Changes in human cortical bone due to thermal stress. An experimental histological approach.

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Author:	Ing. Tristan Krap
Studentnumber:	s1170635
Faculty:	Archaeology
University:	Leiden University
Specialisation:	Human Osteology and Funerary Archaeology
Supervisors:	Dr. A.L. Waters-Rist Drs. F.R.W. van de Goot
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A histological investigation should be conducted as a valuable addition to the macroscopic investigation of cremated remains.
- Prof. Dr. Bernd Herrmann (1977)

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1. Introduction

Burned human skeletal remains are often studied by physical anthropologists, especially in the field of forensics (Ubelaker 2008). The examination of human skeletal remains often deals with questions concerning the identity of the deceased, the vitality of the body before exposure to the fire, and the cause of death. But it can also be relevant to determine the duration and the amount of thermal stress that the skeletal remains have been subjected to (Bohnert *et al.* 1998). There are numerous events that lead to burned human remains. Modern situations include aircraft accidents, explosions, natural disasters, house fires, and in some cases fire is used for suicide or to cover up homicides (Shkrum *et al.* 1992; Valenzuela *et al.* 2000; Sledzik *et al.* 2002; Fairgrieve 2007; Blau *et al.* 2011). Archaeological examples include cultural mortuary practices or disposal of skeletal remains in a domestic cooking context (Baby 1954; Hanson *et al.* 2007).

As a humanistic discipline aimed at reconstructing the lifeways of people that lived in the past, the archaeologist often finds himself investigating skeletal remains often, because it can be the only biological tissue available (Renfrew *et al.* 2004). Skeletal remains contain much biophysical information about the humans or animals they once belonged to. The remains also contain crucial information about the way the bones have been treated and disposed of by people from the past (Jans 2005). Since fire provides light, heat, protection from predators, and means of cooking meat and plant materials, proving the usage of fire and cooking is an important piece of evidence in unravelling ancient behaviour (Hanson *et al.* 2007). In order to interpret the information from archaeological material correctly it is crucial to study the fundamental processes (Fagan 1993) such as the changes that the organic component of bone undergoes due to thermal stress.

Currently there is a distinction in the archaeological and physical anthropological record between cremated remains and inhumation (not cremated remains) (Oestigaard 2000; Koon *et al.* 2010). The last few decades researchers focussed mainly on the macroscopic heat induced changes of bones and teeth (Vark 1974; Dunlop 1978; Shipman *et al.* 1984; David 1990;

Walker *et al.* 2005; Devlin *et al.* 2008; Symes *et al.* 2008). Morphological analysis can indicate whether the remains are of a complete or incomplete cremation, but in combination with taphonomical processes and other environmental factors it can be difficult to differentiate between incomplete cremated, cooked and not cremated remains (Oestigaard 2000; Koon *et al.* 2003). Therefore, macroscopic analysis of burned skeletal remains is not always the best tool for temperature estimation (Thompson 2009). It is even possible that bones that have been exposed to a relatively low amount of thermal stress are considered to be inhumated remains because there are no macroscopic changes, or there is insufficient circumstantial evidence for such a scenario (Oestigaard 2000). However, histological analysis often gives more insight in to the pre-burial treatment of human remains (Herrmann 1977).

Although the morphological and histological changes of bone due to thermal stress have been under investigation for over a half century, many questions remain to be answered (Herrmann 1977; Thompson 2009). Is it possible to determine or estimate low temperatures based on histological changes within cortical bone? Is one of those questions. Morphologically this is a difficult task since taphonomic alterations can mimic discoloration (Shahack-Gross *et al.* 1997) but also previous research using histology found it difficult to detect changes at low temperatures (Hanson *et al.* 2007).

When investigating histological alterations due to thermal stress, the descriptions are mostly based on structural or colour changes of the inorganic component of bone (Shipman *et al.* 1984; Hanson *et al.* 2007). The first histological change begins at 185°C and is described as; *The bone surface becomes more irregular as small, granular asperities, separated from each other by tiny pores and fissures, appear. The bone surface remains intact and continuous* (Shipman *et al.* 1984). This histological change can be easily overlooked or seen as taphonomic since it is not until temperatures reach 285°C that characteristics develop that are not found in bone subjected to taphonomic events (Shipman 1981). Thus, changes at temperatures below 285°C are uncertain.

Therefore, it will be useful to further study histological changes of human bone tissue after it has been exposed to a relatively low amount of

thermal stress. Staining the organic component of bone may give more insight in to the structural changes bone undergoes when it is being exposed to heat because it is less resistant to stress than the inorganic component. It is possible that changes in the organic component can be used in distinguishing thermal stress at a lower temperature rather than using histology of only the inorganic component. The organic component of bone is less well-studied from an archaeological and anthropological point of view, but might prove to be very applicable for remains that are well-preserved, like remains found in a forensic context.

The following section gives insight into the development, morphology and histology of bone. It also provides a historic background and overview of the research that has already been done on the subject followed by the research questions. The next chapter will outline the materials and methods that will be used, the results, discussion and conclusions with recommendations for future research.

1.1 Bone growth, morphology, histology and remodelling.

Bone is produced by cells called osteoblasts and occurs by appositional growth on the surface of already existing bone, connective tissue or cartilage. Bone forms primarily during the embryonic development (Tate 2012). There are two main mechanisms for bone formation; intramembranous and endochondral. During intramembranous ossification, bone forms directly out of connective tissue. Only a few bones of the human skeleton are formed this way, mainly those of the skull. The mesenchymal cells, that are present onsite, can transform into osteoblasts by cellular signalling molecules. Mesencymal cells are multipotent stem cells that can develop in to specific connective tissue cells like osteoblasts. During endochondral ossification, the mesenchymal cells differentiate into cartilage, which is later replaced by bone. Both mechanisms of bone formation, which is the same as modelling, lead to the production of primary or woven bone. Primary bone will be remodelled in to mature or lamellar bone during life (Gilbert 2000; Tate 2012).

Once bone is formed by osteoblasts there are cells that maintain it, called osteocytes. Osteoblasts that become completely surrounded by bone become osteocytes. Osteocytes maintain themselves in small spaces within the bone

matrix called lacunae. The spaces that are used for the cell processes of the osteocytes are called canaliculi. The osteocytes play a major role in the remodelling process by targeting sites that need to be remodelled due to mechanical stress (Noble *et al.* 2000).

To remodel bone, the current structure has to be broken down; osteoclasts are responsible for this part. They produce an acidic environment that decalcifies the bone matrix, by producing hydrons (H^+). The osteoclasts also produce enzymes that are able to break down the protein component of the bone matrix (Gilbert 2000; Tate 2012).

Bone has to be remodelled for several reasons; the most important ones are adjusting the bone to the mechanical stress it is under, repairing fractures within the bone, and the uptake of calcium ions (Ca^{2+}). Calcium is critical for normal muscle and nervous system functions (Berchtold *et al.* 2000); bone is the largest storage site within the body for calcium. Bone remodelling always follows the same sequence: activation > resorption > formation. This alteration of bone is carried out by a complex arrangement of cells called the basic multicellular unit, a BMU, that exists out of; osteoclasts, mononuclear cells, and osteoblasts. The intracortical BMU's move nearly longitudinally through the long bone diaphysis, removing and replacing bone structural units. This process can be seen in figure 1. The BMU leaves a tunnel behind it that is called the haversian canal and has a diameter of approximately 250-300 μm (Gilbert 2000; Robling *et al.* 2008; Tate 2012).

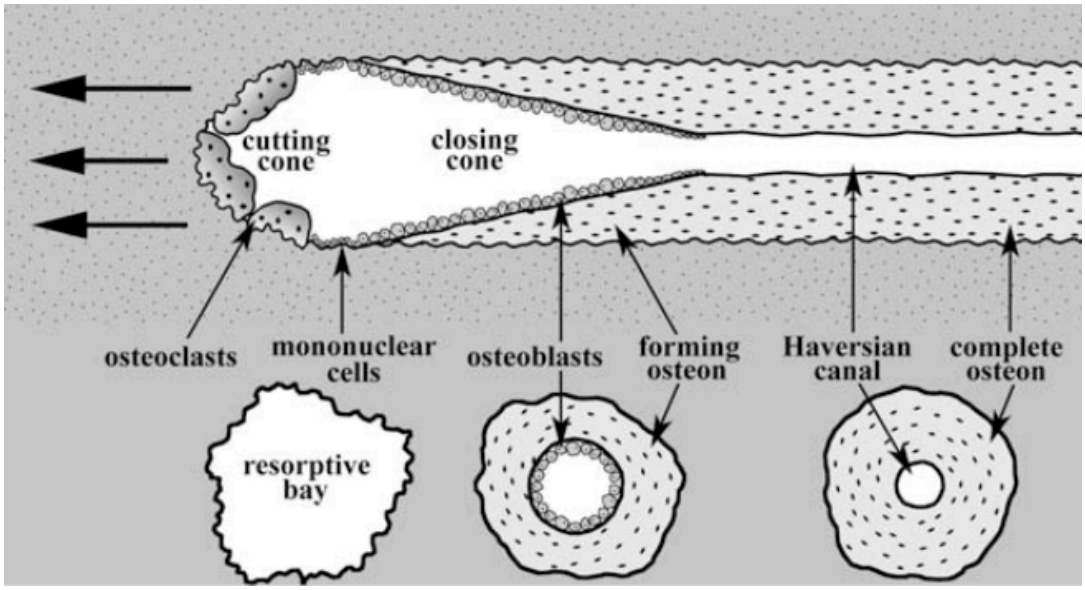

Figure 1. ***Top****: Longitudinal view of a BMU moving from right to left.* ***Bottom****: Transverse sections corresponding to the longitudinal view (Robling et al. 2008).*

Bone consists, by weight, out of approximately 35% organic and 65% inorganic material. The organic part of bone is primarily composed of type 1 collagen and the inorganic part out of calcium phosphate crystals called hydroxyapatite which has the formula $Ca_{10}(PO_4)_6(OH)_2$. The collagen in bone gives it its flexibility and the mineral component of bone gives it its strength (Tate 2012). Bones are classified by their outer shape, there are; long bones, short bones, flat bones, and irregular bones. Table 1 lists examples for each type of bone.

Table 1. Examples of types of bones.

Type of bone:	Examples:
Long bones	Humerus, femur, radius.
Short bones	Carpals, tarsals, patella.
Flat bones	Scapula, parietal, frontal.
Irregular bones	Sacrum, vertebrae, mandible.

The internal structure of the different types of bone is different as well; long bones exist out of cortical bone that serves as the exterior shell of the entire bone and is mainly present at the diaphysis, and cancellous bone that is present at the proximal and distal ends (see appendix A, figure 7 for an overview of the anatomical planes and directions). The diaphysis of long bones can have a medullary cavity which is a large central canal filled with bone marrow or adipose tissue. Short bones exist out of cancellous bone between outer layers of cortical bone, and they are as wide as they are long. Flat bones and irregular bones have the same internal structure as short bones (Tate 2012).

Cortical bone has the main purpose of supporting the organisms weight, protecting vital organs, and plays a major role in the storage and release of elements like calcium (Parfitt 2002). Cortical bone is composed out of different types of structures: concentric lamellae that surround an osteon, circumferential lamellae that extend around the outer surface of the bone, and interstitial lamellae between the osteons (see the transverse cross section of the lateral part of the diaphysis of the femur in figure 2). Cancellous bone has a lower density than cortical bone but a higher surface area. This bone tissue often houses red bone marrow and therefore can be very vascular. It consists

out of trabeculae that connect with each other (see the longitudinal cross section of the proximal end of the femur in figure 2) (Tate 2012).

All bones have a connective tissue membrane around the outer surface of the bone, called the periosteum, that consists of blood vessels and nerves on the outside and a single layer of bone cells, including osteoblasts and osteoclasts, on the inside. The internal surfaces of bones, the cavities like the medullary cavity, contain a layer called the endosteum that consists out of a single layer of cells like osteoblasts and osteoclasts (Tate 2012).

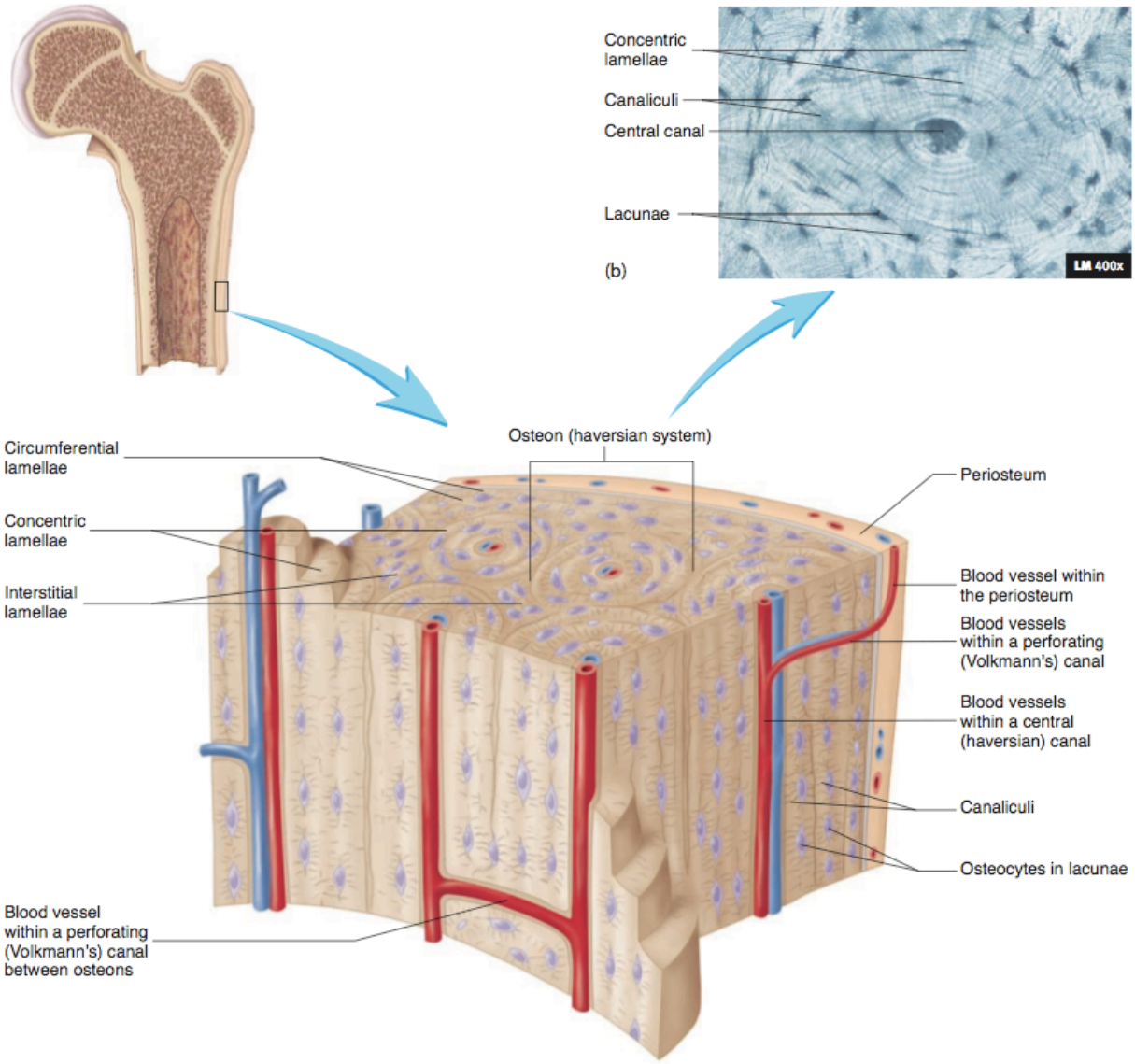

Figure 2. ***Upper left corner:*** *Longitudinal cross-section of the proximal right femur, visible are the cortical bone at the diaphysis and the trabeculae of the cancellous bone.* ***Bottom:*** *Transverse cross-section of the diaphysis of the right femur. From outside inwards; the perosteium, circumferential lamellae, the haversian system and the concentric and interstitial lamellae. There are also blood vessels that either use the canals created by the osteons or non-haversian canals.* ***Upper right corner:*** *a transverse cross-section of the femur, one osteon, made visible by light microscopy, magnitude 400x (Tate 2012).*

1.2 Histological analysis.

The study of histology involves microscopic analysis of cells and tissues. By quantifying the histological structures within the tissue it is possible to apply different statistical analysis upon the generated values. Histology was first recommended for usage by Sir Richard Owen, an anatomist and palaeontologist who lived from 1804 to 1892. The term histology stands for *histos* which means tissue and *logos* which means study (Dwight 1892; Hillier *et al.* 2007).

Histological analysis of bone involves the examination of thin slices of bone to assess the appearance and if possible to quantify the histological structures. Any method involving the quantification of the histomorphology is called histomorphometry. There are different histomorphometrical fields of interest, for example; age at death estimation, species determination, and the degree of diagenetic alteration (Hillier *et al.* 2007; Robling *et al.* 2008; Boer *et al.* 2010).

Using microscopy to analyse histomorphology proves is a powerful tool for physical anthropologists (Simmons 1985). There are multiple ways to visualize histological structures by microscopy but they are all of a destructive nature. Although newly developed methods, like synchrotron x-ray micro-tomography, can give the same resolution as conventional microscopy (Wildenschild *et al.* 2002), these methods are not yet widely applied within the field of physical anthropology due to the rarity and expense of the equipment. The ease and quality of microscopy makes it a highly used method. One of the most applied methods, which can be used for age estimation techniques, requires minimal preparation. A thin transverse slice of bone has to be ground down to 3mm or less, polished, and reflective light can be used to analyse the surface texture by microscopy. In most cases however it is necessary to use light that passes through the sample, which requires thinner sections of less than 80 μm to completely visualize the histological structures.

To analyse the organic component of bone, the thin slice first has to be stained. The main purpose of staining is to make different structures better visible and give the microscopic image more contrast. It is possible to stain transverse thin sections of less than 80 μm, but for a histological investigation

of the organic component of bone, it is desirable to first decalcify the sample. By decalcifying the bone sample it is possible to fixate it and make thin sections of 3 to 10μm. These thinner sections give a less compressed view of the sample and thus a clearer image of the organic structures.

Staining methods are widely applied in histopathology but are less commonly applied in physical anthropology. Many staining protocols are available, giving tissues different colours and thus making it possible to differentiate between different tissues. One of the most common staining methods is Hematoxylin & eosin (H&E), which stains the bone matrix pink, and the osteoclasts, osteoblasts, and collagen fibres purple (Ham *et al.* 1987; An *et al.* 2003), see figure 3 for an example. Other common stains for bone sections include Giemsa, Toluidine blue (often used for ground sections), methylene blue, basic fuchsin and Stains-All (An *et al.* 2003).

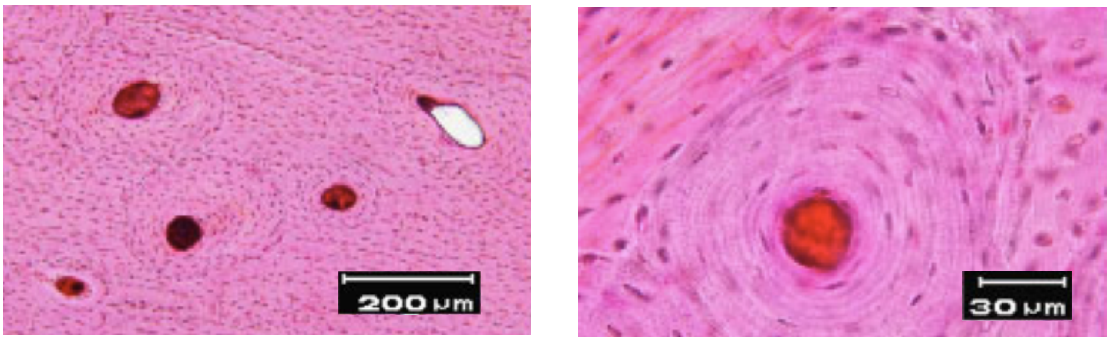

Figure 3. ***Left:*** *Micrograph of a transverse section of an undecalcified human femur, good preservation. The specimen is taken from the diaphysis. Staining: diluted haematoxylin solution and eosin. Bright field.* ***Right:*** *Micrograph of a detail of a transversal section of an undecalfied human femur, stained with the diluted hamatoxylin solution and eosin, good preservation. 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 Haversan system as well as of the interstitial bone is well distinguishable. Microphotographs and description from de Boer et al. (2010) page 5.*

Another way of microscopically visualizing the internal architecture of bone is by polarized light. Collagen fibres of differing orientation can be visualized by polarized light in transverse cut section of at least 80μm. Collagen fibres oriented transversely appear bright and the background dark (Bromage *et al.* 2003). Light is a wave phenomenon. One of its characteristics is its vibration direction, which is always perpendicular to the travel direction. Normal light is randomly polarized. That means the vibration direction of the

light is in all directions, 360° perpendicular to the travel direction. If the vibration is restricted to only one direction, it is referred to as plane polarized light. Light can become partially or totally polarized in a number of ways including reflection, adsorption, and by scattering through an anisotropic material (Bromage *et al.* 2003). Collagen fibres are an anisotropic material and cause a birefringence when light travels through them. When the light, which passes through the sample that causes birefringence, is linear (plane) polarized with an arrangement of two polarizing filters, one vibration direction is isolated and becomes visible (Bromage *et al.* 2003; Kubic *et al.* 2005; Boer *et al.* 2010), see figure 4 for an example. It is possible that changes, due to thermal stress for example, occur first in the orientation of the collagen fibres before they are completely destroyed and are therefore visible when using polarized light.

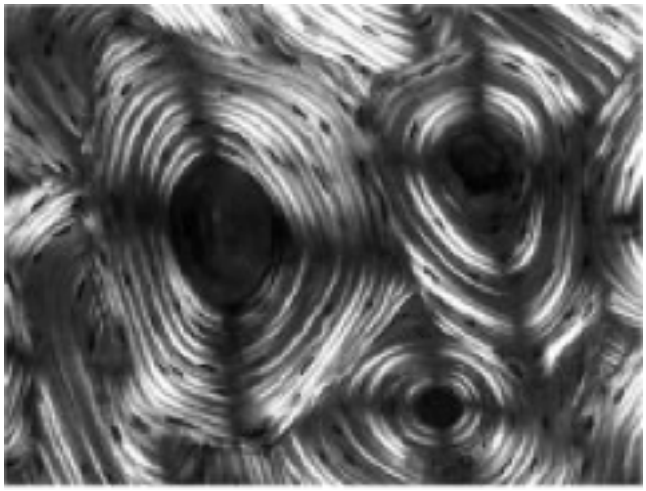

Figure 4. Osteons containing lamellae composed of collagen fibers that cause birefringement and appear bright against a dark background. Linear polarized light microscopy. Micrograph from Bromage et al. (2003) page 159.

1.3 Historical review of studies about burned human remains.

The first publications about burned human skeletal remains where case studies, mainly concerning basic physical anthropological analyses like sex, age and stature (Baby 1954; Buikstra *et al.* 1973; Bennett 1999). The morphological or histological changes that occurred became first of interest in the 1970's in a study by Van Vark (Vark 1974). He was the first to publish information about temperature related shrinkage of the bone dimensions (Ubelaker 2008). After Van Vark others followed with morphological and histological changes due to specific temperatures (Herrmann 1977; Shipman *et al.* 1984; Bennett 1999; Koon *et al.* 2003; Thompson 2009). Bones tend to shrink when exposed to thermal stress. Temperatures lower than 800°C and of minimal duration produce minimal shrinkage (Holland 1989).

Temperatures as low as 300°C can lead to loss of organic components such as proteins (Cattaneo *et al.* 1994). Holden et al. (1995) heated bone samples at selected temperatures in the range of 200-1600°C for periods of 2,12,18, and 24 hours and looked at both macro- and microscopic changes. The microscopic changes where observed by using a scanning electron microscope, which is an expensive method that makes studying tissue at a very strong magnification possible. They reported a progressive combustion of the organic portion of the bone tissue up to 400°C (Holden *et al.* 1995). It is unknown if this change is visible by using normal light microscopy and what happens between 200 and 400°C.

Other structural changes reported by Holden et al. (1995) include the beginning of recrystallization of the bone mineral at 600°C and melting of the bone mineral at 1600°C. Between 800°C and 1400°C new crystals appear with some crystal fusion above 1000°C (Holden *et al.* 1995). Sillen et al. found that char was produced between temperatures of 300°C and 500°C (Sillen *et al.* 1993; Ubelaker 2008).

The colour of bone that is affected by heating is a function of oxygen availability, duration, and temperature (Walker *et al.* 2005). The colour of bone cremated at temperatures as low as 200-300°C begins to change from ivory to dark brown or black. At higher temperatures ranging from 400-500°C, bone becomes black or dark grey due to carbonization. If the temperature rises above 600°C to 900°C the bone will become grey to grey-blue (Shipman *et al.* 1984; Correia 1997). When bone is being exposed to 800°C or more it becomes calcined, it has a chalky consistency, and the colour changes to white (Shipman *et al.* 1984; Walker *et al.* 2005). The full range of colour alterations can be present within one skeleton, but also on one bone fragment. This is certainly the case if soft tissue was present during the exposure to the fire or heat (Symes *et al.* 2008; Ubelaker 2008). In addition to the changing colour, heated bones also display cracking and longitudinal fractures (Correia 1997).

Contact between bones and environmental materials can result in a variety of colours being displayed on the surface of the bone. For example, the presence of copper produces a pink colour in cremated bones, iron a green colour and zinc a yellow colour (Dunlop 1978). But also the burial

environment alters the coloration of skeletal remains, also of heated or burned bones. The soil composition can differ greatly within a burial site thus altering the skeletal remains differently (Devlin *et al.* 2008).

1.4 Research questions

As was made clear in the introduction, further investigation of the organic part of human bone tissue is needed. Since the organic component is reported to burn away at 400°C (Holden *et al.* 1995), it is important to see what happens at and before 400°C. One of the main issues with burned skeletal remains is that the process of change is not only temperature related but also time dependent; therefore it is important to see how the two variables work in the process of altering. Is the alteration more dependant on time or temperature, or are the two variables inseparable? The earlier mentioned method of using circular polarized light microscopy to display the collagen fibres might reveal a specific marker whereby the thermal stress can be identified. This marker might be more specific in indicating the temperature or time that the sample has been exposed to. The central question of this study is what happens with the organic component of bone, at a histological level, when it is exposed to thermal stress? This study will determine if it is possible to quantify the changes, and if so is the alteration significantly dependent on temperature, time or both? The analysis will provide data about if there is a specific alteration that gives a stronger indication of the temperature the bone has been exposed to. It will also try to incorporate this specific alteration into the quantification method. Furthermore, it will also address the question if there is a significant difference between the alteration of the ulna and radius.

The conclusions that can be drawn from these results will contribute to fundamental understanding about the changes of the organic component of bone due to thermal stress. More generally it deals with the question of what happens to bone when it is heated.

2. Materials and methods

2.1 Materials; radii and ulnae.

The skeletal material comes from six embalmed cadavers from the dissection room of the AMC (Amsterdam Medical Centre) that was transported to the MCA (Medical Centre of Alkmaar). The age-at-death and sex of each cadaver is presented in table 2.

Table 2. Age-at-death and sex of cadaveric samples.

Number:	Age at death:	Sex:
085/2009	74	Female
052/2010	56	Male
085/2010	93	Female
091/2010	86	Female
110/2010	88	Female
118/2010	92	Female

The long bone diaphysis where removed from the cadavers by drs. F.R.W. van de Goot, (forensic) pathologist at Symbiant and general manager of the Centrum Forensic Pathology. The bones were defleshed by maceration at 80°C. The periosteum and most of the marrow in the medullary cavity were still present. The bones were kept frozen after they where removed from the mortal remains and fixated in formaldehyde 4%. From the available material only the diapysis from the radii and ulnae are used in this study.

The material was made available for research with approval of the medical ethics committee. Any remaining materials will be cremated at the MCA, following standard protocol for human anatomical material.

2.2 Methods and materials for applying thermal stress.

To apply thermal stress an apparatus is needed and therefore a medium to apply the thermal stress with has to be chosen. For this study the medium to apply thermal stress is plain air and, as a comparison, water. Any oven is suitable for the task of heating air but different ovens have different limitations. Electrical ovens, for household use, usually have a maximum temperature of around 275°C while a gas oven can reach temperatures above

1000°C depending on the gas-oxygen ratio and the type of gas that is used. Further, since water cannot reach a temperature higher than 100°C under normal pressure when boiling, any stockpot or pan is suitable.

For the lower temperatures an electrical oven with a range of 100-250°C and a precision of ±5°C is sufficient. For these experiments a Samsung combi-oven was used. The precision of the electrical oven was calculated by a calibrated infrared thermometer with a range of -50 to 350 ± 0.2°C. The temperatures above 250°C to 400°C are carried out by a gas oven that is heated by a mixture of butane and propane gas and has a thermocouple with a precision of ±10-20°C.

Thermal stress is not only dependent on temperature but also on time. When comparing samples it is important to keep one of the two dependent variables stable, otherwise the comparison is skewed. Both dependants will be taken in to account. In table 3 an example of the temperatures (and the related time) is displayed. The temperature range between 100°C and 300°C will be increased by steps of 50°C to be able to carefully study the alterations caused by both dependants. Since collagen is reported to be burned out of the bone at 400°C, that temperature is the maximum we will apply. We expect that after 20 minutes no collagen fibres are present anymore. It is very well possible that at 300°C already a lot of collagen has been destroyed, that is why the 350°C step is left out and at 300°C we will only apply thermal stress for the same amount of time as for 400°C for a proper comparison. Because of a limitation of the oven, 150°C is replaced by 160°C. See appendix C for the tables of applied thermal stress for each sample.

Table 3. The temperatures and related time the samples will be exposed to.

Temperature/Time:	10' *1	20'	30'	60'
0°C	I			
100°C	*II*	*III*	*IV*	
100°C Boiling		*V*		*VI*
160°C	*VII*	*VIII*	*IX*	*X*
200°C	*XI*	*XII*	*XIII*	*XIV*
250°C	*XV*	*XVI*	*XVII*	*XVIII*
300°C	*XIX*	*XX*		
400°C	*XXI*	*XXII*		

[1]: ' is a symbol for minute.

The chosen number of samples for both radius and ulna for heating by air is five, and two for heating by water.

In this experimental stage the bone samples that will be heated are transverse diaphyseal sections of approximately 2,5mm thickness. The samples will be heated in porcelain cups with a diameter of 5cm. The transverse sections were made with an IsoMet 4000, a low speed precision saw with water-cooling, from Buehler.

2.3 Haematoxylin and eosin staining.

Before the tissues can be stained for histological analysis they have to be pretreated. The first step is decalcification until only the organic component is left. The decalcification is done in a 5-10% hydrochloric acid (HCl) solution that is refreshed on a weakly basis; the samples have to be kept in this solution for several days, depending on the thickness of the sample. The equation between the hydroxyapatite and the protons from the acid is:

$$Ca_{10}(PO_4)_6(OH)_2 + 8H^+ \Leftrightarrow 10Ca^{+2} + 6HPO_4^{-2} + 2H_2O$$

The equation above shows the dissolution of the hydroxyapatite complex, $Ca_{10}(PO_4)_6(OH)_2$ by the protons from HCl. HCl ionizes completely (falls apart in to smaller fragments) in water by splitting in H^+ and Cl^-, forming H_3O in stead of H_2O, which is water. The H_3O wants to get rid of this H^+, which is called a proton, to become H_2O again and therefore it will react with the hydroxyapatite complex that wants to form H_2O and HPO_4^{-2} because these are, under the acidic conditions, more stable components. Afterwards the sample has to be thoroughly washed, in 70-50% ethanol, to make sure that there is no residual decalcifier solution left that might interfere with the next steps (Skinner 2003).

After washing the sample is fixated in formaldehyde and embedded in paraffin. The sample is then ready to be cut into thin slices of approximately 4-10 µm by using a microtome. These thin slices are attached to microscopic glass and the remaining paraffin has to be removed prior to staining. In table 4 the steps prior to staining are explained.

Table 4. Pretreatment of tissue sample prior to staining.

Step:	Material:	Further explanation:
Starting with a decalcified tissue sample.		
Dehydration.	Alcohol series Time: 10 min.	Ascending from 50% to nearly 100% purity. All of the water has to be removed prior to embedding in paraffin.
Removal of alcohol.	Xylene series Time: 10 min.	Ascending from 50% to nearly 100% purity. Because paraffin dissolves in alcohol the alcohol has to be completely removed.
Removal of xylene.	Paraffin baths.	Prior to embedding the xylene has to be removed otherwise there is the chance of incomplete embedding.
The tissue is now embedded in paraffin, ready to be cut into thin slices by using a microtome and can be attached to microscopic glass.		
Deparaffination.	Xylene series Time: 10 min.	Ascending from 50% to nearly 100% purity, to remove all the paraffin.
Removal of xylene.	Alcohol series Time: 10 min.	Ascending from 50% to nearly 100% purity, to remove all the xylene.

Now the tissue is clean and ready to be stained. The tissues will be stained by haematoxylin and eosin. Appendix D shows the materials that are needed in order to stain and table 5 shows the steps that have to be undertaken to obtain stained microscopic slides. It can be used for both decalcified and undecalcified bone samples. The staining is meant to give contrast, making it easier to recognize histological structures. In general, the bone matrix stains pink and the other cellular structures will stain purple or bluish (Jenkins *et al.* 2003).

Table 5. Method for staining with H&E.

Step:	Description:	Time:
1	Place the microscopic slides in distilled water.	
2	Stain with alum haematoxylin.	4 minutes
3	Rinse in tapwater.	
4	Stain with Acidic alcohol untill background is colourless.	
5	Rinse in distilled water.	
6	Stain with eosin.	2 minutes
7	Rinse in distilled water.	
8	Dehydrate using an alcohol series from 50% to 100% purity and cover the microscopic glass with a coverslip.	

2.4 Linear polarized light microscopy.

In order to visualize the histological slides and the collagen fibres by polarized light, a Leica microscope will be used: Leica DM 1000.

To visualize the birefringence of the sample by linear polarizing two elements have to be used. The first, called the polarizer, is placed between the light source and the sample. The second, called the analyzer, is positioned between the sample and the ocular or camera. This method is further referred to as linear polarized light microscopy or abbreviated as LPL.

The microscope is also equipped with a Leica camera, EC3, connected with a stand-alone computer. For the microphotography a program from Leica is used, called Leica Application Suite EZ.

2.5 Interpretation of histology.

In 1995, Hedges et al. introduced an index table to classify the internal structure of bone by histological investigation. By using a classification it is possible to quantify results and apply proper statistics. The index table, in table 6, consists of six classes based upon the amount of intact bone and polarisation characteristics of the collagen fibres still present.

Table 6. Altered classification of histological structures (after Hedges et al. 1995).

Index	Approx. % of intact bone.	Description:
0	< 5	No original features identifiable.
1	< 15	Strong discolouration of tissue. Structures are lost. Strong bands are present. LPL is not present.
2	< 33	Strong discolouration of tissue. Structures are lost. Strong bands are present. LPL is weak.
3	> 67	Discolouration of tissue, in general it is more pale. Bands are forming. LPL is still present but starts to fade.
4	> 85	Only minor discolouration, otherwise generally well preserved. LPL is bright.
5	> 95	Very well preserved, virtually indistinguishable from fresh bone. LPL is very bright.

Although the classification suggested by Hedges was originally intended for the diagenetic alteration of archaeological material it shows clear guidance for the interpretation of the histology of heated bone and quantifying the results.

Since the goal is to investigate histological changes due to thermal stress at different temperatures and times it is important to have an untreated sample, a 'zero', for comparison. The untreated sample will undergo all the same steps as the other samples except it will not be heated and therefore it will normally be scored as an index 5. If the zero is not scored as an index 5 the samples from that specific bone have to be excluded from the study.

2.6 Statistical analysis

The statistics that will be applied are a Levene's test to see if there is a difference between the two groups, which are the radius and ulna, and subsequently multiple paired Students t-Tests to investigate the difference due to the experiments. For ulna and radius the data is collected separately. All results given an index value are treated as ordinal data.

Levene's test

Levene's test assesses the assumption that variances of different groups from which different samples are drawn are equal, which is the null hypothesis. If the p-value of Levene's test is less than 0.05, the null hypothesis is rejected, which means that there is a significant difference between the variance of the samples of the two groups. In this study this concerns the question if the data can be grouped together. If there is no significant difference between the groups then this allows for two groups of five samples to be combined into one group of ten samples.

Paired Student's t-test

When using a paired t-test the means of the different groups will be compared with the untreated samples and with each other. The outcome of the t-test will show us if there is a significant different between the groups. The significance level is $\rho \leq 0.05$. For the correlated paired t-test the significance (ρ) has to be lower than 0.05, which means that there is at least a 95% confidence of a real difference in means between the groups. First a t-test will be carried out comparing the heated with unheated samples. Then a t-test is carried out to see if there is a significant difference between different times at a steady temperature.. The last t-test involves a comparison between every possible combination of groups, excluding the untreated samples.

3. Results

This chapter will start with a thorough description of the histology of the samples at each temperature compared with the untreated samples. None of the samples where excluded on the basis of a poor untreated sample. After the described histology the statistical test will be conducted. For the scoring tables see appendix E, and for the micrographs of the samples that correspond to the different indexes from table six see appendix F.

3.1 Description of the histological image.

Zero

All of the untreated samples the matrix is bright pink and the organic structures are clearly visible. The circular collagen structures surrounding the osteons become very bright when using the polarising filters. Figure 8.a and 8.b from appendix F display this clearly.

Boiling

Boiling for twenty or sixty minutes does not affect the organic component of bone; the histological and polarized images are the same as the untreated samples.

100°C

The amounts of stress at ten, twenty and thirty minutes do not look different than the untreated sample. This corresponds to the results from boiling. The visibility of the collagen fibres by polarized light appears to be normal as well.

160°C

The samples that have been heated to 160°C still look the same as the untreated sample, although at thirty minutes the matrix is less bright pink and a little bit pale, going from index five to four. The collagen fibres still show up clear and bright when using polarized light.

200°C

There is time dependent degradation of tissue that displays as increasing paleness of the pink matrix. If ten minutes of thermal stress is compared with the normal there is a slight discoloration, index four. When comparing thirty minutes of thermal stress with the normal there is a slightly stronger discoloration. There are also irregular dark bands forming from twenty minutes onwards (see figure 5). The amount of fibres that become visible when using LPL is slightly less intense if one compares it with the normal, after thirty minutes it starts to fade completely, index two to one.

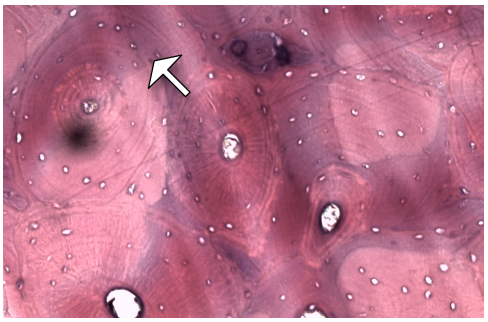

Figure 5. 52-2010-L-X, sample heated at 200°C for twenty minutes. There are dark irregular bands forming, arrow. 10x magnification, cropped.

250°C

After ten minutes of heating there is a slight to medium discoloration when compared with the untreated samples. When comparing thirty minutes of thermal stress with the normal there is a strong discoloration. After ten minutes of thermal stress at 250°C the collagen fibres are still visible but after twenty minutes they are no longer visible, index 3 to 4. The quality of the samples after thirty minutes is poor; there is loss of coherence. The index ranges from class two to zero. After twenty minutes the histology is similar to that of 200°C after twenty minutes, although the organic component of the heated samples at 250°C is less well preserved.

300°C

The colour of the samples, after being heated for ten and twenty minutes, is slightly darker with more contrast when compared with the untreated samples. The earlier noted paleness from 160°C onward is not present in these samples. Interestingly, there appears to be a particular fragmenting pattern resulting in loss of coherence, there are a lot of fragments, but the collagen fibres do light up within these fragments when using polarized light, in some samples even after twenty minutes of being heated.

400°C

There is not much histology left after ten minutes, the fragments that are visible are much darker than the untreated samples. The same fragmented patterns are found as at 300°C. The transversally orientated collagen fibres still show up when using the polarizers. After twenty minutes nothing is left anymore except for some dark spots.

3.2 Statistical analysis

For both the Levene's test, and the Student's paired t-test the statistical formula cannot be calculated if the standard deviation is zero, which is the case for multiple groups, see appendix E. This means for the Levene's test that there is no variance between the two groups, radius and ulna.

According to the Levene's test the null hypothesis is not rejected for any of the groups since the significance is higher than 0.05 in all cases, see table 7. Although the F-value is relatively high in four cases; 200°C/20', 200°C/30', 250/20', and 300°C/20' the variance is not significant. The outcome of the data suggests that the groups can be brought together for the Student's t-test since the variance is considered to be equal.

Table 7. Results from the Levene's test.

		Levene's Test for Equality of Variances	
		F	Sig.
R_160_30	Equal variances assumed	.000	1.000
R_200_20	Equal variances assumed	1.756	.222
R_200_30	Equal variances assumed	1.524	.252
R_250_10	Equal variances assumed	.000	1.000
R_250_20	Equal variances assumed	.590	.464
R_250_30	Equal variances assumed	1.756	.222
R_300_10	Equal variances assumed	.000	1.000
R_300_20	Equal variances assumed	1.969	.198
R_400_10	Equal variances assumed	.000	1.000

There is a significant change between the untreated samples and the samples heated at 200°C for 20’ and 30’, 250°C for 10’ until 30’, 300°C 10’ and 20’, and 400°C for 10’. Since 400°C for 20’ has a standard deviation of zero and also an average score of zero the significant difference is at its maximum. See table 8 for the results of the paired t-test between the unheated samples and the heated samples.

Table 8. Paired Student’s t-test between unheated and heated samples.

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 6	R_0_0 - R_160_30	.200	.422	.133	-.102	.502	1.500	9	.168
Pair 8	R_0_0 - R_200_20	2.400	.699	.221	1.900	2.900	10.85	9	.000
Pair 9	R_0_0 - R_200_30	3.600	.516	.163	3.231	3.969	22.04	9	.000
Pair 10	R_0_0 - R_250_10	1.800	.789	.249	1.236	2.364	7.216	9	.000
Pair 11	R_0_0 - R_250_20	3.100	.738	.233	2.572	3.628	13.28	9	.000
Pair 12	R_0_0 - R_250_30	3.600	.699	.221	3.100	4.100	16.28	9	.000
Pair 13	R_0_0 - R_300_10	2.500	.527	.167	2.123	2.877	15.00	9	.000
Pair 14	R_0_0 - R_300_20	3.500	.707	.224	2.994	4.006	15.65	9	.000
Pair 15	R_0_0 - R_400_10	4.400	.516	.163	4.031	4.769	26.94	9	.000

When taking a closer look at time as the dependant factor there is a significant difference between 200°C for 10’ and 20’, 10’ and 30’, and 20’ and 30’. Prior to this temperature there was a difference between the groups at 160°C but this difference proved not to be significant. For the temperature of 250°C the same applies as for 200°C, there is a significant difference between 10’ and 20’, 10’ and 30’, and 20’ and 30’. There is also a significant difference between 10’ and 20’ at both 300°C and 400°C. See table 9 for the results of the Stundent’s t-test with time as the dependent factor.

When comparing the different temperatures at a pinned time the earliest significant change occurs between 100°C and 250°C after ten minutes. Another early significant change is found between 100°C and 200°C at twenty minutes.

Table 9. Paired Student's t-test with time as the dependent factor.

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 5	R_160_10 - R_160_30	.200	.422	.133	-.102	.502	1.50	9	.168
Pair 6	R_160_20 - R_160_30	.200	.422	.133	-.102	.502	1.50	9	.168
Pair 7	R_200_10 - R_200_20	1.400	.699	.221	.900	1.900	6.33	9	.000
Pair 8	R_200_10 - R_200_30	2.600	.516	.163	2.231	2.969	15.9	9	.000
Pair 9	R_200_20 - R_200_30	1.200	.422	.133	.898	1.502	9.00	9	.000
Pair 10	R_250_10 - R_250_20	1.300	.675	.213	.817	1.783	6.09	9	.000
Pair 11	R_250_10 - R_250_30	1.800	1.033	.327	1.061	2.539	5.51	9	.000
Pair 12	R_250_20 - R_250_30	.500	.527	.167	.123	.877	3.00	9	.015
Pair 13	R_300_10 - R_300_20	1.000	.943	.298	.326	1.674	3.35	9	.008
Pair 14	R_400_10 - R_400_20	.600	.516	.163	.231	.969	3.67	9	.005

There is even a change noticed between 100°C and 160°C after thirty minutes, however this change is not statistically significant. When increasing the temperature at thirty minutes from 160°C to 200°C the change becomes significant.

At 200°C all temperature increases seem to be significant but one, an increase from 200°C to 250°C at thirty minutes seems not to be significant, meaning the damage has already been done after twenty minutes. The same holds for an increase from 250°C to 300°C at twenty minutes, while a temperature increase of 50°C is significant at ten minutes. Interestingly, an increase from 250°C to 400°C at twenty minutes is significant. Going from 300°C to 400°C at ten and twenty minutes results in a significant change as well.

As was noted earlier in this chapter, the samples heated at 300°C for ten and twenty minutes showed better preserved collagen structures than those that where heated at 250°C. This probably explains the insignificant change between 250°C and 300°C at twenty minutes.

Some pairs have been excluded from table 10, which shows the results from the Student's t-test comparing samples at different temperatures at a fixed time. This is due to the fact that absolute scores provide a standard deviation of zero, which makes a comparison incalculable.

Table 10. Student's t-test, comparing samples at different temperatures at a fixed time.

		Paired Differences					t	df	Sig. (2-tailed)
					95% Confidence Interval of the Difference				
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper			
Pair 3	R_100_10 - R_250_10	1.800	.789	.249	1.236	2.364	7.21	9	.000
Pair 4	R_100_10 - R_300_10	2.500	.527	.167	2.123	2.877	15.0	9	.000
Pair 5	R_100_10 - R_400_10	4.400	.516	.163	4.031	4.769	26.9	9	.000
Pair 7	R_100_20 - R_200_20	2.400	.699	.221	1.900	2.900	10.8	9	.000
Pair 8	R_100_20 - R_250_20	3.100	.738	.233	2.572	3.628	13.2	9	.000
Pair 9	R_100_20 - R_300_20	3.500	.707	.224	2.994	4.006	15.6	9	.000
Pair 11	R_100_30 - R_160_30	.200	.422	.133	-.102	.502	1.50	9	.168
Pair 12	R_100_30 - R_200_30	3.600	.516	.163	3.231	3.969	22.0	9	.000
Pair 13	R_100_30 - R_250_30	3.600	.699	.221	3.100	4.100	16.2	9	.000
Pair 15	R_160_10 - R_250_10	1.800	.789	.249	1.236	2.364	7.21	9	.000
Pair 16	R_160_10 - R_300_10	2.500	.527	.167	2.123	2.877	15.0	9	.000
Pair 17	R_160_10 - R_400_10	4.400	.516	.163	4.031	4.769	26.9	9	.000
Pair 18	R_160_20 - R_200_20	2.400	.699	.221	1.900	2.900	10.8	9	.000
Pair 19	R_160_20 - R_250_20	3.100	.738	.233	2.572	3.628	13.2	9	.000
Pair 20	R_160_20 - R_300_20	3.500	.707	.224	2.994	4.006	15.6	9	.000
Pair 22	R_160_30 - R_200_30	3.400	.843	.267	2.797	4.003	12.7	9	.000
Pair 23	R_160_30 - R_250_30	3.400	.966	.306	2.709	4.091	11.1	9	.000
Pair 24	R_200_10 - R_250_10	.800	.789	.249	.236	1.364	3.20	9	.011
Pair 25	R_200_10 - R_300_10	1.500	.527	.167	1.123	1.877	9.00	9	.000
Pair 26	R_200_10 - R_400_10	3.400	.516	.163	3.031	3.769	20.8	9	.000
Pair 27	R_200_20 - R_250_20	.700	.483	.153	.354	1.046	4.58	9	.001
Pair 28	R_200_20 - R_300_20	1.100	1.197	.379	.244	1.956	2.90	9	.017
Pair 29	R_200_20 - R_400_20	2.600	.699	.221	2.100	3.100	11.7	9	.000
Pair 30	R_200_30 - R_250_30	.000	.471	.149	-.337	.337	.000	9	1.000
Pair 31	R_250_10 - R_300_10	.700	.675	.213	.217	1.183	3.28	9	.010
Pair 32	R_250_10 - R_400_10	2.600	.516	.163	2.231	2.969	15.9	9	.000
Pair 33	R_250_20 - R_300_20	.400	1.350	.427	-.566	1.366	.937	9	.373
Pair 34	R_250_20 - R_400_20	1.900	.738	.233	1.372	2.428	8.14	9	.000
Pair 35	R_300_10 - R_400_10	1.900	.568	.180	1.494	2.306	10.5	9	.000
Pair 36	R_300_20 - R_400_20	1.500	.707	.224	.994	2.006	6.70	9	.000

4. Discussion

4.1 Interpretation of the results.

There is no significant difference in between temperatures up to 160°C for 30 minutes when compared with an untreated sample using this method. This corresponds to others that have found no significant change in bone structure or composition below temperatures of 200°C for a relatively short duration (Vark 1974; Shipman *et al.* 1984; Roberts *et al.* 2002). Roberts et al. (2002) already investigated the histological changes bone undergoes when exposed to boiling, from 3 hours up to 81 hours, and reported no changes. But they only looked at the inorganic component of bone. They did report an increase in porosity and decrease in nitrogen content, suggesting structural changes including changes within the organic component of bone (Roberts *et al.* 2002). Further increasing the time that the tissue is exposed to 100°C may lead to histological changes in the organic component of bone.

The results imply that both time and temperature are responsible for the changes within the organic component of bone. A prolonged duration of thermal stress leads to the same change as noticed at a shorter duration but higher temperature. The results also revealed that increasing the time has a bigger impact upon the destruction of the organic component than increasing the temperature (with a step of 50°C). This makes determining a temperature that bones have been exposed to, at these lower temperatures and by this method, rather difficult, if not impossible. It is possible to differentiate between temperatures, based on both the histology and the birefringence. Between 200°C and 250°C it is difficult to differentiate. Although the samples that have been exposed to 250°C are slightly darker than those that are exposed to 200°C the general histology after twenty minutes is too similar in colour and the lack of birefringence of the collagen structures (see figures 13 and 14 in appendix F). Between 250°C and 300°C there is a difference worth investigating in future research, the fragments at 300°C are clearly defined with sharp edges while those at 200°C and 250°C are rounder and there is still some coherence between the fragments.

Taking into account that both time and temperature are correlated to the destruction of the organic component of bone, one important result is that there are almost no recognisable structures left after the bones have been heated at 400°C for twenty minutes. This result corresponds to the results Holden et al. (1995) reported, but adds time as an important dependent since a small amount of structures are visible after being exposed to the heat for only ten minutes (see figure 15 in appendix F).

Another result that might be useful in determining the amount of thermal stress bones have been exposed to is the birefringement of the collagen structures. These results suggest that a prolonged duration of heat at a low temperature results in loss of this characteristic. Combining both the histological view and the polarized light view might prove to be a useful tool for determining time and temperature that bones, recovered in both archaeological and forensic casework, have been exposed to.

That the collagen structures lose their birefringent characteristic after being exposed to thermal stress has not been reported yet. It seems to be a logical step prior to complete destruction, since exposure to temperatures above 190°C leads to complete denaturation of collagen (Wang *et al.* 2001). Therefore, it is interesting to see that the birefringence is better preserved at 300°C after twenty minutes when compared with 250°C after twenty and thirty minutes. A possible explanation for this effect might be the carbonization of the surrounding tissue, as is reported by Sillen et al. (1993) and clearly morphologically visible when examining the samples prior to the pretreatment. The char might absorb some of the heat and provide a structural framework that contains the collagen fibers. Therefore, the morphological aspect, including colour, has to be taken in to account when investigating samples of unknown origin.

4.2 The possibilities for this method in casework

Although much research remains to be done on this topic, it is possible that this method will prove useful in investigating skeletal samples that show evidence of being heated or that come from a context suggesting that they have been heated. For forensic casework this method might be usable since the

samples will be relatively recent, providing histology that has minimal alterations due to secondary external factors such as taphonomic processes.. For archaeological casework taphonomic processes might interfere with the histology. However, De Boer et al. (2010) have shown that it is possible to successfully stain human dry bone with heamatoxylin and eosin, dating from the 13th to 17th century that were excavated at the end of the 20th century, for histological analysis. They found it was possible to identify taphonomic changes of bone and therefore it might be possible to differentiate between the taphonomic and the low temperature alterations based on bone histology (Boer *et al.* 2010). The birefringence of the collagen within the samples De Boer et al. used was still bright, which is a solid indication that it is possible to use this method on archaeological samples.

Another potential for this method is to show that samples have not been heated but might have been discoloured by influences from the environment mimicking characteristics of heated bones. If the histology is preserved well, staining by haematoxylin and eosin shows the organic component of bone is still present, and the polarized light view is bright, then the bones have probably not been heated to temperatures above 200°C. In this way the method is able to answer questions in both archaeological and forensic casework.

Although staining methods appear to be difficult to apply or to interpret, the opposite is true. Most hospitals are capable of processing bone tissue, and the interpretation is not very different from standard histomorphometrical analysis that is often used in physical anthropological research. The staining protocol used in this study can be applied on thicker, not decalcified, transverse sections as well, which makes it even less complicated.

4.3 Limitations of the study

The method using thin slices of 4 um was chosen because this is one way of studying the changes of the organic component of bone on a histological level. During the interpretation process it became clear that there is a significant change in the characteristic birefringence of the collagen structures surrounding the osteons. Unfortunately, the chosen method is not

optimal for studying the birefringence of collagen structures, since the thickness of the sample influences the intensity of the birefringence (Junqueira *et al.* 1982). As well, the use of linear polarized light presents a limitation to the interpretation of the histology, because a phenomenon called "Malthese Crosses" occurs. These form because the analyzer and polarizer filter transmission axes lie outside the vibration plane of light passing through circulair lamellae at these positions (Bromage *et al.* 2003). See figure 6 for an example of a Malthese cross. These Malthese crosses become a serious problem when scoring samples that have been heated at higher temperatures, since the birefringent characteristic starts to fade at 200°C. A circular polarizing filter offers a solution (Bromage *et al.* 2003). Another solution is a microscope table that can rotate.

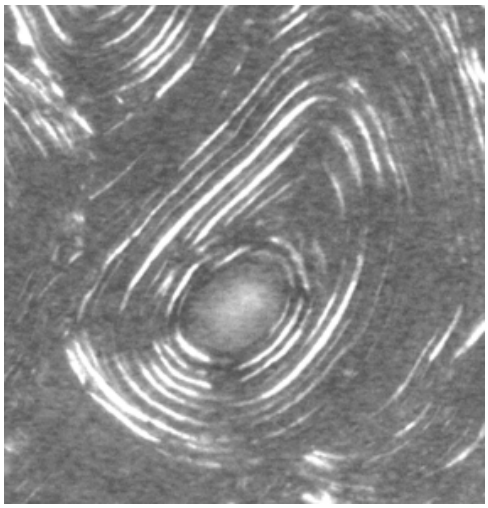

Figure 6. Example of a 'Malthese Cross' in sample 118-2010-K-II. Unheated sample, 10x magnification, cropped, linear polarized light.

Another limitation of the method becomes clear at higher temperatures. The samples are demineralized and thus the bone matrix is lost resulting in loss of context for the remaining organic structures. Carefully comparing it with the untreated sample might not be enough, there is even a chance that organic structures where washed away in the demineralization process or during the embedding in paraffin.

All specimens used in this study are considered to be old adults. This leads to highly remodelled cortical bone with a lot of osteons and circular lamellae. Thus, significant changes due to thermal stress found in this study only apply for highly remodelled cortical bone from old adults. What happens with bones from young adults or even from infants when they are exposed to the same amount of thermal stress is unknown. It is possible that bones of other age groups with less remodelling behave differently under thermal stress.

The sample composition of this study is 80% female and 20% male. The general pattern of long bone adaptation with age includes endosteal

resorption and periosteal apposition of bone tissue. Thus, bone diameter increases, but cortical thickness decreases and thereby the amount of visible osteons decreases as well (Ruff *et al.* 1988; Bouxsein *et al.* 1994). Some studies indicate that both men and women exhibit this redistribution pattern (Smith *et al.* 1964; Ruff *et al.* 1983; Ruff *et al.* 1988), but others report that women undergo geometric changes that lead to decreased bone strength (Garn *et al.* 1967; Martin *et al.* 1980). The cross-sectional geometry of the lower limbs has been studied by several research groups (Smith *et al.* 1964; Ruff *et al.* 1983), but the cross-sectional geometry of the forearm only by a few (Bouxsein *et al.* 1994). According to Bouxsein et al. (1994) the ulna of females does undergo this redistribution of bone, only in the radius it is less pronounced and not significant. Although sexual dimorphism and the just noted difference of bone redistribution does not play a big role in this study, since it is about comparing samples from before and after the application of heat, it is important to realize that the data represents mostly a female population instead of an equally divided population.

The samples that where exposed to thermal stress in this study were all defleshed. It is known that flesh covering bones provides protection against high temperatures. It serves as a buffer for as long as there is organic mass (Haan *et al.* 2001). During a cremation the radius and ulna are reported to start defleshing after twenty minutes, at temperatures ranging from 670°C to 800°C (Bohnert *et al.* 1998). Once the soft tissue has been removed, the bones are exposed directly to the heat. The temperatures the skeletal elements are exposed to before the soft tissue is burned away is unknown but probably lie between 100-500°C. The results from this study are therefore only an indication of the changes resulting from thermal stress upon defleshed bones but can be compared with samples taken from whole body-parts (radii and ulnae) that have been exposed to known amounts of thermal stress.

The last limitation that has to be mentioned it that this method is of a destructive nature. Taking a cross section of a sample is for forensic cases not a real limitation, except if the material is very scarce. However, for archaeological material it might be a problem because of the rarity or exclusiveness of the material.

Conclusion

Based on examining over two hundred microscopic slides this thesis found that, the organic component of bone undergoes several alterations when exposed to thermal stress. First the tissue becomes paler, looses its birefringent characteristic, and looses coherence. At higher temperatures the tissue becomes darker and the birefringent characteristic is better preserved. These alterations are quantifiable by the proposed index table, which is an altered version of Hedges index that is widely applied to score histological preservation.

Both time and temperature play a significant role in the alteration of the organic component of bone based on the quantified data. This makes determination of a specific temperature difficult when examining archaeological or forensic samples. The first significant change starts at 200°C after twenty minutes and there is a specific alteration, the birefringent characteristic of the collagen fibers within the lamellae, that gives a strong indication that a sample has been heated to 200°C or to 250°C. Time plays a significant role at temperatures from 200°C onwards. The temperature dependent is significant from 200°C onwards although there is no significant difference between 200°C and 250°C after thirty minutes, and 250°C and 300°C after twenty minutes. This method can differentiate between temperatures lower than 250°C or higher than 300°C but it is difficult to distinguish between 200°C and 250°C.

After improving and increasing the sample size, and further improving the proposed index table, this method might prove to be useful when analyzing both forensic and archaeological samples. When examining archaeological samples taphonomic alterations have to be taken into account but in many cases these changes will not interfere with the specific alterations due to the induced heat. By using this method it is possible to answer the question if skeletal elements have been exposed to, and to what degree of, thermal stress.

Future research

This method has certain limitations that could be improved by, for example, using thicker slides of approximately 80 μm. Also improving the number of cadaveric samples and sexual distribution is necessary for generating more reliable data. When the previous steps generate data that can be used with the data presented in this study it is possible to start with a whole cadaver experiment to study the difference between fleshed and defleshed bone.

From the results it became clear that there is a difference in the structure of the tissue at that has been exposed to 250°C and 300°C; at 250°C there is slightly more coherence and the edges of the remaining tissue are round while at 300°C there is less coherence between the fragments and the edges are sharp. This characteristic can be incorporated within the index table. Because of this transition area future examined intervals should be closer together, for example 250°C-275°C-300°C. Further it would be interesting to see what happens between 160°C and 200°C since there is a change noticeable at 160°C after thirty minutes, perhaps there is a already a significant change at 180°C after for example twenty minutes.

To be able to use this method in archaeological or in forensic casework on skeletal material that has been subjected to taphonomic processes, it is important to study the effect of taphonomy upon the organic component of bone that has been altered due to thermal stress. This can be done by the same methodology as is used in this study by adding one extra sample group to each group. This sample group will be exposed to diagenetic processes after being heated. In this way, one can compare the heated sample with the not heated sample and than compare the heated sample that is exposed to diagenesis with both.

In general, the organic component of bone deserves more attention. More studies should be conducted on the changes of the organic and the inorganic components of bone; they can give more insight in changes due to thermal stress and due to taphonomy.

Abstract

Estimating the amount and duration of heat that bones have been exposed to is a difficult task, but can be an important question for both forensic and archaeological casework. Temperature related changes can be estimated by histology. For this method most researchers focus on the changes to the inorganic component of bone, because the organic component is often not as well preserved. However, it has proved difficult to estimate lower temperatures based on the inorganic component of bone. Therefore, the organic component of bone should be analysed to determine if it is possible to estimate temperatures below 400 °C.

To answer the question if histological analysis of the organic component of bone can be used to estimates temperatures below 400°C, two hundred transverse sections of six dissection room cadavers, composed of both the radius and ulna, where heated to temperatures ranging from 100°C to 400°C at three different time intervals; ten, twenty, and thirty minutes. The transverse bone sections where then processed into histological slides for microscopic analysis.

The results showed that both time and temperature are significant dependents for the alterations that bone undergoes when it is exposed to thermal stress. Interestingly, time seems to play a bigger role, than temperature, in alterations in the organic component by heat. By using histology to examine the organic component of bone it is possible to differentiate between temperatures ranging between 200°C and 400°C but it was difficult to distinguish between 200°C and 250°C after twenty minutes. The most discriminative feature is the birefringence of the collagen structures, which is greatly reduced when time is extended.

By combing the histological view and the birefringence of the collagen structures it might be possible to estimate temperature and time in the range of 200°C to 400°C. Most important, by using this method it may be possible to exclude samples that, based on colour, have been suggested to be exposed to heat. This method can improve the toolbox of physical anthropologists by answering difficult questions in both archaeological and forensic cases.

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Appendix

Appendix A. Overview of anatomical planes and directions.

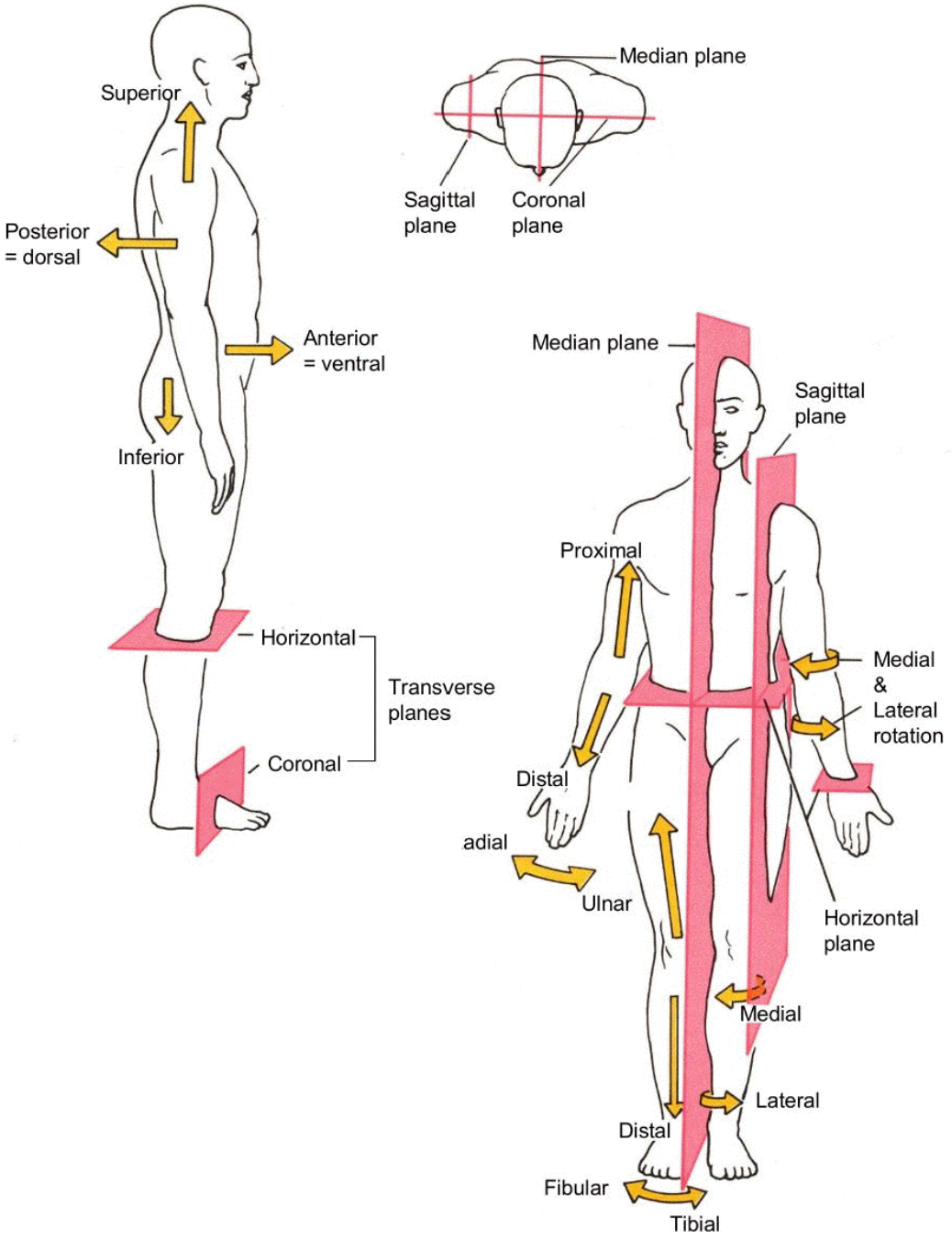


Figure 7. Anatomical planes and directions. Image from Seattle Central Educational Centre.

Appendix B. Paragraph concerning approval of ethics committee

Since the material has been made available for use at the dissection room of the Amsterdam Medical Centre there is already approval to use the cadaveric material for research. The material was removed by and under supervision of a medical doctor, Drs F.R.W. van de Goot, in accordance with the law on corpse delivery (in Dutch 'De wet op Lijkbezoring') that applies since 7 March 1991. The deceased gave permission for dissection in accordance with article 18 paragraph one, 19 and 67. In accordance with standard procedure the remains will be disposed of by cremation after analysis. This research was conducted under supervision of a medical doctor; Drs. F.R.W. van de Goot.

Date: ……..

Name: ……

Signature:

……..

Appendix C. Tables of applied thermal stress

Table 11. Temperature, time and sample numbers for radius.

Temperature	Time	Sample number:				**Radius**
		85-2009	*52-2010*	*85-2010*	*91-2010*	*118-2010*
0°C	0'	i – I	d – I	k – I	h – I	k – II
100°C	10'	i – II	d – II	k – II	h – II	k – III
100°C	20'	i – III	d – III	k – III	h – III	k – V
100°C	30'	i – IV	d – IV	k – IV	h – IV	k – VI
100°C *	20'	I – XIX	-	-	-	k – VII
100°C *	60'	i – V	-	-	-	k – XXIII
160°C	10'	i – XVI	d – V	k – V	h – V	k – XX
160°C	20'	i – XVII	d – VI	k – VI	h – VI	k – XXI
160°C	30'	i – XVIII	d – VII	k – VII	h – VII	k – XXII
200°C	10'	i – VI	d – VIII	k – VIII	h – VIII	k – VIII
200°C	20'	i – VII	d – IX	k – IX	h – IX	k – X
200°C	30'	i – VIII	d – X	k – X	h – X	k – XI
250°C	10'	i – IX	d – XI	k – XI	h – XI	k – XII
250°C	20'	i – X	d – XII	k – XII	h – XII	k – XIV
250°C	30'	i – XI	d – XIII	k – XIII	h – XIII	k – XV
300°C	10'	i – XII	d – XIV	k – XIV	h – XIV	k – XVI
300°C	20'	i – XIII	d – XV	k – XV	h – XV	k – XVII
400°C	10'	i – XIV	d – XVI	k – XVI	h – XVI	k – XVIII
400°C	20'	i – XV	d – XVII	k – XVII	h - XVII	k – XIX

Table 12. Temperature, time and sample numbers for ulna.

Temperature	Time	Sample number:				**Ulna**
		85-2009	*52-2010*	*85-2010*	*91-2010*	*110-2010*
0°C	0'	f – I	L – II	L – I	L – I	f – I
100°C	10'	f – II	L – III	L – II	L – II	f – II
100°C	20'	f – III	L – V	L – III	L – III	f – III
100°C	30'	f – IV	L – VI	L – IV	L – IV	f – IV
100°C *	20'	-	L – VII	L - XIX	-	-
100°C *	60'	-	L – XXIII	L - V	-	-
160°C	10'	f – V	L – XX	L - XVI	L – V	f – V
160°C	20'	f – VI	L – XXI	L - XVII	L – VI	f – VI
160°C	30'	f – VII	L – XXII	L - XVIII	L – VII	f – VII
200°C	10'	f – VIII	L – VIII	L - VI	L – VIII	f – VIII
200°C	20'	f – IX	L – X	L - VII	L – IX	f – IX
200°C	30'	f – X	L – XI	L - VIII	L – X	f – X
250°C	10'	f – XI	L – XII	L - IX	L – XI	f – XI
250°C	20'	f – XII	L – XIV	L - X	L – XII	f – XII
250°C	30'	f – XIII	L – XV	L - XI	L – XIII	f – XIII
300°C	10'	f – XVI	L – XVI	L - XII	L – XIV	f – XIV
300°C	20'	f – XVII	L – XVII	L - XIII	L – XV	f – XV
400°C	10'	f – XVIII	L – XVIII	L - XIV	L – XVI	f – XVI
400°C	20'	f – XIX	L – XIX	L - XV	L - XVII	f – XVII

**: Water instead of air as a medium to apply thermal stress.*

Appendix D. Composition of the staining fluids

Table 13. Materials needed for H&E staining.

Haematoxylin & Eosin staining - Solution 1: Alum haematoxylin.	
Aluminium ammonium sulfate	200 g
Haematoxylin (CL 75290)	20 g
95% Ethanol	40 mL
Sodium iodide	4 g
100% Acetic acid	80 mL
Glycerol	1200 mL
Distilled water	2800 mL
- Solution 2: Acidic alcohol 0,3%	
100% Ethanol	2800 mL
Distilled water	1200 mL
37% Hydrochloric acid	12 mL
- Solution 3: Distilled water	
- Solution 4: Alcoholic acidified eosin/phloxin	
1% Eosine Y (Cl 45380)	400 mL
1% (aq) Phloxin (CL 45405)	40 mL
95% Ethanol`	3100 mL
100% Acetic acid	16 mL

Appendix E. Table of generated data

Table 14. Index scores for the radius samples.

Temperature	Time	Sample number:				**Radius**
		85-2009	*52-2010*	*85-2010*	*91-2010*	*118-2010*
0°C	0'	5	5	5	5	5
100°C	10'	5	5	5	5	5
100°C	20'	5	5	5	5	5
100°C	30'	5	5	5	5	5
100°C *	20'	5	-	-	-	5
100°C *	60'	5	-	-	-	5
160°C	10'	5	5	5	5	5
160°C	20'	5	5	5	5	5
160°C	30'	4	5	5	5	4
200°C	10'	3	4	4	4	4
200°C	20'	2	2	2	3	4
200°C	30'	1	1	1	1	2
250°C	10'	3	2	4	3	4
250°C	20'	2	1	2	3	3
250°C	30'	1	1	1	2	2
300°C	10'	3	2	3	2	2
300°C	20'	0	2	1	0	0
400°C	10'	1	0	1	0	1
400°C	20'	0	0	0	0	0

Table 15. Index scores for the ulna samples.

Temperature	Time	Sample number:				**Ulna**
		85-2009	*52-2010*	*85-2010*	*91-2010*	*110-2010*
0°C	0'	5	5	5	5	5
100°C	10'	5	5	5	5	5
100°C	20'	5	5	5	5	5
100°C	30'	5	5	5	5	5
100°C *	20'	-	5	5	-	-
100°C *	60'	-	5	5	-	-
160°C	10'	5	5	5	5	5
160°C	20'	5	5	5	5	5
160°C	30'	5	5	5	5	5
200°C	10'	4	4	4	4	4
200°C	20'	2	3	3	3	2
200°C	30'	1	2	2	2	1
250°C	10'	4	4	3	3	2
250°C	20'	1	3	2	2	1
250°C	30'	0	2	2	2	1
300°C	10'	3	3	3	2	2
300°C	20'	2	1	2	2	2
400°C	10'	1	1	0	1	0
400°C	20'	0	0	0	0	0

Appendix F: Photo catalogue

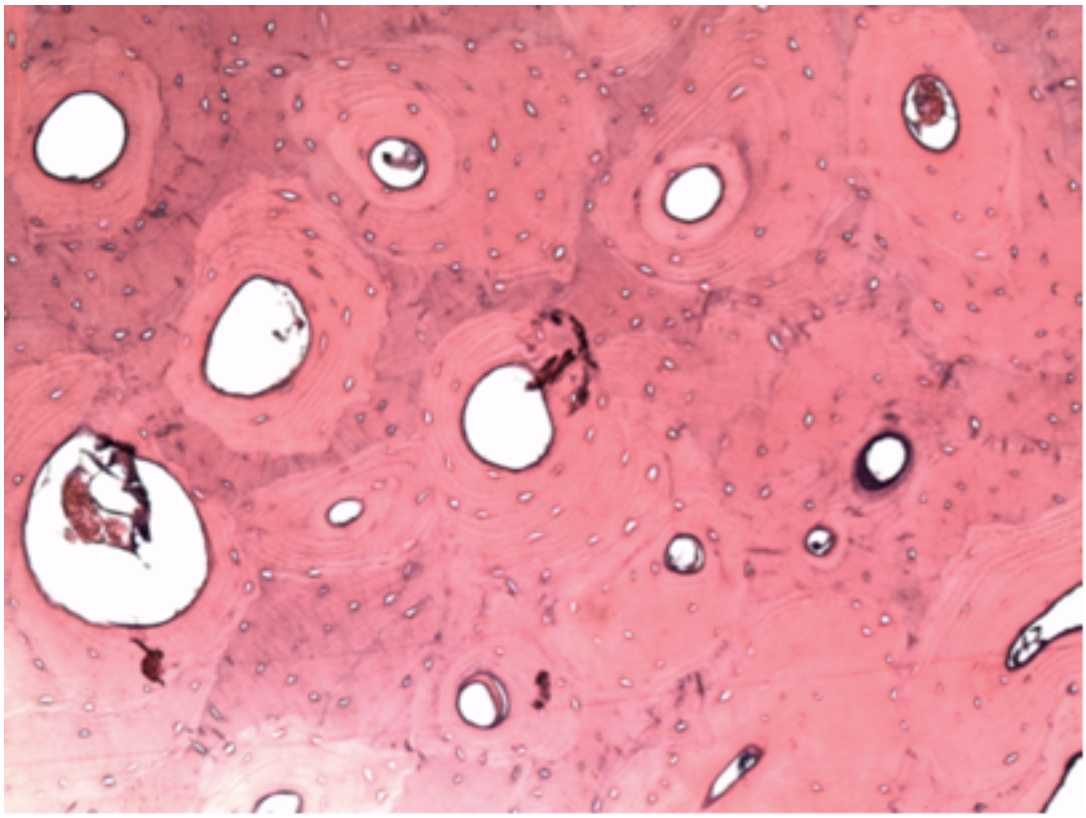


Figure 8.A. Micrograph of 118-2010-k-II. Unheated sample, H&E stained. Clear vision of the osteons, circulair lamellae, and also of the lacunae. 10x magnitude. Index 5.

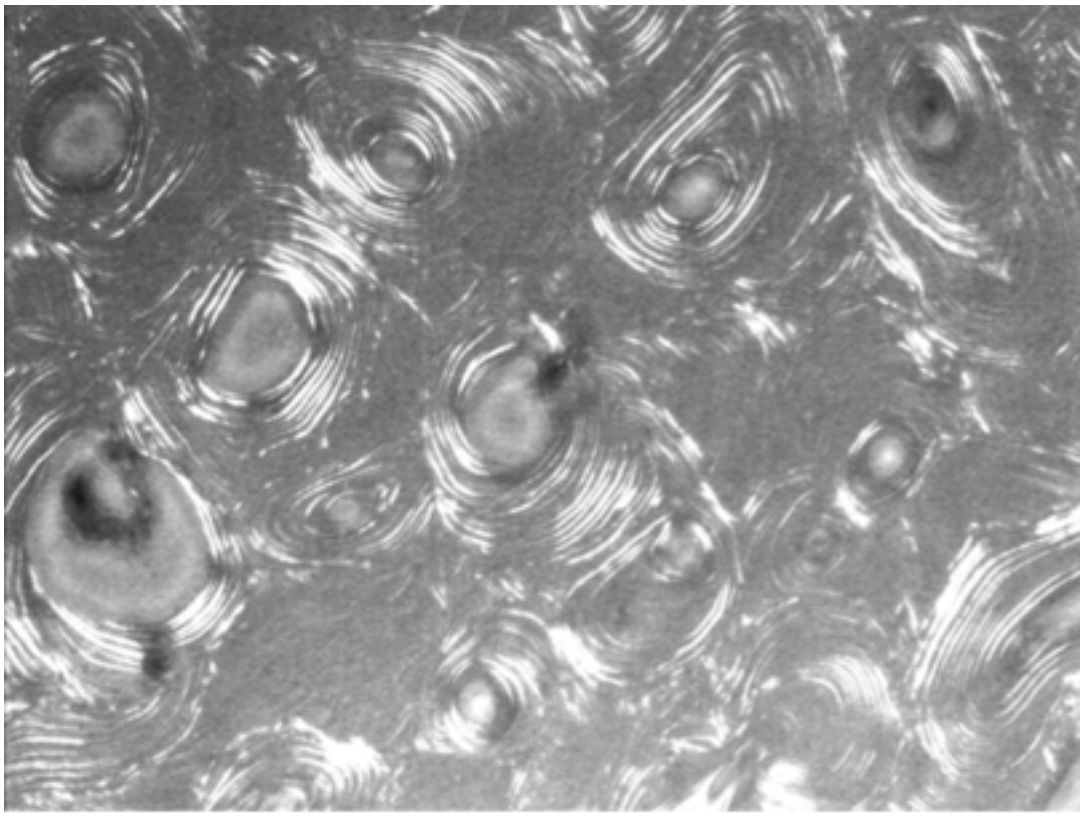


Figure 8.B. Micrograph of 118-2010-k-II. Unheated sample, LPL is bright. Clear vision of the osteons and the collagen fibres within the circulair lamellae. 10x magnitude. Index 5.

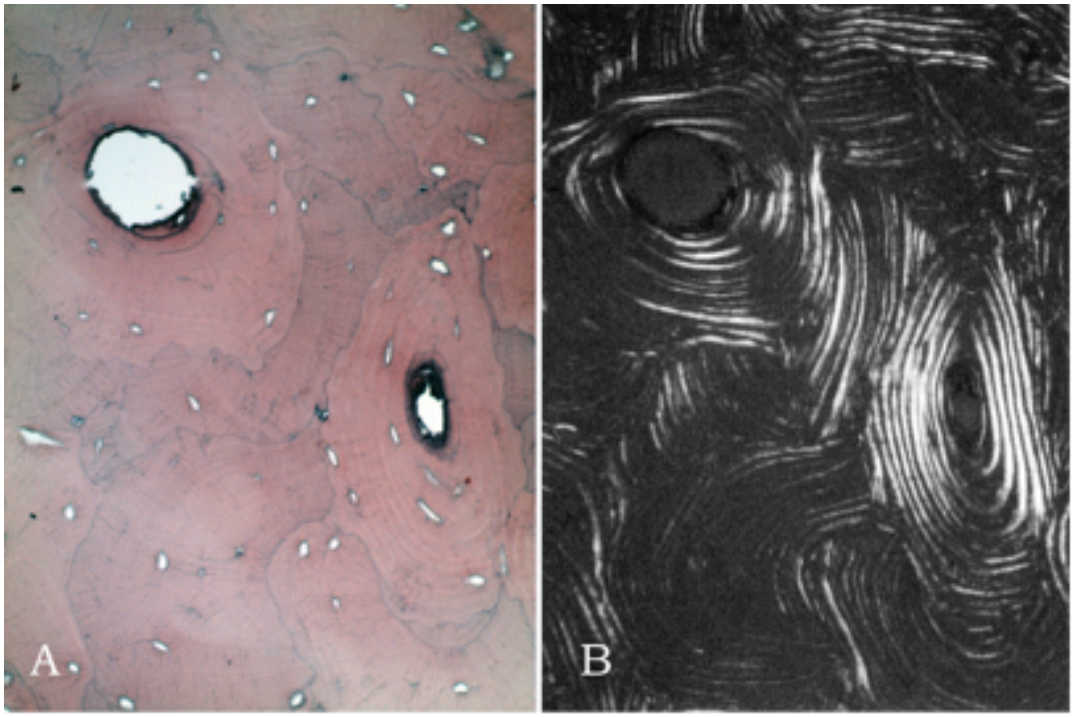


Figure 9.A and 9.B, micrograph of sample 118-2010-k-VII. Sample boiled for twenty minutes. Clear vision of the osteons, matrix slightly pale pink. The collagen fibres within the circulair lamellae appear very bright when using polarized light. 20x magnitude. Index 5.

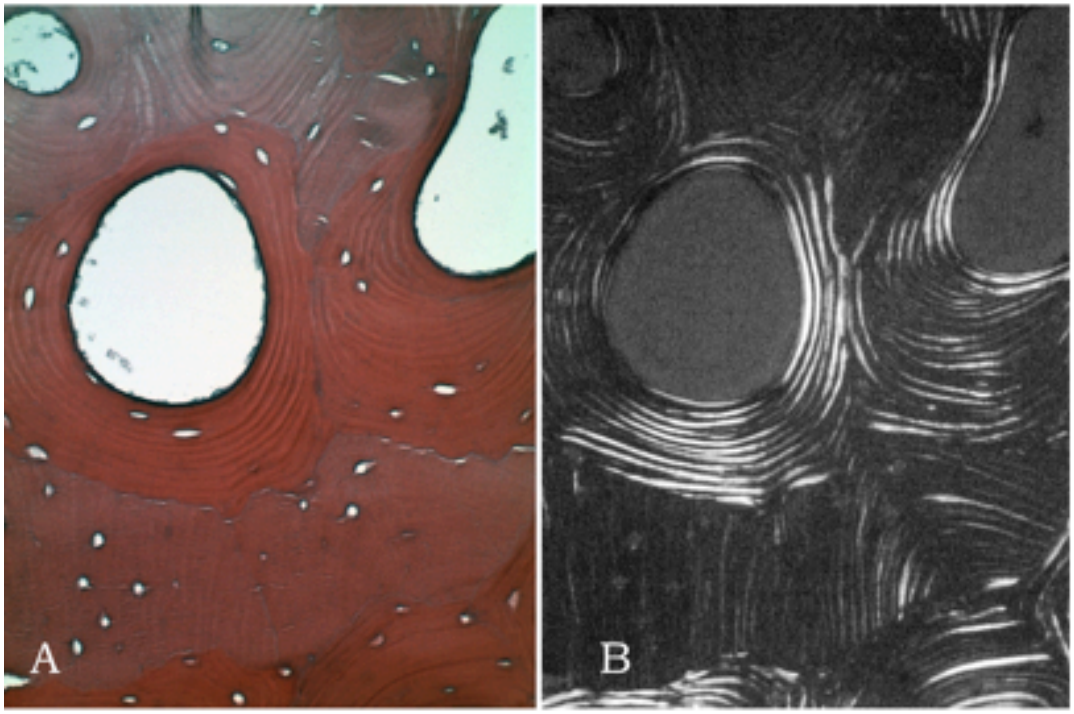


Figure 10.A and 10.B, micrograph of sample 85-2010-L-V. Sample boiled for sixty minutes. Clear vision of the osteons. The matrix is slightly darker than after twenty minutes of boiling. The birefringence is still very bright. 20x magnitude. Index 5.

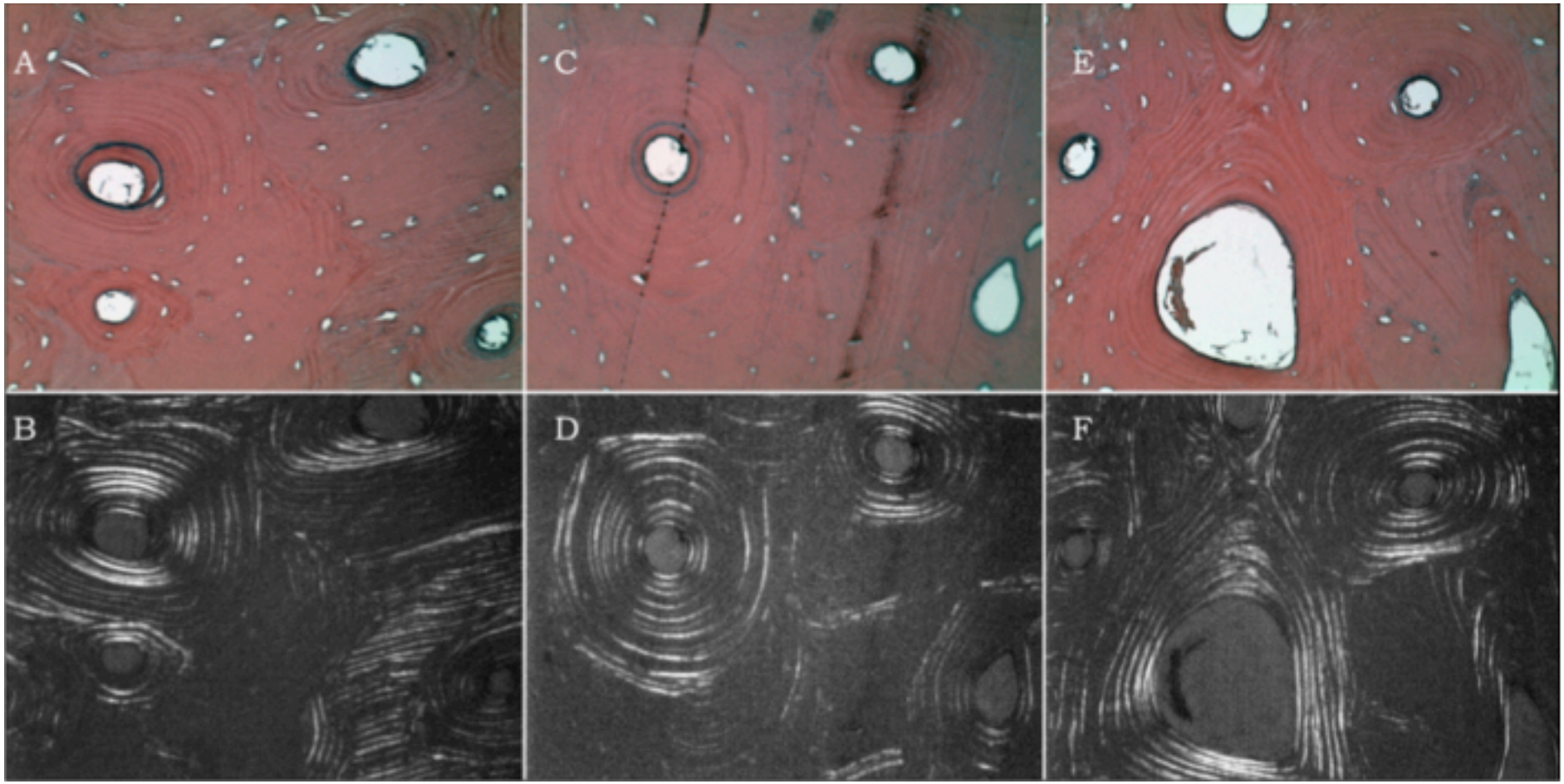


Figure 11.A and 11.B, Micrograph of sample 85-2009-i-II. Sample heated for ten minutes at 100°C. Both the histological image as the polarized light image correspond to the untreated sample, index 5. Figure 11.C and 11.D, 85-2009-i-III. Sample heated for twenty minutes at 100°C. There is no difference noticeable after twenty minutes, index 5. Figure 11.E and 11.F, 85-2009-i-IV. Sample exposed for thirty minutes at 100°C, no noticeable difference between the histological and polarized view between this degree of thermal stress and the untreated sample, index 5.

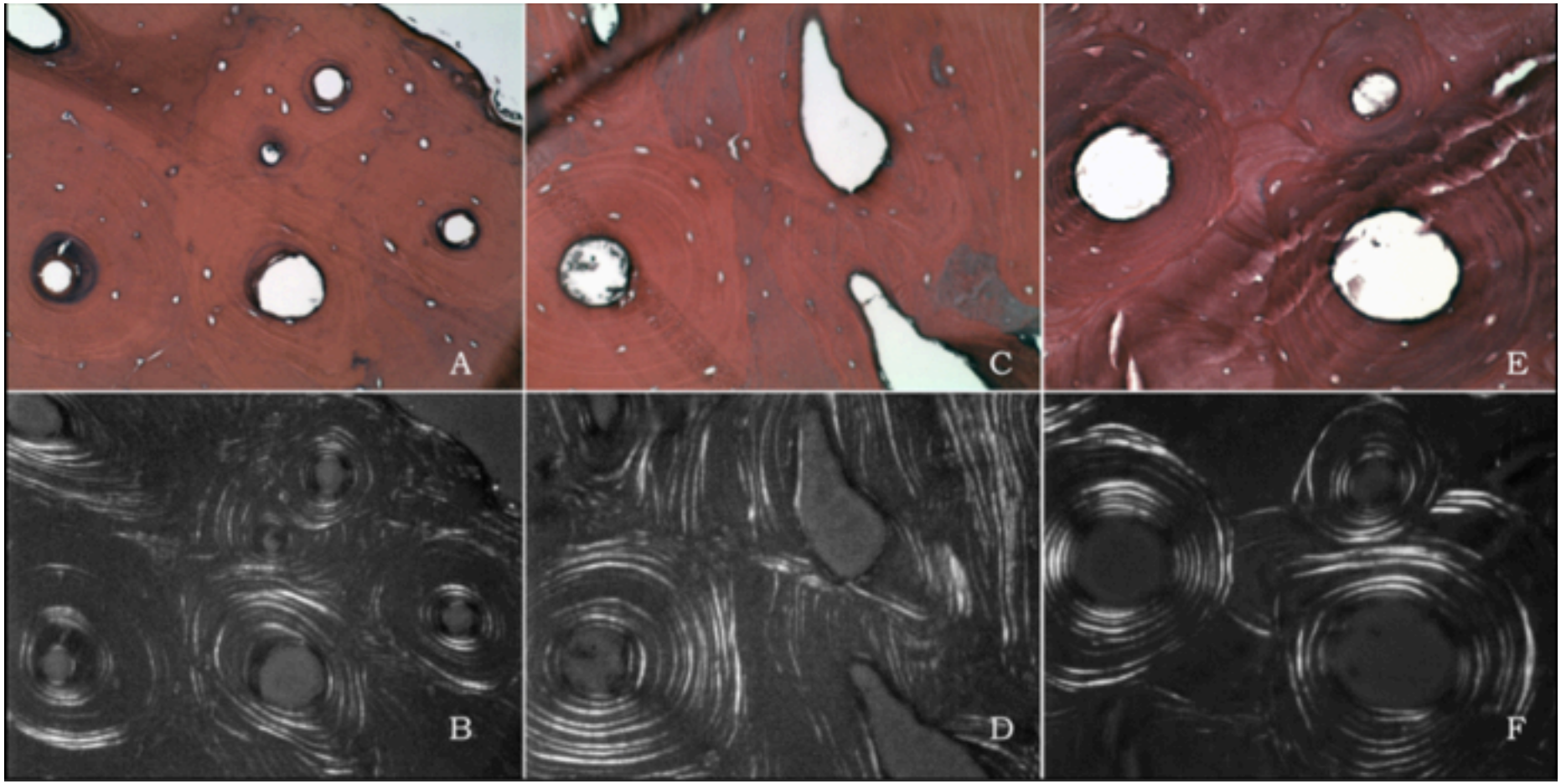


Figure 12.A and 12.B, 52-2010-d-V. Sample heated for ten minutes at 160°C. Both the histological as the polarized light images correspond to the untreated sample, index 5. Figure 12.C and 12.D, 52-2010-d-VI. Sample heated for twenty minutes at 160°C. There is no difference noticeable compared with the untreated sample, index 5. Figure 12.E, and 12.F, 52-2010-d-VII. Sample exposed for thirty minutes at 160°C, the matrix is darker, this also shows as more contrast when using polarized light, but the birefringence is unaffected, index 4 to 5.

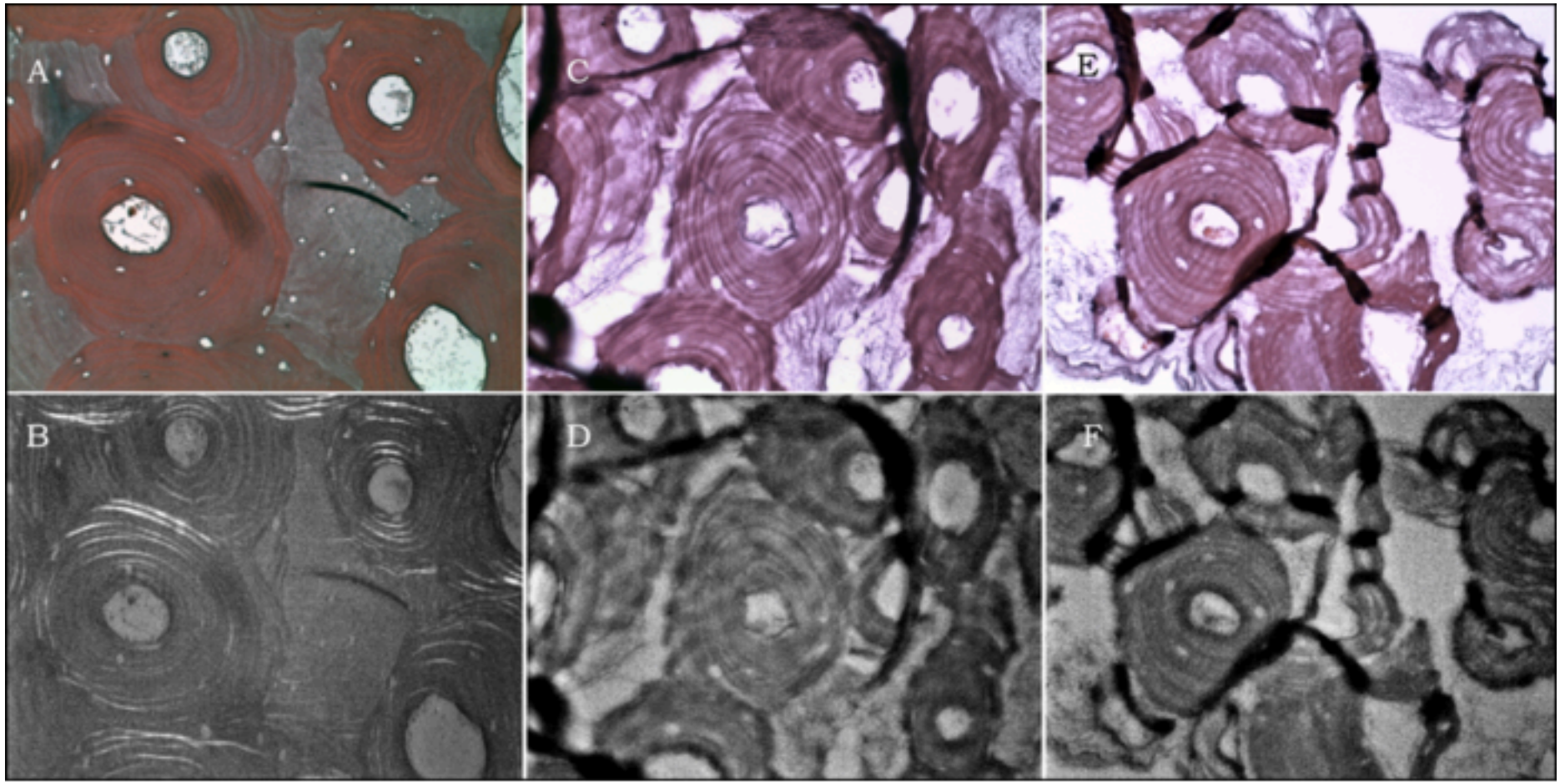
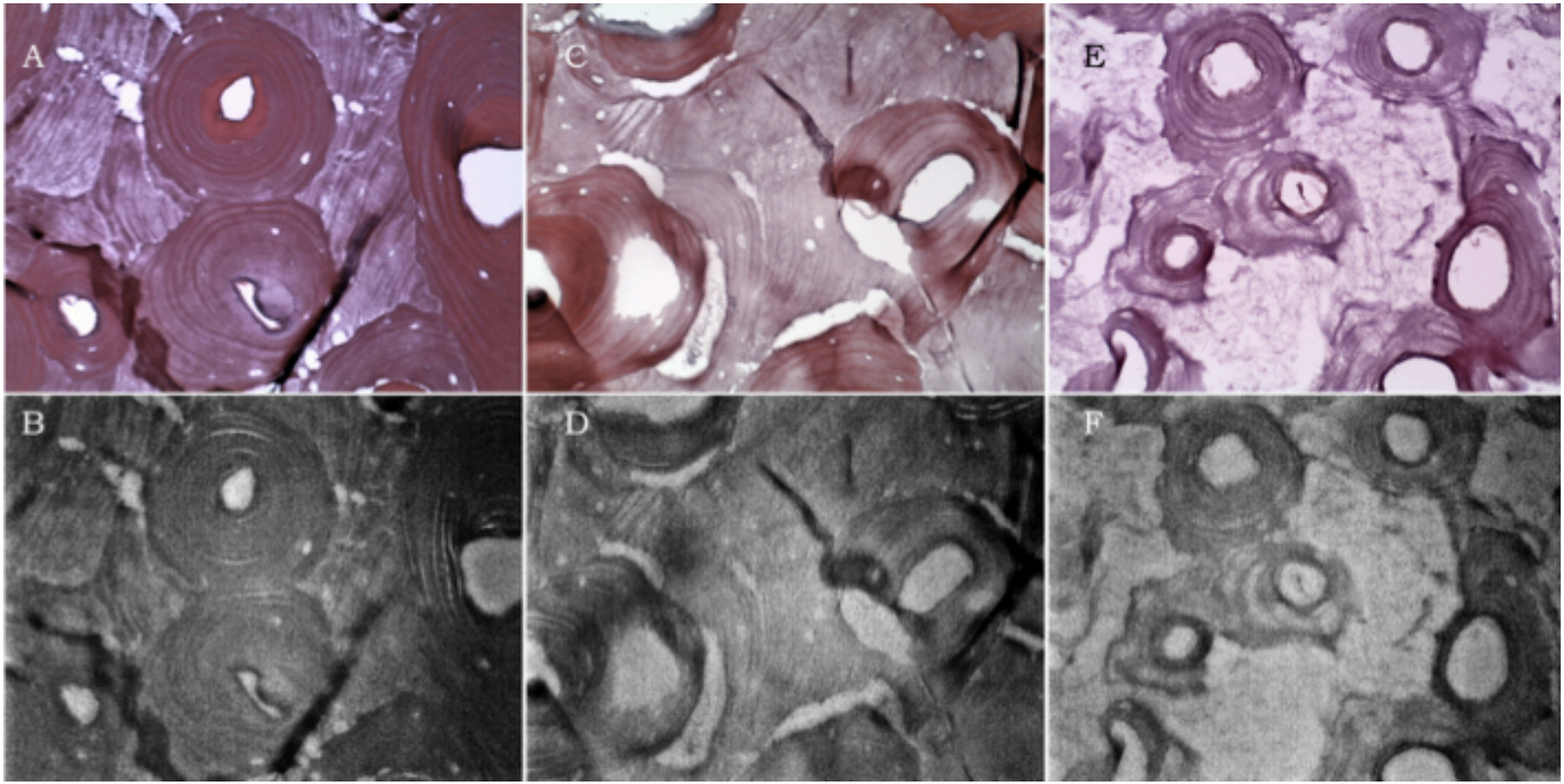
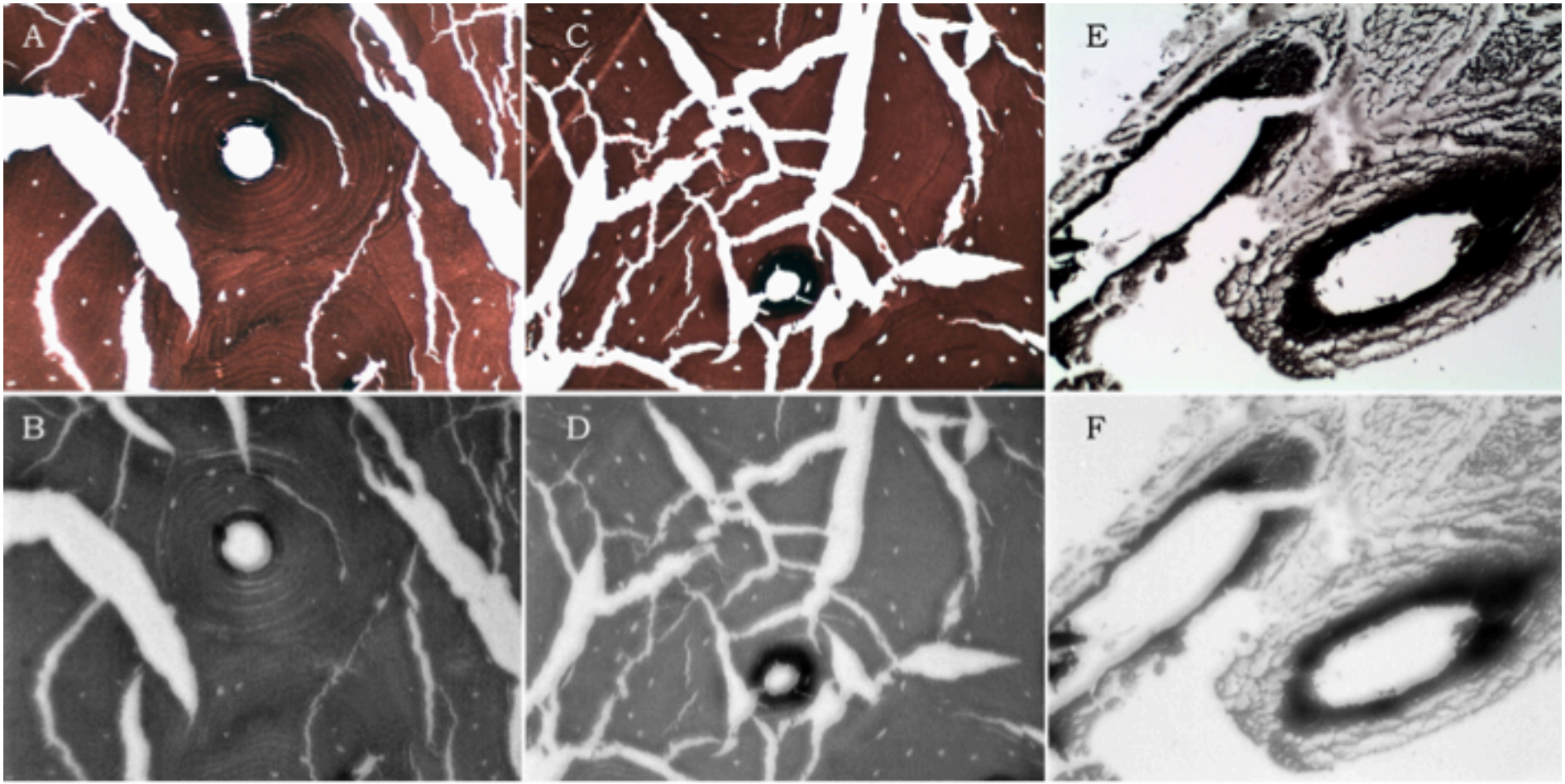


Figure 13.A and 13.B, 85-2010-L-VI. Sample heated for ten minutes at 200°C. The tissue between the osteons is paler, the tissue within the circulair lamellae seems less affected by the thermal stress. The birefringence is decreased, index 4. Micrographs 13.C and 13.D, 85-2010-L-VII. Sample heated for twenty minutes at 200°C. There is a great difference noticeable compared with the untreated sample, the interstitial lamellae start to loose coherence and there is no birefringence anymore around the osteons, index 2 to 3 Figures 13.E, and 13.F, 85-2010-L-VIII. Sample exposed for thirty minutes at 200°C, there is less coherence between the osteons and no birefringence left when using polarized light, index 2.



Figures 14.A and 14.B, 85-2010-k-XI. Sample heated for ten minutes at 250°C. The tissue between the osteons is paler, the tissue within the circulair lamellae seems less affected by the thermal stress. The birefringence is decreased, index 3. Micrographs 14.C and 14.D, 85-2010-K-XII. Sample heated for twenty minutes at 250°C. There is a difference noticeable compared with the untreated sample but compared with 200°C for twenty minutes there is no real difference, the interstitial lamellae start to loose coherence and there is almost no birefringence anymore around the osteons, index 2 to 3 Micrographs 14.E, and 14.F, 85-2010-K-XIII. Sample exposed for thirty minutes at 250°C, there is loss of coherence between the osteons, the interstitial lamellae are gone, and no birefringence left when using polarized light, index 2 to 1.



Figures 15.A and 15.B, 85-2009-f-XVI. Sample heated for ten minutes at 300°C. The tissue seems to be ruptured with sharp edges, the preservation is good. In between the tissue fragments all coherence is lost. The birefringence is decreased, index 3. Micrographs 15.C and 15.D, 85-2009-f-XVII. Sample heated for twenty minutes at 300°C. There is no birefringence anymore around the osteons, the amount of fragments has increased but the remaining tissue structures are recognizable. Index 2 Micrographs 15.E, and 15.F, 85-2010-L-XIV. Sample exposed for ten minutes at 400°C, There are some structures that are still recognizable as osteons but in general most structures are lost. There is no birefringence left. Index 0 to 1. Note: 400°C after twenty minutes is missing because there was no tissue left after the pre-treatment. (decalcification).

Ing. Tristan Krap

4-11-1985, Dutch, male.
Brandaris 283
1503 CD Zaandam
Nederland
0031 (0)6-30381319
info@tristankrap.nl

Rational and ambitious. Passionate about research.

EDUCATION

9.2011 – present	**MSc. Human Osteology and Funerary Archaeology** University Leiden, Faculty of Archaeology. Second specialization: **Forensic Science** University of Amsterdam, Faculty of Science.
9.2007 – 7.2011	**BASc, Forensic Science** The Amsterdam University of Applied Sciences. With a minor in applied psychology.

(RELATED) EXPERIENCE

1.2012 – 7.2012	**Master thesis: Changes in human cortical bone due to thermal stress. An experimental histological approach.** Supervisors: Drs. F.R.W. van de Goot, dr. A. Waters-Rist.
1.2011 – 7.2011	**Researcher VUmc** Supervisors: Drs. F.R.W. van de Goot, prof. dr. H.W.M. Niessen. Department: Pathology in cooperation with the Dutch Forensic Institute and the Centrum Forensic Pathology. – Research towards the lethality of catecholamines on cardiomyocytes. – Research towards cytokines as a marker for chronological injury dating.
9.2010 – 1.2011	**Bachelor thesis: Solubility of soft tissue and bone in strong acids and bases.** Supervisor: Drs. F.R.W. van de Goot.
1.2007 – present	**Freelance photographer, www.tristankrap.nl** Independently without staff. Kvk nummer: 51724758 (2011). Services: Forensic and medical photography.

COURSES AND CONFERENCES

23.2.2012	**IGBA Symposium, VU, Geo- and Bioarchaeology** Topics: Forensic Archaeology and Botany Present as a participant.
10.3.2011	**CSI in Dutch court.** Organized by Verilabs and the Centrum for Forensic Pathology. Present as a participant.
2.2.2011	**Internal training immunohistochemistry, VUmc**
2.11.2010	**Themeday digital criminal investigation.** Organized by the Amsterdam University of Applied Sciences. Present as speaker and co-organizer.
29.5.2010	**Workshop: Crime Scene** Organized by the dutch police academy.

OTHER SKILLS

- Driver license A + B
- Microsoft Office, Adobe Photoshop and Lightroom, SPSS.