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Dry Bone Histology

Technicalities, diagnostic value and new applications

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DRY BONE HISTOLOGY
TECHNICALITIES, DIAGNOSTIC VALUE AND NEW APPLICATIONS

Proefschrift

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de graad van Doctor aan de Universiteit Leiden,
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Dr. V. Soerdjbalie-Maikoe	Nederlands Forensisch Instituut

In the long history of humankind (and animal kind too),
those who learned to collaborate and improvise most effectively have prevailed.

Charles Darwin

Voor mijn ouders

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Chapter 1

GENERAL INTRODUCTION

Physical anthropology, forensic anthropology and palaeopathology

Physical anthropology (also known as biological anthropology) is the academic discipline that studies man as a biological species. As such it studies human origin, evolution and variability, the latter both in relation to time and space (geography). For comparative reasons, physical anthropologists sometimes extend their research to other mammals, primarily primates.

Physical anthropologists work in all scientific fields in which human anatomy, variability and adaptation are central themes. Well-known examples are palaeoanthropology, which studies human evolution, and comparative anatomy, which compares human anatomy with that of other mammals. Also human growth and development, as well as geographical phenotypical variation and sexual dimorphism (i.e. the anatomical differences between males and females) are part of the physical anthropological domain. The research in this thesis focuses on two subspecialties of physical anthropology: forensic anthropology and palaeopathology. Due to the wide scope of the discipline, the contemporary physical anthropologist can be found as a colleague among archaeologists, biologists and medical doctors.

Forensic anthropologists apply physical anthropological knowledge in a forensic setting (e.g. Reichs, 1998). An example hereof is the identification of human remains that are unsuitable for 'regular' identification methods such as facial recognition, odontology, finger printing or straightforward DNA-profiling. In such cases, forensic anthropologists use knowledge on human anatomical variation with regard to sex, age at death, stature and ancestry to define a biological profile. This may lead to a positive identification. Because of their specialized knowledge, forensic anthropologists are often asked to participate in 'disaster victim identification' (DVI) units, in which they help to identify for instance airplane crash victims. Furthermore, they are often requested to assist forensic pathologists and scene of crime officers by analyzing decomposed or cremated human remains related to an unnatural death. In those cases, thorough knowledge on anatomical and pathological (including traumatological) patterns are essential.

Palaeopathology is the study of diseases in ancient remains. Palaeopathologists combine knowledge on human variability, effects of ageing and pathological processes to diagnose diseases in skeletonized or mummified remains. Subsequently, demographic knowledge on the individuals is used to formulate epidemiological

hypotheses. By diagnosing and studying diseases in the past, palaeopathologist not only provide a valuable glimpse into the living conditions of past populations, but they also improve our understanding of disease mechanisms (e.g. Ortner, 2003). This in turn might aid in managing disease in the present and future (e.g. Donoghue *et al*. 2004, Halperink, 2004). Although palaeopathology may also encompass diseases in animals, this thesis focuses on human palaeopathology.

Forensic anthropologists and palaeopathologists differ with regard to the context in which they operate (i.e. forensic versus historical), but they share a common ground. Both work with human remains, often in an advanced state of decomposition. The remains may be completely skeletonized, incomplete or commingled. Sometimes they are combusted or even deteriorated to a degree almost beyond recognition. Secondly, both share a focus on anatomical (age at death, sex, stature, ancestry, anomalies) and pathological (disease, trauma, cause of death) features. Lastly, both are frequently confronted with a general lack of objective contextual information.

Since forensic anthropologist and palaeopathologists encounter the same challenges, approaches and applied methods are often very similar. The principles used for age at death and sex assessment are for instance the same. This also holds for methods on the identification of diseases or on the analysis of trauma. Advancements in forensic anthropological methods have therefore often a direct bearing on palaeopathological practice and vice versa. This can be illustrated by the use of the same scientific journals and the attendance at the same scientific conferences. Nevertheless, only a few workers practice both disciplines in combination. The situation in the Netherlands is no exception thereof.

Due to analytical limitations associated with forensic anthropological and palaeopathological research, workers combine multiple methodological approaches to increase the reliability of their diagnoses. The research in this thesis centers around one of those diagnostic modalities: the use of the light microscope. To understand its potential we will review the histology of bone tissue in short.

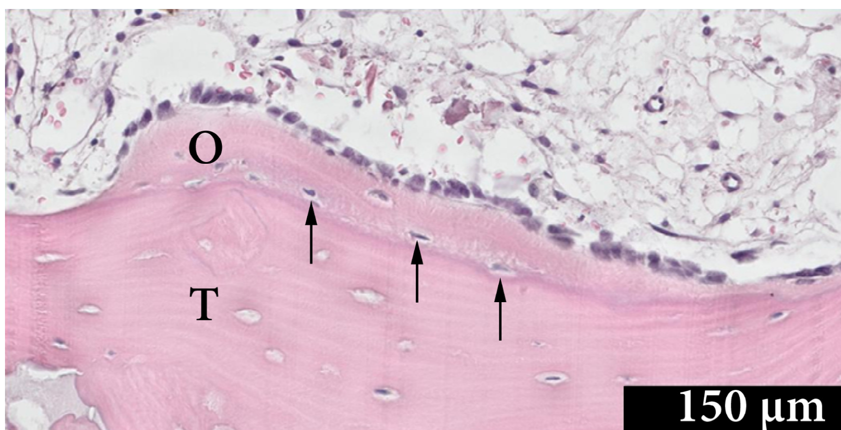
Bone tissue histology

Bone tissue is not a static entity but a metabolically active tissue, constantly communicating with its surroundings and adapting to the physical demands. Julius Wolff already identified this relation in the 19th century when studying trabecular bone morphology. He came to the conclusion that bone tissue has a ‘well motivated architecture, which grants every one of its trabeculae a mechanical meaning […] as a building block […] in the grand structure that is the bone’ (Wolff, 1870). This insight

was eloquently conmemorated and summarized by Paul Ernst-Heidelberg in 1931 when he stated that 'Struktur [verhält zich] zu Funktion wie Form zu Kraft. […] Es besteht zwischen Struktur und Funktion ein Abhängigkeitsverhältnis zweier veränderlicher Größen, demzulfolge eine Veränderung der einen Größe eine Veränderung der andern bedingt, wodurch über ihr Kausalitätsverhältnis nog nichts ausgesagt ist' (Ernst-Heidelberg, 1931). Harold Frost further refined and elaborated on this idea in the 'Utah paradigm of bone physiology' (Frost, 1960; 2000). In summary the basic idea was unchanged: bone morphology is regulated by, and thus a direct effect of biomechanical demands. The underlying regulatory mechanisms are complex and not fully understood, and are beyond the scope of this thesis. Still, bone tissue growth and adaptation is primarily a cellular and microarchitectural one. A major role is reserved for the three types of bone tissue cells. These cells and their main functions will be introduced shortly. For a more detailed description the reader is referred to a general histology book (e.g. Ross *et al.*, 1995).

Bone tissue is formed by osteoblasts, mononuclear cells from the fibroblast cell lineage (Fig. 1). Osteoblasts produce an extracellular bone matrix progenitor called osteoid, which is subsequently mineralized by the same osteoblasts. After

Figure 1.

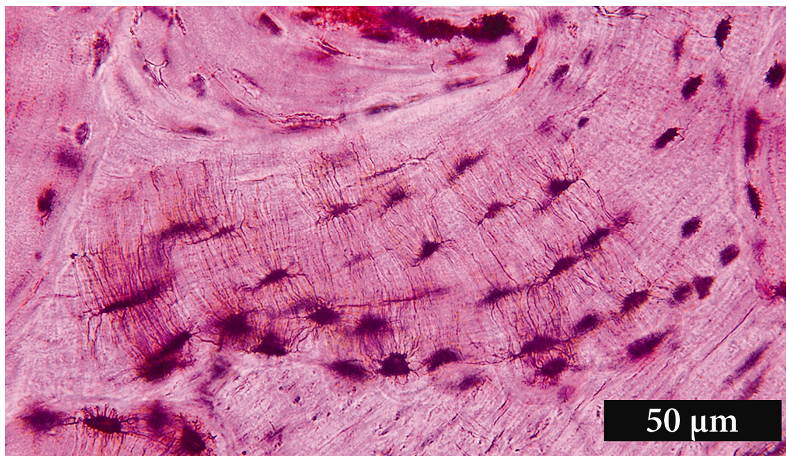


Decalcified section of fresh human bone trabecula, stained with haematoxylin and eosin. Bar indicates size. The trabecula (T) is covered with osteoid (O). The osteoid was produced by the lining string of osteoblasts. Apparently, a number of osteoblasts have become incorporated by their own product (arrows). After mineralization of the surrouding osteoid, these osteoblasts differentiate into osteocytes. They reside in savings called osteocyte lacunae.

mineralization the bone tissue matrix is 'ready', for instance for mechanical loading and metabolic demands. Due to their matrix production, osteoblasts become incorporated within their own product. Once incorporated, osteoblasts differentiate into osteocytes. Osteocytes reside in (matrix) lacunae and communicate with each other by their surrounding cellular processes that project through the matrix in so-called 'canaliculi' (i.e. little canals, see Fig. 2). One of the main functions of the osteocytes is the monitoring of their surrounding bone tissue matrix. As such, they play a major role in monitoring bone turnover (see below), whereas recent results suggest they also play a role in maintaining endocrine homeostasis (Noble, 2008). Bone tissue is resorbed by osteoclasts. These are polynuclear cells from the mononuclear phagocytic lineage. Once activated, these multinuclear cells adhere to the bone matrix surface and excrete erosive hydrogen ions. As a result, the adjacent bone tissue dissolves, creating a resorption bay (Fig. 3). Such a resorption bay is called a 'Howship's lacuna', named after the 19th century British anatomist John Howship.

In mature trabecular bone, resorption and apposition take place at the trabecular surface, while in mature cortical bone they mainly take place by means of 'basic multicellular units' (BMUs). A BMU is essentially a drilling and filling unit that

Figure 2.

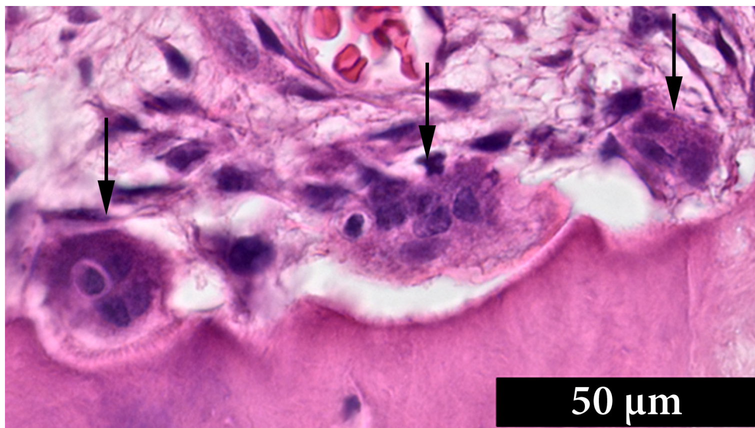


Undecalcified ground section of human dry bone, stained with haematoxylin and eosin. Due to the accumulation of grinding debris in the remaining space after cell decomposition, osteocyte lacunae and their interconnecting canaliculi become clearly visible. During life, the canaliculi contain cellular projections by which the osteocytes monitor their surroundings.

transverses through cortical bone, remodeling it on the way. At the front end of the unit, osteoclasts reabsorb bone tissue. The interior of the thus extending tunnel is subsequently lined by osteoblasts, which deposit and mineralize osteoid in a lamellar way at the rear end. The result is a 'stave' of bone tissue, composed of concentrically deposited lamellar bone fibres, called a Haversian system (or osteon). The Haversian system is essentially a thick walled cylinder of 'new' bone with a narrow central canal, the Haversian canal. The latter contains blood vessels and a little connective tissue. In a transverse microscopy section, the perimeter of a Haversian system shows a cement line, which demarcates the end of the initial osteoclastic resorption. In a normal situation, bone tissue is continuously replaced by newly formed bone. This balanced resorption and apposition process annually replaces approximately 10% of the total bone mass, thus gradually increasing the number of Haversian systems during life.

The degree of osteoclastic and osteoblastic activity is tightly regulated by many factors, such as by hormonal changes (e.g. by the parathyroid hormone), electrolyte changes (e.g. of calcium and phosphate) and mechanical changes (see e.g. Vigorita, 2007). Osteoblastic and osteoclastic activity can also be affected by pathological conditions, such as by trauma, vitamin deficiencies or hormonal imbalances.

Figure 3.



Polynuclear osteoclasts, adherent to a bone trabecula (arrows). By secreting erosive substances, the osteoclasts resorb the underlying bone tissue. This creates scalloped reabsorption pits, so-called 'Howship's lacunae'. Decalcified section of fresh human bone, stained with haematoxylin and eosin.

Irrespective of the causal factor, bone tissue reaction is limited to an osteoblastic, osteoclastic and osteocytic response. With these mechanisms in mind, the potential of the light microscope for the investigation of bone tissue can be understood.

Histology of dry bone tissue in forensic anthropology and palaeopathology

The potential of microscopic investigation of dry bone tissue did not remain unnoticed by forensic anthropologists and palaeopathologists. After a somewhat hesitant start, the use of histology gradually increased over the past decades and is nowadays applied for a variety of purposes. An example is the study of microarchitectural differences to differentiate between human and animal bone (e.g. Hillier and Bell, 2007; Cuijpers, 2009). Another application is the use of the life-long remodeling process and its resulting increase in Haversian systems within cortical bone to estimate the age at death of individuals (e.g. Kerley, 1965; Stout and Simmons, 1979; Maat *et al.*, 2006). The histological research on the gradual decomposition of human bone by soil, ground water, insects and microbes became applicable to assess the postmortem time interval of interred and non-interred corpses (e.g. Hedges *et al.*, 1995; Jans *et al.*, 2004).

Such examples illustrate that histology can be a useful and valuable tool in the study of human remains. But, like all methods, microscopy has its diagnostic limitations and technical challenges. Many workers, unfamiliar with the microscope as a tool, assume that histological research is expensive, difficult and probably of little additional value to gross anatomical and pathological study. Yet, this thesis will focus on these principal aspects to demonstrate the various methodological approaches, as well as the potential and restrictions of dry bone histology.

Part 1: Technicalities associated with dry bone histology

If a pathology laboratory produces histological sections of 'fresh' bone tissue, the material is decalcified and in most cases paraffin-embedded, after which it is sectioned with a microtome. Such an approach is unsuitable for dry bone material, as decalcification will result in the total dissolving of the specimen. The production of sections of undecalcified dry bone material is problematic, since its hardness and brittleness will cause it to shatter upon direct sectioning. Several costly and time consuming methods have been proposed to overcome these problems, but they were scarcely accepted (e.g. Xipell *et al.*, 1974, Wallin *et al.*, 1985). The introduction of a

‘cheap and quick’ method by Maat *et al.* in 2001 overcame a great deal of the objections and made histological research of dry bone more accessible to all (Maat *et al.*, 2001).

Chapter 2 of this thesis will focus on an useful addition to this latter method, viz. the use of histochemical staining. Traditionally, forensic anthropologists and palaeopathologists produce and perform their investigations on unstained bone sections. This in contrast to pathologists, who always apply stainings to improve the visibility of microarchitecture and cells. We will discuss the potential of histochemical staining of dry bone, and propose a relatively easy method to stain undecalcified dry bone sections.

Chapter 3 tackles a ‘shortcoming’ of Maat’s method, as it was thought to be less suitable for the production of sections of fragile/trabecular bone material (Beauchesne and Saunders, 2006). Several earlier approaches to handle extremely fragile bone tissue were proposed, but their production times and costs appeared to run out of proportion (e.g. Schultz, 1998). Besides, these methods did not allow for histochemical staining. In this chapter we provide a relatively quick and easy method to produce embedded sections of dry bone, with the optional possibility to apply histochemical stains.

Part 2: The diagnostic value of dry bone histology

The lack of soft tissue in dry bone remains poses a huge problem for diagnosing disorders, since all characteristic soft tissue architecture and cytonuclear characteristics are missing. This problem is further increased by the usual lack of contextual data on the case, such as medical history details, resulting in an ongoing discussion on the diagnostic usefulness of dry bone histology (Waldron, 2009; Weston, 2009; Schutskovski and Fernandez-Gil, 2010; Van der Merwe *et al.*, 2010).

In chapter 4 we briefly review the varying opinions on the value of dry bone histology diagnoses. Then, the existing literature on the histopathological diagnoses of various groups of disorders in dry bone material is reviewed and reflected on in the light of up-to-date knowledge on the pathogenesis of these disorders. By doing so, we aim to define which diseases do or do not have a pathognomonic dry bone histomorphology.

In chapter 5 we summarize these findings and propose, specifically for archaeologists, robust methods for section production and histological age assessment, in order to make dry bone histology more accessible to those less familiar with microscopy.

A recent case of how dry bone histology can aid in palaeopathological and archaeological analysis is illustrated in chapter 6. It describes the investigation of alleged scurvy in crew members of the lost Franklin Expedition of 1845.

Part 3: The use of histology for the detection of features of mechanical injury in dry bone

Traumatic lesions are amongst the most common findings in human archaeological and forensic remains. Consequently, a large body of literature exists on their interpretation. In a substantial number of papers a 'best guess' is made on the time relation between the moment of a traumatic event and eventual death, as it may shed light on to what extend the traumatic event may have affected the individual's life. Usually, lesions are only described as being antemortem, postmortem and -in indifferent cases- perimortem (Lovell, 1997). In antemortem lesions, a further analysis of the bone changes could lead to an estimation of the time laps between the traumatic event and eventual death. This 'posttraumatic time interval' is generally only roughly characterized by mentioning whether the lesion is healing (usually interpreted as a short posttraumatic time interval) or healed (usually interpreted as a long posttraumatic time interval) (e.g. Brickley, 2006). A more detailed 'dating' of a lesion would be desirable, as it would aid in the interpretation of facets such as medical status, medical care and the timing/sequence of multiple traumas. In forensic practice, the interpretation of alleged cases of torture and child abuse would benefit from such a method.

Chapter 7 examines the feasibility and objectivity of a more detailed assessment of the posttraumatic time interval in dry bone tissue. The study extrapolates on recent forensic pathological practice, in which healing features are used as an intrinsic indicator of posttraumatic time interval (Maat, 2008; Maat and Huls, 2010). The study assesses which microscopic and radiologic features are still reliably detectable in dry bone material.

The potential of the approach is illustrated in chapter 8 en 9. Chapter 8 describes the analysis of mechanical traumas in a 19th century mining population from Kimberly, South Africa. Chapter 9 focuses on a selection of mechanical traumas found in soldiers of the army of Napoleon from his 1812 field campaign in Russia.

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Part 1:
Technicalities associated with dry bone histology

Chapter 2

STAINING GROUND SECTIONS OF NATURAL DRY BONE TISSUE FOR MICROSCOPY

From: *De Boer, H.H., Aarents, M.J., Maat, G.J.R., 2012. Staining ground sections of natural dry bone tissue for microscopy. International Journal of Osteoarchaeology 22(4), 379–386.*

Abstract. The histological staining of bone tissue is of great value in identifying structural changes in human bone tissue when examined microscopically, and is therefore routinely used by clinical pathologists. However, for archaeological and other types of undecalcified dry bone tissue, histochemical staining is currently not widely used. The purpose of this paper is to describe a new method developed for the staining of undecalcified archaeological bone which may be a valuable addition to the paleopathological toolkit. Sections were ground by hand and stained with haematoxylin and eosin. The microstructure and architecture of the stained sections becomes clearly visible without compromising total tissue envisioning. In addition, staining enhances differentiation between taphonomically affected and non-affected bone tissue. This paper accordingly describes a reproducible stepwise method for the production of ground stained sections. An additional troubleshooting paragraph discusses the most often encountered problems and provides solutions.

Introduction

Light microscopy has for an extended period of time been an important tool for diagnosing diseases in clinical practice, and is potentially so in identifying palaeopathological changes in archaeological dry bone tissue. Although microscopic assessment of decalcified sections from 'fresh' human bone is routinely done clinically, osteoarchaeologists, paleopathologists and forensic anthropologists have to frequently deal with undecalcified sections from dry skeletonised remains. In contrast to the processing of 'fresh' bone from living or recently deceased individuals, decalcification of dry bone material would destroy the specimen, making diagnosis impossible (Schultz, 2001).

Frost (1958) introduced a quick method for the preparation of thin undecalcified sections for light microscopical investigation of fresh bone tissue. This method is

especially valuable since it does not need any embedding medium, for instance resin, to support the specimen during processing, making it quick and easily accessible. Although Frost developed this approach 'to provide a means for the microscopic observation of bone that appears close to the ideal of observation *in vivo*', it was demonstrated that if adapted for dry bone tissue it is also suitable for the microscopic assessment of osteoarchaeological and forensic remains (Maat *et al.*, 2001, 2006). This was tested and confirmed in 2006 (Beauchesne and Saunders, 2006).

After the adaptation of Frost's method, an increasing number of researchers have used it to extend their arsenal of diagnostic tools. For instance the method of using polarization filters on bone sections to envision bone fiber direction is now easily accessible. Nonetheless, after publication of Frost's method and its reintroduction by Maat *et al.* (2001, 2006), very few researchers explored the potential of this method from a histochemical point of view. This is peculiar, since staining of dry bone sections may be of potential great importance in diagnosing pathologic changes in bone, or in enhancing/replacing the current methods. For example, the use of polarized light gives information on tissue fiber direction, but only does so with respect to fifty percent of section surface. The other half is more or less 'invisible' due to the 'Maltese crosses' that show in Haversian systems (Bromage, 2003). Therefore an incomplete image of the section is visible. Secondly, the use of polarized light can be confusing, since it enhances tissue fiber direction, irrespective of tissue histochemistry. The abovementioned set-back holds also for a red quartz 'hilfsobject'. Histochemical staining is a valuable addition to microscopy in paleopathology since it should result in total and complete envisioning of tissue architecture and fiber orientation and could thus provide additional information in comparison to unstained sections.

Extensive literature research surfaced only a few papers on efforts of dry bone staining technology. In the few cases where a histochemical staining on natural dry bone tissue was used, the description of the applied protocol was either unclear or lengthy embedding of the specimen was needed. (Bain *et al.*,1990; Bancroft and Stevens, 1996; Hahn, 1991; Hermann, 1993; Romeis, 1989; Stout, 1976; Villanueva, 1974; Watanabe, 1998). Some authors argued if the staining of dry bone was even feasible (Locke, 2004). Therefore, a simple and robust protocol for the staining of unembedded ground sections of undecalcified dry bone tissue will be described for general histomorphological use. The section preparation method of Maat *et al.* (2001) will be the basis of this method, since it does not make use of any embedding resins that could hamper the impregnation with dye. Hematoxylin-eosin (HE) staining was chosen for its clear and all-round tissue differentiation (Ross, 2006). In addition, HE can be used as a counter staining to other more specific stainings. Hopefully, this

stepwise manual for the staining of undecalcified dry bone tissue will further expand the diagnostic power of the toolkit of paleopathologists.

Materials

Bone material

Ten dry human femora and three humeri of adults were randomly chosen from the archaeological depot of the Leiden University Medical Centre (LUMC). The samples were taken from 13 different individuals that were buried during the 13th-17th century in the city of Delft, The Netherlands. All bones were excavated at the end of the twentieth century. In addition to these bone specimens that did not show any pathology, we used a healed fractured femur of an adult to show the additional value of staining from a paleopathological point of view. During life, the traction of thigh muscles had made the lower part of the femoral shaft translocate laterally to the upper part, where it became fixed by callus formation. The microscopy sections made from this femur encompass shaft and external callus.

In our sample preservation phases varied between phase 1 (strong complete bone) and 2 (fragile bone). All preservation phases in this article refer to the standards of Gordon and Buikstra (1981). No demographic data of the originating individuals was known.

Equipment

Equipment as listed in the 'manual for the preparation of ground sections for the microscopy of natural bone tissue' (Maat *et al.*, 2001, 2006) was used, with the addition of the following:

- Waterproof abrasive paper for a second smoothening of the sections (grit P1200A; Klingspor Germany®).
- Four 500 cc plastic specimen containers.
- A small soft artist's paint brush.
- Small perforated plastic section holders (Klinipath B.V.; Nr. 2020).
- Perforated porcelain staining cups (W. Haldenwanger, Technische Keramik, Gmbh & CoKG; Tiegel 82a-nr. 3; Berlin, Germany).
- Two glass funnels and two sheets of filtration paper.
- Haematoxylin (Merck®; Darmstadt, Germany). Solution according to Mayer (Romeis, 1989).
- Eosin Y Solution: 20 grams of Eosin Y dissolved in 192 ml demineralized water, 710 ml ethanol 100% and 100ml saturated picric acid solution.
- Demineralized water.

- Formalin solution 4%.
- Aquatex®, an aqueous medium for mounting the sections only stained with haematoxylin (Merck®; Darmstadt, Germany).

Methods

In the following description we provide a manual for the preparation and subsequent staining of dry bone tissue. Since the use of eosin is only functional in bone sections containing living cells, the manual provides a method both for staining sections by haematoxylin only and by haematoxylin and eosin. A great deal of these two staining methods is the same and therefore the instructions are integrated.

Although the entire production of the stained section takes a time span of roundabout two weeks, the actual time spent on the handling of the sections is less than two hours. The preparation of the ground sections themselves is dealt with in the manual of Maat *et al*. (2001, 2006). Thus steps that are exactly the same are only mentioned in brief. If detailed knowledge on the grinding process is wanted for, a manual can be ordered from Barge's Anthropologica. Throughout the preparation, it is very important to handle and transport the section with care, preferably only using a small painter's brush. The moist section will easily adhere to the brush.

Figure 1.

A 2 mm thick section is taken out of the diaphysis of the femur. Two parallel cuts transverse to the axis of the shaft are made with a hacksaw. The section can be easily taken out of the shaft by breaking its attachment by bending it sideways with a knife. It is not necessary to cut a wedge or to cut the femur in half.

1. Use a hacksaw to cut sections of approximately 2 mm from the desired part of the bone (Fig. 1). Remove any dirt or sand with a soft brush.

2. Grind the section by hand to the desired thickness using tap water and waterproof abrasive paper of a coarseness of grit P220. A sheath of the abrasive paper is stuck on a Vaseline® buttered thick glass slab to function as a grinding platform. It is very important to regularly turn the section and to frequently check the evenness of the section.

3. Rinse the section gently using tap water and a soft painter's brush.

4. After initial grinding, polish the section using tap water and waterproof abrasive paper of lesser coarseness (P1200A) than at step 2. This abrasive paper is attached and moistened as mentioned in Maat's manual. The polishing is done by grinding the section in a circular motion with your bare index and middle finger without applying pressure. It goes without saying that both sides of the section must be polished.

5. In contrast to the suggestion in the manual of Maat, the resulting thin section is not dehydrated using ethanol. Instead, without allowing the section to dry, it is thoroughly cleaned in a series of four 500 cc plastic containers filled with 400 cc of demineralized water. In the first two of these containers a droplet of detergent is added to prevent brushed-off dirt from surfacing and reattaching to the section surface during transportation between containers. Two small painter's brushes are used for transportation and cleaning: one is used to hold the section in place, whilst the other is used to gently brush off the immersed section.

6. Subsequent to cleaning and again without allowing the section to dry, the sections are fixated in a formalin solution (4%) for a week. The formaline solution is used to coagulate any remaining protein to ensure for an evenly stained section. For the use of formalin a fumehood is mandatory.

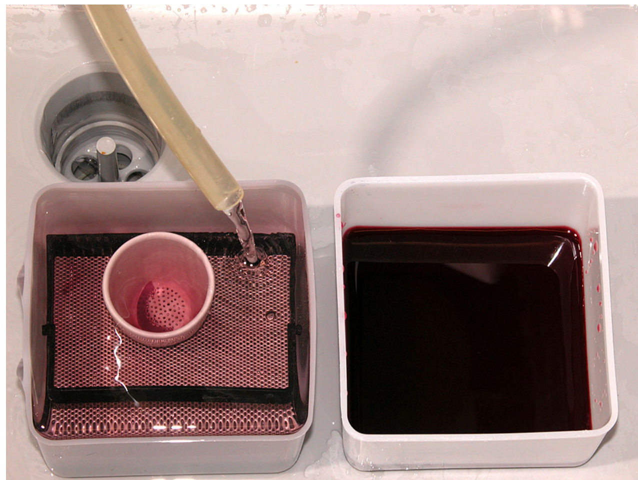
7. The section is placed in a small perforated plastic section holder and is rinsed overnight, using gently running tap water. The water jet must not be aimed directly onto the section. After rinsing overnight, the section is ready for staining. Bear in mind that between staining and rinsing, the section does not

dry. If necessary, the section can stay for an unlimited time submerged in demineralized water.

8. For staining, a volume of 100 cc of the standard haematoxylin solution according to Mayer (Romeis, 1989) is diluted with demineralized water to 25% of the original solution of the recipe. The diluted haematoxylin must be filtered by means of a funnel and folded filtration paper. The filtered solution (400 cc) is collected in a 500 cc plastic container. Be sure to filter the stain just prior to proceeding, hereby diminishing the chance that any airborne dust will contaminate the stain. If the solution is to be stored, it also must be filtered after staining. The section itself is placed from the perforated plastic section holder into a perforated porcelain staining cup by means of a soft painter's brush. Staining of multiple sections is possible. In that case, each section should have its own staining cup but alteration of staining times or volumes is not needed.

9. The section stays in haematoxylin 25% for 4 minutes. During staining, the porcelain cup is moved a few times in a gentle way to allow for an evenly distribution of stain throughout the section.

Figure 2.



Rinsing the section for ten minutes with running tap water after staining with the diluted haematoxylin solution. The water jet should not be aimed directly on the section in the porcelain staining cup. To guarantee a proper rinsing, the cup must be stirred mildly within the container a few times.

10. After staining, the porcelain staining cup is placed in an empty 500 cc plastic container and is rinsed under gently running tap water for 10 minutes (Fig. 2). The porcelain cup is moved in a gently stirring motion for a few times during rinsing. If an additional staining with eosin is needed, 400 cc of eosin can be filtered in the meantime. As with the haematoxylin, it is done with a glass funnel and filtration paper. The filtered eosin is collected in a third 500 cc plastic container.

11. In addition to the first rinsing, the porcelain staining cup containing the section is now placed in a fourth 500 cc plastic container filled with 400 cc of demineralized water and is moved gently with a stirring motion for 1 minute. Subsequently, all excessive haematoxylin is removed. If an additional staining with eosin is needed, skip step 12 and proceed with step 13. When the haematoxylin-stain is considered sufficient, continue and end with step 12.

12. The stained section is now ready for mounting on a microscopy glass slide holder with Aquatex® and a glass cover slip. The section is mounted as quickly as possible as it must not be allowed to dry. The Aquatex® is left to dry in a horizontal position for half an hour. After this short period of time, the section is ready for inspection with a light microscope. If the mounted section is to be stored, the edges of the glass cover slip must be sealed with Entalan® to avoid a reaction between the Aquatex® and airborne water and to increase the shelf-life of the stain. To allow the Entalan® and Aquatex® to dry, the sealed section must dry horizontally for a week. Any mounting medium with similar chemical characteristics can be used, but this was not tested by the authors.

13. The rinsed section in the porcelain cup is placed in the filtrated eosine solution for 90 seconds. Also in this step, the gentle stirring motion in which the porcelain cup is moved allows for a proper and evenly distribution of the stain. The eosine solution can be stored if needed. In that case, filtration of the solution after staining is required.

14. After this second staining, the section is rinsed in demineralized water in a fifth 500 cc plastic container for 30 seconds, also whilst the cup is gently moved in a stirring motion.

15. For dehydration, the stained section, still kept in the porcelain staining cup, is led through two containers filled with ethanol (100%) for five minutes each. Excessive eosin will blush the ethanol, but this is considered to be normal and does not interfere with staining quality of the section.

16. To facilitate immersion of the section with the mounting medium Entalan® (step 16) the porcelain staining cup and its content is led through three containers with xylene for five minutes each. During these fifteen minutes, preparations for the mounting of the section can be made.

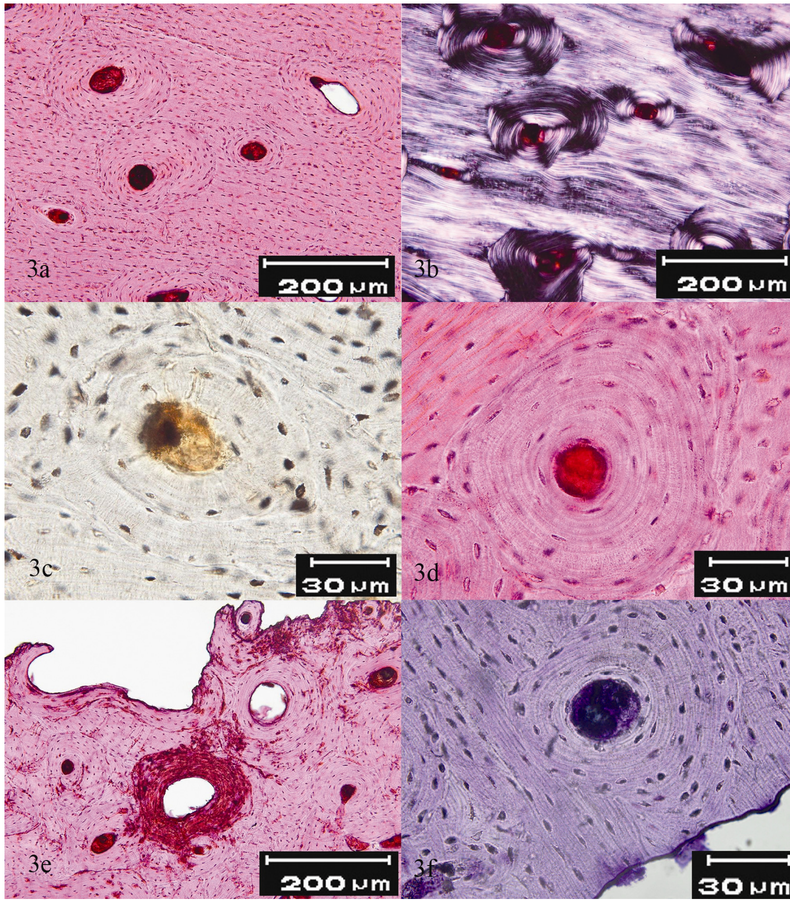
17. The section is mounted on a microscopy glass slide holder and is allowed to dry for a week.

Results

By using the abovementioned procedure, satisfactory stained sections are produced within two weeks of time. In the sections without pathology, an adequate staining without any artefacts is achieved (Fig. 3a). At the same time, the sections remain ready for analysis with polarized light (Fig. 3b). Staining provides a better differentiation between the different histological elements of bone material in comparison to unstained sections (Figs. 3c and 3d). Details can be better outlined and distinguished and all histological elements of bone material, e.g. Haversian and non-Haversian bone, osteocyte lacunae and Volkmann's canals are clearly visible under bright field microscopy. Cement lines between the Haversian systems and even canaliculi radiating from the lacunae can be seen. In some sections taphonomic changes like tunneling from bacteria or fungi may be present (Fig. 3e). In those cases the related amorphous tissue appears dark red, whereas the not-affected bone is brightly stained making differentiation between affected bone and not-affected bone easier. The differences in staining intensity is due to the capillary action of the dye in the tunnelings made by the microorganisms. In the sections stained by haematoxylin only, the separate lamellae are even better distinguishable than in those stained by both haematoxylin and eosin (Fig. 3f). Results were consistent throughout all used femora and humeri.

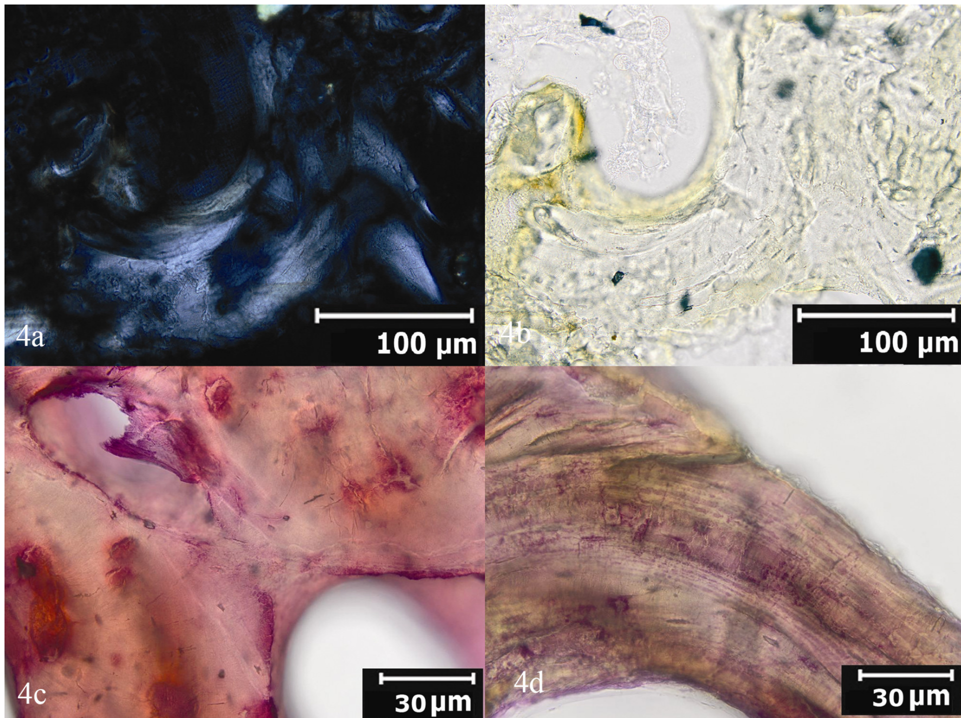
Abovementioned results were again noted in stained sections of the cortex and its adjacent external callus of a healed fractured femur though in these and in unstained sections of the fracture, abundant taphonomical alterations hampered visibility (Fig. 4a, 4b, 4c, 4d). Where tissue architecture was less profoundly altered by taphonomy, tissue architecture appeared better visible due to staining.

Figure 3.



(3a) Micrograph of a transverse section of an undecalcified human femur, preservation phase 1. The specimen was taken from the diaphysis. Staining: diluted haematoxyline solution and eosine (see text). Bright field. **(3b)** Same area as shown in figure 4a, but now viewed through polarizing filters. The fiber direction is emphasised due to anisotrophy of the different bone lamellae. The osteocyte lacunae can be seen as little purple spots between the lamellae. **(3c)** Micrograph of an unstained transverse section of an undecalcified archeological sample from a human femur. Preservation phase 1. Bright field. Haversian system with adjacent lamellar bone. The border between the different histo-architectual types of bone is hardly distinguishable. Bone lamellae are not easy demarcated. Osteocyte lacunae are visible as colorless oval structures. The lacunae that are filled with amorphous dust from the grinding process are black. **(3d)** Micrograph of a detail of a transversal section of an undecalcified human femur, stained with the diluted haematoxylin solution and eosin. Preservation phase 1. Bright Field. Cement lines are clearly visible at the edge of the Haversian system. Osteocyte lacunae are easily noticeable. The orientation of the bone lamellae both in the Haverisan system as well as of the surrounding interstitial bone is well distinguishable. **(3e)** Micrograph of a transverse section of a human femur, stained with the diluted haematoxylin solution and eosin. Preservation phase 2. Bright field. Taphonomic tunneling is clearly visible within the Haversian system in the centre of the micrograph. The process has affected a Haversian system in total. **(3f)** Micrograph of a detail of a transverse section of human a femur, stained with the diluted haematoxylin solution. Preservation phase 1. Bright Field. The separate lamellae are well distinguishable without the use of polarized light.

Figure 4.



(4a) Micrograph of a transverse section of a healed fractured human femur. Preservation phase 2. Polarized light. A highly magnified part of the hard callus, adjacent to the outer cortex is shown. Tissue fiber direction is hardly enhanced by the polarized light and the polarizing filters make inspection of only the half of the section surface possible since the other half remains dark. No additional information can be derived from this image in comparison to figure 4b. The blue color is due to the polarizing filters. **(4b)** The same part as shown in figure 4a. Bright field. Osteocyte lacunae are distinguishable as grey oval patches. Bone lamellae and cement lines are hardly/not visible. Taphonomical alteration of the tissue has given the section surface a chaotic and crackled appearance. The yellowish color is due to staining by groundwater and soil. **(4c)** Another part of the same callus as shown in the previous figures, now stained with both haematoxylin and eosin. Preservation phase 2, bright field. Cement lines are seen as white bands between adjacent deposited bone. The visibility of the surrounding bone tissue is blurred since the microscope is focused on the level of the cement lines. Also, taphonomical alteration of the bone makes staining somewhat irregular. **(4d)** Another part of the same callus as shown in figure 4a and 4b, now stained only with a diluted haematoxylin dye. Preservation phase 2, bright field. Although the preservation of this bone hampers microscopic visibility and interacts with the dye, all aspects of the bone lamellae can be clearly seen.

This was especially clear at higher magnifications (Fig. 4b, 4c and 4b). Polarized light gave clear information on fiber direction at low magnifications, but at higher magnifications it no longer enhanced the orientation of tissue fiber (Fig. 4a). Staining

of the sections showed lamellar orientation throughout the unaltered bone tissue at high magnifications (Fig. 4c), but also enhanced fiber structure and direction in those parts of the sections that did not show natural birefringence. Staining also made cement lines visible (Fig. 4c).

Troubleshooting

Fissures and 'craquelé'

If a section appears damaged, a differentiation must be made between coarse superficial scratches, already noticeable at low magnification and a meshwork of microfissures, the so-called 'craquelé' that can only be seen at high magnification. In the case of the large superficial scratches, the most likely cause is too short polishing of the section. It is advised to elongate the second grinding step (step 4) with the abrasive paper of grit P1200. If the superficial scratches maintain nonetheless, they are probably caused by rough handling of the section during cleaning and/or grinding. The recommended handling of the section with only a small painter's brush must therefore be considered mandatory. Even the use of tweezers or sliding the section over a table surface may cause such damage.

Small microfissures (craquelé), are most likely caused by drying strain within the section. Therefore sections must be kept wet from grinding until the eventual mounting on the glass slide. Forced dehydration before staining is unnecessary and harmfull, and may lead to the same craquelé-effect. If the small microfissures remain in spite of abovementioned recommendations, they are probably caused by taphonomic alteration of the bone tissue, as described by Grupe and Dreses-Werringloer (1993).

Dirty sections

Dirty sections can be due to various causes. The most likely cause is the remaining of dust from the grinding process. During microscopy this dust is visible as black or glass-like crystals on the section surface. Since the (grinding) dust is usually highly eosinophilic these improperly cleaned sections will stain irregular. As a solution, it is recommended to elongate step 4. If this does not produce a satisfying result, reattachment of dust from the surface of water is a liable cause. Adding a drop of detergent to the water that is used for cleaning will prevent this reattachment. Keeping the sections submerged in water until staining will prevent any other kind of dirt or dust to attach to the section. If all these precautions do not work, make sure that both your hematoxylin and eosin are filtered properly. If there are indications that the staining solution is dirty, a new one should be prepared. Bear in mind that

some taphonomic changes may resemble dirt. Postmortem fungal invasion for instance appears as dark focal tunneling along the endosteal or periosteal surface, or infiltrates from the Haversian or Volkmann's canals (Hackett, 1981).

Unevenly stained sections

If a section is unevenly stained, one should first assess whether the staining cup was stirred enough during staining. Due to its light weight and thinness, the section has the tendency to stick to the inner surface of the porcelain staining cup. The gentle stirring motion during staining and rinsing usually prevents this from happening. If the section nonetheless sticks to the staining cup, it must be removed with a soft painter's brush. If this is done directly, it is not necessary to alter the given time for staining or rinsing. If staining intensity fades or darkens along the entire periosteal and/or endosteal surface, this is probably the effect of postmortem tissue oxidation. If the aforementioned is not the case, the cause of an improper stain distribution must be sought in the characteristics of the bone tissue itself. Both the unevenly dispersion of remaining marrowfat and other organic substances may cause the section to stain unevenly. Therefore the section must be fixated with the formalin solution for a longer period. The rinsing of the section after fixation (step 7) must then be elongated proportionally. Eventually, an unevenly stained section can also be caused by taphonomical processes. In that case the uneven stain is intrinsic to the altered architecture of the bone.

Discussion

By using the presented protocol it is possible to stain unembedded dry bone tissue within reasonable time in an easy and cheap way. It assures a consistent staining throughout the section. Both information on general bone architecture and histological details can be obtained in a similar or better way than without staining. If desired, one may combine the use of polarized light and histological staining, hereby expanding the amount of information that can be obtained from one section.

The method presented here is new in the field of histological examination of dry bone tissue in several ways. Expensive machinery and labour intensive handling of the section is no longer necessary. For instance, a motorized microtome, an automated grinding system, cleaning by ultrasonic bath or tissue support with an embedding medium is not needed, in spite of suggestions by several authors (Bain *et al.*, 1990; Caropreso, 2000; Mohsin *et al.*, 2006; Sterchi and Eurell, 1989; Wallin *et al.*, 1985; Wong, 1985). Artefacts that are normally the case in microtome-cutted sections (Maat *et al.*, 2001; Schultz, 2001) are no longer an issue. The dehydration process by

ethanol or acetone is not needed in the sections that are only stained with haematoxylin. Thereby strain on the specimen by forced dehydration is avoided. In addition, the use of Aquatex makes it possible to inspect the section already after half an hour.

The additional information that can be derived from stained sections is useful in many ways. Since staining enhances differentiation between different types of bone architecture and simultaneously remains available for inspection under polarized light, it becomes of use for more reliable diagnoses of various diseases. Especially diseases in which bone structure is affected, e.g. remodeling, apposition, lysis or invasive growth are of interest. As illustrated by Figure 4, the better visibility of tissue texture together with the visibilty of cement lines can for instance help examine how bone is deposited after a trauma. Cement lines show resorption depth (reverse lines) and subsequent deposition of bone tissue, which indicates one of the phases of healing.

The taphonomical alterations in Figure 4 show that invasion of microorganisms into the microarchitecture hampers visibility of tissue architecture. The use of stain helps to differentiate between altered and unaltered parts of bone. This may be helpful in assessing the seriousness of taphonomic processes after burial.

Since our staining results stayed consistent while the specimens originated from randomly chosen skeletal elements of different individuals, from different populations and from different periods, we assume that such factors are hardly of any influence on the final results.

Even in spite of the differences in preservation quality between specimens, it was possible to stain satisfactorily by using a standardized method. Theoretically, the staining protocol should also apply to more fragile bone. Of course, because of our grinding method reasonably preserved cortical bone is most suitable (Beauchesne and Saunders, 2006) if it meets the preservation phases 1 or 2 of Gordon and Buikstra (1981).

Like every method, the method stays open to improvement and testing in due time.

Conclusion

Histochemically stained sections of human dry bone tissue can be produced in a quick and easy way. By using a simple haematoxyline or haematoxlyine-eosin stain if compared to viewing with polarized light only, extra information on bone texture can be obtained in comparison to the use of unstained sections. Resulting extended knowledge will be helpful to better corroborate palaeopathological diagnoses.

Staining also improves visualization of taphonomic processes. Stained sections remain available for inspection under polarized light. Staining results showed to be consistent for different types of cortical bone.

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Chapter 3

MANUAL FOR THE PREPARATION AND STAINING OF EMBEDDED NATURAL DRY BONE TISSUE SECTIONS FOR MICROSCOPY

From: *De Boer, H.H., Aarents, M.J., Maat, G.J.R., 2013. Manual for the preparation and staining of embedded natural dry bone tissue sections for microscopy. International Journal of Osteoarchaeology 23(1), 83–93.*

Abstract. In the last decade, the use of light microscopy has been firmly established for the investigation of exhumated human bone tissue. As a rule, these remains cannot be decalcified, thus they are most commonly prepared for microscopic analysis as ground thin sections. These ground sections are of great value in diagnosing disease, for age estimation or in assessing taphonomic alteration. As bone is sometimes fragile and can be damaged by the grinding process, the specimen is occasionally supported by an embedding medium.

In contrast to the vast amount of research conducted on embedded and unembedded unstained bone material, the use of histological stains on undecalcified dry bone tissue has been long neglected. In this article a new method for embedding, sawing and grinding dry bone tissue is presented. The produced sections are subsequently stained with haematoxylin. The results show that even ground sections of fragile bone can be made in a quick and easy manner. Staining these sections enhances the envisioning of microarchitecture and taphonomical processes. In addition, the sections stay open for inspection under polarized light. The results were consistent throughout the used bone material. To keep the method as accessible and comprehensive as possible, a step-wise manual is provided. An additional troubleshooting paragraph discusses the most often encountered problems and provides solutions.

Introduction

In paleopathology, bioarchaeology and forensic anthropology, the past can be reconstructed by investigating human remains. With the exception of a few cases, in

which soft tissue may be preserved, researchers mainly work with skeletonized remains.

The architecture of 'dry' bone is the result of continuous adaptation of the skeleton during life and, as such, provides valuable information about an individual (Frost, 1985). Since most of the changes that shape bone during life act on a microscopic level, light microscopy is one of the most apt instruments to study bone tissue (Frost, 1985). Consequently, researchers have incorporated different methods of microscopic investigations in standard skeletal analyses including light microscopy (Frost, 1985; Stout and Paine, 1992; Weinstein *et al.*, 1981), microradiography (Blondiaux *et al.*, 1994; Stout and Simmons, 1979) and plain microscopy (Cuijpers, 2009; Hanson and Cain, 2007; Hedges *et al*., 1995; Jans *et al*., 2004). Investigations have also utilized different filters, more commonly the polarizing filter (Maat *et al.*, 2006b) and the *quartz hilfs-object* (Schultz, 2001).

When compared to techniques widely used in clinical medical practice, the relative absence of histochemical staining in anthropological investigations is remarkable. In clinical pathology, staining is used on a regular basis to diagnose or enhance tissue architecture. Tissue specimens are usually decalcified and cut by a microtome (Romeis, 1989) or embedded undecalcified and cut with a high-duty microtome (Grill *et al*., 1995; Gruber and Mekikian, 1991; Wallin *et al*., 1985). In those cases in which a grinding technique is used, the resin is removed prior to staining with strong solutes (Derkx and Birkenhager-Frenkel, 1995; Romeis, 1989; Xipell *et al*., 1974).

Archaeological bone is not suited to these kinds of methods for two reasons: first, it cannot be decalcified (Schultz, 2001) and second, it is usually less tough than fresh dry bone tissue. In general, the decalcification and microtome cutting of archeological bone is considered obsolete. More recently, researchers rely on (automated) grinded sections (Garland, 1989; Maat *et al.*, 2001; Schultz, 2001; Stout and Simmons, 1979). If more fragile or trabecular material is grinded, a supporting embedding medium is used (Bancroft and Stevens, 1996; Guarino, 2006; Romeis, 1989; Schultz, 2001). However, the embedding procedure hampers the utility of histochemical staining (Morrow, 2002).

Despite these difficulties, some researchers remained focused on the staining of archeological specimens. Garland, for example, describes several histochemical stains that may be applied on a theoretical basis, but did not include experimental data (Garland, 1989). Some researchers stained dry bone tissue, but did so with the use of an unsuited microtome (Stout, 1976) or they used destructive decalcification (Antonutto *et al.*, 1979; Guarino *et al*., 2000; Morrow, 2002). Methods in which decalcification was not used and the bone material was not embedded appear

unsuitable for fragile bone tissue (De Boer *et al*., 2012; Guarino, 2006; Hermann, 1993). Although each method has its shortcomings, the outcome of the abovementioned efforts suggest that additional value may be gained from histochemistry.

While staining of archeological bone has produced promising results, no procedure has been developed that bypasses all technical problems, such as those by decalcification and microtome cutting. Thus, the implementation of histochemistry within anthropological analyses has remained limited.

This article presents a simple method to produce and stain sections of dry bone tissue to advance histological and histochemical research. In addition, we elucidate the additional value of staining when compared to the sole use of bright field and polarized light.

Materials

Bone material

Thirteen archeological sternums were randomly selected from individuals originating from three different populations (Tab. 1). All samples belonged to the archaeological collection of the Leiden University Medical Centre (LUMC). Preservation phases differed between 1, 2 and 3 according to the standards introduced by Gordon and Buikstra (Gordon and Buikstra, 1981). Phase one represents 'strong complete bone', phase 2 'fragile bone' and phase 3 'fragmented bone'. All sternums were completely fused. Consequently all individuals were at least 14 years of age (Maat and Mastwijk, 2007). No further information on for example sex, age at time of death or pathological conditions, was known.

Equipment

This protocol is an extrapolation on previous work. Therefore, only additions to the previously published manual (De Boer *et al*., 2012) are mentioned. All required equipment is readily available at the local hardware store or in any histology laboratory. For the embedding, sectioning and staining of dry bone tissue the following is needed:

- Electronic balance, for weighing the components of the epoxy resin (e.g. Sartorius MC1 laboratory 2200P, Göttingen, Germany).
- LX-112 epoxy resin embedding kit, containing LX-112 resin, dodecenyl succinic anhydride (DDSA), nodic methyl anhydride (NMA) and dimethyl aminophenol (DMP-30) (Ladd Research, Williston, VT , USA.).
- Four disposable plastic pipettes for preparation and stirring of the embedding medium.

- A glass bottle for containing the prepared embedding medium.
- Ethanol solutions of different percentages (70%, 80%, 90% and 100%) for dehydration.
- Acetone for cleaning tools and table surfaces after working with the epoxy resin.
- A vacuum system for the proper distribution of the epoxy resin within the specimen.
- One preparation needle for positioning the specimen in the embedding medium.
- Plastic embedding containers, one for each specimen. (e.g. Peel-a-Way Scientific South el Monte, CA, U.S.A).
- A stove for polymerization (e.g. Incubat 85, Melag, Berlin, Germany).
- Cyano-acrylate glue (e.g. Pattex Classic®, Henkel, Nieuwegein, The Netherlands).
- A pair of disposable latex laboratory gloves.
- A single-edge razorblade for removing excess glue (e.g. GEM Scientific, West-Yorkshire, UK).

Table 1. Used bone material.

Sternum (no.)	Origin	Burial period	Year of exhumation	Preservation phase[1]
1	Meer en Berg, Santpoort-Zuid, NL[2]	1890-1935	2009	2
2	Meer en Berg, Santpoort-Zuid, NL	1890-1935	2009	2
3	Meer en Berg, Santpoort-Zuid, NL	1890-1935	2009	1
4	Meer en Berg, Santpoort-Zuid, NL	1890-1935	2009	1
5	Meer en Berg, Santpoort-Zuid, NL	1890-1935	2009	2
6	Meer en Berg, Santpoort-Zuid, NL	1890-1935	2009	2
7	Meer en Berg, Santpoort-Zuid, NL	1890-1935	2009	2
8	Meer en Berg, Santpoort-Zuid, NL	1890-1935	2009	3
9	Meer en Berg, Santpoort-Zuid, NL	1890-1935	2009	2
10	Meer en Berg, Santpoort-Zuid, NL	1890-1935	2009	1
11	Meer en Berg, Santpoort-Zuid, NL	1890-1935	2009	2
12	Oude Nieuwe Gasthuis, Delft, NL	1265-1652	1993	2
13	Koningsveld, Delft, NL	1252-1572	2003	2

[1]According to Gordon and Buikstra (1981).
[2] The Netherlands.

Methods

For the development of this protocol we used a systematic trial-and-error method. The protocol is 'stand-alone'. Nevertheless, it is recommended that prior to experimentation a few articles discussing the embedding or grinding of sections are studied (De Boer *et al.*, 2012; Garland, 1989; Maat *et al.*, 2001; Maat *et al.*, 2006a; Schultz, 2001).

Fixation and embedding

1. Clean the specimen by using a dry soft brush. Use a band saw or hacksaw to cut slices approximately 2 cm thick out of the desired part of the cancellous/spongy bone. The maximum size of the specimen depends on the size of the available plastic embedding container. If the specimen is extremely fragile and could damage on contact with the saw blade, consider embedding the entire specimen. If the section is not to be stained, skip step 2 and 3 and continue with step 4.

2. Immerse the specimen in a 4% formalin solution for seven days.

3. Gently rinse the specimen under running tap water for at least 12 hours. Make sure that the water jet is not directly aimed on the specimen, as it may easily damage the exposed trabeculae.

4. Dehydrate the specimen in consecutive ethanol solutions of 70, 80, 90 and 100 percent. Each dehydration step should take least one hour. Longer dehydration does not negatively affect the embedding process. Overnight dehydration is an option.

5. After dehydration, let the specimen air dry for approximately 30 minutes. When entirely dry, the specimen is ready for embedding. It is advisable to embed the specimen immediately after drying in order to prevent airborne water or dust from contaminating the specimen. We found it convenient to prepare the embedding medium during the 30 minute drying period. If using a previously prepared embedding medium, proceed to step 7.

6. When preparing the embedding medium, it is preferable to measure the components gravimetrically rather than a volumetrically, since even a slight deviation from the recipe will seriously affect the elasticity of the embedding

medium and the further handling of the section. An embedding medium that is too hard will shatter or break easily, whilst a medium that is too elastic will not provide sufficient tissue support. Therefore, a highly sensitive balance must be used to weigh the different components. The best elasticity/hardness ratio is reached with the proportions as stated in Table 2. In contrast to the manual of the epoxy resin kit, it is not necessary to make two separate mixes before combining the two. If the embedding medium is used at once and the remaining medium stays deep frozen, all the components can be mixed in one glass container or bottle. First weigh and mix the LX-112 resin, the NMA and the DDSA, each with a new disposable plastic pipette. Stir or shake thoroughly, using one of the plastic pipettes, before adding the epoxy accelerator (DMP-30). When the DMP-30 is added and the entire mixture is stirred thoroughly a second time, the embedding medium is ready for use. Continue with step 8.

7. If a previously made, deep frozen medium is used. It is important that the embedding medium is allowed to reach room temperature before opening the bottle, as this prevents the development of water condensation on the medium. In the meantime, pre-heat the stove to 60°C.

8. Draw a vacuum on the opened epoxy resin bottle to remove any air bubbles caught in the medium during mixing or storage.

Table 2. Recipe for 100 ml of LX-112 embedding resin.

Component	Amount (gr.)
Glycide-ether (LX-112)	56,60 g
D.D.S.A.[1]	31,00 g
M.N.A.[2]	29,20 g
D.M.P.30[3]	1,17 g

[1] Dodecenyl succinic anhydride.
[2] Nodic methyl anhydride.
[3] Dimethyl aminophenol 30.

9. Place the bone specimen in the plastic embedding container with the trabecular surface facing upwards. Keep the specimen separated from the base of the container by using two pieces of a wooden toothpick cut to size. This allows for the proper distribution of medium around the specimen. A previously polymerized layer of embedding medium on the bottom of the embedding container can also be used. Make sure that the specimen is placed in the middle of the container, so it is equally supported at all sides.

10. Gently pour the medium onto the trabecular surface of the specimen. Continue to pour the medium into the plastic embedding container until the specimen is entirely submerged. Next, draw a vacuum over the embedded specimen. The specimen and toothpicks will probably float due to the air trapped between the trabeculae. Reposition the specimen and the toothpicks with the preparation needle.

11. As the viscosity of the medium will prevent some trapped air bubbles from being drawn out of the specimen by the vacuum, place the specimen in the pre-heated polymerization stove for five minutes. The warmth of the stove will lower the viscosity of the medium, allowing for a better distribution.

12. Remove the embedding container from the stove, position the specimen upright and in the centre of the embedding container and quickly draw a vacuum over it while the medium is still warm. This will release excess air from the specimen. After the air bubbles have been released, turn the specimen upside down and repeat the vacuum. Place the embedding container back in the stove for another five minutes and repeat the process of drawing a vacuum and of turning the section until there is no more air left in the specimen. Usually the cycle of heating, drawing a vacuum, turning the section and drawing a second vacuum must be repeated three times to remove all the trapped air.

13. To polymerize the medium, place the container in the stove at 60°C for at least 12 hours. A longer polymerization period does not negatively affect the process.

14. Let the specimen cool. Peel away the plastic embedding mold. Cooling down does not take much time and there is no need to use running water or refrigeration to hasten the process.

15. The encased specimen can be cut in every desired direction by means of a band saw or hacksaw. Make sure that these initial 'thick' slices are not thinner than 1 mm, as this will unnecessarily complicate the grinding process and can eventually result in an uneven or broken section. Do not apply force on the block during sawing, since this may cause the saw to deviate, resulting in a skewed section.

Grinding

16. Grind one side of the thick slice by hand on a moistened piece of waterproof grinding paper with grit P220. When grinding, make sure that the surface of the specimen is entirely smooth and even. For additional information on the grinding method, see Maat *et al.* (2001, 2006a) or De Boer *et al*. (2010).

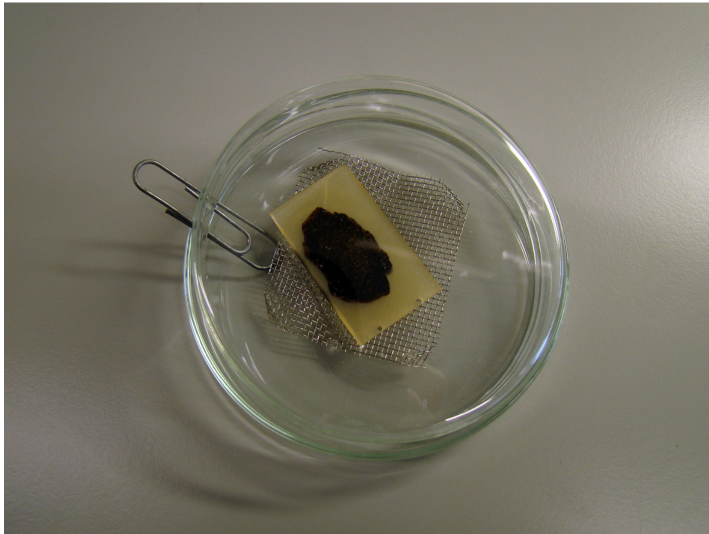
17. After grinding, hand polish the section on a moistened piece of waterproof grinding paper with grit P1200. It is not necessary to apply much pressure, since the polishing is only meant to smoothen the surface, not to make the section any thinner. The best way to assess the thinness and evenness of the section is by inspection under grazing light.

18. Clean the ground and polished surface of the section using three 500 cc containers of demineralized water and a soft painter's brush. Add a drop of detergent to the first container of water. Clean the section by gently brushing it off, leading it through all three containers.

19. Let the section air dry with the smooth and clean surface positioned upwards. For this step we used two Petri dishes and a piece of folded iron meshwork. The two Petri dishes are kept open with a paperclip (Fig. 1), to prevent airborne dust from attaching to the section whilst drying.

20. Subsequent to drying, wipe the specimen surface clean with a clean cotton cloth, moistened with 100% ethanol. Do the same for the surface of a glass microscopy slide holder and let both surfaces dry. We found it convenient to use an oversized glass section holder that provides plenty space for the section, since this will make the following grinding process easier.

Figure 1.



By means of a Petri-dish, a piece of bended iron mesh and a paperclip, a simple device is constructed that enables the section to dry without becoming dusty.

21. Position the glass microscopy section holder on an even table surface or a thick glass slab and place a drop of cyano-acrylate glue with a diameter of ca. 4mm on the clean surface of the holder. Mount the smooth and cleaned surface of the specimen on the microscopy slide holder as if mounting a section with mounting medium (Fig. 2a, 2b and 2c). Be careful to prevent air bubbles from being trapped beneath the section. When the glue is distributed evenly, apply gentle pressure on the specimen to remove any excess glue. Any trapped air bubbles can also be pushed outwards by pressing gently on the middle of the section. Let the glue dry under applied pressure for approximately two minutes.

22. With a tightly fitting latex glove on the dominant hand of the researcher, turn the microscopy slide holder with the attached section upside down and wipe the back with a cloth moistened with 100% ethanol. Let the ethanol dry.

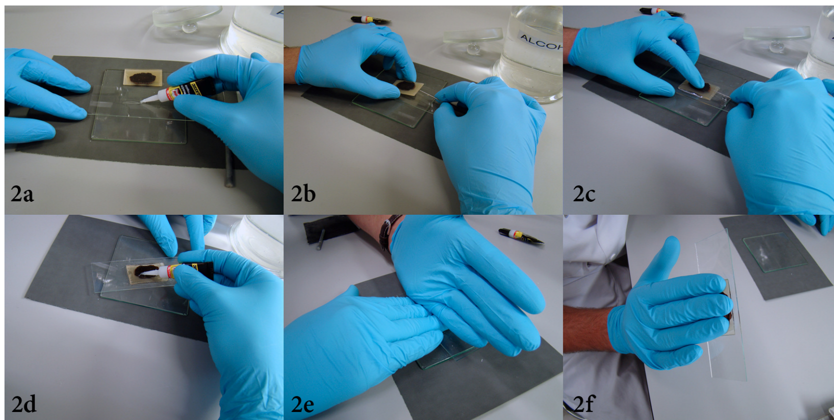
23. Put a drop of cyano-acrylate glue onto the middle of the back of the section holder (Fig. 2d). Using the fingertips of index- middle- and ring finger of a gloved hand, immediately distribute the glue by making circular motions.

After five seconds, apply firm pressure, glueing the fingertips of the glove onto the middle of the back of the section holder. Apply additional pressure with the ulnar side of the other hand (Fig. 2e). Let the glue dry (approximately 40 seconds). As a result of the abovementioned step, the section will be glued to the microscopy section holder, which itself is glued to the latex glove on the dominant hand of the researcher (Fig. 2f). In this way, the exposed and unground surface of the section can be ground, whilst the attached glove provides sufficient grip during the grinding process.

24. On a moistened piece of waterproof grinding paper of grit P220, evenly grind the specimen down to the desired thickness. With a slight tilting of the wrist, a particular area of the section can be ground, so fine adjustments to the evenness and thickness of the specimen can be made. If the glove comes off during grinding, repeat steps 22 and 23.

25. After grinding, remove the latex glove from your hand and detach it from the section holder with a single-edged razor blade. Any remaining glue can also be easily scraped off with this razor blade.

Figure 2.



Glueing the section on a glass microscopy slide holder and the holder on a laboratory glove. After applying a line of glue (2a) the section is placed into the glue (2b) and the section is lowered onto the glass holder (2c). Then, the holder is glued to the gloved hand: a drop of cyano acrylate glue is placed on the backside of the holder (2d). The glue is spread for a few seconds. Then the middle three fingertips are pressed on the holder. Additional pressure is applied with the ulnar side of the other hand (2e). After 15 seconds, the glove will be attached firmly to the holder (2f).

26. Cut a strip of approximately 2 cm width and 10 cm length from the waterproof grinding paper of grit P1200. Moisten the tip of the grinding paper with some tap water. This moist surface can now be used to polish the section by making small circles on the specimen surface. Begin by applying gentle pressure and gradually lessen the applied force to smooth the surface as much as possible. Make sure that the paper and the section stay moist throughout the process.

27. When the grinding and polishing is completed, rinse the section under running tap water, thereby removing most of the remaining grinding dust.

28. Subsequent to rinsing, clean the section as mentioned in step 18.

29. When cleaned thoroughly, let the section air dry. If the section does not need to be stained, it is ready for cover-slipping (see step 32). If additional histochemical staining of the section is desired, proceed to step 30 without cover-slipping. If attention and care is paid, immediate inspection/photographing of the uncovered section is possible. It is imperative that the section is kept clean when not cover-slipped.

Staining

30. Filtrate 400cc of the Mayer's Haematoxylin according to Mayer by using a funnel and filtration paper in a 500cc plastic container. If the staining solution is to be stored, also filtrate the solution afterwards. If a batch of ready prepared haematoxylin is purchased, filtration prior to first use is not necessary.

31. Position the microscopy section holder with the glued section upright in the plastic staining container. Ensure that the entire section is submerged. Stain the sections for eight minutes.

32. Rinse the stained sections in a plastic container under running tap water for ten minutes.

33. Rinse the sections for one minute in a plastic container filled with demineralized water. The section must be completely submerged.

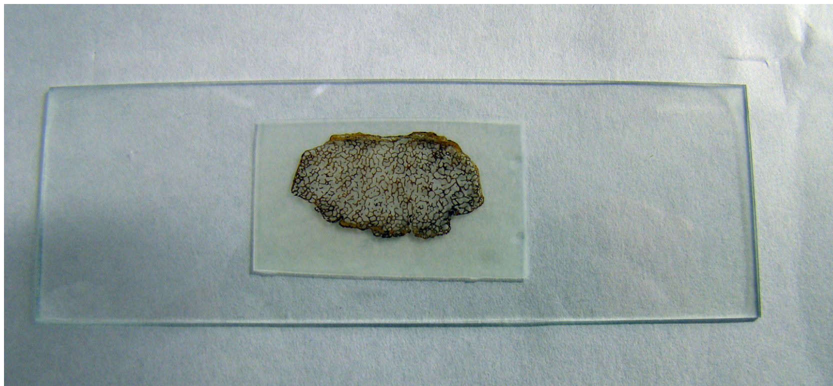
34. Remove the excess demineralized water by tapping a corner of the section holder on a paper towel. Let the section dry on air. Make sure the section is completely dry before cover-slipping.

35. Mount the section using Entalan® and a glass cover slip. Also, LX-112 can be used as a mounting medium. It the latter case, LX-112 must be allowed to polymerize by placing the section in the stove at 60°C overnight.

Results

Using the abovementioned protocol, it is possible to produce stained and/or unstained sections of embedded trabecular dry bone within nine days. Production of unstained sections takes no longer than two days. True impregnation has been discarded as an embedding option in favor of merely supporting the specimen with epoxy resin, as this allows even the most fragile parts of bone to be kept intact and in place, whilst the bone surface stays open for staining (Fig. 3).

Figure 3.



Unstained transverse section of an archaeological sternum. The thickness of this section is approximately 80μm. The macroscopic and microscopic structure of the specimen is intact. The production of such a section takes only nine days. If the fixation step is skipped, the production can be completed within two days. Unstained sections are open for inspection under bright light, polarized light or other filters.

The resulting specimen can be inspected both stained and unstained (Figs. 4a and 4b). An unstained specimen, not covered with a glass cover slip, remains available for subsequent staining after inspection.

With the use of a haematoxylin stain, information on bone microarchitecture can be obtained. Cement lines (C), bone lamellae and osteocyte lacunae (O) are clearly discernable (Figs. 4a and 4c). In some areas the canaliculi that connect various osteocytes are visible. The differentiation between taphonomically altered and unaltered bone is enhanced in comparison to unstained sections (Figs. 4b and 4d), since the surface staining does not stain the tunnels made by microorganisms. Both stained and unstained specimens are still useable for inspection under polarized light or other filters (Figs. 4e and 4f).

In addition, the staining of bone tissue enhances the microstructure in those cases in which this is no longer visible with plain light or polarized light. In those parts where natural birefringence is lost, staining can enhance the visibility of lamellae or cement lines (Figs. 5a-5d). Staining intensity did not differ between different samples, but in sections more affected by taphonomical alteration visibility of general tissue morphology and microarchitectural elements is hampered (5d).

Troubleshooting

Resin-related problems

When an embedding medium does not polymerize as expected, several causes must be considered. In the case of a brittle medium, water contamination by insufficient dehydration or condensation from airborne water into the resin is the most likely cause. Solve this problem by elongating the dehydration step and letting the resin reach room temperature before opening the bottle or mixing it. If the specimen is more elastic or harder than usual, the ratio between the different components may be incorrect. Any deviation from the normal resin color is also an indication of a wrong mixture. In this case, a new mixture should be made. In all cases it is advised that a new mixture is prepared as often as possible.

The presence of air bubbles inside the epoxy resin after polymerization is a frequently occurring problem. A single draw of the vacuum on the section is often insufficient. Step 11 and 12 may be repeated.

Problems with grinding the section

Several authors have reported problems with hand-grinding (Beauchesne and Saunders, 2006; Frost, 1958; Maat *et al*., 2001). To facilitate a quick start, the most commonly encountered problems are briefly discussed.

Figure 4.

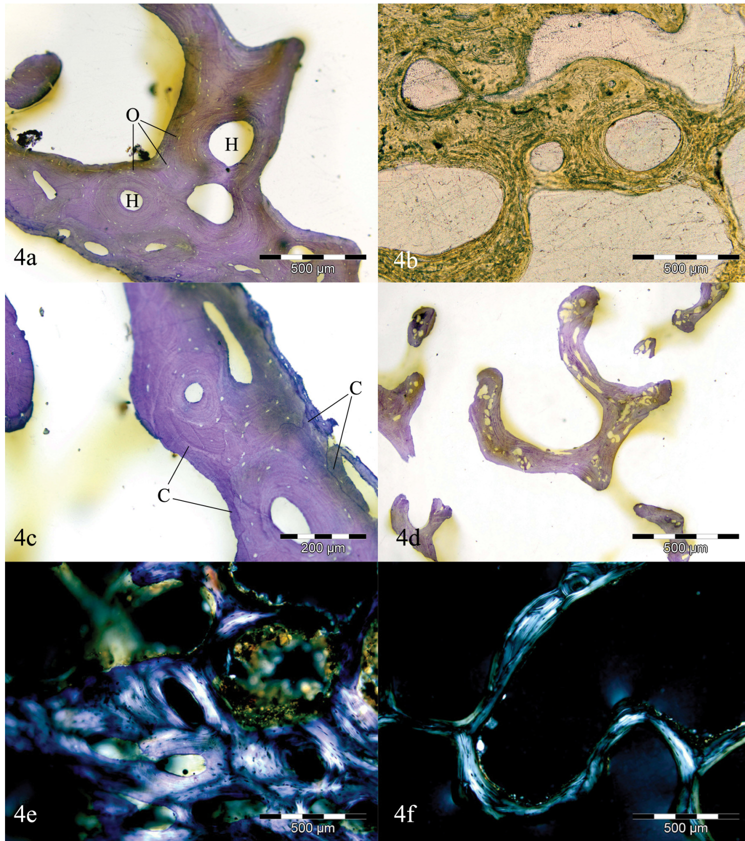
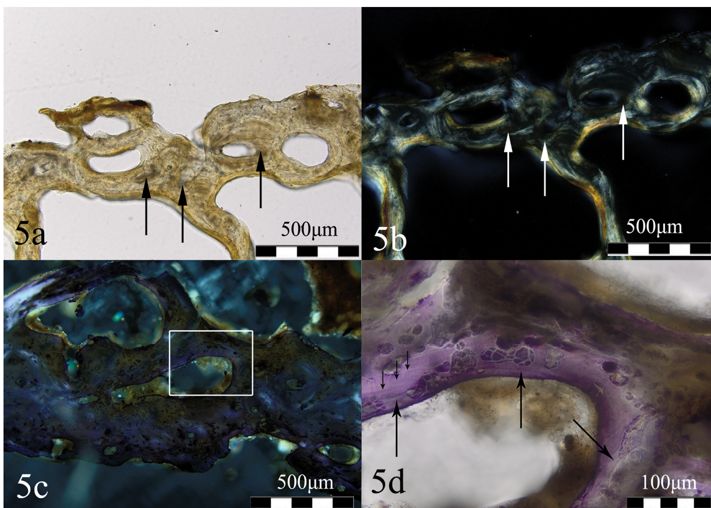


Figure 5.



Micrographs of stained and unstained transverse sections of an embedded, undecalcified human sternum. **(4a)** Detail of a human sternum, Haematoxylin. Bright field. Bar indicates size. The microarchitecture of the specimen is clearly visible. Lamellae are discernible as concentric lines around a Haversian canal (H). Osteocyte lacunae (O) are visible as unstained ellipsoid spots between the lamellae. Cement lines can be seen, but are better visible at higher magnification. The yellow shadows in some parts of the section are caused by underlying unstained bone material. **(4b)** Detail of an unstained human sternum. Bright field. Bar indicates size. This specimen was not covered by a glass cover slip during inspection. The specimen's microstructure is hardly visible. Haversian canals can be seen, but the adjacent lamellae are barely perceptible. In the lower part of the micrograph, taphonomic alteration by fungal or bacterial ingrowth is visible. **(4c)** Detail of a human sternum. Haematoxylin. Bright Field. Bar indicates size. The cement lines that demarcate the edges of the places where bone modeling started (reverse lines) are clearly visible (C). Osteocyte lacunae are visible as unstained spots. **(4d)** Detail of a human sternum. Haematoxylin. Bright Field. Bar indicates size. In this specimen, fungal or bacterial ingrowth has 'tunneled' the surface of the specimen. Due to staining, the differentiation between affected and non-affected bone is enhanced. **(4e)** Detail of a human sternum. Haematoxlyin. Polarized light. Bar indicates size. Fiber direction in the specimen is enhanced by natural birefringence. **(4f)** Detail of an unstained human sternum. Polarized light. Bar indicates size. Fiber direction is enhanced by the natural birefringence of the bone tissue.

Micrographs of taphonomically altered sternums, stained and unstained. **(5a)** Detail of a human sternum, bright field. Bar indicates size. The microarchitecutre of the bone is hardly visible in plain light. Due to taphonomical alterations, the bone tissue appears chaotic and irregular. **(5b)** Same detail as shown in fig. 5a, polarized light. Bar indicates size. The white arrows show parts in which microbial ingrowth and remineralisation has destroyed bone microarchitecture, and thus natural birefringence is lost. The white arrows correspond with the black arrows in fig 5a. **(5c)** Detail of a human sternum. Haematoxlyin. Bar indicates size. The loss of birefringence in a large part of this section suggests severe taphonomic alteration. The microarchitecture in the middle is no longer enhanced by polarized light. On the left side, birefringence is still visible. **(5d)** Detail of fig 5c. Haematoxylin. Bright field. Bar indicates size. Although microarchitecture was no longer visible with polarized light, staining with haematoxylin enhances architectural elements such as cement lines (long arrows) and lamellae (short arrows). Visibility is hampered in large parts of the section due to total destruction of the microarchitecture.

The inability to keep the section even is one of the most encountered problems. Although familiarity and greater experience with the grinding process will largely solve this problem, there are a few guidelines that can help the more inexperienced researcher. First: the larger the section, the more difficult it will be to keep the section even. It is advisable to start with thick slices, since a specimen that is sawn too thin or obliquely during step 15 will leave little room for corrective measures. During grinding, the application and distribution of pressure is of great importance. When the section becomes thinner, the glass microscope slide and specimen will have a tendency to bend under the applied pressure, resulting in a section that will become uneven. Therefore it is important to regularly rinse the section and check on the progress being made. Feeling the edges and corners of the section with a fingertip of the non-grinding hand will give a good indication of section evenness.

If the section has become skewed, particular areas of the section can be ground by tilting the wrist, applying pressure with only one or two fingers and turning the hand. If the section is not sloping but is irregular, the only way to correct this is to take a small strip of grinding paper and grind specific areas of the section as done when polishing the section. This method allows for a very precise correction.

Problems during inspection of the section

An extensive description of problems occurring during the inspection of unstained or unembedded and stained specimens has been given by several authors (Hahn, 1991; Hanson and Cain, 2007; Jans, 2005; Maat *et al*., 2001). Therefore only artefacts that are caused by embedding are mentioned here. Air bubbles are sometimes seen during inspection. In general, more cautious glueing, mounting and vacuum procedures will solve the problem. In some sections certain parts may appear darker or have a shaded, yellowish background. This is most likely due to shading by bone tissue underneath the (stained and microscope-focussed) surface. A thinner section can help to solve this problem. However the problem can never be solved entirely, since sections are usually between 50 and 100 micrometers thick and do not consist of single cell layers. Another cause of darker parts in the section may be residual soil that has been embedded with the specimen. This occurs frequently in sections that have been affected by 'tunneling' (Hackett, 1981) or that have very dense trabeculae. Also, taphonomical alterations themselves may cause artefacts. Therefore proper knowledge on the microscopical characteristics of taphonomic changes is important.

Discussion

The results show that it is possible to produce a satisfactory stained section in less than nine days. The possibility of inspecting and photographing the section both stained and unstained makes the method even more efficient, as only one section suffices for different kinds of inspection. If no staining is needed, the fixation process can be discarded and the production of a section may take no more than two days. Although the production times of other methods is often not mentioned, they can be estimated to be seven days or longer. The only paper that describes the production process into detail states at least three weeks (Schultz, 2001) but may take up to 6 weeks (Roumelis, 2007). This method was cited as the 'most suitable one for archaeological bone' (Roumelis, 2007). It can be concluded that the method provided in this manuscript is faster than the methods currently used.

In all sections prepared in this study, both the microarchitecture of the inspected bone tissue and, if present, the taphonomic alterations, were clearly visible. The enhancement of all bone lamellae, without losing sight on half of the fibres, as unavoidably is the case when using polarized light, is of great value, for example in the investigation of diseases or conditions that affect bone deposition, resorption and remodeling. Each Haversian system, consisting of a Haversian canal and its concentric oriented lamellae, is outlined by a cement line. These lines depict where bone resorption was completed and new bone formation began (reverse lines). In our sections these lines are now visible as darker stained lines. In several diseases these lines have a distinct pattern, for instance in Paget's disease, where the cement lines have a ruffled appearance and are characteristically irregular (Vigorita, 2007).

In cases in which taphonomical alteration is present, staining enhances visibility in two different ways. First of all, since specimen are surface stained, the 'tunnels' made by microorganisms are clearly visible since they remain unstained or filled with excess stain. This increases differentiation between affected and non-affected bone and thus makes inspection of tunneling and its specific characteristics possible.

Secondly, staining can increase the visibility of tissue architecture around taphonomically altered tissue. Whereas altered tissue loses its natural birefringence and polarizing light is no longer helpful in envisioning fiber direction, staining can reveal some important remnants of tissue architecture such as cement lines and lamellar fragments. However, sometimes parts of taphonomically altered tissue kept natural birefringence, while staining did not enhance tissue architecture. It can therefore be concluded that polarized light and staining should both be used in case of badly preserved tissue.

With regard to the preservation status of our choice of samples and the fact that only preservation phases 1, 2 and 3 were included in this study, we argue that only these three phases consider bone material that allows for meaningful histological investigation. Bone material included in these phases varies from 'intact' to 'fragmented, but still recognizable and reconstructible'. Preservation phases 4 and 5 show decay beyond the recognition of normal and pathological conditions (Gordon and Buikstra, 1981). These types of poorly preserved remains are mostly totally remineralized to a degree of amourphousness that makes histological research impossible.

The staining intensity was similar in all sampled material. As the samples were randomly taken from different historical periods and most probably from individuals of different age, this suggests that such differences are of no fundamental importance for the staining procedures. Also, the thickness of the section is of almost no importance, as long as the specimen is sufficiently translucent under light microscopy (De Boer *et al.*, 2012).

We chose to stain with haematoxylin only since this is one of the most used stains for envisioning overall tissue morphology. There are a number of relative simple stainings that could enhance visibility of bone microstructure (Bain *et al.*, 1990; Derkx and Birkenhager-Frenkel, 1995; Grill *et al.*, 1995; Gruber and Mekikian, 1991; Xipell *et al.*, 1974). Testing of these could present interesting results.

The simplicity and speed of our method is mainly due to the avoidance of impregnation steps (Schultz, 1988; Spurr, 1969). Our specimens were not impregnated, only supported by resin. The use of manual techniques and simple laboratory materials, makes the method accessible to a wider range of researchers. This does not implicate that the usage of machinery is incompatible with our method. For the mass production of sections, grinding machinery, such as produced by Buehler, Strues or Leco, can be used (An and Gruber, 2003).

The used embedding resin was chosen on the following basis. We compared the LX-112 resin with that recommended by Spurr (Romeis, 1989; Spurr, 1969) and methyl methacrylate (MMA) (Romeis, 1989). Since MMA is much softer and shrinks more than the standard epoxy resin or Spurr's, it was not considered for testing. During our experiments, the overall hardness, thermal hardening characteristics and relative inertia to ethanol after polymerization favoured the LX-112 resin. The higher viscosity in comparison to Spurr's medium did not pose a problem since heating of the medium prior to drawing a vacuum compensated for this 'setback'.

We chose to embed and stain dry human sternums, since this is one of the most fragile bones of the body. With its thin cortex and fragile trabeculae it is easily damaged during grinding, whilst the densely packed spongiosa and surrounding

cortex hampers the distribution of the embedding medium. Because the method provided a satisfactory result, even when employed on sternums, it is assumed that it is useful for all cancellous bone with or without compact bone.

Although our protocol eliminates many shortcomings, it has its disadvantages. By using a band saw or hacksaw a small component of the specimen is lost. For every section made, at least 1 or 2 mm of the specimen is destroyed. Therefore, it is necessary to be very selective in the manner in which the 'thick' section is made.

Of course, our method remains open for further improvement and testing.

Conclusion

Staining embedded ground sections of undecalcified natural dry bone tissue can be done in nine days. The resulting sections can be inspected both stained and unstained and are also open to inspection with additional filters. Staining enhances the visibility of bone microarchitecture, especially of the orientation of the lamellae and of cement lines/reverse lines. Differentiation between taphonomically altered and unaltered bone is improved by histochemical staining. Also, staining can enhance the visibility of tissue architecture in cases in which polarized light can no longer be used. The staining characteristics of the sections are consistent regardless of bone preservation. Our method is accessible, comprehensive and inexpensive.

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Part 2:
The diagnostic value of dry bone histology

Chapter 4

THE DIAGNOSTIC VALUE OF MICROSCOPY IN DRY BONE PALAEOPATHOLOGY: A REVIEW

From: *The diagnostic value of microscopy in dry bone palaeopathology: a review. De Boer, H.H., Van der Merwe, A.E., Maat, G.J.R., 2013. InternationalJjournal of Paleopathology 3(2), 113-121.*

Abstract. Over recent decades histology has increasingly been used as a diagnostic tool in human dry bone palaeopathology. Still, the use of histology in human dry bone is associated with various problems, including a lack of pathognomonic histomorphology and a need for more experimental data. Consequently, the value of histology as diagnostic tool in human dry bone remains a subject for debate.

Here we review all published palaeohistopathological research in human dry bone. A systematic search identified 3363 articles, with the 64 most relevant citations studied in depth. We specifically focused on the interpretation of histomorphological parameters and the use of comparative fresh bone tissue and/or experimental data.

Our literature review shows that only a few disorders demonstrate a 'specific' histomorphology: Paget's disease, osteoporosis, hyperpara-thyroidism and possibly osteomalacia. In all other cases, histology may aid during the differential diagnostic process, but alone it is unable to confirm a definitive diagnosis. The histological diagnostic process and consequential recommendations for the use of histology are discussed per following disease categories: metabolic disease, neoplasm, infectious disease and trauma.

Introduction

Numerous papers have been published in which the palaeopathological diagnosis of disease was supported by histological investigation. However, no recent reviews regarding the impact of these efforts have been published. Here we discuss the limitations of palaeohistopathological investigation in general and systematically review palaeohistopathological diagnostic efforts and/or potential per disease category. Special emphasis will be placed on the difficulties associated with the

interpretation of palaeohistopathological images. This paper will also touch on the interplay between palaeopathological findings, fresh tissue pathology and experimental research. Our aim is to add depth to ongoing debates about the diagnostic value of microscopy in dry bone tissue.

Limitations of palaeohistopathology

In palaeopathology, the identification of gross anatomical processes in human skeletal remains and their interpretation are considered challenging (Wood *et al*., 1992; Aufderheide and Rodriguez-Martín, 1998; Ortner, 2003). The same challenges are relevant in palaeohistopathology. Dry bone remains are void of soft tissue and bone tissue cells, and it is just these components that provide fresh tissue pathologists with the pathognomonic features leading to a reliable diagnosis (e.g. Ross *et al*., 1995; Vigorita, 2007; Rosai and Ackermans, 2011). This problem is further complicated by the nature of bone tissue, which only reacts to a stimulus (disease) in three ways visible on a microscopic level: resorption of bone tissue (an osteoclastic bone response), deposition of new bone tissue (an osteoblastic bone response) or a combination of the two (Frost, 1985). Only a small subset of diseases can be associated with such characteristic histological alterations that they can be regarded as pathognomonic. For all other diseases, microscopic changes appear to be similar, and as a result various authors have downplayed the value of palaeohistopathology as a diagnostic tool (Putschar, 1966; Stout and Simmons, 1979; Waldron, 2009).

Bianco and Ascenzi (1993) stated that the lack of pathognomonic histological information derived from skeletal remains, in combination with a lack of independent extra source information, such as medical data, posed a fundamental problem to the advance of palaeohistopathology. According to these authors, palaeohistopathologists risk 'making nonscientific statements', i.e. statements that cannot be proven false; a problem that could be minimized by new knowledge on the visibility of changes that specific diseases show in dry bone. They stressed the need for research in which palaeohistopathological diagnoses are supported by experimental research or comparative research with the use of current documented dry bone reference specimens.

Palaeohistopathological research in the past decades

The popularity of histology as a diagnostic tool for archaeological remains has increased over recent decades. In 2001, pronouncements on the value of microscopy

as a diagnostic tool were generally optimistic (Schultz, 2001). Palaeohistopathology was said to be a dependable tool in the differentiation between tumors, metabolic disorders and infectious diseases since specific histoarchitectural characteristics could be linked to specific diseases. This latter statement contrasted sharply with earlier consensus, which was generally more skeptical (Putschar, 1966; Stout and Simmons, 1979; Bianco and Ascenzi, 1993). Several examples were presented to prove the specificity of morphological features, such as those for the diagnosis of syphilis in dry bone tissue (Schultz, 2001; Schultz, 2003; Von Hunnius *et al*., 2006).

However, the diagnostic power of these features has been challenged. For instance, Weston (2009) and Van der Merwe *et al*. (2010) showed that microarchitectural features alone were by no means diagnostic for specific diseases such as treponematosis, and Schutkowski and Fernandez-Gil (2010) came to the same conclusions for tuberculosis and leprosy. All in all, more recent publications challenged the diagnostic value of microscopy, a debate that is pressing due to the questioned acceptance of destructive sampling.

Methods

To avoid bias from expert-based reviewing, we used a systematic and sequential inclusion-exclusion strategy to select relevant articles. Ten keynote articles were selected prior to key word article retrieval to test the adequatcy of the search (Table 1). Since palaeohistopathological papers are distributed widely throughout the medical, physical anthropological and archaeological literatures, a general search was executed for both Pubmed and Web of Science references. The search strategy consisted of the key word combination of 'physical anthropology AND histology'. The search details can be found in Table 2. In order to ensure that no relevant articles were missed, publications from the American Journal of Physical Anthropology, Journal of Archaeological Science, International Journal of Osteoarchaeology and International Journal of Paleopathology with the terms 'histolog*' or 'microscop*' in the title were also included. A total of 4155 articles were automatically retrieved of which 792 duplicates could be excluded.

To continue in the study sample, the remaining articles had to comply with the following: 1) the article was written in English, 2) histological investigation was performed on human dry bone, 3) microscopic investigation (light microscopy, microradiography or scanning electron microscopy) was used as a palaeopathological diagnostic tool, 4) the article was an original research paper. An exception for the latter criterion was made for the review article by Schultz (2001), since it added a substantial amount of key data from outside the English literature.

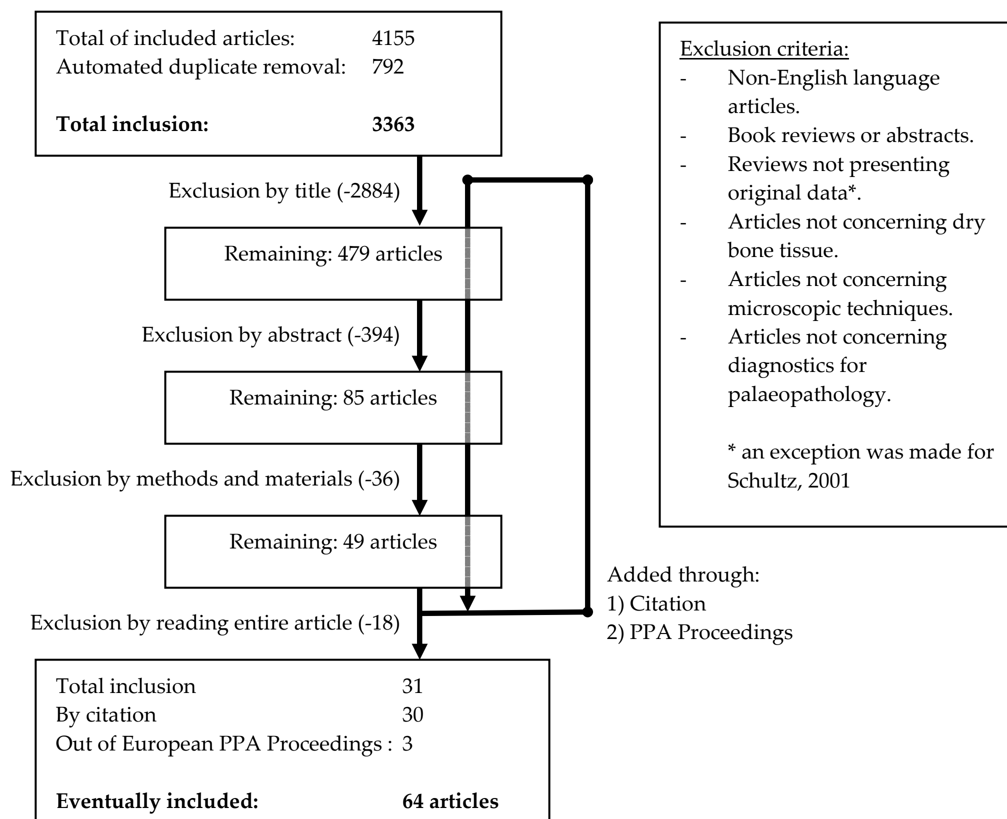
Table 1. Key-note articles used for testing the sensitivity of the inclusion criteria.

Authors	Year	Title	Journal
Aaron *et al.*	1992	Paleohistology of Pagets-Disease in 2 Medieval Skeletons.	Int. J. of Osteoarchaeology
Blondiaux *et al.*	1994	Microradiographs of leprosy from an osteoarchaeological context.	Int. J. of Osteoarchaeology
Cuijpers	2009	Distinguishing between the bone fragments of medium-sized mammals and children. A histological identification method for archaeology.	Anthropol. Anzeiger
Guarino *et al.*	2006	Bone preservation in human remains from the Terme del Sarno at Pompeii using light microscopy and scanning electron microscopy.	J. of Archaeological Science
Von Hunnius *et al.*	2006	Histological identification of syphilis in pre-Columbian England.	Am. J. of Phys. Anthrop.
Hackett	1981	Development of caries sicca in a dry calvaria.	Virchows Archiv
Hanson & Chester	2007	Examining histology to identify burned bone.	J. of Archaelogoical Science
Maat *et al.*	2001	Manual preparation of ground sections for the microscopy of natural bone tissue: update and modification of Frost's 'rapid manual method'.	Int. J. of Osteoarchaeology
Schultz	2001	Paleohistopathology of bone: a new approach to the study of ancient diseases.	Yearb. of Phys. Anthrop.
Stout & Teitelbaum	1976	Histomorphometric determination of formation rates of archaeological bone.	Calcif. Tiss. Res.

Table 2. Search strategies per database.

Database	Strategy
PubMed	((‘Anthropology, Physical’[mesh] NOT ‘Fossils’[mesh]) OR paleopathology OR ‘forensic anthropology’ OR ‘physical anthropology’ OR paleopatholog* OR ‘biologic anthropologic’ OR archaeology [mesh] OR archaeology [tw] OR archaeological [tw]) AND (‘histology’[tw] OR ‘histology’[mesh] OR ‘microscopy’[mesh] OR ‘microscopy’[tw] OR ‘histological techniques’[mesh])).
Web of Science	TS=((paleopathology OR forensic anthropology OR physical anthropology OR paleopatholog* OR biologic anthropologic OR biologic anthropology OR forensic anthropologic OR physical anthropologic OR archaeologic*) AND (histology OR histologic OR histolog* OR Histological OR Histological Techniques OR Autoradiography OR Bone Demineralization Technique OR Decalcification Technique OR Histocytochemistry OR Immunohistochemistry OR Histocytochemical OR Immunohistochemical OR Histocytological Preparation Techniques OR Microdissection OR Microtomy OR Replica Techniques OR Staining and Labeling OR Tissue Embedding OR Tissue Preservation OR microscopy OR Diagnostic Imaging OR Photomicrography OR Age Determination by Skeleton OR age determination OR Fluoroscopy OR Microradiography OR Sex Determination by Skeleton OR sex determination OR Radionuclide Imaging OR Spectroscopy)).

Table 3. Total count of references in- and exclusion.



Other relevant citations were also included, for instance those connected to the Proceedings of the European Meetings of the Palaeopathological Associations beginning in 1982. The procedures for article selection are illustrated in Table 3.

The histological diagnostic features used in the articles were grouped by diagnosis. Their value was assessed by evaluating whether the features were rooted in experimental data, fresh tissue specimen of known cases or a rigorous biomedical discussion of pathogenesis.

Results and discussion

The seemingly small number of remaining articles, when compared to the large body of literature that was initially identified (see Table 3), was in line with statements by Schultz (2001) and Weston (2009) that the histology of dry bones had

not been frequently used for palaeopathological diagnoses. Nevertheless, the growing popularity of palaeohistopathology was illustrated by a steady increase in the number of journal articles per decade (see Table 4), with the majority being published within the last twenty years, 14% with American first authors and 81% with European first authors (see Figure 1).

This literature included metabolic, neoplastic, infectious and traumatic pathological conditions. These conditions will be discussed in the following sections with respect to the diagnostic value of their microscopic features.

Table 4. Published articles per decennium.

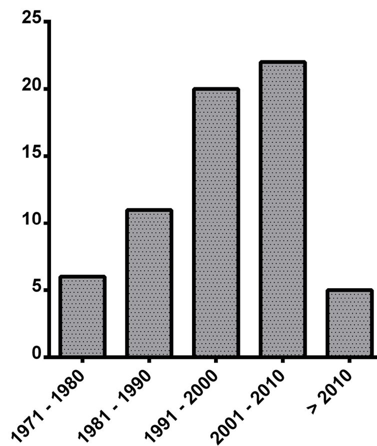
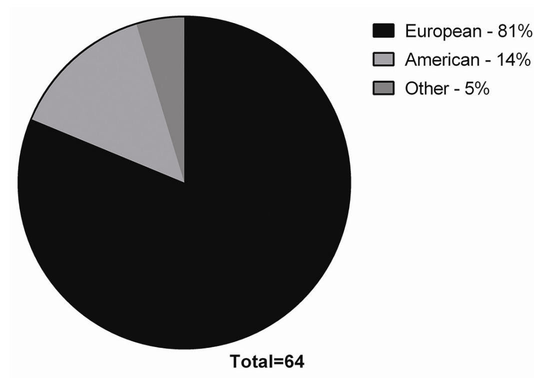


Figure 1.



Nationality of first authors.

Possible and potential diagnostic features

Metabolic disorders

Osteoporosis and osteopenia

Osteoporosis is not a disease in itself but a symptom, which may be associated with various conditions such as malnutrition, hormonal imbalance or disuse. It is defined as a decrease in bone tissue volume and primarily leaves the gross anatomical bone outline intact. It is routinely assessed clinically by radiographic measurement of the bone mineral density (BMD) (Resnick, 1996; Brown, 2006). Histomorphometric parameters such as trabeculae number, cortical thickness and mean trabecular width may also be used during the diagnostic process (Vigorita, 2007; Barger-Lux and Recker, 2009). Due to soil contamination, radiology may not be the method of choice for the assessment of osteoporosis in archaeological skeletal remains, and therefore palaeopathologists tend to use histomorphological parameters such as cortical thickness, cortical area and trabecular bone mass as diagnostic measures (Richmann and Ortner, 1979; Martin and Armegalos, 1979, 1985; Gonzales-Reimers and Arnay-de-la-Rosa, 1992; Velasco-Vazquez *et al.*, 1999; Paine and Brenton, 2006; Cho and Stout, 2011). The utility of these diagnostic features was corroborated by related clinical reports and fresh tissue pathology, which led researchers to conclude that dry bone microscopy allowed for differentiation between various causes of osteoporosis (Richmann and Ortner, 1979; Martin and Armegalos, 1979, 1985) and that the occurrence of osteoporosis could be linked to social structure, life style and dietary customs (Gonzales-Reimers and Arnay-de-la-Rosa, 1992; Velasco-Vazquez *et al.*, 1999; Paine and Brenton, 2006).

In examples where bone is imperfectly preserved, a lack of intact tissue may hamper a histomorphological assessment. Therefore, some researchers resorted to (subjective) qualitative assessment of osteoporotic changes. Roberts and Wakely (1992) used scanning electron microscopy (SEM) to detect microfractures in fragile osteoporotic bone. The authors suggested that SEM may detect osteoporosis at an earlier stage than radiography. However, they admitted that the sole presence of microfractures was a non-specific change, since there was no information available on its relationship with the severity of osteoporosis. Schultz (1999) diagnosed osteoporosis in a fractured Neanderthal ulna by virtue of its enlarged Haversian canals and the replacement of cortical shaft tissue by cancellous bone. The unilateral presence suggested disuse as the cause. While these results are tantalizing, we argue that the diagnosis of osteoporosis is most convinving when using a histomorphometrical approach, since osteoporosis is by definition, a quantitative change (Raisz, 2005).

Scurvy

Since scurvy leaves histomorphological traces in the skeletal record (Resnick, 1996; Cotran *et al*., 1999; Ortner, 2003; Fain, 2005), histology has been used to corroborate gross anatomical palaeopathological diagnosis (Maat and Uytterschaut, 1984; Schultz, 2001; Maat, 2004; Van der Merwe *et al*., 2010; Mays *et al*., 2012). Maat (2004) described several histological features which may be indicative of active and healed scurvy, i.e. denaturized hemoglobin in black maculas around metaphyseal endofractures and longstanding ossified hematomas (Maat and Uytterschaut, 1984; Maat, 2004). In contrast to periosteal reactions of an infectious or neoplastic nature, ossified hematomas do not affect the periosteal surface (also see Schultz, 2001). Stratification of such ossified hematomas indicates recurrent episodes of scurvy.

The criteria of Maat (2004) and Schultz (2001) were built on earlier studies of soft tissue pathology and experimental data (Murray and Kodicek, 1949; Van Wersch, 1953) and were recently confirmed by Van der Merwe *et al*. (2010), who added that gradual ossification and remodeling of a hematoma occurred in three phases in which the radiating structure of the appositional bone stayed visible for a considerable period of time. Maat (2004), Schultz (2001) and Van der Merwe *et al*. (2010) did not report increased resorption parameters and defective osteoid formation while others describe these (non-pathognomonic) features to be associated with scurvy (Fain, 2005; Waldron, 2009).

Isolated ossified hematomas are not pathognomonic for scurvy (Schultz, 2001; Van der Merwe *et al*., 2010). Their appearance in true scorbutic cases is dominated by gross anatomical symmetry, especially in the lower extremities. Therefore, it can be concluded that although histology aids in the identification of ossified hematomas, additional gross anatomical features are needed for a definitive scurvy diagnosis.

Rickets and osteomalacia

Fresh bone from active rickets and osteomalacia has a distinct histomorphology showing the accumulation of osteoid and increased resorptive activity (Mankin, 1974; Vigorita, 2007). Nevertheless, the identification of these features in dry bone is difficult. Stout and Teitelbaum (1976) suggested that increased resorption in suspected cases might indicate osteomalacia. Yet, they admitted that increased osteoclastic activity is by no means specific. Schultz (2001) stated that rickets can cause porotic hyperostosis of the vault and can be differentiated from other causes by microscopic features, such as restricted thickening of the vault, splintered internal and external lamina and a 'totally changed microstructure'. However, the vague description of these features and a lack of fresh bone tissue diagnoses and experimental data made these criteria of little practical value.

In an attempt to describe identifiable diagnostic features, Schamall *et al*. (2003) used histomorphometry and qualitative histology on documented museum specimens of rickets and osteomalacia. In both, diminished bone tissue volume (osteopenia) was noted, together with resorptive features such as fields of Howship's lacunae and enlarged osteocyte lacunae. Backscattered electron microscopy and microradiographs showed hypomineralization. These results corresponded to those found by Brickley *et al*. (2007), who studied osteomalacic skeletons by means of regular scanning electron microscopy. In addition, Brickley *et al*. described distinct ring-like structures, so-called 'defective cement lines', which most likely resulted from the postmortem loss of osteoid. It was suggested that 'defective cement lines' were pathognomonic for osteomalacia. All results of Schamall *et al*. (2003) and Brickley *et al*. (2007) were thoroughly supported by clinical case studies in which osteomalacia was diagnosed based on fresh bone tissue specimens.

Osteopenia and resorptive features can be seen in various diseases, such as infection or hyperparathyreoidism. Hypomineralisation can be caused by any process that induces rapid growth and remodeling (Grynpas, 1993). We therefore conclude that Schamall *et al*.'s (2003) sole finding of described features in rachitic and osteomalacic specimens cannot be regarded as pathognomonic. Yet, in cases where these histological features can be associated with the characteristic pattern of gross anatomical changes observed in cases of the two diseases (e.g. bowed long bones in case of rickets and Looser zones in case of osteomalacia), they will strongly support the diagnosis.

The 'defective cement lines' described by Brickley *et al*. (2007) might be a pathognomonic finding, but it has not been reported by other workers. Yet, it is thoroughly supported by comparative research and biomedical literature (e.g. Boyde *et al*., 1986; Parfitt, 1998). Future research is required to establish its usefulness.

Hyperparathyroidism

Histomorphological features of hyperparathyroidism develop when the increased activity of the parathyroid glands stimulates profuse bone resorption, which is indicated by an increased number of Howship's lacunae and the enlargement of Haversian canals. The disease may be primary, secondary to hypocalcaemia, or even tertiary (in the case of long-lasting secondary hyperparathyroidism). Resorption is particularly noticeable in (sub)periosteal bone tissue and cancellous bone, which in the latter causes 'dissecting osteitis' or 'tunneling resorption' (Cotran, 1999; Vigorita, 2007; Fraser, 2009; McCarthy, 2010). These features, especially 'tunneling resorption', remain detectable in well-preserved palaeopathological material (Weinstein *et al*., 1981; Cook *et al*., 1988; Zink *et al*., 2005; Mays *et al*., 2007; Waldron, 2009;).

Although osteoporosis and hypovitaminosis D may mimic hyperparathyroidism histologically (Weinstein *et al*., 1981; Cook *et al*., 1988; Mays *et al*., 2007;), tunneling resorption is widely accepted as pathognomonic (Vigorita, 2007; McCarthy, 2010). Therefore, we can conclude that this disease can be diagnosed palaeohistopathologically in well-preserved cases. However, as this rare affliction appears without any gross anatomical changes of the skeleton, it will often remain undiagnosed in palaeopathology.

Paget's disease

On a microscopic level, Pagetic bone lesions develop in three phases: an initial osteolytic stage, followed by a mixed osteoclastic-osteoblastic stage, and ultimately evolving into a quiescent osteosclerotic stage (Cotran, 1999), which is characterized by a distinctive 'mosaic' pattern of woven and lamellar tissue, demarcated by numerous convoluted cement lines in trabecular and cortical bone tissue - a pathognomonic 'patchwork' architecture (Ralston, 2008). Stout and Teitelbaum (1976) hypothesized that this distinct histomorphology would remain visible in archaeological remains. Bell and Jones (1991) and Aaron *et al*. (1992) confirmed this, when they analyzed pagetic bone with SEM and light microscopy, respectively. Their findings concurred with those seen in a macerated specimen of a reported case of Paget's disease. Additionally, diagnosis was proved possible in poorly preserved archaeological bone material (Bell and Jones, 1991; Roches *et al*., 2002).

As noted by Aaron *et al*. (1992) and Roches *et al*. (2002), some types of neoplasm may mimic pagetic histomorphology. Osteogenic sarcoma, which can be a complication of Paget's disease (Ortner, 2003), and osteoblastic carcinoma (e.g. metastases of prostate or mamma carcinoma) should be considered in the differential diagnoses. However, these do not present the same gross anatomical and radiological changes (Wells and Woodhouse 1975; Ortner, 2003). Consequently, histology is an appropriate method to confirm Paget's disease in dry bone material.

Hereditary and acquired hemolytic anemia

Anemia is not a disease, but like osteoporosis it is a symptom resulting from pathological conditions such as malaria, iron deficiency, sickle cell disease or thalassaemia to name a few. In some chronic cases (hereditary and acquired haemolytic types), an attempt to increase the production of red blood cells results in bone marrow hyperplasia. As a result, radial enlargement of adjacent cancellous bone occurs at the cost of (porotic) thinning of the external cortical lamina (Middlemiss and Raper, 1966; Stuart-MacAdam, 1987; Resnick, 1996; Tyler, 2006; Ejindu, 2007). Consequently, marrow hyperplasia may cause cranial porotic hyperostosis, which

was studied via SEM by Marcsik *et al*. (1984), Maat and Baig (1990) and Maat (1991). The expansion of the marrow cavity at the cost of the outer table has shown to be visible in archaeological remains. SEM has even allowed for the establishment of a conclusive diagnosis when malformed erythrocytes, pathognomonic for sickle cell disease, can be identified (Maat and Baig, 1990; Maat, 1997). However, such a finding is rare. Schultz (2001) and Wapler *et al*. (2004) confirmed the detectability of marrow hyperplasia in cranial porotic hyperostosis and cribra orbitalia by means of light microscopy. Furthermore, Schultz (2001) suggested six developmental phases, ranging from minor changes to the exuberant hair-on-end appearance.

When differentially diagnosing cranial porotic hyperostosis and cribra orbitalia, infectious disease, rickets or scurvy should be considered (Marcsik *et al*., 1984; Schultz, 2001; Wapler *et al*., 2004). Schultz (2001) and Wapler *et al*. (2004) state that infectious lesions usually present a relatively more destructive (lytic) and irregular microarchitecture, indicated by numerous Howship's lacunae and irregular new bone formation (Schultz, 2001; Wapler *et al*., 2004). However, these statements were not corroborated by documented cases or experimental data. Marscik and colleagues (1984) theorized that in principle, rickets and scurvy primarily affect the outer surface of the external bone table and do not cause an expansion of the diploë (Marcsik *et al*., 1984). Their theory was in line with more recent clinical findings (Resnick, 1996; Tyler, 2006; Vigorita, 2007).

It can be concluded that the histomorphology of marrow hyperplasia allows for an accurate histological identification. Histology can be a useful tool to narrow the differential diagnosis associated with porotic hyperostosis and cribra orbitalia.

Neoplasms

Primary tumors

In a clinical context, fresh tissue pathology is the basis for the diagnosis of bone tissue tumors. As a result, a large amount of literature exists on their histomorphology (e.g. Cotran, 1999; Vigorita, 2007; Rosai and Ackermans, 2011). In contrast, only a small number of primary bone tumors have been studied palaeohistopathologically.

In the majority of cases, histology is used to identify whether the tumorous process is benign or malignant. In general, the differentiation of bone tumor tissue is inversely correlated to its malignancy grade. Benign lesions can be recognized by their overall regular lamellar tissue architecture. They do not grow invasively and therefore rarely show osteolytic activity (Strouhal *et al*., 1996; Hershkovitz *et al*., 1999; Vyhánek *et al*., 1999; Eshed *et al*., 2002). Conversely, malignant tumors are invasive by

definition. Malignant tissue is characteristically amorphous, often demonstrating both abundant osteolytic and osteoblastic activity (Suzuki, 1987; Schultz, 1991; Strouhal *et al*., 1997). The erosive invasive areas are characterized by numerous Howship's lacunae, and any new bone is deposited as an atypical 'patchwork' of lamellar and woven bone with little or no remodeling.

The differentiation between benign and malignant tissue is only the first step in the differential diagnostic process. Eventually the conclusive, most likely diagnosis will be based on data such as the age of the individual and macroscopic and radiological observations, e.g. anatomical distribution pattern, size, contour, density etc. In many cases, the additional value of histology is often limited. For example, although Suzuki's (1987) histological observations on a tumor were compatible with a diagnosis of osteosarcoma, the individual's age at death and the tumor's location and radiological appearance had already been strong diagnostic indicators of the diagnosis. Similar situations occurred in the case of a paranasal carcinoma (Schultz, 1991), the case of an osteosarcoma of the skull described by Strouhal and colleagues (1997), and a case of a multiple myeloma reported by Wakely *et al*. (1998). All these examples had a high *a priori* likelihood of diagnosis, which reduced the additional value of histology. In the case of a suspected meningioma, histology is considered completely redundant (Campillo, 1991).

Nevertheless, in some specific cases, histology may very well have additional value, particularly when dry bone histomorphology is compared to fresh tissue specimens. For example, Schamall *et al*. (1999) used a comparative histological approach to differentiate between a possible osteosarcoma, meningioma or hemangioma in a cranial lesion observed in a young female. Hershkovitz *et al*. (1999) and Eshed *et al*. (2002) showed that histology may aid in the differentiation between similar looking benign lesions of the skull. If a tumor contains preserved soft tissue, histology may provide a conclusive diagnosis (Strouhal, 1976; Strouhal and Němečková, 2004). However, such findings are rare.

In conclusion, the histological analysis of primary tumors is useful for differentiation between benign and malignant cases. It can, however, corroborate suspected diagnoses in only a few specific examples.

Secondary tumors (bone metastases)

In the description of metastases, palaeopathologists adopted the fresh tissue pathology approach for categorizing metastases as being either osteolytic, osteoblastic or a combination of both (for fresh tissue pathology see Vigorita, 2007; Roodman, 2011, for palaeopathology cases see Anderson *et al*., 1992; Campillo and Marí-Balcells, 1984; De La Rua *et al*., 1995; Grupe, 1988; Molnar *et al*., 2009; Šefčáková

et al., 2001; Schultz, 1993; Schultz *et al*., 2007; Tkocz and Bierring, 1984; Wakely *et al*., 1995). Since metastases are by definition malignant, the histomorphology of osteoblastic and osteolytic lesions is similar to those found in malignant primary bone tumors (see above). The hematogenous dissemination of the original tumor may be sometimes disclosed by osteoblastic apposition within Haversian canals (Anderson *et al*., 1992; Wakely *et al*., 1995).

Palaeohistopathologists use metastatic tissue type to differentiate between various original tumors. However, this approach has limitations. Firstly, the vast majority of metastases are osteolytic (Vigorita, 2007). Secondly, medical practice shows that both osteolytic and osteoblastic lesions are seen in the majority of patients with bone metastases (Roodman, 2011). Thirdly, there is no fixed relationship between the original tumor and the histological nature of its metastases (Roodman, 2011). This has resulted in conflicting statements by palaeopathologists, for instance when Tockz and Bierring (1984) stated that lung, kidney and thyroid gland metastases were osteoblastic, whereas Grupe (1988) suggested that they were osteolytic.

Histologically, osteoblastic lesions may mimic Paget's disease, fluorosis and osteopetrosis (Anderson *et al*., 1992; Schultz, 2001; Schultz *et al*., 2007; Tkocz and Bierring, 1984). Osteoclastic and mixed-type lesions can mimic lytic infectious lesions, although tumors should have a 'more regular trabecular microarchitecture' (Schultz, 2001). However, the relevance of this feature is limited, due to its subjective nature.

We conclude that histology is an apt tool to identify the three different types of metastastic tissue (osteolytic/osteoblastic/mixed). Yet, if used in isolation, histology is unable to provide a conclusive diagnosis. For identification of the origin of the malignant process, the demographic profile of the individual and epidemiological knowledge on the different types of bone metastases is pivotal.

Infectious diseases

General characteristics of non-specific infections.

In fresh tissue, acute bone infection is histologically characterized by hypervascularisation, influx of acute inflammatory cells, small vessel thrombosis and edema, which eventually results in ischaemia and necrosis (Kahn and Pritzker, 1973; Lew and Waldvogel, 1997; Vigorita, 2007; Calhoun, 2012). If not eradicated, the infection changes into a chronic state, with necrotic bone (sequestrae) and reactive new bone (involucrum) as hallmarks (Kenan *et al*., 1993; Vigorita, 2007; Calhoun, 2012). The deposition of reactive bone tissue by osteoblasts is triggered by periosteal irritation and the presence of growth factors (Kenan *et al*.,1993; Vigorita, 2007). Throughout the clinical and pathological literature, authors emphasized that the

degree of bone reaction is defined by many variables such as age and immune status of the host, bone type and site of infection.

In palaeohistopathological investigations, the diagnosis of infectious lesions has been based primarily on the osteolytic destruction of original bone and the reactive formation of new bone tissue (Hackett, 1981a; Blondiaux *et al*., 1994; Schultz, 2001; Schultz and Roberts, 2002; Wapler *et al*., 2004; Von Hunnius *et al*., 2006; Flohr and Schultz, 2009a, 2009b; Weston, 2009; Van der Merwe *et al*., 2010; Nicklisch *et al*., 2012). The osteolytic component of the infection demonstrates omnipresent Howship's lacunae and 'lytical' resorption cavities (Hackett, 1981a; Blondiaux *et al*., 1994; Schultz, 2001; Wapler *et al*., 2004; Flohr and Schultz, 2009a; Van der Merwe *et al*., 2010;). The anatomical boundaries/site of bone tissue destruction defines whether an infection should be called periostitis, osteitis or osteomyelitis. Appositional reactive bone may vary from chaotic and speculated (e.g. Blondiaux *et al*., 1994; Schultz, 2001; Wapler *et al*., 2004) to regular, sclerotic and dense (e.g. Hackett, 1981a; Schultz and Roberts, 2002; Flohr and Schultz, 2009b;). Often, vestiges of hypervascularisation (i.e. vascular channels) are visible at the periosteal surface (Hackett, 1981a; Blondiaux *et al*., 1994; Schultz, 2001; Von Hunnius *et al*., 2006). Extensive remodeling may hamper the differentiation between appositional and original bone (Schultz, 2001; Van der Merwe *et al*., 2010).

Throughout the palaeohistopathological literature, authors appear to have no difficulty differentiating between infected lesions and taphonomical processes (Schultz, 2001; Wapler *et al*., 2004; Flohr and Schultz, 2009a) or ossified hematomas (Schultz, 2001; Maat, 2004; Van der Merwe *et al*., 2010). However, differentiation from primary bone tumors or (osteolytic) metastases is challenging (Schultz, 2001). Gross anatomical and radiological analysis is needed in those cases.

The identification of specific infectious diseases

In addition to non-specific infections, the histomorphology of specific infectious diseases has also been studied, with many workers focused upon syphilis (Hackett, 1981a; Schultz, 2001; Von Hunnius *et al*., 2006; Weston, 2009; Van der Merwe *et al*., 2010) and leprosy (Blondiaux *et al*., 1994; Schultz, 2001; Schultz and Roberts, 2002). The findings in some studies led to the assertion that these diseases may present distinct, pathognomonic histomorphological features (Schultz, 2001; Schultz and Roberts, 2002). However, recent developments have added nuance or even refuted these assertions (Von Hunnius *et al*., 2006; Weston, 2009; Van der Merwe *et al*., 2010). If the pathogenesis of infectious bone lesions is considered, the inability to identify pathognomonic histomorphology can be understood.

Both the osteolytic and osteoblastic component of the infected bone tissue is nonspecific. Osteolytic bone destruction is caused by the nonspecific recruitment and activation of osteoclasts by inflammatory cytokines (Lew and Waldvogel, 1997; Phan, 2004). Reactive new bone formation is caused by the nonspecific excretion of growth factors and the lifting and irritation of the periosteum, both of which result in osteoblastic apposition of new bone tissue (Calhoun, 2012; Kenan *et al*., 1993).

Therefore, the eventual histomorphology of an infectious process will be an index of the biological activity and anatomical location of the infectious process, the patient's age and metabolic state, and the thickness and firmness of attachment of the periosteum (Kahn, 1973; Kenan, 1993). As a result, the infectious microorganism does not correlate with one typical histomorphology, and one microorganism can create a wide range of bone lesion phenotypes. Thus, those features once thought to be pathognomonic for syphilis and leprosy are not specific for infection with treponema pallidum or mycobacterium leprae, respectively. If anything, the features indicate a slowly developing and recurrent infection. The same holds for mycobacterium tuberculosis infections.

Mechanical trauma

The identification of traumatic lesions in dry bone material is usually based on gross anatomical and radiological investigation. As a result, microscopy has seldom been used for trauma analysis. This general lack of palaeohistopathologicial investigation of traumatic lesions contrasts with recent suggestions that it may aid in the assessment of posttraumatic survival time or could aid in the differentiation between peri- and antemortem lesions (Blondiaux, 2000; Maat, 2006a, 2008; Cattaneo *et al*., 2010; De Boer *et al*., 2012a, 2013a,). Lagier and Baud (1980) analyzed a juxtacortical osteoblastic lesion on a femur, secondary to mechanical trauma. Light microscopy showed an intact femoral cortex, which excluded a diagnosis of malignant tumor or infection. Their comparison with fresh tissue histology of known cases established a diagnosis of myositis ossificans.

Conclusion

Histology has become an essential and integral part in the investigation of human dry bone. It may aid in the differentiation between human and animal bones (Owsley *et al*., 1985; Cuijpers *et al*., 2009; Hincak *et al*., 2009), the study of taphonomy (Hackett, 1981b; Garland, 1989; Hedges *et al*., 1995; Jans *et al*., 2004), age determination (Kerley,

1965; Stout, 1976; Maat *et al*., 2006b;) or can usefully supplement analyses of ancient DNA (Guarino *et al*,. 2000, 2006) and ancient proteins (Haynes *et al*., 2002; Schmidt-Schultz *et al*., 2004). As our review shows, histology is also useful for a wide range of diagnoses in palaeopathology.

In our analysis of the palaeohistopathological literature, we found that histological features were often described in a vague and ambiguous manner. Moreover, supporting biomedical literature was rarely cited. This restricts the rigor of palaeohistopathological papers, may discourage investigators to pursue histological methods and may hamper the evaluation of results.

Because of its value, histological investigative methods should be accessible to all palaeopathological investigators. For decades, the preparation of sections of undecalcified bone tissue was restricted to well-equipped histological laboratories. Fortunately, is it now possible for high-standard histological sections to be made with relative ease by means of accessible and inexpensive equipment (Maat *et al*., 2001; Beauchesne and Saunders, 2006; De Boer *et al*., 2012b, 2013b).

Thus, we see that microscopy is used for the palaeopathological diagnosis of a wide variety of diseases and conditions. In almost all cases, the histological analysis of bone tissue aids in differential diagnosis, but in only a few diseases can a definitive pathognomonic histomorphology be identified. In general, histological analysis is of value if used in comparison to fresh tissue specimens of 'known cases' and if combined with gross anatomical and radiological results.

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Chapter 5

HUMAN DRY BONE PATHOLOGY FOR ARCHAEOLOGISTS

From: *The histology of human dry bone (a review). De Boer, H.H., Maat, G.J.R., Cuadernos de Prehistoria (in press).*

Abstract. Despite archaeological preservation conditions, the histomorphology of human dry bone still contains useful information on the physiological and pathological status of deceased individuals. Histology can therefore be a helpful addition to routine archaeological methods. But practice has shown that, for many archaeologists, unfamiliarity with this technique shaped a pointless obstacle to add it to their tool box.

Thus, after having addressed the restrictions associated with histological analysis in general, we will show that the preparation of sections/slides does not need to be difficult, expensive or time-consuming. Then we will provide an introduction to the histological application of assessing age at death of the deceased. Its theoretical basis, its value in comparison to other methods and its limits are discussed.

Finally, we will elaborate on the effectiveness of histology as an indicator of pathological processes, and explain that only a small number of disorders have distinct 'pathognonomic' microscopic features. In all other cases, the histological findings must be combined with gross anatomical and radiological findings from the same individual to come to a conclusive diagnosis or to a shortened list of differential (alternative) diagnoses.

Introduction

On a light microscopic level, bone tissue contains useful physiological and pathological information. Investigation of bone by means of microscopy can therefore aid in the understanding of the effect that various phenomena such as ageing, mechanical strain, nutrition, genetics, general health and acquired diseases have on bone tissue (Frost, 1985; Vigorita, 2007). For archeologists this is of special interest, since over the past century it was shown that in dry bone tissue microarchitecture remains for a great deal intact, despite archaeological preservation conditions (Stout and Simmons, 1979).

To meet the specific demands of archaeological research, investigators extrapolated on routine histological methodology developed by the study of normal, physiological bone tissue or of the postmortem alterations thereof in the first place. For instance, methods originally applied to study the bone remodeling process were further developed into methods to estimate the age at death of the deceased (e.g. Kerley, 1965; Stout and Simmons, 1979; Stout and Paine, 1992; Maat, 2006a). Furthermore, microscopic studies on the biology of microorganisms have proven to be of value to determine the extent and cause of taphonomical processes in excavated bone material (e.g. Hackett, 1981a; Bell, 1990, Hedges *et al.*, 1995; Nielsen-Marsh and Hedges 2000, Nielsen-Marsh *et al.*, 2000b; Jans, 2004). Also a long tradition of microscopic investigation of pathological human remains exists. 'Histopalaeopathologists' focused, much in line with their medical counterparts, on the microscopic diagnosis of diseases. All in all, light microscopy has become an important tool in the investigation of human remains from an archaeological context.

Despite the valuable contribution that light microscopy may have on archaeological research, many archaeologist are reluctant to use histological techniques. To some extent, this might be due to the understandable averseness toward invasive section-taking. However, it might also be due to unfamiliarity with histological techniques and the histomorphology of normal and pathological bone tissue. In this article we offer an introduction into the most basic techniques necessary to execute the histological analysis of human dry bone. In addition we provide a concise overview of histological analytical methods that are useful for osteoarchaeologists.

A discussion of the histological differentiation between human and animal bone or the histomorphology of taphonomical processes is beyond the scope of this article. For a recent review on the former see Hillier and Bell (2007). The latter is comprehensively discussed in various other publications (e.g. Hedges *et al.*, 1995; Collins *et al.*, 2002; Jans *et al.*, 2002).

Restrictions with respect to the histology of human dry bone material

Due to the specific nature of dry bone, its histology is considered challenging. By definition, dry bone remains are void of any remaining soft tissue and bone cells, leaving only the mineralized 'framework' for analysis. As medical pathologists use the soft-tissue components to come to an eventual diagnosis in the first place, absence of soft tissue severely hampers the interpretation of the remaining dry bone tissue.

Furthermore, postmortem processes such as weathering, microbial destruction, protein degradation and mineral replacement (fossilization) can modify the original

histomorphology. These 'taphonomical alterations' can subsequently lead to focal destruction, presence of included material, microfissures and loss of birefringence (Jans *et al.*, 2002). As a result, taphonomical processes may lead to partial, or even total destruction of bone histomorphology. Histological analysis may therefore be of little value in badly preserved remains. Finally, during life, bone tissue is a dynamic structure that is continuously adapting to a multitude of stimuli (Frost, 1985). Among others, growth, ageing, disease and nutrition all have their effect on bone (histo)morphology. As bone of the living can only react to a stimulus in a limited number of ways (see below), and as bone of the just deceased only represents a static end-point, and as taphonomic processes will even further alter that end-point status, reconstruction of all past processes is indeed demanding.

The abovementioned dynamics make in-depth knowledge of the growth, ageing and pathology of bone tissue a prerequisite. Although most of this knowledge can be easily acquired from medical histology textbooks (Ross, 1995; Rosai, 2011), or from specialized orthopaedic pathology books such as Vigorita (2007), consultation of an (orthopaedic) pathologist is often wanted for.

The preparation of sections/slides for microscopy

The methods used for the preparation of sections for microscopy originate from those used in laboratories for anatomy and pathology. Still, the specific nature of dry bone does not allow for the direct application of these routinely used histological methods. For instance, the usual prior decalcification of the specimen is not an option, as this will destruct (chemically dissolve) it. Undecalcified tissue on the other hand, does not allow for sectioning by knife-microtome. The brittleness of the bone mineral 'framework' will cause the unembedded specimen to pulverize, or will at least create 'wash board' section surface deformities.

Osteoarchaeologists introduced various methods to tackle these problems. Unfortunately, the majority of these methods is time-consuming, costly and need specialized (automated) equipment. See for instance: Stout and Teitelbaum, 1976; Sturmer, 1979; Wolf *et al.*, 1983; Schultz, 1988; Wallin *et al.*, 1985. For archaeologists on a low budget and without access to a fully equipped histology laboratory, the manual methods as proposed by Maat *et al.* (2001) and De Boer *et al.* (2013a) are good alternatives. These methods were widely tested (Beachesne and Saunders, 2006; Martiniakova, 2006; Van der Merwe, 2010; Turner-Walker and Mays, 2008; Haas, 2011 pers. comm.) and proved to be suitable for cortical and even trabecular/fragile bone. The methods are swift, cheap, and easy to learn and are widely applied by starters

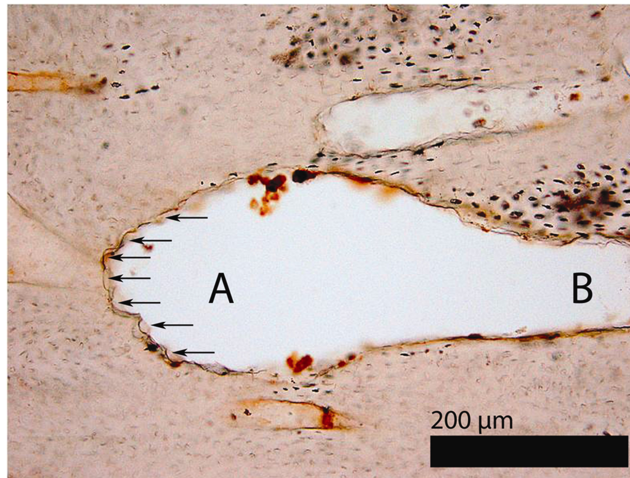
and experienced workers. Detailed manuals can be directly requested for free from the authors.

Histological age at death estimation

Individual age at death assessment is one of the most basic analyses osteoarchaeologists have to deal with. Routinely used gross anatomical methods, such as dental eruption status, epiphyseal closure, cranial suture obliteration, or the alterations of the face of the pubic symphysis, the iliac auricular surface, the cancellous tissue in the proximal ends of long bones and of the fourth rib end, all share the precondition that they need gross skeletal anatomy for a great deal intact. In case of microscopic methods only a small, but essential portion of a bone is needed (the central shaft of the femur or the clavicle, a rib, etc.).

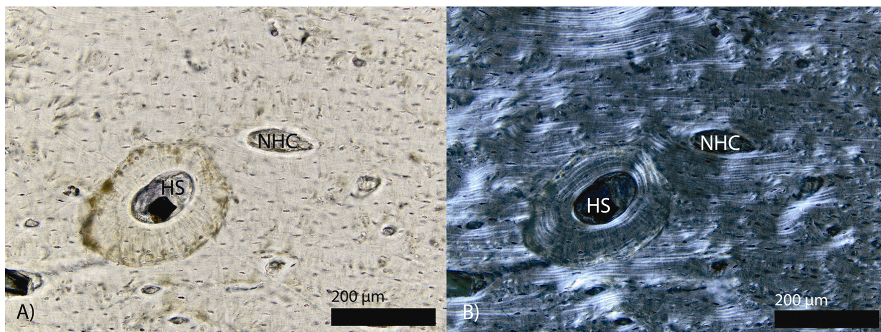
Kerley was the first to make a useful attempt to utilize microscopy for age-at-death assessment, when she published her seminal work on 'the microscopic determination of age in human bone' in 1965 (Kerley, 1965). The method was based on the principle that during life, cortical bone tissue of the shaft of a long bone undergoes gradual conversion/remodeling from circumferential lamellar bone (i.e., bone that was circumferentially deposited during the first anlage of the cortex, embedding pre-existing blood vessels, so-called non-Haversian canals) into new lamellar bone of Haversian systems (osteons with a central blood vessel). The 'older', the more mature the bone, the more the original circumferential lamellar bone with its non-Haversian canals is replaced by Haversian systems. Replacement is executed by Basic Multicellular Units or BMUs (Figure 1). A BMU consists of a 'cutting cone' at its head end and a 'closing cone' at its tail end that respectively 'drills' and replaces existing bone tissue. On a cellular level, the cutting is done by osteoclasts in the head end cone, 'drilling' a longitudinal channel. In the rear end cone, osteoblasts deposit new layers of lamellar bone, thereby 'closing' the channel. The result, in a transverse microscopic slide, is a Haversian system (osteon) bordered by a jagged cement line that abruptly halts/cuts the original circumferential lamellae. This latter feature differentiates it from non-Haversian canals, which borders are flowing in line with the original circumferential lamellar bone and that miss a cement line (Figure 2). The rate of remodeled bone gives an estimation of the age at time of death. Kerley defined this in various bone specific regression formulas (Kerley, 1965; Kerley and Ubelaker, 1978). Initially, her method used diaphyseal cross sections of femoral, tibial and fibular cortices.

Figure 1.



The 'cutting' end of a Basic Multicellular Unit (BMU), as visible in human dry bone. Longitudinal hand ground section of a tibia. Bright Field. Bar indicates scale. The head end cone (A) of the BMU is characterized by its wide 'drilling head' and its numerous Howship's lacunae (arrows). Although the rear end cone of the BMU is not visible, its sloping aspect towards the closing end can be appreciated (B). This is caused by the apposition of bone tissue by osteoblasts. Photograph on courtesy of G.J.R. Maat.

Figure 2.



Differentiation between a non-Haversian canal and a Haversian system (osteon). Transverse section of a human femur. Hand-ground section. Bar indicates scale. **(A)** Viewed with bright field. The circumference of the non-Haversian canal (NHC) is not delineated by a cement line, and its borders (seem to) flow in line with the circumferential lamellae. The border of a Haversian system (HS) is demarcated by a cement line, which indicates the extend of osteoclastic resorption before the subsequent osteoblastic apposition by the Basic Multicellular Unit. As a result, a Haversian system abruptly halts the circumferential lamellae. **(B)** Same section as (A), viewed under polarized light. The polarization enhances the visibility of the differences between the Non-Haversian canal (NHC).

Several authors confirmed the correlation between histomorphology and age at these locations (Ahlqvist and Damsten, 1969; Stout and Gehlert, 1982; Thompson, 1979; Ericksen, 1991; Maat, 2006a). Although the method also proved to be useful in mandibulae (Singh and Gunberg, 1970), in ribs and clavicles (Stout and Paine, 1992) and in humeri and ulnae (Thompson, 1979), the femoral mid-shaft remained to be the most utilized (Maat, 2006a).

In her original publication, Kerley used per defined surface area: osteon counts (counts of intact Haversian systems), counts of fragmented/'older' osteons, the percentage of 'original' circumferential lamellar bone and counts of non-Haversian canals for her regression formulas. However, the use of these features showed to cause definition and identification problems (e.g. Stout and Stanley, 1991), and thus generated substantial inter-observer bias (Lynnerup *et al.*, 2006). Various authors tried to tackle this problem by using other derivatives to define the developmental status of the remodeling process, e.g. by counting aggregations of osteons plus osteon fragments thus outlining the total amount of remodeled bone (Ahlqvist and Dahmsten, 1969; Thompson, 1979; Ericksen, 1991), or inversely, counting the percentage of bone surface occupied by original circumferential lamellar bone plus non-Haversian systems thus outlining the total amount of unremodeled bone (Maat, 2006). In this way the definition problems could be circumvented to a high degree.

Kerley (1965) counted at four positions in a mid-shaft cross section of the femur (anterior, posterior, medial and lateral). Alhqvist and Damsten (1969) also used four fields, but positioned them between those of Kerley, thereby avoiding the linea aspera, the insertion site of thigh muscles. Mechanical strain by these muscles produces a histomorphology unrelated to age. To avoid muscle insertions and complete transsection of a femur, others used only the anterior parts of the shafts (Ericksen, 1991; Maat, 2006a). Thompson (1979) proposed an even less invasive method, by using only a small core of bone tissue, at the expense of a higher statistical bias. Irrespective of which method is adopted, spatial variation in histomorphology within a single transverse slide is high (Saunders, 1987). Also, counting results between bones within a single individual differ (Stout and Stanley, 1991). Sampling and counting must therefore be done, exactly as prescribed in the original manuscripts.

Although the overall results of histological age assessment showed to be acceptable, a few considerations need to be addressed. Bone remodeling is indeed defined by age to a great extent. Nevertheless, inter-individual variation in pace of ageing exists. Other factors have an effect too, for instance: disease, nutrition and mechanical stress (Frost, 1985). Correlation rates (r^2) between microscopic ageing features and true age at death of around 0.7-0.8 and standard errors of circa 10 years

seem to reflect the highest possible accuracy (Maat, 2006a). In spite of suggestions by some, difference between the sexes appears to be neglectable (Uberlaker, 2005; Maat, 2006a)

The gradual increase in the amount of remodeled bone, or the decrease of the percentage of unremodeled bone tissue over age, is not a linear one, but a curvilinear process that comes asymptotically to an end when the last fragment of unremodeled bone tissue is remodeled (Kerley, 1965; Maat, 2006). Most regression-formulas did not take this into account and adopted an unnatural linear correlation.

The degree of inter-observer bias was addressed in several studies (Baccino *et al.*, 1999; Lynnerup *et al.*, 2006). Yet, in cases in which the method was deployed by experienced microscopists, inter-observer bias was shown to be low (Maat, 2006a). Ideally, the histological approach should be used alongside other age assessment methods (Baccino *et al.*, 1999).

The histological diagnosis of disease in general

On a gross anatomical level, the identification of pathological processes and their interpretation in fresh human skeletal remains is complicated. Their histology/histo-palaeopathology in dry bone tissue is even more difficult. Bone reacts to a stimulus/disease in a very limited number of ways. This restricts the differential diagnostic power of microscopy. On a microscopic level, the reaction of bone tissue may be osteoclastic/resorptive, osteoblastic/depositional or a combination of both. Only a very few diseases present with a 'pathognomonic', specific, histomorphology. In all other cases, histomorphology alone is unable to produce a final diagnosis. More often, the combination of gross anatomical/radiological characteristics and distinct (but on itself not pathognomonic) histomorphology may provide a conclusive diagnosis. In most cases, histology must be regarded as a tool to reduce the list of differential diagnoses, or to support an established diagnosis. Below, the few disorders with pathognomonic microscopic features are discussed.

Disorders with pathognomonic histomorphologic features

Paget's disease of bone is an excellent example of a disease with a pathognomonic histomorphology. In this disease, the normal physiological bone remodeling rate is elevated and chaotic. Microscopy shows alternating fields of excessive osteoclastic and osteoblastic activity. Although the earliest phase of the disease demonstrates non-specific resorption, the osteoclastic-osteoblastic interplay eventually produces a

‘mosaic’ pattern of woven and lamellar tissue, demarcated by numerous convoluted cement lines in trabecular and cortical bone tissue (Ralston *et al.*, 2008). Several palaeopathological cases of this ‘Pagetic’ histomorphology have been reported (Stout and Teitelbaum, 1976; Bell and Jones, 1991; Aaron *et al.*, 1992; Roches *et al.*, 2002).

Hyperparathyreoism is caused by elevated blood serum levels of the hyperparathyroid hormone, an activator of widespread osteoclastic activity. Though osteoclastic resorption by itself is not a specific process, its pattern in case of hyperparathyreoidism is particularly outspoken in (sub)periosteal and in cancellous bone tissue (Vigorita, 2007; Fraser, 2011). In the latter, it causes the typical and pathognomonic ‘dissecting osteitis’ or ‘tunneling resorption’. Hyperparathyreoidism is very rare and comes without any external gross anatomical bone changes. Still, various palaeopathological cases have been discovered in well-preserved dry bone skeletons (Weinstein *et al.*, 1981; Cook *et al.*, 1988; Zink *et al.*, 2005).

The histomorphology of osteomalacia (the adult form of hypovitaminosis D) in dry bone tissue is dominated by an overall increased osteoclastic activity, as the living bone attempts to keep blood serum calcium levels within a normal range (Stout and Teitelbaum, 1976; Schamall *et al.*, 2003; Brickley *et al.*, 2007). The pathognomonic histomorpholoy of osteomalacia is not based on its specific osteoclastic pattern but on microscopic vestiges/remnants of calcification defects of osteoid. Osteoid is the ‘to-be-mineralized’ bone matrix substance. Brickley reported that these vestiges may show as so-called ‘defect osteoid lines’ in dry bone Haversian systems. They are caused by the postmortem decomposition of osteoid (Brickley *et al.*, 2007). It should be noted that this feature has not been reported by other palaeopathologists. Nevertheless, knowledge on these vestiges was supported by comparative fresh tissue specimens of known disease cases.

Osteoporosis is a symptom, not a disease/final diagnosis and is defined as a decrease in total bone volume. As such, its histomorphology is pathognomonic by definition. Palaeopathologists used metric (Richmanna and Ortner, 1979; Martin and Armegalos, 1979, 1985; Gonzales-Reimers and Arnay de-la-Rosa, 1992; Velasco-Vazques *et al.*, 1999; Paine and Brenton, 2006; Cho and Stout, 2011) and non-metric histomorphological methods to establish its existence (Roberts and Wakely 1992; Schultz, 1999). As osteoporosis is a disorder in terms of quantity, theoretically a metric diagnostic approach should be preferred.

Finally, accurate diagnoses can sometimes be made when ‘dry bone’, assumed to be composed of its mineral component only, still appears to contain remnants of soft tissue/cells, even if fossilized. In such cases, the combination of dry and soft tissue histology may provide a sound diagnosis. Examples are, for instance: a calcified myoma uteri (Strouhal, 1976), a sacral neurolemmoma (Strouhal and Nemecková,

2004) and sickle cell anaemia (Maat and Baig, 1990; Maat, 1991). Although such findings are rare, they illustrate that histology may yield unexpected results.

The complementary value of histology in the diagnosis of other diseases

The disorders in this category miss pathognomonic histomorphologic characteristics. Nonetheless, histological analysis may yield considerable corroborative information.

In scurvy (a deficiency of vitamin C) the increased tendency to develop subperiosteal hematomas may lead to their ossification if fresh supplies of vitamin C become available to the sufferer (Maat and Uytterschaut, 1984; Ortner, 2003; Maat, 2004). Gross anatomically, this pathologic change may look similar to that of infectious and tumorous lesions or to that of ossified hematomas from mechanical trauma or to hypertrophic (pulmonary) osteoarthropathic depositions. But in contrast to the latter two options, scurvy has a distinct/specific distribution pattern of depositions (Maat, 2004). And in contrast to the first two alternatives, ossified hematomas leave the periosteal surface intact (Schultz, 2001; Maat, 2004). This feature can only be properly visualized by microscope. Thus, ossified hematomas are not pathognomonic for scurvy. But if they occur symmetrically at anatomical predilection sites, i.e. especially at the metaphyses in the lower extremities, and come with other scorbutic characteristics, then the diagnosis becomes almost certain.

Like in case of osteoporosis, 'cribra orbitalia' and 'porotic hyperostosis' are non-specific disease features. Histology may help to differentiate between their potential causes: infection, taphonomy, chronic anaemia (hereditary or acquired haemolytic forms; Marscik *et al.*, 1984; Maat and Baig, 1990, 1997; Schultz, 2001; Wapler *et al.*, 2004; Walker, 2009). In the latter case, a radiating hypertrophy of hematopoietic bone marrow will expand the amount of cancellous bone at the cost of the external cortical lamina. In infections, the histomorphology is dominated by irregular lytic changes and abundant fields of Howship's lacunae. Taphonomic causes demonstrate an absence of osteoclastic or -blastic processes and a disregard for microscopic anatomical dimensions/borders. Although none of these features are pathognomonic, the 'overall histological image' can be indicative for either one of the causes.

In primary bone tumours, histology can help to identify whether the growth process is benign or malignant. The former demonstrates a more regular/organized texture, grows non-invasive and is predominantly osteoblastic (Strouhal *et al.*, 1996; Vyhanek *et al.*, 1999; Hershkovitz *et al.*, 1999; Eshed *et al.*, 2002). Malignant lesions are

invasive by definition, and present both osteoblastic and osteoclastic activity. In malignant lesions, the erosive areas feature numerous fields of Howship's lacunae, whereas the new appositional bone is an irregular combination of lamellar and woven bone. It shows little to no signs of regular physiologic remodeling (Suzuki, 1987; Schultz in: Strouhal 1991; Strouhal *et al.*, 1997). None of these features is pathognomonic, not even for tumours. Still, in combination with gross anatomical and radiological data, microscopy will cut back the list of differential diagnoses. Histology has proven to be especially useful if comparative fresh tissue samples of documented cases are available (Schamall *et al.*, 1999; Eshed *et al.*, 2002).

Secondary bone tumours (metastases) are malignant by definition. Their histomorphology is therefore similar to that of malignant primary bone tumours. In addition, the apposition of new bone in Haversian canals may suggest a hematogenous dissemination (Anderson *et al.*, 1992; Wakely *et al.*, 1995). In concordance to clinical practice, metastases are defined as either osteoblastic, osteoclastic or a combination thereof (examples of fresh tissue pathology references: Roodman, 2011; examples of palaeohistopathology references: Tkocz and Bierring, 1984; Campillo and Marci-Balcells, 1984; Grupe 1988; Anderson *et al.*, 1992; Schultz, 1993; Wakely *et al.*, 1995; De la Rua *et al.*, 1995; Šefčákova *et al.*, 2001; Schultz, 2007; Molnar *et al.*, 2009). Histology is an apt instrument for such differentiation. Yet, the differentiation may be of little practical value for the diagnosis of the type of tumour, since there is no fixed relationship between the histology of the metastasis and the original/primary tumour. Clinical practice shows that a primary tumour may have both osteoblastic and osteoclastic metastases (Roodman, 2011).

In all tumorous lesions (primary or secondary), the likely diagnosis depends on data such as age at death, sex of the individual, distribution pattern of the lesions and the gross anatomical/radiological appearance (e.g. Suzuki, 1987; Schultz, in Strouhal, 1991; Wakely *et al.*, 1998).

In the same way, the histomorphology of infectious lesions is not pathognomonic. Generally, infectious lesions demonstrate abundant osteolytic/resorptive destruction of the original bone tissue architecture and the formation of reactive new bone tissue (Hackett, 1981a; Blondiaux *et al.*, 1994; Schultz, 2001; Schultz and Roberts, 2002; Wapler *et al.*, 2004; Von Hunnius *et al.*, 2006; Flohr and Schultz, 2009a, 2009b; Weston, 2009; Van der Merwe *et al.*, 2010;). As such, histology can be used to differentiate between infectious lesions and taphonomic alterations. Differentiation between infectious and tumorous lesion is however misleading with histology alone.

Whether specific infections, such as tuberculosis, lepra or treponematoses produce pathognomonic histomorphologies has been studied on several occasions (Hackett, 1981a; Blondiaux *et al.*, 1994; Schultz, 2001; Schultz and Roberts, 2002; Von Hunnius *et*

al., 2006; Weston, 2009; Van der Merwe *et al.*, 2010). Palaeopathologists nowadays generally agree that no such pathognomonic histomorphology exists (Weston, 2009; Schutskowski and Fernandez-Gil, 2010; Van der Merwe *et al.*, 2010). The histomorphological features previously described as pathognomonic of for instance treptonematosis (see e.g. Schultz, 2001) must be regarded as indicative for a chronic, slowly developing infectious disease. The diagnosis of specific infectious diseases should therefore be based on the combination of gross anatomical, radiographic and sometimes histological analysis.

With respect to the diagnosis of mechanical traumas (ante- and postmortem fractures, amputations and ossified hematomas after long standing subperiosteal bleedings), practise shows that the diagnosis of fractures and amputations is seldom based on their histological analysis. Still, histology can be of use, since the microscopically assessed phase of their healing process can be linked to a certain minimum posttraumatic survival time (Blondiaux, 2000; Maat, 2006; De Boer *et al.*, 2012, 2013b). Microscopy is also helpful to differentiate between ante- and postmortem traumas (Maat, 2008; De Boer, 2013b). Finally, ossified subperiosteal hematomas show a very characteristic radiating architecture, that tends become solid over time by the 'filling in' of interradiate spaces, and finally by the regular remodelling process (Van der Merwe *et al.*, 2010).

Conclusion

The nature of dry bone tissue excludes the possibility to apply microscopic section/slide preparation methods routinely used in (medical) histology laboratories. Nonetheless, sections can be made manually in a quick and cheap manner, by means of very basic tools, making histology accessible to all archaeologists.

Histological analysis of human remains may aid in the assessment of age-at-death and palaeopathological disorders. But only few disorders present specific, pathognomonic, features. In all other cases, histological findings must be combined with gross anatomical and radiological findings to come to a conclusive diagnosis or to a shortened list of differential (alternative) diagnoses.

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Chapter 6

SCURVY AS A FACTOR IN THE LOSS OF THE 1845 FRANKLIN EXPEDITION TO THE ARCTIC: A RECONSIDERATION

From: *Scurvy as a factor in the loss of the 1845 Franklin expedition to the Arctic: a reconsideration. Mays, S., Maat, G.J.R., De Boer, H.H., 2013. International Journal of Osteoarchaeology (in press).*

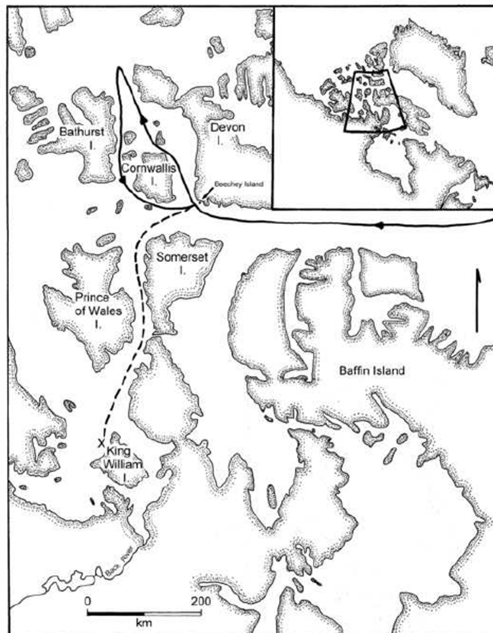
Abstract. In 1845, an expedition, commanded by Sir John Franklin, set out to discover the Northwest Passage. The ships entered the Canadian Arctic, and from September 1846 were beset in ice off King William Island. A note left by the expedition in May 1847 reported all was well, but by April 1848, 24 of the 129 men had died. The ice-locked ships were deserted in April 1848, but the 105 survivors were so weakened that all perished before they could reach safety. The causes of the morbidity and mortality aboard the ships have long been debated, and many commentators have argued that scurvy was an important factor. This study evaluates the historical evidence for the likely effectiveness of anti-scorbutic precautions taken on polar voyages at that time, and investigates whether the skeletal remains associated with the expedition provide evidence for scurvy. Skeletal remains available for study were carefully examined for pathological changes, and lesions potentially consistent with scurvy were subject to histological analysis. Where remains were no longer accessible, use was made of published osteological work. It is argued that the anti-scorbutic measures customarily taken on mid 19th century British naval polar voyages were such that there is no *a priori* reason to suppose that scurvy should have been a problem prior to the desertion of the ships. The analysis of the skeletal evidence provided little in the way of bony lesions consistent with the disease, and cannot therefore be used to support the presence of scurvy. Factors other than scurvy may have been the main causes of morbidity and mortality in the eleven months prior to the desertion of the ships.

Introduction

In May 1845 two ships, HMS *Erebus* and HMS *Terror*, set out from England on a Royal Navy voyage to the Arctic. The ships were under the command of Sir John Franklin. The purpose of the expedition was to map the route of the Northwest Passage, the seaway connecting the north Atlantic with the Pacific via the Canadian Arctic. The expedition called at Disko Bay on the west coast of Greenland in July 1845, and later that month entered the Canadian archipelago via Lancaster Sound with 129 men. None would return alive.

The expedition wintered in 1845-6 at Beechey Island in the Canadian High Arctic (Fig. 1). Three men died during this period and were buried in the permafrost (Beattie and Geiger, 1987). The following summer, the ships sailed south and west in search of the Passage. In September 1846, the ships were once more beset in ice, northwest of King William Island. The ice failed to release them the following summer. A note, left in a canister on King William Island, dated May 1847, indicated that all was well. However an addendum, dated April 1848, indicated that 24 men (nine officers, 15

Figure 1.

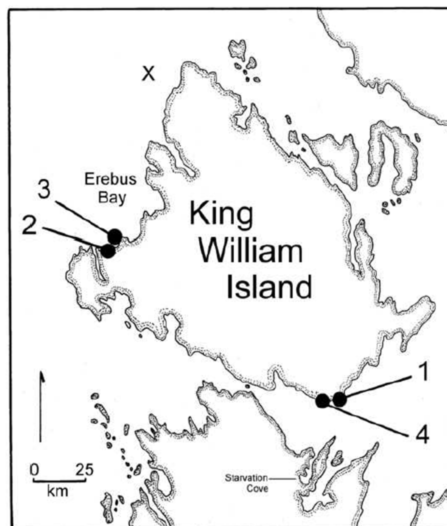


Canadian Arctic, showing Franklin's 1845 route (solid line), and presumed 1846 route (dashed line).

lower ranks) had died by that time, and the remainder would attempt to reach safety overland via the Back River. Surface scatters of artifacts and human remains show that many died on the march along the western and southern coasts of King William Island (Cyriax, 1939; Owen, 1978). The furthest point reached was Starvation Cove on the Canadian mainland, barely 400km along the presumed route, and still more than 1000km from the nearest Hudson Bay Company post via the Back River (Gibson, 1937).

Cut-marks on human remains (Beattie, 1983; Beattie and Savelle, 1983; Keenleyside *et al*., 1997) confirm Inuit accounts of cannibalism in the final throes of the expedition, and leave little doubt that those who perished attempting to make the Back River suffered from starvation. The small distance that the men had managed to travel from the ships suggested that they were already in a severely weakened state and hence that problems began before this. This is supported by the number of deaths that occurred prior to the desertion of the ships. The 24 deaths that had occurred by April 1848 represent 19% of the ships' companies. The Franklin voyage was very well equipped, and members included men experienced in polar conditions.

Figure 2.



King William Island, showing location of finds of the material listed in Table 1. Find spots: 1, Booth Point, 2, Erebus Bay, 3, Islet in Erebus Bay, 4, Near the mouth of the Pfeffer River. X indicates the location of the ships when deserted in April 1846.

Yet the death rate up to April 1848 was markedly greater than that in any other Royal Navy polar expedition (Woodman, 1991). In eight British Naval expeditions between 1819 and 1836 the overall death rate was only 3% (Cookman, 2000). In an 1839 voyage to the Antarctic, under the command of James Clark Ross, aboard HMS *Erebus* and HMS *Terror*, the deaths after 3 years amounted to just 4% (Ross, 1994).

Reasons for the deaths in the period May 1847 - April 1848, and for the weakened state of those who deserted the ships, have been debated for the last 150 years. Starvation seems unlikely at that stage of the expedition. Franklin indicated that his supplies were sufficient for five years, and if necessary could be made to last for seven (Beattie and Geiger, 1987). In addition, firearms had been taken with the intention of taking birds or other game as the opportunity arose (Owen, 1978), and bones of wildfowl at the Franklin camp at Beechey Island testify that this occurred (Cyriax, 1939). Different theories involving health problems have been suggested. Research in the 1980s and 90s reported high lead levels in the human bones from King William Island (Beattie, 1985; Kowal *et al*., 1989, 1991; Keenleyside *et al*., 1996), and in tissue samples from the Beechey Island bodies, which were exhumed in the 1980s (Amy *et al*., 1986; Kowal *et al*., 1991). These findings have been used to support suggestions that lead poisoning, either from the expedition's canned food supply (Beattie and Geiger, 1987; Kowal *et al*., 1989, 1991) or other sources (Battersby, 2008) contributed. Another suggestion was that food poisoning through inadequate canning of the tinned meat supplied to the expedition, or from shot game, was a factor (Cookman, 2000; Horowitz, 2003). However, what is perhaps the oldest theory holds that the men were suffering the effects of scurvy.

Scurvy arises due to a deficiency of vitamin C in the diet. Prime sources of vitamin C are fresh fruit and vegetables, but it is also present to a lesser extent in fresh animal products. The vitamin oxidises on exposure to air so, unless adequate precautions are taken, the vitamin C content of foods gradually diminishes during storage. Scurvy was always a potential threat on polar voyages due to the lack of locally available fresh fruit and vegetables.

In 1859, an expedition under Francis McClintock found the first traces, in the form of human remains and artifacts, relating to the final march of Franklin's men along the western and southern shores of King William Island. Noting that the rapidity of the disaster that overtook them meant that the men must have been in a severely weakened condition when they left the ships, he ascribed this to scurvy (McClintock, 1908). Gibson (1937) felt that it was scurvy that caused the men to abandon the ships and to seek the Back River route to safety because game was known to be abundant in that area and fresh meat was understood to be beneficial for scurvy. Two years later, Cyriax, writing what has become the classic account of the expedition, felt able

to state that there had been 'general assent' to the scurvy theory (Cyriax, 1939). Subsequent major works on the voyage have largely concurred that scurvy was an important factor in the mortality and morbidity suffered by the expedition between May 1847 and April 1848 (e.g. Neatby, 1970; Owen, 1978; Beattie and Geiger, 1987; Berton, 1988; Woodman, 1991; Ross, 1994; Lambert, 2009; Brandt, 2011).

Despite this general consensus, there was for some time no direct evidence to support the scurvy theory. There is testimony from Inuit that expedition members they encountered on their final march along the south coast of King William Island had blackened mouths (Woodman, 1991). Darkening of the gums could be consistent with scurvy (Aschoff and Koch, 1919), but is rather non-specific – for example it may also result from chronic lead ingestion (Lockheart, 1981). The Inuit accounts were related second-hand more than 20 years later so it is difficult to know what weight to give them. In any event, they do not tell us anything about the condition of the men whilst they were still on the ships. The lack of any direct evidence for scurvy appeared to change with the examination of skeletal remains from King William Island recovered during the 1980s. Owen Beattie and his co-workers conducted the first modern archaeological surveys in this area, and recovered and studied 36 bones representing a minimum of 3 individuals.

Deficiency of vitamin C leads to weakening of blood vessel walls, so haemorrhage is a prominent and characteristic feature of scurvy. If haemorrhage occurs close to bone it may potentially lead to osteological changes, in adults principally subperiosteal deposits of new bone (Brickley and Ives 2008). Beattie reported periosteal reactions on some bones from Erebus Bay on the west coast of King William Island, and from the vicinity of Booth Point, on the south coast (Beattie, 1983; Beattie and Savelle, 1983). Periosteal reactions may arise as a response to a multitude of conditions other than scurvy (Weston, 2008). Beattie (1983) acknowledged this, but observed that their occurrence supported the presence of scurvy. In 1992, another King William Island Franklin site, on an islet in Erebus Bay, was located. More than 300 human bones were retrieved and subject to osteological study (Keenleyside *et al*., 1997). One bone showed periostitis, but rather than inferring scurvy, the authors felt the lesion suggested trauma or infection.

Since the publication of Beattie's work, major works on the Franklin tragedy have used his skeletal evidence to support the scurvy hypothesis. Some have greatly exaggerated the evidence, or else attributed to the findings an unwarranted degree of diagnostic certainty. For example, Woodman states that changes attributable to scurvy 'were universally found in the remains from King William Island' (Woodman, 1991), and for Lambert, the presence of scurvy has been 'confirmed by forensic science' (Lambert, 2009). The purpose of the current work is firstly to review the anti-

scorbutic precautions followed on British naval Arctic expeditions at the time of the Franklin voyage, and secondly to evaluate the osteological evidence from remains associated with the expedition to determine whether, as has been claimed, they provide evidence for the presence of scurvy.

Scurvy and the Franklin expedition

Following the pioneering experiments of 18th century British naval surgeon James Lind that demonstrated the value of citrus fruit in combating scurvy, and the advocacy of Sir Gilbert Blane, another naval physician, orders were issued in 1795 that lemon juice be carried on board every ship in the Royal Navy (Sauberlich, 1997). The use of lemon juice virtually eliminated the problem of scurvy in the British navy for the next 50 years (Carpenter, 1986; Harvie, 2002), and it was routinely used at the time the Franklin expedition set sail.

Victualling records indicate that the Franklin expedition was supplied with 9300lbs of lemon juice (Cyriax, 1939). When it was introduced in 1795, the daily lemon juice ration was three-quarters of an ounce per man (Brown, 2003). However, in view of the special threat of scurvy in polar regions, this was increased to one ounce on Arctic voyages (Cyriax, 1939). On this basis, there would have been sufficient lemon juice on board for full Arctic rations for at least three years and two months, i.e. until July 1848, three months after the date at which the ships were deserted.

Although the cause was not understood, it was well-recognised by the 19th century that lemon juice gradually lost its antiscorbutic properties upon exposure to air. The usual way to preserve it was in containers with olive oil poured into the top, and which were then corked and sealed, a system first devised in the 17th century (Carpenter, 1986). It is not known exactly how the lemon juice was stored aboard the *Erebus* and *Terror* (Beattie, 1985), but given the care with which the expedition was equipped and victualled (Cyriax, 1939; Beattie and Geiger, 1987) it seems unlikely that these routine and elementary precautions against its deterioration, which were used on other mid 19th century polar expeditions (e.g. Armstrong, 1858), would have been neglected.

That men could be kept healthy on prolonged naval voyages under polar conditions is illustrated by contemporary accounts. John Ross's 1818 Arctic expedition spend four winters on the ice without suffering scurvy (Houston, 1990). In the same commander's 1829 expedition, his men were 'fresh and good humoured' after three winters on the ice (Neatby, 1970), and only one man died of scurvy during the whole four-year voyage (Cookman, 2000). In Parry's 1821 Arctic expedition, slight symptoms of scurvy were reported after 27 months but no-one succumbed (Houston,

1990). In 1854, the crew of Collinson's *Enterprise* emerged from three winters in the Arctic in excellent health (Neatby, 1970). Aboard McClure's *Investigator*, in a three year Arctic expedition beginning in 1850, only three men were lost to scurvy, and that was toward the end of the expedition, some months after the lemon juice ration had been halved (Armstrong, 1858; Harvie, 2002). However, scurvy was a greater problem on some other polar voyages. The 1848 expedition in search of Franklin under James Clark Ross suffered from scurvy in their first winter (Owen, 1978). George Back's 1836 Arctic voyage was afflicted by scurvy after only 6 months (although, as he noted, other voyages were by that time usually exempt from the disease). The narrative of the expedition (Back, 1838) suggests that provision of lemon juice was inadequate in quantity, and anti-scorbutics did not appear to have been taken daily.

It would seem that although polar expeditions were not always free of scurvy, the disease could be effectively combatted for prolonged periods with proper dosage of lemon juice. There is therefore no *a priori* reason to assume that scurvy was a problem for the Franklin expedition in the 11 months between May 1847 and April 1848. In addition, the scurvy hypothesis fails to account for the disproportionate numbers of deaths that befell the officers during that period (9 out of 24 officers (38%) died compared with only 15 of 105 (14%) lower ranks). The remainder of this paper attempts to investigate the extent to which skeletal remains from the voyage can be used to support the scurvy hypothesis.

A difficulty with interpreting skeletal evidence for scurvy is that signs of the disease in the adult skeleton are rather subtle, and certainly less pronounced than in the growing skeleton, and none are pathognomonic (Joffe, 1961; Ortner, 2003; Brickley and Ives, 2008; Mays, 2008). The most important skeletal changes are connected with osteological responses elicited by haemorrhage. The bleeding and swelling of the gums that is such a prominent soft tissue feature of scurvy may potentially lead to changes in the alveolar bone (Ortner, 2003; Van der Merwe *et al*., 2010a). However, alveolar bone changes arise through a great many other causes which have nothing to do with vitamin C deficiency (Hillson, 1986), and some alveolar bone pathology is almost universal in adults in British pre-modern skeletal populations (Kerr, 1998). Alterations to alveolar bone are therefore of little value in the identification of scurvy. Diffuse osteopaenia may occur in scurvy (Joffe, 1961) and may predispose to fracture, especially of the ribs (Aschoff and Koch, 1919) and vertebrae (Grusin and Kincaid-Smith, 1954). However, a great many diseases other than scurvy can predispose to fracture, and, obviously, fracture may also occur without a predisposing skeletal condition. Subperiosteal haemorrhages occur in scurvy, often in response to minor trauma. In infants and young children, bony

alterations due to haemorrhage characteristically occur in the craniofacial skeleton (Ortner, 2003). These may sometimes be seen in adults (Geber and Murphy, 2012), but the more typical lesions occur postcranially. In the scorbutic adult, subperiosteal haemorrhages occur most often in the lower limb (Hess, 1920) where they may provoke an osteoblastic response resulting in deposition of new bone upon the normal bone surface (Hess, 1920; Wolbach and Howe, 1926; Grusin and Kincaid-Smith, 1954; Joffe, 1961; Aufderheide and Rodríguez-Martín, 1998; Weinstein *et al*., 2001; Fain, 2005). In the absence of vitamin C intake, haemorrhage, indicated by ecchymoses (darkening caused by escape of blood into subcutaneous tissue) may appear on the lower limb in as little as 1-2 months, even in individuals who were replete with the vitamin at the time the vitamin C-free diet was begun (Hodges *et al*., 1971).

The current work re-evaluates the skeletal evidence for scurvy by, as far as is possible, re-examination of the skeletal material from King William Island. The focus will be principally on the evidence for periosteal new bone in the remains.

Materials

The human remains recovered from King William Island since 1980, and upon which there is published scientific work, are listed in Table 1, and the find spots are shown in Fig. 2. Remains from three of the four locations were scatters of disarticulated remains partially exposed upon the ground surface. They principally comprise bones from the lower limb, upper limb long-bones and cranial vault fragments. The remains interred beneath the Franklin Memorial in Greenwich were recovered from King William Island in 1869 as an articulated burial in which most major elements were present. All remains were recovered from locations lying on the presumed route of the final march of Franklin's men, so it is likely that they mainly or entirely represent remains of men who perished after the desertion of the ships in April 1848. Of the remains in Table 1, those described by Keenleyside *et al*. (1997) are no longer available for study, but they were subject to detailed osteological analysis by Keenleyside's team. The other material in Table 1 has been examined for the present work.

Methods

The remains recovered by Beattie's team in 1981/2, and the skeleton interred beneath the Franklin memorial, were carefully examined under strong light for

Table 1. Human remains recovered from King William Island since 1980.

Location	Date recovered	Nature of material	No. of bones[a]	MNI[b]	Reference	Material studied in present work
Booth Point	1981	Surface scatter	8	1	Beattie (1983) Beattie & Savelle (1983)	Yes
Erebus Bay	1982	Surface scatter	28	2	Beattie (1983)	Yes
Islet in Erebus Bay	1993	Surface scatter	304	11	Keenleyside *et al*. (1997)	No
Near mouth of Pfeffer River	1869/2009[c]	Articulated burial	69	1	Mays *et al*. (2011)	Yes

[a] Cranium counted as one element. Full skeletal inventories are given in the cited references.

[b] MNI = Minimal Number of Individuals.

[c] Recovered from King William Island in 1869 and interred beneath Franklin Memorial, Greenwich England, from where they were exhumed in 2009.

evidence of pathology, paying particular attention to evidence of periostitis. Any instances of subperiosteal new bone thus identified were subject to histological study with the aim of distinguishing periosteal new bone formation as a response to haemorrhage (and hence potentially due to scurvy) from that arising from other causes (van der Merwe *et al*., 2010b).

For histological study, a transverse section of bone was removed by making two, closely spaced parallel cuts of sufficient depth to penetrate the full thickness of the cortical bone. The saw blade was twisted to break the section free; care was taken not to cause any flaking of the bone surface.

The cut sections were processed according to earlier published methods (Maat *et al*., 2001, De Boer *et al*., 2013). For reasons of readability the procedures will be summarized here. Prior to further processing, the sections were embedded in LX-112 epoxy resin to prevent any damage to the fragile subperiosteal bone. After overnight curing, the sections were cut by means of a hacksaw and ground down by hand to a thickness of approximately 80μm. The sections were cover-slipped using the same resin as was used for embedding. Histological analysis was done by means of a standard light microscope, using bright light and polarized light.

Results

Gross observations

The Greenwich Memorial skeleton was examined prior to its reburial in 2009. The specimens collected by Beattie from Booth Point and Erebus Bay were studied at the Canadian Museum of Civilization in Ottawa. All were present in the collection, save for a partial left scapula and a small cranial fragment, both from Erebus Bay, which had been destroyed for lead analysis (Beattie, 1985; Kowal *et al*., 1991); neither had been identified by Beattie (1983) as pathological. Some small samples had also been cut from a few other elements for lead content analysis. The condition of the material in general was good. Most subperiosteal surfaces were undamaged, but a few showed flaking and / or cracking due to weathering.

In general, there was little evidence for pathological changes. None of the specimens examined showed *in vivo* fractures or gross suggestions (in the form of thinned cortices or rarified trabecular bone) of osteopaenia, nor were there any potentially haemorrhagic changes in the cranial bones. Similarly, none of these indications was reported by Keenleyside *et al*. (1997) in the material they examined, but one specimen, a right tibia, was reported by them as showing periosteal new bone. None of the bones from the skeleton from the Franklin Memorial in Greenwich show evidence of periosteal new bone, but three postcranial specimens from Beattie's fieldwork appeared to show minor, well remodelled periosteal reactions:

Booth Point, Beattie specimen number 81-22. The Booth Point remains comprise parts of at least eight skeletal elements - cranium, left fibula, left tibia, right femur, left and right radii, left and right humeri - probably from a single individual. Specimen 81-22 is the left tibia. It is the diaphysis in two parts. The proximal part is 14cm long, the distal part 17cm; both ends are missing (Beattie, 1983: Fig. 3). This bone has had a small piece removed from the midshaft for lead analysis. At the midshaft there is a slightly porous area on the postero-medial angle of the bone (Fig. 3a). It extends along the middle third of the bone shaft. It appears slightly raised above the surrounding bone.

Erebus Bay, specimen 82-1. This specimen was found as part of a scatter on the south side of the bay. It is a right tibia (Beattie, 1983: Fig. 10), complete but for three samples that have been removed for lead analysis at the proximal and distal metaphyses and at the midshaft. Its maximum length is 34.2cm. The specimen bears a small, raised area of bone on the medial surface of the proximal metaphysis (Fig. 4a). This area shows a little pitting, and its posterior border is fairly distinct and slightly raised above the surrounding bone.

Erebus Bay, specimen 82-12. This specimen was an isolated find on the south side of the bay. It is a left tibial shaft (Beattie, 1983: Figs 7 and 10), both ends missing, 22.2cm long. It is not from the same individual as 82-1. There is a pitted, slightly roughened area extending from the midshaft to the broken distal end of the bone on the posterior part of the medial surface (Fig. 5a).

In arctic burial conditions, remains of haematomas, even if unossified, may survive as dark staining on the bones (Maat, 1982, 2004). Neither the Greenwich skeleton, nor the material collected by Beattie, showed evidence for such staining. Keenleyside *et al*. (1997) noted no such alterations in the material they studied.

Histological observations

Transverse sections of the periosteal lesions identified on specimens 81-22, 82-1 and 82-12 were removed and histological slides prepared. Each specimen showed excellent preservation of microstructural features.

The microscopic section from specimen 81-22 (Fig. 3b, 3c) appears to show a very well consolidated, localised deposit of new bone, with a maximum thickness in the cut section of about 2mm. The original subperiosteal surface is discernible, particularly under polarised light (Fig. 3c), but the circumferential lamellae are no longer continuous, having been partly obliterated by remodelling. There is no evidence for any lytic changes nor of any alterations to the original periosteal surface that might suggest infection. There is no sign of any pathological process active at time of death. The appearance of the lesion is suggestive of an extensively remodelled ossified subperiosteal haematoma (phase III remodelling on the scale of van der Merwe *et al*. (2010b)).

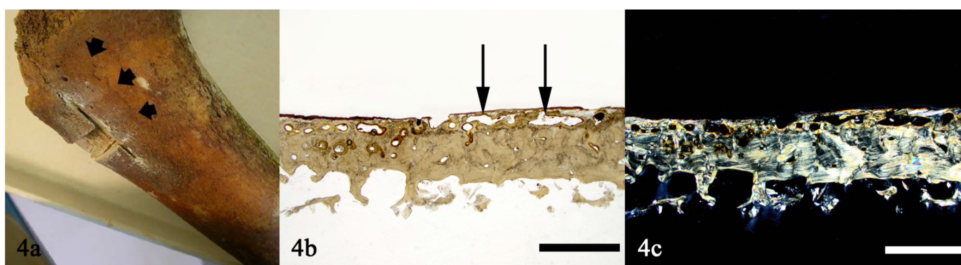
Specimen 82-1 (Fig. 4b, 4c). There are sinuous cavities beneath the intact periosteal surface which may be relics of an earlier infectious process. There is no sign of haematoma formation and no evidence of any abnormal process active at time of death. The evidence suggests an old remodelled lesion which arose as a result of a non-haemorrhagic process.

The section from specimen 82-12 (Fig. 5b, 5c) presents a similar appearance to that from 81-22. There appears to be a well-remodelled deposit of subperiosteal new bone upon an intact cortical surface. The original circumferential lamellae have been partially removed by remodelling. There is no evidence for any abnormal process active at time of death. The lesion appears to represent an extensively remodelled ossified haematoma.

Figure 3.

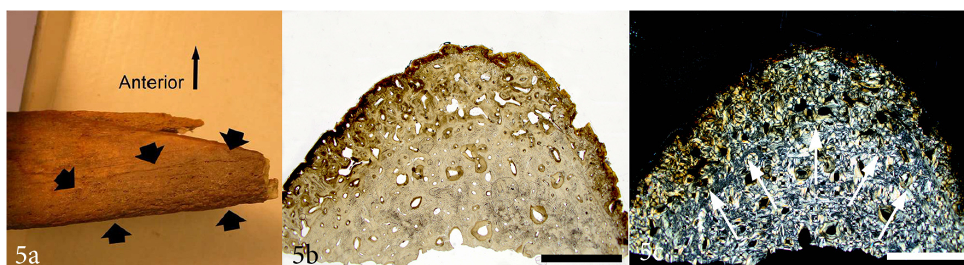
Booth point, specimen 81-22 **(3a)** Left tibia diaphysis, medial view. The arrows denote the raised, slightly pitted area towards the posterior margin of the medial surface. **(3b and c)** Transverse section of the lesions, under plain (b) and polarized light (c). Scale bar 2 mm. Arrows (c) indicate the line of the orginal subperiosteal surface.

Figure 4.



Erebus bay, specimen 82-1. **(4a)** Proximal part of right tibia medial surface. The arrows indicate the raised, slightly pitted area of bone. A sample of bone had been cut for lead content analysis. **(4b and c)** Transverse section of the lesion, under plain (b) and polarized light (c). Scale bar 2 mm. Arrows (b) indicate two large sinous cavities.

Figure 5.



Erebus Bay, specimen 82-12. **(5a)** Distal part of the left tibia shaft, medial view. There is an area of slightly roughened bone at the posterior part of the medial surface, toward the distal end of the bone (arrows). **(5b and c)** Transverse section of the lesion, under plain (b) and polarized light (c). Scale bar 2mm. Arrows in image (c) indicate the line in of the original subperiosteal surface.

Discussion

In two of the three specimens showing gross signs of abnormality, histological study suggested the presence of very well remodelled ossified haematomas. Ossified haematomas may arise due to trauma of the periosteum in undiseased individuals (Resnick and Niwayama, 1988), as well as in scurvy. Lesions due to the former cause are usually isolated and often occur on bones such as the tibia where there is only a thin covering of soft tissue (Zimmerman and Kelley, 1982; Aufderheide and Rodríguez-Martín, 1998). In scurvy, the potential for widespread haemorrhage upon minimal trauma means that ossifying haemorrhagic lesions are often multiple, and may involve more than one bone. Given the nature of the remains, the question of whether multiple bones were affected in these individuals cannot be answered conclusively, but a few points can be made. None of the other bones available from the Booth Point individual show periosteal new bone (a femur did show slight porosity over its general surface, some of which at least was antemortem, but there appeared to be no new bone deposition and the porosity appeared within the bounds of normal variation). It is not known whether left tibia 82-12 and the right tibia from the site on the small islet in Erebus Bay, described by Keenleyside *et al*. (1997) as showing periosteal new bone, come from the same individual but given the difference in location it seems unlikely. The lesions in 81-22 and 82-12 are located on subcutaneous surfaces vulnerable to trauma. Trauma in a healthy individual would appear to be as likely an interpretation as haemorrhage associated with scurvy. Both the ossified haematomas examined here were very well remodelled. Whatever the cause, these haemorrhagic lesions occurred long before death, potentially prior to the expedition rather than on it.

It appears that some vitamin C is needed for individuals to be capable of mounting a significant osteoblastic response, and hence for ossification of subperiosteal haematomas. It might therefore be suggested that we would not expect to see periostitis in those who failed to recover from scurvy. Experimental work with guinea pigs, which, like humans, are unable to synthesise vitamin C and hence require a dietary intake, indicates that in the absence of vitamin C bone formation is severely retarded, but when some vitamin C is restored to diets bone deposition does occur. Ossification of subperiosteal haemorrhages occurs in partially vitamin C deficient guinea pigs, and only small amounts of vitamin C are needed to enable an osteoblastic response – only about 2-5% of the dose required to maintain vitamin C saturation in a healthy animal (Bourne, 1942, 1943; Murray and Kodicek, 1949a, b). Observations in man show that radiographically visible periosteal new bone deposition in association with haemorrhage adjacent to bone occurs in acute as well

as in chronic cases of scurvy (Joffe, 1961), presumably because, even though insufficient to prevent acute scurvy, enough vitamin C was present to enable an osteoblastic response.

Given the effectiveness of lemon juice for preventing scurvy in 19th century sea voyages, for scurvy to have been a problem, either the anti-scorbutic properties of Franklin's supplies of lemon juice must have deteriorated due to breakdown of the vitamin during storage, or the men were put on short rations to try and eke out the supply. In the first case, the juice would still have contained some vitamin C even if it eventually became insufficient to prevent scurvy. If the men were put on short rations and the juice administered in higher quantities only to the sick (as for example occurred aboard *Investigator* when supplies of lemon juice ran low (Armstrong, 1858)), some vitamin C would still have been ingested by those with scurvy. In addition, such fresh meat as could be hunted would also have supplied some vitamin C.

We would not expect to find skeletal evidence in any cases of scurvy beginning during the final throes of the expedition on the march along the shores of King William Island. At that time the men were suffering from starvation, so there would presumably have been a complete absence of vitamin C. If scurvy did break out prior to this, aboard the ships, it is likely to have been due to insufficient vitamin C rather than its total absence, so had this occurred we would expect to see skeletal evidence.

There are a number of archaeological assemblages representing populations specifically known from documentary sources to be suffering from scurvy in the years immediately prior to death. In those from 19th century prisoners from Quebec (Cybulski, 1988), early 17th century French pioneers in Maine (Crist *et al*., 2005) and a 19th century Great Famine population from Ireland (Geber and Murphy, 2012) bony signs of the disease were present. However this is not invariably the case. Among 17th century Dutch whalers buried at Spitsbergen (Maat, 1982), although soft tissue signs of scurvy were present, bony changes on the whole were not, presumably suggesting a diet for most individuals totally lacking vitamin C. If the three cases of subperiosteal new bone described here are added to the tibia described by Keenleyside *et al*. (1997), they give an overall prevalence of periosteal reactions of 1% for the 409 bones listed in Table 1. Unfortunately, the changes in the known scurvy populations described above are not quantified in ways which permit direct comparison with the disarticulated remains from the Franklin expedition. However a few studies of 19th century skeletal remains from England report periostitis prevalences calculated by bone rather than by individual. Among remains of retired Royal Navy personnel interred in the 18th and 19th centuries at Royal Hospital Greenwich, London, about 4% of total bones examined showed periosteal reactions

(Boston *et al*., 2008). At least some of these men probably suffered (and recovered) from scurvy at some point in their lives, probably many years before their deaths. The rate at which periosteal reactions are removed by remodelling is not known, and this complicates comparison with the Franklin remains. If scurvy was present on the Franklin voyage it would clearly have affected in men in the last years and months of their lives. Hence there would be less opportunity for lesions to be removed by remodelling, so if scurvy was present we might expect a high prevalence of lesions, but in fact, their prevalence among the Franklin remains is lower than at Greenwich. Among osteological studies of 19th century skeletal remains from England from burial grounds where there is no reason to believe scurvy was an especial problem, studies of larger assemblages (N=1458-18970 bones), give figures in the range 2.3-7.5% (Boulter *et al*., 1998; Boston *et al*,. 2009; Western and Kausmally, 2010). Although comparisons are not straghtforward, if scurvy was a widespread problem on the Franklin expedition, we might expect an elevated prevalence of periosteal reactions. In fact, the King William Island remains show little periostitis compared with contemporaneous English skeletal series.

The paucity of periosteal reactions or other bony signs of scurvy means that the skeletal remains provide little support for the idea that scurvy was a serious problem for the expedition prior to the desertion of the ships. Absence of evidence does not of course equate to evidence of absence. For one thing, the evidence is based on a small amount of skeletal remains – the material listed in Table 1 represents the very partial remains of a minimum of 15 out of the probable 126 expedition members surviving when the ships were beset in September 1846. Nevertheless, it does show that, contrary to claims in the literature, the remains do not provide support for the scurvy hypothesis.

Conclusion

The Franklin expedition set sail at a time when the Royal Navy had, in the form of lemon juice, an effective means of combatting scurvy. The Franklin expedition was provided with adequate quantities for the voyage. Effective procedures to prevent deterioration of anti-scorbutic properties of lemon juice were routine at that time. There is therefore no *a priori* reason to assume that scurvy should have been a problem. Gross and microscopic examination of extant skeletal remains from King William Island showed only two bones with changes consistent with subperiosteal haematoma. Both lesions were well remodelled and so may have formed prior to, rather than on, the expedition. In addition, they may well represent old traumatic injuries unconnected with scurvy.

The skeletal remains produced little evidence for scurvy. Although this does not provide evidence of its absence, it may be that other factors, for example lead poisoning, for which there is significant skeletal evidence (Beattie, 1985; Kowal *et al*., 1989, 1991; Keenleyside *et al*., 1996), may have been more important as causes of morbidity and mortality aboard Franklin's ships between May 1847 and April 1848.

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Part 3:
The use of histology for the detection of features of mechanical injury in dry bone

Chapter 7

ASSESSING POSTTRAUMATIC TIME INTERVAL IN HUMAN DRY BONE.

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Abstract. The posttraumatic status of antemortem fractures in human dry bone remains is currently defined as being either 'healing' or 'healed'. However, detailed 'dating' of the related posttraumatic time interval would be desirable, since it would aid in assessing individual medical status and care at the time of death. Within forensic pathology practice, fresh tissue healing phases are routinely used as an intrinsic parameter for the length of the posttraumatic time interval. Unfortunately, the direct application of such a method is hampered when applied to dry bone skeletal material.

This study explores the possibility of applying a fracture dating system, drawn forth from the traditional forensic pathology method, on dry bone remains. More specifically, the aim is to establish the extent to which various histomorphological features indicative of specific time intervals of healing are consistently detectable. Human dry bones with fractures and amputations in various phases of healing were studied.

Results show that the complementary use of radiological and histological investigation techniques improves the differentiation between various healing phases and thus allow for a more detailed dating of lesions. For future use, healing features that have proven to be consistently detectable and their related post traumatic time intervals are listed. The system aids in demarcating a considerably more clear posttraumatic time interval than usual.

Introduction

It is generally agreed upon that studying trauma in human remains can yield interesting individual, cultural and environmental information (Krogman and İşcan, 1986; Auferheide and Rodríguez-Martin, 1998; Ortner, 2003;). Indeed, a large pool of literature exists describing and interpreting gross anatomical traumatic lesions

observed in skeletons such as fractures, amputations and dislocations (e.g., Berryman and Haun, 1996; Grauer and Roberts, 1996; Mays, 1996; Lovell, 1997; Alvrus, 1999; Galloway, 1999; Judd and Roberts, 1999; Nakai *et al.*, 2001; Anderson, 2002; Holt *et al.*, 2002; Mitchell, 2006; Djuric *et al.*, 2006; Lovell, 2008; Van der Merwe *et al.*, 2009; Redfern, 2009).

Notwithstanding its acceptance, investigating traumatic lesions in dry bone tissue is still challenging, since palaeopathologists, and sometimes forensic pathologists and anthropologists, are confronted with the investigation of material that lacks soft tissue (Uberlaker and Adams, 1995; Grauer and Roberts, 1996; Lovell, 1997; Sauer, 1998; Rodríguez-Martin, 2006; Ortner 2003). Traditionally, the moment that lesions are inflicted is defined as being either 'antemortem' (prior to death), perimortem (occurring around the time of death) or 'postmortem' (after death). Antemortem lesions are defined by the presence of healing features (e.g. callus formation), whereas postmortem lesions are defined by the lack of any healing features. For postmortem lesions that occurred during excavation, the colour difference between fracture surface and periosteal surface may also aid in identifying postmortem lesions (Van der Merwe *et al,*. 2010). Perimortem lesions are generally those that cannot be reliably assigned to either the ante- or postmortem group. A more detailed discussion of the differentiation between ante- peri- and postmortem lesions is beyond the scope of this publication, but can be found in various comprehensive publications (e.g. Quatrehomme and Iscan, 1997; Wheatley, 2008; Wieberg and Wescott, 2008).

Describing lesions as being either antemortem, perimortem or postmortem has severe limitations as it gives no estimation of the length of the time period between the moment of the traumatic insult and the time of death (the posttraumatic time interval). Usually the status of antemortem lesions is expressed as being 'healing' or 'healed' (see for instance Brickley, 2006). However, a more detailed temporal specification of antemortem time would be desirable, since a more precise 'dating' would aid palaeopathologists and forensic anthropologists in interpreting facets such as medical status and medical care at the time of death. More specific information on differences in the timing of multiple injuries in a specific individual may also be essential in evaluating evidence for child abuse and torture (Maat, 2008). Moreover, it would assist in determining the sequence of multiple traumas observed in a single individual. The later could especially be an addition to the growing body of research that studies the occurrence of trauma recidivism (Judd, 2002; Martin *et al.*, 2010).

Theory and practice of dating traumatic lesions

In forensic pathological investigations, estimation of the posttraumatic interval is usually done by analyzing the local soft tissue response (healing), since healing is

supposed to be an intrinsic and reliable time indicator (Oehmichen, 2004). Although in forensic pathology practice dating is mostly applied to soft tissue lesions such as cutaneous wounds, the principle can also be utilized for bone tissue injuries, since bone tissue response also follows a strict time dependant developmental sequence irrespective of complex variables such as the type of lesion, location, age at death and health status (Todd and Iler, 1927; Frost, 1989a; Frost, 1989b; Tosounidis *et al.*, 2009; Vigorita, 2007).

The usefulness of healing as an indicator of posttraumatic survival time was, for instance, illustrated in an investigation of war crimes. Following an extensive literature review on fracture healing, a time table was constructed and applied that linked the elapsed time after bone tissue injury to radiographic and histological healing features (Maat, 2008). The approach proved to be adequate for cases regarding adult and paediatric individuals (Maat and Huls, 2010).

Despite the fact that the approach was designed for fracture dating, it should also be suitable for amputations, since stages and timing of bone healing are supposed to be similar (Barber, 1930). Comparisons between Maat's timetable (2008), based on a literature review of microscopic and radiologic observations, and the work by Barber (1929, 1930, 1934), based on gross anatomical and radiological observations in macerated material, supports this statement.

Application in palaeopathology and forensic anthropology

It goes without saying that forensic pathological trauma dating has great potential for implementation in palaeopathology. This was for instance demonstrated by Mays (1996) who applied Barber's gross anatomical approach in a case study regarding healed amputations (Barber, 1929, 1930, 1934; Todd and Barber 1934). Recently, Cattaneo *et al.* (2010) also showed that useful histological markers for the dating of traumatic lesions were even traceable in macerated bone. However, macerated bone material, like fresh forensic material, is usually better preserved than archaeological dry bone material. It therefore allows for 'better' analysis. As a result, the consistency in the detectability of healing features as used in the approaches of Maat (2008) and Barber (1929, 1930, 1934) are still left to be tested in case of dry bone material.

This study will therefore specifically attempt to: (1) evaluate which features as described by Barber (1929, 1930, 1934) and Maat (2008), are adequately assessable in traumatic lesions in dry bone material, and (2) determine to what extent they allow for estimation of the 'age' of an injury.

Material

Dry bone specimens from various collections were assembled in order to ensure a sufficiently large sample for investigation. Only traumas from presumably healthy individuals were included. Because of the destructive nature of histological methods we were not permitted to sample cranial bone material. Therefore only postcranial material was included in the study. There is however no indication that the healing process and its timing would be different in cranial bones from that in postcranial ones (Frost, 1989a, 1989b; Vigorita, 2007).

A total of 22 specimen with fractures and nine amputations in various phases of healing were included in this study. They were excised from 21 individuals. Twelve fractures and seven amputations were obtained from the Gladstone skeletal collection from Kimberley, South Africa (Van der Merwe *et al.*, 2009). One fracture originated from a 17th century Dutch male whaler exhumed on Spitsbergen, Norway (Maat, 1981), and two fractures from a 19th century population from Bloemendaal, The Netherlands. The sample was further extended with six fractures and one amputation from the dissection hall collection of the Leiden University Medical Centre (LUMC). Three intentionally sawn/broken specimens from the Bloemendaal collection were used as controls.

Throughout our assemblage, wherever possible, a (contra lateral) control sample was included for comparison of bone density and histological architecture. General information on the studied bone material is listed in Table 1.

Methods

All lesions were photographed and studied gross anatomically, in order to define whether they are either ante-, peri- or postmortem. For this, we used standard anthropological methods (Van der Merwe *et al.*, 2010). Subsequently, conventional plain radiographs in an anterior-posterior and a medio-lateral direction were taken. The lesion plus a representative length of unaffected bone was imaged together with its contra lateral control specimen (whenever available). To prevent bias by specimen recognition, each image was anonymized by random numbering.

After radiological imaging, histology samples, so called ‘thick slices’, were excised from the centre of each lesion and its control (i.e. perpendicular to the fracture/amputation plane). A new random numbering blinded the identity of these ‘thick slices’. From each thick slice, two thin sections were produced with a minor adaptation according to the method of De Boer *et al.* (2013): i.e., the embedding medium was also used for cover slipping.

Table 1. Skeletal material.

	Individual No.[1]	Origin	Bone	Gross anatomical dating[2]	Preservation phase[3]
Fractures	N74.5	Kimberly, SA	Hu	Antemortem	1
	N38.1	Kimberly, SA	Ri	Perimortem	1
	N38.2	Kimberly, SA	Fe	Antemortem	1
	N38.2	Kimberly, SA	Ti	Antemortem	1
	N38.2	Kimberly, SA	3th MCP	Antemortem	1
	N38.3	Kimberly, SA	Ra	Antemortem	1
	N74.4	Kimberly, SA	Ul	Antemortem	1
	S2.3	Kimberly, SA	Fe	Perimortem	2
	S2.3	Kimberly, SA	Ra	Antemortem	2
	S2.9	Kimberly, SA	8th Ri	Antemortem	1
	S3.5	Kimberly, SA	Fe	Perimortem	2
	Sk. 1	Unknown, NL	Fi	Antemortem	1
	Sk. 2	Unknown, NL	Ti	Antemortem	1
	Sk. 2	Unknown, NL	Fi	Antemortem	1
	Sk. 4	Unknown, NL	Fe	Antemortem	1
	Sk. 5	Unknown, NL	Hu	Antemortem	1
	Sk. 5	Unknown, NL	Ra	Antemortem	1
	541	Spitsbergen, NO	Fe	Antemortem	1
	1033	Bloemendaal, NL	Ri	Antemortem	2
	1033	Bloemendaal, NL	Ri	Antemortem	2
	H 1	Bloemendaal, NL	St	Postmortem	2
	H 2	Bloemendaal, NL	Fe	Postmortem	2
Amputations	N34.3	Kimberly, SA	Ti	Antemortem	2
	N38.2	Kimberly, SA	Fe	Perimortem	1
	N8.1	Kimberly, SA	Ti	Antemortem	2
	S2.6	Kimberly, SA	Ti	Antemortem	1
	S2.7	Kimberly, SA	Hu	Perimortem	2
	S2.7	Kimberly, SA	Hu	Antemortem	2
	S2.7	Kimberly, SA	Ra+Ul	Perimortem	2
	Sk. 3	Unknown, NL	Fe	Antemortem	1
	H 2	Bloemendaal, NL	Fe	Postmortem	2

[1] Numbering used in prior published articles.
[2] According to Van der Merwe *et al.* (2010).
[3] According to Gordon and Buikstra (1981).
Abbreviations: SA= South Africa; NO= Norway; NL= The Netherlands; Hu= Humerus; Fe= Femur; Ra= Radius; Ul= Ulna; Ri= Rib; Ti= Tibia; Fi= Fibula; MCP= Metacarpal; St= Sternum.

Table 2. Healing features and associated posttraumatic time intervals, combined from Barber (1929, 1930, 1934), Maat *et al.* (2008, 2010) and Maat (pers. comm.).

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

[1] Features visible by plain radiographic analysis.
[2] Features visible by histological analysis.

One of the thin sections remained unstained; the other was stained with haematoxylin to enhance the visibility of tissue architecture. All sections were microscopically investigated using bright field and polarized-light.

By combining the work of Barber (1929, 1930, 1934), Maat (2008; pers. comm.) and Maat and Huls (2010), a table was constructed in which healing features were linked to time intervals (Table 2). Features that are impossible to assess in archaeological material, such as soft tissue changes, were not taken into account. In the few cases in which descriptions given by Maat and Barber did not agree (some minor gross anatomical changes), preference was given to the publication of Maat, since it included more recent data. The merged data were then used to develop a multiple choice questionnaire that addressed the consistency in detectability by examiners of each of the designated healing features.

This questionnaire was then used to assess the radiographs and histological sections. By letting three examiners assess the images and sections independently, we diminish inter-observer bias to a minimum. In the questionnaire, each healing feature could be marked as: 'yes' (the described feature was present), 'no' (the described feature was not present), a question mark (the provided image/section did not allow for a conclusive answer), or as 'NA' (not applicable). Agreement between the three examiners, and thus consistency in the detection of healing features, was measured by calculating a one way random Intraclass Correlation Coefficient (ICC) for each healing feature for each modality. Prior established categories were used to interpret the ICC. According to Landis and Koch (1977), an ICC greater than 0.6 is considered to reflect substantial agreement between examiners.

Results

In the following text, all healing features will be printed in *italics*. Due to the nature of dry bone tissue, two features of healing described by Maat (*scattered bone spiculae* and *fields of calcified cartilage at sites of callus formation)* were not present in any of our samples. Furthermore, as expected, healing features were absent in the postmortem control samples included in this study.

Results of radiographic analyses

In analyses of the plain radiographs, several common healing features were detected with substantial interobserver agreement (See Table 3). Agreement on the presence of *clearly visible callus* at the lesion site, both at the *endosteal* and *periosteal* aspect was substantial (ICC of 0.756 and 0.770).

Table 3. Intraclass Correlation Coefficients of healing features from microscopic and radiographic analyses.

Healing feature	Unstained histology ICC (95% CI)	Stained histology [1] ICC (95% CI)	X-ray analysis ICC (95% CI)
Common healing features.			
Smoothening of the lesion margins.	.806 (.629-.917)	.863 (.726-943)	.416 (.125-.693)
Absorption of cortical bone adjacent to the lesion.	NA	NA	.275 (.052-.632)
Presence of endosteal callus.			
- Distant from the lesion site.	.652 (.470-.798)	.393 (.178-.608)	.345 (.131-.580)
- At the lesion site.	.736 (.583-.851)	.709 (.546-.834)	.756 (.611-.863)
Presence of periosteally situated callus.			
- Distant from the fracture site.	.678 (.505-.815)	.649 (.455-.799)	.343 (.126-.568)
- At the lesion site.	.912 (.848-.953)	1.000[2]	.770 (.629-.871)
Firm attachement of periosteally situated callus.	.900 (.766-.967)	.739 (.484-.902)	.154 (.000-.532)
Local osteoporosis of the cortex.	.664 (.484-806)	.706 (.540-.832)	.341 (.116-.574)
Margin of the lesion appears more sclerotic.	NA	NA	.421 (.204-632)
Endosteal callus becomes indistinguishable from the cancellous bone in the marrow cavity.	.845 (.740-.916)	.370 (152-.590)	.837 (.728-.911)
Features specific for fractures[2].			
Union by bridging of the cortical bone discontinuity by primary woven bone.	.882 (.779-.945)	.629 (.403-.807)	.608 (.378-.794)
Union by bridging of the cortical bone discontinuity by secondary lamellar bone.	.443 (.191-.684)	.764 (.590-.884)	.939 (.882-.972)
Smoothening of the callus outline.	.510 (.118-.838)	.348 (.013-.715)	.838(.652-.942)
Features specific for amputations[3].			
Visibility of cut marks on the amputation surface.	1.000[4]	.733 (.409-.924)	0.000
Start of 'capping' of the medullary cavity.	1.000[4]	1.000[4]	1.000[4]
Complete closure of the medullary cavity.	1.000[4]	.857 (.350-.996)	1.000[4]

[1]Haematoxylin stained, according to De Boer *et al*. (2013).

[3] N= 22.

[4] N= 9.

[4] An ICC of 1.000 indicated no difference in observation between the examiners.

Abbreviations: ICC= Intraclass Correlation Coefficient. CI= Confidence Interval. NA= Not Applicable.

The examiners also agreed upon the existence of *remodeling of endosteal callus* making it *indistinguishable from the cancellous bone* (ICC of 0.837). Internal and external callus formation at some distance from the lesion site produced low Intraclass Correlation Coefficients (ICC of 0.345 and 0.343). Also the evaluation of *absorption of the cortical bone adjacent to the lesion* (ICC of 0. 275), *more sclerotic lesion margins* (ICC of 0.421), *osteoporosis of the cortex* (ICC of 0.341), the presence of *smoothening of lesion margins* (ICC of 0.416) and *firm attachment of periosteal callus to the cortex* (ICC of 0.154) did not produce substantial interobserver agreement.

In fractures, examiners agreed upon the detectability of the *smoothening of the callus outline* (ICC of 0.838). High Intraclass Correlation Coefficients were also seen with regard to the *union by bridging of the cortical bone discontinuity*, irrespective of whether this union was constructed by means of primary woven or secondary lamellar bone (ICC of 0.608 and 0.939).

When considering amputations, no differences in observation were seen between examiners regarding *the start of 'capping' of the medullary cavity* with newly formed bone, or the *complete capping of the medullary cavity* (both an ICC of 1.000). In contrast to histological analysis there was no substantial agreement on the detectability of *cut marks on the amputation surface* in radiological analyses.

Results of histological analysis

On a microscopic level, a number of healing features were detected with substantial interobserver agreement, irrespective of the use of histochemical staining (See Table 4). The presence of *frayed bone lamellae at the lesion margins* (ICC of 0.881 and stained 0.763), *smoothening of the lesion margins* (ICC of 0.806 and stained 0.863), and the presence of *clearly visible callus formation* at the lesion site, both *endosteally* (ICC of 0.736 and stained 0.709) and *periosteally* (ICC of 0.912 and stained 1.000) showed substantial agreement. Also, *firm attachment of callus on the periosteal surface* (ICC of 0.900 and stained 0.739) and *quiescent appearance* of the lesion site after healing has concluded (ICC of 1.000 and stained 0.741) were generally agreed upon in both unstained and stained sections. Agreement regarding early indications of *periosteal osteogenesis at distance from the fracture margins* were considered 'borderline' (ICC of 0.678 and stained 0.649). The examiners did not agree convincingly on the detectability of *endosteal osteogenesis* at some distance from the fracture site (ICC of 0.625 and stained 0.393).

Agreement on the detectability of some common healing features (fractures and amputations) was lower in haematoxylin stained sections. In stained sections, agreement was lower on the presence of *Howship's lacunae* (ICC of 0.700 vs. stained 0.575) and *cutting and closing cones* (ICC of 0.965 vs. stained 0.577). Also the final stage

of endosteal callus remodeling, when *endosteal callus becomes indistinguishable from the cancellous bone*, was better detectable in unstained sections (ICC of 0.845 vs. stained 0.370). The agreement on detectability of *transition of primary woven bone into secondary lamellar bone* in callus was higher in haematoxylin stained sections, both in endosteally (ICC of 0.491 vs. stained 1.000) and periosteally situated callus (ICC of 0.242 vs. stained 1.000).

In fractures, differences existed in the ICCs between observations done in unstained and stained sections. There was, however, substantial agreement upon the detectability of *union by bridging of the cortical bone discontinuity* by primary woven bone in both unstained and stained sections (ICC of 0.882 and stained 0.629). In contrast, agreement on the presence of *union by bridging of the cortical bone discontinuity* by secondary lamellar bone was reduced in unstained if compared to stained sections (ICC of 0.443 vs. stained 0.764). The *smoothening of callus outline* was inconsistently assessed in fractures, irrespective of the application of haematoxylin (ICC of 0.510 and stained 0.348).

When considering unstained and stained sections of amputations, features such as the presence of *cut marks* (ICC of 1.000 and 0.825), the *start of 'capping' the medullary cavity* with newly formed bone (both an ICC of 1.000) and eventual *complete 'capping' of the medullary cavity* (ICC of 1.000 and 0.875) were all detected with substantial interobserver agreement.

Table 4. Intraclass Correlation Coefficients of healing features from microscopic analysis.

Healing feature	Unstained histology ICC (95% CI)	Stained histology[1] ICC (95% CI)
Frayed bone lamellae at the lesion margins.	.881 (.797-.936)	.763 (.620-.868)
Howship's lacunae at the lesion cleft.	.700 (.533-.828)	.575 (.376-.745)
Howship's lacunae at the periosteal surface.	.814 (.695-.989)	.599 (.405-.762)
Howship's lacunae at the endosteal surface.	.903 (.832-948)	.503 (.290-.695)
Cortical cutting and closing cones oriented towards the lesion.	.965 (.922-.977)	.577 (.376-.747)
Remodeling of endosteal callus into secondary lamellar bone.	.491 (.205-.742)	1.000[2]
Remodeling of periosteal callus into secondary lamellar bone.	.242 (-.099-.627)	1.000[2]
Quiescent histomorphological appearance, indicating ended healing.	1.000[2]	.741 (.583-.856)

[1]Haematoxylin stained, according to De Boer *et al*. (2013).

[2] An ICC of 1.000 indicates no difference in observation between the examiners.

Abbreviations: ICC= Intraclass Correlation Coefficient. CI= Confidence Interval.

Table 5. Consistently detected healing features visible in dry bone material.

Healing feature	Time interval	Unstained histology	Stained histology[1]	Plain radiography
Common healing features.				
Frayed bone lamellae at the lesion margins.	Before 48 hours	x	x	
First Howship's lacunae at the lesion margins.	After 4-7 days	x		
Smoothening of the lesions margins.	After 4-7 days	x	x	
Start of periosteal callus formation, distant from the lesion margins, separable from the cortex.	After 7 days	x	x	
Endosteal callus formation clearly visible.	After 10-12 days	x	x	x
Osteoporotic appearance of the cortex.	After 12 days	x	x	x
Start of the transition of primary woven bone into secondary lamellar bone.	After 14 days		x	
Cortical cutting and closing cones orientated towards the lesion.	After 14-21 days	x		
Clearly visible periosteally situated callus.	After 15 days	x	x	x
Endosteal callus becomes indistinguishable from the cancellous bone in the marrow cavity.	After 17 days	x		x
Periosteal callus becomes firmly attached (inseparable) to the cortex.	After 6 weeks	x	x	
Features specific for fractures.				
Union by bridging of the cortical bone discontinuity.	After 21-28 days	x	x	x
By primary woven bone.		x	x	
By secondary lamellar bone.			x	
Smoothening of the callus outline.	After 2-3 months			x
After adequate immobilization: quiescent appearance indicating subsided healing.	After 1-2 years	x	x	
Features specific for amputations.				
Visibility of cut marks on the amputation surface.	Before 13 days	x	x	
Start of 'capping' of the medullary cavity.	'After not many weeks'	x	x	x
Complete capping of the medullary cavity.	'After several months'	x	x	x

[1]Haematoxylin stained, according to De Boer *et al*. (2013).

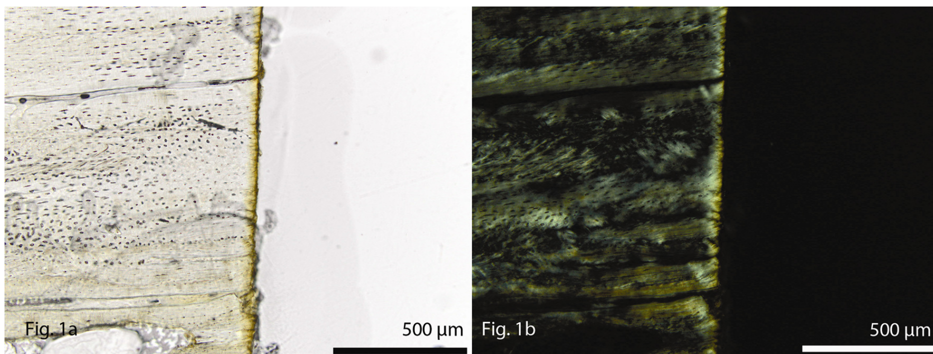
Discussion

The results show that a considerable amount of healing features are still reliably detectable in dry bone tissue. Throughout the results, features indicating the same level of healing were in agreement with each other, supporting the statement that healing happens in an orderly, sequential fashion (Vigorita, 2007). The results will now be discussed in the time sequence in which they are reported to appear during the healing process (Barber 1929, 1930, 1934; Maat, 2008; Maat and Huls, 2010). This sequence is also described in Tables 2 and 5.

Frayed bone lamellae at the lesion margins are reported to only be observable within the first 48 hours after injury. Due to the morphological character and dimensions of this feature, it could only be viewed by histological analyses. The use of histochemical staining did not affect the consistency of the detection. However, it was noted that the use of polarized light dramatically enhanced the visibility of frayed margins (Fig. 1)

After four to seven days, lesion margins will start to become eroded by osteoclasts. Their activity is indicated by the presence of *Howship's lacunae*. These are only visible on a microscopic level. The use of histochemical staining impeded interobserver agreement. This might be due to confusion in microscopic image interpretation from the histotechnique of section surface staining. This leaves deeper situated tissue within the section unstained.

Figure 1.



Micrographs of the frayed lesion margin of an amputated femur (S38.2), undecalcified and unstained section. Bar indicates scale. **(1a)** The cutting margin at the amputation end shows a frayed, brush-like appearance due to the fanning of bone lamellae. The yellow discolouring at the margin is caused by taphonomical processes. **(1b)** Same section as 1a, now viewed with polarized light. The use of polarized light clearly enhances the visibility of the frayed margin.

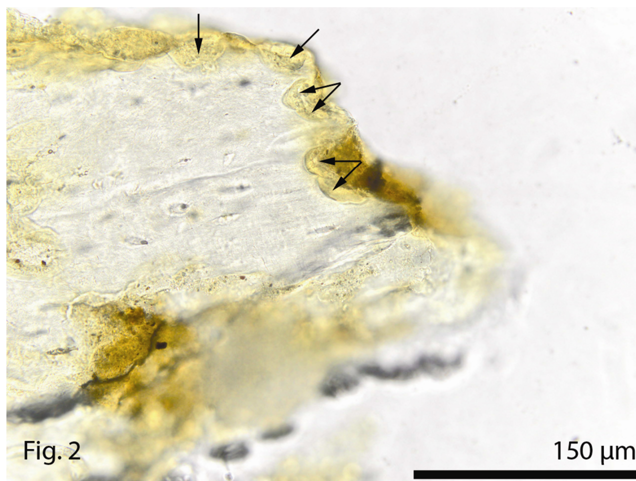
Osteoclastic activity on the lesion margins results in a *smoothening of the lesion margins*, at the earliest reported to be visible after four days. Good interobserver agreement was obtained irrespective of section staining. The unsatisfactory agreement in case of radiological analysis might be explained by difference in magnification and resolution (ICC of 0.416).

Due to a combination of taphonomic processes and excavation tissue damage, the next step in healing sequence, the formation of *bone spiculae between the lesion margins* (becoming visible after four to seven days) can not be found in dry bone specimens.

It was expected that by natural *absorption of cortical bone adjacent to the lesion margins*, margins would appear less opaque (see Table 2). However, this feature did not perform well in terms of interobserver agreement (ICC of 0.275). This might be explained by the difficulty to differentiate between antemortem healing changes and postmortem taphonomic alterations of the cortex.

After initial osteogenesis in the form of bone spiculae, callus formation starts both on the endosteal and periosteal aspect of the fractured bone. Minor *periosteal osteogenesis* (callus formation, visible after seven days) usually starts *at some distance* from the lesion site (Maat, pers. comm.) i.e. in the 'corner' where the periosteum is

Figure 2.

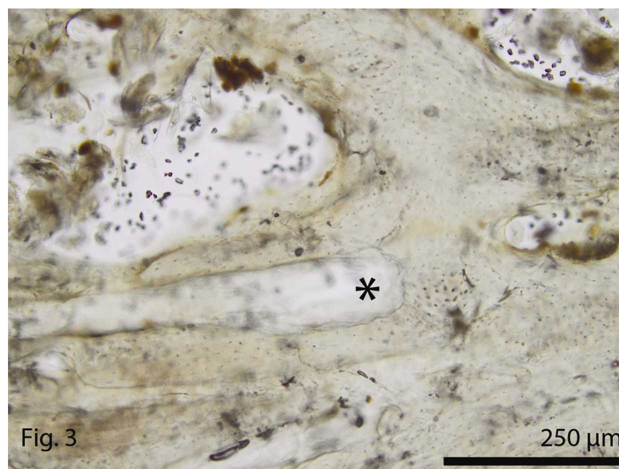


The fracture margin of a third metacarpal (N38.2), undecalcified and unstained section. Bright light. Bar indicates scale. The lesion shows Howship's lacunae as numerous bite-like indentations (arrows). They are caused by resorption of bone tissue by osteoclasts.

being lifted from the bone by the subperiosteal hematoma, resulting in an appearance similar to the radiological 'Codman's triangle' visible in osteosarcomas. From there it progresses towards and unites with callus formation at the lesion site. Since this phenomenon only exists for a short period of time, it was unfortunately not observed in our sample. As a result, we examined periosteal callus at some distance as a part of the callus formation at the lesion site. This most likely caused the borderline interobserver agreement of the feature (ICC of 0.678 and stained 0.649). However, it is expected that if cases of early periosteal callus formation had been included in our collection, this could very well have shown to be consistently detectable.

Endosteal osteogenesis (callus formation) may start after seven days, and is usually *clearly visibl*e after ten to twelve days. It was consistently detected in both stained and unstained histological sections as well as during radiological analysis. In addition, we analyzed whether endosteally situated callus, like periosteal callus, also started at some distance from the site of lesion. Related Intraclass Correlation Coefficients were, however, low with broad 95% Confidence Intervals, both histologically (unstained ICC of 0.625 and stained ICC of 0.393) and radiologically (ICC of 0.345). This is

Figure 3.



Micrograph taken near the fracture site of a fractured radius (N38.3), undecalcified and unstained section. Bright light. Bar indicates scale. In the centre, a 'cutting cone' running/drilling from left to right is indicated (asterisk). The cutting cone is characterized by Howship's lacunae. The closing cone is situated to the left, outside the field of photography. Undecalcified, unstained section.

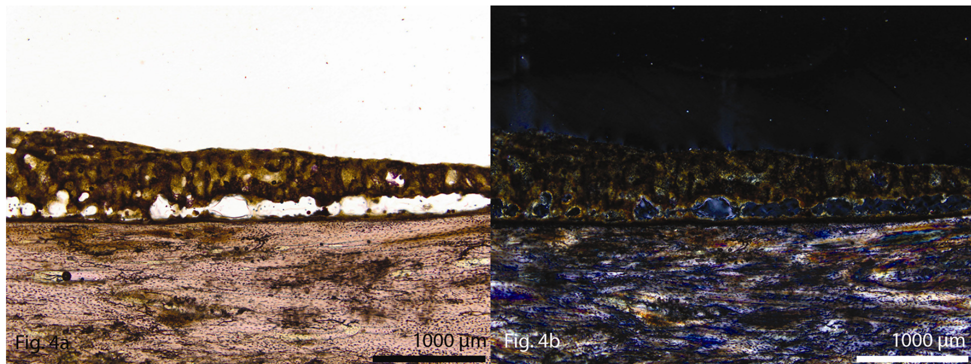
probably due to the difficulty to differentiate between early endosteal callus formation and the naturally present surrounding cancellous bone.

As healing progresses, *local osteoporosis of the cortex* may be observable. This is usually not visible before twelve days. Osteoporosis within our sample was only consistently detected in stained and unstained histological sections, and not during radiological investigation (ICC of 0.341). The latter might be due to differences in technical resolution and magnification, resulting in more debatable findings in plain radiographs. This seems to especially hold true for early stages of local osteoporosis. In advanced phases, local osteoporosis becomes clearly visible on plain radiographs. See Figure 7.

As new bone formation progresses, the *margin of the lesion appears more sclerotic* after 12-20 days (see Tab. 2). The related ICC, however, did not present substantial interobserver agreement (ICC of 0.421). As in the case of *local osteoporosis of the cortex*, this may be caused by differences in resolution and magnification.

In amputations, the presence of *cut marks* can be seen up till about 13 days after the traumatic event (see Table 2). This feature was only consistently reported in unstained and stained histological analysis, and was not consistently detectable with

Figure 4.



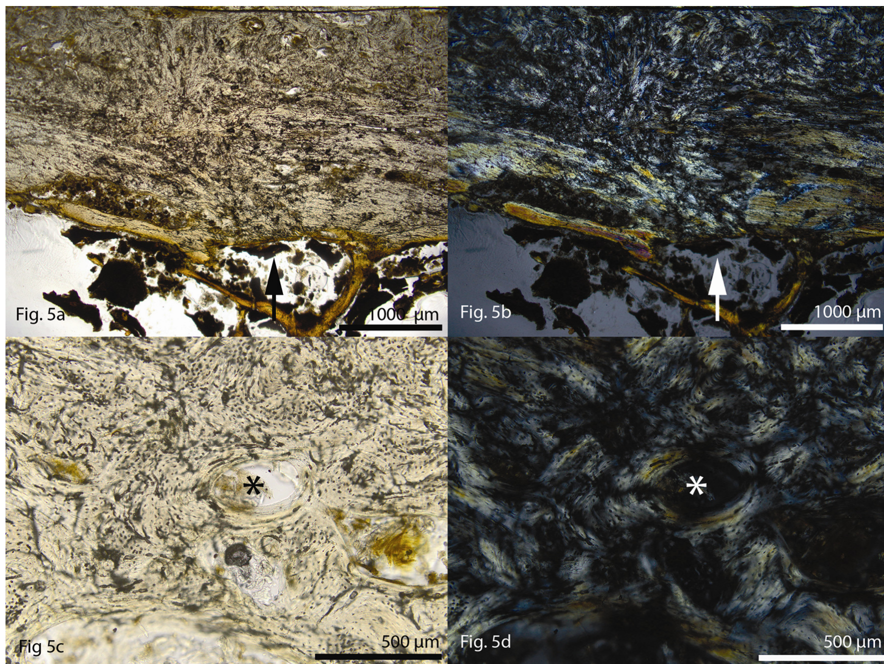
Micrographs of so-called 'separable periosteal callus' in a fractured third metacarpal (N.38.2), Undecalcified section, stained with haematoxilin. Bar indicates scale. **(4a)** Section viewed with bright light. The separable periosteal callus is only connected to the periosteal aspect of the cortex by small pillars of bone tissue. The sloping aspect of the callus illustrates the build-up of callus towards the fracture site. **(4b)** Same section as shown in Figure 4a, now viewed with polarized light. Extensive taphonomic alteration of the periosteal callus hampers the visibility of the microarchitecture of the callus. The callus in this healing phase constitutes mainly of primary woven bone, in contrast to the secondary lamellar bone of the underlying cortex.

radiography (ICC of 0.000).

As stated earlier, loose microscopic *bone tissue spiculae* are undetectable due to postmortem decomposition. The same holds for the *fields of calcified cartilage* that only will be seen in fresh material after fourteen days.

After 14 days, the newly formed endosteal and periosteal callus starts its *transition from primary woven bone into secondary lamellar bone*. The histological detectability hereof was primarily analyzed with the use of polarized light, both in stained an unstained sections. However, our results suggest that staining increased consistent detection above the 0.6 threshold (endosteal: ICC of 0.491 vs. 1.000 and periosteal:

Figure 5.

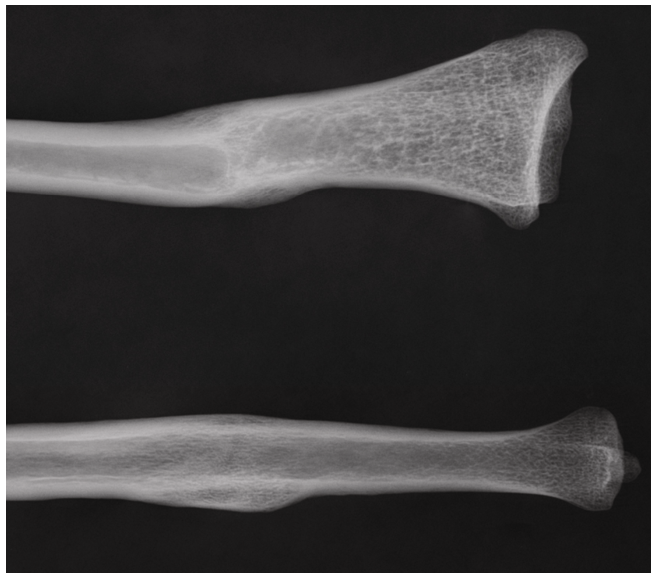


Micrographs showing the remodeling of callus in a fractured radius (N38.3), undecalcified, unstained section. Bar indicates scale. **(5a)** Section viewed with bright light. Overall, the callus has a disorganized appearance and consists mainly of primary woven bone. The lesion site is indicated by an arrow. **(5b)** Same section as shown in Figure 5a, now viewed with polarized light. The use of polarized light enhances the random orientation of the primary woven bone fibers. Also, the discontinuity of the cortex at the lesion site (arrow) is enhanced by polarized light. **(5c)** Detail of the callus as shown in 5a and 5b. Viewed with bright light. A higher magnification shows one of the Haversian canals (asterisk), indicating that advanced remodeling of the callus has started. **(5d)** Same section as shown in Figure 5c, viewed by polarized light. The detectability of Haversian systems, organized around a Haversian canal (asterisk), becomes undisputed by the use of polarized light.

ICC of 0.242 vs 1.000). The phenomenon of improved visibility of separate bone lamellae by the use of haematoxylin was noted before by De Boer *et al.* (2012, 2013). The improvement in the ICC between stained and unstained sections may therefore be the result of a cumulative effect of staining and polarized light.

The remodeling process is accompanied by an increased number of *cortical cutting and closing cones*, observable two to three weeks after injury. Our results suggest a better detectability in unstained sections (ICC of 0.965) if compared to stained sections (ICC of 0.577). As cortical cutting and closing cones are recognized by their characteristic two end conical shape with Howship's lacunae at their 'cutting' end, the previously noted negative effect of staining on the visibility on Howship's lacunae might have hampered detectability.

Figure 6.



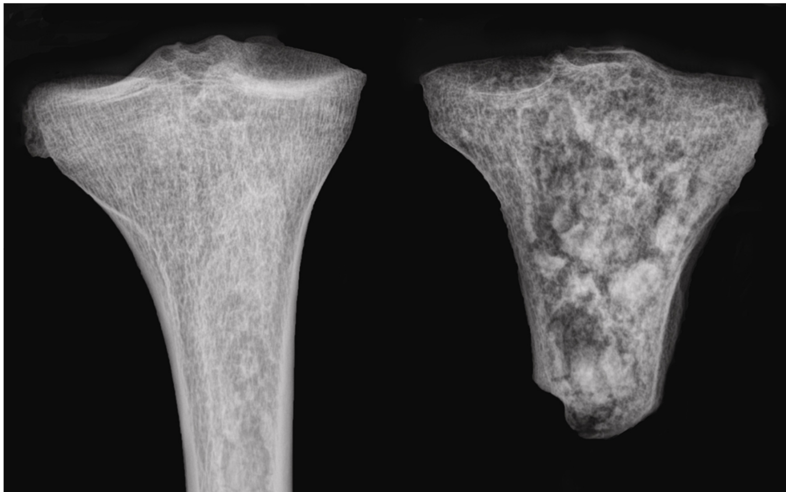
Radiographs of remodeled calluses. **Top:** The X-ray of a fractured radius (N38.3) shows both an endosteally and a periosteally situated callus. The periosteal callus has a smoothened outline. Continuity of the cortex is restored, although its bone density at the lesion site is still decreased. **Below:** This X-ray of a fractured ulna (N74.4) shows a smoothened periosteally situated callus. The continuity of the cortex is almost totally restored and is slightly less radiodense at the fracture site. The internal callus is almost indistinguishable from the surrounding cancellous bone of the marrow cavity, indicating advanced remodeling.

As remodeling progresses, *the endosteal callus becomes indistinguishable from the cancellous bone in the medullary cavity* (first visible after 17 days). In radiological analysis, this was consistently detected. On a microscopic level, interobserver agreement was much higher in unstained than in stained sections (ICC of 0.845 vs. 0.370). This might be due to the above discussed confusing effect that histochemical staining may have on the visibility of overall bone tissue architecture.

According to Table 2, *periosteal callus becomes clearly visible* after about 15 days, observable both in stained and unstained histology, and radiological analysis. As callus formation progresses, the cortical defect in fractures is eventually bridged by callus after 21-28 days. This *union by bridging of the cortical bone discontinuity* was consistently detected in both (un)stained histological and radiological analyses.

Subsequently, the *periosteal callus becomes firmly attached (inseparable) to the cortex*. This is observable after six weeks and was only consistently detected in histological analysis, irrespective of the use of staining. Due to the earlier mentioned low

Figure 7.



Radiograph of an amputated tibia (N34.3). When compared to its contralateral control, The amputated stump shows diminished density of the cancellous bone and a thinned cortex. This indicates osteoporosis due to disuse. The medullary cavity at the amputation end is almost closed by new bone formation, indicating advanced capping. The mottled appearance of the tibial stump is partly due to dirt.

resolution and magnification in plain radiographic images the ICC was very low (ICC of 0.015). The *smoothening of the callus outline* in fractures (after two to three months) was only consistently detected during radiological investigation (ICC of unstained 0.510 and stained 0.348 vs. radiology 0.838). The low magnification of routine radiographs now proved to be in favor of tissue overview if compared to histology.

In amputations, healing eventually progresses towards the *start of 'capping' of the medullary cavity* and the eventual *complete 'capping'* thereof. This was consistently detected in unstained and stained sections, as well as in radiological analysis. In fractures, healing eventually subsides, leaving a quiescent histomorphological appearance after 1-2 years. Both stained and unstained sections proved to be consistent.

The authors used interobserver agreement, assessed by means of Intraclass Correlation Coefficient (ICC) calculation, as an indicator for the consistency in detection of a healing feature. Cohen's Kappa is the most used statistical calculation for assessing interobserver agreement, but for comparisons between more than two examiners, ICC is regarded superior (Berk, 1979). The Intraclass Correlation Coefficient is defined as the proportion of true variance relative to total variance. In other words, a high ICC indicates that the method of analysis does not add variance to the total variance between subjects. Since calculation of the ICC uses total sample variation, its value is sensitive to the extensiveness of values in the sample. As a result, calculated ICC values are only 'reliable' in a heterogeneous population. As this research uses bone material with sufficient variability between healing phases, interobserver agreement on the presence of a parameter can be reliably calculated. When the Intraclass Correlation Coefficient is used as an indicator for consistency in observation, its calculated degree of agreement does not clarify whether a feature is detectable or undetectable. It only expressed the degree in consistency between examiners with respect to the detection of healing features.

We are aware of the limited sample size with regard to the number of amputations. Amputations are not frequently found in archaeological populations, and if so, not in varying phases of healing. Nevertheless, since fractures and amputations generally share a similar healing process (Barber, 1930), it is believed that the combined assembly allows for meaningful interpretation. For those features in which logical inferences resulted in a limited number of examined specimens (e.g. complete closure of the medullary cavity in amputations), Intraclass Correlation Coefficient must be interpreted with caution, due to sample homogeneity.

Overall, our results suggest that not every healing feature as described by Barber (1929, 1930, 1934), Maat (2008) and Maat and Huls (2010) was consistently detected. Nevertheless the results from this study suggest that if only those with substantial

interobserver agreement are used, a fair estimation of the minimal and maximal time lapsed after an injury can still be made. This is a substantial improvement in diagnosis if compared to conclusions such as 'healing' or 'healed'. For those who do not routinely 'date' fractures or amputations, a table was made showing only the consistently detected features together with its modality (Table 5). Furthermore, some reference figures are given (Figs. 1-7).

We recommend using plain radiographic and histological analysis. Histology yields the best results if both unstained and haematoxylin stained sections are used, in combination with polarized light. And, as usual, the value of negative observations is limited, in contrast to positive observations. Time intervals should be adjusted to specific conditions (e.g. shortened in case of children (Maat, 2008; Maat and Huls, 2010). It goes without saying that our approach stays open for improvement and reliability will increase with experience.

Conclusion

By using complementary radiological and (un)stained histological investigation methods, a differentiation can be made between features that indicate various stages of the healing process after trauma as may be observed in human dry bone material. The consistency in the detection of healing features indicate that the bone healing process can be used to estimate the posttraumatic time interval of fractures and amputations.

Acknowledgements

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Chapter 8

SURVIVAL TIME AFTER FRACTURE OR AMPUTATION IN A 19TH CENTURY MINING POPULATION AT KIMBERLEY, SOUTH AFRICA

From: *Survival time after fracture or amputation in a 19th century mining population at Kimberley, South Africa. De Boer, H.H., Van der Merwe, A.E., Maat, G.J.R., 2013. South African Archaeological Society Goodwin series* 11, 52-60.

Abstract. At the end of the 19th century, the discovery of diamonds resulted in a massive influx of migrant mine workers to Kimberley, South Africa. The skeletons of some of these migrant workers were excavated in 2003 and the analysis of these remains provided an interesting glimpse into their living conditions. The high prevalence of antemortem fractures and amputations within the population sample suggested harsh living conditions, but also indicated that basic medical and social care was available to at least some of the individuals.

Plain radiographical and histological analyses were conducted of the excavated migrant worker's antemortem fractures and amputations to assess posttraumatic survival time as a means of providing additional information on their living conditions and medical status around the time of death.

Results showed a wide range of posttraumatic survival times in amputations and fractures. This finding supports the availability of medical care for the mine workers. Furthermore the assessment of posttraumatic survival time allowed for assumptions on the cause of death, reasons of hospitalization, and trauma sequence. In addition, the results showed that histology may have additional value in differentiating between ante- and perimortem lesions, if compared to sole gross anatomical assessment.

Introduction

Mechanical traumatic injuries are frequently encountered when dry human skeletal remains are studied by osteoarchaeologists and palaeopathologists. Notwithstanding technical and diagnostical difficulties, a substantial number of

publications exist that discuss and interpret them (e.g. Maples, 1980; Grauer and Roberts, 1996; Lovell, 1997; Rodriguez-Martin, 2006; Brickley, 2006; Van der Merwe *et al.,* 2010; Cornish, *et al.* 2010). The majority of these publications suffice by reporting basic epidemiological parameters, such as the frequency of fractures per bone type or per subpopulation (e.g. Alvrus, 1999; Judd, 1999; Djuric *et al,.* 2006; Mitchell, 2006). Also, whether a lesion was caused antemortem, perimortem or postmortem is frequently discussed (e.g. Lovell, 1997; Redfern, 2009; Van der Merwe *et al.,* 2010). However, the relationship between the moment of injury and death in antemortem lesions has received little attention, irrespective of the meaningful extra detail that it may give. This disregard for fracture dating may be due to the misunderstanding that the accuracy of radiographical and histological analyses, as often performed in a forensic pathological context, is low when applied to human dry bone. Yet, recent papers suggest that their use provides satisfying posttraumatic time intervals when assessing only dry bone tissue (Maat, 2008; Maat and Huls, 2010; De Boer *et al.,* 2012).

This study focuses on posttraumatic time interval as assessed in (antemortem) fractures and amputations observed in human skeletons dating from the end of the 19th century. The individuals at the focus of this study were excavated in 2003, when several unmarked graves were accidentally disturbed next to the fenced Gladstone cemetery in Kimberley, South Africa. This cemetery was opened in 1883 and facilitated the burial of individuals who died in Kimberley and surroundings. At the end of the 19th century local diamond mining was exponentially growing when migrant workers flocked into the region. The skeletal remains studied in this sample are believed to be those of mine workers that died in the Kimberley hospital between 1897 and 1900 (Van der Merwe, 2010).

Van der Merwe *et al.* (2010), one of the co-authors of the present study, already reported on the large number of traumas in this population. She suggested that this high frequency could most likely be attributed to the strenuous and dangerous labour conditions associated with mining. Interpersonal violence was believed to be another major contributor. Contemporary historical documents and the high frequency of cranial fractures in the community supported the latter statement (Van der Merwe *et al,.* 2009). Despite the alleged difficult living conditions, the presence of well healed fractures suggested that basic medical care and social support was available to at least some of the individuals. The presence of amputations and historical records furthermore substantiated this assumption (cited from Van der Merwe *et al.,* 2010).

The aim of this study is to assess the posttraumatic time intervals of fractures and amputations in the Gladstone skeletal sample. This information could provide additional insight about the degree and effectiveness of medical care and health status of these 19th century labourers.

Material

The total Gladstone cemetery population consists of 107 individuals. Within this sample 28 individuals presented a total of 36 fractures. The sample presented a total of seven amputations. Two individuals presented a 'secondary' amputation, i.e. a amputation more proximal to the initial lesion site. In one individual this was a re-amputation, another individual presented an amputation of a previous fracture.

Not all fractures and amputations were included in this study. Due to the destructive methods needed for the assessment of the posttraumatic time interval, all cranial lesions were excluded. Poor preservations also led to the exclusion of a few specimens.

As a result, a total of nineteen gross anatomical traumas identified from the post cranial remains of thirteen individuals were included in this study. Wherever possible, a contra-lateral control specimen was studied for comparison.

Demographic details regarding the individuals and the gross anatomical status of their injuries as observed by Van der Merwe *et al.* (2010) are to be found in Table 1.

Methods

Posttraumatic time interval was assessed histologically and radiologically according to an earlier proposed method by De Boer *et al.* (2012). This method uses earlier established healing features derived from Barber (1929, 1930 and 1934), Maat (2009) and Maat and Huls (2010) that are still consistently detectable in archaeological remains (De Boer *et al*., 2012). These healing features and their temporary appearance during healing can be found in Table 2 and Figure 1. The used method can be readily found (De Boer *et al.*, 2012), but will be summarized here for reasons of clarity.

For roentgenographic evaluation, antero-posterior (AP) and lateral (L) plain radiographs were taken of all lesions and control specimens. For histological evaluation, a 'thick slice' of approximately 1 cm thickness was cut from the centre of each lesion, perpendicular to the fracture/amputation line. Additional 'thick slices' were excised from the control specimens. All 'thick slices' were processed into (un)stained histological sections using embedding, hand-grinding and staining methods as described by De Boer *et al.* (2013). At least two histological sections were produced from each 'thick slice': one unstained and one haematoxylin stained. In order to limit bias by recognition of samples, all radiographical images and histological sections were anonymised by random numbering.

First, X-ray images were analysed in numerical order on the department of Radiology of the Leiden University Medical Center. Subsequently, the unstained and

stained histological sections were evaluated separately, also in numerical order. Bright light and polarized light were used complementarily.

The control samples were analyzed in order to rule out any systemic diseases that could impede healing. The lesions were analysed for the presence of radiological or histological healing features. If a healing feature was identified, the from literature known moment of onset of its development was adopted as the minimum amount of elapsed posttraumatic time. During the interpretation of the images and sections, equivocal healing features were not taken into account as sole positive or negative observations. In other words, if the presence/absence of a healing feature could not be reliably assessed, the survival time was calculated in the 'safest' way. Furthermore, it was assumed that preservation had not affected the visibility of healing features, i.e. the absence of a healing characteristic was believed to be unrelated to bad visibility. In line with these assumptions, we used the absence of (not yet developed) healing features to identify the maximum stretch of elapsed posttraumatic time.

Table 1. Skeletal material.

	Skeletal related lesion no.[1]	**Sex**	**Age**	**Bone**	**Gross anatomical status of injury**[2]
Fractures	N38.1	Male	YA	Left rib	Perimortem
	N38.2-1	Male	YA	Right femur	Antemortem
	N38.2-2	Male	YA	Right tibia	Antemortem
	N38.2-3	Male	YA	Right 3rd metacarpal	Antemortem
	N38.3	Male	MA	Right radius	Antemortem
	N74.4	Male	MA	Left ulna	Antemortem
	N74.5	Male	MA	Left humerus	Antemortem
	S2.3-1	Male	YA	Right Radius	Antemortem
	S2.3-2	Male	YA	Right femur	Perimortem
	S2.9	Male	MA	Left 8th rib	Antemortem
	S3.2	Male	MA	6th rib	Antemortem
	S3.5	Male	YA	Left femur	Perimortem
Amputations	N34.3	Male	YA	Left tibia	Antemortem
	N38.2-4	Male	YA	Right femur	Perimortem
	N8.1b[3]	Male	A	Left tibia	Perimortem
	S2.6	Male	MA	Left tibia	Antemortem
	S2.7b-1	U	U	Humerus	Antemortem
	S2.7b-2[3]	U	U	Humerus	Perimortem
	S2.7c[3]	U	U	Right radius + ulna	Perimortem

[1] Numbering adopted from Van der Merwe *et al*. (2010). Suffixes were added to indicate multiple traumas in one person.

[2] Adopted from Van der Merwe *et al*. (2010).

[3] Discarded (distal) amputation parts.

Abbreviations: A= adult, 20< years. YA = Young adult, 20-35 years. MA = Middle adult, 36-49 years. U= unknown.

After separate evaluation, the results of radiological and histological analysis were combined in order to establish a definitive posttraumatic time interval.

Results

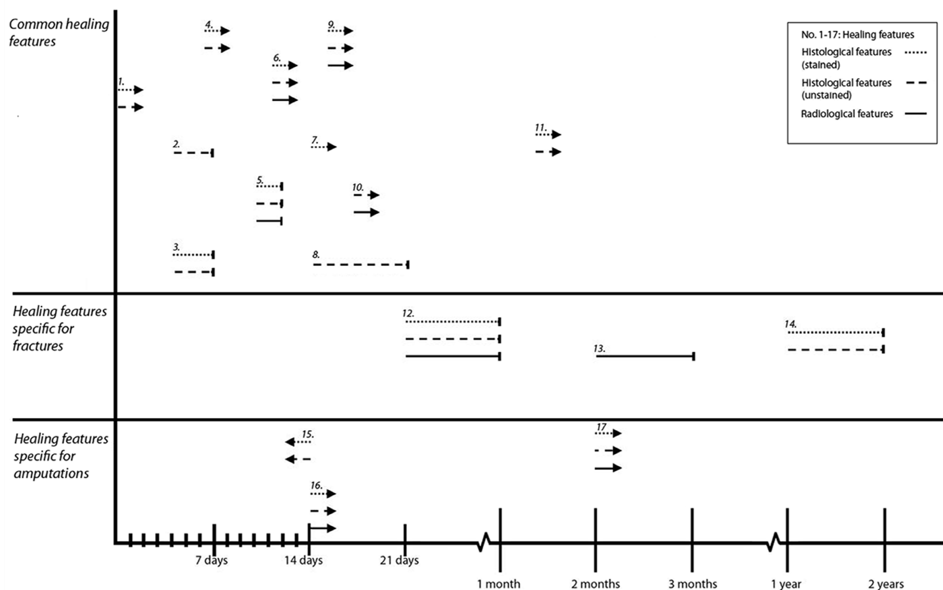
The radiological and histological analysis of the control samples did not present any abnormal findings. For fractures, the healing features are listed in Tables 3 and 4. The corresponding posttraumatic time intervals can be found in Figure 2. Two fractures (S2.3-1 and S2.3-2) presented with no signs of healing, which indicated a posttraumatic survival time up to seven days. In four fractures (N38.1, S3.5, N38.2-1 and N38.2-2) radiological healing features were absent, but histology displayed early signs of healing. The presence of Howship's lacunae indicated a posttraumatic

Table 2. Healing features for fractures and amputations, adopted from De Boer *et al.* (2012).

Healing feature	Time interval
Common healing features.	
Frayed bone lamellae at the lesion margins.	From start
First Howship's lacunae at the lesion margins.	Starts after 4-7 days
Smoothening of the lesions margins.	Starts after 4-7 days
Start of periosteal callus formation, distant from the lesion margins, separable from the cortex.	Starts after 7 days
Endosteal callus formation clearly visible.	Starts after 10-12 days
Osteoporotic appearance of the cortex.	Starts after 12 days
Start of the transition of primary woven bone into secondary lamellar bone.	Starts after 14 days
Cortical cutting and closing cones orientated towards the lesion.	Starts after 14-21 days
Clearly visible periosteally situated callus.	Starts after 15 days
Endosteal callus becomes indistinguishable from the cancellous bone in the marrow cavity.	Starts after 17 days
Periosteal callus becomes firmly attached (inseparable) to the cortex.	Starts after 6 weeks
Features specific for fractures.	
Union by bridging of the cortical bone discontinuity.	Starts after 21-28 days
By primary woven bone.	
By secondary lamellar bone.	
Smoothening of the periosteal callus outline	Starts after 2-3 months
After adequate immobilization: quiescent appearance indicating subsided healing.	Starts after 1-2 years
Features specific for amputations.	
Visibility of cut marks on the amputation surface.	Visible before 13 days
Start of 'capping' of the medullary cavity.	Starts after 'not many weeks'
Complete capping of the medullary cavity.	Starts after 'several months'

survival time of at least four days. The absence of endosteal callus formation limited the posttraumatic survival time to less than twelve days. In a fractured metacarpal (N38.2-3), the presence of 'cutting and closing cones' and distinct periosteal callus formation resulted in a minimum posttraumatic survival time of fifteen days. Due to the absence of union of the fracture ends, the maximum posttraumatic survival time was set at one month.In four fractures (N38.3, N74.4, N74.5 and S2.9), radiological analysis indicated a minimum posttraumatic survival time of two months due to the presence of smoothening of the periosteal callus outline. In lesion N38.3 and N74.4, histological presence of an inseparable attachment of the periosteal callus set posttraumatic time at six or more weeks, whereas the absence of signs of subsided healing set the upper limit at two years. In lesion N74.5 and S2.9, one of the histological sections showed signs of subsided healing, whereas the other section did not. Posttraumatic survival time was thus set between one and two years. For amputations, the detected healing features are listed in Tables 5 and 6. The corresponding posttraumatic time intervals can be found in Figure 3. Four amputations did not present any healing features (N8.1b, N38.2-4, S2.7b-2 and S2.7c),

Figure 1.



Healing features and the timing of their onset. Data from De Boer et al. (2012b). Arrowhead represent infinite time limit expansions. Numbers are adopted from Table 2.

indicating a posttraumatic survival time of up to seven days. Based on the distinct presence of periosteal callus, one lesion (S2.7b-1) gave a radiologically and histologically postoperative survival time of at least fifteen days. The inseparable attachment of the periosteal callus in lesion N34.3 indicated a posttraumatic survival time of at least 6 weeks. Indicated by the completed capping of the medullary cavity, lesion S2.6 led histologically and radiologically to a posttraumatic survival time of at least two months.

Table 3. Healing features for fractures, using histological assessment.

H.	N74.5		N38.1		N38.2-1		N38.2-2		N38.2-3		N.38.3	
	U	S	U	S	U	S	U	S	U	S	U	S
1	N	N	N	N	N	N	N	N	N	N	N	N
2	N	N	N	N	Y	Y	Y	Y	Y	Y	N	N
3	/	/	Y	Y	Y	Y	Y	Y	Y	Y	/	/
4	N	N	N	N	N	N	N	N	Y	Y	N	N
5	N	N	N	N	N	N	N	N	N	N	Y	Y
6	N	N	N	N	N	N	N	N	N	N	N	N
7	Y	Y	/	/	/	/	/	/	N	N	Y	Y
8	N	N	N	N	N	N	N	N	Y	Y	Y	Y
9	Y	Y	N	N	N	N	N	N	Y	Y	Y	Y
10	Y	*	N	*	N	*	N	*	N	*	N	*
11	Y	Y	/	/	/	/	/	/	N	N	Y	Y
12	Y	Y	N	N	N	N	N	N	N	N	Y	Y
14	Y	?	N	N	N	N	N	N	N	N	N	N

Abbreviations: H=healing feature (coded according to Fig. 1 and Tab. 2). U=unstained histology. S=Stained histology. Y=Yes, feature is visible. N=No, feature is not visible. ?=No conclusive answer is possible. *=Not consistently detectable in this modality. /=Not applicable.

(Table 3, continued).

H.	N.74.4		S2.3-1		S2.3-2		S2.9		S3.5	
	U	S	U	S	U	S	U	S	U	S
1	N	N	Y	Y	Y	Y	N	N	N	N
2	N	N	N	N	N	N	N	N	N	N
3	/	/	N	N	N	N	/	/	Y	Y
4	N	N	N	N	N	N	N	N	N	N
5	Y	N	N	N	/	/	N	N	N	N
6	N	N	N	N	N	N	N	N	N	N
7	Y	Y	N	N	/	/	Y	Y	/	/
8	Y	Y	?	?	N	N	N	N	N	N
9	Y	Y	N	N	N	N	Y	Y	N	N
10	N	*	N	*	N	*	Y	*	N	*
11	Y	Y	/	/	/	/	Y	Y	/	/
12	Y	Y	N	N	N	N	Y	Y	N	N
14	N	N	N	N	N	N	Y	?	N	N

Table 4. Healing features for fractures, using radiological assessment.

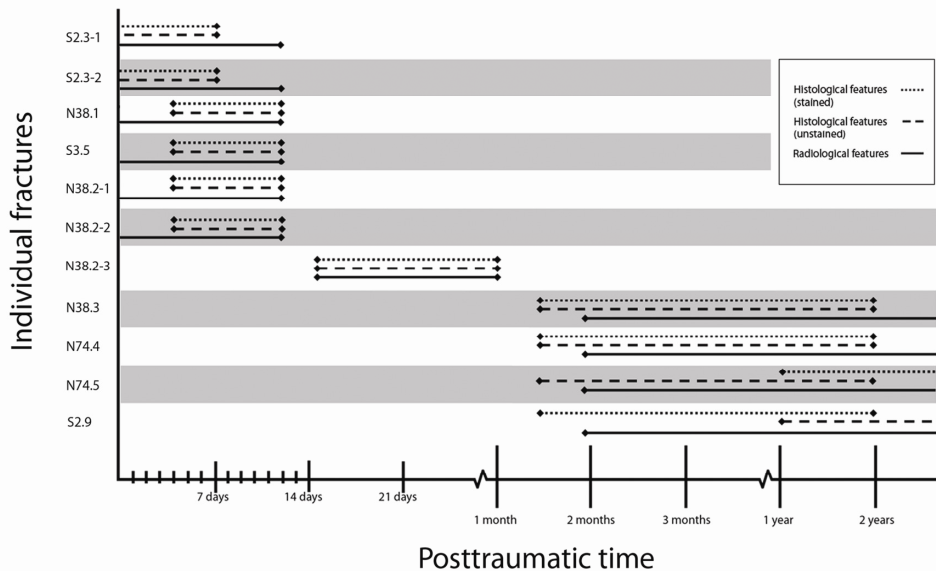
H.	N74.5	N38.1	N38.2-1	N38.2-2	N38.2-3	N.38.3
5	N	N	N	N	Y	Y
6	N	N	N	N	?	N
9	Y	N	N	N	Y	Y
10	N	N	N	N	N	N
12	Y	N	N	N	N	Y
13	Y	N	N	/	N	Y

Abbreviations: H=healing feature (coded according to Fig. 1). Yes, feature is visible. N=No, feature is not visible. ?=No conclusive answer is possible. /=Not applicable

(Table 4, continued).

H.	N.74.4	S2.3-1	S2.3-2	S2.9	S3.5
5	Y	N	N	N	N
6	N	N	N	N	N
9	Y	N	N	Y	N
10	N	N	N	Y	N
12	Y	N	N	Y	N
13	Y	/	/	Y	/

Figure 2.



The final conclusions on the posttraumatic time interval of all lesions are summarized in Table 7.

We furthermore compared the above presented results with the original gross anatomical assessment of the injury statuses as listed in Table 1. (See Tab. 8). A few discrepancies were noted. Two fractures (N38.1 and S3.5) were originally diagnosed as perimortem but showed a posttraumatic survival time of four to twelve days. Of two fractures that were originally described as antemortem, one did not display a fracture (S3.2), whereas the other did not present any healing features (S2.3-1). In amputations, no differences were noted between gross anatomical injury status and the radiological and histological results.

Table 5. Healing features for amputations, using histological assessment.

H.	N34.3		N38.2-4		N8.1b		S2.6		S2.7b-1		S2.7b-2		S.2.7c	
	U	S	U	S	U	S	U	S	U	S	U	S	U	S
1	N	N	Y	Y	Y	Y	N	N	N	N	Y	Y	Y	?
2	N	N	N	N	N	N	Y	Y	Y	Y	N	N	N	N
3	Y	Y	N	N	N	N	Y	Y	Y	Y	N	N	N	N
4	Y	Y	N	N	N	N	N	N	Y	?	N	N	N	N
5	?	?	N	N	N	N	N	N	Y	?	N	N	N	N
6	Y	Y	N	N	N	N	N	N	N	?	N	N	N	N
7	Y	Y	/	/	/	/	/	/	?	?	/	/	/	/
8	N	N	N	N	N	N	N	N	N	N	N	N	N	N
9	Y	Y	N	N	N	N	N	N	Y	?	N	N	N	N
10	Y	*	N	*	N	*	Y	*	N	*	N	*	N	*
11	Y	Y	/	/	/	/	/	/	N	/	/	/	/	/
15	N	N	N	N	N	N	N	N	N	N	N	N	N	N
16	Y	Y	N	N	N	N	Y	Y	N	N	N	N	N	N
17	?	?	/	/	/	/	Y	Y	/	/	/	/	/	/

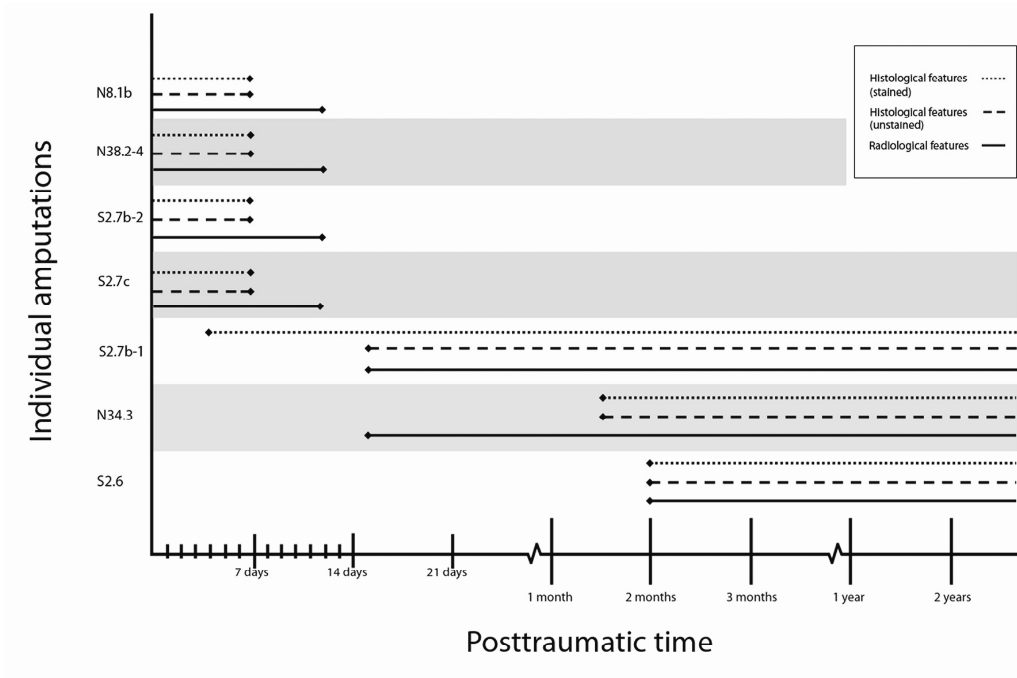
Abbreviations: H=healing feature (coded according to Fig. 1). U=unstained histology. S=Stained histology. Y=Yes, feature is visible. N=No, feature is not visible. ?=No conclusive answer is possible. *=Not consistently detectable in this modality. /=Not applicable.

Table 6. Healing features for amputations, using radiological assessment.

H.	N34.3	N38.2-4	N8.1b	S2.6	S2.7b-1	S2.7b-2	S2.7c
5	N	N	N	N	?	N	N
6	?	N	N	Y	N	N	N
9	Y	N	N	N	Y	N	N
10	N	N	N	Y	N	N	N
16	Y	N	N	Y	N	N	N
17	N	/	/	Y	/	/	/

Abbreviations: H.=healing feature (coded according to Fig. 1). Y=Yes, feature is visible. N=No, feature is not visible. ?=No conclusive answer is possible. /=Not applicable.

Figure 3.



Discussion

Throughout our results, it appeared that individual posttraumatic survival time was either relatively 'short', i.e. less than 12 days, or relatively 'long', i.e. more than 6 weeks. This division held for fractures and amputations alike (see Figs. 2 and 3). Healing features coded 7, 8, 9, 10 and 12 in Table 2 exclude that this could be attributed to lack of diagnostic healing features of the intermediate period (See Fig. 1). The resulting subgroups, i.e. 'short survival' versus 'long survival', will be discussed below to deepen our understanding of the health status and medical care of the deceased in the Gladstone cemetery. Many lesions, for instance fractures S2.3-1, S2.3-2, S3.5, N38.1, S3.5, N38.2-1 and N38.2-2, represented a relative short posttraumatic survival time, i.e. less than twelve days. Apparently, this first period was critical to life expectancy. The related patients probably succumbed immediately (in case of no healing features) or shortly thereafter (in case of only early healing features) from consequences related to the trauma event. This holds especially in individuals presenting evidence of a complication, such as the secondary infections that were indentified in individuals N38.2 and N8.1b (Van der Merwe *et al.* 2010). Yet, a possibility stays that some may have died from unrelated causes, especially in cases with minor affections, like a sole rib fracture (N38.1). More than half of the

amputations failed to show any healing features (N8.1b, N38.2-4, S2.7b-2 and S2.7c) and thus seemed to have had a posttraumatic survival time of maximal seven days. However, of these amputations, the large majority represented only the discarded distal amputated parts. By definition, these cannot show healing features and their analysis did not permit new insights with regard to posttraumatic survival time.

Individuals with a relatively long posttraumatic survival time generally survived for at least six weeks (Figs. 2 and 3). Apparently, once the first two posttraumatic weeks had passed, the patient was likely to survive for at least another four weeks. In contrast to lesions with a relatively short posttraumatic survival time, individuals with these 'old' lesions probably died from disorders unrelated to the healing or healed trauma injury. Any suggestion on the cause of their death would be highly speculative. With respect to those with a fracture, survival time indicated that medical care was not limited to traumas only.

Amputations with a relatively long posttraumatic survival showed that at least some amputation procedures were carried out successfully. The re-amputation in individual S2.7b-1, which consisted of a more proximal amputation due to infection of the initial amputation site (Van der Merwe *et al*., 2010), demonstrated prolonged postoperative care. The eventual survival after this secondary amputation could not be assessed, as only the diaphyseal humeral part between the amputation and fracture was excavated (see also Van der Merwe *et al.* 2010). The very long

Table 7. Posttraumatic time interval per lesion.

Individual No.	Bone type	Lesion type	Posttraumatic time interval
N34.3	Left tibia	Amputation	More than 6 weeks
N38.1	Left rib	Fracture	Between 4 and 12 days
N38.2	Right femur	Fracture	Between 4 and 12 days
	Right tibia	Fracture	Between 4 and 12 days
	Right metacarpal	Fracture	Between 15 days and 1 month
	Right femur	Amputation	Less than 7 days
N38.3	Right radius	Fracture	Between 2 months and 2 years
N74.4	Left ulna	Fracture	Between 2 months and 2 years
N74.5	Left humerus	Fracture	Between 1 and 2 years
N8.1b	Left tibia	Amputation	Less than 7 days
S2.3	Right radius	Fracture	Less than 7 days
	Right femur	Fracture	Less than 7 days
S2.6	Left tibia	Amputation	More than 2 months
S2.7b	Humerus	Amputation	More than 15 days
	Humerus	Amputation	Less than 7 days
S2.7c	Right radius and ulna	Amputation	Less than 7 days
S2.9	Left 8th rib	Fracture	Between 1 and 2 years
S3.5	Left femur	Fracture	Between 4 and 12 days

posttraumatic survival times in cases N34.3 and S2.6 indicated that hospitalization was probably unrelated to prior amputation. Medical care was thus apparently provided to disabled members of the community.

Interestingly, posttraumatic survival time assessment allowed for the reconstruction of a chain of medical events in the case of individual N38.2 (lesions N38.2-1, N38.2-2, N38.2-3 and N38.2-4). Fifteen to 30 days prior to death, this patient fractured a metacarpal. This lesion did not require hospitalization, because ten days later he experienced a major trauma event that caused both his right tibia and femur to fracture. A few days later, the femoral lesion became infected, causing osteomyelitis, an infection of cortical bone and marrow cavity. The latter was indicated by the presence of lytic/infectious new bone formation and the presence of abundant osteolysis by osteoclasts, resulting in numerous Howhsip's lacunae (Fig. 6). In an attempt to stop the further spread of the infection, a leg amputation was performed. As the amputation surface did not show any healing features or changes from infection, and the individual was buried together with the amputated femoral parts, the amputation probably coincided with the demise of the individual. The gross anatomical, radiographical and histological findings that allowed for this reconstruction are shown in Figures 4, 5 and 6.

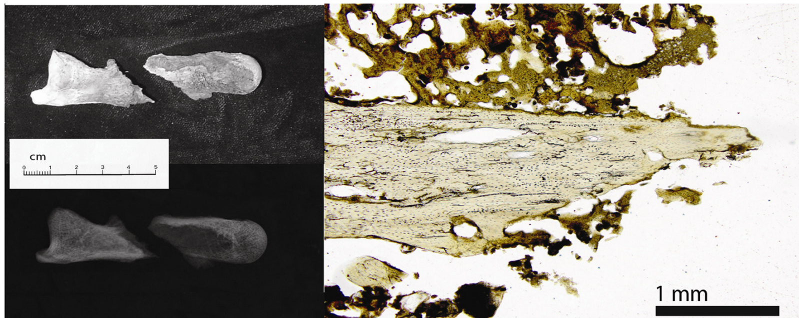
Table 8. Comparison of earlier gross anatomical dating[1] with current results.

	Skeletal related lesion no.	Gross anatomical status of injury	Posttraumatic time interval
Fractures	S2.3-2	Perimortem	Less than 7 days
	N38.1	Perimortem	4 - 12 days
	S3.5	Perimortem	4 - 12 days
	S3.2	Antemortem	No fracture
	S2.3-1	Antemortem	Less than 7 days
	N38.2-1	Antemortem	4 - 12 days
	N38.2-2	Antemortem	4 - 12 days
	N38.2-3	Antemortem	Between 15 days and 1 month
	N38.3	Antemortem	Between 2 months and 2 years
	N74.4	Antemortem	Between 2 months and 2 years
	N74.5	Antemortem	Between 1 and 2 years
	S2.9	Antemortem	Between 1 and 2 years
Amputations	S2.7b-2	Perimortem	Less than 7 days
	S2.7c	Perimortem	Less than 7 days
	N38.2-4	Perimortem	Less than 7 days
	N8.1b	Perimortem	Less than 7 days
	S2.7b-1	Antemortem	More than 15 days
	N34.3	Antemortem	More than 6 weeks
	S2.6	Antemortem	More than 2 months

[1] According to Van der Merwe et al. (2010).

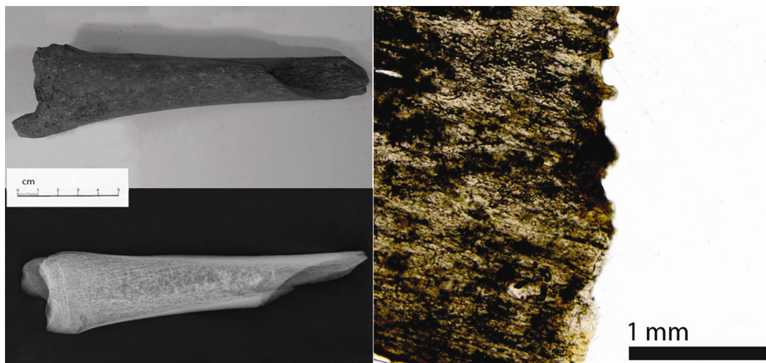
When looking at Figures 2 and 3, some differences between the histological and radiological results can be observed. Especially in relatively 'young' lesions, the histological analysis appears to be able to define a more clear posttraumatic time interval. This is an expected result, as the time length of the initial series of histological changes due to healing are relatively short if compared to those of the 'advanced' period of healing. In addition, many of the initial histological changes stay 'invisible' for radiological detection. Not all healing characteristics are reliably

Figure 4.



Photograph, radiograph and micrograph of a fractured third metacarpal, lesion N38.2-3. Bars indicate magnification. The photograph and radiographs show the presence of periosteal callus. The micrograph of an unstained ground section shows that the periosteal callus (arrows) had not yet become inseperably attached to the cortex. Furthermore, the smoothened appearance of the lesion margin can be appreciated.

Figure 5.

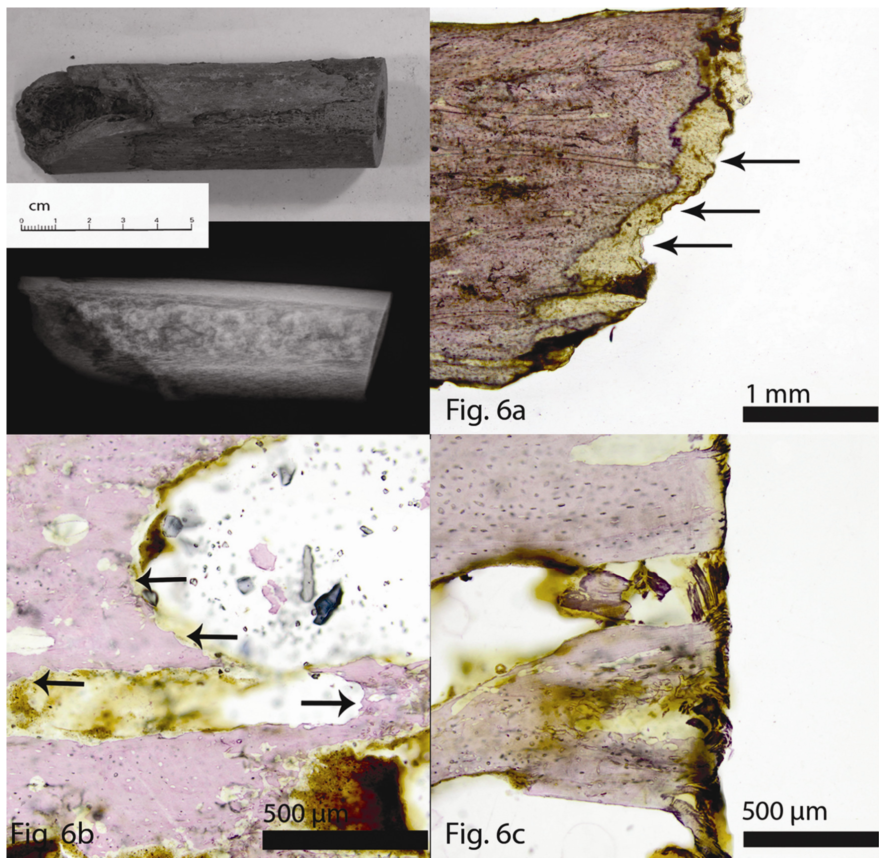


Photograph, radiograph and micrograph of a section of the fractured tibia, lesion N38.2-2. Bars indicate magnification. The photograph and radiograph show absence of healing features. Although taphonomical alteration hampers visibility, the micrograph of an unstained ground section allows for appreciation of the smoothened appearance of the lesion margins.

assessable with radiology and histology alone, the complementary use of radiology and histology is therefore preferred.

In the majority of the lesions, the conclusion on their status by Van der Merwe *et al.* (2010) could be confirmed. Yet in two cases (N38.1 and S3.5), the histological assessment indicated a posttraumatic survival time of four to twelve days, where gross anatomical analysis had defined the lesion as perimortem. This is understandable, since the earliest signs of healing are only visible at the microscopic level. Lesion S3.2, gross anatomically defined as antemortem rib fracture, did not

Figure 6.



Photograph, radiograph and three micrographs (6a, 6b and 6c) of a femur, lesions N38.2-1 and N38.2-4. The photograph and radiograph show the porotic fracture end. The reactive formation of bone tissue is visible. On the micrographs of haematoxylin stained ground sections, the fracture end (Fig 6a) has a smoothened appearance. The cortical bone tissue adjacent to the fracture end is osteolytic (infectious), with abundantly present Howhip's lacunae (arrows) (6b). The amputation end shows frayed bone fibers, indicating no healing (6c).

demonstrate features of a (healing) fracture (see Tab. 7). This is probably due to the fact that ribs may present quite acute anatomical angles that can be misinterpreted as malaligned healed fractures. Lesion S2.3-1 was diagnosed by Van der Merwe as ante mortem (Van der Merwe *et al*., 2010), but no healing characteristics were found. This might be due to unrepresentative section-taking for histology and misinterpretation of radiological imaging.

These discrepancies suggest that sole gross anatomical analysis, as well as sole histological and/or radiology might cause erroneous results. Yet these findings must be interpreted with caution, as the gross anatomical analysis and section-taking was not done by the same researcher that performed the radiological and histological analysis.

Although our findings might have been affected by some unknown degree of selection bias from the exclusion of some poorly preserved postcranial specimens and the exclusion of crania, we believe that meaningful insight could be made regarding populational and individual posttraumatic survival time.

Conclusion

Posttraumatic survival time assessment in the Gladstone cemetery skeletal sample substantiated the provision of medical care to the workers of the Kimberley diamond mines at the end of the nineteenth century. The first two posttraumatic weeks appeared to be a high risk period, but if survived, patients were likely to live for at least another four weeks. The results also suggest that medical care was provided to individuals with minor fractures, major traumas and to patients without recent skeletal lesions. Furthermore, amputations were carried out, some of them successfully. Amputees apparently received postoperative care. Disabled individuals were not excluded from medical care. Finally, radiological and histological analysis has shown to produce an increased degree of trauma information on a populational and individual level, and may avoid gross anatomical misinterpretation in the perimortem and antemortem domain.

Acknowledgements

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Chapter 9

TRAUMA AND POSTTRAUMATIC SURVIVAL TIMES DURING THE FIELD CAMPAIGN OF NAPOLEON'S ARMY IN 1812: A REPORT ON TEN CASES FROM THE KOENINGSBERG MASS GRAVE

From: *Trauma and posttraumatic survival times during the field campaign of Napoleon's army in 1812: a report on ten cases from the Koeningsberg mass grave*. *De Boer H.H., Van der Merwe A.E., Berezina, N., Maat, G.J.R., Dutour, O., Buzhilova, A.P. In: Anthropology and archaeology of the Russian Napoleonic campaign. Buzhilova, A., Dutour,O. (Eds.). (in press).*

Abstract. In 2006, a French-Russian collaboration team excavated a mass grave in the former Koeningsberg, presently the Russian enclave Kaliningrad. Found artefacts and historical records suggested that the excavated individuals were soldiers of Napoleon's 'Grande Armee', who died at the end of the Russian field campaign of 1812. A substantial number of traumatic lesions was noted on the remains (Buzhilova *et al.*, 2009).

To provide physical, in addition to historical, information on the health conditions of these soldiers gross anatomical and radiological analyses were performed on twelve randomly selected antemortem traumatic lesions from ten individuals (skeletons), i.e. ten fractures and two amputations. Features indicative of healing were investigated for each trauma in an attempt to define the minimum and maximum posttraumatic survival time of the related victims.

Results indicated posttraumatic survival times consistent with historical knowledge on battlefield medical care as provided in Napoleons 'Armee', viz. adequate evacuation from the battlefield and (on-the-spot) medical care. A considerable amount of fractures showed a minimum posttraumatic survival time of two months, indicating that the related individuals coped with their injuries throughout the retreat. The analyzed amputations showed no features indicative of healing. Apparently, surgery was still performed till the final days of the campaign. Furthermore, the results allowed for some surmises on the cause of death of some of the individuals.

Introduction

Napoleon's 1812 campaign

The French invasion of Russia by Napoleon's 'Grande Armee' in the autumn of 1812 is regarded as a turning point in Napoleon's reign over Europe (Von Clausewitz, 1996). Its outcome severely shook his reputation as a military genius and weakened his military strength. The resulting shift in European alliances eventually lead to the war of the 6th coalition that resulted in Napoleon's abdication and his banishment to Elba in 1814.

Napoleon's Russian campaign started when his massive army crossed the Neman River on the 24th of June (Riehn, 1991). In the first months, Napoleon found little resistance from the ever retreating Russian army. The Russians were unable to set up a proper defensive position against the quickly moving French, but nevertheless managed to block the way to the Russian capital, St. Petersburg. As a result, Napoleon marched on to Moscow. Just in sight of the city, the Russians managed to set up a defensive position at Borodino. The Battle of Borodino was the bloodiest of the campaign, with at least 70 000 casualties in one day. Although victorious, Napoleon's exhausted army was unable to give the Russians a decisive blow. The heavy losses on Napoleon's side gave the Russians time to retreat and evacuate Moscow unconditionally. On the 14th of September, Napoleon moved into an empty and stripped city. Fires broke out that same day, eventually destroying almost 80% of the city. Without Russian capitulation and without supplies to replenish his tired troops, Napoleon eventually decided to withdraw.

On the 20th of October, the French army left Moscow. The restrengthened Russian army forced the French to use the same route as they came by, that was by then stripped of all useful supplies. Starvation, disease and constant attacks further weakened the French. After the heavy battle at the banks of the Berezina river from 26 to 29 november, Napoleon abandoned his troops to handle a *coup d'etat* in France. The campaign officially ended when the decimated troops left Russia by crossing the Neman river on December the 14th. They reached Koenigsberg (now Kaliningrad) on the 18th of December.

Medical care in the Napoleonic army

Mostly due to the work of the Surgeon-in-Chief Dominque-Jean Larrey (1766-1842), medical standards of the Napoleonic army were considered to be high. Because of his pioneering work on the improvement of the medical care for soldiers, he is still looked upon as one of the inventors of modern battlefield medicine (Pearce, 2002; Baker *et al.*, 2005; Welling *et al.*, 2010; Welling and Rich, 2013).

During the early 19th century, wounded soldiers were often left on the battlefield unattended for, only to receive medical care after the fighting had ended. Larrey recognized the need for immediate medical care and invented the so-called 'ambulances volantes', or flying ambulances (Baker, 2005). These chariots, equipped with the necessary supplies and trained medical personnel, made quick evacuation or on-the-spot surgery possible. Larrey also improved injury prognosis by performing rigorous 'debridement' and pro-active amputation. In his memoires he states that "it is necessary to […] take advantage of the favorable moment to do the amputation, without waiting until the dead tissue is established" (Larrey, 1812; in Welling *et al.*, 2010). By the improvement of amputation techniques, he minimized operation time. In the battle of Borodino, he stated to have performed 200 amputations within 24 hours. Larrey opposed the then usual doctrine of supplying medical care on the basis of hierarchy. Instead, he ordered to treat the most severely wounded first, irrespective of rank or distinction (Welling and Rich, 2013). This attitude effectively laid the foundation for the modern triage systems. In the pre-antiseptic and pre-antibiotic era, Larrey's exertions and insights were considered groundbreaking and it is believed that his approach greatly improved the prognosis for men with battlefield injuries (Pearce, 2002; Baker *et al.*, 2005). In contrast to Napoleon, Larrey was still with the French army when they arrived in Koeningsberg. Soon after their arrival, he started organizing medical care for those who had survived (Welling *et al.*, 2010).

The Koeningsberg mass grave

In 2006, construction workers stumbled upon an archaeological site in former Koeningsberg. The site consisted of remnants of the old fortification of Koeningsberg, with in its vicinity twelve grave pits containing numerous human skeletons (Buzhilova *et al.*, 2009). The pits were organized in two rows, with the number of interred individuals per pit varying from a few dozen to hundreds. Artefacts such as buttons and fragments of uniforms identified the remains as those of soldiers from Napoleon's Russian field campaign, and dated the mass graves at late 1812, early 1813 (Buzhilova *et al.*, 2009). Historical evidence suggested that the excavated individuals were patients of a military hospital (lazarette) within the city walls (Buzhilova *et al.*, 2009).

Posttraumatic survival time in palaeopathology

Mechanical traumas are a common finding in skeletonized human remains. A large amount of literature has established the value of its analysis when reconstructing the health conditions of past populations (e.g. Lovell, 1997). At the very base of 'palaeotraumatology' is the assessment of the time laps between a

traumatic event and eventual death, as it defines to what extend an individual's health was affected by the lesion. Lesions are therefore described as either being antemortem, postmortem or -in indefinite cases- perimortem. In antemortem lesions, a further analysis of the lapsed time between trauma and death (the so-called posttraumatic survival time) is meaningful.

Posttraumatic survival time is generally only roughly estimated by designating a lesion to be healing (having a short posttraumatic time interval) or healed (having a long posttraumatic time interval) (e.g. Brickley, 2006). A more detailed estimation of the posttraumatic survival time could aid in interpreting facets such as medical status, medical care and the timing/sequence of multiple traumas (De Boer *et al.*, 2013). For such a detailed estimation, an approach based on the fixed relationship between specific healing features and minimum and maximum posttraumatic survival time has been proposed (De Boer *et al.*, 2012).

Aim of this study

This article describes a selected number of cases of mechanical traumas as found in individuals excavated from the 1812 Koeningsberg mass grave. Our primary aim was to gain insight in the medical care and the health status of the soldiers by assessing the related posttraumatic survival times. Due to the historical value of the skeletal material, no (destructive) histological investigation was allowed. The standard approach for trauma dating (De Boer *et al.*, 2012), was therefore extended with Computed Tomography (CT) scanning. Secondarily the value of this technical extension was assessed.

Material

The excavation of the Koeningsberg mass grave by a French-Russian collaboration team surfaced a minimum number of ca. 600 individuals (Buzhilova *et al.*, 2009). Within this sample, a notable number of mechanical injuries were identified in varying stages of healing. After excluding badly preserved and postmortem lesions, twelve antemortem ones were randomly selected from ten individuals, i.e. ten fractures and two amputations (Table 1). No demographic information was available for the related individuals but it may be assumed that the individuals were young males (Buzhilova *et al.*, 2009).

Methods

The injuries were gross anatomically investigated according to generally accepted palaeopathological standards (Lovell, 1997). Trauma type and the resulting bone deformity (e.g. angulation, shortening) was documented and all lesions were photographed.

The traumas were radiologically imaged by plain X-rays in an anterio-posterior and lateral direction. Supplementary, 80 and 140 kV Computed Tomography (CT) scans were performed on a Philips Microtron CT-scanner with a slide thickness of 0.4 mm. All imaging was performed at the Moscow State University (MSU) by the Department of Anthropology in collaboration with the Department of Medicine.

Table 1. Bone material.

Lesion type	Lesion no.	Bone type	Lesion side
Fractures	C11-30a	Ulna	Right
	C11-30b	Radius	Right
	C29	Ulna	Right
	E1-6	Femur	Left
	Gvr14	Ulna	Left
	Gvr15	Humerus	Left
	HS50	Ulna	Left
	K9-107	Tibia	Left
	Kvr19	Humerus	Right
	X27	Humerus	Right
Amputations	Ivr5	Tibia	Right
	Kvr13	Humerus	Right

Table 2. Gross anatomical and radiological healing features[1].

	Healing feature appearance	Posttraumatic time interval
General features	Endosteal callus formation clearly visible.	Starts after 10-12 days
	Periosteal callus formation distant from the lesion margins.	Starts after 7 days
	Osteoporotic appearance of the cortex.	Starts after 12 days
	Clearly visible periosteally situated callus.	Starts after 15 days
	Endosteal callus becomes indistinguishable from the cancellous bone in the marrow cavity.	Starts after 17 days
Features specific for fractures	Union by bridging of the cortical bone discontinuity.	Starts after 21-28 days
	Smoothening of the callus outline.	Starts after 2-3 months
	Pseudoarthrosis development.	Starts after 6-9 months
Features specific for amputations	Cut marks on the amputation surface.	Visible before 13 days
	Start of 'capping' of the medullary cavity.	Starts after 'not many weeks'
	Complete 'capping' of the medullary cavity.	Starts after 'several months'

[1] Adopted from De Boer *et al.* (2012).

The posttraumatic time interval was estimated according to De Boer *et al.* (2012). This approach utilizes gross anatomical, radiological and histological healing features from Barber (1929, 1930 and 1934), Maat (2009) and Maat and Huls (2010). As no microscopic investigation was allowed by the owners, only gross anatomical and radiologic features were taken into account (see Table 2).

During interpretation, it was assumed that preservation had not affected the visibility of healing features, i.e. the absence of a healing feature was believed to be unrelated to bad visibility. In line with this assumption, the presence of a healing feature was interpreted as an indicator of the minimum posttraumatic survival time, and the absence of (not yet developed) healing features was used to identify the maximum posttraumatic survival time.

Survival time was calculated 'conservatively'. For example, if the presence/absence of a healing feature could not be conclusively assessed, it was not taken into account. After separate evaluation of the plain X-rays and CT scans, both conclusions were combined to establish a final posttraumatic time interval.

Results

The gross anatomical and radiological findings on the lesions are listed in Table 3 and 4. The corresponding posttraumatic time interval per lesion can be found in Table 5.

Concerning the fractures, four lesions (Gvr14, Gvr15, HS50 and Kvr19) displayed an obvious periosteally situated callus, indicating a minimum posttraumatic survival time of 15 days. The lack of smoothening of the callus contour in lesion Gvr14, Gvr15 and HS50 limited the posttraumatic survival time to three months. Since Kvr19 displayed signs of reactive bone formation due to infection, assessment of callus contour smoothening was impossible and no maximum posttraumatic survival time could be assessed.

Fracture C11-30b showed union by bridging of the cortical bone discontinuity, indicating a posttraumatic survival time of at least 21 days. Absence of smoothening of the callus contour maximized the posttraumatic survival time to three months. In lesion C11-30a, the traumatic event did not cause a total discontinuity of the cortex, and thus this healing feature was noted as 'not applicable'. However, since lesion C11-30a was found in the same individual as C11-30b and was supposedly caused by the same traumatic event (see discussion), it was assumed to have had the same posttraumatic time interval.

A more prolonged posttraumatic survival time was seen in fractures C29, E1-6, K9-107 and X27. The smoothened callus outlines indicated that these individuals

Table 3. Results from gross anatomical and plain X-ray evaluation.

Healing feature no.[1]	Lesion no.							
	C11-30a	C11-30b	C29	E1-6	Gvr14	Gvr15	HS50	K9-107
1	●	●	●	●	●	●	●	●
2	●	●	●	●	●	●	●	●
3	●	●		●	●	●	●	
4	●	●	●	●	●	●	●	●
5								
6	N/A	●		●				●
7			●	●				●
8								
9								
10								
11								

[1] Healing features as coded in Table 2.
● = Healing feature was present. * = Only assessable gross anatomically. N/A = Not applicable, because no cortex discontinuity was present.

(Table 3, continued).

Healing feature no.[1]	Lesion no.			
	Kvr-19	X27	Ivr5	Kvr13
1	●			
2	●			
3	●			●
4	●			
5		●		
6		●		
7		●		
8				
9			●*	●*
10				
11				

survived for at least two months after the trauma. The lack of osteoporosis in K9-107 and X27 indicated that the related extremities were functionally used. For these lesions, the posttraumatic survival time probably exceeded the minimum of two months.ue to non-union in the presence of abundant callus formation. possible pseudoarthrosis development in case of lesion C29 was considered. Although gross anatomical analysis suggested corticalisation of the fracture ends, plain X-rays did not show fracture margin sclerosis (see Figure 1). The lesion was therefore not interpreted as a stabilized pseudoarthrosis and thus minimum posttraumatic survival time was kept at two months.

Table 4. Results from CT-scan evaluation.

Healing feature no.[1]	Lesion no. C11-30a	C11-30b	C29	E1-6	Gvr14	Gvr15	HS50	K9-107
1	●	●	●	●	●	□	●	●
2	●	●	●	●	●	●	●	●
3	●	●		●	●	●	●	
4	●	●	●	●	●	●	●	●
5				□				
6		●		●				●
7			●	●				●
8								
9								
10								
11								

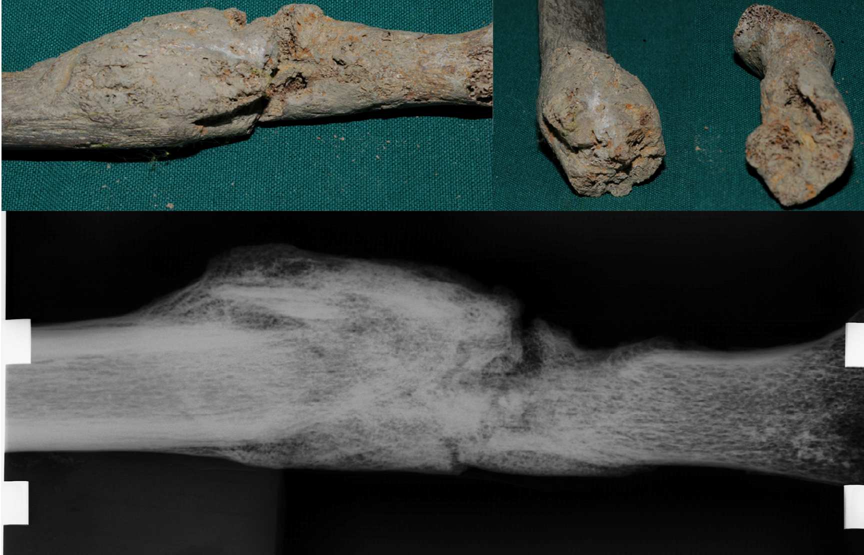
[1] Healing features as coded in Table 2.
● = Healing feature was present. * = Only assessable gross anatomically. N/A = Not applicable, because no cortex discontinuity was present.

(Table 4, continued).

Healing feature no.[1]	Lesion no. K9-107	Kvr-19	X27	Ivr5	Kvr13
1	●	●			
2	●	●			
3		●			●
4	●	●			
5			●		
6	●		●		
7	●		●		
8					
9					
10					
11					

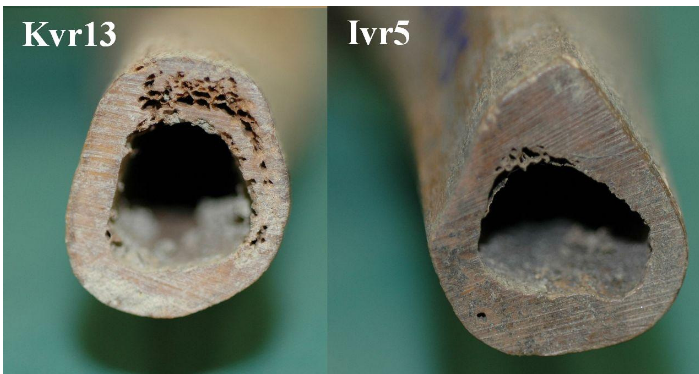
No healing features were identified in the amputations Ivr5 and Kvr13. The lack of endosteal callus formation indicated a maximum posttraumatic survival time of 12 days. In accordance, the presence of saw marks on the cut surfaces suggested that the amputation had not preceded death by more than 13 days (see Figure 2). The osteoporotic cortex noted in lesion Kvr13 was attributed to disuse related to lesion Kvr19, and thus was not taken into account as a time indicator.

Figure 1.



Malunion in a fractured right ulna (lesion C29). A smoothened callus contour without union of the fracture ends suggested pseudoarthrosis development. Although gross anatomical analysis (above) indicated ongoing corticalisation of the fracture ends, plain X-rays (below) showed no fracture end sclerosis. Therefore the lesion was not interpreted as a completely stabilized pseudoarthrosis.

Figure 2.



The presence of saw marks on these amputation surfaces indicated a posttraumatic survival time of maximally 13 days. Note the gradual shift in the angle at which the bone was sawn.

Discussion

Although the small sample size and possible selection bias did not allow for conclusions on a population level, the assessment of posttraumatic survival time in the Koeningsberg sample deepened our understanding of the health conditions in Napoleon's army during the 1812 field campaign.

In fractures, a substantial amount of posttraumatic survival time was noted. All lesions showed a posttraumatic survival of at least 15 days. Even, almost 50 percent of the individuals, showed a posttraumatic survival time of at least two months (see Table 5). Assuming that all studied fractures were battlefield injuries, our results support intentional systematic care such as by the accessibility of adequate evacuation from the battlefield and medical care on-the-spot. This would be in line with several published reconstructions of the actual medical services during the field campaign (e.g. Wellin, 2010). In two of the three found major traumas of the lower leg (e.g. E1-6), posttraumatic survival time assessment showed that, despite transportation problems such as the increasing shortage of horses (Von Clausewitz, 1996), disabled soldiers were able to keep up with the retreat of the 'Armee' to Koeningsberg. The success of medical care was furthermore suggested by lesions that showed a lack of osteoporosis in the injured extremity, as this indicated continuous functional use of the related limb. The number of fractures with posttraumatic survival times of at least two months implied that soldiers could cope with injuries inflicted during or prior to the encampment in Moscow, and thus throughout the retreat to Koeningsberg. In this context it is interesting to note that none of the fractures with a minimum posttraumatic survival time under two months were lower-limb related lesions.

Table 5. Posttraumatic survival time per lesion.

Lesion no.	Posttraumatic time interval
C11-30a	21 days – 3 months
C11-30b	21 days – 3 months
C29	More than 2 months
E1-6	More than 2 months
Gvr14	15 days – 3 months
Gvr15	15 days – 3 months
HS50	15 days – 3 months
K9-107	More than 2 months
Kvr19	More than 15 days
X27	More than 2 months
Ivr5	0 days – 12 days
Kvr13	0 days – 12 days

In contrast to the fractures, the two amputations showed no healing features and thus, maximum posttraumatic survival time was set at twelve days. Apparently, they had been carried out in the final days of the retreat. This conclusion is in line with historical reports stating that Larrey constantly tried to provide medical care throughout the retreat (Welling, 2013). The short time laps between the amputations and death seemed also to point to a direct relationship between the two. This should not come as a surprise, as it has been reported that mortality rates at the Battle of Waterloo in 1815 were 70 to 80% (Seymour, 2002). Other contemporary reports presented mortality rates between 49 and 92% (McCallum, 2008). Larrey, known for his amputation skills, produced mortality rates of (only) approximately 35% during a campaign in Saxony (Kirk, 1944). Nevertheless, the bad pre-surgery condition of the retreating soldiers might have raised the mortality rates in Koeningsberg.

Theoretically, in case of lesions with a relative long posttraumatic survival time, eventual death could very well be unrelated to the prior traumatic event. Several historical reports emphasized that the high mortality during the field campaign was primarily due to infectious diseases, such as typhus and dysentery (e.g. Allen, 1998; Conlon, 2006; Raoult *et al.*, 2006). Buzhilova *et al.* already suggested that infectious diseases played a major role in the death of the individuals in the Koeningsberg mass grave (Buzhilova *et al.*, 2009).

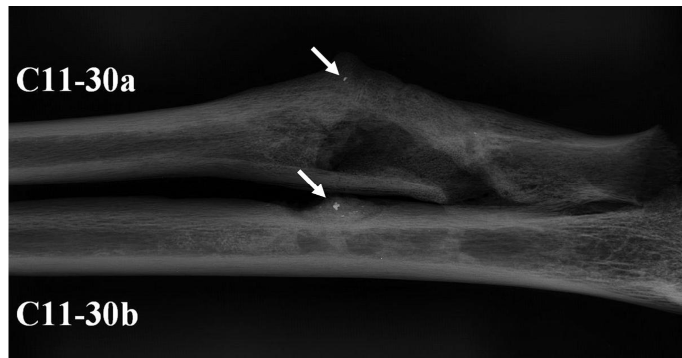
In 1944, Kirk reconstructed Larrey's indications for immediate amputation during the 1812 campaign. He believed them to be: 1) compound fractures and gunshot wounds of the long bones 2) intra-articular fractures or gunshot wounds 3) extensive loss of soft tissue parts with or without fracture or, 4) destruction of the main blood supply of a limb. Our results showed that these reconstructed instructions were not always followed. For instance, radiologic analysis of the fractured ulna and radius in one of the skeletons (lesions C11-30a and C11-30b) identified bullet fragments in the callus (Figure 3), but no amputation was carried out during the minimum of three weeks the individual survived. In lesion E1-6, also bullet fragments were noted, but again no amputation was done. The compound fracture of a humerus (Kvr19) was also not immediately amputated. Instead, the reactive bone formation indicated a secondary infection of the fracture end. An amputation was only performed at least 15 days after initial trauma. Such findings also seem to contradict the assertion from contemporary military surgeon Blackadder that Larrey suffered from 'amputation mania' (Wangensteen and Wangensteen, 1978). Perhaps circumstances forced Larrey to postpone the indicated amputation. Or perhaps the traumatic lesion was initially not considered to be life threatening, but the subsequent infection was.

Several historical reports emphasized the extreme speed by which Larrey performed his amputations (e.g. Kirk, 1944; Pearce, 2002; Welling and Rich, 2013).

Part of his amputation methodology was detectable in lesion Kvr19. The radiographs showed that the saw cut stopped at approximately 85% of the bone diameter (Figure 4). Time was probably saved by breaking the remaining cortical continuity, after which the soft tissues could be severed.

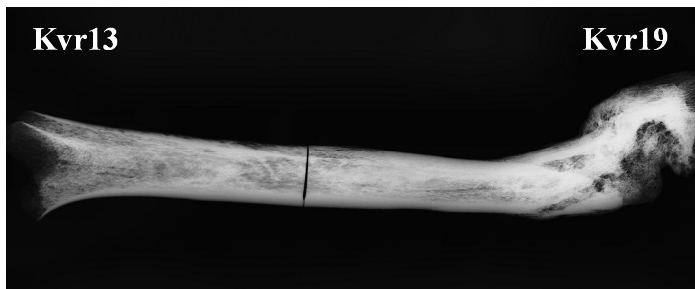
Finally a few technicalities should be discussed. It was noted that the CT images improved the visibility of osteoporosis, although they did not alter conclusions on final posttraumatic survival times. Further, detection of endosteal callus (healing

Figure 4.



Plain X-ray of bullet fragments in a fracture of the right forearm. In the callus of this fracture radiodense particles, bullet fragments (arrows), were noted. The bullet shattered the ulna (above), while only the cortical surface of the adjacent radius was damaged. Both lesions clearly show an external and internal callus.

Figure 5.



Kvr 19: the abundant reactive bone formation at the fracture at the distal humerus indicated the presence of a secondary infection. The callus formation and the osteoporotic aspect of the cortex indicated a minimum posttraumatic survival time of 15 days prior to amputation. Kvr 13: the amputation saw cut in the shaft of this humerus only transversed approximately 85% of the cortex diameter. The remaining bone had been broken off, finalizing the amputation.

features no. 1 and 5) proved to be difficult to assess with CT if compared to plain radiography. This might be due to lack of tissue mass in the thin CT-scan slices. Nevertheless, although CT results stayed inconclusive in some cases (see Table 3 and 4), its use improved 'general' trauma analysis.

The above results showed that even without histological analysis, a fair estimation of the posttraumatic survival time can still be made. Though, from earlier studies we know that histology would have notably improved the results as it potentially could have differentiated between ante- or perimortem lesions (De Boer *et al.*, 2012; De Boer *et al.*, 2013). It might also have added accuracy to the assessment of the posttraumatic survival time in case of lesions with more 'advanced' healing features, for instance by addressing the presence and status of callus remodeling. Therefore, whenever possible, we advise to use gross anatomy, radiology and histology complementary.

Conclusion

During Napoleon's 1812 field campaign, (extensive) mechanical injuries showed not to be (immediately) lethal per se. The relative long posttraumatic survival time in case of a notable number of the lesions in the Koeningsberg sample suggested that at least some adequate battlefield evacuation and provision of (on-the-spot) medical care was available throughout the 1812 campaign. The presence of two amputations with a short posttraumatic survival time indicated that surgery was still carried out in the Koeningsberg lazarette during the final days of the military retreat. Furthermore our results allowed for some surmises on the cause of death of a few individuals. The use of Computed Tomography was helpful for general traumatological analysis, but did not effectively contribute to the estimation of posttraumatic survival times.

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Chapter 10

SUMMARY AND GENERAL DISCUSSION

Physical anthropology, forensic anthropology and palaeopathology

Physical anthropology (also known as biological anthropology), studies mankind as a biological species. As such it encompasses many academic disciplines in which human evolution and human anatomical variation play a central role. The research in this thesis focused on two subspecialties of physical anthropology, namely forensic anthropology and palaeopathology.

Forensic anthropology uses physical anthropological knowledge in a forensic context. In the majority of cases, this concerns the identification of human remains when advanced decomposition or cremation makes the material unfit for standard identification methods. Forensic anthropologists are often consulted by forensic pathologists and Scene Of Crime Officers (SOCOs) to analyze decomposed remains related to an unnatural death, for instance in case of mechanical trauma.

Palaeopathology is the study of disease in an archaeological context. As such it provides insight into the (public) health status of individuals and gives information on the development of diseases. Palaeopathologists often deal with remains in an advanced state of decomposition such as skeletons, mummies or cremated remains. Even though palaeopathology may also encompass animal diseases, this thesis concentrates on human material.

Although forensic anthropologists and palaeopathologists work in different contexts, they share a common ground. Both combine anatomical features of age at death, stature, sex and ancestry with features of disease, trauma and cause of death. Often, both have to deal with a general lack of background information, like missing medical data. As a result, forensic anthropologists and palaeopathologists are confronted with the same methodological and diagnostic challenges.

This thesis focuses on one of these issues, namely on the value of microscopical/histological analysis of skeletonized human remains, that is of so-called ‘dry bone’. To date, forensic anthropologists and palaeopathologists mainly use dry bone histology to distinguish human from animal material, to estimate the age at death or to evaluate the phase of decomposition. Apart from these applications, dry bone histology is not generally accepted as a reliable research tool. This thesis addresses the possibilities and impossibilities of dry bone histology.

Part 1: Technical aspects associated with dry bone histology

Chapter 1 introduces the technical difficulties that are associated with dry bone histology. After all, to make it suitable for analysis by light microscope, the material has to be processed into very thin, translucent sections. In case of 'fresh' material, the bone tissue is decalcified, embedded in paraffin or plastic and sectioned with a microtome. This approach is however unsuitable for dry bone material as it no longer contains sufficient bone protein, in particular collagen. To overcome this set-back, Maat introduced a simple, rapid and inexpensive production method for hand ground unstained sections (Maat *et al.*, 2001). Due to its robustness and reliability, this method was gradually applied by laboratories around the world (see for example Martiniakova *et al.*, 2006 or Turner-Walker and Mays, 2008).

Chapter 2 describes a valuable extension of the method of Maat, namely the application of histochemical staining. By means of a trial-and-error approach, we developed a protocol for staining with haematoxylin alone, and with haematoxylin and eosin. The use of these dyes improved the visibility of features that aid in the distinction between woven bone and lamellar bone. Also, it improved the visibility of the cement lines that line the Haversian systems. Furthermore, the visibility of the destruction of bone tissue by microbes was enhanced.

Although Maat's approach can also be used for the production of sections of fragile bone material, some consider it to be less suitable for this particular material (Beauchesne and Saunders, 2006). Prior to 2001, several researchers tried to circumvent this problem by immersing the bone tissue with resin (see e.g. Schultz, 1988). However, these methods proved to be time consuming, required expensive specialized equipment and could not be used in conjunction with histochemical staining.

A simple and inexpensive alternative is presented in **chapter 3**. In contrast to earlier approaches, the fragile bone tissue was not impregnated, but only surrounded/supported by resin. After curing, a slice with a thickness of approximately 1 mm was sawn off, which was then easily processed by hand into a section with a thickness of approximately 80 micrometres. In this way, the bone tissue at the surface of the slide was free of resin and thus remained suitable for histochemical staining. The slide could be examined before and after staining and the embedding did not limit the applicability of a polarizing filter. The bone tissue remained intact during the procedure, regardless of fragility and decomposition phase. The new method dramatically shortened the production process if compared with the existing methods, viz. from six weeks to two days for non-stained sections.

Part 2: The diagnostic value of dry bone histology

In 'fresh' bone material, a histological diagnosis is based on the cytonuclear, biochemical and architectural characteristics of bone fibres and surrounding soft tissues. By definition, these hallmarks are no longer available in dry bone. Consequently, the diagnosis of disorders in dry bone is problematic. As a result, the value of histological examination of dry bone remains a subject of ongoing discussion among palaeopathologists .

Chapter 4 adds depth to this discussion, as it presents an overview of all literature in which the diagnosis of bone disorders was at least partially based on dry bone histology. These diagnostic efforts were tested against the current knowledge on disease histomorphology. The results showed that only a limited number of disorders present a pathognomonic histomorphology in the remaining dry bone material. These disorders were osteoporosis, hyperparathyroidism, Paget's disease and possibly osteomalacia. In all other cases, such as tumours and infections, a definitive diagnosis could not be made on the histological analysis alone. In these cases the histological efforts only aided in the differential diagnostic process, for instance to reject or support a particular diagnosis.

For those who are unfamiliar with dry bone histology, **chapter 5** provides an accessible introduction in the histological aspects of the microscopic anatomy and dynamics of bone tissue. For a quick start, convenient methods for the production of sections of dry bone tissue are described. These methods are specifically suitable for those situations in which a fully equipped anatomy or pathology laboratory is not available. Furthermore it is shown how the continuous remodelling of bone tissue during life allows for a histological assessment of the age at death of an individual. The various parts of the skeleton on which this approach can be applied are introduced, together with the confounding influence of muscle activity during life and decomposition after death. Also, the necessary attention is given to the real, yet sometimes limited value of dry bone histology in the diagnosis of disorders.

How histology of dry bone material can be of use in an archaeological context is illustrated in **chapter 6**. Herein bone fragments from members of the famous and dramatic Franklin expedition of 1845 were analysed (Mays *et al.*, 2013). The expedition, which aimed to find a northern passage from the Atlantic to the Pacific Ocean, disappeared a few months after departure in the polar ice of northern Canada. Reconstructions suggested that the demise of the crew was probably due to scurvy. Macroscopic examination of skeletal elements of several expedition members seemed to confirm this. However, histological examination showed no signs of active scurvy.

The combination of these findings with a revision of several historical sources, showed that scurvy probably played no major role in the end of the expedition.

Part 3: The use of histology for the detection of features of mechanical injury in dry bone

The last part of this thesis describes the use of dry bone histology in the investigation of mechanical traumas, i.e. fractures and amputations. Traditionally, lesions are considered to be either antemortem (having occurred before death), or postmortem (having occurred after death). If a lesion can not be attributed to one of these domains, a lesion is usually classified as perimortem (i.e. inflicted 'around' death). In antemortem lesions, a further specification of the time span between the traumatic event and eventual death is important, as this 'posttraumatic survival time' can aid for instance to determine the relation between death and trauma. Since the healing processes of bone tissue follows a time-dependent and sequential pattern, determination of the healing phase can be used to estimate the length of the posttraumatic survival time.

In **chapter 7**, it was tested to what extent defined features of healing processes after bone traumas are still reliable detectable in dry bone material. For this, fractures and amputations from different healing phases were assessed microscopically and radiologically by three observers. The results showed sufficient consistency between the observations to justify the use of healing features to estimate posttraumatic survival time. This is an improvement to current practice, in which only a distinction is made between 'non-healing', 'healing' and 'healed' lesions. Determination of the posttraumatic survival time is of practical importance for forensic examinations, for instance in cases suspect of torture or child abuse.

Chapter 8 illustrates the usefulness of this approach when analysing fractures and amputations from an archaeological skeleton collection. It describes the examination of the remains of individuals exhumed from the Gladstone cemetery in Kimberley, South Africa (Van der Merwe *et al.*, 2010). At the end of 19th century, the related individuals worked in the diamond mines. The radiological and histological assessment of various posttraumatic survival times identified several interesting trends, such as the apparent medical importance of the first two weeks after trauma for the life expectancy of the wounded. In addition, earlier conclusions regarding medical care and causes of death, based on historical evidence only, could be confirmed. In one individual with multiple lesions, it was even possible to determine

the order in which the traumas had occurred. The radiological and histological analysis also aided in the differentiation between ante- and perimortem injuries.

Chapter 9 describes the assessment of posttraumatic survival time in the deceased soldiers of Napoleon's army, specifically of those who took part in the Russian campaign of 1812. These individuals were exhumed in 2006 in Kaliningrad, formerly Koeningsberg, the terminus of the for Napoleon disastrous campaign (Buzhilova *et al.*, 2009). Due to the historico-cultural value of this collection, invasive histological examination was not allowed. This was reason to extend the plain radiological examination with computed tomography (CT) scanning. The analyses showed that the sole application of gross anatomical and radiological analysis still allowed for a reasonable estimation of the posttraumatic survival time, be it less accurate than with additional histology. The results corroborated that at least some adequate historically documented battlefield evacuation and provision of (on-the-spot) medical care was available throughout the military campaign. In addition they provided insight into medical decision making with respect to amputations.

Conclusion

This thesis presents an easy, rapid and inexpensive supplement to the well-known method of Maat *et al.* (2001). This new method allows for the histochemical staining of dry bone material, enhancing the visibility of important hallmarks of dry bone histomorphology. In addition, this thesis provides a new, easy, rapid and inexpensive method for the production of sections of fragile dry bone tissue, histochemically stained or not.

Furthermore we show that dry bone histology is a valuable tool in the diagnosis of bone disorders. Although the majority of bone disorders have no pathognomonic dry bone histomorphology, histology may still have a considerable value for the differential diagnostic process. Finally, when studying mechanical trauma, dry bone histology is a valuable tool for the estimation of posttraumatic survival time, especially when used in conjunction with gross anatomical and radiological analyses.

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Hoofdstuk 11

SAMENVATTING EN ALGEMENE DISCUSSIE

Fysische antropologie, forensische antropologie en paleopathologie

Fysische antropologie, ook wel biologische antropologie genoemd, bestudeert de mens als biologische soort. Deze wetenschap omvat een groot aantal deelgebieden, waarin de evolutie van de mens en de menselijke anatomische variabiliteit een centrale rol spelen. Het onderzoek in dit proefschrift richtte zich op twee deelgebieden van de fysische antropologie, namelijk de forensische antropologie en de paleopathologie.

De forensische antropologie gebruikt fysisch antropologische kennis om forensische vraagstukken te beantwoorden. Meestal betreft dit identificatie-onderzoek van geskeletteerde of gecremeerde lichamen als de gebruikelijke identificatiemethoden niet meer uitvoerbaar zijn. Daarnaast worden forensisch antropologen regelmatig geconsulteerd door technisch rechercheurs en forensisch pathologen om de omstandigheden rond het overlijden van een individu te verduidelijken, bijvoorbeeld als er sprake is geweest van mechanisch letsel.

De paleopathologie houdt zich bezig met het diagnosticeren van ziekten in een archeologische context. Hierdoor wordt inzicht in de toenmalige (volks)gezondheidstoestand en informatie over de ontwikkeling van ziekten verkregen. Ook hierbij gaat het veelal om onderzoek aan lichamen in verregaande staat van ontbinding zoals skeletten, mummies of crematieresten. Hoewel de paleopathologie zich in principe ook bezighoudt met dierziekten, beperkt dit proefschrift zich tot menselijk materiaal.

Ook al verschilt de context waarin forensisch antropologen en paleopathologen werken, toch zijn er veel overeenkomsten in hun onderzoekswijzen. Beiden combineren anatomische kenmerken van leeftijd bij overlijden, lichaamslengte, geslacht en herkomst met kenmerken van ziekte, trauma en doodsoorzaak. Beiden werken veelal met (te) weinig achtergrondinformatie, bijvoorbeeld door het ontbreken van klinische gegevens. Bijgevolg staan forensisch antropologen en paleopathologen vaak voor dezelfde methodologische en diagnostische uitdagingen.

Dit proefschrift richt zich op een van die problemen, namelijk het nut van het microscopisch/histologisch onderzoek van geskeletteerde menselijke resten, het zogenoemd ‘droog botweefsel’. Tot op heden wordt droog botweefselhistologie door forensisch antropologen en paleopathologen voornamelijk gebruikt om onderscheid

te maken tussen menselijk en dierlijk materiaal, om een schatting te maken van de leeftijd bij overlijden en om de staat van ontbinding vast te stellen. Behalve ten aanzien van deze toepassingen wordt de microscopie niet algemeen geaccepteerd als een betrouwbaar onderzoeksinstrument. In dit proefschrift werden de mogelijkheden van droog botweefselhistologie nader onderzocht.

Deel 1: Technische aspecten van het histologisch onderzoek van droog botweefsel

Hoofdstuk 1 introduceert de technische problematiek rond het onderzoek van droog botweefsel. Immers, ten behoeve van microscopisch onderzoek moet het te bestuderen materiaal verwerkt worden tot zeer dunne coupes. De standaardmethode voor 'vers' botweefsel bestaat uit decalcificatie van het materiaal, inbedding in paraffine of kunststof en het vervaardigen van coupes met een microtoom. Deze benadering is echter niet geschikt voor geskeletteerd droog botmateriaal, omdat dit niet meer voldoende boteiwit, met name collageen, bevat. Om dit bezwaar te overkomen werd in 2001 een simpele, snelle en goedkope methode geïntroduceerd voor de productie van ongekleurde, handgeslepen coupes (Maat *et al.*, 2001). Door zijn robuustheid en betrouwbaarheid werd deze methode gaandeweg toegepast door laboratoria over de gehele wereld (zie bijvoorbeeld Martiniakova *et al.*, 2006; Turner-Walker en Mays, 2008).

Hoofdstuk 2 beschrijft een waardevolle uitbreiding van de 'methode Maat', namelijk de toepassing van histochemische kleuringen. Door middel van een trial-and-error onderzoek werd een protocol voor kleuring met haematoxyline en met haematoxyline én eosine ontwikkeld. Het gebruik van deze kleuringen verbeterde de zichtbaarheid van kenmerken die het onderscheid tussen weefbeen en lamellair botweefsel mogelijk maken en van de cementlijnen die de Haverse systemen omringen. Bovendien maakten zij destructie van botweefsel door microorganismen beter zichtbaar.

Hoewel de 'methode Maat' (2001) reeds snel coupes van fragiel materiaal kan produceren, wordt deze toch door sommigen minder geschikt geacht voor gecremeerd botmateriaal en trabeculair botweefsel (Beauchesne and Saunders, 2006). Vóór 2001 probeerde men dit probleem te omzeilen door gebruik te maken van een immersiemedium (zie bijvoorbeeld Schultz, 1988). Deze methode bleek echter tijdrovend, vereiste kostbare gespecialiseerde apparatuur en kon niet gebruikt worden in combinatie met histochemische kleuringen.

Hoofdstuk 3 presenteert hiervoor een eenvoudige en goedkope oplossing. Hierbij werd het botweefsel niet geïmpregneerd, maar omringd door inbedmedium. Na

uitharding werd een plak, met een dikte van circa 1 mm afgezaagd, die eenvoudig met de hand verder kon worden afgeslepen tot een dikte van ongeveer 80 micrometer. Op deze manier bleef het weefsel gelegen aan het oppervlak van de coupe geschikt voor histochemische kleuring. De coupe kon zowel voor als na kleuring en met of zonder polarisatiefilter worden onderzocht. De botweefselsamenhang bleef tijdens deze bewerking goed intact, onafhankelijk van de fragiliteit en van de staat van ontbinding van het bot. De inbedding beperkte de toepasbaarheid van een polarisatiefilter niet. Vergeleken met de bestaande inbeddingsmethoden verkortte de nieuwe methode het productieproces dramatisch, namelijk van zes weken naar twee dagen voor ongekleurde coupes.

Deel 2: De waarde van histologie voor het diagnosticeren van ziekten in droog botweefsel

In 'vers' botweefsel wordt een histologische diagnose gebaseerd op de cellulaire kenmerken van het botweefsel zelf en op die van de omringende 'weke delen'. In droog botweefsel zijn beiden niet meer aanwezig, waardoor de diagnostiek problematisch is. Vandaar dat de waarde van histologisch onderzoek van droog botweefsel een punt van discussie is onder paleopathologen.

In **hoofdstuk 4** wordt op deze controverse ingegaan. Hiervoor werd alle literatuur verzameld waarin de diagnostiek van botaandoeningen (mede) was gebaseerd op histologisch onderzoek van droog botweefsel. De gestelde diagnosen werden getoetst aan de huidige kennis omtrent de histomorfologie. Slechts bij een beperkt aantal aandoeningen bleek het resterende droge botweefsel een specifiek histologisch beeld te laten zien. Deze aandoeningen waren respectievelijk osteoporose, hyperparathyreoidie, de ziekte van Paget en mogelijk osteomalacie. In alle overige gevallen, zoals bij tumoren en infecties, kon een definitieve diagnose niet worden gesteld. In deze gevallen waren de histologische bevindingen wel van toegevoegde waarde voor het differentiaal diagnostisch proces, bijvoorbeeld voor het verwerpen van een diagnose of voor de ondersteuning van een waarschijnlijkheidsdiagnose.

Hoofdstuk 5 bespreekt enkele belangrijke histologische aspecten van de microscopische anatomie en de dynamiek van botweefsel voor diegene die daarmee niet vertrouwd zijn. Tevens wordt een aantal handzame methoden beschreven voor het vervaardigen van coupes van droog botweefsel, die ook uitvoerbaar zijn als de faciliteiten van een specifiek anatomisch/pathologisch laboratorium niet beschikbaar zijn. Om de dynamiek van het weefsel te illustreren wordt uiteengezet hoe de continue ombouw van botweefsel gedurende het leven een histologische bepaling van de leeftijd bij overlijden mogelijk maakt. De verschillende onderdelen van het

skelet waarop deze methode toepasbaar is worden besproken, alsmede de mogelijk verstorende invloed van spieractiviteit tijdens het leven en van ontbindingsprocessen na de dood. Ook wordt aandacht besteed aan de reële, maar beperkte waarde die de droog botweefselhistologie heeft voor het diagnosticeren van botaandoeningen.

De toegevoegde waarde van droog botweefselhistologie in een archeologische context wordt geïllustreerd aan de hand van een casus in **hoofdstuk 6**. Enkele botfragmenten, afkomstig van leden van de beroemde en dramatisch verlopen Franklin-expeditie van 1845 (Mays et al., 2013) werden hiervoor onderzocht. De expeditie, die tot doel had om een noordelijke doorvaarroute van de Atlantische Oceaan naar de Grote Oceaan te vinden, verdween enkele maanden na vertrek in het niet in het poolijs ten noorden van Canada. Reconstructies suggereerden dat scheurbuik hierbij een belangrijke rol had gespeeld. Macroscopisch onderzoek van skeletelementen van enkele later gevonden expeditieleden leek dit te bevestigen. Doch bij het in het kader van dit proefschrift verrichte histologisch onderzoek werden geen tekenen van recent opgetreden scheurbuik gevonden. De combinatie van deze bevinding met kennis uit historische bronnen, leidde tot de conclusie dat scheurbuik hoogstwaarschijnlijk geen rol heeft gespeeld bij de teloorgang van de expeditie.

Deel 3: De toepassing van droog botweefselhistologie bij de bestudering van mechanisch letsel

Het laatste deel van dit proefschrift beschrijft het gebruik van droog botweefselhistologie bij het onderzoek van mechanisch trauma, zoals dat optreedt in het geval van botbreuken en amputaties. In het forensisch antropologische of paleopathologische onderzoek van traumata wordt traditioneel onderscheid gemaakt tussen letsels van voor en na het overlijden, dat wil zeggen tussen zogenaamde ante- en postmortem letsels. Als geen onderscheid gemaakt kan worden, wordt een letsel geclassificeerd als perimortem (dus van 'rond' het overlijden). Bij antemortem letsels is een specificering van de tijdsduur tussen het ontstaan van het letsel en het optreden van de dood van belang. Deze 'posttraumatische overlevingstijd' kan bijvoorbeeld gebruikt worden om te beoordelen in hoeverre het overlijden verband houdt met het trauma. Omdat genezingsprocessen volgens een vast patroon verlopen, kan door bepaling van de aangetroffen genezingsfase de posttraumatische overlevingstijd worden geschat.

Hoofdstuk 7 beschrijft in hoeverre de genezingsprocessen die het gevolg zijn van botfracturen en amputaties waarneembaar zijn in droog botweefsel, en of zij te stageren zijn. Dit werd gedaan aan de hand van een verzameling botmonsters die

radiologisch en microscopisch door drie waarnemers werden geanalyseerd. De resultaten bleken voldoende consistent om het gebruik van botgenezingskenmerken voor het schatten van de posttraumatische overlevingstijd te rechtvaardigen. Dit is een verbetering ten opzichte van de huidige praktijk, waarbij meestal slechts onderscheid wordt gemaakt tussen 'niet genezende', 'genezende' en 'genezen' letsels. Vaststelling van de posttraumatische overlevingstijd is van praktisch belang voor het forensisch onderzoek, bijvoorbeeld bij verdenking op mishandeling voorafgaand aan de dood.

Hoofdstuk 8 illustreert de meerwaarde van deze benadering bij het onderzoek van traumata, aangetroffen in archeologisch skeletmateriaal. Het onderzoek betrof de overblijfselen van individuen opgegraven uit de Gladstone begraafplaats in Kimberley, Zuid-Afrika (Van der Merwe *et al.*, 2010). Aan het eind van 19^{e} eeuw werkten deze arbeiders in de diamantmijnen ter plekke. Door de bepaling van de posttraumatische overlevingstijd werden interessante trends zichtbaar. Zo bleken de eerste twee weken na het trauma voorspellend voor de levensverwachting. Ook werden eerdere, op historisch onderzoek gebaseerde conclusies met betrekking tot medische zorg en de vastgestelde doodsoorzaken bevestigd. In één individu met multipele letsels was het zelfs mogelijk om de volgorde waarin de letsels waren opgetreden vast te stellen. De methode bleek eveneens van waarde voor het maken van onderscheid tussen *ante*- en *perimortem* letsels.

Hoofdstuk 9 beschrijft de toepassing van paleopathologisch onderzoek van traumata bij soldaten van het leger van Napoleon, die deelnamen aan de Russische veldtocht in 1812. Deze individuen werden in 2006 opgegraven in Kaliningrad (voorheen Koeningsberg): het eindstation van de voor Napoleon desastreus verlopen campagne (Buzhilova *et al.*, 2009). Vanwege de historische/museale waarde van de menselijke resten, werd invasief histologisch onderzoek niet toegestaan. Radiologische onderzoek was wel geoorloofd en werd bovendien uitgebreid met computed tomography-scanning. De resultaten lieten zien dat bij toepassing van uitsluitend macroscopisch en radiologisch onderzoek een, weliswaar minder nauwkeurige, maar toch redelijke schatting van de posttraumatisch overlevingstijd mogelijk was. Zo konden ook in deze populatie verbanden tussen letsels en overlijden worden gelegd. Daarnaast werd inzicht verkregen in de militair geneeskundige besluitvorming omtrent amputaties.

Conclusie

In dit proefschrift wordt een technisch eenvoudige, snelle en goedkope aanvulling beschreven op de droog botweefselhistologie volgens de 'methode Maat' (Maat *et al*., 2001). Met behulp van deze methode kunnen zeer dunne botcoupes histochemisch gekleurd worden, waardoor het weefsel beter beoordeelbaar wordt. Voorts wordt een nieuwe, eenvoudige methode gepresenteerd, die het mogelijk maakt om ook fragiel droog botweefsel te bewerken tot al dan niet histochemisch gekleurde coupes voor histologisch onderzoek.

Aangetoond wordt, dat deze techniek in het geval van enkele botaandoeningen een histologische diagnose mogelijk maakt dan wel ondersteunt. Tenslotte blijkt dat bij de bestudering van mechanische traumata droog botweefselhistologie van waarde is om in combinatie met macroscopisch en radiologisch onderzoek een schatting te maken van de posttraumatische overlevingstijd.

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Appendices

I. List of publications by the author

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Lieve Vivianne, bedankt dat je er altijd voor me bent.

‘s Gravenhage, april 2014

III. Curriculum vitae

Hans de Boer was born on the 24th of August 1986 in Tynaarlo, The Netherlands. In 2004 he graduated from the Zernike College (pre-university secondary education/VWO, *'Voorbereidend Wetenschappelijk Onderwijs'*) in Haren, after which he left for Leiden to study medicine at the Leiden University Medical Center (LUMC).
During his first year of academic study (2004-2005) he became a teaching assistant at the dissection hall of the Anatomy Department, a position that he would keep to the end of his study in 2011. In 2006 he completed the summer course 'Introduction to Physical Anthropology'. After assisting in a PhD-project at the LUMC Department of Radiology, he started with physical anthropological research under supervision of prof.dr. G.J.R. Maat in January 2008.
During medical training, he attended the LUMC Honours Class and an extracurricular course on epidemiology. He was secretary of the executive board of the Leiden Medical Students Association (M.F.L.S., '*Medische Faculteit der Leidse Studenten'*), member of the student council of the LUMC and general board member of the Leiden University Fund (LUF, *'Leids Universiteits Fonds'*). He graduated *cum laude* as Medical Doctor (MD) in December 2011.
After spending a month at the Emergency Department of the Mulago Hospital in Kampala, Uganda, he worked as a 'resident not in training' (ANIOS, *'Arts-assistent Niet In Opleiding tot Specialist'*) at the General Surgery Department of the Medical Center Haaglanden (MCH) in The Hague. In January 2013, he started his pathology residency at the Academic Medical Center (AMC) in Amsterdam under supervision of prof.dr. M.J. van de Vijver.
Currently, he is vice-president of the Dutch Association for Physical Anthropology (NVFA, *'Nederlandse Vereniging voor Fysische Antropologie'*), board member of the Dutch Foundation for Anthropobiology and is a member of the physical anthropological research and teaching unit 'Barge's Anthropologica' in Leiden and Amsterdam.