The use of histological methods to distinguish between burned remains of human and non-human bone.

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Year: 2021

Date of submission: 14 February 2022

DECLARATION

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ABSTRACT

As part of a medico-legal analysis it is necessary to identify if bone tissue is animal or human in nature. This process is complicated when bone is highly fragmented or burned. Previous research has established the ability to differentiate human from non-human bone histologically, however, further research is necessary to determine if this is still applicable in the case of burned remains. In South Africa, approximately 500 deaths and 15 000 fire related injuries occur annually in Cape Town and such fires ranged between 600°C to 1000°C. The aim of this research was to study the qualitative and quantitative characteristics of femur bone microstructure of human and animal bones exposed to different temperatures and to determine the possibility of distinguishing them. The study consisted of 17 femoral bone samples collected from four different species namely; humans (*Homo sapiens*), pig (*Sus scrofa*), wildebeest (*Connochaetes gnou*) and cow (Bos taurus). Unburned samples were compared to bone samples burned at 600°C, 700°C, 800°C and 900°C in a muffle furnace for 20 minutes. Bone samples were processed into thin sections for histological analysis. During analysis, each bone specimen was divided into four quadrants and two periosteal regions. For histomorphometric analysis, quantitative characteristics were assessed by measuring the area, perimeter, and minimum and maximum diameter of the Haversian system and Haversian canals as well as osteon circularity and osteon density. According to the qualitative results, the main structural bone tissue observed in all quadrants and two periosteal regions of unburned animal bone was primary vascular plexiform bone and irregular Haversian bone. Human bone consisted of dense Haversian bone. Quantitative results indicated a statistically significant difference in most parameters between species within burned as well as unburned samples (p<0.001). Statistically significant differences in quantitative parameters within human and wildebeest bone were noted at different burn temperatures (p<0.001). Overall, the results showed that heat exposure to bones can affect the bones' quantitative and qualitative characteristics but human and non-human bones can still be differentiated. This histological method can be used in forensic fire cases.

Keywords: Burned bones; Bone histology; Fire; Species differentiation, Osteon.

DEDICATIONS

This Masters degree is dedicated to my late sister, Lerato Paulinah Nkatswang (Morwa). Thank you for everything you've done for me, more especially for being proud of me. Thank you very much Big Sis, I love you!!

ACKNOWLEDGEMENTS

I, Masego Jessica Sebolai (the researcher) would like to acknowledge the following people and funders who contributed to the success of this research:

- The UCT Master's financial aid and the Health and Welfare Sector Education and Training Authority (HWSETA) for funding my studies.
- My supervisor, Mr Calvin Mole for his mentorship, patience, understanding and encouragement during the research process.
- Lisa Alberts for assisting me with the animal bone preparation and photography.
- My sisters; Bontle Louisa Sebolai, Refilwe Vivian Sebolai & Tshegofatso Goarona Sebolai and my dear friend Motlhalefi Alpheus Sebolaaneng for their continuous support throughout my studies.

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CHAPTER 1: LITERATURE REVIEW

1. INTRODUCTION

In forensic and archaeological contexts, biological/ forensic anthropologists may be called to determine whether bone or bone fragments originated from human or nonhuman bones. In cases where skeletal remains are highly fragmented, burned, degraded or unidentifiable, macroscopic analysis for species differentiation is often not possible to perform especially in cases where articular surfaces of such bones are absent. DNA analysis can be used to detect the species origin of such bones; however, such techniques are costly and there are cases where DNA cannot be obtained due to poor preservation of DNA in highly burned or fragmented bones (Andronowski *et al*., 2017).

Cases involving fragmented or burned bones include, but are not limited to, mass disasters, fire, explosions, or homicide case where the body is burned in order to conceal the crime committed (Schwark *et al*., 2011). The burning of bones alters its physical and chemical structure. The physical changes observed in burned bones include bone shrinkage, a change in the bone's colour, alterations to microscopic features or morphology and dehydration (Ubelaker *et al*., 2009). Dehydration in bones is caused by the loss of water and moisture due to heat which eventually causes deformation and fragmentation of the bones (Shipman *et al*., 1984). The chemical modification observed in burned bone includes combustion and pyrolysis of the chemical substances present in the bones and the more the bones are exposed to heat or higher temperatures, the more DNA degrades in the bones (Imaizumi, 2015). Thus, DNA analysis is not always possible to assist in identifying the origin of the human or non-human burned remains.

In cases where no identifiable macroscopic landmarks are present to use to differentiate between burned or unburned human and non-human bones, it may be possible to use a histologically based determination method which involves examining the microstructure of unburned bones and comparing it with the microstructure of burned sample (Absolonova *et al*., 2012; Crescimanno and Stout, 2012). Histological analysis in such cases includes the examination of thin-block sections of compact bone under a microscope to analyse histomorphologic and histomorphometric characteristics (Hillier *et al*., 2007). Based on histological examinations, the possibility of observing variations between bones of different species has been established (Martiniakova *et al*., 2007; Absolonova *et al*., 2012; Crescimanno and Stout, 2012; Brits *et al*., 2014; Carroll and Squires, 2020). However,

limited research has assessed the degree to which microscopic bone structures may be affected by burning and thus the applicability of histological differentiation of species. This literature review will provide a brief discussion of histological structures of bone followed by a review of the literature relating to the effect of temperature on bone and microscopic comparison between human and non-human bones.

1.1 GENERAL BONE HISTOLOGY & MORPHOLOGICAL CHARACTERISTICS OF BONES

The histological structure of bone is used in forensic anthropology and archaeology to assist in analysing fragmented bones for determining bone trauma and healing, age estimation prior to death, and to differentiate human and non-human bone fragments (Brits *et al*., 2014). In addition, it is important to note that biological and environmental factors play a major role in bone development and the development of bone microstructures (Martiniakova *et al*., 2007).

Bone may be viewed as a hierarchical structure consisting of four levels (Rho *et al*., 1998). The first level is known as the nanoscale level, at this level the bone is made up of extrafibrillar mineral as well as the mineralised collagen fibre. The second level is called the microscale, it contains information on bone microstructure or the structures of bone which can only be observed under a microscope. The third level is known as the mesoscale, this level provides information that assists in understanding the relationship between cancellous and cortical bone. The fourth level is the macro scale which looks at the whole bone. This study will only be focusing on the microscopic level of bones (the second level).

The osteoclasts, osteoblasts and osteocytes are the main cells involved in the maintenance and the formation of bone (White *et al*., 2012). The Osteoblasts are non-divided cells that play a major role in the build-up of bones. The cells secrete a collagen fibre which assists in the development of the bone matrix and the calcification process. Osteoclasts are the largest bone cells and they're located in the endosteum of bone. Osteoclasts originate as the products of diffusion of monocytes and their role is to breakdown the collagen and the salts of the matrix, in the end destroying the bone cells. The osteocytes are the mature bone cells located in the lacunae and connected by the canaliculi in order to provide nutrients, assist in waste and gas exchange and in maintaining the overall bone tissue (Zylberberg, 2004; Currey, 2012; White *et al*., 2012). Bone development occurs during the endochondral ossification process or intramembranous process. Once the skeletal structure is formed, bone begins to grow and

change shape to allow for bone movement or for the accommodation of internal organs. This process of bone modelling and/or bone growth allows for bone formation or bone resorption to take place on a given bone surface (Langdahl *et al*., 2016; Allen and Burr, 2019). Bone remodelling is a bone repair process involving the activity of osteoblasts and osteoclasts to resorb and replace the existing bone. This process of bone-resorbing osteoclasts and bone-forming osteoblasts is known as basic multicellular unit (BMU) of remodelling (Lad *et al*., 2019; Crowder and Stout, 2012). Bone remodelling takes place in all bone surfaces such as the endosteum and trabeculae and the histomorphology of bone remodelling is often observed as secondary osteon in cortical bone (Crowder and Stout, 2012). As indicated by Wolff's law: "Bone is deposited where it is needed and resorbed where it is not needed." (White *et al*., 2012). This balance occurs because of the roles played by the osteoclasts, osteoblasts and osteocytes. Wolff's law is important to note because human and non-human bones have different arrangement in their structure and this structure can change during mechanical, biological and chemical functions. Such functions include structural support or body weight support, protection, storage of water and minerals, movement and healing (Rho *et al*., 1998). Wolff's law mainly focuses on the development of the trabecular orientation in long bones especially the proximal part of the femur (Ruff *et al*., 2006).

Microscopically, mammalian bone can be divided into fibrolamellar, lamellar bone, woven bone and Haversian bone (Currey, 2012). At microstructure level, there are mineralized collagen fibres which form into lamellae (about 3µm thick) (Rho *et al*., 1998). There are different types of lamellae (bone tissue layers) found in compact bone and they are as follows; the outer circumferential lamellae which covers the bone circumference, the inner circumferential lamellae which is observed near the medullar cavity beneath the endosteum and the interstitial lamellae which is found between the osteons. In some cases, the lamellae can be present around a vascular canal, forming what is known as secondary osteon or Haversian system, which is the basic unit of compact bone. The lamellae found in spongy bone is known as the irregular lamellae and they form the trabeculae seen in spongy bone. The bone cells (osteoclasts, osteoblasts and osteocytes) are observed to be round in the central cavity. When the osteoblasts deposit bone, they form lamellae and the Haversian canal and the collective activity of a Bone Multicellular Unit (BMU) are formed during the coordinated activity of osteoclasts and osteoblasts (Crescimanno and Stout, 2012; Allen and Burr, 2019). Woven bone is initially formed from collagen. It forms rapidly and can eventually differentiate into compact bone through osteoclastic and osteoblastic activity. During the skeletal growth

and maturation stages, the woven bone gets replaced by an organized primary lamellar bone as well as a secondary osteonal bone (Rho *et al*., 1998; Currey, 2012; Andronowski *et al*., 2017).

The vascular canals (Haversian canals and Volkmann's canals) are observed at the microstructure level, they have a sequence of microscopic tubes throughout the cortical bone. The arrangement of vascular canals within the bone can give rise to different types of bone such as the Haversian bone and the plexiform bone (Andronowski *et al*., 2017). Haversian bone is made up of primary and secondary osteons. Primary osteons have lamellae which merge/ combine smoothly with the surrounding bone (Crescimanno and Stout, 2012). Secondary osteons (Haversian systems) are cylindrically shaped microstructural units present in the outermost surface of the cortical walls in the long bones. They are formed during bone remodelling process which takes place in six phases namely activation, resorption, reversal, formation, mineralization and reversible growth/arrest state and this process becomes a success due to a complex arrangement achieved by the osteoclasts and osteoblasts working together. During the reversal phase, mononuclear cells smooths the resorptive bay, this process then forms the cement lines observed in the secondary osteons (Crescimanno and Stout, 2012; Brits *et al*., 2014). Osteons include a central (Harversian) canal which contains blood vessels observed to be parallel to the long axis of a bone and are surrounded by lamellae or a concentric ring of matrix. Furthermore, between the rings, there are spaces known as lacunae and osteocytes are located in those spaces. The small channels known as the canaliculi, emit from the lacunae to the Haversian canal to create passageways through the hard matrix. The dense middle portion of such a Haversian bone is known to be made up of approximately 50% of secondary osteons and 50% of interstitial lamellae, however, the composition does change and this depends on the specie and the stage of bone growth (Crescimanno and Stout, 2012). The main difference between the primary and secondary structures of osteons is that, unlike secondary osteons, primary osteons are not surrounded by a reversal (cement) line or concentric lamellae; such a line is observed at the remodelling site of the bone (Martiniakova *et al*., 2007; Crescimanno and Stout, 2012). Plexiform bone (also known as a fibrolamellar bone) is a laid down bone with a dense network of vascular canals which when observed under a microscope, consist of a dense network of vascular canals set out longitudinally, circumferentially and radially, giving a "brick-wall" appearance (Enlow *et al*., 1958). Small mammals and primates (including humans) adults typically have Haversian bone, while most medium- to large-sized mammals have plexiform bone (Hillier *et al*., 2007; Martiniakova *et al*., 2007; Cattaneo *et al*., 2009).

When looking at adult skeletal bone at a macrostructure level, mature compact bone (cortical bone) and spongy bone (cancellous bone) are the two main types of bones present in both human and non-human bones. Compact bone is lamellar in structure and the structure is penetrated by the Harversian system which carries blood vessels for the osteocytes (White *et al*., 2012). The outer layer of the compact bone tissue (substantia compacta) makes up the shafts of the long bones and its extremities, the short bones as well as the outer and inner layer of the skull in mammals (Martiniakova *et al*., 2007). Spongy bone is made up of interconnected bony spicules known as trabeculae which are made by the irregular lamellae present in the bone and this type of bone is mostly found in the head of the long bones and between the flat bones (White *et al*., 2012). The bone microstructure observed in the spongy bone mainly contains irregular, sinuous convolutions of lamellae while the microstructure of compact bone not only contains regular, cylindrically shaped lamellae but also provides more bone microstructures for reliable species differentiation (Rho *et al*., 1998).

Compact bone comprises 80% of the human skeletal bones while the spongy bone only makes up 20% of the human skeletal bones, thus, compact bone is commonly used in forensic science research studies (White *et al*., 2012). When comparing spongy bone to compact bone, spongy bone is found to be extra porous and less dense than the compact bone. In cases where the forensic science studies involve burning of bones or load-bearing of bones, compact bone would be the preferred bone to study because the bone is less fragile and will therefore often remain intact in such conditions (Rho *et al*., 1998).

A study done by Crescimanno and Stout (2012) states that sexual dimorphism in humans can contribute to the shape and size of the human bone microstructure such as the size of the Haversian canals and the osteon diameter and area in femur bones. The study further reports that other studies have contradictory results on the sizes of the Haversian canals between males and females and this led to the sex of an individual not being used in such research or added as a factor in the microscopic analysis used for bone differentiation and identification. Other studies argue that since, although rare, plexiform bone may be present when children experience large growth spurts and plexiform bone is not present in all animals, it should not be the main microscopic differentiation used to differentiate between human and non-human bones (Crescimanno and Stout, 2012). However, a study by Andronowski *et al*. (2017) states that plexiform bone in non-human bones has a distinguishable alternating sheet of woven and lamellar bone. Therefore, this plexiform-type arrangement of fibrolamellar bone is unique to non-human bones and is rarely seen in adult human bones (Andronowski *et al*., 2017).

Bone histomorphology observed between mammals differs among species mainly due to factors relating to the rate of growth and development, forms of locomotion, environmental conditions and adaptation, nutrition, responses, total body size and mechanical influences. Furthermore, particular bone features can be affected by pathology, nutritional deficiencies and abnormal posture and weight changes. Such factors can affect the general histology of such bones (Brits *et al.*, 2014).

1.2 THE EFFECT OF TEMPERATURE ON BONE

A research study by Thompson (2005) reported dehydration, decomposition, inversion and fusion as the four stages of heat-induced transformations in bone. Dehydration of the bone is marked by weight loss of the bone and fracture patterns observed on the bones at burning temperatures ranging between 100°C and 600°C. Bone decomposition is characterised by the change in colour and porosity of the bone, the loss of mechanical bone strength and weight loss at burning temperatures ranging between 300°C and 800°C. Bone fusion is observed when there is an increase in the bone's mechanical strength and crystal size and a reduction in the dimension of the bone as well as a change in bone porosity at burning temperatures of above 700°C. Inversion is marked by an increase in the crystal size at burning temperatures ranging from 500°C and 1100°C (Thompson, 2005). A study by Castillo *et al*. (2013) reported that during bone dehydration between burning temperatures of 100°C and 300°C, the bone size can decrease by one to two percent of its original volume. As the temperature increases from 300°C to 600°C, a change in the primary structural features begins and the contraction of bone structure increases. At higher temperatures (above 800°C), the crystals melt and become larger and the bone structure fragility takes place. The melting point recorded in this study was approximately 1630°C. In addition, a study by Ellingham and Sandholzer (2020) reported that an individual's age, sex and health status contributes to the degree of shrinkage and the change in the pattern of the bone tissue during bone burning.

Macroscopic analysis of burned bone can be successfully performed in bone exposed to low temperatures (between 100°C to 300°C) and little damage is observed in the physical and chemical structure of bone. However, high temperature exposure to bones is known to alter the shape, size and colour of the bone and the elasticity of bones which plays a factor in how the bone can burn (Castillo *et al*., 2013; Ellingham *et al*., 2015). Such damage can cause difficulties in utilising macroscopic analysis for bone identification and differentiation in

forensic anthropology and archaeological cases and the alternative would be to use DNA analysis (which is costly) or histological methods for biological profiling and bone identification and differentiation (Mulhern *et al*., 2001; Thompson, 2005; Andronowski *et al*., 2017).

At the histological level, investigation into heat induced changes in bone has produced contradictory findings which are well summarised by Carroll and Squires (2020). A study by Bradtmiller *et al*. (1984) discovered that burning human femur bones in a small electric oven at higher temperatures (600°C) causes the burned bone's osteons size to be uniformly larger than the unburned femur bones. However, a study by Nelson (1992) performed a similar experiment and discovered that the burned bone's osteon size actually decreases at the same temperature (600°C). In addition, a study by Absolonova *et al.* (2012) discovered that burning human ribs at temperature between 700°C and 800°C causes rib bone's osteon and Haversian canal size to decrease. More research on the burned bone microstructure discovered that not only is bone carbonisation observed at temperatures between 300°C and 800°C, the bone microstructure is preserved or observable at temperatures between 600°C to 900°C (Herrmann, 1977; Squires *et al*., 2011; Castillo *et al*., 2013). A study by Squires *et al*. (2011) reported that the bone microstructure such as the Haversian system and the Volkmann's canals are preserved and observed at burning temperatures between 300°C and 900°C and as the temperature increases (> 900°C) the above-mentioned bone microstructure cannot be observed. What is observed is the complete fusion of the hydroxyapatite crystals. The fusion and recrystallisation of the hydroxyapatite are known to cause microscopic changes in the bone tissue. Crystallinity of the bone mineral increases at the burning temperature of 600°C, revealing a glass-like structure which is suspected to be the cause of the bone remains losing their steadiness at that temperature. At burning temperatures of 700°C to 800°C, the crystalline structure enlarges and appears round in shape. This is known to be caused by recrystallisation and the calcium phosphate changing into tricalcium phosphate and in the end causing greater hardness, mechanical resistance and an increase in bone steadiness. As the temperatures approaches 900°C the large crystal structure begins to disappear or becomes unidentifiable and as the temperature continue to rises (above 900°C), the bone matrix converts into completely crystalline and becomes amorphous and granular in shape (Castillo *et al*., 2013). Ultimately, the recrystallisation phenomena of the compounds obtained from the hydroxyapatite thermal hydrolysis causes bone fracture and retraction, the bursting of Haversian canals and the cluster

and bone matrix formation observed during histological examinations of burned bones (Castillo *et al*., 2013)**.**

A study by Lemmers *et al*. (2020) burned fleshed archaeological bones (buried for approximately 80 years) and recent bones (never buried) at different temperatures (300°C,500°C,700°C and 900°C) and durations (20,40,90 and 160 minutes). The results indicated that bone microstructure of the bones burned at different temperatures and durations was still visible under the microscope except for archaeological bones burned at 500°C which had high bone carbonation or blackening. The result show that the presence or absence of soft tissue in bones may have little to no effect on the visibility of bone microstructure in bones burned at high temperatures (>300°C).

Deaths due to fire is a common global public health issue (Melez *et al*., 2017). Fire is an exothermic oxidation reaction which requires fuel, oxygen, heat and a chemical oxidation and it can occur in flaming and/ or smouldering form (Schmidt and Symes, 2015). Fire exposure to the human body can cause skin burns, body alterations and death (Fanton *et al*., 2006). The burning of human remains can be observed in homicidal cases, accidental cases such as household fires, aircraft and road traffic accidents, and in fire explosions and the fire damage to the human remains depends on the temperature exposure and the type of bone burned (Schwark *et al*., 2011; Schmidt and Symes, 2015). The World Health Organization reported that approximately 265 000 deaths due to burns caused by fire occur every year and in 2004, 11 million people required medical treatment for fire burns (World Health Organization, 2008; Peck, 2011). The highest fire mortality rates per 100,000 population per year were recorded in Southeast Asia (11.6 deaths), the Eastern Mediterranean (6.4 deaths) and Africa (6.1 deaths) (Melez *et al*., 2017). In addition, South Africa has recorded high fire burn mortality rates in its four metropolitan centres (Van Niekerk *et al*., 2009). Approximately 500 fire related deaths and 15 000 fire related injuries occur annually in Cape Town and of the 18 504 fire incidents recorded between the period of 1990 and 2004, 47% of them occurred in informal settlements (Western Cape Government, 2015). The fire spreads rapidly in such settlements due to shacks and household structures being in close proximity to one another. Furthermore, the use of paraffin stoves (a known risk factor for fire) within these structures is prevalent (Walls *et al*., 2017). The temperatures recorded in shack fires ranges between 600°C and 800°C and may exceed 1000°C (Walls *et al*., 2017).

While numerous studies have investigated the differentiation of species using histological techniques, limited research has examined the effect of temperature on the microstructure of bone which can assist in fire cases for identification and to differentiate between human and non-human burned remains. A few studies have performed microscopic analysis or histological methods on burned human and non-human bones and reported the differences between the human and non-human bone microstructure as well as the preservation and the visibility of the Haversian system and Volkmann's canals in human and non-human bones exposed to temperatures between 400°C and 600°C and 700°C and 1000°C (Hanson and Cain 2006; Martiniakova *et al*., 2007; Absolonova *et al*., 2012; Brits *et al*., 2014; Carroll and Squires, 2020).

It is important to keep in mind the challenges that can rise when performing qualitative analysis on burned bone microstructure. Accurate results of the qualitative analyses on burned bones highly relies on the examiner's knowledge and experience (Carroll and Squires 2020).

1.3 MICROSCOPIC COMPARISON BETWEEN HUMAN AND NON-HUMAN BONES

Histological approach involving comparing qualitative and quantitative microstructural traits of human and non-human bones is used to assist in differentiating burned human and non-human bones. Differences in osteon histomorphology and histomorphometry has been found to exist between animal and human bones (Martiniakova *et al*., 2007). Haversian bone has been observed in different mammalian species including humans as well as in reptiles and birds (Currey, 2003). In most primates, herbivores and carnivores' primary vascular plexiform bone is initially laid down during development. This is, however, soon replaced by Harvesian bone while in other mammalian group such as the cattle (*Bovidae*) and deer (*Cervidae*), primary vascular plexiform bone is retained and Haversian bone is only seen in small regions of such mammalian bones. Different animals display different types of plexiform bone based on the rate of deposition (Martiniakova *et al*., 2007).

Histomorphometric analysis is used for the quantification of bone microstructure for age estimation and to differentiate between human and non-human bones. Typical measurements include, but are not limited to, area, volume, length, circularity, diameter, perimeter as well as osteon density of Haversian systems and Haversian canals (Crowder and Stout, 2012).

A South African study by Brits *et al*. (2014) used femur bones of commonly known South African species namely; human (***Homo sapiens)***, impala (***Aepyceros melampus)***, cat ***(Felix catus)***, dog ***(Canis familiaris)***, cow ***(Bos taurus)***, pig ***(Sus scrofa Domestica)***, Horse ***(Equus ferus caballus)***, donkey ***(Equus africanus asinus)***, vervet monkey ***(Chlorocebus pygerythrus)*** and baboon (***Papio ursinus)*** to study and compare their histomorphology**.** The study focused on the qualitative analysis of human and non-human bones by particularly studying bone vascularisation patterns which are established on the arrangements of vascular canals, primary osteons and/or the secondary osteons (Haversian systems). Following histological analysis of the periosteal surface of each bone no bone microstructure similarities were reported between human bones and the impala, cat, dog, cow and pig bones and when comparing the avascular bone of the adult human bone and the non-human primate bone (baboon) microscopically (100 X magnification), the human bone had an isolated Haversian system towards the periosteum while the non-human primate contained a few isolated longitudinal canals towards the periosteum. If the Haversian system was absent in the human bone and the longitudinal canals were absent in the non-human primate bone, it would have been difficult to differentiate the two species.

Another study by Martiniakova *et al*. (2007) focused on microscopically studying the femur bone of four various species in order to analyse their compact bone tissue microstructure qualitatively and quantitatively. The species studied included adult pigs (*Sus scrofa domestica*), adult cows (*Bos taurus*), sheep (*Ovis aries*) and adult rabbits (*Oryctolagus cuniculus*). The qualitative analysis was performed by looking at the anterior, posterior, medial and lateral parts of the thin sectioned bones. Non-vascular bone was only seen in the cow bone, resorption lacunae were identified between the secondary osteon of the pig bone, irregular Haversian bone was observed only in the sheep bone's periosteal and endosteal borders, only the rabbit bone had primary vascular longitudinal bone tissue and plexiform bone was observed in all large animals. The only similarity found between all the animals studied was the Haversian bone tissue observed in the new or younger bone tissue area of all animals. The quantitative results recorded the following differences; the size of the secondary osteons, the primary osteon's vascular canals, the Haversian canals were higher in the cow bone than the rest of the animals studied and the pig bone had the highest measurements when it came to measuring the area of the primary osteon's vascular canals as well as the minimum diameter of the primary osteon's vascular canals and the secondary osteons. A study by Martiniakova *et al*. (2007) further states that other research studies have observed few quantitative differences between some

mammalian species and the quantitative differences are connected to the variables of the Haversian canals but mainly the secondary osteons diameters. Therefore, the presence of Haversian bone alone should not be used to differentiate between human and non-human bones.

Osteon circularity has been reported to be more circular in non-human bones than human bones due to specific skeletal elements such as connective tissue, the hard structures, semirigid structures, elastic structure, hydrostatic structures and buoyancy force (Crescimanno and Stout, 2012). In addition, osteon circularity has been reported to be higher in areas of the bone under high strain (Crescimanno and Stout, 2012). A study by Dominguez *et al*. (2012) reported that when focusing on the bone's osteon circularity, the human osteon is observed to be elliptical in shape, while the non-human osteons is seen to be circular in shape.

Osteon bands are the linearly orientated primary and/or secondary osteonal systems in bone histology (Andronowski *et al*., 2017). A study by Mulhern *et al*. (2001) reported that osteon banding is more regularly observed in non-human bones such as in sheep and the goat femora than in humans (specifically the occurrence of numerous bands). Another study by Andronowski *et al*. (2017) reported that there are studies which have observed osteon banding in human subadults but since such information is not well documented, researchers have not been using osteon banding as one of the bone microstructures to differentiate between human and non-human bones. More research is currently taking place using different microscopical techniques to investigate if osteon banding can be present or absent in human adult bones (Andronowski *et al*., 2017).

1.4 CONCLUSION

It is well established that microscopic methods can be used to differentiate between human and non-human bones, however, limited research has been done to differentiate between human and non-human burned bones. Since that is the case, deductions can be drawn from research focusing on bone microstructure and bone burning (Bradtmiller *et al*., 1984).

Research shows that there is a great variation between the histomorphology of human and non-human bones even between various animal species such as the cow, pig, deer and sheep (Brits *et al*., 2014). In addition, quantitative variables such as the vascular canals of primary osteon, the area, perimeter, maximum and minimum diameters of the Haversian canals and the

secondary osteons can be used to differentiate between human and non-human bones (Martiniakova *et al*., 2007).

While some research has investigated the quantitative changes as a result of burning such as the changes in the size of the Haversian system and Haversian canals, it is unclear if these changes would affect species identification (Bradtmiller *et al*., 1984; Nelson 1992; Squires *et al*., 2011; Absolonova *et al*., 2012).

While substantial research has been conducted by looking at the histomorphology/ histomorphometry of bone in human and non-human bone, little research has investigated if differentiation is still possible when bone is burned. The burning of bones causes changes to bone macro/ micro structure which can interfere with the ability to differentiate between such bones.

Currently research has focussed more on qualitative analysis of bone microstructure than investigating the quantitative analysis of bone microstructure to differentiate between human and non-human bones and both analyses should be treated as equally important for accurate species identification and differentiation (Crescimanno and Stout, 2012).

In conclusion, more studies need to be conducted to investigate the quantitative and qualitative analysis of bone microstructure between human and non-human bones especially in South Africa and more studies need to be done on the microscopic examination of human and non-human bones burned at higher temperatures in order to implement affordable histological methods to assist in forensic cases where skeletal remains are highly fragmented, burned, degraded or unidentifiable and macroscopic analysis is not an option or often not possible to perform. The positive outcomes will then be used to differentiate between burned and unburned human and non-human bones using such histological methods and this study aims to achieve that.

1.5 AIMS AND OBJECTIVES

The primary aim of this study was to determine the effect of burning on bone histomorphology and morphometry and the secondary aim was to determine if burned human bone can be histologically differentiated from burned non-human bone.

This was achieved through the following objectives:

1. To describe the effects of burning at different temperatures on bone histomorphology and morphometry in human and non-human bone.
2. To compare bone histomorphology and morphometry in burned human and non-human bone.

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CHAPTER 2: JOURNAL ARTICLE

ABSTRACT

Species differentiation using histology is well established, however, difficulties may arise when bone is altered due to burning. The aim of this study was to determine the effect of burning on bone histomorphology and morphometry and the secondary aim was to determine if burned human bone can be histologically differentiated from burned non-human bone. Femur bones of four different species (human (*Homo sapiens*), wildebeest (*Connochaetes gnou*), cow (*Bos taurus*) and pig (*Sus scrofa*)) were analysed during this study. The mid shaft of each bone was equally sectioned into five parts for analysis. One section was used as a control and remained unburned, other sections were burned in an electric furnace at different temperatures (600°C, 700°C, 800°C and 900°C) for 20 minutes. Following burning, bones were embedded in epoxy resin and thin ground bone sections were created for histological analysis. The histological structure of each bone specimen was described and histomorphometric data were recorded. A total of 846 secondary osteons were measured. Species differed histomorphologically at different quadrants. ANOVA testing showed an overall statistically significant difference between the burned and unburned human and non-human bones studied. The statistically significant difference was observed in most parameters measured at different temperatures. Most parameters increased in size at temperatures 0°C-900°C and decreased in size at temperatures 0°C-700°C, 0°C-800°C and 700°C-800°C. Overall, the results showed that heat exposure to bones can affect quantitative and qualitative characteristics of bone but human and non-human bone can still be differentiated under such conditions. This histological method can be used in forensic fire cases.

Keywords: Burned bones; Bone histology; Fire; Species differentiation, Osteon.

1. INTRODUCTION

The identification of human skeletal remains in forensic cases is of outmost importance because not only does is assist in building a biological profile of the deceased but it also assists in determining the cause and manner of death (Carroll and Squires, 2020). However, bone modification due to fire exposure can cause difficulties in the anthropological or morphological interpretation of bones. The burning of human remains can occur in a variety of circumstances including due to accidents such as household fires, aircraft and road traffic accidents, fire explosions, and more rarely as a means of homicide to conceal/ destroy evidence (Fanton *et al*., 2006; Gonçalves *et al*., 2011; Schwark *et al*., 2011). High temperature exposure to human and animal remains can lead to misidentification of bones due to the colour change, bone shrinkage, dehydration, bone fractures or bone fragmentation and in such cases human bones may be misidentified as animal bones or vice versa (Thompson, 2005; Robbins *et al*., 2015). DNA techniques can assist in the identification and differentiation of burned human remains from animal remains. However, such techniques are expensive which is why there are currently histological methods being implemented to assist in analysing burned bones (Andronowski *et al*., 2017).

The two main types of bones present in human and non-human adult skeleton are mature compact bone and spongy bone. Most histological research focuses on using compact bone microstructure instead of spongy bone microstructure for species differentiation as the bone microstructure observed in spongy bone is not as complex as compact bone microstructure (Rho *et al*., 1998; Martiniakova *et al*., 2006a; White *et al*., 2012; Andronowski *et al*., 2017). Spongy bone microstructure is made up of trabeculae which are made by irregular, sinuous convolutions of lamellae while the compact bone microstructure is lamellar in structure and the structure consists of vascular canals (Haversian canals and Volkmann's canals) which carry blood vessels and can give rise to different types of bone such as the Haversian system and the plexiform bone (Rho *et al*., 1998; Martiniakova *et al*., 2006a; White *et al*., 2012; Andronowski *et al*., 2017). The Haversian system consists of secondary osteons which are made by the concentric lamellae, a central Haversian canal, lacunae and canaliculi (Martiniakova *et al*., 2006a). The lacunae are the spaces in the osteon containing osteocytes and the canaliculi connects the osteocytes (Currey, 2012; Andronowski *et al*., 2017). Primary osteons are the vascular canal with no surrounding of the Haversian lamellae, while secondary osteons are surrounded by the Haversian lamellae which makes them the main structural and

functional unit of compact bone (Martinova *et al*., 2006a). Plexiform bone is observed to have a dense network of vascular canals arranged longitudinally, circumferentially and radially and can be laid down in a highly organized and rapid way to accommodate juvenile growth (Currey, 2003; Mulhern and Ubelaker, 2003; Brits *et al*., 2014).

Numerous studies have been conducted to differentiate between human and non-human bone microstructure (Martiniakova *et al*., 2007; Brits *et al*., 2014; Carroll and Squires, 2020). For example, two studies by Martiniakova *et al*. (2006a, b) analysed the difference between the histomorphology and histomorphometry of the femur bone sections or the bone microstructure of adult pigs (*Sus scrofa domestica*), cows (*Bos taurus*), rats (*Rattus nor-vegicus)*, sheep (*Ovis aries)*, rabbits *(Oryctolagus cuniculus)* and human (*Homo sapiens)*. Focusing on the first study by Martiniakova *et al*. (2006a) which only compared the femur bone microstructures of the cows and the pigs, the histomorphology of the study reported that the cow's non-vascular bone tissue formed the inner layer of the medullary cavity and the layer contained concentric lamellae with no presence of vascular canals or the primary and secondary osteons. Moving towards the anterior and posterior sides of the same bone section, dense Haversian bone was observed and replaced at the periosteal surface by a primary vascular plexiform bone tissue which had isolated secondary osteons and numerous primary osteons. Furthermore, the lateral side of the thin bone section only had a non-vascular bone present.

On the other hand, the pig's femur thin bone section displayed the presence of primary vascular plexiform bone tissue which surrounded the medullar cavity and the presence of primary osteons and scattered Haversian system in the same region. The resorption lacunae were observed in the anteromedial and posterior sides of the thin bone sections and dense Haversian system was observed towards the periosteal surface. In addition, primary vascular plexiform bone tissue together with numerous primary osteons and scattered secondary osteons were present and they created the pig's periosteal bone.

The histomorphometry of the studies reported the measurements of the species' Haversian canal, Haversian system and the vascular canals of the primary osteons. The histomorphometry appeared to be similar in the area, perimeter and minimum diameter of the Haversian system, Haversian canals and the primary canals of the vascular canals and statistically significant difference was observed in the measurement of the maximum diameter of the Haversian system, Haversian canals and the primary canals of the vascular canals.

The second study by Martiniakova *et al*. (2006b) added femora of human, sheep, rats and rabbits bones for comparison purposes. When comparing the histomorphology and histomorphometry of the adult human bone to that of the cow and the pig bone, the human thin bone section consisted mainly of dense Haversian system with various secondary osteons while the cow and the pig bone had numerous primary osteons, isolated secondary osteons and primary vascular plexiform bone tissue. The cow's dense Haversian system gets replaced by primary vascular plexiform bone tissue at the periosteal surface. In addition, the area, perimeter, maximum and minimum diameter of the Haversian canals and secondary osteons in human femur bones appeared to be significantly different from the pig and the cow femur bones.

There is a large number of scientific studies focusing on the investigation of the macroscopic changes in burned remains (Mulhern *et al*., 2001; Thompson, 2005; Gonçalves *et al*., 2011; Castillo *et al*., 2013; Schmidt and Symes, 2015). These studies highlight colour change, bone shrinkage, dehydration, bone fractures or bone fragmentation as the morphological changes that occur during heat exposure to human and animal bones (Thompson, 2005; Robbins *et al*., 2015; Schmidt and Symes, 2015). The colour change in bone ranges from ivory/tan colour in unburned bones to black/brown colour in charred bones to grey/white in calcined bones (Schmidt and Symes, 2015). The studies further indicate how macroscopic analysis can be successfully performed in bones exposed to low temperatures (between 100°C-300°C) due to the little damage observed in the physical and chemical structure of bone (Hanson and Cain 2006). However, in forensic anthropological cases involving high temperature exposure to bones, using macroscopic analysis for biological profiling can lead to human bones being misidentified as animal bones due to high bone fragmentation which is known to occur in such conditions (Mulhern *et al*., 2001; Thompson 2005; Castillo *et al*., 2013).

Since bone microstructure of compact bone is used in most studies to assist in differentiating between human and non-human bones by studying the qualitative and quantitative analysis of compact bone microstructure, a similar technique can be used to assist in differentiating between burned human and non-human bones. However, more research needs to be done on the histomorphometric analysis of human and non-human bone in order to fully establish the circumstances under which human bone can be differentiated from animal bone (Martiniakova *et al*., 2007; Brits *et al*., 2014).

This study provides a detailed histological analysis of the burned and unburned human and non-human femoral compact bones. The primary aim of this study was to determine the effect of burning on bone histomorphology and morphometry and the secondary aim was to determine if burned human bone can be histologically differentiated from burned non-human bone. curreying objectives; to describe the effects of burning at different temperatures on bone histomorphology and morphometry in human and non-human bone and to compare bone histomorphology and morphometry in burned human and non-human bone.

2. MATERIALS AND METHODS

This study utilised 17 femoral bone samples collected from 4 different species namely:

1. Humans (*Homo sapiens*) (four femur bones)
2. Wildebeest (*Connochaetes gnou*) (five femur bones)
3. Pig (*Sus scrofa*) (five femur bones)
4. Cow (*Bos taurus*) (three femur bones).

All bone specimens (human and non-human) were obtained from adults except pig (10-12 months) and cow (20-22 months). Human femora (> 20 years old) were obtained from the Department of Human Biology, University of Cape Town and the human femora were sourced from donated cadavers. Cow and pig bones were obtained from local butcheries and wildebeest bones (>20 years old) were obtained from a local hunter. Prior to the study, the bones were de-fleshed using a scalpel, sectioned into five cross-sections of two centimetres thick using a hacksaw along the mid-diaphysis and then stored in a freezer at -20°C. For the preparation of the study, bones were removed from the freezer and allowed to thaw for 24 hours prior to further processing. Previous research studies have not indicated any significant effects on the bone microstructure due to the thawing and freezing of bones (Lander *et al*., 2014).

Ethical clearance for the use of human (HREC REF: 426 /2021) and animal (AEC REF: 021_014) bones specimen was granted for this study by the Faculty of Health Science Human Research Ethics Committee and the Faculty of Health Science Animal Research Ethics Committee at the University of Cape Town. No animals were killed for the purpose of this study.

2.1 Bone burning and thin bone section preparation

The bone specimens from four different species were divided into five different sections. The first bone section was unburned and used as the control section and the four remaining sections were burned at different temperatures (600℃, 700℃, 800℃ and 900℃) in a muffle furnace. Bones were placed in the furnace at the desired temperature and burned for 20 minutes. Following this, bones were removed from the furnace and allowed to cool naturally. Once cooled, bones were embedded in a clear epoxy resin for bone preservation and stabilisation prior to cutting.

The control bone sections were cut into thinner sections (~2mm) using a precision saw (Beuhler Ltd., Lake Bluff, Il, USA), and thin sections were prepared using Frost's rapid manual method as described by Maat *et al*. (2001). Briefly, sections were ground down using an iterative process of different grit waterproof sandpaper (grit 200, 600, 800 and 1200), sunlight liquid soap and water until they appeared semi-translucent (~ 0.5mm thick). Bones were then placed in a petri dish full of water using tweezers and were cleaned using a paint brush and placed on paper towel to dry. The dried bone sections were mounted on glass microscopic slides using Entellen (Merck) and then covered with a glass cover slip. The slides were left to dry overnight before being viewed under a microscope.

Burned bone samples were embedded in clear epoxy resin (KRUSTAL 3O, Advanced Material Technology Pty Ltd., South Africa) prior to be sectioned. The hardener and resin were mixed following the manufacturers guidelines at a ratio of 33:100 by weight. Specimens were left for approximately 24 hours to dry. Once the epoxy dried, thin sections (~2mm) were cut at high-speed precision saw (Beuhler Ltd., Lake Bluff, Il, USA), and sections were prepared in the same manner as for unburned bone.

2.2 Analysis

For analysis, bone specimens were divided into four quadrants (anterior, posterior, lateral and medial) and two periosteal regions (anteromedial and posterolateral), see figure 1. The four quadrants were used for both histomorphology and histomorphometry analysis while the periosteal regions were only used for histomorphology analysis to avoid recalculations of secondary osteons during histomorphometry analysis. All of the measurements were taken from the periosteal region within each quadrant. Different quadrants were examined to ensure

coverage of the whole bone based on expected differences in morphology and morphometry in the different regions as seen by for example Brits *et al*. (2014).

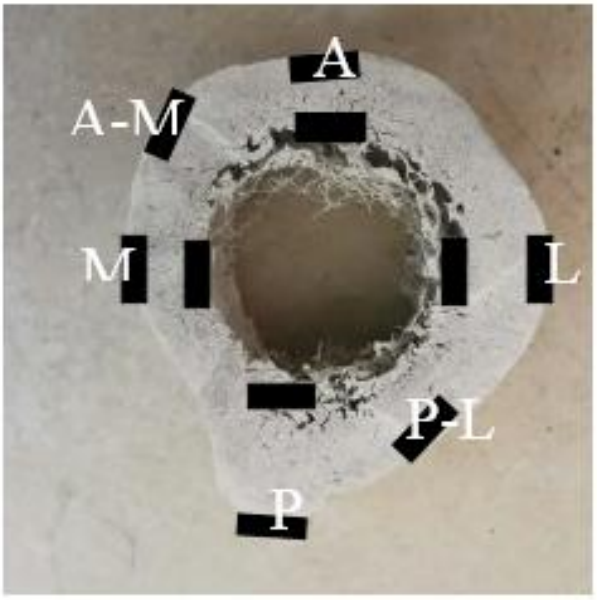

Figure 1: The four quadrants (A-anterior, P-posterior, L-lateral and M-medial as well as the two periosteal regions: A-M (anteromedial) and P-L (posterolateral) viewed under the microscope.

Slides were viewed and photographed using the Leica DM500 compound microscope and software (Leica Microsystem Pty Ltd., Australia). The images for the qualitative and quantitative analysis of all four species were microscopically observed and captured using 4 X magnification.

Histomorphology analysis of the species studied included determining the presence or absence of carbonation, the type of bone (is it a lamellar bone or plexiform bone?), the presence or absence of Haversian system, primary osteons, secondary osteons, central Haversian canals and Volkmann's canal and also the type of Haversian system present (is it dense or regular?). This prevented complete destruction of the bone sample (Crowder and Stout, 2012; Burr *et al*., 2014; Alunni *et al*., 2018).

Histomorphometric analysis of the species studied included measuring the osteon density (n/mm2), osteon circularity (μm^2), area (μm^2), perimeter (µm), maximum and minimum diameter (µm) of the Haversian canals and the Haversian system of a given sample. All parameters were measured using the measuring tool (straight line and polygon selections) in ImageJ version 1.530 (NIH – National Institute of Health, U.S. Department of Health). The

minimum and maximum Haversian canal and Haversian system diameters were determined by multiple measurements spanning the centre of the Haversian canal and Haversian system until the equivalent minimum and maximum was detected.

The following formulae were used to calculate:

Osteon Circularity = 4π (area/perimeter^2)

Osteon density = Number of secondary osteons present in the image/the area of the image or field view.

2.3 Statistical Analysis

The normality of data was assessed using the Shapiro Wilk test. Differences between species within unburned bones and bones burned at 600℃, 700℃, 800℃ and 900℃ were analysed using one-way analysis of variance (ANOVA) for normally distributed data and the Kruskal Wallis test for non-normally distributed data. Differences within a species following burning at different temperatures was assessed using a repeated measures ANOVA. Pairwise differences were assessed post-hoc following Bonferonni correction. Significance of all tests were measured at $\alpha = 0.05$. All statistical tests were conducted using IBM SPSS Statistics for windows, version 25 (IBM Corp, Armonk, N.Y., USA).

3. RESULTS

3.1 Histomorphological analysis of the burned & unburned bones

All the quadrants including the two selected periosteal regions in the human (*Homo sapiens sapiens*) bone specimens consisted of a dense Haversian bone with closely packed secondary osteons.

The anterior, medial and lateral quadrants as well as the anteromedial periosteal region of the cow (*Bos taurus*) bone specimens consisted of primary vascular plexiform bone and irregular Haversian bone with scattered secondary osteons some containing Haversian canals and others Volkmann's canals. The posterior quadrant and the posterolateral periosteal region of the cow bones only consisted of primary vascular plexiform bone.

The anterior, lateral and posterior quadrants of the wildebeest (*Connochaetes gnou*) bone specimens consisted of primary vascular plexiform bone found near the periosteal region and

irregular Haversian bone with closely packed secondary osteons consisting of Haversian canals and Volkmann's canals. In addition, avascular bone was found at the endosteal region of the above-mentioned quadrants. The medial quadrant and anteromedial periosteal region of the wildebeest bone consisted only of the primary vascular plexiform bone. The posterolateral periosteal region consisted of primary vascular plexiform bone and irregular Haversian bone with secondary osteons.

In the pig (*Sus scrofa*) bone, all the quadrants studied including the two selected periosteal regions specimens consisted only of primary vascular plexiform bone.

Human and non-human bones burned at 600℃ had the highest carbonation and the carbonation was observed more in animal bones than in human bones. Bone microstructure at this temperature was visible in human bones and not in animal bones. All human and non-human bones burned at 700℃ had carbonation and bone fragmentation present but that did not block the visibility of the bone microstructure. The human and non-human bones burned at 800℃ had high carbonation and bone fragmentation present in all bone samples which blocked the visibility of the bone microstructure in most but not all bone samples. In addition, the secondary osteons were more visible than the primary vascular plexiform bone in animal bones burned at 800℃. The human and non-human bones burned at 900℃ had less carbonation and the highest bone fragmentation than the lower temperatures selected in this study. Most of the secondary osteons appeared to have melted into the lamellar bone, however, this did not occur in all the selected regions and did not block the appearance of bone microstructure in all samples.

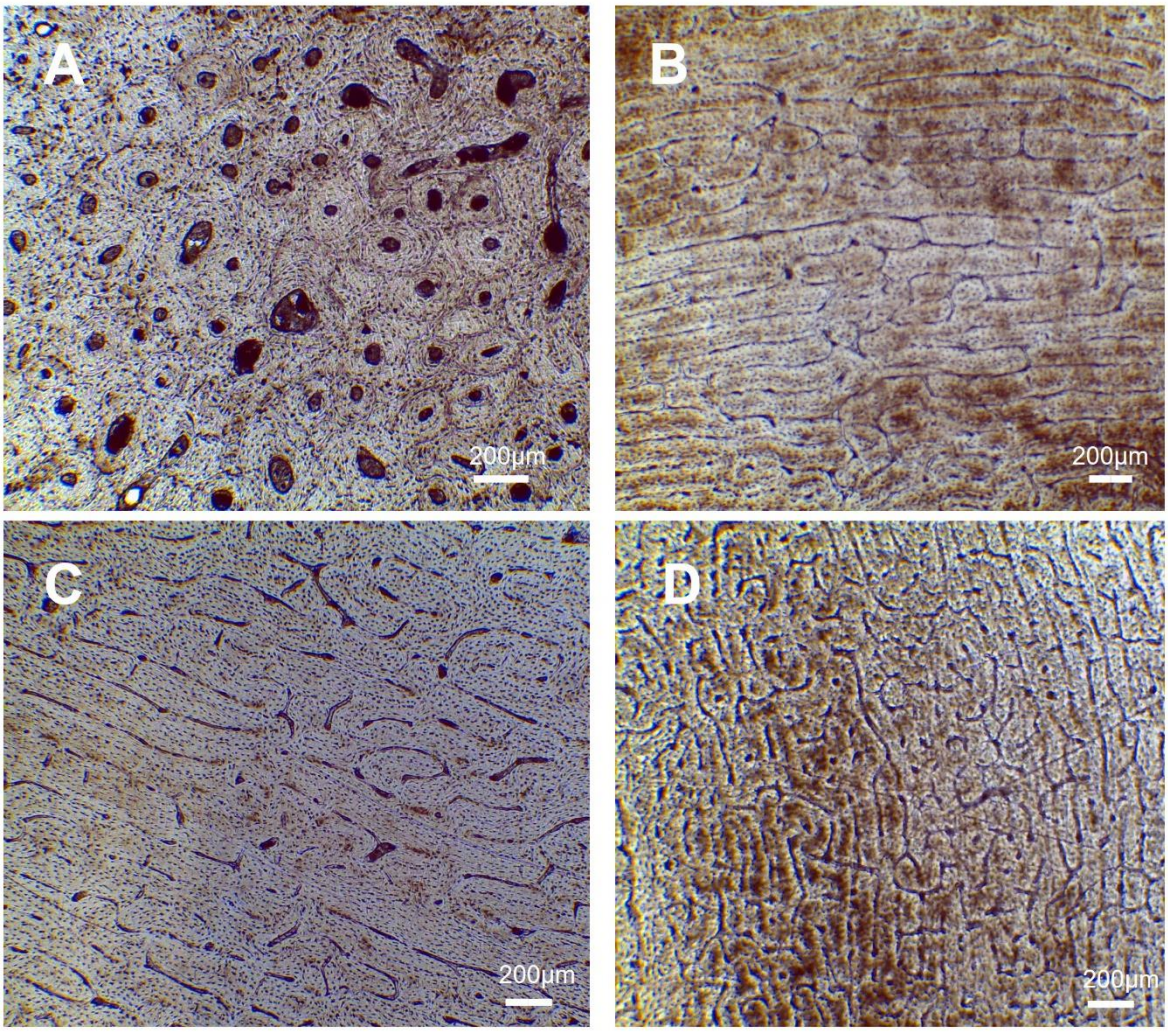

Figure 2: The general bone structure of the unburned human and non-human bones (A-human, B-wildebeest, C-cow and D-pig) studied at 4 X magnification.

3.2 Histomorphometric analysis of the burned & unburned bones

Histomorphometric analysis of the species studied included measuring the osteon density (n/mm2), osteon circularity (μm^2), area (μm^2), perimeter (µm), maximum and minimum diameter (µm) of the Haversian canals and the Haversian system of a given sample. These variables were calculated in each species at temperatures 0°C, 600°C, 700°C, 800°C and 900°C and since the pig samples only showed plexiform bone with no secondary osteons, the pig samples were not included in the quantitative analysis. Comparison of the differences between the species and the analysis of the differences within a species at various temperatures .took place. A total of 234 secondary osteons were measured in the unburned samples. 65 secondary osteons were measured from the human bone samples burned at 600℃ and due to no visibility

or the absence of secondary osteons in the animal bones burned at 600℃, no comparison or quantitative analysis of human and non-human bone microstructure took place under this temperature. A total of 254 secondary osteons were measured in human and non-human bones burned at 700℃, 101 secondary osteons were measured in the human and non-human bones burned at 800℃ and 196 secondary osteons were measured in the human and nonhuman bones burned at 900℃. Different numbers of secondary osteons measured or observed during analysis was due to the presence of high carbonation in most burned samples which hindered the visibility of secondary osteons and due to brittleness of the bones after burning or during bone preparation, most samples were lost.

Table 1: Number of secondary osteons analysed

Names of Species	**Human**	**Pig**	**Cow**	**Wildebeest**	**Total per section**
Unburned bone sections	147	0	21	66	**234**
Burned bone sections at 600℃	61	0	0	0	**61**
Burned bone sections at 700℃	76	0	106	72	**254**
Burned bone sections at 800℃	79	0	9	13	**101**
Burned bone sections at 900℃	47	0	70	79	**196**
Total per species	**410**	**0**	**206**	**230**	

The difference in the means of the measured parameters of the unburned human and non-human femur bones were observed in the species studied. The cow femur bone had the highest minimum and maximum Haversian diameter, the wildebeest femur bone had the highest minimum and maximum canal diameter and the human femur bone had the highest area and perimeter of Haversian system and canal (Table 2). Means of the measured parameters of the unburned human and non-human bones differed from the means of the measured parameters observed in human and non-human bones burned at 700℃, 800℃ and 900℃. The size of the maximum and minimum Haversian and canal diameter decreased from 0℃ to 700℃ while the size of the area and perimeter of Haversian system and canal increased. At 700℃, the human femur bone had the highest mean in all the parameters measured (Table 3). The size of the maximum and minimum Haversian and canal diameter of the human femur bone decreased a lot from 0℃ to 800℃ while there was a slight decrease in the size of the maximum and minimum Haversian and canal diameter of the cow and wildebeest femur bones. In addition, human, cow and wildebeest femur bones had a decrease in the size of the area and perimeter of the Haversian system and an increase in the size of the area and perimeter of canal. Wildebeest femur bone had the highest minimum and maximum Haversian diameter while the cow femur bone had the highest minimum and maximum canal diameter as well as the area and perimeter of Haversian system and the human femur bone had the highest area and

perimeter of canal. The size of the maximum and minimum Haversian and canal diameter of the human and cow femur bone slightly decreased at 0℃ to 800℃ while the size of the maximum and minimum Haversian and canal diameter of the wildebeest femur bone slightly increased. The size of the area of Haversian system of the cow and human femur bones increased while the size of the area of Haversian system of the wildebeest femur bone decreased. In addition, the area of canal and perimeter of canal and Haversian system of human, cow and wildebeest femur bones increased (Table 4). At 900℃, the cow femur bone had the highest minimum and maximum Haversian and canal diameter and the human femur bone had the highest area and perimeter of Haversian system and canal (Table 5).

Table 2: Quantitative analysis of unburned femur bones (secondary osteons) of different species

Name of species & parameters measured:	No. secondary osteons Presen	Variables				
Maximum Haversian diameter (µm)		Mean	Standard Deviation	Minimum	Maximum	Median
Human	147	133.186	32.164	51.470	217.303	133.215
Cow	21	137.737	21.450	99.436	175.852	139.825
Wildebeest	66	121.343	42.830	56.975	210.456	121.139
Minimum Haversian diameter (µm)						
Human	147	119.850	33.946	40.421	209.886	118.364
Cow	21	124.370	21.067	91.173	171.545	122.114
Wildebeest	66	100.328	41.748	32.529	174.729	100.706
Maximum canal diameter (µm)						
Human	147	60.688	38.272	8.624	226.176	55.694
Cow	21	80.465	33.438	16.564	142.091	78.347
Wildebeest	66	93.286	30.304	35.750	176.038	87.394
Minimum canal diameter (µm)						
Human	147	50.055	36.217	4.159	207.675	42.684
Cow	21	60.682	26.936	15.032	106.437	55.551
Wildebeest	66	69.864	28.549	8.963	153.879	64.658
Area of Haversian system (µm)^2						
Human	147	32904.877	12143.616	9312.599	93951.636	32596.221
Cow	21	20489.241	12031.651	7611.496	48066.281	16912.672
Wildebeest	66	27071.823	9497.115	9616.431	51543.165	24889.320
Area of canal (µm)^2						
Human	147	3269.687	2864.241	128.465	21909.892	2606.651
Cow	21	1311.351	567.509	278.,6723	2492.561	1195.250
Wildebeest	66	767.286	1046.168	2.268	7770.258	459.275
Perimeter of Haversian system (µm)						
Human	147	669.904	123.187	374.968	1127.901	669.160
Cow	21	520.427	156.079	329.304	863.522	474.627
Wildebeest	66	610.755	108.882	372.160	879.574	601.559
Perimeter of canal (µm)						
Human	147	209.233	80.540	67.352	575.739	203.320
Cow	21	140.855	31.083	69.497	194.811	139.859
Wildebeest	66	107.502	47.059	44.131	361.897	93.360

Table:3 Quantitative analysis of femur bones (secondary osteons) of different species burned at 700°C						
Name of species & parameters measured:	**No. secondary osteons present**	**Variables**				
Maximum Haversian diameter (µm)		**Mean**	**Standard Deviation**	**Minimum**	**Maximum**	**Median**
Human	76	94.782	38.200	13.222	203.212	83.197
Cow	106	83.250	30.403	22.525	155.373	82.424
Wildebeest	72	82.419	28.209	15.404	179.574	83.507
Minimum Haversian diameter (µm)						
Human	76	95.944	43.082	16.344	200.138	87.658
Cow	106	88.155	30.678	30.709	164.759	82.554
Wildebeest	72	89.028	29.483	26.827	166.113	90.407
Maximum canal diameter (µm)						
Human	76	102.186	77.746	11.707	243.885	63.248
Cow	106	103.928	81.183	4.813	245.705	91.492
Wildebeest	72	81.440	61.175	5.997	219.917	73.177
Minimum canal diameter (µm)						
Human	76	104.856	83.144	10.262	250.865	61.425
Cow	106	98.784	78.124	4.842	250.241	92.269
Wildebeest	72	88.097	68.023	5.495	238.942	75.109
Area of Haversian system (µm)^2						
Human	76	37881.231	18461.659	10786.733	91564.757	35231.520
Cow	106	24153.571	9548.747	10252.525	56221.304	21569.958
Wildebeest	72	25080.088	8440.318	6238.042	47228.301	25150.360
Area of canal (µm)^2						
Human	76	5403.059	4780.524	35.217	18566.063	3924.819
Cow	106	1844.152	1286.351	319.100	8617.998	1554.178
Wildebeest	72	1709.439	1335.514	287.876	7011.072	1240.480
Perimeter of Haversian system (µm)						
Human	76	709.839	178.751	382.304	1140.147	704.194
Cow	106	577.127	108.755	383.955	892.374	554.265
Wildebeest	72	589.725	99.693	311.858	807.644	596.024
Perimeter of canal (µm)						
Human	76	272.834	105.683	97.292	508.211	248.972
Cow	106	172.086	48.345	92.581	349.404	163.712
Wildebeest	72	164.377	50.402	90.573	323.726	149.258

Table:4 Quantitative analysis of femur bones (secondary osteons) of different species burned at 800°C						
Name of species & parameters measured:	**No. secondary Osteons Present**	**Variables**				
Maximum Haversian diameter (µm)		**Mean**	**Standard Deviation**	**Minimum**	**Maximum**	**Median**
Human	79	65.898	19.438	28.826	108.701	68.473
Cow	9	110.665	38.963	70.620	165.749	85.062
Wildebeest	13	126.695	32.309	83.971	200.755	119.745
Minimum Haversian diameter (µm)						
Human	79	70.345	20.434	30.621	116.215	70.384
Cow	9	117.481	35.734	78.729	172.056	101.985
Wildebeest	13	123.103	33.252	73.479	178.271	122.901
Maximum canal diameter (µm)						
Human	79	44.117	15.448	14.397	83.702	44.142
Cow	9	90.732	43.712	42.860	154.395	69.753
Wildebeest	13	80.610	27.082	36.045	123.779	73.522
Minimum canal diameter (µm)						
Human	79	42.641	16.528	15.168	83.821	44.134
Cow	9	90.252	40.259	43.838	152.230	67.114
Wildebeest	13	83.038	970.857	31.159	31.077	87.500
Area of Haversian system (µm)^2						
Human	79	13627.466	7275.123	1306.408	37843.762	12448.519
Cow	9	17000.280	8471.389	7364.599	30306.452	16334.529
Wildebeest	13	11662.878	6462.618	2911.972	20941.840	9816.557
Area of canal (µm)^2						
Human	79	4807.532	4418.538	281.000	24381.117	3708.870
Cow	9	3876.415	2944.027	677.561	7993.446	2770.028
Wildebeest	13	1119.819	845.911	81.934	2887.613	770.621
Perimeter of Haversian system (µm)						
Human	79	427.782	115.618	144.069	715.000	427.098
Cow	9	479.994	121.824	327.189	650.422	491.415
Wildebeest	13	398.528	112.555	221.419	551.779	384.328
Perimeter of canal (µm)						
Human	79	251.988	99.067	79.886	579.032	240.758
Cow	9	239.581	91.052	118.224	341.280	204.430
Wildebeest	13	132.482	45.171	54.592	207.628	128.410

Table:5 Quantitative analysis of femur bones (secondary osteons) of different species burned at 900°C						
Name of species & parameters measured:	**No. secondary Osteons Present**	**Variables**				
Maximum Haversian diameter (µm)		**Mean**	**Standard Deviation**	**Minimum**	**Maximum**	**Median**
Human	47	116.063	19.123	90.419	180.443	111.587
Cow	70	122.091	22.088	71.710	180.598	120.945
Wildebeest	79	115.678	24.685	68.704	210.418	115.235
Minimum Haversian diameter (µm)						
Human	47	115.445	20.194	88.107	189.840	111.614
Cow	70	120.537	21.148	71.415	176.672	117.643
Wildebeest	79	116.887	24.828	71.039	208.378	117.441
Maximum canal diameter (µm)						
Human	47	104.991	36.454	63.419	244.338	98.273
Cow	70	120.302	37.607	32.066	224.743	118.262
Wildebeest	79	103.898	27.301	55.78125	225.562	102.024
Minimum canal diameter (µm)						
Human	47	104.987	41.748	60.722	240.602	95.773
Cow	70	118.055	36.491	34.044	211.716	117.682
Wildebeest	79	102.224	28.178	39.611	233.875	100.476
Area of Haversian system (µm)^2						
Human	47	39223.391	18148.493	13952.898	111938.437	35589.868
Cow	70	21533.239	9203.120	5499.036	61394.703	19561.466
Wildebeest	79	20531.660	5897.718	9807.345	35635.244	19984.308
Area of canal (µm)^2						
Human	47	5591.167	4931.991	1494.629	24025.202	4409.043
Cow	70	1090.887	961.882	215.121	6424.506	798.047
Wildebeest	79	991.685	642.735	251.703	3308.420	782.320
Perimeter of Haversian system (µm)						
Human	47	725.910	155.277	443.823	1248.589	700.734
Cow	70	550.254	115.328	287.092	952.440	531.757
Wildebeest	79	544.240	77.582	383.267	712.972	546.900
Perimeter of canal (µm)						
Human	47	271.997	99.911	157.402	585.480	258.007
Cow	70	138.917	44.646	76.116	346.746	129.581
Wildebeest	79	138.003	39.384	72.464	266.784	134.560

Differences in the means of the measured parameters of the burned and unburned human and non-human femur bones were observed at different quadrants (anterior, medial, lateral and posterior). The mean values of the different parameters at different quadrants and different temperatures for different species can be seen in tables 11-14 (Appendix A).The medial quadrant of the wildebeest femur bone had no secondary osteons to measure. The errors observed under SD (standard deviation) in table 13 are values which were divided by zero.

The mean osteon density varied among the species at different temperatures (Table 6). The human's osteon density was the highest in unburned bone samples and bones burned at 800℃ and the cow's osteon density was the highest in bones burned at 700℃ and 900℃. The errors observed under SD (standard deviation) in table 6 are values which were divided by zero.

Table:6 Comparison of osteon densities measured at different quadrants of different species						
Osteon Density						
Unburned Bones						
Species:	**Human**		**Cow**		**Wildebeest**	
Quadrants:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	1.827	0.141	0.843	Error	1.406	Error
Medial	1.827	0.494	0.843	Error	0.000	0.000
Lateral	2.460	0.070	0.632	0.211	1.312	0.351
Posterior	1.593	0.047	0.000	0.000	1.968	0.497
Bones burned at 600°C						
Species:	**Human**		**Cow**		**Wildebeest**	
Quadrants:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	1.054	0.351	0.000	0.000	0.000	0.000
Medial	1.335	0.351	0.000	0.000	0.000	0.000
Lateral	1.195	0.632	0.000	0.000	0.000	0.000
Posterior	0.703	0.281	0.000	0.000	0.000	0.000
Bones burned at 700°C						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	0.703	0.162	1.897	0.070	0.843	Error
Medial	0.890	0.124	1.687	Error	0.000	0.000
Lateral	0.843	0.281	1.078	0.727	0.984	Error
Posterior	0.843	0.057	1.499	0.204	1.462	0.322
Bones burned at 800°C						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	0.750	0.261	0.351	0.211	0.000	0.000
Medial	0.843	0.293	0.141	Error	0.000	0.000
Lateral	1.406	0.081	0.422	Error	0.703	Error
Posterior	1.171	0.169	0.000	0.000	0.562	0.141
Bones burned at 900°C						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	0.562	0.152	1.265	0.000	0.562	0.000
Medial	0.422	0.081	1.546	Error	0.000	0.000
Lateral	0.515	0.124	1.195	0.122	1.335	0.306
Posterior	0.515	0.248	1.124	0.281	1.359	0.124

The osteon circularity of different species varied at different temperatures (Table 7). The human's osteon circularity was the highest in unburned bone samples and bones burned at 700℃ and 900℃ followed by cow's osteon circularity and wildebeest's osteon circularity respectively. Under the 800℃ temperature, the cow had the highest osteon circularity followed by human's osteon circularity and wildebeest's osteon circularity respectively.

Table: 7 Comparison of different species' osteon circularity at different temperatures				
Osteon Circularity: Unburned Bones				
Species	**Osteon Circularity**	**Standard Error**	**Lower Range**	**Upper Range**
Human	0.89782	0.00292	0.89205	0.90358
Cow	0.88304	0.01129	0.85948	0.90660
Wildebeest	0.87758	0.00533	0.86696	0.88821
Osteon Circularity: Bones burned at 600°C				
Species	**Osteon Circularity**	**Standard Error**	**Lower Range**	**Upper Range**
Human	0.89877	0.00410	0.89057	0.90696
Cow	0	0	0	0
Wildebeest	0	0	0	0
Osteon Circularity: Bones burned at 700°C				
Species	**Osteon Circularity**	**Standard Error**	**Lower Range**	**Upper Range**
Human	0.89510	0.00430	0.88653	0.90367
Cow	0.87864	0.00298	0.87273	0.88455
Wildebeest	0.87728	0.00382	0.86966	0.88490
Osteon Circularity: Bones burned at 800°C				
Species	**Osteon Circularity**	**Standard Error**	**Lower Range**	**Upper Range**
Human	0.86180	0.00532	0.85063	0.87298
Cow	0.87018	0.01609	0.84552	0.89484
Wildebeest	0.84573	0.01199	0.81961	0.87185
Osteon Circularity: Bones burned at 900°C				
Species	**Osteon Circularity**	**Standard Error**	**Lower Range**	**Upper Range**
Human	0.89417	0.00516	0.88378	0.90457
Cow	0.86049	0.00481	0.85089	0.87009
Wildebeest	0.85303	0.00401	0.84505	0.86101

3.2.1 Differences within a species following burning at different temperatures

To determine if there is a significant change in the size of the microstructure of human and non-human bones burned at different temperatures (600℃, 700℃, 800℃ and 900℃), a repeated measures ANOVA was performed on the selected parameters within each species.

For the human bones analysed, repeated measures ANOVA was statistically significant (p<0.001) indicating differences in microstructures between the temperatures analysed. When comparing the means of all parameters of the human bones at different temperatures, the maximum Haversian diameter, maximum and minimum canal diameter, area of canal, area of Haversian system, perimeter of canal and perimeter of Haversian system showed a statistically significant difference at different temperatures (p<0.05). The minimum Haversian diameter decreased at temperatures 700℃ and 800℃, the maximum canal diameter increased at 600℃, 700℃ and 900℃., the minimum canal diameter highly increased at 600℃ and decreased at

800℃., the area of Haversian system decreased at 600℃ and 800℃., the perimeter of canal increased at 700℃., 800℃ and 900℃, the size of the perimeter of the Haversian system decreased at 600℃ and 800℃ and the size of the perimeter at the two temperatures mention were almost the same. The means of the same parameters with no statistically significant differences at different temperatures are reported in table 8 below.

Table 8: Means of parameters with no statistically significant differences at different temperatures in human femur bones

Parameters measured	Between Temperatures:	*p* -value
Maximum Haversian Diameter (µm)	0°C & 900°C	0.721
Minimum Haversian Diameter (µm)	0°C & 900°C	1
Maximum Canal Diameter (µm)	0°C & 700°C	0.129
Maximum Canal Diameter (µm)	600°C & 800°C	0.122
Maximum Canal Diameter (µm)	700°C & 900°C	0.143
Area of Haversian System (µm)^2	0°C & 700°C	0.166
Area of Haversian System (µm)^2	0°C & 900°C	0.364
Area of Haversian System (µm)^2	700°C & 900°C	1
Area of Canal (µm)^2	0°C & 600°C	1
Area of Canal (µm)^2	0°C & 800°C	1
Area of Canal (µm)^2	0°C & 900°C	0.091
Perimeter of Haversian system (µm)	0°C & 700°C	0.978
Perimeter of Haversian system (µm)	0°C & 900°C	1
Perimeter of Haversian system (µm)	600°C & 800°C	0.057
Perimeter of Haversian system (µm)	800°C & 900°C	1
Perimeter of Canal (µm)	0°C & 600°C	0.457
Perimeter of Canal (µm)	0°C & 800°C	1
Perimeter of Canal (µm)	0°C & 900°C	0.153

For the cow bones analysed, repeated measures ANOVA was statistically significant ($p<0.05$) indicating differences in microstructures between the temperatures analysed analysed except within the perimeter of canal ($p=0.039$) and perimeter of Haversian system ($p=0.059$) and minimum Haversian diameter ($p=0.053$). When comparing the means of all parameters of the cow femur bones at different temperatures, the maximum Haversian diameter, maximum canal and minimum canal diameter, area of canal and area of Haversian system showed a statistically significant difference at different temperatures ($p<0.05$). The maximum Haversian diameter decreased at temperatures 700℃, 800℃ and 900℃, the minimum canal diameter highly increased at 900℃., the area of canal decreased at 900℃ and the area of Haversian system slightly decreased at 700℃ and 900℃. The means of the same parameters with no statistically significant differences at different temperatures are reported in table 9 below.

Table 9: Means of parameters with no statistically significant differences at different temperatures in cow femur bones		
Parameters measured	**Between Temperatures:**	***p* -value**
Maximum Haversian Diameter (µm)	0°C & 800°C	0.452
Maximum Haversian Diameter (µm)	700°C & 800°C	1
Maximum Haversian Diameter (µm)	700°C & 900°C	0.102
Maximum Canal Diameter (µm)	0°C & 700°C	1
Maximum Canal Diameter (µm)	0°C & 800°C	1
Maximum Canal Diameter (µm)	0°C & 900°C	1
Maximum Canal Diameter (µm)	700°C & 800°C	1
Maximum Canal Diameter (µm)	700°C & 900°C	1
Maximum Canal Diameter (µm)	800°C & 900°C	0.665
Minimum Canal Diameter (µm)	0°C & 700°C	1
Minimum Canal Diameter (µm)	0°C & 800°C	1
Minimum Canal Diameter (µm)	700°C & 800°C	1
Minimum Canal Diameter (µm)	800°C & 900°C	0.665
Area of Haversian System (µm)^2	0°C & 700°C	0.095
Area of Haversian System (µm)^2	0°C & 800°C	0.0601
Area of Haversian System (µm)^2	0°C & 900°C	1
Area of Haversian System (µm)^2	700°C & 800°C	1
Area of Haversian System (µm)^2	700°C & 900°C	0.234
Area of Haversian System (µm)^2	800°C & 900°C	0.078
Area of Canal (µm)^2	0°C & 700°C	0.68
Area of Canal (µm)^2	0°C & 800°C	0.199
Area of Canal (µm)^2	0°C & 900°C	1
Area of Canal (µm)^2	700°C & 800°C	1
Area of Canal (µm)^2	700°C & 900°C	0.357

For the wildebeest bones analysed, repeated measures ANOVA was statistically significant ($p<0.05$) indicating differences in microstructures between the temperatures analysed except within the maximum Haversian diameter ($p=0.388$), maximum canal diameter ($p=0.446$), area of canal ($p=0.232$) and perimeter of canal ($p=0.438$). When comparing the means of all parameters of the wildebeest bones at different temperatures, the minimum Haversian diameter, area of Haversian system and perimeter of Haversian system showed a statistically significant difference at different temperatures ($p<0.05$).The minimum Haversian diameter decreased at 700°C, the area of Haversian system decreased significantly at 800°C and 900°C and the size of the perimeter of Haversian system decreased a lot at 800°C and 900°C. The means of the same parameters with no statistically significant differences at different temperatures are reported in table 10 below. The minimum canal diameter had a *p*-value of 0.048, however, no statistically significant difference was observed between the different temperatures.

Table 10: Means of parameters with no statistically significant differences at different temperatures in wildebeest femur bones		
Parameters measured	**Between Temperatures:**	***p* -value**
Minimum Haversian Diameter (µm)	0°C & 800°C	0.554
Minimum Haversian Diameter (µm)	0°C & 900°C	0.132
Minimum Haversian Diameter (µm)	700°C & 900°C	0.226
Minimum Haversian Diameter (µm)	800°C & 900°C	1
Area of Haversian System (µm)^2	0°C & 700°C	1
Area of Haversian System (µm)^2	0°C & 900°C	1
Area of Haversian System (µm)^2	800°C & 900°C	0.233
Perimeter of Haversian system (µm)	0°C & 700°C	1
Perimeter of Haversian system (µm)	800°C & 900°C	0.118

3.2.2 Differences between the species of burned and unburned bone

Differences between species' parameters within unburned bones and bones burned at 700℃, 800℃ and 900℃ were analysed. The results of the unburned samples indicated that for all the parameters analysed there was a statistically significant difference between the species (p<0.05) except within the maximum Haversian diameter (p=0.076).

Post hoc tests revealed the pairwise differences between the human and wildebeest's maximum and minimum canal diameter (p<0.001), wildebeest and human's area of canal (p<0.001), cow and human's area of Haversian system (p<0.001), cow and human's maximum and minimum canal diameter (p<0.05), wildebeest and human's perimeter of canal (p<0.001),cow and human's perimeter of Haversian system (p<0.001), the area of canal of wildebeest-cow (p<0.05) and cow-human (p<0.001), the area of Haversian system of cow-wildebeest (p=0.024) and wildebeest-human (p<0.001), the perimeter of canal of wildebeest-cow (p<0.05) and cow-human (p<0.001) as well as the perimeter of the Haversian system of cow-wildebeest (p<0.05) and wildebeest-human (p<0.001).

No statistically significant differences were found between the maximum canal diameter of the cow and wildebeest (p=0.166) and the minimum canal diameter of cow and wildebeest (p=0.226).

The results of the human and non-human bone samples burned at 700℃ indicated that for all the parameters analysed there was a statistically significant difference between the species' area and parameter of canals and Haversian systems (p<0.05). No statistically significant difference was observed within the species' maximum Haversian diameter (p=0.202), minimum Haversian diameter (p=0.855), maximum canal diameter (p=0.138) and minimum canal diameter (p=0.232).

Post hoc tests revealed the pairwise differences between the wildebeest and human's area and perimeter of canals and Haversian systems (p<0.05) and the cow and human's area and perimeter of canals and Haversian systems (p<0.05).

No statistically significant differences were found between the cow and wildebeest's area of canal (p=0.253), area of Haversian system (p=0.238) and the wildebeest and cow's perimeter of canals (p=0.209) and perimeter of Haversian systems (p=0.218).

The results of the human and non-human bone samples burned at 800℃ indicated that for all the parameters analysed there was a statistically significant difference between the species(p<0.05) except within the area of Haversian system(p=0.322) and perimeter of Haversian system(p=0.336).

Post hoc tests revealed the pairwise differences between the human and cow's minimum and maximum Haversian diameter (p<0.001) and the minimum and maximum canal diameter (p<0.001) and the human and wildebeest' minimum and maximum Haversian diameter (p<0.001), minimum and maximum canal diameter and area and perimeter of canal (p<0.001) as well as the wildebeest and cow's area of canal (p<0.05) and perimeter of canal (p<0.05).

No statistically significant differences were found between human and cow's perimeter of canal (p=0.837) as well as the cow and wildebeest's maximum Haversian diameter (p=0.266), minimum Haversian diameter (p=0.737), maximum canal diameter (p=0.860) and minimum canal diameter (p=0.899).

The results of the human and non-human bone samples burned at 900℃ indicated that for all the parameters analysed there was a statistically significant difference between the species (p<0.05) except within the maximum Haversian diameter (p=0.168) and minimum Haversian diameter (p=0.072).

Post hoc tests revealed the pairwise differences between the human and cow's maximum and minimum canal diameter (p<0.001), area and perimeter of canals and Haversian systems (p<0.001), the human and wildebeest's area and perimeter of canals and Haversian systems (p<0.001) and the wildebeest and cow's minimum and maximum canal diameter (p<0.001).

No statistically significant differences were found between the human and wildebeest's maximum canal diameter (p=0.533), the wildebeest and cow's area of canal (p=0.810), area of Haversian system (p=0.837), perimeter of canal (p=0.901) and perimeter of Haversian system (p=0.991).

The r-values for the ANOVA size effect of the unburned human and non-human bones indicated a lesser effect in all parameters; maximum Haversian diameter (r = 0.027), minimum Haversian diameter(r = 0.064), maximum canal diameter (r = 0.149), minimum canal diameter (0.068), area of canal (r = 0.203), area of Haversian system (r = 0.109), perimeter of canal (r = 0.318) and perimeter of Haversian system (r = 0.122).

The r-values for the ANOVA size effect of the human and non-human bones burned at 700℃ also indicated a lesser effect in all parameters; maximum Haversian diameter (r = 0.028), minimum Haversian diameter(r = 0.010), maximum canal diameter (r = 0.017), minimum canal diameter (0.007), area of canal (r = 0.251), area of Haversian system (r = 0.192), perimeter of canal (r = 0.314) and perimeter of Haversian system (r = 0.168).

The r-values for the ANOVA size effect of the human and non-human bones burned at 800℃ indicated a lesser effect in all parameters: the maximum Haversian diameter (r=0.479), minimum Haversian diameter(r = 0.428), maximum canal diameter (r = 0.397), minimum canal diameter (r=0.407), area of canal (r = 0.080), area of Haversian system (r = 0.026), perimeter of canal (r = 0.145) and perimeter of Haversian system (r = 0.024).

The r-values for the ANOVA size effect of the human and non-human bones burned at 900℃ indicated a lesser effect in all parameters: the perimeter of canal (r = 0.470), maximum Haversian diameter (r=0.018), minimum Haversian diameter(r = 0.009), maximum canal diameter (r = 0.051), minimum canal diameter (r=0.041), area of canal (r = 0.379), area of Haversian system (r = 0.334) and perimeter of Haversian system (r = 0.314).

Differences between the species' osteon density at different temperatures (0℃, 700℃, 800℃ and 900℃) were analysed and the results showed a statistically significant difference between the species at all different temperatures studied (p<0.05).

For the unburned samples, post hoc tests revealed pairwise differences between the osteon density of the cow and human (p<0.05) and no statistically significant difference was found between the osteon density of human and wildebeest (p=0.217) and cow and wildebeest (p=0.058).

For the samples burned at 700℃, post hoc tests revealed a pairwise differences between the osteon density of human and cow (p<0.05) and no statistically significant difference was found between the osteon density of human and wildebeest (p=0.145) as well as cow and wildebeest (p=0.349). For the samples burned at 800℃, post hoc tests showed a pairwise differences

between the osteon density of human and cow (p<0.05) and no statistically significant difference was found between the osteon density of human and wildebeest (p=0.208) and cow and wildebeest (p=0.333).For the samples burned at 900℃, post hoc tests revealed the pairwise differences between the osteon density of the cow and human (p<0.001), human and wildebeest (p<0.05) and wildebeest and cow (p<0.05).

The r-values of the ANOVA size effect for the burned and unburned human and non-human bones studied showed a lesser effect in the osteon density at all the different temperatures. For the unburned samples r-value = 0.455, for samples burned at 700℃ r-value =0.302, for samples burned at 800℃ r-value =0.449 and for samples burned at 900℃ r-value =0.451.

Differences between the species' osteon circularity at different temperatures (0℃, 700℃, 800℃ and 900℃) were analysed and the results showed a statistically significant difference between the species at all different temperatures studied (p<0.05) except at 800℃ (p=0.291).

For the unburned samples, post hoc tests revealed pairwise differences between the osteon density of the wildebeest and human *(*p<0.001) and no statistically significant difference was found between the osteon density of wildebeest and cow (p=0.410) as well as human and cow (p=0.244).

For the samples burned at 700℃, post hoc tests revealed a pairwise differences between the osteon density of human and cow (p<0.001) and human and wildebeest (p<0.001) and no statistically significant difference was found between the osteon density of wildebeest and cow (p=0.900).

For the samples burned at 800℃, post hoc tests showed no statistically significant difference between the osteon density of human and wildebeest (p=0.121), cow and wildebeest (p=0.257) and human and cow (p=0.928).

For the samples burned at 900℃, post hoc tests revealed the pairwise differences between the osteon density of the cow and human (p<0.001), human and wildebeest (p<0.001) and no statistically significant difference was found between the osteon density of wildebeest and cow (p=0.187).

The r-values of the ANOVA size effect for the burned and unburned human and non-human bones studied showed a lesser effect in the osteon circularity at all the different temperatures. For the unburned samples r-value = 0.052, for samples burned at 700℃ r-value =0.053, for samples burned at 800℃ r-value =0.014 and for samples burned at 900℃ r-value =0.164.

Overall, differentiation was possible between the human and non-human unburned femur bones and the differentiation was also observed at temperatures 700℃, 800℃ and 900℃ with exception for some measured parameters at temperatures 800℃ and 900℃. At 800℃, no differentiation was observed between the perimeter of canal of human and cow femur bones, minimum and maximum Haversian and canal diameters of cow and wildebeest femur bones as well as the osteon circularity of human and non-human femur bones. At 900℃, no differentiation was observed between the maximum canal diameter of the human and wildebeest femur bones, area and perimeter of canal and Haversian system of the cow and wildebeest femur bones as well as the osteon circularity of cow and human femur bones.

4. DISCUSSION

Fire cases can lead to the difficulty of identifying and examining human remains, for example, a fire in homicidal cases is often used to destroy forensic evidence and, in such cases, caution is needed during the recovery of remains to avoid extra bone damage or fragmentation (Ubelaker, 2009; Schwark *et al*., 2011; Donlon *et al*., 2020). Studies focusing on the investigation of the macroscopic changes in burned human and animal remains highlight colour change, bone shrinkage, dehydration, bone fractures or bone fragmentation as the morphological changes that occur during heat exposure to the remains (Thompson, 2005; Robbins *et al*., 2015). Most of these studies have indicated that a fire with a temperature above 900°C exposed to human remains for a longer period is expected to cause high bone fragmentation and fragility as compared to bones exposed to fires below the temperature of 400°C (Mulhern *et al*., 2001; Thompson, 2005; Ubelaker, 2009; Castillo *et al*., 2013; Robbins *et al*., 2015; Andronowski *et al*., 2017). It has been discovered that heat exposure to bones causes shrinkage of collagen fibers and loss of water (dehydration) bound to bone crysticals, therefore, causing bone shrinkage (Herrmann, 1977; Thompson, 2005.) A study by Bradtmiller *et al*. (1984) indicated that human femur bones burned at high temperatures (600°C) in a small electric oven have osteons size which are uniformly larger than the unburned femur bones. However, a study by Nelson (1992) performed a similar experiment and discovered that the burned bone's osteon size actually decreases at the same temperature (600°C). A current study by Ellingham and Sandholzer (2020) used three dimensional micro-CT measurements to measure the change in volumetric shrinkage of bones exposed to different temperatures and found the results to be almost similar to the two dimensional measurements in the study by Herrmann (1977). A study by Ellingham and Sandholzer (2020) recorded no change in

volumetric shrinkage at temperatures between 400°C and 600°C and a slight increase in volumetric shrinkage from 700°C to 1000°C. The degree of skeletal tissue shrinkage depends on numerous conditions such age, health status, sex, mineral distribution to bones as well as the type of bone (Gejvall, 1965; Thompson, 2005).

In South Africa, approximately 500 deaths and 15 000 fire-related injuries occur annually in Cape Town and of the 18 504 fire incidents recorded between the period of 1990 and 2004, 47% of them occurred in informal settlements. The fire deaths were predominantly linked to shack fire spread and due to the presence of flammables such as candles and heat stoves, such fires ranged between temperatures of 600°C to 1000°C (Western Cape Government, 2015; Walls *et al*., 2017).

Thus, this study aimed to determine if certain histological methods could assist in differentiating between human and non-human bones burned at different temperatures.The temperatures selected for this study were in correspondence to fire temperatures recorded from the fire-mortality crisis in the Western Cape Province (Cape Town) in South Africa (Western Cape Government, 2015).

In this study, the burning process took place in an electric furnace under a controlled environment and the bones were defleshed. In a forensic context, fire cases mostly involve burned remains which before the fire were fleshed. Furthermore, such remains are found in houses, cars or bush fires and the temperatures of such fires vary and depend on accelerants used and flammables present or nearby such as wood, paper, paraffin and petrol. Thus, the results presented in this study may not represent a complex or dynamic burn environment, however, to fully understand the effect of temperature on bone microstructure it is necessary to conduct research under controlled conditions. A study by Ubelaker (2009) stated that fire exposure to fleshed bodies can cause soft tissue changes to occur and depending on the temperature exposure and duration this can cause more bone fractures than if bones were defleshed.

4.1 Histological comparison of burned and unburned human and non-human bones

In this study, the qualitative analysis revealed the main structural bone tissue observed in all quadrants and two periosteal regions of unburned animal (pig, cow and wildebeest) bones to be the primary vascular plexiform bone and an irregular Haversian system which was absent

in unburned human bones. The main structural bone tissue observed in all quadrants and two periosteal regions of the human bone is dense Haversian system which was absent in the animal bones studied. Secondary osteons were found in all species except the pig bone which only displayed primary osteons. The medial and the posterior quadrant of the pig, cow and wildebeest femur bones display no similarities with human bone and this can assist in differentiating between human and non-human bones. The differences observed in bone histomorphology of the different species studied can be due to the rate of bone growth and development, forms of locomotion, environmental conditions, adaptation, nutrition and the total body size. In addition, bone features can also be affected by pathology, nutritional deficiencies and abnormal posture and weight changes which also affect the general histology of such bones (Rho *et al*., 1998; Brits *et al*., 2014).

Most bone microstructures documented from the unburned human and non-human bones were still visible in bones burned at 700°C, 800°C and 900°C, however, due to high carbonation and bone fragmentation, bone microstructure was not visible in animal bones burned at 600°C but the presence of secondary osteons was seen in human bones burned at 600°C.

In bone histology, osteon bands are the linearly orientated primary and/or secondary osteonal systems (Andronowski *et al*., 2017). A study by Mulhern *et al*. (2001) reported that osteon banding is more regularly observed in non-human bones than in human bones especially when looking at the occurrence of numerous bands present in the primary and secondary osteons. In this study, no osteon bands were observed in any of the species studied and this method could not be used to differentiate between the human and non-human bones studied.

When comparing the histomorphology analysis results of the unburned human, cow, wildebeest and pig bones of this study to those reported in the Brits *et al*. (2014) and Martiniakova *et al*. (2006b) studies, the analysis of the human bone recorded in this study corresponds to that reported in the study by Martiniakova *et al*. (2006b). The results in both studies displayed dense Haversian bone tissue as the basic structural pattern observed in human bone. However, in the study by Brits *et al*. (2014), the human bone was described to have irregular and dense Haversian bone. In addition, both studies including this study observed Haversian canals and Volkmann's canals in the human bone tissue. Focusing on the animal bones and keeping in mind the biological and genetic differences between animals in South Africa (used in this study and Brits *et al*. (2014) study) and animals in Germany (used in the Martiniakova *et al*. (2006b) study) the basic structural unit observed in the animal bones in all

three studies was the primary vascular plexiform bone. In the study by Martiniakova *et al*. (2006b), avascular bone was observed in the endosteal region at the posterior and anterior view of the cow bone while in this study the avascular bone was observed in the endosteal region at the posterior quadrant of the wildebeest bone. The pig bone in this study and the Brits *et al*., (2014) study displayed primary osteons while the study by Martiniakova *et al*. (2006b) observed secondary osteons. In addition, in all three studies, the animals with scattered and/or closely packed secondary osteons also had an irregular Haversian bone. Human bones in all the three studies were adults. The pig (10-12 months) and cow (20-22 months) bone age in this study as well as in the Brits *et al*. (2014) study were the same while all the animals in the Martiniakova *et al*. (2006b) study were adults (age not specified).

A study by Castillo *et al*. (2013) reported a successful performance of utilising macroscopic analysis on bones burned at 100°C-300°C due to the little damage present in the bone's physical and chemical structure. The study also highlighted the difficulties of utilising macroscopic analysis on bones exposed to higher temperatures because such temperatures alter the shape, size and colour of the bone and the elasticity of bones. The current study exposed human and non-human bones to higher temperatures (600°C, 700°C, 800°C and 900°C) and performed microscopic analysis to study their bone microstructure and to differentiate them. During the analysis, high carbonation and bone fragmentation played a significant role in the ability to observe and photograph the bone microstructure of the species studied. Bone burned at 600°C displayed extensive carbonation which inhibited the ability to visualise and photograph structures. This was particularly noticeable in animal bones. Since the bones burned at 600°C were not as fragile as the bones burned at higher temperatures and bone microstructure of the human bones burned at 600°C was visible, this finding suggests that human bones burned at 600°C may carry less carbonation than animal bones burned at the same temperature.

A quantitative comparative analysis of the unburned human and non-human femur bones (secondary osteons) was performed in this study and the study done by Martiniakova *et al*. (2006b). The parameters analysed were the area and perimeter of the Haversian canals and Haversian system as well as the minimum and maximum canal and Haversian diameters. In this study, the quantitative results of the unburned samples using the Kruskal Wallis test indicated that for all the parameters analysed there was a statistically significant difference between the species ($p<0.001$) except within the maximum Haversian diameter ($p=0.076$) while in the study done by Martiniakova *et al*. (2006b) the quantitative results of the unburned samples using the Scheffe test indicated that for all the parameters analysed there was a

statistically significant difference between the species only within the perimeter of the Haversian system ($p<0.05$) and the maximum Haversian diameter ($p<0.001$) and not the other parameters studies.

A study by Absolonova *et al*. (2012) focused on the qualitative and quantitative analysis of the human rib bones burned at 700°C and 800°C and the only visible histological variables observed were osteons and Haversian canals, some intact and some fragmented. This study also observed intact and fragmented Haversian system and Haversian canals but from the human femoral bones burned at 600°C,700°C, 800°C and 900°C. Focusing on the comparison of the unburned bones and bones burned at 700°C and 800°C for both studies, the results in the study by Absolonova *et al*. (2012) showed a decrease in the mean of the area of Haversian system, area of canal, perimeter of canal, maximum and minimum canal diameter of the human rib bone as the temperature increased. In this study, the mean of the above-mentioned parameters increased from 0°C to 700°C then decreased from 700°C to 800°C. This study utilised adult human femur bones (older than 20 years) and the study by Absolonova *et al*. (2012) used adult human rib bones (age range of 19-95 years).

In a study by Crescimanno and Stout (2012), osteon circularity was reported to be more circular in non-human bones than in human bones due to specific skeletal elements such as connective tissue, the hard structures, semirigid structures, elastic structure, hydrostatic structures and buoyancy force. Focusing only on the femur bones in both studies and keeping in mind possible biological and genetic differences between animals in South Africa (used in this study) and animals in the United States (used in the Crescimanno and Stout (2012) study), this study showed that the human femur bone is more circular (0.897) than the cow (0.883) and the wildebeest (0.877) femur bones while in the study by Crescimanno and Stout (2012), the femur bones of the animals (dog (*Canus*)(0.874), pig (*Sus*)(0.870) and deer (*Odocoileus*)(0.871)) were almost as circular as the human femur bone (0.867). The pig bone in this study had no visible secondary osteons present to measure their osteon circularity and compare them with that of humans and the results of the study done by Crescimanno and Stout (2012). The absence of secondary osteons in the pig bone samples may be due to the age of the pigs (Mulhern and Ubelaker, 2003). The pig femur bone used in this study had an age range of 10-12 months while the study by Crescimanno and Stout (2012) only mentioned that the animals used in their study were sexually matured without specifying the age range. The osteon circularity measurements for human femur bones (older than 20 years) were higher in this study

than the study done by Crescimanno and Stout (2012), which studied right femur bones of males and females at the age range of 54-78 years.

4.2 Limitations and future work

The process of bone burning and thin bone section preparation was cost-effective but labour intensive. What is observed on a slide under the microscope depends on the level of bone grinding that took place and level of charring of the bone. For example, the posterior quadrant of the unburned cow bone displayed only primary vascular plexiform bone and the other regions showed few secondary osteons while in burned samples, secondary osteons appeared to be more in the cow bone samples and they were also visible in the posterior quadrant. Alternatively, this can be more due to the different locations in the bone shaft of the different sections analysed. To limit variation of this nature an attempt was made to ensure that bones burned at the same temperature were from segments in the same shaft section.

Since burned bone samples are brittle and fragile, the grinding process might have contributed to further bone fragmentation which led to more fragmented secondary osteons which could not be measured for quantitative analysis. While embedding the samples in epoxy resin did help to stabilise the bone samples, some fragmentation during the grinding process still occurred. This resulted in some sections being damaged or not being thin enough to view the bone microstructures under the microscope. This was most notable in the bones burned at 600°C and external lighting was necessary for such burned samples to record the visibility of bone microstructures present. Future studies should include a method that does not increase bone fragmentation. Due to high carbonation in burned bones and the difficulty to grind bone embedded in hard clear epoxy resin, secondary osteons were not visible enough to capture under a microscope without extra external lighting, in our case a torch was used. Future studies might use equipment, microscope or three-dimensional non-destructive methods. Future studies might use equipment or microscope which provide enough lighting to capture more visible bone microstructure for observation and measuring.

Recent advances in imaging technology such as high resolution micro–Computed Tomography (Micro-CT) and synchrotron based methods have been highly beneficial for the non-destructive assessment of materials (Akhter and Recker, 2021). Such systems may be utilised to visualise trabecular and cortical bone structures in three-dimensions and have previously been used for bone morphometric analyses to measure parameters such as bone volume fraction, bone surface

and trabecular number, thickness and separation (Nogueira *et al*., 2010; Particelli *et al*., 2012; Leszczyński *et al*., 2014; Obata *et al*., 2020). While micro-CT systems are becoming more accessible with benchtop instruments becoming more powerful, synchrotron-based CT remains difficult to access (Akhter and Recker, 2021). These techniques are expensive, require specialised instrumentation and expertise, and only allow a limited number of specimens to be imaged at a time. In contrast, the creation of histological thin sections, although time consuming, is effective and relatively inexpensive.

Histological analysis of burned and unburned bone requires the preparation of thin sections for the bones to be observed and analysed under a microscope. Not only are the available protocols for compact thin bone sections time consuming, but it is also difficult to use the protocol on fragile or burned bones. However, the protocols allow for the bone microstructure such as the Haversian system to be visible and studied under the microscope for species identification and differentiation (Martiniakova *et al*., 2007; Maggio *et al*., 2019). In this study the burning process took place in an electric furnace under a controlled environment, future studies may utilise methods and procedures to study the heat exposure in different environments.

This study focused on adult human femur bones, therefore, more research needs to be done to compare burned juvenile human remains to animal remains as they possess a lot of similarities.

5. CONCLUSION

In this study the qualitative and quantitative analysis took place mainly to try and differentiate between burned human and non-human femur bones using histological methods. According to the results, each species differed histomorphologically, especially when examining the unburned bone microstructure using the four quadrants and two periosteal regions. With quantitative analysis the assessment showed that the human femur bone is significantly different from the animal bones, in this case the cow and wildebeest bone. Overall, the results showed that different temperatures exposure to bone can affect the quantitative and qualitative characteristics of bone. These findings assisted in quantitatively and qualitatively differentiating between burned and unburned human and non-human bones studied and the histological methods used can be used in forensic anthropological cases involving remains being exposed to higher temperatures. Both qualitative and quantitative characteristics can be used to provide full and accurate information when differentiating between human and non-human burned and unburned bones.

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APPENDIX A: Tables for different parameters measured at different quadrants and different temperatures for different species

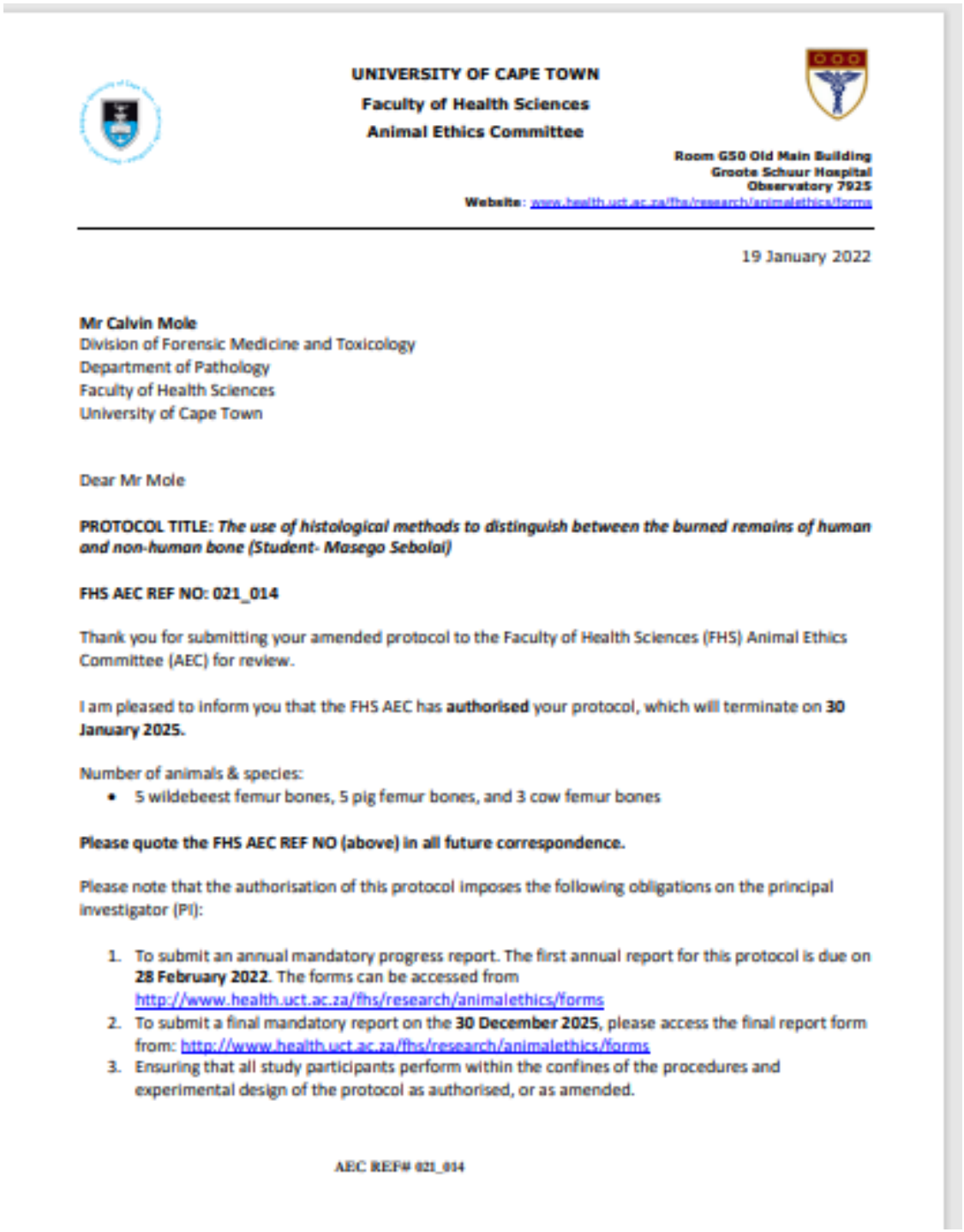
Table:11 Comparison of parameters measured at different quadrants of different speci						
Unburned Bones						
Maximum Haversian Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Quadrants:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	134.541	63.655	132.446	40.529	159.688	20.630
Medial	130.980	63.503	135.027	53.296	0.000	0.000
Lateral	119.778	65.207	143.072	42.578	117.308	31.418
Posterior	150.068	58.479	0.000	0.000	100.310	34.459
Minimum Haversian Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	121.922	58.963	120.950	46.897	136.391	15.713
Medial	112.376	62.914	128.350	47.976	0.000	0.000
Lateral	106.835	60.717	123.996	35.999	98.588	30.001
Posterior	137.341	58.607	0.000	0.000	76.836	32.781
Maximum Canal Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	58.062	30.858	65.031	17.635	136.204	7.978
Medial	55.872	30.022	74.998	28.920	0.000	0.000
Lateral	48.376	27.585	123.996	35.999	83.058	15.175
Posterior	81.731	33.008	0.000	0.000	85.259	25.689
Minimum Canal Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	47.517	22.228	56.132	10.067	118.502	6.240
Medial	43.123	20.794	59.094	21.261	0.000	0.000
Lateral	40.382	20.960	94.398	19.820	59.111	15.802
Posterior	71.046	26.678	0.000	0.000	58.710	13.187
Area of Haversian System						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	33673.538	1644.273	20774.365	5741.403	29341.002	3438.178
Medial	30553.620	1671.183	12537.271	3439.727	0.000	0.000
Lateral	37400.649	2538.200	25600.473	3606.200	25630.479	1654.334
Posterior	30906.879	2034.955	0.000	0.000	28495.725	1965.916
Area of Haversian Canal						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	2835.018	278.785	1524.492	257.056	626.202	100.678
Medial	3223.476	285.299	1231.538	155.061	0.000	0.000
Lateral	4432.320	706.522	1222.466	215.889	789.111	152.936
Posterior	2624.455	516.244	0.000	0.000	887.527	275.496
Perimeter of Haversian System						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	676.747	17.100	529.333	74.690	630.424	39.427
Medial	646.437	19.216	410.307	48.578	0.000	0.000
Lateral	710.909	23.089	587.903	43.277	600.020	18.483
Posterior	646.762	21.115	0.000	0.000	627.332	21.907
Perimeter of Haversian Canal						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	198.537	11.951	153.841	11.831	100.483	7.703
Medial	217.149	9.331	139.953	8.955	0.000	0.000
Lateral	240.988	17.043	132.798	12.426	106.758	7.855
Posterior	179.735	15.199	0.000	0.000	112.023	11.676

Table: 12 Comparison of parameters measured at different quadrants of different specie						
Bones burned at 700°C						
Maximum Haversian Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Quadrants:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	95.613	37.526	75.295	37.320	52.891	37.338
Medial	104.165	28.308	79.245	41.414	0.000	0.000
Lateral	79.998	24.732	88.023	48.674	72.348	41.846
Posterior	97.922	41.063	89.536	42.486	88.538	37.060
Minimum Haversian Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	98.813	34.150	77.907	38.118	73.026	45.892
Medial	100.389	28.872	87.616	47.843	0.000	0.000
Lateral	81.555	26.458	93.038	49.794	78.220	40.799
Posterior	101.423	41.656	93.696	41.488	93.785	37.367
Maximum Canal Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	108.230	16.530	68.337	33.884	59.817	35.705
Medial	99.150	13.801	140.525	44.139	0.000	0.000
Lateral	79.952	26.024	119.130	35.677	42.082	22.790
Posterior	117.487	26.372	95.584	34.135	94.532	43.265
Minimum Canal Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	108.621	10.732	80.742	33.630	67.896	38.234
Medial	97.063	11.981	127.248	47.370	0.000	0.000
Lateral	85.790	16.666	98.071	38.420	44.769	22.069
Posterior	122.972	18.501	93.173	41.435	102.094	44.835
Area of Haversian System						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	31883.309	2606.272	26224.535	2430.849	18792.369	3999.550
Medial	38468.113	4586.394	22817.857	1807.528	0.000	0.000
Lateral	43868.416	4882.986	25297.573	1733.562	21065.724	2007.593
Posterior	36674.930	3905.491	22585.729	1359.743	26886.385	1100.536
Area of Haversian Canal						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	4578.087	718.801	1672.023	180.465	2437.346	477.501
Medial	5426.432	973.981	1669.154	224.413	0.000	0.000
Lateral	5980.533	1350.104	2236.407	419.727	1542.390	241.089
Posterior	6332.991	1010.752	1838.699	170.579	1670.425	199.517
Perimeter of Haversian System						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	654.421	28.589	595.215	26.990	502.893	55.947
Medial	715.333	47.748	561.050	20.650	0.000	0.000
Lateral	770.642	45.121	591.496	18.968	539.203	27.223
Posterior	287.370	22.011	563.596	17.033	613.346	11.762
Perimeter of Haversian Canal						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	252.549	18.211	168.257	8.765	198.717	20.028
Medial	268.198	24.586	161.590	8.287	0.000	0.000
Lateral	275.248	30.363	178.900	14.058	163.058	12.652
Posterior	694.524	35.693	178.292	6.972	160.769	7.040

Table:13 Comparison of parameters measured at different quadrants of different species						
Bones burned at 800°C						
Maximum Haversian Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Quadrants:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	40.846	18.996	136.775	28.181	0.000	0.000
Medial	59.721	30.915	82.102	33.882	0.000	0.000
Lateral	75.915	33.881	76.670	33.584	138.699	32.389
Posterior	74.359	31.677	0.000	0.000	119.193	30.737
Minimum Haversian Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	43.133	21.076	142.190	27.390	0.000	0.000
Medial	68.250	33.925	78.729	24.498	0.000	0.000
Lateral	78.825	35.744	89.218	38.982	136.622	39.940
Posterior	79.094	35.764	0.000	0.000	114.654	37.124
Maximum Canal Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	29.356	12.108	121.889	13.544	0.000	0.000
Medial	42.504	16.282	42.860	9.663	0.000	0.000
Lateral	50.237	16.557	54.762	20.242	78.829	18.000
Posterior	47.382	18.315	0.000	0.000	81.723	23.935
Minimum Canal Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	24.101	8.624	117.285	11.345	0.000	0.000
Medial	44.154	17.049	43.838	6.199	0.000	0.000
Lateral	47.750	14.433	60.668	20.824	89.987	22.599
Posterior	47.288	19.403	0.000	0.000	78.694	25.838
Area of Haversian System						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	12312.375	676.012	13932.067	4420.695	0.000	0.000
Medial	13936.442	929.053	22173.509	Error	0.000	0.000
Lateral	15975.205	2151.185	20389.561	3534.849	14392.450	3527.632
Posterior	19097.471	1744.311	0.000	0.000	9956.896	1850.510
Area of Haversian Canal						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	2900.813	465.787	2820.513	1246.511	0.000	0.000
Medial	4710.297	536.304	2269.607	Error	0.000	0.000
Lateral	11745.371	1914.474	6171.855	1543.748	714.374	256.548
Posterior	3410.212	339.811	0.000	0.000	1373.222	325.544
Perimeter of Haversian System						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	413.431	107.260	427.177	55.210	0.000	0.000
Medial	443.086	15.637	577.416	Error	0.000	0.000
Lateral	592.185	63.427	535.548	40.470	440.100	60.352
Posterior	227.497	9.422	0.000	0.000	372.546	34.104
Perimeter of Haversian Canal						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	198.362	14.461	193.409	37.383	0.000	0.000
Medial	259.346	18.044	195.027	Error	0.000	0.000
Lateral	494.306	57.265	331.385	8.624	107.626	18.282
Posterior	512.914	22.811	0.000	0.000	148.017	15.107

Table:14 Comparison of parameters measured at different quadrants of different specie						
Bones burned at 900°C						
Maximum Haversian Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Quadrants:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	118.125	18.726	134.570	11.294	121.389	11.033
Medial	115.923	12.791	97.962	15.009	0.000	0.000
Lateral	112.361	13.068	122.216	16.817	114.465	12.661
Posterior	116.234	21.644	123.702	15.691	114.903	7.506
Minimum Haversian Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	118.181	17.927	132.834	10.323	119.892	9.510
Medial	115.685	12.800	97.595	14.095	0.000	0.000
Lateral	111.119	12.676	123.089	16.451	114.418	12.341
Posterior	114.765	21.614	120.022	12.214	118.878	7.506
Maximum Canal Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	104.901	6.793	134.664	8.708	109.008	5.907
Medial	109.086	7.247	75.469	11.141	0.000	0.000
Lateral	95.646	8.376	130.992	12.704	99.358	11.742
Posterior	108.671	18.880	122.508	10.456	107.733	7.315
Minimum Canal Diameter						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	108.899	9.393	135.115	9.734	109.308	5.048
Medial	107.744	9.655	68.212	11.242	0.000	0.000
Lateral	95.763	6.688	124.327	12.364	98.277	12.483
Posterior	104.086	14.910	123.661	10.139	104.465	5.684
Area of Haversian System						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	34254.426	2150.623	20646.082	1883.649	18777.192	1605.613
Medial	31346.597	4777.932	20527.088	0.978	0.000	0.000
Lateral	37769.190	4116.666	21588.924	2489.460	18936.083	834.737
Posterior	55517.572	7563.355	22620.315	2221.641	23348.402	1125.902
Area of Haversian Canal						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	4835.574	558.560	1098.657	238.609	1458.441	185.079
Medial	5111.925	1942.702	712.437	165.656	0.000	0.000
Lateral	3654.294	815.861	1029.462	125.412	1027.996	115.722
Posterior	8754.169	1966.772	1302.025	255.885	750.965	74.671
Perimeter of Haversian System						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	687.135	80.248	534.697	23.852	522.980	21.730
Medial	646.350	46.648	538.930	21.513	0.000	0.000
Lateral	720.340	37.960	556.599	33.834	523.273	11.336
Posterior	866.429	56.552	562.618	25.245	580.512	14.307
Perimeter of Haversian Canal						
Species:	**Human**		**Cow**		**Wildebeest**	
Regions:	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Anterior	264.091	13.670	138.083	11.503	167.042	10.653
Medial	249.501	36.185	115.675	8.588	0.000	0.000
Lateral	227.962	23.046	142.089	7.078	141.055	6.966
Posterior	342.021	37.429	147.947	10.965	121.988	4.948

APPENDIX B: ETHICS CERTIFICATES



UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Animal Ethics Committee

Room G50 Old Main Building
Groote Schuur Hospital
Observatory 7925
Website: www.health.uct.ac.za/fhs/research/animalethics/forms

19 January 2022

Mr Calvin Mole
Division of Forensic Medicine and Toxicology
Department of Pathology
Faculty of Health Sciences
University of Cape Town

Dear Mr Mole

PROTOCOL TITLE: ***The use of histological methods to distinguish between the burned remains of human and non-human bone (Student- Masego Sebolai)***

FHS AEC REF NO: 021_014

Thank you for submitting your amended protocol to the Faculty of Health Sciences (FHS) Animal Ethics Committee (AEC) for review.

I am pleased to inform you that the FHS AEC has **authorised** your protocol, which will terminate on **30 January 2025.**

Number of animals & species:

- 5 wildebeest femur bones, 5 pig femur bones, and 3 cow femur bones

Please quote the FHS AEC REF NO (above) in all future correspondence.

Please note that the authorisation of this protocol imposes the following obligations on the principal investigator (PI):

1. To submit an annual mandatory progress report. The first annual report for this protocol is due on **28 February 2022**. The forms can be accessed from http://www.health.uct.ac.za/fhs/research/animalethics/forms
2. To submit a final mandatory report on the **30 December 2025**, please access the final report form from: http://www.health.uct.ac.za/fhs/research/animalethics/forms
3. Ensuring that all study participants perform within the confines of the procedures and experimental design of the protocol as authorised, or as amended.

AEC REF# 021_014

4. Ensuring that all study participants comply with all applicable national legislation, UCT policies, FHS AEC policies and standard operating procedures (SOPs) and national standards (SANS 10386: 2008).
5. Ensuring compliance with DAFF Section 20 requirements.
6. Ensuring that you as the PI immediately alert the FHS AEC to any event involving the welfare of the animals which has occurred during the course of the study, as well as the actions that were taken to respond to these events.
7. Ensuring that you as the PI alert the FHS AEC to any new or unexpected ethical issues that arose during the course of the study, and how these issues were addressed.
8. Ensuring that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals or will be performing the procedures under the direct and continuous supervision of SAVC-registered veterinary professionals or SAVC-registered para-veterinary professionals.
9. If the PI or any study participant is in any way uncertain how to respond to any of these obligations or deal with any of the issues referred to above, they must consult with FHS AEC.
10. All animals found dead must be reported to the RAF on the appropriate form: http://www.health.uct.ac.za/fhs/research/animalethics/forms
11. All animals found in distress must be reported to the RAF on the appropriate form.

My best wishes for successful research and /or teaching endeavour.

Yours sincerely

PROF G. LOUW
CHAIR, FHS AEC

AEC REF# 021_014

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room 650- Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: hrec-submissions@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

12 July 2021

HREC REF: 426/2021

Mr Calvin Mole
Division of Forensic Medicine & Toxicology
FHS
Email: Calvin.mole@uct.ac.za
Student: mamisebolai@gmail.com

Dear Mr Mole

PROJECT TITLE: THE USE OF HISTOLOGICAL METHODS TO DISTINGUISH BETWEEN THE BURNED REMAINS OF HUMAN AND NON-HUMAN BONE-MPHIL CANDIDATE-MS MASEGO SEBOLAI-SUB-STUDY-584/2016.

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

This approval is subject to strict adherence to the HREC recommendations regarding research involving human participants during COVID -19, dated 17 March 2020 & 06 July 2020.

Approval is granted for one year until the 30 July 2022.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

The HREC acknowledge that the student: Ms Masego Sebolai will also be involved in this study.

Please quote the HREC REF 426/2021 in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

HREC/REF426/2021ss

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FACULTY OF HEALTH SCIENCES HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
NHREC-registration number: REC-210208-007

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2020), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI), and Declaration of Helsinki (2013) guidelines. The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

HREC/REF426/2021ss