cellular components in skeletal remains, such as the blood cell components.
This experimental research of this thesis (presented in chapter 6) was focused on the observation and understanding of how blood components decompose and deteriorate in cadavers and in skeletonized remains. Only passing through a basic experimental approach on taphonomy such as this, in the future it will be possible to conduct applied research in which to employ the molecular methodologies suitable for the search of biomarkers as a key to the interpretation of trauma in skeletal remains. This third research line - absolutely novel - has provided important information on blood taphonomy and opens the way to further research on the use of cell biomarkers also for the diagnosis of lesions in skeletal remains, a fundamental implication in forensic anthropology.

In conclusion, the PhD project highlights the importance of having known reference osteological and skeletal collections, through which to conduct a valuable scientific research and construct theoretical models, some still absent. Similar collections represent an invaluable source for applied scientific research, not only for their potential in validation and verification of methods and techniques used currently in forensic anthropology, but also for the evaluation of important issues still unsolved. Both, applied and experimental scientific research, are fundamental for the acquisition of knowledge and for the improvement of best theoretical models to follow. Also in forensic anthropology research and multidisciplinarity have proven to be essential as highlighted by these projects (Chapter 7). In this global overview, taphonomy seems the main protagonist of many of the problems encountered, of which both the effects and the implications have to be inevitably comprehended.
Finally, taphonomy plays a key role in FA, revealing itself as the first antagonist not only in the interpretation of trauma, but also in the interpretation of other anthropological aspects (highlighted in chapter 7) although marginal with respect to the main subject of the thesis. The loss of soft tissue, the deterioration of the components of bone tissue and the progressive reduction of tissue constituents and the moisture content, are all processes which manifest themselves after death and need to be explained, as they are responsible for changes which are difficult to assess, and sometimes prevent a correct anthropological interpretation.
This thesis has therefore provided several stepping stones for future research in skeletal trauma analysis. Both for antemortem and perimortem trauma it has stressed, through scientific studies, the inadequacy of current methods and has set the fundamentals for possible solutions to these crucial problems which need to be assessed in the future with

further scientific projects. Only in this manner will it be hopefully possible, both in criminal and humanitarian scenarios, to ascertain torture, maltreatment, previous trauma with id potential and cause and manner of death on skeletal or putrefied human remains.

Chapter 9

REFERENCIES:

1. Dayal MR, Kegley ADT, Strkalj G, Bidmos MA, Kuykendall KL, The history and composition of the Raymond A. Dart Collection of human skeletons at the University of the Witwatersrand, Johannesburg, South Africa, *Am J Phys Anthropol*, 2009, 140:324–35

2. Hunt DR, Albanese J, History and demographic composition of the Robert J. Terry Anatomical Collection, *Am, J, Phys, Anthropol*, 127 (2005) 406–17

3. Eliopoulos C, Lagia A, Manolis S, A modern documented skeletal collection from Greece, *Homo* 58 (2007) 221–8

4. L'Abbé EN, Loots M, Meiring JH, The Pretoria bone collection: a modern South African skeletal sample, *HOMO J Com Hum Biol*, 56 (2004) 197–205.

5. Bosio LA, García Guraieb S, Luna LH, Aranda C, Chacarita project: conformation and analysis of a modern and documented human osteological collection from Buenos Aires – theoretical, methodological and ethical aspects, *HOMO – J Comp Hum Biol*, 2012, 63:481-92

6. Roberts CA, Why study human remains from archaeological sites?, In: Roberts CA (Ed), Human Remains in Archaeology: A Handbook Practical Handbooks in Archaeology N. 19, *Cambridge University Press*, New York, 2009, 1–16

7. Tobias PV, On the scientific, medical, dental, and educational value of collections of human skeletons, *Int J Anthropol*, 1991, 6:277–80

8. Komar DA, Grivas C, Manufactured populations: What do contemporary reference skeletal collections represent? A comparative study using the Maxwell Museum Documented Collection, *Am J Phys Anthropol*, 2008, 137: 224–233.

9. Cardoso HFV, The Collection of identified human skeletons housed at the Bocage Museum (National Museum of Natural History), Lisbon, Portugal, *Am J Phys Anthropol*, 2006, 129:173–6

10. Giraudi R, Fissore F, Giacomo G, The collection of human skulls and postcranial skeletons at the Department of Human Anatomy of the University of Torino (Italy), *Am J Phys Anthropol*, 1984, 65:105–7

11. Usher B, Reference samples: the first step in linking biology and age in the human skeleton. In: Hoppa, R.D., Vaupel, J.W. (Eds.), Paleodemography. Age Distributions from Skeletal Samples, *Cambridge University Press*, Cambridge, 2002, 29–47

12. Albanese J, A metric method for sex determination using the hipbone and femur, *J Forensic Sci*, 2003, 48(2):263-73

13. Falsetti AB, Sex assessment from metacarpals of the human hand, *J Forensic Sci*, 1995, 40(5):774-6

14. Hart GO, Fracture pattern interpretation in the skull: differentiating blunt force from ballistics trauma using concentric fractures, *J Forensic Sci*, 2005, 50(6):1276-81

15. Meadows Jantz L, Jantz RL, Secular change in long bone length and proportion in the United States, 1800-1970, *Am J Phys Anthropol*, 1999, 110(1):57-67

16. Ubelaker DH, Volk CG, A test of the Phenice method for the estimation of sex, *J Forensic Sci*, 2002, 47(1):19-24

17. Yoder C, Ubelaker DH, Powell JF, Examination of variation in sternal rib end morphology relevant to age assessment, *J Forensic Sci*, 2001, 46(2):223-7

18. Snodgrass JJ, Sex differences and aging of the vertebral column, *J Forensic Sci*, 2004, 49(3):458-63

19. Ross AH, Slice DE, Ubelaker DH, Falsetti AB, Population affinities of 19th century Cuban crania: implications for identification criteria in South Florida Cuban Americans, *J Forensic Sci*, 2004, 49(1):11-6

20. Prince DA, Ubelaker DH, Application of Lamendin's adult dental aging technique to a diverse skeletal sample, *J Forensic Sci*, 2002, 47(1):107-16

21. Buck TJ, Vidarsdottir US, A proposed method for the identification of race in sub-adult skeletons: a geometric morphometric analysis of mandibular morphology, *Journal of Forensic Sciences*, 2004, 49(6):1159-64

22. Byrd JE, Adams BJ, Osteometric sorting of commingled human remains, *Journal of Forensic Sciences,* 2003,48(4):717-724.

23. Lovejoy CO, Meindl RS, Pryzbeck TR, Mensforth RP. Chronological metamorphosis of the auricular surface of the ilium: a new method for the determination of adult skeletal age at death, *Am J of Phys Anthropol*, 1985, 68(1):15-28.

24. Meindl RS, Lovejoy CO, Ecto suture closure: a revised method for the determination of skeletal age at death based on the lateral-anterior sutures, *Am J of Phys Anthropol*, 1985, 68(1):57-66.

25. Mann RW, Symes SA, Bass WM, Maxillary suture obliteration: aging the human skeleton based on intact or fragmentary maxilla, *J Forensic Sci*, 1987, 32:148–57

26. Rogers TL, A visual method of determining the sex of skeletal remains using the distal humerus, *J Forensic Sci*, 1999, 44: 57–60

27. Bidmos MA, Asala SA, Discriminant function sexing of the calcaneus of the South African whites, *J of Forensic Sci,* 2003, 48(6):1213-8.

28. Bidmos MA, Asala SA, Sexual dimorphism of the calcaneus of South African blacks, *J Forensic Sci*, 2004, 49(3):446-50

29. Dayal MR, Bidmos MA, Discriminating sex in South African blacks using patella dimensions, *J Forensic Sci*, 2005, 50(6):1294-7,

30. Cunha E, Testing identification records: evidence from Coimbra identified skeletal collection (nineteenth and twentieths centuries), In: Herring, A., Saunders, S. (Eds.), Grave Reflections: Portraying the Past through Skeletal Studies, *Canadian Scholar's Press*, Toronto, 1995, 179–98

31. Cunha E, Umbelino C, What can bones tell about labour and occupation: the analysis of skeletal markers of occupational stress in the Identified Skeletal Collection of the Anthropological Museum of the University of Coimbra (preliminary results*), Antropología Portuguesa*, 1995, 13:49–68

32. Santos AL, Roberts CA, Anatomy of a serial killer: differential diagnosis of tuberculosis based on rib lesions of adult individuals from the Coimbra Identified Skeletal Collection, Portugal, *Am J Phys Anthropol*, 2006, 130:38–49

33. Cox M, Life and Death in Spitalfields 1700 to 1850, *CBA*, York, 1996.

34. Molleson T, Cox M, The Spitalfields Project, The Middling Sort: The Anthropology, vol. 2, *CBA*, York, 1993

35. Reeve, J, Adams M, The Spitalfields Project, Across the Styx, vol. 1. *CBA*, York, 1993

36. Bedford ME, Russell KF, Lovejoy CO, Meindl RS, Simpson SW, Stuart-Macadam PL, Test of the multifactorial aging method using skeletons with known ages-at-death from the grant collection, *Am J Phys Anthropol,* 1996, 99:287–97,

37. Hens SM,Rastelli E, Belcastro G, Age Estimation from the Human Os Coxa: A Test on a Documented Italian Collection*, J Forenisc Sci*, 2008, 53(5):1040-3

38. Szilvassy J, Kritscher H, Estimation of chronological age in man based on the spongy structure of long bones, *Anthropol Anz*, 1993, 91(3): 289–98

39. Ion A, A brief overview of "Francisc J. Rainer" Human Osteological Collection, *Ann Roum Anthropol*, 2011, 48:24-32

40. Alceda SA, Desandolo B, Garçia Mancuso R, Plischuk M, Inda AM, The 'Prof. Dr. Romulo Lambra Collection: An Argentinian Sample of modern skeleons, *HOMO- Journal of Comparative Human Biology*, 2012, 63:275-81

41. Barrio, PA, Trancho GJ, Sánchez JA, Metacarpal sexual determination in a Spanish population, *J Forensic Sci*, 2006, 51:990–5

42. Bidmos, MA, Asala, SA, Discriminant function sexing of the calcaneus of the South African whites, *J Forensic Sci*, 2003, 48:1213-18

43. Trancho, GJ, Robledo B, López-Bueis I, Sánchez JA, Sexual determination of the femur using discriminant functions, Analysis of a Spanish population of known sex and age, *J Forensic Sci*, 1997, 42:181-5

44. Steyn M, Iscan MY, Osteometric variation in the humerus: sexual dimorphism in South Africans, *Forensic Sci Int*, 1999, 106: 77–85

45. Oettle AC, Steyn M, Age estimation from sternal ends of ribs by phase analysis in South African Blacks, *J Forensic Sci*, 2000, 45:1071–79

46. Wu L, Sex determination of Chinese femur by discriminant function, *J Forensic Sci*, 1989, 34: 1222–7

47. Ericksen MF, How 'representative' is the Terry collection? Evidence from the proximal femur, *Am J Phys Anthropol*, 1982, 59: 345–50

48. Wood J, Milner G, Harpending H, Weiss K,. The osteologi- cal paradox: problems of inferring prehistoric health from skeletal samples [and comments and reply], *Curr Anthropol*, 1992, 33:343–70

49. Bocquet-Appel J, Masset C, Paleodemography: expectancy and false hope, *Am J Phys Anthropol,* 1996, 99:571–83

50. Buikstra J, Konigswald L,. Paleodemography: critiques and controversies, *Am Anthropol,* 1985, 87:316–33

51. Meindl R, Russel K,. Recent advances in method and theory in paleodemorgraphy, *Annu Rev Anthropol*, 1998, 27:375–99

52. Wright L, Yoder C, Recent progress in bioarchaeology: approaches to the osteological paradox, *J Archaeol Res*, 2003, 11:43– 70

53. Knight B, Forensic pathology, 2nd Ed, *Arnold*, 338 Euston Road, London NW1 3BH, 1996

54. Cunha E, Cattaneo C, Forensic anthropology and forensic pathology. The state of the art, In: Schmitt A, Cunha E, Pinheiro J, Forensic anthropology and Medicine, *Humana Press*, Totowa, New Jersey 07512, 2006, 3: 39-53

55. N Sauer, The timing of injuries and manner of death: distinguishing among antemortem, perimortem and postmortem trauma, In: K Reichs, Forensic Osteology, Advances in the identification of human remains*,* 2nd Ed, *Charles C Thomas Publisher LTD*, Springfield, Illinois, USA, 1998

56. Symes SA, Berryman HE, Smith OC, Saw marks in bone: introduction and examination of residual kerf contours, In: Reichs KJ, Forensic Osteology: Advances in the identification of human remains, 2nd Ed, *Charles C Thomas Publisher LTD*, Springfield, Illinois, USA, 1998

57. Hart GO, Fracture pattern interpretation in the skull: differentiating blunt force from ballistics trauma using concentric fractures, *J Forensic Sci*, 2005, 50(6):1276-81

58. Semeraro D, Passalacqua N, Symes SA, Gilson T, Patterns of trauma induced by motorboat and Ferry Propellers as illustrated by three known cases from Rhode Island, *J Forensic Sci*, 2012, 57(6): 1625-29

59. Galloway A and Zephro L, Skeletal Trauma Analysis of the lower Extremity, In: Rich J, Dean DE, Forensic Science and Medicine Forensic Medicine of the Lower Extremity: Human Identification and Trauma Analysis of the Thigh, Leg, and Foot, *Powers RH © The Humana Press Inc, Totowa, NJ*, 2005, 253-77

60. Cruess RL, Healing of bone, tendon and ligament, In: Fractures in adults, Rockwood, CA, Dean DP(ed), Philadelphia, *JB Lippincott Co*, 1984, 1:147-55.

61. DJ Ortner, WGJ Putschar, Identification of pathological conditions in Human Skeletal Remains, Washington DC, Contribution to Anthropology, *Smithsonian Institution Press*, 1981

62. M Doblaré, JM Garcìa, MJ Gòmez, Modelling bone tissue fracture and healing: a review, *Engineering Fracture Mechanics*, 2004, 71: 1809-40

63. Hendrix RW, Fracture Healing. In: Rogers LF (ed), Radiology of Skeletal Trauma, vol. 1, 3rd ed, *Philadelphia, Churchill Livingstone*, 2002, 203-30.

64. Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R Toward a molecular understanding of skeletal development, *Cell*, 1995, 80:371–8

65. Rodan GA, Introduction to bone biology, *Bone*, 1992, 13(1):S3–S6

66. Aubin JE, Turksen K, Heersche JMM Osteoblastic cell lineage. In: Noda M (ed.) Cellular and Molecular Biology of Bone, *Academic Press*, San Diego, CA, U.S.A., 1993, 1–46

67. Dimitriou R, Tsiridis E, Giannoudis PV, Current concepts of molecular aspects of bone healing, *Injury Int J Care Injured*, 2005, 36: 1392-404

68. Hufnagel, An Investigation of time since injury: A radiographic study of fracture healing, *Thesis*. *The University of Wisconsin at La Crosse*, 2005

69. McKibbin B, The biology of fracture healing in long bones, *J Bone Joint Surg*, 1978, 60 (2):150-62

70. Einhorn TA, The cell and molecular biology of fracture healing, *Clin Orthop*, 1998; 35(5S):7-21

71. Brighton CT, Hunt RM. Early histological and ultra structural changes in medullary fracture callus, *J Bone Joint Surg* [Am], 1991, 73 (6):832–47

72. Ham AW, Histology, *JB Lippincott Company, Philaddelphia, Montreal*, 4th edition

73. Collins DH, Pathology of Bone, *London, Butterworths*, 1966

74. Frost MD, The biology of fracture healing. An overview for Clinicians. Part 1, *Clinical Orthopedic and related Research*, 1989, 248: 283-93

75. Paton DF, Fractures and Orthopaedics, *Edinburgh, Churchill Livingstone*,1992

76. Sevitt S, Bone Repair and Fracture Healing in Man, *Edinburgh: Churchill Livingstone*, 1981

77. Frost MD, The biology of fracture healing. An overview for Clinicians. Part 2, *Clinical Orthopedic and related Research,* 1989, 248: 294-309

78. Philips AM, Overview of the fracture healing cascade, *Injury Int J Care Injured*, 2005, 36s: s5-7

79. Toal RL, Mitchell SK, Fracture Healing and Complications, In: Thrall DE (ed), Textbook of Veterinary Diagnostic Radiology, 4th ed, *WB Saunders Co* , Philadelphia, 2002, 161-78

80. Oehmichen M, Vitality and time course of wounds, *Forensic Sci International*, 2004, 144: 2-3

81. Todd TW, Iler DH, The phenomena of early stages in bone repeair, *Annuals of Surgery*, 1927, 86(5): 715-36

82. Vigorita VJ, Orthopedic pathology, *Lippincott-Williams and Wilkins*, Philadelphia, New York, Baltimore, 2009

83. Tosounidis T, Kontakis G, Nikolau V, Papathanassopoulos A, Giannioudis PV, Fracture healing and bone repair: an update, *Trauma*, 2009, 11(3):145-56

84. Barbian LT, Sledzik PS, Healing following cranial trauma, *J Forensic Sci*, 2008, 53(2):263–8

85. Maat GJR, Dating of fractures in human dry bone tissue. The Berisha case, In: Kimmerle E, Baraybar JP, Identification of traumatic skeletal injuries resulting from human rights abuses and armed conflicts, *Taylor and Francis Group, Boca Raton*, FL, 2008, 245-54

86. Maat GJR, Huls N, Histological fracture dating on fresh and dried bone tissue. In: Bilo RAC, Robben SGF, van RiJn RR, Forensic aspects of Pediatric Fractures. Differentiating Accidental Trauma from Child Abuse, *Springer-Verlag Berlin Heidelberg*, 2010, 194- 201

87. O'Connor JF, Cohen J, et al, Dating Frcatures, In: Kleinmark PK, Diagnostic images of child abused, 2nd Ed, *Mosby,* 1988, 168-77

88. Prosser I, Maguire S, Harrison SK et al, How old is this fracture? Radiologic dating of fractures in children: a systematic review, *AM J Roentgenol*, 2005, 184 (4): 1282-6

89. Islam O, Sobeleski D, Symons S et al, Development and duration of radiographics signs of bone healing in children, *AM J Roentgenol*, 2000, 175 (1): 16-9

90. William PL, Warwick R, Dyson M et al, Gray's Anatomy. The response of bone to injury, 37th Ed, *Churchill Livingstone*, 1989, 313-5

91. Heppenstall RB, Fracture healing, In: Heppenstall RB (ed), Fracture treatment and healing, *Saunders*, 1980, 41-64

92. Buckwalter JA, Einhorn ThA, Bolander ME et al, Healing of musculoskeletal tissue, In: Rockwood ChA, Bucholz RW, Green DP et al (eds), Fracture in adults, 4th ed, *Lippincott-Raven*, 1996, 267-83

93. De Boer HH, van der Merwe AE, Hammer S, Steyn M, Maat GJR, Assessing post-traumatic time interval in human dry bone, *Int J Osteoarchaeology*, 2012, DOI: 10.1002/oa.2267

94. Steyn M, De Boer HH, van der Merwe AE, Cranial trauma and the assessment of posttraumatic survival time, *Forensic Sci Int*, 2014, 244:e25-9

95. Ortner D, Differential diagnosis of skeletal injurie, In: Kimmerle EH, Baraybar JP, Skeletal trauma: identification of injuries from human rights abused and armed conflict, *CRC Pres*, 2008, 21-93

96. Ubelaker DH, Taphonomic applications in forensic anthropology, In: Haglund WD, Sorg MH, (ed) Forensic taphonomy: the postmortem fate of human remains, *Boca Raton*, FL, CRC Press, 1997, 77–90

97. Ubelaker DH, Adams BJ, Differentiation of perimortem and postmortem trauma using taphonomic indicators*, J Forensic Sci*, 1995, 40(3):509–12

98. Wieberg DAM, Wescott DJ, Estimating the timing of long bone fractures: correlation between the postmortem interval, bone moisture content, and blunt force trauma characteristics, *J Forensic Sci*, 2008, 53(5):1028–34

99. Dirkmaat DC, Cabo LL, Ousley SD, Symes SA, New perspectives in forensic anthropology, *Am J Phys Anthropol*, 2008, 51:33–52

100. Evans FG, Stress and strain in bones, Springfield, *IL: Charles C. Thomas*, 1957

101. Johnson E, Current developments in bone technology, *Adv Archaeol Method Theory*, 1985, 8:157–235.

102. Moraitis K, Eliopoulos C, Spiliopoulou C, Fracture Characteristics of Perimortem Trauma in Skeletal Material, *The Internet Journal of Biological Anthropology*, 2009, 1-3

103. Symes SA, L'abbé EN, Stull KE, Lacroix M, Pokines JT, Taphonomy and the timing of bone fractures in trauma analysis, in: Pokines JT, Symes SA (ed), Manual of forensic taphonomy, *CRC, CRC Press, Taylor & Francis Group*, 2014, pp: 341-65

104. Harkess JW, Ramsey WC, Harkess JW, Principles of fractures and dislocations, In: Rockwood CA Jr, Fractures in adults, Green DP (ed), 4th edition, *JB Lippincott Company*, Philadelphia, 1996, 3–210

105. Wheatley B. Perimortem or postmortem bone fractures? An experimental study of fracture patterns in deer femora, *J Forensic Sci,* 2008;53:69–72.

106. Ubelaker DH, Perimortem and postmortem modification of human bone. Lessons from forensic anthropology, *Antropologie,* 1991, 29:171–4

107. Calce SE, Rogers TL, Taphonomic changes to blunt force trauma: a preliminary study, *J Forensic Sci*, 2007, 52(3):519–27

108. Galloway A, Symes SA, Haglund WD, et al. The role of forensic anthropology in trauma analysis, In: Galloway A (ed), Broken Bones: Anthropological Analysis of Blunt Force Trauma, Springfield, *IL: Charles C. Thomas*, 1999, 5–31

109. Galloway A, The biomechanics of fracture production, In: Galloway A (ed), Broken Bones: Anthropological Analysis of Blunt Force Trauma, Springfield, *IL: Charles C. Thomas*, 1999, 35–62117-118

110. Karr LP, Outram AK, Bone degradation and environment: understanding, assessing and conducting archeological experiments using modern animal bones, *Int J Osteoarcheol*, 2012, doi:10.1002/oa.2275

111. Karr LP, Outram AK. Tracking changes in bone fracture morphology over time environment, taphonomy and the archeological record, *J Archeol Sci*, 2012; 39:555–9

112. La Croix M, A study of the impact of weathering upon the minimal force required to fracture bone. *Unpublishd M.S. Thesis , Boston University, Boston, MA*, 2013

113. Maples W, Trauma analysis by the forensic anthropologist. In: Reichs KJ (ed), Forensic Osteology: Advances in the identification of human remains, *Springfield, IL, Charles C Thomas*, 1986, pp 218-228

114. Quatrehomme G, Alunni V, Bone trauma, in: Siegel and Saukko, Encyclopedia of Forensic Science, Second Edition, *Elsevier Science & Technology*, 2013, 89-96

115. Villa P, Mahieu E, Breakage patterns of human long bones, *J Hum Evol,* 1991, 21:27–4

116. Bonnichsen R, Pleistocene Bone Technology in the Beringian Refugium, *Archaeological Survey of Canada*, 1979, 89:1-280

117. Morlan RE, Toward the definition of criteria for the recognition of artificial bone alterations, *Quaternary research*, 1984, 22: 160-71

118. Berryman HE, Symes SA, Smith OC, Moore SJ, Bone fracture II: Gross examination of fractures. *Proceeding of the 43th Annual Meeting of the American Academy of Forensic Sciences*, Anaheim, CA

119. Madea B, Saukko P, Oliva A, Musshoff, Molecular pathology in forensic medicine- Introduction, *Forensic Sci Int*, 2010, 203 (1-3): 3-14

120. Kondo T, Ishida Y, Molecular pathology of wound healing, *Forensic Sci Int,* 2010, 203 (1-3): 93:8

121. Hernandez-Cueto C, Girela E, Sweet D, Advances in the Diagnosis of Wound Vitality: A Review, *Lippincott Williams and Wilkins Inc*, 2000, 21(1): 21-31

122. Clark RAF, (ed) The molecular and cellular biology of wound repair, 2nd ed, *New York: Plenum Press*, 1996

123. Singer AJ, Clark RA, Cutaneous wound healing, *N Engl J Med*, 1999, 347:738-46

124. Grellner W, Dimmeler S, Madea B, Immunohistochemical detection of fibrinectin in post-mortem incised wounds of porcine skin, *Forensic Sci Int*, 1998, 97:109-16

125. Schmidt-Bleek K, Schell H, Kolar P, Pfaff M, Perka C, Buttgereit F, Duda G, Lienau J, Cellular composition of the fracture hematoma compred to a muscle hematoma:a study in sheep, *J Orthop Res*, 2009, 1127: 47

126. Raekallio J, Timing of wounds in forensic medicine, *Jpn J Legal Med*, 1976, 30: 125-36

127. Betz P, Nerlich A, Wilske J, Tübel J, Penning R, Eisenmenger W, Analysis of the immunohistochemical localization of collagen type III and V for the time-estimation of human skin wounds, *Int J Legal Med,* 1993, 105: 329–332

128. Takamiya, M., Saigusa, K., and Aoki, Y, Immunohistochemical study of basic fibroblast growth factor and vascular endothelial growth factor expression for age determination of cutaneous wounds, *Am J Forensic Med Pathol,* 2002, 23: 264–7

129. Hayashi T, Ishida Y, Kimura A, Takayasu T, Eisenmenger W, Kondo T. Forensic application of VEGF expression to skin wound age determination, *Int. J Legal Med,* 2004, 118: 320–5

130. Cattaneo C, Andreola S, Marinelli E, Poppa P, Porta D, Grandi M, The detection of microscopic markers of hemorrhaging and wound age on dry bone, *Am J Forensic Med Pathol,* 2010, 31(1):22-26

131. Kondo T, Ohshima T, Mori R, Guan DW, Ohsima K, Eisenmenger, Immunohistochemical detection of chemokines in human skin wounds and its applicationto wound age determination, *Int J Legal Med*, 2002, 116: 87-91

132. Kolar P, Schmidt-Bleek K, Schell H, Gaber T, Toben D, Schmidmaier G, Perka C, Buttgereit F, Duda GN, The early fracture hematoma and its potential role in fracture healing, *Tissue engineering*, 2010, 16 (4): 427-34

133. Huser CJ, Zhou J, Joshi P, Cuchens MA, Kregor P, Devidas M, Kennedy RD, Poole GV, Hughes JL, The immune microenvironment of human fracture/soft tissue haematomas and its relationship to systemic immunity, *J trauma*, 1997, 42: 895-904

134. Thachiana T, Matsubara T, Mizuno K, Hirohata K, Enhancement of new bone formation by hematoma at fracture site, *Nippon Seikeigeka Gakkai Zasshi*, 1991, 65: 349:58,

135. Simmons DJ, Fracture healing perspectives, *Clinical orthopaedics and related research*, 1985, 200:100-15

136. Cox M, Scott A, Evaluation of the obstetric significance of some pelvic characters in an 18th century British sample of known parity status, *American Journal of Physical Anthropology,* 1992, 89: 431-40

137. White TD, Human Osteology (2nd ed), *Academic Press: New York*, 2000

138. Phenice TW, A newly developed visual method of sexing the os pubis, *American Journal of Physical Anthropology,* 1969, 30: 297–302

139. Bone KE, Bias in skeleton sexing, *American Journal of Physical Anthropology,* 1993, 16: 59

140. Meindl and Lovejoy Meindl RS, Lovejoy CO, Mensforth RP, Don Carlos L, Accuracy and direction of error in the sexing of the skeleton: Implications for paleodemography, *American Journal of Physical Anthropology* 1985, 68: 79-85

141. Brook ST, Suchey JM, Skeletal age determination based on the os pubis: a comparison of the Acsadi-Nemeskéri and Suchey-Brooks methods, *Human Evolution*, 1990, 5: 227-38

142. Lovejoy CO, Meindl RS, Pryzbeck TR and Mensforth RP, Chronological metamorphosis of the auricular surface of the ilium: a new method for the determination of adult skeletal age at death, *American Journal of Physical Anthropology,* 1985, 68: 15-28

143. Beauthier JP, Lefèvre P, Meunier M, Orban R, Polet C, Werquin JP, Quatrehomme G, Palatine Sutures as an Age Indicator: A Controlled Study in Elderly, *J Forensic Sci*, 2010, 55:153-58

144. Iscan Işcan MY, Loth SR, Wright RK, Age estimation from the ribs by phase analysis: White males, *Journal of Forensic Sciences,* 1984, 29: 1094–104

145. Işcan MY, Loth SR, Wright RK, Age estimation from the ribs by phase analysis: White females, *Journal of Forensic Sciences,* 1985, 30: 853–63

146. Rougé-Maillart C, Vielle B, Jousset N et al, Development of a method to estimate skeletal age at death in adults using the acetabulum and the auricular surface on a Portuguese population, *Forensic Sci Int*, 2009, 188(1-3):91-5

147. Dwight T. Methods of estimating the height from part of the skeleton, *Medical Record,* 1894, 46:293-6

148. Dupertius CW, Hadden JA, On the reconstruction of stature from long bones, *American Journal of Physical Anthropology,* 1951, 9: 15-53

149. Trotter M, Gleser GC, Estimation of stature from long bones of American Whites and Negroes, *American Journal of Physical Anthropology,* 1952, 10: 463-514

150. Trotter M, Gleser GC, A re-evaluation of estimation of stature based on measurements of stature taken during life and of long bones after death, *American Journal of Physical Anthropology* 1958, 16: 79-123.

151. Olivier G, Aaron C, Fully G, Tissier G, New estimations of stature and cranial capacity in modern man, *Journal of Human Evolution,* 1978, 7: 513-8

152. Feldesman MR, Femur/stature ratio and estimates of stature in children, *American Journal of Physical Anthropology,* 1992, 87: 447-459

153. Mendonça MC, Estimation of height from the length of long bones in a Portuguese adult population, *American Journal of Physical Anthropology* 2000, 112: 39-48.

154. Steele DG, The estimation of sex on the basis of the talus and calcaneus, *American Journal of Physical Anthropology,* 1976, 45: 581–8,

155. Chandra NS, Nath S, Reconstruction of femoral and humeral bone lengths using a fragmentary bone measure, *Indian Journal of Physical Anthropology and Human Genetics*, 1985, 11: 123-8

156. Jacobs K, Estimating femur and tibia length from fragmentary bones: an evaluation of Steele's (1970) method using a prehistoric European sample, *American Journal of Physical Anthropology*, 1992, 89: 333–345

157. Cappella A, Amadasi A, Gaudio D, Gibelli D, Borgonovo S, Di Giancamillo M, Cattaneo C, The application of cone-beam CT in the aging of bone calluses: a new perspective, *Int J Legal Med,* 2013, 127: 1139–44

158. Barber CG, Immediate and eventual features of healing in amputated bones, *Ann Surg,* 1929; 90: 985–92

159. Mays SA, Healed limb amputations in human osteoarchaoelogy and their causes: a case study from Ipswitch, UK, *Int. J Osteoarchaeol*, 1996, 6: 101–13

160. Malone CA, Sauer, NJ, and Fenton TW, A radiographic assessment of pediatric fracture healing and time since injury, *J Forensic Sci,* 2008, 56: 1123–30

161. Simmons T, Adlam RE, Moffatt C, Debugging decomposition data: comparative taphonomic studies and the influence of insects and carcass size on decomposition rate, *J Forensic Sci*, 2010, 55(1):8-13

162. Setzer TJ, Sundell IB, Dibbley SK, Les C, Technical Note: A histological technique for detecting the cryptic preservation of erythrocytes and soft tissue in ancient human skeletonized remains, *Am J Phys Anthropol*, 2013

163. Stout SD, Teitelbaum SL, Histological analysis of unde- calcified thin sections of archaeological bone, *Am J Phys Anthropol,* 1976, 44:263–9

164. Schweitzer MH, Wittmeyer JL, Horner JR, Soft tissue and cellular preservation in vertebrate skeletal elements from the Cretaceous to the present, *Proc R Soc B*, 2007, 274:183–97

165. McNamara ME, Orr PJ, Kearns SL, Alcalà L, Anadòn P, Penalver-Mollà E, High-fidelity organic preservation of bone marrow in ca. 10 Ma amphibians*, Geology*, 2006, 34:641– 4

166. Charlier P, Georges P, Bouchet F, Huynn-Charlier I, Carlier R, Mazel V, Richardin P, Brun L, Blondiaux J, Lorin de la Grandmaison G, The microscopic (optical and SEM) examination of putrefaction fluid deposits (PFD). Potential interest in forensic anthropology*. Virchows Archiv,* 2008, 453:377–86

167. Penttilä A, Lahio K, Autolytic changes in blood cells of human cadavers. II. Morphological studies, *Forensic Sci Int* 1981, 17(2):121-32

168. Brecher G, Bessis M, Present status of spiculed red cells and their relationship to the discocyte-echinocyte transformation: a critical review, *Blood*, 1972, 40:333-4

169. Hess JR, Red cell changes during storage, *Transfus Apher Sci,* 2010, 43:51-9

170. Hess JR, Red cell storage, *J Proteomics*, 2010b, 73:368-373

171. Lim GHW, Wortis M, Mukhopadhyay R, Stomatocyte-discocyte-echinocyte sequence of the human red blood cell: evidence for the bilayer-couple hypotesis from membrane mechanics, *PNAS*, 2002, 99(26):16766-69

172. Sheetz MP, Singer SJ, Biological membranes as bilayer couples, *P Natl Acad Sci USA*, 1974, 71(11):4457-61

173. Simpson LO, Nondiscocytic erythrocytes in myalgic encephalomyelitis. New Zeal Med J, 1989, 102:126-7

174. Vermehren C, Hanses HS, Shape changes in the erythrocyte membrane induced by the absorption enhancer didecanophosphatidylcholine, *Int J Pharm*, 1998, 174:1-8

175. Hanson MS, Stephenson AH, Bowle EA, Sridharan M, Adderley S, Sprague RS, Phosphodiesterase 3 is present in rabbit and human erythrocytes and its inhibition potentiates iloprost-induced increases in cAMP. American Journal of Physiology, *Am J Physiol Heart Circ Physiol*, 2008, 295: H786-H793

176. Kheirolomoom A, Satpathy GR, Török Z, Banerjee M, Bali R, Novaes RC, Little E, Manning DM, Dwyre DM, Tablin F, Crowe JH, Tsevtkova NM, Phospholipid vesicles increase the survival of freeze-dried human red blood cells, *Cryobiology,* 2005, 51:290-305

177. Simpson LO, Herbison MD, Herbison GP, The results from red cell shape analysis of blood samples from members of myalgic encephalomyelitis organization in four countries, *J Orthomol Med*, 1997, 12(4):221-6

178. Maccoll AJ, James KAC, Booth CL, Erythrocyte morphology and filterability in rats fed on diets containing different fats and oils, *Brit J Nutr*,1996, 76:133-40

179. Minetti G, Seppi G, Ciana A, Balduini C, Low PS, Brovelli A, Characterization of the hypertonically induced tyrosine phosphorilation of erythrocyte band 3, *Biochem,* 1998, J 335:305-11

180. Vromen J, McCane B, Red blood cell segmentation from SEM images, *24th International Conference Image and Vision Computing New Zealand*, 2009

181. Masaphy S, External ultrastructure of fruit body initiation in *Morchella*, *Mycol Res,* 2005, 109(4):508-2

182. Rydjord B, Namork E, Nygaard UC, Wiker HG, Hetland G, Quantification and characterization of IgB binding to mould spores by flow cytometry and scanning electron microscopy, *J Immunol Methods*, 2007, 323:123-31

183. Kovarik C, Stewart D, Cockerell C, Gross and histologic postmortem changes of the skin, *Am J Forensic Med Pathol*, 2005, 26(4):305-8

184. O'Brien TG, Kuehner AC, Waxing grave about adipocere: soft tissue change in an aquatic context, *J Forensic Sci*, 2007, 52(2):294-301

185. Voss SC, Forbes SL, Dadour IR, Decomposition and insect succession on cadavers inside a vehicle environment, *Forensic Sci Med Pathol,* 2008, 4(1):22-32

186. Lee Goff M, Early post-mortem changes and stages of decomposition in exposed cadavers, *Exp Appl Acarol*, 2009, 49(1-2):21-36

187. Quatrehomme G, Işcan MY, Postmortem skeletal lesions, *Forensic Sci Int*, 1997, 89(3):155-65

188. Marchiafava V, Bonucci E, Ascenzi A, Fungal osteoclasia: a model of dead bone resorption, *Calcif Tissue Res*, 1974, 14(3):195-210

189. Hackett CJ, Microscopical focal destruction (tunnels) in exhumed human bones, *Med Sci Law*, 1981, 21(4):243-65

190. Jans, MME, Nielsen-Marsh CM, Smith CI, Collins MJ, Kars H, Characterisation of microbial attack on archaeological bone, *J Archaeol Sci*, 2004; 31(1): 87-95

191. Dent BB, Forbes SL, Stuart BH, Review of human decomposition processes in soil, *Environ Geol*, 2004; 45(4): 576-85

192. Hare PE, Organic geochemistry of bone and its relation to the survival of bone in the natural environment, In: Behrensmeyer AK, Hill A, Fossils in the making: vertebrate taphonomy and paleoecology, *University of Chicago Press*, 1988

193. Goffer Z, Archaeological chemistry: a sourcebook on the applications of chemistry to archaeology, *Wiley, New York*, 1980

194. Janaway RC, The decay of buried human remains and their associated materials. In: Hunter J, Roberts C, Martin A (eds) Studies in crime: an introduction to forensic archaeology, *Routledge, London*, 1997, pp 58–85

195. van den Berg H, Kluin PM, Vossen JM, Early reconstitution of haematopoiesis after allogeneic bone marrow transplantation: a prospective histopathological study of bone marrow biopsy specimens, *J Clin Pathol*, 1990, 43(5):365-9

196. Thiele J, Kvasnicka HM, Zankovich R, Diehl V. Relevance of bone marrow features in the differential diagnosis between essential thrombocythemia and early stage idiopathic myelofibrosis, *Haematologica*, 2000, 85(11):1126-34

197. Thiele J, Kvasnicka HM, Zankovich R, Diehl V, The value of bone marrow histology in differentiating between early stage Polycythemia vera and secondary (reactive) Polycythemias, *Haematologica*, 2001, 86(4):368-74

198. Wain J, Pham VB, Ha V, Nguyen NM, To SD, Walsh AL, Parry CM, Hasserjian RP, HoHo VA, Tran TH, Farrar J, White NJ, Day NP, Quantitation of bacteria in bone marrow from patients with typhoid fever: relationship between counts and clinical features*, J Clin Microbiol*, 2001, 39(4):1571-6

199. Van der Merwe AE, Maat GJR, Steyn, M, Ossified haematomas and infectious bone changes on the anterior tibia: histomorphological features as an aid for accurate diagnosis, *Int J Osteoarchaeol*, 2010, 20(2):227-39

200. Wilkins BS, Erber WN, Bareford D, Buck G, Wheatley K, East CL, Paul B, Harrison CN, Green AR, Campbell PJ, Bone marrow pathology in essential thrombocythemia: interobserver reliability and utility for identifying disease subtypes, *Blood*, 2008, 111(1):60-70

201. Schultz M, Paleohistopathology of bone: a new approach to the study of ancient diseases, *Am J Phys Anthropol*, 2001, 33:106-47

202. von Hunnius TE, Roberts CA, Boylston A, Saunders SR, Histological identification of syphilis in pre-Columbian England, *Am J Phys Anthropol*, 2006, 129(4):559-66

203. Schultz M, Light microscopic analysis of macerated pathologically changed bones. Bone histology. An anthropological perspective. *CRC Press, Boca Raton*, 2012, 253-96

204. Dokgöz H, Arican N, Elmas I, Fincanci SK, Comparison of morphological changes in white blood cells after death and in vitro storage of blood for the estimation of postmortem interval, *Forensic Sci Int*, 2001,124(1):25-31

205. Chen Y, Cai J, Membrane deformation of unfixed erythrocytes in air with time lapse investigated by tapping mode atomic force microscopy, *Micron*, 2006, 37(4):339-46

206. Strasser S, Zink A, Kada G, Hinterdorfer P, Peschel O, Heckl WM, Nerlich AG, Thalhammer S, Age determination of blood spots in forensic medicine by force spectroscopy, *Forensic Sci Int*, 2007, 170(1):8-14

207. Wu Y, Hu Y, Cai J, Ma S, Wang X, Chen Y, Pan Y, Time-dependent surface adhesive force and morphology of RBC measured by AFM, *Micron*, 2009, 40(3):359-64,

208. Cappella A, Stefanelli S, Caccianiga M, Rizzi A, Cattaneo C. Blood or spores? The difficult issue of interpreting cellular debris on human skeletal remains, *10th congress of FASE Symposium, Heidelberg*, 2013

209. Maat GJR, Baig MS, Fossilized human blood cells from a Hellenistic settlement, *Int J Anthropol*, 1990, 5(3): 277-80

210. Maat GJ, Ultrastructure of normal and pathological fossilized red blood cells compared with pseudopathological biological structures, *Int J Osteoarchaeol*, 1991, 1(3-4): 209-14

211. Luna LG, AFIP: Manual of Histological Staining Methods. 3rd ed, *New York, NY: McGraw-Hill Book Company*, 1968

212. Schultz M, Microscopic investigation of excavated skeletal remains: A contribution to paleopathology and Forensic Medicine. In: Haglund WD and Sorg MH, Forensic Taphonomy. The post-mortem fate of human remains, *CRC Press, Taylor and Francis Group, Boca Raton*, FL, 2006, pp 200- 222

213. Cattaneo C. and Baccino E. A call for forensic Anthropology in Europe, *Newsletter of the Int J Leg Med*, 2002, 116: N1-N2

214. Ritz-Timme S, Cattaneo C, Collins MJ, Waite ER, Schutz Hw, Kaatsch HJ, et al. Age estimation: the state of the art in relation to the specific demands of forensic practice, *Int J Legal Med*, 2000, 113:129-36

215. Cunha E, Baccino E, Martrille L, Ramsthaler F, Prieto J, Schuliar Y, Lynnerup N, Cattaneo C. The problem of aging human remains and living individuals: A review*, Forensic Science International,* 2009, 193: 1-13

216. Kagerer, G. Grupe, Age-at-death diagnosis and determination of life-history parameters by incremental lines in human dental cementum as an identification aid, *Forensic Sci Int*, 2001, 118(1):75–82

217. Lamendin H, Observations on teeth roots in the estimation of age, Int. J. Forensic Dent, 1973, 1:4–7

218. Lamendin H, Baccino E, Humpert JF, Tavernier JC, Nossintchouk RM, Zerilli A, A simple technique for age estimation in adult corpses: the two criteria dental method, *J Forensic Sci*, 1993, 37 (5) 1373–9

219. Logan WHG, Kronfeld R, Development of the human jaws and surrounding structures from birth to the age of fifteen years, *JADA*, 1933, 20:379–427

220. Schour I, Massler M, Rate and gradient of grown in human deciduous teeth with special reference to neonatal ring, *J Dent Res*, 1937, 16:349–50

221. Schour I, Massler M, The development of the human dentition*, J Am Dent Assoc*, 1941, 28:1153–60

222. Anderson DL, Thompson GW, Popovich F, Age of attainment of mineralization stages of the permanent dentition, *J Forensic Sci*, 1976, 21(1):191–200

223. Ubelaker DH, Estimating age at death from immature human skeletons: an overview, *J Forensic Sci*; 1987, 32 1254–63

224. Smith SL,. Buschang PH, Variation in longitudinal diaphyseal long bone growth in children three to ten years of age, *Am J Hum Biol*, 2004, 16:648–57

225. Smith SL, Buschang PH, Longitudinal models of long bone growth during adolescence, *Am J Hum Biol*, 2005, 17:731–45

226. Meindl RS, Lovejoy CO, Ectocranial sutures closure:a revised method for the determination of skeletal age at death based on the lateral-anterior sutures, *Am J Phys Anthropol*, 1985, 57-66

227. Masset C, Age estimation on the basis of cranial sutures, In: Iscan MY editor, Age markers in the human skeleton, *Springfield: Charles C, Thomas*; 1989

228. Nemeskeri J, Harsanyi H, Acsadi G, Methoden zur diagnose des lebensalters von skelettfunden, *Anthropol Anzeiger*, 1960, 24:70–95

229. Baker RK, The relationship of cranial suture closure and age analyzed in a Modern Multi-Racial sample of males and females, *Thesis for the Degree Master of Arts in Anthropology Fullerton, California State Universit*y, 1984

230. Todd TW, Age changes in the pubic bone I: the male white pubis, *Am J Phys Anthropol*, 1920, 3:285–334

231. Todd TW, Age changes in the pubic bone VI: the interpretation of variations in the symphyseal area, *Am J Phys Anthropol*, 1921, 4:407–24

232. Brooks ST, Skeletal age at death: reliability of cranial and pubic age indicators, *Am J Phys Anthropol*, 1955, 3:567–97

233. Iscan MY, Loth SR, Wright RK, Racial variation in the sternal extremity of the rib and its effect on age determination, *J Forensic Sci*, 1987, 32(2):452-66

234. Kerley ER, The microscopic determination of age in human bone, *Am J Phys Anthropol*, 1965, 23:149–63

235. Kerley Er, Ubelaker DH, Revisions in the microscopic method of estimating age at death in human cortical bone, *Am J Phys Anthropol*, 1978, 49:545-6

236. Ahlqvist J, Damsten O, A modification of Kerley's method for the microscopic determination of age in human bone, *J Forensic Sci*, 1969, 14 (2)205–12

237. Stout SD, Paine RR, Histological age estimation using rib and clavicle, *Am J Phys Anthropol*, 1992, 87:111–15

238. Samworth R, Gowland R, Estimation of adult skeletal age-at-death: statistical assumptions and applications, *Int J Osteoarch*, 2007, 17:174-88.

239. Franklin D, Forensic age estimation in human skeletal remains: Current concepts and future directions, *Legal Medicine*, 2010, 12: 1-7

240. Lovejoy CO, Meindl RS, Mensforth RP, Barton TJ, Multifactorial determination of skeletal age at death: a method and blind tests of its accuracy, *Am J Phys Anthropol*, 1985, 68:1-14

241. Martrille L, Ubelaker DH, Cattaneo C, Seguret F, Tremblay M, Baccino E, Comparison of four skeletal methods for the estimation of age at death on white and black adults, *J Forensic Sci*, 2007, 52:302-7

242. Saunders SR, Fitzgerald C, Rogers T, Dudar C, McKillop H, A test of several methods of skeletal age estimation using a documented archaeological sample, *Can Soc Forensic Sci J*, 1992, 2:97-118

243. Bedford ME, Russell KF, Lovejoy CO, Meindl RS, Simpson SW, Stuart-Macadam PL, Test of the multifactorial aging method using skeletons with known ages-at-death from the Grant collection, *Am J Phys Anthropol*, 1993, 91:287-97

244. Nagar Y, Hershkovitz I, Interelationships between various aging methods, and their relevance to palaeodemogography, *Hum Evol*, 2004, 19:145-56

245. Schmitt A, Murail P, Cunha E, Rougé D, Variability of the pattern of aging on the human skeleton: evidence from bone indicators and implications on age at death estimation, *J Forensic Sci*, 2002, 47:1203-09

246. Baccino E, Schmitt A, Determination of adult age at death in the forensic context, In: Schmitt A, Cunha E, Pinheiro J, editors, Forensic anthropology and medicine, *New York: Humana Press*, 2006, p. 259-80

247. Katz D, Suchey JM, Races differences in pubic symphyseal aging patterns in the male, *Am J Phys Anthropol*, 1989, 80:167-72

248. Murray KA, A test of the auricular surface aging technique, *J Forensic Sci*, 1991, 36:1162-69

249. Schmitt A, Age at death assessment using the os pubis and the auricular surface of the ilium: a test on an identified Asian sample, *Int J Osteoarcheol*, 2004, 14:1-6

250. Schmitt A, Murail P, Is the first rib a reliable age indicator of age at death assessment? Test of the method elaborated by Kunos et al (1999), *Homo*, 2004, 54:207-14

251. Cattaneo C, Porta D, De Angelis D, Gibelli D, Poppa P. and Grandi M, Unidentified bodies and human remains: An Italian glimpse through a European problem, *Forensic Sci In*t, 2010, 195:161-67

252. http://www.interno.gov.it/mininterno/export/sites/default/it/assets/files/27/2013_10_02_Aggiornamento_della_Relazione_2012_con_dati_al_giugno_2013.pdf

253. Garvin HM, Passalacqua NV, Current Practices by Forensic Anthropologists in Adult Skeletal Age Estimation, *Journal of Forensic Sciences*, 2012, 57: 427–33

254. Baccino E, Tavernier JC, Lamendin H, Frammery D, Nossintchouk R, Humbert JF, Recherche d'une méthode multifactorielle simple pour la détermination de l'âge des cadavres adultes [in French], *J Med Leg Droit Med*, 1991, 34:27-33

255. Taylor RE, Suchey JM, Payen LA, Slota PJ Jr., The use of radiocarbon (C-14) to identify human skeletal materialsof forensic science interest. *J Forensic Sci,* 1989, 34(5): 196-205

256. Cook SF, Heizer RF, Archaeological dating by chemical analysis of bone, *Southwestern Journal of Anthropology*, 1953, 9:231-38

257. Cook SF, Dating Prehistoric bone by chemical analysis. In: Heizer R, Cook SF (eds) The Application Of Quantitative Methods In Archaeology, *Viking Fund Publications in Anthropology*, Chicago, 1968, pp 223-45

258. Bell LS, Skinner MF, Jones SJ, The speed of post-mortem change to the human skeleton and its taphonomic significance, *Forensic Sci Int*, 1996, 82(2):129-40

259. Yoshino M, Kimijima T, Miyasaka S, Sato H, Seta S, Microscopical study on estimation of time since death in skeletal remains, *Forensic Sci Int*, 1991, 49(2):143-58

260. Berg S, The determination of bone age. In: Landquist F (ed) Methods of forensic sciences, vol. 2, *Interscience*, 1963, pp 231-52.

261. Knight B, Lauder I () Methods of dating skeletal remains, *Hum Biol*, 1969, 41(3):322-41

262. Knight B, Lauder I, Practical methods of dating skeletal remains: a preliminary study. Med Sci Law, 1967, 7(4): 205-209

263. Castellano MA, Villanueva EC, von Frenckel R () Estimating the date of bone remains: A multivariate study, *J Forensic Sci*, 1984, 29(2):527-34

264. Facchini F, Pettener D, Chemical and physical methods in dating human skeletal remains, *Am J Phys Anthropol*, 1977, 47(1):65-70

265. Ramsthaler F, Ebach SC, Birngruber CG, Verhoff MA, Postmortem interval of skeletal remains through the detection of intraosseal hemin traces, A comparison of UV-fluorescence, luminol, Hexagon-OBTI ®, and Combur® tests. *Forensic Sci Int,* 2011, 209(1-3):59-63

266. Introna FJr, Di Vella G, Campobasso CP, Determination of postmortem interval from old skeletal remains by image analysis of luminol test results, *J Forensic Sci,* 1999, 44(3):535-38

267. Ramsthaler F, Kreutz K, Zipp K, Verhoff MA, Dating skeletal remains with luminol-chemiluminescence. Validity, intra- and inter-observer error, *Forensic Sci Int*, 2009, 187(1-3):47-50

268. Creamer JI, Buck AM, The assaying of haemoglobin using luminal chemiluminescence and its application to the dating of human skeletal remains, L*uminesce*nce, 2009, 24(5):311-16

269. Taylor RE, Problems in Radiocarbon Dating of bone, In: Currie LA (ed), Nuclear and chemical dating techniques: interpreting the environmental record, *American Chemical Society, Washington, DC*, 1982, pp453-73

270. Ubelaker DH, Buchholz BA, Stewart JE, Analysis of artificial radiocarbon in different skeletal and dental tissue types to evaluate date of death, *J Forensic Sci*, 2006, 51(3):484-88

271. Ubelaker DH, Buchholz BA, Complexities in the use of bomb-curve radiocarbon to determine time since death of human skeletal remains, *Forensic Science Communications*, 2006, 8(2):1-9.

272. Ubelaker DH, Parra RC, Radiocarbon analysis of dental enamel and bone to evaluate date of birth and death: perspective from the southern hemisphere, *Forensic Sci Int*, 2011, 208 (1-3):103-7

273. Cardoso HF, Puentes K, Soares AM, Santos A, Magalhães T, The value of radiocarbon analysis in determining the forensic interest of human skeletal remains found in unusual circumstances, J Forensic Leg Med, 2012, 19(2): 97-100

274. Hoke N, Grigat A, Grupe G, Harbeck M, Reconsideration of bone post-mortem interval estimation by UV-induced auto-fluorescence, *Forensic Sci Int*, 2013, 228(1-3)176.e1-e6

275. McLaughlin G, Lednev IK, Potential application of Raman spectroscopy for determining burial duration of skeletal remains, *Anal Bioanal Chem*, 2011, 401(8): 2511-8

276. Howes JM, Stuart BH, Thomas PS, Raja S, O'Brian C, An investigation of model forensic bone in soil environments studied using infrared spectroscopy, *J Forensic Sci*, 2012, 57(5): 1161-7

277. Patonai Z, Maasz G, Avar P, Schmidt J, Lorand T, Bajnoczky I, Marl L, Novel dating method to distuingish between forensic and archaeological human skeletal remains by bone mineralization indexes, *Int J Leg Med*, 2013, 127(2): 529-533

278. MacLaughlin-Black SM, Herd RJ, Willson K, Myers M, West IE, Strontium-90 as an indicator of time since death: a pilot study investigation, *Forensic Sci Int,* 1992, 57(1): 51-6.

279. Swift B, Dating human skeletal remains: investigating the viability of measuring the equilibrium between 210Po and 210Pb as a means of estimating the post-mortem interval, *Forensic Sci Int*, 1998, 98(1-2):119-26

280. Neis P, Hille R, Paschke M, Pilwat G, Schnabel A, Niess C, Bratzke H, Strontium90 for determination of time since death*, Forensic Sci Int*, 1999, 99(1): 47-51

281. Swift B, Lauder I, Black S, Norris J, An estimation of the post-mortem interval in human skeletal remains: a radionuclide and trace element approach*, Forensic Sci Int*, 2001, 117(1-2): 73-87

282. Schrag B, Uldin T, Mangin P, Froidevaux P, Dating human skeletal remains using a radiometric method: biogenic versus diagenetic 90Sr and 210Pb in vertebrae, *Forensic Sci Int*, 2012, 220(1-3): 271-8

283. Yoshino M, Microscopic and chemical examinations for estimating the time since death of skeletal remains its application to forensic cases, *Nihon Hoigaku Zasshi*, 1991, 45(5-6): 379-92

284. Trueman CN, Martill DM, The long-term survival of bone: the role of bioerosion. Archaeometry, 2002, 44 (3): 371-82

285. Hedges REM, Millard AR, Measurements and relationships of diagenetic alteration of bone from three archaeological sites, *J Archaeol Sci*, 1995, 22(2): 201-9

286. Weber K, Die Anwendung der Chemilumineszenz des Luminols in der gerichtlichen Medizin und Toxikologie, *Deutsch Zeitschr Gesamtegerichtl Med*, 1966, 57(3): 410-23

287. Calcagnile L, Quarta G, Cattaneo C, D'Elia M, Determination of the radiocarbon content in different human tissues: implications for the application of the 14c bomb spike dating in Forensic Medicine, *Radiocarbon, University of Arizona*, 2013, Vol. 55 Nr. 2-3, 1845-9

288. Quarta G, Butalag K, Calcagnile L, D'Elia M, Arthur P, Tinelli M, Caramia A, IBA analyses of major and trace elements of AMS 14C dated bones from two medieval sites in Italy, *Nuclear Instruments and Methods in Physics Research B*, 2008, Vol. 266 pp. 2343-7

289. Stuiver M and Polach HA, Discussion: Reporting of 14C data, *Radiocarbon*, 1977, 19(3):335-63

290. Haynes CV, Bone organic matter and radiocarbon dating in Radiocarbon Dating and Methods of Low Level Counting, *International Atomic Energy Agency*, Vienna, 1967, pp. 163–8

291. Olsson IU, Farid M, El-Daoushy AF, Abdel-Mageed AI, Klasson M, A comparison of different methods for pretreatment of bones, *Geol. Forensics* (Stockholm), 1974, 96(2):171–81

292. Hoke N, Burger J, Weber C, Benecke N, Grupe G, Harbeck M, Estimating the chance of success of archaeometric analyses of bone: UV-induced bone fluorescence compared to histological screening, *Palaeogeogr Palaeoclim Palaeoeco*l, 2011, 310:23-31

293. Lynne S, Bell, Mark F, Skinner, Sheila J, Jones, The Speed of Post Mortem Change to the Human Skeleton and Its Taphonomic Significance*, Forensic Sci Int*, 1996, 82: 129-40

294. Quickenden TI, Creamer JI, A study of common interferences with the forensic luminol test for blood*, Luminescence*, 2001, 16: 295–8

295. Creamer JI, Quickenden TI, Apanah MV, Kerr KA, Robertson P. A comprehensive experimental study of industrial, domestic and environmental interferences with the forensic luminol test for blood*, Luminescence*, 2003, 18: 193-8

296. Galloway A, (Ed), Broken Bones: Anthropological Analysis of Blunt Force Trauma, *Charles C Thomas, Springfield*, 1999,

297. Symes SA, L'Abbe´EN, Chapman EN,Wolff I, Dirkmaat DC, Interpreting traumatic injury to bone in medicolegal investigations, in: Dirkmaat DC (Ed), A companion to forensic anthropology, *Wiley-Blackwell, Chichester*, 2012, pp. 340–89,

298. Dirkmaat DC (Ed), A companion to forensic anthropology, *Wiley-Blackwell, Chichester*, 2012,

299. Klotzbach H, Delling G, Richter E, et al., Post-mortem diagnosis and age estimation of infants' fractures, *Int. J. Legal Med*, 2003, 117: 82–9

300. Gaudio D, Di Giancamillo M, Gibelli D, Galassi A, Cerutti E, Cattaneo C, Does actually Cone Beam ameliorate stab wounds analysis in bone?, *Int J Legal Med*, 2013, 128 (1): 151-9

301. Yang F, Jacobs R, Willems G, Dental age estimation through volume matching of teeth imaged by cone-beam CT, *Forensic Sci Int* , 2006, 159(1):78–83

302. Von See C, Bormann KH, Schumann P et al, Forensic imaging of projectiles using cone-beam computed tomography. *Forensic Sci Int*, 2009, 190(1–3):38–41

303. Lee WJ, Wilkinson CM, Hwang HS, An accuracy assessment of forensic computerized facial reconstruction employing cone-beam computed tomography from live subjects, *Forensic Sci*, 2012, 57(2): 318–27

304. Murphy M, Drage N, Carabott R, Adams C, Accuracy and reliability of cone beam computed tomography of the jaws for comparative forensic identification: a preliminary study, *J Forensic Sci*, 2012, 57(4): 964–8

305. Cappella A, Castoldi E, Sforza C, Cattaneo C, An Osteological revisitation of autopsies: comparing anthropological findings in exhumed skeleatons to their respective autopsy reports in seven cases, *Forensic Sci Int*, 2014