A multidisciplinary approach to investigate the manifestations of Diffuse Idiopathic Skeletal Hyperostosis (DISH) in modern South African skeletal remains

by

Rachel Lucy Victoria Holgate

Submitted in partial fulfilment of the requirements for the degree

Doctor of Philosophy

PhD (Anatomy)

In the Faculty of Health Sciences

Department of Anatomy

University of Pretoria

Pretoria

2020

Supervisors

Professor Ericka N. L'Abbé

Professor Maryna Steyn

Declaration of original work

I, Rachel Lucy Victoria Holgate, declare that this thesis, which I hereby submit for the degree Doctor of Philosophy in Anatomy at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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DATE: __14.07.2020_____

Acknowledgements

There are many people whose support was instrumental in the completion of this thesis. Firstly, thanks go to my supervisors Ericka and Maryna, without their guidance and support this research would not have been possible. Their proof reading and comments were invaluable throughout the development and eventual completion of the project.

Thanks go to the following people for their assistance during this research project: Grant Hall, University of Pretoria, for his help in the isotopic analysis; Gabrielle Kruger, University of Pretoria, for her help in gaining access to the Pretoria Bone Collection, as well as being a great friend; Mandi Alblas, Stellenbosch University, for her help in gaining access to the Kirsten Collection; Nicholas Bacci, University of the Witwatersrand, for his assistance throughout my analysis of the Raymond A. Dart Collection of Human Skeletons; Brendon Billings, University of the Witwatersrand, for his help in gaining access to the Raymond A. Dart Collection of Human Skeletons; and finally not but least, thanks must go to Jakobus "Kobus" Hoffman, and Lunga Bam from The South African Nuclear Energy Corporation (NECSA), for their technical assistance in accessing and using the micro-CT scanner at the Radiography & Tomography (RADTOM) facility.

I want to thank Clarisa Sutherland, not only for keeping me sane throughout the entirety of my PhD and for being a great person to talk over ideas with, (relevant to the research or not) but also for being a truly wonderful friend.

Lastly, I want to thank my husband Ryan, without whose support, I never would have had the opportunity to start my PhD. He gave me the strength to persevere through the things that life throws at you (particularly our wedding, moving home and country three times in the last two years and the birth of our beautiful daughter).

Abstract

Diffuse idiopathic skeletal hyperostosis (DISH) is a pathological condition that primarily affects the spine. The focus of this study was to investigate the possible link between a diet rich in animal proteins and DISH using carbon and nitrogen isotope analysis, while also making observations on the development and underlying structure of the spinal manifestations of individuals diagnosed with DISH using micro-computed tomography (micro-CT).

This investigation into DISH was undertaken in three steps. Firstly, a macroscopic analysis of DISH was conducted to identify individuals diagnosed with DISH according to accepted diagnostic criteria from three cadaver-derived South African skeletal populations (including the Pretoria Bone Collection, The Raymond A. Dart Collection of Human skeletons, and the Kirsten Collection of Human remains). Secondly, stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) were measured from human bone collagen samples (rib and femur), to investigate the relationship between diet and DISH, specifically a diet high in animal protein. The isotopic values ($\delta^{13}C$ and $\delta^{15}N$ respectively) were compared for both the rib and femur samples jointly and separately, between the DISH group and a control group, to see if differences existed according to sex, ancestry, BMI, BMI and ancestry combined and collection source. Thirdly, the underlying structure associated with the calcification/ossification associated with DISH (in the spine) was assessed using micro-CT scanning.

Across all three skeletal collections, 127 (77% male and 23% female) individuals had characteristics associated with DISH (3.3%), with no difference in prevalence between males and females. DISH was found to be most prevalent among white South African males, indicating something (perhaps genetic or cultural) within the group, giving rise to their propensity to developing the disease. No association between BMI classification and prevalence of DISH was found. Stable isotope analysis indicated that while individuals with DISH generally had elevated $\delta^{15}N$ values, but $\delta^{15}N$ was also high among the control group, with both groups displaying a mean of 13.0‰. Most black South Africans diagnosed with DISH were enriched in $\delta^{13}C$, but relatively depleted in $\delta^{15}N$, while the white and coloured South Africans showed a positive correlation between $\delta^{15}N$ enrichment and $\delta^{13}C$ enrichment. Within the DISH group, overweight individuals were generally more enriched in $\delta^{15}N$ and depleted in $\delta^{13}C$, while the underweight individuals were more enriched in $\delta^{13}C$ and depleted in $\delta^{15}N$. The isotope analyses presents a complex narrative between diet and DISH. While elevated $\delta^{15}N$ values among individuals diagnosed with DISH could be interpreted as consumption of animal

protein, it was not exclusive to those with DISH and was also noted in non-DISH individuals. Any variations in the $\delta^{13}C$ and $\delta^{15}N$ values found between the DISH and control groups are most likely reflecting different dietary patterns, associated with temporal changes, cultural practices, religious and economic factors, independent of DISH. Observations from the micro-CT scans corroborate some of the clinical interpretations within the literature, such as flowing ossification being limited to the right lateral portion in the anterior aspect of contiguous vertebrae (except in one case where situs inversus was a possibility) and retention of the intervertebral disc space. In contrast to the literature, a possible erosive/inflammatory process was noted in the spinal development of DISH, preceding new bone formation. This was characterised by the destruction of the original vertebral wall underneath the fluid ossification/new bone formation. In summary, the finding from the micro-CT scans is worth further exploration in future research, while the isotopic analysis lends no support to the theory that the presence of DISH is associated with a diet high in protein.

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Abbreviations

aDNA – Ancient deoxyribonucleic acid

ALL – Anterior longitudinal ligament

AS – Ankylosing spondylitis

BMI – Body mass index/indices

CAM - Crassulacean acid metabolism

CE – Common era

CT – Computed tomography

DDD – Degenerative disc disease

DISH – Diffuse idiopathic skeletal hyperostosis

KC – Kirsten Collection

MRI – Magnetic resonance imaging

NECSA – The South African Nuclear Energy Corporation

PBC – Pretoria Bone Collection

PLL – Posterior longitudinal ligament

RDC – Raymond A. Dart Collection of Human Skeletons

SES – Socioeconomic status

SIJ – Sacroiliac joint

UK – United Kingdom

VO – Vertebral osteophytes

WHO – World Health Organisation

1. INTRODUCTION

Humans are shaped by their environment, both physically and biochemically, over a relatively lengthy lifespan. Osteologists, biological anthropologists and palaeopathologists seek to understand long term bony changes in ancient and modern human skeletons from an individual level to within and between populations. This is to address issues such as sexual dimorphism, age related changes, bone's response to disease and injury, as well as attempting to reconstruct health and wellbeing.

Standard macroscopic techniques limit the assessment of diseases in the human skeleton, as well as interpretations at a population level, providing less details. Macroscopic analysis is the primary method for exploring skeletal manifestations of disease and can be very informative about the general incidence of disease in a population; however macroscopic analysis of skeletal material is the most basic evaluation of a population's health status. Biochemical and microscopic techniques are employed for a deeper analysis of bone pathology and are often used to bolster or indeed to expand on a macroscopic evaluation. Over the last few decades, the emergence and advancement of auxiliary techniques such as micro computed tomography (micro-CT) scanning, isotopic analysis, ancient DNA (aDNA), scanning electron microscopy (SEM) and radiography have contributed significantly to our ability to gather information from human skeletal remains and to evaluate the manifestation of disease in the human skeleton (Ortner, 2003; Roberts and Manchester, 2010).

Chronic disease and lifestyle have been shown to affect the skeletal system in both past and present populations. Human palaeopathological research evaluates, amongst other things, skeletal manifestations of a general classification and/or particular disease among a group of people as a means to interpret health and behaviour within a population (Ortner, 2003). This avenue of analysis also seeks to expand on our understanding of past peoples using materials such as pots, tools and ornaments of adornment (*e.g.* beads and other types of jewellery), which can be limited. It has been said that "Human remains are the most tangible evidence for understanding how people lived in the past…it is a unique repository for social information concerning the lifestyles and lifeways of past people.." (Gowland and Knüsel, 2006, page ix). Once we start to look deeper into the health status of individuals within a population, inferences can be made about their lifestyle. By looking at the overall health status of a population,

palaeopathologists are able to start to understand the nuances of peoples' lives, bringing the past back to life and putting the flesh back onto the bones.

Palaeopathologists over the last century have been able to identify a variety of factors influencing population health from the study of skeletal remains, contributing to our understanding of health and disease in modern populations (Roberts, 2016). Roberts argues that "…understanding the long history of disease could be claimed to be essential for understanding the origin and evolution of society" (Roberts, 2016, page 3); an important aspect of medicine, that aids in our comprehension of the distribution of diseases in modern populations and how best to tackle them. A tangible example of how palaeopathology is helping in our understanding of diseases in modern societies is in the sequencing of ancient genomes and comparing them to modern examples. This is particularly important in understanding the origin of diseases with a bacterial aetiology and how these bacteria have evolved over time (Trueba, 2014). However, it can be seen as a circle of knowledge; clinical understanding aids in our analysis of disease in archaeological populations, and understanding the health status of individuals in the past, feeding back into our understanding of diseases that are still prevalent in society today.

We know from developments in medicine that health and nutrition are closely linked, for example, humans subsisting on diets deficient in ascorbic acid (vitamin C) can lead to the development of scurvy, while vitamin D deficient diets lead to diseases such as rickets in children, or osteomalacia in adults (Ortner, 2003). A disease with bony manifestations that possibly relates to lifestyle choices such as poor diet and nutrition is diffuse idiopathic skeletal hyperostosis (DISH) (Rogers and Waldron, 2001; Müldner and Richards, 2007; Spencer, 2008). This disease occurred in the past and is relevant today, although its aetiology is still poorly understood.

DISH has been discussed in the clinical literature with great interest, but with little agreement between clinicians on the disease's pathogenesis, diagnosis, patient risk factors and aetiology (Forestier and Rotés-Querol, 1950; Littlejohn and Hall, 1982; Resnick and Niwayama, 1988; Shirakura et al., 2000; Kiss et al., 2002a; Sencan et al., 2005; Denko and Malemud, 2006; Eckertova et al., 2009; Reuven Mader et al., 2009; Ikeda et al., 2011; Zincarelli et al., 2012; Haddad et al., 2013; Mader et al., 2013, 2015; Arad et al., 2017; Mader et al., 2017). Patients diagnosed with DISH have reported symptoms such as back and/or neck pain, restriction of mobility of the spine, peripheral joint affection, obesity, and resulting

cardiovascular comorbidities, while some individuals are asymptomatic (Artner et al., 2012). Links between various metabolic conditions and DISH have been explored and include comorbidities of obesity, adult onset type 2 diabetes mellitus, gout, and hyperuricemia with DISH (Littlejohn and Hall, 1982; Shirakura et al., 2000; Kiss et al., 2002a; Sencan et al., 2005; Denko and Malemud, 2006; Eckertova et al., 2009; Mader et al., 2009; Ikeda et al., 2011; Zincarelli et al., 2012; Haddad et al., 2013; Mader et al., 2013). DISH also has a propensity to affect males more than females, with varied reported ratios, but with males consistently being more affected than females (Rogers and Waldron, 1995; Ortner, 2003; Roberts and Manchester, 2010). Another interesting development which has made academics and clinicians alike, rethink their understanding of DISH, is the suggestion that the ossification could be preceded by a local inflammatory process (Mader et al., 2015; Arad et al., 2017; Mader et al., 2017). This is contrary to the widely accepted understanding of DISH being a non-inflammatory condition, however, further investigation is needed to corroborate findings (Mader et al., 2017). Within the palaeopathological literature, DISH has been described as a type of joint disease or simply as a miscellaneous disease (Rogers and Waldron, 1995; Ortner, 2003; Roberts and Manchester, 2010), while clinically, DISH has featured regularly within journals that centre on rheumatic diseases (Mader et al., 2017).

The treatment for DISH focusses on pain management and “stiffness”, so as to improve the patients’ general quality of life. This is achieved through the prescription of nonsteroidal anti-inflammatory drugs for pain management and physical therapy. Other treatments focus on weight reduction and increased physical activity for the patient, with a diet low in saturated fats and carbohydrates, aiding in the prevention and management of comorbidities most frequently observed in clinical cases of DISH (Mader et al., 2017). However, it is not clear if early and effective treatment of associated conditions such as type 2 diabetes mellitus and obesity can prevent the formation of the entheses, or if there is indeed any link at all (Mader et al., 2013). Osteological analysis can help in exploring some reasons for the manifestation of DISH that may be useful for application in a clinical setting.

Osteologically, DISH primarily affects the spine and presents with a flowing ossification (a 'candle wax' appearance) along the anterior longitudinal ligament (ALL) on the right anterolateral surface of contiguous vertebrae. Ossification along the ALL may lead to eventual ankylosis of the spine (Forestier and Rotés-Querol, 1950; Figure 1.1). There is discussion as to whether the ALL is the structure that ossifies to create the 'candle wax'

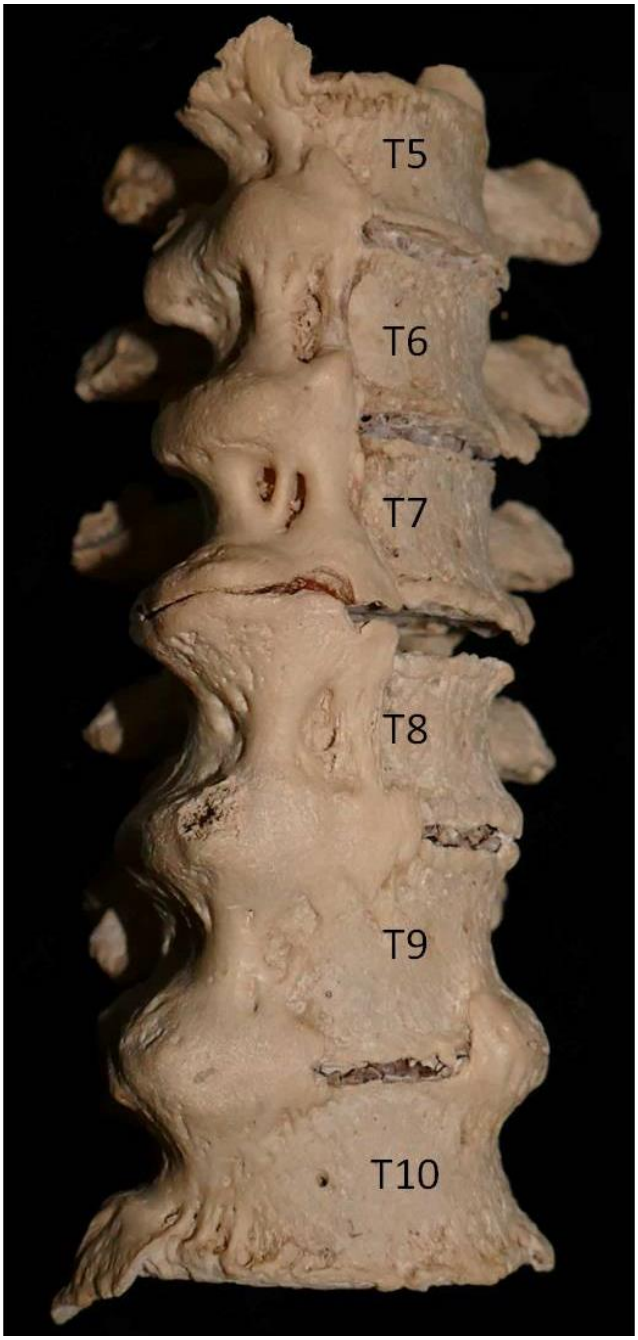

Figure 1.1: An osteological case of DISH. Note the 'candle wax' appearance of the ossification. Individual AN427, KC, Stellenbosch University. Source: Author.

appearance or whether it is entirely new bone formation. In the first descriptions of the disease, authors investigating DISH noted that the new bone formation or hyperostosis was "…independent of the anterior vertebral common ligament…" (Forestier and Rotés-Querol, 1950, page 328) and "…that the ligamentum longitundinale anterius is partially integrated in these bony proliferations at the level of the vertebral edges and the predisc spaces (although

this ligament remains free in front of the actual vertebral bodies)…" (Forestier and Lagier, 1971). The confinement of the ossification to the right anterolateral surface of the vertebral bodies has been attributed to the pulsation of the descending aorta, which is said to prevent ossification (Forestier and Lagier, 1971). DISH is not limited to the spine alone (Resnick et al., 1975), with the condition also being associated with the ossification/calcification of tendons, ligaments and capsule insertions (entheses) occurring at multiple peripheral sites (Resnick et al., 1975; Resnick and Niwayama, 1988; Rogers and Waldron, 2001; Mader et al., 2009; Roberts and Manchester, 2010).

Although the incidence of DISH is reported to be relatively high in archaeological groups, the prevalence varies markedly among modern populations as reported in the clinical literature (Cassim et al., 1990; Weinfeld et al., 1997; Mazières and Rovensky, 2000; Sarzi-Puttini and Atzeni, 2004; Gorman, 2005). This may be because clinical diagnosis relies on radiography (Resnick et al., 1975) which is not always so readily available or conducted: it may be that particular patients who have DISH do not meet the requirements for a clinician to justify an individual's exposure to the ionising radiation present in medical exposure (The Royal College of Radiologists, 2000). This varied prevalence in modern populations may also be due to the condition going undetected for a long time as many patients are asymptomatic (Artner et al., 2012); or it may be that the symptoms that are recorded as associated with DISH (Artner et al., 2012) are shared with other diseases/conditions and can be difficult to differentiate diagnostically. This provides palaeopathologists with the unique opportunity to use human skeletal remains to tackle research questions that have relevance in a clinical context, as well as to evaluate previous interpretations of archaeological remains.

The exact aetiology of DISH is unknown, but type 2 diabetes mellitus, obesity and increasing age (+50 years) have been highlighted as risk factors (Cassim et al., 1990; Kiss et al., 2002a; Eckertova et al., 2009; Reuven Mader et al., 2009; Zincarelli et al., 2012). The current investigation explores aspects of a presumed diet (specifically, high levels of animal protein) as a possible causative agent of DISH (Rogers and Waldron, 2001; Mader et al., 2009; Roberts and Manchester, 2010). This idea of DISH being related to individuals with high protein/high calorie diets began with the investigation of skeletons discovered at the late medieval archaeological site of Merton Priory (1140 – 1540 CE) in Surrey, England (Waldron, 1985). During archaeological excavations and osteological analysis, high incidences of individuals were observed to have had DISH. Waldron, in his 1985 paper entitled "DISH at

Merton Priory: evidence for a "new" occupational disease?" suggested that the condition could be aligned to the eating habits of the monks working in the monastery. The use of occupation in this context seems more like a phrasing intended to represent a lifestyle choice, which could in this case, be linked to diets of excess. The diets of the monks of the late medieval period in England consisted of foodstuffs such as fish, pigs, swans, quails, pigeons, geese, ducks and pheasants, to name but a few (Waldron, 1985; Bishop, 2001; Rogers and Waldron, 2001). These diets were synonymous with gluttony and from the description of the foodstuffs eaten, it has been suggested that these diets would have exceeded the recommended allowance (by today's standards) of protein and calorific intake, perhaps resulting in the development of obesity and/or type 2 diabetes mellitus, both identified in the literature as being linked to the development of DISH (Cassim et al., 1990; Kiss et al., 2002a; Eckertova et al., 2009; Reuven Mader et al., 2009; Zincarelli et al., 2012). This would suggest that an investigation into the dietary components of individuals diagnosed with DISH would be useful in assessing a possible dietary aetiological source. Other archaeological sites that have been under investigation for a possible link between diet and DISH, using stable isotope analysis, includes the Gilbertine priory of St. Andrews, Fishergate (York, UK), Blackfriars Friary (Gloucester, UK), St. James Abbey (Northampton, UK), Royal Mint Site (London, UK), Blackfriars Friary (Ipswich, UK), The Hospital of St. James and St. Mary Magdalene (Chichester, UK), Hereford Cathedral (Hereford, UK) (Müldner and Richards, 2007; Spencer, 2008) and the Carmelite friary (Aalst, Belgium) (Quintelier et al., 2014).

Stable light isotope analysis is the main tool used by archaeologists (and other academics in similar fields), to assess the dietary components of past populations. At the most basic level, analysis is primarily achieved by comparing the carbon and nitrogen stable isotope ratios in a consumer's tissue/s, to the carbon and nitrogen isotope ratios present in their food sources (Fry, 2006; Roberts and Manchester, 2010; Reitsema, 2012). However, stable isotope analysis cannot establish a list of the specific foodstuffs eaten or their proportions in an individual's diet (Spencer, 2008). Through the use of stable carbon isotope analysis ($\delta^{13}C$), we are able to decipher whether diets were from a predominantly terrestrial or marine based ecosystem (Chisholm et al., 1982; Schoeninger and DeNiro, 1984), while the use of nitrogen stable isotopes ($\delta^{15}N$) in assessing collagen can give an indication of a consumers trophic level and dietary protein sources (Ambrose and Norr, 1993; Howland et al., 2003). Unfortunately, no differentiation can be made between the source of protein from the same animal when meat is compared to dairy products (O'Connell and Hedges, 1999). Stable isotope analysis has been

applied to investigate the potential link between diet and DISH, with no definitive answer as to their association (Müldner and Richards, 2007; Spencer, 2008; Quintelier et al., 2014).

1.1. Aim

The aim of this research is to assess a possible link between diet and DISH, while also making observations on the development and underlying structure of the spinal manifestations of individuals diagnosed with DISH.

Stable light isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) will be used to assess the relationship between diet and DISH, while micro-CT analysis will be used to make observations on the development of the spinal manifestations and to record features in the underlying structures. The macroscopic observations and dietary analysis will be used to assess differences that may exist within the individuals diagnosed with DISH according to sex, ancestry and BMI classification (where the information exists).

1.2. Objectives

The main objectives set out in this research study to achieve the abovementioned goals are as follows:

1. To specify criteria by which to diagnose DISH, taking into consideration clinical and osteological recommendations.
2. To analyse and make a macroscopic diagnosis of DISH across the three major skeletal collections in South Africa, namely the Pretoria Bone Collection, housed at the University of Pretoria; the Kirsten Collection, housed at the University of Stellenbosch; and the Raymond A. Dart Collection of Human Skeletons, housed at the University of Witwatersrand – the majority of skeletons within each collection date from the 20th century.
3. To assess the incidence of DISH between the sexes and different populations, to gain more information on its development and propensity to affect certain groups over others.
4. To take samples of bone (rib and femur) for isotope analysis. The results from individuals diagnosed with DISH will be compared to a control group to assess a possible dietary hypothesis for the development of DISH.

5. To statistically analyse the isotopic data ($\delta^{13}C$ and $\delta^{15}N$) for both the rib and femur samples separately and jointly, between the DISH group and control group, to see if differences exist according to sex, ancestry, BMI, BMI and ancestry combined and collection source.
6. To utilise micro-CT scanning of individuals diagnosed with DISH to see if any observations on features associated with the ossification/calcification and new bone growth associated with DISH (in the spine) can contribute to our understanding of the disease.

While the population being investigated is considered modern, the stance of this study is very much a bioarchaeological one, mainly because the primary source of evidence is that of the skeletons themselves.

2. LITERATURE REVIEW

This chapter will cover a wide range of topics, starting with an explanation of DISH, how it is diagnosed in clinical and palaeopathological contexts respectively, as well as discussing the differential diagnosis of DISH in human skeletal remains. Studies using different imaging modalities and their contribution to understanding DISH will also be discussed. One section in this chapter will cover the basic principles of stable isotope analysis as well as the basic chemistry of carbon and nitrogen stable light isotope analysis. Stable light isotopes are a major tool of this study because they allow for the evaluation of parts of an individual's diet to be explored. The section on stable light isotope analysis will discuss a variety of factors that can account for variation in values such as fractionation rates, variations in sampling different bodily tissues and the generally expected pattern of carbon and nitrogen isotopic values for different foodstuffs. Using all of the aforementioned information on stable light isotope analysis, the author discusses the use of carbon and nitrogen isotope analysis for interpreting diet using three example studies. Finally, this chapter will give an overview of the presumed diets of South Africans groups during the 20th century.

2.1. What is DISH?

Previously referred to as Forestier's disease and ankylosing hyperostosis in both clinical and palaeopathological literature, DISH was first described as a specific disease in 1950 (Forestier and Rotés-Querol, 1950). In the medical literature, the condition was described as early as the 19th century (Smythe and Littlejohn, 1998) and is truly a disease of antiquity, with positively identified cases of DISH in Neanderthal skeletal remains and cases from ancient Egypt (Crubézy and Trinkaus, 1992; Hussein et al., 2009; Rogers and Waldron, 1995; Saleem and Hawass, 2014).

DISH is characterised by excessive production of bone at entheses and joint margins, and is seen particularly in the spine. Ossification of the ALL produces the smooth and flowing 'candle-wax' appearance on the right anterolateral surface of the spine, and is pathognomonic of the disease. The ALL is a ligament that runs down the anterior surface of the spine, from the base of the skull to the sacrum, as shown in red in Figure 2.1. It does not cover the entire surface of the vertebral bodies, but is restricted to the anterior surface (roughly 2.5cm wide) where it blends into the periosteum or attaches directly to the anterior surface of each vertebrae (Deer et al., 2009; Netter, 2014). In more advanced stages of DISH, the spine (and ALL) eventually

fuses with the underlying cortex of the vertebral bodies remaining intact (Smythe and Littlejohn, 1998).

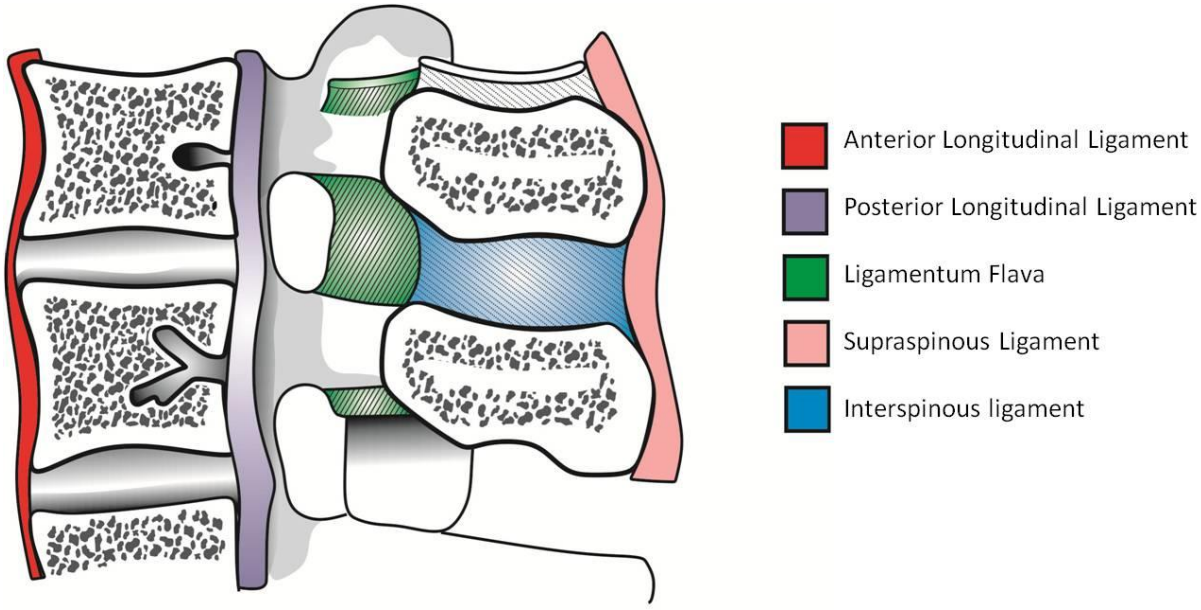

Figure 2.1: Sagittal cross section of the spine and its associated ligaments. Source: redrawn from Theakston (2020).

With the ALL, the ossification of the spine (specifically located in the thoracic region) is often limited to the right side of the anterior surface of the vertebral bodies. Many researchers have attributed this characteristic ossification to the pulsation of the abdominal aorta, which may prevent ossification on the left side of the anterior surface of the vertebral bodies (Mader, 2002; Sarzi-Puttini and Atzeni, 2004). In Figure 2.2, an example of DISH is shown; note the candlewax appearance along the anterior vertebral spine and the lack of involvement of the superior and inferior articular facets of the vertebrae (red circle) as well as the articulation of the vertebral bodies (blue oval).

The ossification of the posterior longitudinal ligament (PLL) is also discussed as a fairly frequent characteristic of DISH, and is described in the medical literature (Resnick and Niwayama, 1976; Resnick et al., 1978; Pouchot et al., 1987; Trojan et al., 1992; Ehara et al., 1998; Vigorita, 2008). However, the PLL is rarely addressed in palaeopathology. Whether this is because the location of the PLL makes observations difficult to assess, especially when ankylosis takes place, or because the feature has not been observed (or noted) in palaeopathological individuals before, is not clear.

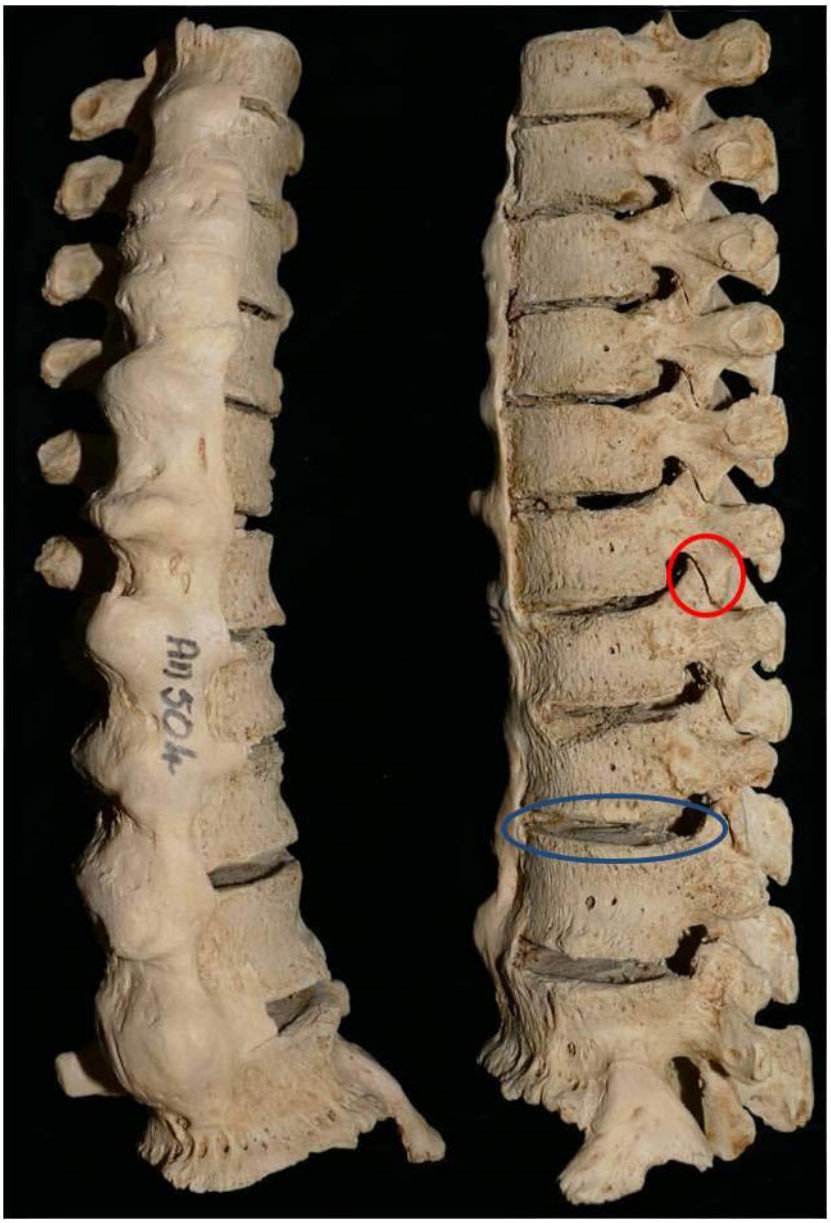

Figure 2.2: Anterior view (left) and left anterolateral view (right) of T4-L1 showing a clear delineation of the ossification/new bone growth, attributed to the pulsation of the aorta. Note the retention of the intervertebral disc space (blue oval) and the apophyseal joint space (red circle). Individual AN504 from the KC, Stellenbosch University. Source: Author.

2.1.1. DISH in a clinical context

Some patients with DISH can be asymptomatic. In a study on DISH, 5 individuals who were meant to be incorporated into a "healthy" control group were discovered to have clinical manifestations of DISH. The patients were completely unaware (Mata et al., 1997). Symptoms of DISH include back and/or neck pain, restriction of mobility of the spine, peripheral joint affection, dysphagia due to distortion or compression of the oesophagus, foreign body

sensation, hoarseness, myelopathy due to ossification of the PLL, hyperuricemia, obesity, hypercholesterolemia, and resulting cardiovascular comorbidities (Artner et al., 2012).

Many studies have established a connection between various metabolic conditions and DISH. The extent to which metabolic diseases play an aetiological role in the development of DISH appears to be very complex and is being further investigated by clinicians. Pillai and Littlejohn (2014) published an extensive overview of clinical data, focussing on the association between metabolic factors such as cardiovascular diseases, and DISH. A trend in the comorbidities of obesity, adult onset type 2 diabetes mellitus, glucose intolerance, adipokines (leptin), gout, hyperinsulinemia and hyperuricemia have all been identified with DISH (Littlejohn and Hall, 1982; Shirakura et al., 2000; Kiss et al., 2002a; Sencan et al., 2005; Denko and Malemud, 2006; Eckertova et al., 2009; Mader et al., 2009; Ikeda et al., 2011; Zincarelli et al., 2012; Haddad et al., 2013; Mader et al., 2013). Many of these conditions have shared associations, for example, adipokines (cytokines secreted by adipose tissue), are increased in obese individuals and leptin levels (an adipokine) have been shown to have an effect in promoting an increase in cortical bone (Li et al., 2007). Of all the aforementioned diseases linked to DISH, the most frequently investigated are Type 2 diabetes and obesity (Julkunen et al., 1971; Boachie-Adjei and Bullough, 1987; Mata et al., 1997; Coacciolo et al., 2000; Kiss et al., 2002a; Sarzi-Puttini and Atzeni, 2004; Spencer, 2008; Mader et al., 2009; Zincarelli et al., 2012; Pillai and Littlejohn, 2014). The link between obesity and type 2 diabetes is a close one, with obesity frequently being the most common risk factor for type 2 diabetes (World Health Organization, 2016). Type 2 diabetes is a disease with increasing prevalence in modern societies with the number of cases in 2014 at 422 million, compared to 108 million in 1980. As expected, the trend in obesity is much the same, with a worldwide report in 2016 finding 39% of adult men and 40% of adult women obese (World Health Organization, 2016).

Generally, the treatment for DISH focuses on pain management and mobility but treatment guidelines are often tailored specifically for patient's needs (Mader et al., 2017). Nonsteroidal anti-inflammatory drugs (NSAIDs) are prescribed for pain and physical therapy to improve the range of movement. Weight reduction and physical activity with a diet low in saturated fat and carbohydrates are other non-invasive therapeutic interventions in the prevention and management of comorbidities most frequently observed in clinical cases of DISH. In severe and rare cases, a patient may require surgical intervention to remove excess bone at joint margins (Mader et al., 2017). However, it is not clear if early and effective

treatment of associated conditions such as type 2 diabetes mellitus can prevent the formation of the entheses, or if there is indeed any link at all (Mader et al., 2013).

In a clinical setting, the diagnosis of DISH is difficult and primarily relies on radiological evidence of bone forming bridges across vertebral bodies, with thoracic vertebrae being most commonly involved (Smythe and Littlejohn, 1998). Additionally, the superior and inferior vertebral facets are never involved (no evidence of bony ankylosis) and the intervertebral disc space is retained (Taljanovic et al., 2009).

No specific classification exists for DISH as its aetiology is officially unknown (idiopathic), but a relationship with type 2 diabetes mellitus, obesity and age has been suggested (Rogers and Waldron, 2001; Mader et al., 2009; Roberts and Manchester, 2010). A genetic component to the disease has also been explored, with little success in finding a definitive answer (Pappone et al., 1996; Sarzi-Puttini and Atzeni, 2004; Gorman, 2005; Spencer, 2008). As well as a suggestion that individuals with DISH could be "bone-formers" (Rogers et al., 1997; Waldron, 2009), especially when one looks at the co-occurrence of DISH and other disorders such as hyperostosis frontalis interna (Smythe and Littlejohn, 1998; Wilczak and Mulhern, 2012), however, further investigation is needed. What is certain is that the prevalence of DISH increases with age, with males over 45 years being more vulnerable to the disease than their female counterparts (Forestier and Rotés-Querol, 1950; Pappone et al., 1996; Rogers and Waldron, 2001; Ortner, 2003; Roberts and Manchester, 2010). Another interesting development which has made academics and clinicians re-examine their clinical understanding of DISH is the suggestion that a local inflammatory process could precede the ossification (Resnick and Niwayama, 1976; Mader et al., 2015; Arad et al., 2017; Mader et al., 2017). Resnick and Niwayama (1976) noted in their description of DISH that "hypervascularity and an occasional mild chronic inflammatory cellular infiltrate surrounding the ALL adjacent to the degenerating annulus". This is contrary to the previous understanding of DISH being a non-inflammatory condition, however, further investigation is needed to corroborate the suggestion of an inflammatory response (Mader et al., 2017).

A higher frequency of DISH has been shown in certain population groups over others, with the disease being commonly found in Japanese and Pima Indian populations, but relatively rare in African American, Native American and Asian groups (Weinfeld et al., 1997; Mazières and Rovensky, 2000). In a study of lateral chest radiographs of 1500 black South Africans, the prevalence of DISH was 3.9% (3.8% in males and 4.2% in females), with 52.4% of individuals

diagnosed with DISH (using the modified Resnick and Niwayama, 1976 criteria) while also having type 2 diabetes mellitus (Cassim et al., 1990). DISH had a prevalence of 25% in males over the age of 50 years and 15% in females over the age of 50 years among American Midwesterners (Weinfeld et al., 1997; Vigorita, 2008), while other studies report 3–6% prevalence of the population over the age of 40 and 11% over the age of 70 (Sarzi-Puttini and Atzeni, 2004; Gorman, 2005). The large variation in the reported incidence could be associated with the methods used to assess and record the disease, but most probably reflects the varying underlying predisposition for the disease in various groups. The role that genetics may play (and its aetiological significance) in the development of DISH is still not completely understood (Pappone et al., 1996; Sarzi-Puttini and Atzeni, 2004; Gorman, 2005; Spencer, 2008).

2.1.2. DISH in the palaeopathological literature

Palaeopathological studies of DISH have focused on linking its prevalence in past populations to certain socioeconomic levels in society. Several studies have assessed the frequency of DISH between monastic and lay populations and a pattern has emerged showing a higher frequency of DISH amongst individuals buried in monastic cemeteries (Waldron, 1985; Rogers and Waldron, 2001; Jankauskas, 2003; Verlaan et al., 2007), suggesting that people living in monasteries (monks/priests) had a distinguishable contributing factor to developing DISH. This factor was mostly suggested to be a generally better, but much richer, diet high in animal protein and fats. To illustrate the association of signs of DISH with individuals who are well or over nourished, four of these studies are discussed in detail below.

In a study of the monastic site of Merton Priory (1140 – 1540 CE) in Surrey, England, a prevalence of 8.6% of DISH was calculated from 35 skeletons at the site. Waldron (1985) did not state what criteria were used to make a positive diagnosis of DISH; however, a reference was made to the extra spinal manifestations described by Resnick et al. (1975). All individuals from Merton Priory, with the exception of the one female found at the site (presumed to be a benefactress of the priory), are assumed to have been priors (Waldron, 1985).

In a similar analysis of two archaeological cemeteries (Wells Cathedral and the Royal Mint in London) by Rogers and Waldron (2001), a trend between monastic cemeteries and DISH was beginning to emerge. The sample of skeletons discussed by Rogers and Waldron (2001) came from excavations (1978 and 1982) at the Wells Cathedral in England where DISH

was analysed from three groups: individuals from the lay cemetery (n=93), individuals from the 13th century Lady chapel (n=15) and individuals from the 16th century Stilington's chapel (n=13). The prevalence of DISH was assessed using the Rogers and Waldron (1995) criteria and was reported as 7%, 13% and 23% for each of the groups, respectively. The second group of skeletons to be analysed were excavated from the Royal Mint site in London, England, and were representations from the lay cemetery (n=99) and individuals buried in the church and chapels (n=52). No DISH was diagnosed amongst the individuals in the lay cemetery, but a prevalence of 12% was found in individuals representing the church and chapels (Rogers and Waldron, 2001). This led to the interpretation that individuals who were buried inside the church and chapels were of higher status/wealthier individuals and as such, would have been able to afford a richer diet than the lay people. This reinforced the previously suggested interpretation that DISH was linked to diets higher in calories/animal proteins and was highlighted by the fact that little evidence of DISH was present in the lay population.

In another similar study, skeletal material (n=458) from several archaeological sites spanning from the 1st millennium CE to the 2nd millennium CE in Lithuania were studied for the incidence of DISH (Jankauskas, 2003). The diagnostic method used was not specified. The total sample was divided into three categories of social status dependant on where an individual was buried. If an individual was interred inside a church, they were considered to be of high status ("rich"), if an individual was excavated from an urban cemetery then they were assumed to have been of 'middle to lay' status ("lay"), and if an individual was found in a rural area they were assumed to be of low or peasant status ("poor"). With the sexes pooled, a prevalence of 27% (36% males, 5% females) of DISH was found within the individuals considered to be of a 'rich' social status, 12% (12% males, 3% females) of individuals were diagnosed with DISH from the 'lay' social status and 7% (17% males, 2% females) from those considered to be from a 'poor' social status (Jankauskas, 2003). Jankauskas (2003) concluded that DISH was significantly linked with advancing age, elite social status (determined from the burial context), and being male. He was unable to make such an assessment for the prevalence of DISH in the female cohort. No similarities were detected in the social status of males and females. It appears that while males in Lithuanian Iron Age society were shown to be of higher social status with access to more protein rich diets, for females, age-at-death played a large role in their place in society's hierarchy (Jankauskas and Kozlovskaya, 1999). The explanation for this was primarily linked to the evidence for funerary artefacts incorporated into the graves of females. Richer female burials were predominantly correlated to the graves of women of peak

reproductive age (females in their twenties). Of course, DISH is a disease that has consistently been linked to individuals of older age, so there is no surprise that DISH was not prevalent in this burial context. Women whose age-at-death was 40 years or older (individuals more susceptible to developing DISH where age is a factor), were found to be significantly poorer, a conclusion made by their lack of funerary artefacts (Jankauskas, 2003).

An analysis of 42 adults (275-1795 CE) excavated from the Pandhof site in the city of Maastricht, the Netherlands, also continued the investigation into this trend of ancient clergymen, and their apparent predisposition towards DISH. Seventeen individuals were diagnosed with DISH, providing a 40% prevalence rate of DISH at the site (Verlaan et al., 2007). Of the 17 individuals diagnosed with DISH (all adults), 10 were male, 5 were female and 2 were of indeterminate sex.

All the above studies showed a strong correlation between the prevalence of DISH among males and those with increasing age. Also, the main factor identified in the development of DISH was a generally rich diet or a diet specifically higher in animal protein than those of other contemporary individuals. This had led to the consideration that DISH is a disease of lifestyle, mainly acquired by individuals of a higher socioeconomic status (SES), who could afford to eat a diet rich in animal protein and calories, such as monks in medieval Europe (Waldron, 1985; Rogers and Waldron, 2001; Jankauskas, 2003; Verlaan et al., 2007). It should be kept in mind that the converse may also be a relevant interpretation - an archaeological skeleton diagnosed with DISH does not imply that the individual was of high SES (Rogers and Waldron, 2001). The intricacies of socio-economic status across different countries and how it has changed over time cannot be explored here, but it is worth noting that while access to food stuffs can be interpreted as being directly correlated with an individual's SES in an archaeological context, there are more factors to take into consideration in modern populations such as life expectancy, geographical residence, income, education, access to healthcare and family circumstances, to name but a few.

Certain lifestyles may be a precursor to, but not the definitive causative agent of DISH. The relationship between certain lifestyles (*i.e.* over-nourishment/obesity) is a plausible explanation that needs further validation in contemporary populations. However, the comparison between archaeological and clinical studies cannot be fully explored until the issue of a standardised diagnostic method is addressed.

2.1.2.1. Diagnosing DISH in skeletal remains

The diagnosis of DISH in skeletal remains is problematic, mainly due to the lack of information on the time it takes for bony characteristics of the disease to manifest in the human skeleton. The main issue in the skeletal diagnosis of DISH is the lack of consensus among academics on previously seen (but not always deemed synonymous with) characteristics of DISH.

A comprehensive analysis of various diagnostic criteria for DISH evaluated the merits and disadvantages of these methods when applied to the examination of archaeological skeletons (Van der Merwe et al., 2012). The four diagnostic guidelines for DISH evaluated by the Van der Merwe et al. (2012) study included Resnick and Niwayama (1976), Arlet and Maziéres (1985), Utsinger (1985), and Rogers and Waldron (2001) and are discussed below.

The Resnick criteria (Resnick and Niwayama, 1976) are widely used as a diagnostic tool for DISH. As a clinical diagnostic method which primarily uses radiography, the Resnick criteria have been adapted for diagnosing DISH in skeletal material, both in archaeological and forensic contexts. Below is a brief description of the 3 main features of the diagnostic criteria:

1. Flowing bony ossification along the anterolateral aspect of at least four contiguous vertebral bodies.
2. Relative preservation of the intervertebral disc height in the involved segment.
3. Absence of apophyseal joint bony ankylosis and sacroiliac joint erosion.

The retention of the intervertebral disc height is an interesting feature that has not been frequently mentioned in diagnostic criteria subsequent to Resnick and Niwayama's (1976) seminal article. With the emergence of newer research (post 1980s) on DISH and its associated risk factors, it seems that the intervertebral disc height can be affected by other comorbid factors such as obesity. Although no clear correlation has been established, obesity has been cited as a probable risk factor for the development of DISH (Julkunen et al., 1971; Waldron, 1985; Kiss et al., 2002a; Sarzi-Puttini and Atzeni, 2004; Spencer, 2008). However, in a study of morbidly obese patients, weight loss was found to result in an increase in intervertebral disc height (Lidar et al., 2012), which suggests that individuals who have a BMI above normal might already have a narrowing of the joint space. Therefore, a compression of the intervertebral disc space due to an individual being overweight or obese, and ankylosis

occurring due to DISH, could result in the original disc space height (as suggested in the above diagnostic criteria) not being retained.

Further possible difficulty with the Resnick diagnostic criteria (Resnick and Niwayama, 1976) lies in the diagnosis of less advanced cases of DISH, as the characteristic flowing ossification may take many years to fully form and for several contiguous vertebrae to fuse. Individuals are commonly observed to present with extra-spinal characteristic bony changes associated with DISH, and with little spinal involvement (Rogers and Waldron, 2001).

Arlet and Mariéres (1985) attempted to devise a method to address the development of the ossification of the ALL along the different regions of the spine of individuals with DISH. Diagnosing DISH according to the Arlet and Mariéres (1985) method, involves the following:

1. Flowing ossification of the ALL of three contiguous vertebral bodies in the lower thoracic region.
2. No intra-articular joint erosion or ankylosis of the sacroiliac joint occurs; however, para-articular ossification of the iliolumbar or sacroiliac ligaments may be observed, with retention of the sacroiliac joint surface.
3. The cervical vertebrae may be affected with ossification of the ligamentum apicis dentis (apical ligament), with retention of the intervertebral disc space and apophyseal joints (as seen in the thoracic and lumbar regions).

A probable case of DISH can be diagnosed in individuals who:

1. Display only two affected vertebrae,
2. Have spinal changes but also show the presence of degenerative disc disease,
3. Although showing no evidence of lower thoracic vertebrae involvement, conforms to the rest of the criteria.

Arlet and Mariéres' (1985) method is more flexible with the number of contiguous vertebrae affected by the ossification of the ALL, and the characteristics associated with a diagnosis of probable DISH. The diagnostic criteria attempt to alleviate the ambiguous nature of the disease's development and the wide array of characteristics of DISH that have been noted in the literature.

Utsinger's (1985) method also tries to confront this issue by providing descriptions for diagnosing cases of definite DISH, probable DISH and possible DISH, while also expanding on the extra-spinal manifestations that are most relevant to the diagnosis of DISH.

Utsinger's (1985) method is described as below:

1. For a definite DISH diagnosis, the ossification of the ALL across four contiguous vertebrae must be present, (with or without ankylosis) in the thoraco-lumbar region. The intervertebral disc space and apophyseal facets are retained with no evidence of ankylosis.
2. A diagnosis of probable DISH is given to individuals displaying two vertebrae affected by the ossification of the ALL, in conjunction with the presence of bilateral extra-spinal enthesophytes at the ulnae (insertion of the *m. triceps brachii*), patellae (insertion of the *m. quadriceps femoris*) and calcanea (insertion of the *m. triceps surae*). Enthesophytes are bony projections at the site of tendon or ligament origin or insertion.
3. If an individual possesses two fused vertebrae, characteristic of ossification of the ALL but has no extra-spinal enthesophytes, then a diagnosis of possible DISH is given.

Utsinger's (1985) diagnostic criteria also provide flexibility with the three classifications of DISH and he stated that according to his criteria, those who were diagnosed with probable DISH would in time, advance to definite DISH.

The diagnostic criteria of DISH according to Rogers and Waldron (1995) include the following features:

1. Ossification of the ALL across at least three contiguous vertebrae, (with or without ankylosis) on the right side (unless *situs inversus* is present) in the thoracic region. There should be retention of the intervertebral disc space and apophyseal facets with no evidence of ankylosis.
2. Evidence of bilateral extra-spinal manifestations should be present and include enthesophytes at one or all of the ulnae (insertion of the *m. triceps brachii*), patellae (insertion of the *m. quadriceps femoris*), calcanea (insertion of the *m. triceps surae*), tibial tuberosities and ossification of the ligamentum flavum.
3. The absence of vertebral osteoarthritis and degenerative disc disease should not be used for diagnostic purposes.

4. Where there is no sufficient evidence in the spine for a diagnosis of DISH, but the rest of the criteria are met, an individual can be classed as possible DISH.

This method is flexible in a diagnosis of DISH, considering the fact that progression of the disease is still not fully understood. Rogers and Waldron (2001) comment on the frequency with which vertebral osteoarthritis (VO) and degenerative disc disease (DDD) affect individuals of an older age. This is important to note as DISH is primarily a disease of individuals over 45 years of age, who could also be susceptible to developing VO and DDD. Therefore, older individuals with DISH might also present with evidence of other diseases such as VO and DDD, which is why the absence of such conditions should not be used for diagnostic purposes.

Other diagnostic criteria for DISH are circulating in the academic literature (Crubézy, 1990; Crubézy and Crubézy-Ibanez, 1993; Kacki and Villotte, 2006); however, the abovementioned criteria are the most utilised and discussed in various studies (Spencer, 2008; van der Merwe et al., 2012; Holgate and Steyn, 2016).

2.1.2.2. Extra spinal manifestations

DISH is not limited to the spine alone, with ossification of tendon, ligament and capsule insertions (entheses) occurring at multiple peripheral sites, a concomitant characteristic of the spinal disease (Resnick et al., 1975; Resnick and Niwayama, 1988; Rogers and Waldron, 2001; Mader et al., 2009; Roberts and Manchester, 2010). Extra-spinal manifestations of DISH have been noted on the calcaneus, patella, ulna and os coxae (Figure 2.3); however, their diagnostic relevance has been disputed.

A small proportion of the population has been shown to be susceptible to the ossification of soft tissues, particularly at the site of entheses (Rogers and Waldron, 1995; Rogers et al., 1997; Waldron, 2009). This development has been attributed to an individual's susceptibility to respond to minimal repetitive trauma, eliciting an ossifying response (Waldron, 2009). As extra-spinal manifestations play a significant role in the diagnosis of DISH, it is not clear whether it is a clear concomitant manifestation of DISH, or whether it is an underlying cause, triggering a tendency to develop enthesophytes in individuals who also have DISH. Either way, some researchers have suggested a connection between an individual's susceptibility to DISH and their status as possible 'bone formers' (Rogers and Waldron, 1995; Rogers et al., 1997; Waldron, 2009).

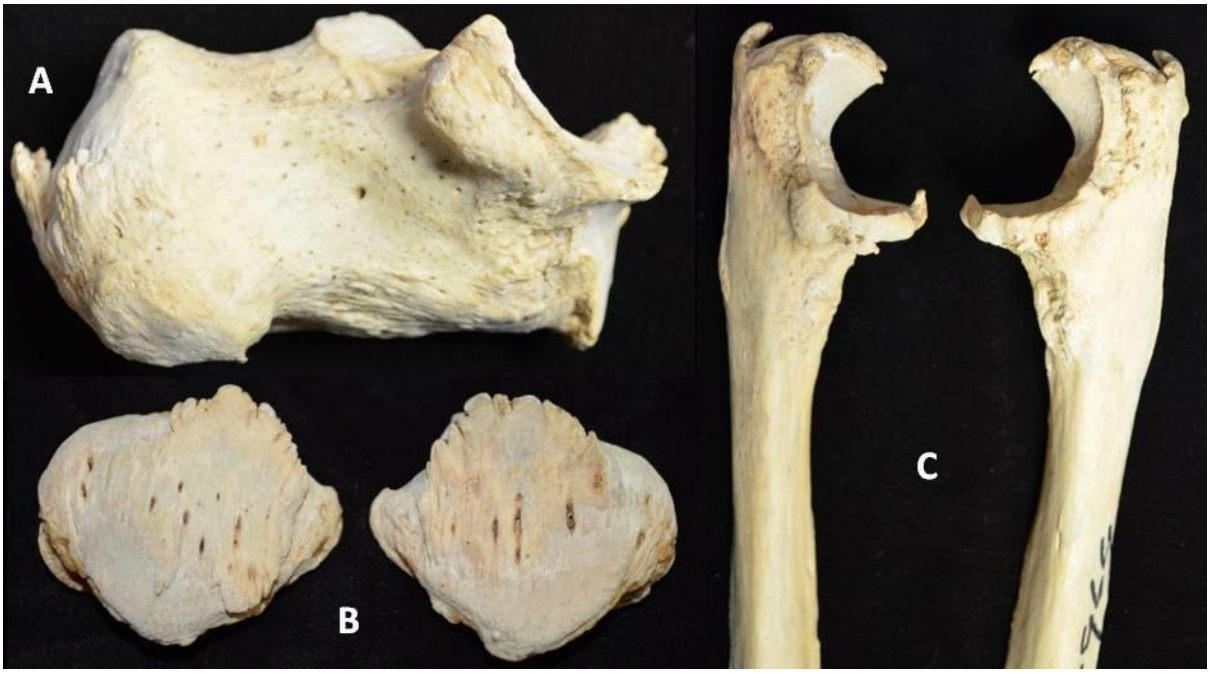

Figure 2.3: Frequently observed enthesophytes associated with DISH. (A) calcaneal spurring, (B) patella tufting (enthesophyte), and (C) enthesophytes on the superior surface of the olecranon process. Source: Holgate and Steyn (2016).

Interestingly, enthesophytes have been shown to have a positive correlation with osteophyte formation in a study of 337 adult individuals across archaeological sites. The study covered 14 ligament insertion sites to assess enthesophyte formation and 14 spinal and peripheral joint sites to assess osteophyte formation (Rogers et al., 1997). Some of the 14 ligament insertion sites (enthesophytes) studied by Rogers and her colleagues overlap with a few of the enthesophyte formation sites that have been suggested to be a concomitant feature of DISH. In Table 2.1, a summary of different enthesophytes suggested to be correlated with DISH are provided, along with their literary source. However, the spinal joint sites for osteophyte formation included cervical facet joints, thoracic facet joints and lumbar facet joints, but excluded any analysis of osteophyte formation at the end plate (Rogers et al., 1997). This should not pose any difficulties when considering individuals with prominent spinal manifestations of DISH, but may be a problem for individuals in the early stages (skeletal manifestation) of DISH – individuals without contiguous ossified vertebrae.

Table 2.1. The various enthesophytes suggested to be associated with DISH within the literature.

Bone	Landmark	Enthesophyte location	Reference
Scapula	coracoid	not specified	(Beyeler et al., 1995)
Humerus	"at shoulder" no other specification	not specified	(Resnick et al., 1975)
	shaft	not specified	(Beyeler et al., 1995)
	greater and lesser tubercles/ rotator cuff	not specified	(Jankauskas, 2003)
	intertubercular groove	insertion of *m. pectoralis major*	(Fuentes-Sánchez et al., 2016)
	deltoid tuberosity	insertion of *m. deltoidius*	(Jankauskas, 2003; Fuentes-Sánchez et al., 2016)
Ulna	olecranon process	insertion of *m. triceps brachii*	(Resnick et al., 1975; Utsinger, 1985; Littleton, 1999; Rogers and Waldron, 2001; Jankauskas, 2003; Mader, 2003; Fuentes-Sánchez et al., 2016; Foster et al., 2018; Ventades et al., 2018)
	supinator crest	origin of *m. supinator*	(Fuentes-Sánchez et al., 2016)
	tuberosity	insertion of *m. brachialis*	(Fuentes-Sánchez et al., 2016)
Radius	radial tuberosity	insertion of *m. biceps brachii*	(Jankauskas, 2003; Fuentes-Sánchez et al., 2016)
	interosseous crest	interosseous membrane	(Fuentes-Sánchez et al., 2016)
	pronator tuberosity	insertion of *m. pronator teres*	(Fuentes-Sánchez et al., 2016)
Axis	dens	apical ligament	(Arlet and Mazieres, 1985)
Rib	not specified	not specified	(Mader, 2008)
Vertebrae	lamina	insertion and origin of ligamentum flava	(Utsinger, 1985; Ehara et al., 1998; Rogers and Waldron, 2001; Kim et al., 2018)
	body	ALL	(Resnick et al., 1975; Utsinger, 1985; Littleton, 1999; Rogers and Waldron, 2001; Jankauskas, 2003; Mader, 2003; Fuentes-Sánchez et al., 2016; Foster et al., 2018;

			Ventades et al., 2018)
		PLL	(Ehara et al., 1998; Kim et al., 2018)
	spinous process	supraspinous ligament	(Forestier and Lagier, 1971)
Os coxae	not specified	not specified	(Resnick et al., 1975; Littlejohn and Hall, 1982; Mader, 2008)
	iliac crest	not specified	(Littleton, 1999; Jankauskas, 2003; Slonimsky et al., 2016)
	sacro-iliac joint	sacroiliac ligaments	(Resnick et al., 1978; Jankauskas, 2003; Fuentes-Sánchez et al., 2016)
	ischial spine	sacrospinous	(Littleton, 1999)
	ischial tuberosity	not specified	(Jankauskas, 2003)
Femur	not specified	not specified	(Resnick et al., 1975)
	trochanters	not specified	(Jankauskas, 2003)
		insertion of *m. iliopsoas* (lesser trochanter)	(Fuentes-Sánchez et al., 2016)
	intertrochanteric crest	insertion of *m. quadratus femoris*	(Fuentes-Sánchez et al., 2016)
	gluteal tuberosity	insertion of *m. gluteus maximum*	(Fuentes-Sánchez et al., 2016)
	linea aspera	not specified	(Jankauskas, 2003)
		origin of *m. vastus medialis*	(Fuentes-Sánchez et al., 2016)
Patella	anterior surface/base/apex	*m. quadriceps femoris*/patellar ligament	(Resnick et al., 1975; Littlejohn and Hall, 1982; Utsinger, 1985; Littleton, 1999; Rogers and Waldron, 2001; Jankauskas, 2003; Mader, 2008; Foster et al., 2018; Ventades et al., 2018)

Tibia	tibial tuberosity	insertion of *m. quadriceps femoris*/patellar ligament	(Resnick et al., 1975; Littlejohn and Hall, 1982; Utsinger, 1985; Rogers and Waldron, 2001; Jankauskas, 2003; Foster et al., 2018)
	soleal line	*m. soleus*	(Jankauskas, 2003; Fuentes-Sánchez et al., 2016)
Calcaneus	tuberosity	origin of *m. flexor digitorum brevis*/plantar fascia	(Littlejohn and Hall, 1982; Rogers and Waldron, 2001; Mader, 2003)
	posterior surface	Insertion of *m. triceps surae*/achilles tendon	(Resnick et al., 1975; Littlejohn and Hall, 1982; Utsinger, 1985; Littleton, 1999; Rogers and Waldron, 2001; Jankauskas, 2003; Mader, 2003, 2008; Foster et al., 2018; Ventades et al., 2018)

2.1.2.3. Differential diagnosis

A differential diagnosis is necessary when diagnosing any pathology and DISH is no exception. While DISH is relatively unique and easily identifiable in advanced cases, some of the skeletal manifestations in the early developmental stages of the disease can be similar to other diseases such as ankylosing spondylitis (AS), fluorosis, osteophytosis, discarthrosis and other variants of spinal arthritis (Tsukamoto et al., 1977; Crubézy and Trinkaus, 1992; Rogers and Waldron, 1995; Littleton, 1999; Ortner, 2003; Aliabadi et al., 2006; Olivieri et al., 2009). However, the distribution and appearance of lesions throughout the skeleton and the bilateral characteristic bony projections (enthesophytes) at sites such as the calcaneus, patella, ulna and os coxae can contribute to the positive diagnosis of DISH (see Figure 2.3).

2.1.2.3.1. DISH versus Ankylosing Spondylitis

AS is clinically characterised as a chronic inflammatory rheumatic disease, and like DISH has some similar entheseal involvement. AS is described as a seronegative spondyloarthropathy, while also being strongly associated with a subtype of the human

leukocyte antigen B27 (HLA-B27) (Rogers and Waldron, 1995; Ortner, 2003; Aliabadi et al., 2006; Baraliakos et al., 2013; Sieper and Braun, 2014).

Differences in the skeletal manifestations and patterning between DISH and AS can be used for positive identification in palaeopathological cases, and this distinction has been highlighted in a clinical setting (Aliabadi et al., 2006). AS usually begins in the lumbar spine and sacroiliac joints, while DISH occurs in the thoracic region of the vertebrae. Although there may be sacroiliac involvement observed in both AS and DISH, AS usually presents with bilateral involvement, whereas DISH is reported to be more unilateral in the sacroiliac region. In individuals with AS the sacroiliac joint will reduce in space, usually as a result of cartilage destruction, and will ultimately result in ankylosis between the auricular surface of the ilium and sacrum, complete obliteration of the joint and a continuity in trabecular bone at the junction (Rogers and Waldron, 1995; Ortner, 2003). The SIJ has been highlighted as an important landmark for differential diagnosis of DISH in a clinical context, and while its assessment in living patients requires the use of imaging modalities such as magnetic resonance imaging (MRI) and CT (Latourte et al., 2018), palaeopathologists have the advantage of being able to study the intra-articular surface directly.

The macroscopic appearance of the spinal involvement of each disease when fully formed are very different, with DISH frequently being referred to as ‘candle-wax’ in appearance and the syndesmophytes of AS (bony outgrowths originating at the margins of the vertebral bodies) having been said to resemble “bamboo” (Rogers and Waldron, 1995; Ortner, 2003). Pathologically speaking, syndesmophytes are similar to osteophytes, however it is the amalgamation and general pattern of other pathological lesions (such as the ossification of the intervertebral disc space and SIJ involvement as seen in AS) that will help to differentiate between the two diagnostically. The abovementioned bamboo effect (Figure 2.4) is created from a sequence of events, namely inflammation of the region, followed by erosion of the structural bone and cartilage, and finally an invasion of repair tissue and the subsequent ossification of the repair tissue (Sieper and Braun, 2014). As previously stated, AS begins in the lumbar region and can extend superiorly through the spine to involve the thoracic vertebrae and the costo-vertebral joints, potentially progressing to the cervical spine – with no ‘skipped’ lesions (Waldron, 2009). In DISH the costo-vertebral joints are less affected by the progression of the disease throughout the spine. The development of syndesmophytes in AS can include the entire vertebral body, whereas in DISH the ossification is limited to the right anterolateral

side of the thoracic vertebrae. This unique presentation has (speculatively) been attributed to the pulsation of the aorta, preventing the ossification (see Figures 1.1 and 2.2).

AS also tends to affect younger individuals, with a peak onset being between 20 and 30 years of age (Aliabadi et al., 2006; Sieper and Braun, 2014). In contrast, DISH usually occurs in individuals aged 40+ years, with the disease increasing in severity with advancing age (Rogers and Waldron, 1995; Aliabadi et al., 2006; Mader, 2008; Roberts and Manchester, 2010). Therefore, age-at-death (in palaeopathology specifically), can (when appropriate) be used as a diagnostic tool.

There are some cases where DISH and AS have been reported to have occurred in the same individual simultaneously, but this is rare (Tischler and Yaron, 1992; Moreno et al., 1996).

2.1.2.3.2. DISH versus Vertebral Osteophytes

Vertebral osteophytes (VO) are extremely common, and similar to DISH have an increasing prevalence with age (Rogers and Waldron, 2001; Ortner, 2003). Vertebral osteophytes take origin from where the fibres of the annulus fibrosus of the intervertebral discs attach and have been known to develop horizontally with vertical inclinations possibly occurring over time (Figure 2.4). This is different from the formation of the syndesmophytes seen in AS, which tend to be more vertically inclined (Rogers and Waldron, 1995). They occur for a variety of reasons, one of which is as a result of mechanical stress on the intervertebral disc as primary weight bearing structures (Maat et al., 1995). VO can also occur with disc changes in vertebral osteoarthritis, which also has a correlation with increasing age (Maat et al., 1995; Ortner, 2003; van der Kraan and van den Berg, 2007), and may result in a narrowing of the intervertebral disc height. Some might argue that VO are a typical feature of growing old and have no diagnostic significance (Rogers and Waldron, 1995; van der Kraan and van den Berg, 2007). However, many cases exist where VO can develop secondary to other conditions or diseases. Some examples include the development of VO after trauma to the spine, VO occurring after the osteolytic lesions have formed in the spine due to infectious diseases such as tuberculosis or malignant neoplasms (primary or metastases) that affect the bone (Rogers and Waldron, 1995; Ortner, 2003). In these examples, we are likely to see evidence of the primary condition/disease which caused the development of the VO, and of

course as with the diagnosis of any disease, the appearance and distribution of lesions throughout the skeleton will help with a positive diagnosis.

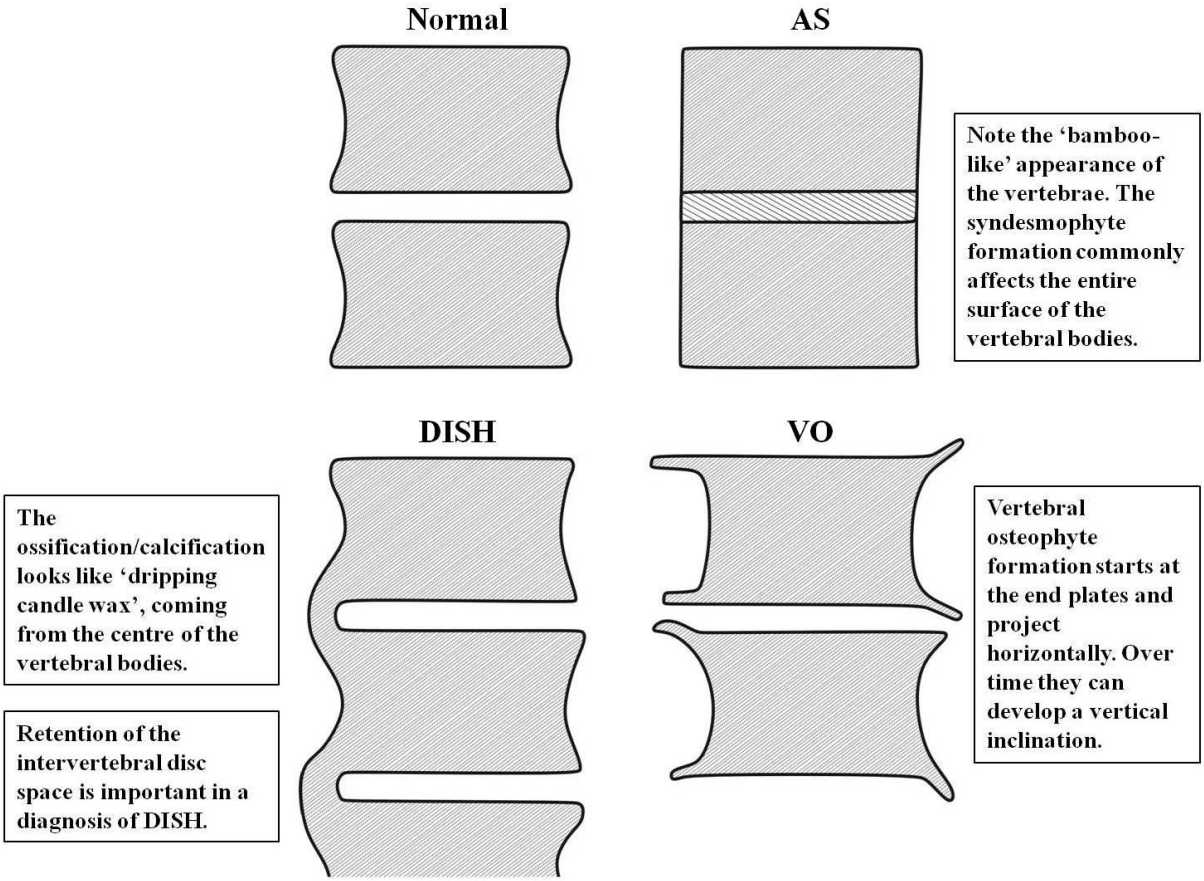

Figure 2.4: The difference in the development and presentation of spinal ossification between ankylosing spondylitis (AS), diffuse idiopathic skeletal hyperostosis (DISH) and vertebral osteophytes (VO). Source: redrawn from Rogers and Waldron (1995: p22).

As previously mentioned, VO formation has been linked to enthesophyte formation, therefore it is suggested that in diseases such as DISH, where individuals are more likely to develop enthesophytes, a high incidence of VO formation is also possible (Rogers et al., 1997; Benjamin et al., 2006). In Figure 2.4, a brief visual summary of the characteristic spinal changes in DISH, AS and VO are provided which can help aid in differential diagnosis.

2.1.3. Imaging modalities and DISH

Within the last decade, different imaging modalities have been used to assess the pathogenesis of DISH in living patients, beyond the method of conventional radiography (Mader et al., 2017). Some studies have been using imaging modalities such as CT and MRI to

identify prevalent features of DISH for differential diagnostic purposes (Saleem and Hawass, 2014; Weiss, 2015; Slonimsky et al., 2016; Braun et al., 2018; Kim et al., 2018), while others have used them to better understand the developmental mechanisms behind the bony manifestations (pathogenesis) of DISH in patients (Mader et al., 2015; Arad et al., 2017). In a study using ultrasonography to assess the peripheral entheses in patients diagnosed with DISH, an interesting discovery was made: the findings suggested that a local inflammatory process might predate enthesophyte formation. This is most interesting as DISH is primarily seen as a non-inflammatory condition (Mader et al., 2015). Another study using MRI scans of the spines of patients diagnosed with DISH, also concluded that a local inflammatory process was perhaps responsible for osteophyte formation, due to the presence of bone marrow oedema and fat deposition (Arad et al., 2017). In both cases, the suggestion was made that more studies are needed to explore and potentially corroborate the findings.

A longitudinal study by Yaniv et al., (2014) used CT examinations (collected over a 10-year period) in an attempt to understand the development of the flowing ossification (noted in the study as bridging osteophyte formation) in patients diagnosed with DISH, by using a semi-quantitative scoring system. Using the data collected from their semi-quantitative scoring system, the authors estimate that it takes approximately 10 years for the flowing ossification to form across vertebrae. Two bridging patterns were identified: bridging osteophytes with ossified ALL and bridging osteophytes without any apparent ALL involvement. The scoring method could be seen as a little confusing, as it clearly shows the development of osteophyte formation (coming from the end plate of vertebrae) with or without ALL involvement, rather than ossification coming from the middle of the vertebral body on the right anterolateral side (Yaniv et al., 2014: p1953). No mention is made in the study that the osteophyte bridging or ossification of the ALL is restricted to the right anterolateral surface of the vertebral bodies. This is unusual as ossification confined to the right anterolateral side of the spine is specified in the literature as a defining feature of DISH that aids in differential diagnosis (Rogers and Waldron, 1995). The Resnick criteria were mentioned to have been used, except for the analysis of the sacroiliac joint due to poor quality CT scans, or the absence thereof. This study could be an example of how complex the pathogenesis of DISH is and how VO could play a much more significant role than previously thought. However, the possibility that the high incidence of VO is due to the age of individuals included in the study should be considered.

2.2. DISH and obesity

Several studies have noted an association between DISH and obesity, in both a clinical (Julkunen et al., 1971; Boachie-Adjei and Bullough, 1987; Mata et al., 1997; Coacciolo et al., 2000; Kiss et al., 2002a; Mader et al., 2009; Zincarelli et al., 2012) and palaeopathological context (Waldron, 1985; Maat et al., 1995; Rogers and Waldron, 2001; Verlaan et al., 2007; Giuffra et al., 2010).

The first and most well-known archaeological studies investigating the relationship between DISH and over-nourishment/obesity was during the analysis of the skeletons of 35 monks, from the Merton Priory Site in Surrey, UK, dated from around the 12th to 16th century (Waldron, 1985). From a combination of contemporary literary resources, archaeological and bioarchaeological evidence, the interpretation that the consumption of food - particularly over-nourishment - was a lifestyle factor missing from the lives of lower SES groups. As such, the theory for DISH being linked to an excess in food consumption (obesity) was established (Waldron, 1985). Interestingly, while one may consider monastic orders to live under an austere regime, especially when it came to the consumption of food, there are many literary resources that suggest that monks and priests worked hard at avoiding dietary regulations. For example, it had been noted that monks would take on shifts to enter the infirmary to contract an illness as there was an exemption for sick monks to eat meat (Henisch, 1976). Another report of how monks avoided dietary restrictions on meat was from 13th century France, where monks were only allowed to eat meat from animals that had been hunted, particularly game meat. To create a loop-hole, the monks would encourage dogs (smuggled onto monastery grounds) to chase and kill pigs from local farms, therefore creating an available supply of “hunted” meat (Henisch, 1976). These examples highlight the suggested gluttony of individuals that lived at monastic sites and reinforces the interpretation of how readily available was food high in animal fat and protein.

The skeletal remains at other archaeological excavations, where DISH was diagnosed, were examined to try and test for this new theory of the disease being related to higher social status, with a focus on its link to over-nourishment/obesity. One of these archaeological sites was the medieval city of Dordercht in the Netherlands, where 176 skeletons were examined (Maat et al., 1995). The general SES of the individuals were estimated to be high - an interpretation made from the relatively large stature of individuals, especially when compared to their 17th and 18th century counterparts. A crude prevalence rate of 14.5% was estimated for

those diagnosed with DISH. The conclusion drawn from the results was that there was a higher prevalence of DISH in individuals with high SES (Maat et al., 1995).

A further examination into the relationship between obesity and DISH was one that looked at two archaeological cemeteries, namely Wells Cathedral and the Royal Mint in London (Rogers and Waldron, 2001). By the time this next study came out, there had been clinical studies suggesting a link between DISH, obesity and type 2 diabetes (Julkunen et al., 1971; Boachie-Adjei and Bullough, 1987; Mata et al., 1997; Coacciolo et al., 2000). The skeletons from both sites were representative of burials from inside the church or chapels, with the assumption that these individuals were of a high SES, as well as burials from the lay cemetery, with the assumption that these individuals would represent individuals of relatively lower SES. When the data from both sites were combined and analysed, there was a statistically significant difference in the prevalence of DISH among the individuals buried in the churches and chapels than in the lay cemetery (Rogers and Waldron, 2001). The interpretation of the results of the study built on those that Waldron had reported in 1985, as the study provided a comparative population with a control for SES with the incorporation of lay cemeteries. The statistically significant prevalence of DISH among the individuals considered to be of high SES was again linked to their access to rich diets, especially when compared to the general population (likely to be farmers and peasants). This reinforced the previously suggested interpretation that DISH was linked to diets higher in calories and animal proteins and was highlighted by the fact that very little evidence of DISH was present in the lay population (Waldron, 1985; Rogers and Waldron, 2001).

An analysis of 42 adults (275-1795 CE) excavated from the Pandhof site in the city of Maastricht, in the Netherlands, continued the investigation into the trend of individuals living within monastic orders, and their apparent lifestyles, predisposing them to DISH (Verlaan et al., 2007). Seventeen (40%) of the 42 individuals were diagnosed with DISH, all of whom were interred between 450-1795 CE. During this time, the authors note that there were no reported shortages of food, and that food may have been plentiful. Again, the main conclusion of the study and the relatively high percentage of individuals diagnosed with DISH was attributed to the abundance of food that may have led to the development of metabolic factors such as obesity and type 2 diabetes mellitus, both risk factors for DISH (Verlaan et al., 2007).

The last archaeological examples that will be discussed is the case studies of two individuals from the Medici family who were interred in the Basilica of San Lorenzo in

Florence, Italy (Giuffra et al., 2010). While this example does not give the overall presentation of DISH within the wider population, the skeletons of the two individuals, the Grand Duke Cosimo I (1519-1574) and his son Ferdinand I (1549-1609) are further examples of DISH and its possible relationship to obesity. Both individuals displayed skeletal manifestations of the disease according to the Rogers and Waldron (2001) diagnostic criteria and were both obese. The evidence of obesity in these two cases are not skeletal, but is evident in historical archives and illustrated portrayals, as well as writings describing their over indulgence in the consumption of meat and wine and dietary supplementation of eggs and cheese. While the evidence for the relationship between DISH and obesity is strong in these two cases (Giuffra et al., 2010), a possible genetic link cannot be overlooked.

An important aspect to note when it comes to the interpretation of DISH in archaeological populations being related to over-nourishment/obesity, is the absence of clinical data on the metabolic status of the individuals under study. As seen above, in the majority of archaeological sites, SES is being used as a proxy for the interpretation of over-nourishment or obesity. In many cases, the conclusion of DISH being linked to obesity is being made with the assumption that individuals of higher SES would have had access to more foodstuffs and as such, may have readily indulged in over eating, resulting in the individual being overweight or obese. The intricacies of SES across different countries and how it has changed over time cannot be explored here, but it is worth noting that while an individual's SES has been used to interpret their access to foodstuffs in an archaeological context. Perhaps there are many factors to consider in modern populations. Another point that has been made is the over diagnosis of DISH in archaeological populations, perhaps causing an unconscious bias in interpreting observations in line with contemporary expectations/hypotheses (Rogers and Waldron, 2001).

Clinical studies help to navigate and bolster the interpretations of bioarchaeological research, where certain data (such as an individual's metabolic status) is missing. Among the earliest, well-known clinical studies suggesting a relationship between DISH and obesity is a study conducted in Finland (Julkunen et al., 1971). In the study, 12,858 individuals from six communities across East and West Finland were examined and a strong association between increased weight and DISH was discovered. The percentage of obese individuals for both men and women was significantly higher among the patients with DISH, when compared to the non-DISH group (Julkunen et al., 1971).

During the analysis of 75 spines during autopsy (Boachie-Adjei and Bullough, 1987), authors found 21 individuals conformed to a diagnosis of DISH, using the description provided by Forestier and Rotes-Querol (1950). The average weight of individuals among the non-DISH group was noted at 65kg, while the average weight for individuals diagnosed with DISH was 20kg heavier, at 85kg. The conclusion was that a strong association between obesity and DISH exists (Boachie-Adjei and Bullough, 1987), a similar interpretation to the study by Julkunen and colleagues (1971) discussed above.

A comprehensive controlled clinical study evaluating the relationship between DISH and obesity across three different groups, again showed a strong association (Mata et al., 1997). One of the three groups comprised of 56 patients diagnosed with DISH (using the Resnick criteria), the second included 43 individuals diagnosed with spondylosis and the final group contained 31 healthy subjects as a control. A combination of weight, BMI and waist circumference were used as markers for obesity and all three were significantly higher among the DISH group when compared to the patients in both the control and spondylosis groups. The weight of the patients was recorded at various markers. The results showed that there were no significant differences in the amount of weight gained for patients diagnosed with DISH, suggesting that early onset obesity may be a risk factor for developing DISH (Mata et al., 1997).

Another clinical study, looked at the prevalence of DISH among individuals who were affected by type 2 diabetes mellitus and obesity (Coacciolo et al., 2000). In total, one hundred and thirty patients, with a control of 40 individuals were analysed. The percentage of DISH among individuals in a group that consisted of 32 obese individuals without type 2 diabetes, was recorded at 37.5%. While in a group of 30 obese patients with type 2 diabetes mellitus, the percentage of DISH was recorded as 40%. There was a 2.5% rate of DISH within the control group. The authors concluded that there was a high prevalence of DISH among obese subjects (Coacciolo et al., 2000).

In a comparison between 131 individuals with DISH (69, males and 62 females) and 131 individuals with spondylosis (69, males and 62 females), who were also matched for age, obesity was found to have a strong association with DISH (Kiss et al., 2002a). The authors found that the connection between high BMI and DISH was more complex than a simple cause-correlation relationship. The authors suggested that a "significantly higher level of serum uric acid was found in the DISH group", but this elevated concentration was not associated with BMI, indicating that obesity was not the cause of the higher serum uric acid levels (Kiss et al.,

2002a: 29). The result of the uric acid levels is interesting as it is suggested that dietary components can affect the concentration of uric acid. For example, one particular study showed that the consumption of large quantities of meat and fish (that contain high amounts of purine), lead to an increase in the concentration of uric acid (Choi et al., 2005). This is particularly interesting when one considers the interpretation made from the archaeological examples discussed above (Waldron, 1985; Rogers and Waldron, 2001; Verlaan et al., 2007; Giuffra et al., 2010) that established a possible link between diets high in animal fat and protein to DISH. Regardless of the serum uric acid levels, it was concluded that there was a strong correlation between early onset obesity, as well as the development of obesity at a later age, and the development of DISH (Kiss et al., 2002a).

A clinical study that focused on the extraspinal manifestations of DISH, also found a strong association between the disease and obesity (Mader et al., 2009). A total of 95 patients were examined, including 47 individuals diagnosed with DISH and 48 individuals without DISH who were matched in age and sex to those with the disease. The results revealed that individuals with DISH had a significantly larger waist circumference and higher BMI, but no difference was found in the serum uric acid levels between the two groups. Overall, there was a significantly higher percentage of obese individuals (BMI >30) within the DISH group than there was within the control group (Mader et al., 2009).

The last clinical study that will be discussed is one where DISH was analysed among 521 individuals who were diagnosed with a range of cardiovascular diseases (Zincarelli et al., 2012). While this comprehensive study included a wide range of clinical data, the authors concluded that the presence of obesity among individuals with DISH was statistically significant, indicating that obesity puts people at a higher risk of developing DISH (Zincarelli et al., 2012).

The abovementioned clinical studies have all provided examples of how DISH and obesity seem to have a strong association. The exact nature of this relationship, as demonstrated above, is not definitively known and in some cases, suggested to be complex.

2.2.1. Body mass indices (BMI) as an indicator of obesity

As mentioned above, being obese has been described as a strong risk factor in the development of DISH (Kiss et al., 2002a; Oxenham et al., 2006) and BMI have been shown to

be a robust indicator of a person's body composition. An individual's BMI is a universal mathematical indicator of energy ("fatness") and nutritional status in adults (Bogin and Beydoun, 2007). BMI (kg/m^2) is calculated using the following formula:

$$\mathbf{BMI}(\mathbf{kg/m^2}) = \left(\frac{\text{weight in kilograms}}{\text{height in meters}^2}\right) \text{x } 100$$

From the BMI value, institutions like the World Health Organisation (WHO) have issued recommendations to adult men and women on their optimal weight for maintaining a healthy lifestyle. There are four main categories calculated from the BMI which are used to classify a healthy/unhealthy weight range. If an individual has a BMI value under 18.5 (kg/m^2), they are considered underweight with possible malnourishment. A BMI value between 18.5 and 24.9 (kg/m^2) is defined as a healthy weight range for young and middle-aged adults. If an individual has a BMI value that falls between 25.0 and 29.9 (kg/m^2), they are considered overweight, with individuals possessing a BMI over 30.0, being considered obese. There are subcategories within the underweight and obese categories, such as severely underweight, obese class 1 or obese class 2.

An increase in an individual's BMI value (mainly within the overweight and obese categories) has been associated with an increased risk of developing type 2 diabetes mellitus, certain cancers, high blood pressure, cardiovascular disease and other chronic diseases, some of which have an association with DISH. This is because an increase in BMI is assumed to be directly correlated to an increase in adiposity (% fat). BMI is related to weight and only provides an approximation for an individual's total body fat, often overestimating the body fat for individuals such as pregnant women, body builders and some athletes and generally underestimating body fat for the elderly and physically disabled where muscle atrophy might be prevalent. Individuals such as these would be considered a minority within a population and are of course extreme examples (Bogin and Beydoun, 2007). Although waist circumference is a preferred indicator of an individual's health risk factors over BMI, the BMI will serve a particular purpose for the current study, namely a broad and robust measurement to indicate the general health of an individual as well as an epidemiological marker of fatness (Bogin and Beydoun, 2007). It is worth noting that estimating body mass from skeletal remains can be problematic and unreliable as the relationship between body mass and skeletal variables have not been demonstrated to an adequate degree of reliability (Elliott et al., 2016). For this reason,

any BMI classifications were estimated from post-mortem weight and height estimates, before individuals were macerated to expose their skeletal remains.

2.3. The presumed, general diets of South Africans

As elements of diet are being explored in this study, we must understand what kind of food the individuals being assessed are presumed to have consumed. This is especially important as the skeletal collections primarily consist of individuals of low SES, and the majority of the palaeopathological information centred on diet and DISH implies that individuals affected by DISH will have been from a high SES – with interpretations centred on the rich foods that these people would have been able to afford as a possible cause for the disease. This is problematic, particularly in modern populations as a diet high in animal protein is not always indicative of high SES, and one can still be obese on foods low in nutrition and high in fat.

Some farmers in South Africa are able to subsist on their crops/animals, using their farms for self-sustainability rather than for commercial gains. In this regard, we may see individuals (who may have been poor), consuming large amounts of protein (obtained from their livestock), which would generally be interpreted as evidence of a high SES status in an archaeological context (Steyn, pers comm). This may vary across regions of the country, depending on climatic and environmental conditions, making them suitable for different types of farming.

Results of the 2011 census, across all provinces, demonstrated that the majority of agricultural households were involved in subsistence or smallholder farming. Subsistence farming refers to consumption by the household and smallholder refers to consumption by the household and small-scale market consumption (Lehohla, 2013). Acquiring such records contemporary to the skeletal collections are uninformative or non-existent, making it unclear whether the general use of agricultural households was similar around the entire time period of the skeletal collection. It is clearly very difficult to make general assumptions about the diets of people growing up and living during the time that spans from the late 20th to early 21st century. The average year-of-death/year -of-accessioning for individuals from the PBC is 1995, while the average within the RDC is 1979. Communication with Professor Maryna Steyn stated that one of her parents grew up poor on a farm in the Southern Free State (early/mid-20th century), and its arid environment made it difficult for crops to grow. Therefore, they relied on

their farming of sheep to feed and support themselves, regularly eating mutton (Steyn, pers comm). This is contrary to the interpretation that access to a regular meat source is indicative of wealth. Many rural black and white South Africans, ending up as migrant workers around the Witwatersrand (and perhaps ending up in skeletal collections) may have consumed very different diets, high in plant-based foods. The situation is even more complex when it comes to people from urban areas, who may have consumed very varied diets. While the majority of research and studies focus on a particular ancestral group, it is extremely difficult and not advisable to make sweeping assumptions about diets based on a person's ancestry.

Other aspects of DISH and diet go beyond SES such as the link between obesity and DISH (Julkunen et al., 1971; Waldron, 1985; Boachie-Adjei and Bullough, 1987; Maat et al., 1995; Mata et al., 1997; Coacciolo et al., 2000; Rogers and Waldron, 2001; Kiss et al., 2002a; Verlaan et al., 2007; Mader et al., 2009; Giuffra et al., 2010; Zincarelli et al., 2012), as discussed above. A higher risk of cardiovascular disease and type 2 diabetes (obesity being a risk factor for both) was reported in individuals of low SES in developed countries such as South Africa (Bourne et al., 2002). The link between obesity across all population groups in South Africa, particularly those from poorer households, has been made (Kruger et al., 2005). Fast foods and other foods high in fat that would be readily available for individuals of low SES, (making them prone to obesity and other metabolic conditions), are relatively cheap when compared to the cost of foods needed to support a healthier lifestyle. However, cultural factors affecting individuals' perception of food and food preference play key roles in dietary habits. Access to a global market economy has led to the consumption of meat and dairy products containing high levels of saturated fats over a more traditional diet of low-fat and high-fibre (Kruger et al., 2005). A meta-analysis on South African diets (living in an urban environment) over a 50-year period (starting in 1940), showed that total energy from fat consumption increased from 16.4% to 26.2%, with a decrease in carbohydrate intake (Bourne et al., 2002). Although total protein intake did not shift much over the 50 years, a significant increase in animal protein consumption was noted (Bourne et al., 2002).

The general perception of African food consumption is one of a low-fat, high-fibre diet (O'Keefe et al., 2015). Some South Africans subsist on what can be deemed traditional foodstuffs, such as potatoes, lentils, samp (corn), cabbage, bananas, root plants, grains, greens, beans, onions, peppers, tomatoes and pap (prepared from *mealie* (corn) meal), coming from a long line of traditional foods (Puoane et al., 2006; O'Keefe et al., 2015). Other South Africans will also frequently eat such foods in various quantities (Viljoen and Gericke, 2001). However,

the geographical region where an individual is resident may also influence their access to foodstuffs, with studies frequently identifying an association with urban environments and high fat-intake diets (Bourne et al., 2002; Kruger et al., 2005; Puoane et al., 2006). In rural environments it is important to recognise there are land where people inhabit, but very few crops can grow, with the presence of little natural fodder to sustain the keeping of animals.

A qualitative study looking at the socio-cultural factors influencing the diets of South Africans in an urban township showed that people had a large dependence on the consumption of meat (particularly red meat) as a perceived necessary dietary habit (Puoane et al., 2006). The consumption of meat was directly linked to the individuals' association of meat with high SES, which lead individuals seeking to eat meat on a daily basis (Puoane et al., 2006). Whether people were able to do so and how frequently is another matter that wasn't clearly addressed, but the views on eating meat are clear. The following quotes from different individuals show how meat is important within the black South African communities, particularly in men's diets:

- "My husband will never eat food if there is no meat in the plate." (Puoane et al., 2006, page 90)
- "I am a man, I have to eat meat every day. Eating fish or chicken is like having a starter to a men" (Puoane et al., 2006, page 90)
- "Lean meat and black tea is only used during mourning period. When we celebrate we need fatty meat and white tea." (Puoane et al., 2006, page 91)
- "I can't eat without meat. The throat just longs for meat. We are used to meat. We learned that as children." (Puoane et al., 2006, page 91)

This trend in peoples' perception of high SES being linked to their meat/protein consumption is prevalent in other population studies (Wong et al., 1984; Belk, 2000). They generally state that a correlation can be seen between the bettering of individuals' family circumstances (an increase in family income) and an increase in individuals' meat/protein consumption (Wong et al., 1984; Belk, 2000; Puoane et al., 2006).

Other foodstuffs typically found within traditional African diets such as soured milk, maize, lentils, pap and other cereals/grains also play a large and important role in the diet of most South Africans, however, moving from a rural to an urban environment changes an individual's perceptions of and access to food. Foods like corn, root plants, greens and beans become associated with poverty and individuals of low SES, while those who enjoy meat and

fast foods are considered to be of high SES. It is an irony of sorts as the fatty meat and fast foods that fuels this perceived higher status is also cheaper than the food that would be bought and consumed for a complete, healthy and varied lifestyle; lean meats, fresh fruits and vegetables (Temple and Steyn, 2009; Mchiza et al., 2015).

Documented evidence for the generalised dietary habits of South Africans is limited. A study on the dietary habits of servicemen showed two dietary trends. Some servicemen (n=120, 55%) ate three meals a day (breakfast, lunch and dinner), but the majority (n=328, 70%) would have two meals a day (Viljoen and Gericke, 2001). This seemed to be closely aligned to different cultural practices. Indeed the habit of two meals a day has been documented to be followed by some communities in South Africa (Vorster et al., 1997). However, the abovementioned study is limited by the fact that it was carried out on individuals within the army between 1993 and 1994, and only included males (Viljoen and Gericke, 2001). It is assumed that the dietary habits explored for the aforementioned servicemen are generally applicable to the wider population (men and women), although other factors such as employment and salary can constrain or liberate access to food. For the individuals who preferred a two-meal-a-day plan, their dietary pattern included a mid-morning snack (missing breakfast) and dinner as their main meals, with a lighter lunch snack. These individuals' midmorning snack usually consisted of fricadels, Vienna sausages, polony or eggs, while lunch consisted of bread and dinner consisted of meat and vegetables (Langenhoven et al., 1988a, 1988b; Viljoen and Gericke, 2001). Individuals who traditionally partook in a three-meal-a-day plan would also have snacks between meals. As with other studies, the diets of the servicemen indicated a high preference for meat (beef, mutton and chicken) rich dishes (Viljoen and Gericke, 2001).

A study looking at the diets of individuals living in the Cape peninsula (Western Cape) over a 24 hour recall period concluded that their diets consisted of high animal protein, high animal fat intake, high sugar intake, low fibre consumption, and low intake of vitamins and minerals (Langenhoven et al., 1988a; Steyn et al., 1990). This was characterised by low consumption of fruit and vegetables, when compared to the ingestion of large portions of meat (Langenhoven et al., 1988b). Other studies looking at the dietary patterns of South Africans in urban areas also point to a diet rich in animal fats and proteins, as well as consuming large quantities of sugar, jams and other sweetened foods (Vorster et al., 1997). A diet low in micronutrients and energy, but high in animal fat and protein was shown to dominate the consumption patterns of communities living in both an urban or rural environment within the

Western Cape (Langenhoven et al., 1988a, 1988b; Steyn et al., 1990; Vorster et al., 1997). A high fat diet would increase the propensity to developing conditions such as obesity and adult onset type 2 diabetes mellitus, in turn increasing their possible susceptibility to DISH, as these have been identified as risk factors for the disease (Kiss et al., 2002a; Jankauskas, 2003; Artner et al., 2012; Pillai and Littlejohn, 2014).

Some conditions/diseases could be overlooked when analysing skeletal remains that are pertinent to an individual's health and dietary status, as they leave no observable lesions on bones. One such condition is alcoholism. The direct effects of alcoholism on the skeleton can be extremely difficult to diagnose macroscopically as they are usually associated with soft tissue changes or secondary conditions that can lead to skeletal changes that in turn have multiple differential diagnoses. But alcoholism does dramatically and detrimentally affect an individual's health status and wellbeing through diet. There is well documented evidence that there is a high prevalence of foetal alcohol spectrum disorders (FASD) in South Africa, with it being among the highest world-wide (Olivier et al., 2016), indicating a generalised misuse of alcohol, culminating in its excessive consumption. Excessive consumption of alcohol has been noted to result in a loss of skeletal muscle protein as well as changes in protein metabolism (Bunout, 1999; Preedy et al., 1999; Hong-Brown et al., 2001). There are no studies on what affect this might have on isotopic values, however, one might theorise that (if chronic) this would lead to tissue enriched in $\delta^{15}N$. An excess of alcohol consumption also results in an increase in calorific intake, however, this does not seem to affect the BMI of heavy, chronic alcohol drinkers (Gruchow et al., 1985). In fact, individuals' weight was shown to be reduced in those with excessive chronic alcohol consumption and excessive protein metabolism, indicating that the calories ingested through ethanol consumption were not used/under-utilised in the body (Preedy et al., 1999). Two studies on alcohol consumption in university students showed that the affect that alcohol consumption will have in weight gain is a positive one (Nies et al., 2012; Booranasuksakul et al., 2019), but these studies have little focus on chronic alcohol consumption. There are other factors that contribute to the relationship between alcohol and BMI (French et al., 2010), but these will not be explored here.

The acquisition of the skeletal material assessed during this study across all three of the skeletal collections spans the late 20th to early 21st century. This makes it extremely difficult to make any definitive presumptions on the generalised diets of South Africans, especially when one takes into consideration temporal changes in dietary practice. Other critical factors such as cultural practices, religion, economic factors and whether an individual is living in an

urban or rural area, have been shown to affect individuals' perception and preference to different foodstuffs. The majority of the research on the diets of South Africans is relatively new and as such can only indicate recent dietary trends that are assumed to be relevant to the individuals under study. In summary, a vast variety of dietary habits exist among South Africans, but many individuals in urban areas partake in diets high in fat, protein and sugar, making them susceptible to conditions that have been linked to DISH such as obesity.

2.4. Stable light isotopes

2.4.1. The basic principles of stable light isotope analysis

Some elements occur in several forms and these variations (changes in atomic mass) are known as isotopes, for example ^{2}H (also known as deuterium or D) and ^{3}H (also known as tritium or T) are isotopes of hydrogen (H). Tritium is a radioactive isotope of the element hydrogen, while deuterium is not radioactive, does not decay and as such is termed a stable isotope. Isotopes differ only in the number of neutrons they possess in their nucleus. As the mass of an element is determined by the number of neutrons and protons present, variation exists in the atomic mass of isotopes of the same element (Katzenberg, 2008; Schoeninger, 2010; Hoefs, 2015).

Isotopes of an element have slight differences in their chemical properties, reacting at different rates with variations in the strength of their bonds due to their differing mass (Hoefs, 2015; Katzenberg, 2008). In general, lighter isotopes (*e.g.* H, ^{12}C, ^{14}N, ^{16}O) react faster than their respective heavier (and stable) counterparts (*e.g.* D, ^{13}C, ^{15}N, ^{18}O) (Fry, 2006; Schoeninger, 2010). This is important, because as isotopes circulate naturally in the biosphere, differences in the reaction rates result in products with relative amounts of isotopes, different to the starting components (substrate) of a reaction. Chemical reactions such as photosynthesis in plants are an example of the kind of reaction that can have an effect on the relative abundance of isotope ratios - particularly $^{12}C/^{13}C$ - in the biosphere (substrate) against the tissues of the plant (product). The term fractionation is used to describe the difference in isotope ratios between a product and substrate and is the basis for stable isotope variation in biological systems (Fry, 2006; Katzenberg, 2008; Schoeninger, 2010). The bio-molecular composition of an organism's tissues (product) is in part sourced from their diet (substrate), and different elements are incorporated into the tissues through various metabolic processes (Leatherdale, 2013). From this, interpretations can be inferred about the contributions made by certain

elements in the tissue of interest (*e.g.* human bone, human tooth enamel, human nail and hair etc.) from their original source (*e.g.* dietary and environmental, etc.). For the purpose of this study, the original source of investigation is the diet of individuals, with particular interest to the analysis of protein in their diet.

On account of the very small variations in isotopic ratios measured in different tissues and their environment, the ratios are expressed using the delta (δ) notation. Therefore, isotope ratios such as $^{13}C/^{12}C$ are represented with the δ-notation and written as $\delta^{13}C$. This ^{13}C portion of the annotation represents the proportion of ^{13}C relative to ^{12}C (Schoeninger, 2010). Delta measures the divergence from a known standard (internationally acknowledged), with units known as 'parts per thousand' or 'parts per million' (‰). Due to the small variations in the relative abundance of isotopes, the 'parts per mil' notation magnifies the data into values that are easier to work with. The values for $\delta^{13}C$ and $\delta^{15}N$ are calculated using the following formulae (Fry, 2006).

$$\delta^{13}C = \left[\frac{(^{13}C/^{12}C)_{Sample}}{(^{13}C/^{12}C)_{Standard}} - 1\right] \times 1000$$

$$\delta^{15}N = \left[\frac{(^{15}N/^{14}N)_{Sample}}{(^{15}N/^{14}N)_{Standard}} - 1\right] \times 1000$$

Depending on the isotope of interest to a researcher, internationally recognised standards have been determined and the values can be obtained through the National Bureau of Standards (NBS) or the International Association of Atomic Energy (IAEA) (Gonfiantini et al., 1995; Katzenberg, 2008). In the case of measuring $\delta^{13}C$, the international standard is Vienna Pee Dee Belemnite (VPDB) and for $\delta^{15}N$, it is Ambient Inhalable Reservoir (AIR). Standards will have a δ value of 0‰ and if the isotope abundance ratio from a sample is equal to the ratio from the standard, then the δ value of that sample will be zero (Fry, 2006). This is true in all stable light isotope laboratories around the world as the samples need to be comparable so as to have academic significance (Katzenberg, 2008). Generally speaking, biological samples contain less $\delta^{13}C$ relative to the international standard (VPDB); therefore, the values will be negative. The $\delta^{15}N$ values in biological samples are generally more enriched relative to the international standard (AIR) used, commonly showing a more positive value (Fry, 2006; Mays, 2000). Interestingly, many of the international (primary) reference standards

were either virtual, or have now been exhausted. Due to this, laboratories routinely use inter-laboratory standards and in-house standards (inter-comparison materials). These internal standards can be natural or synthetic compounds that have been calibrated using the average value from data generated across several laboratories, running multiple samples over several days (Carter and Barwick, 2011). Inter-comparison materials will cover a wide range of isotopic abundance ratios, chosen usually to bracket the material of interest for isotopic analysis. Not only is it important that all isotopic ratios obtained are consistent within a laboratory, but that the results are directly comparable to those generated by other laboratories, regardless of geography. Measurements of in-house standards need to be carefully calibrated against the primary reference standards through the globally accepted calibration materials. This is an important form of quality control that ensures validity and reliability for researchers and their data (Gonfiantini et al., 1995; Katzenberg, 2008; Carter and Barwick, 2011).

2.4.2. Stable carbon isotopes ($\delta^{13}C$)

The two naturally occurring stable isotopes of carbon are ^{12}C and ^{13}C, with relative abundances of 98.9% and 1.1%, respectively. The two main sources of $\delta^{13}C$ in human diets are through terrestrial and aquatic food webs. The $\delta^{13}C$ values in marine environments (oceans) are derived primarily from dissolved carbonate; where the majority of the active cycling carbon is sequestered, while the values of $\delta^{13}C$ in terrestrial environments are derived from the carbon dioxide (CO_2) in the atmosphere. There is an exchange between the oceanic carbon and atmospheric CO_2 by equilibrium isotope fractionation, with oceanic carbon being enriched in $\delta^{13}C$ relative to atmospheric CO_2 (Schoeninger, 2010). It has been calculated that CO_2 in the ocean surface has an approximate $\delta^{13}C$ value of 1‰, while atmospheric CO_2 has a value of around -8‰ (Katzenberg, 2008; Schoeninger, 2010; Wahlen, 1994).

Carbon stable isotope ratios ($\delta^{13}C$) in plants differ according to the photosynthetic pathway of the plant. There are three photosynthetic pathways, C_3 (Calvin-Benson pathway), C_4 (Hatch-Slack pathway) and crassulacean acid metabolism (CAM - uses both C_3 and C_4 pathways) (Leatherdale, 2013; Reitsema, 2012; Smith and Epstein, 1971). C_3 plants produce 3-chain carbon compounds and account for the largest proportion of the worlds plants (phosphoglycerate), whereas C_4 plants produce 4-chain carbon compounds (Oxaloacetate). The recognised carbon isotopic signatures of C_4 plants (tropical and salt grasses) range between -9‰ and -14‰, while C_3 plants (herbaceous plants and trees) show a carbon isotopic signature that falls between -20‰ to -35‰ (Ballentine et al., 1998; Hoefs, 2015; Katzenberg, 2008).

Examples of C_3 plants include rice, wheat, rye, barley, peanuts, potatoes, soybean and most vegetables. Examples of C_4 plants include maize, sorghum, millet, sugar cane and other tropical grasses (Smith and Epstein, 1971; Bender et al., 1981; Katzenberg, 2008; Reitsema, 2012). Plants that follow the Calvin-Benson photosynthetic pathway (C_3) fix carbon using the enzyme RuBisCo (ribulose biphosphate carboxylation). The carbon fixing process involving RuBisCo preferentially incorporates ^{12}C over ^{13}C, resulting in plant tissue that is more depleted in $\delta^{13}C$ relative to plants that fix carbon using the Hatch-Slack photosynthetic strategy (C_4). Plants that follow the Hatch-Slack photosynthetic pathway (C_4) fix carbon through an enzyme called phosphoenolpyruvate carboxylase. The carbon fixing process in C_4 plants does not take preference over which isotope to incorporate, resulting in plant tissue that is more enriched in $\delta^{13}C$ relative to plants that fix carbon using the Calvin-Benson photosynthetic strategy (C_3). The photosynthetic strategy CAM, does not refer to the plant's ability to use a separate carbon fixing pathway to C_3 and C_4 plants, but rather the plant's ability to utilise both C_3 and C_4 pathways (Bender et al., 1981; Katzenberg, 2008). As a result, the tissues of plants that are classified under CAM usually display $\delta^{13}C$ values between C_3 and C_4 plants (Bender et al., 1981; Katzenberg, 2008; Reitsema, 2012).

After experimental studies looking at $\delta^{13}C$ values in animals, it was shown that there is a direct relationship between the consumer and the $\delta^{13}C$ value of their diets (DeNiro and Epstein, 1978). The $\delta^{13}C$ values between diet and the consumer have a difference of 1‰, which results in a very similar $\delta^{13}C$ value between animals and the plants they consume in any given environment. This generally refers to homogenised samples, while different bodily tissues from the same individual, for example, bone collagen vs. bone apatite, can produce different isotopic values (Yesner et al., 2003; Kellner and Schoeninger, 2007). For example, bone collagen $\delta^{13}C$ values are approximately +5‰ relative to dietary $\delta^{13}C$, however, this is not an absolute value (Katzenberg, 2008; Van Der Merwe and Vogel, 1978). This is due to the different metabolic processes in place, routing the $\delta^{13}C$ from different portions of the diet to the different bodily tissues at different rates. There are two suggested models explaining the routing of macronutrients into different bodily tissues, such as bone collagen, apatite and tooth enamel. The first is the 'linear mixing model' which assumes that macronutrients from diet to tissue (no matter their source, be it protein, fat or carbohydrate) are all incorporated into the different bodily tissues equally. The second model is the 'routing model' which assumes that macronutrients are incorporated into comparative bodily tissues. For example, diet protein would be routed into bone collagen (tissue protein) and diet energy, (the lipid and carbohydrate

portion of the diet) would be routed into bone apatite (Krueger and Sullivan, 1984; Spencer, 2008; Reitsema, 2012). It is suggested that within the 'routing model', the $\delta^{13}C$ values from the diet (protein, fat or carbohydrate) become over-represented in the corresponding bodily tissues, even if the macronutrients only account for a small part of the diet (Ambrose and Norr, 1993; Spencer, 2008; Reitsema, 2012).

Previous studies investigated the routing of $\delta^{13}C$ into bodily tissues with the premise that the $\delta^{13}C$ values in bone collagen ($\delta^{13}C_{collagen}$) reflected the dietary protein ($\delta^{13}C_{diet\ protein}$), whereas the $\delta^{13}C$ values in bone carbonate ($\delta^{13}C_{apatite}$) better reflected the values of dietary energy ($\delta^{13}C_{diet\ energy}$) (Krueger and Sullivan, 1984; Ambrose and Norr, 1993; Katzenberg, 2008; Kellner and Schoeninger, 2007; Schoeninger, 2010). However, a study amalgamating the results from previous isotopic dietary studies has supported a slightly different interpretation of the carbon routing model (see Kellner and Schoeninger, 2007 for full list of studies analysed). The meta-analysis showed consistently, that although the $\delta^{13}C_{collagen}$ is more closely correlated with $\delta^{13}C_{diet\ protein}$, as previously thought, the $\delta^{13}C_{\ diet}$ can be correlated with the $\delta^{13}C_{apatite}$, rather than $\delta^{13}C_{diet\ energy}$ (Krueger and Sullivan, 1984; Kellner and Schoeninger, 2007; Schoeninger, 2010; Froehle et al., 2012). The abovementioned studies include mice and rats, which are suggested to be comparable in understanding the role of macronutrient routing in human dietary reconstructions as these animals are considered to have similar digestive systems to humans.

2.4.3. Stable nitrogen isotopes ($\delta^{15}N$)

Nitrogen occurs naturally in the environment in two stable isotopes, ^{14}N and ^{15}N, with relative abundances of 99.6% and 0.4%, respectively. The atmosphere accounts for the majority of nitrogen, as it is reservoired there. Nitrogen in the atmosphere (N_2) enters the biological system through nitrogen-fixing plants (legumes). Nitrogen fixing plants such as legumes will reflect $\delta^{15}N$ values closer to 0‰. Bacteria in the soil use nitrogenase enzymes to combine N_2 and hydrogen to produce ammonia (NH_3), which is then converted to organic compounds. Plants absorb the nitrogen in the soil through their roots, and subsequently animals eat the plants and ingest the organic nitrogen, where it is incorporated into their bodily tissues. Soil nitrogen (NH_3) has more positive $\delta^{15}N$ values relative to atmospheric N_2, therefore most plants (excluding legumes as discuss) are also enriched in $\delta^{15}N$ relative to N_2.

As with the research on $\delta^{13}C$, studies have shown that there is a positive correlation between the $\delta^{15}N$ values in tissues of the consumer and $\delta^{15}N$ values in their diets (DeNiro and Epstein, 1981; Katzenberg, 2008; Schoeninger, 2010; Schoeninger and DeNiro, 1984). The main source of $\delta^{15}N$ in dietary reconstructions is measured from bone collagen (Schoeninger and DeNiro, 1984; Ambrose, 1990; Ambrose 2000; Schoeninger, 2010). Depleted nitrogen is excreted from the body (via urea) as the main product of protein metabolism which results in higher $\delta^{15}N$ values in the remaining tissue (Schoeninger and DeNiro, 1984; Reitsema, 2012). The process reflects the trophic level of the consumer, providing an indication of an individual's protein intake (Schoeninger et al., 1983; Phillips and Koch, 2002). An increased trophic level shift between mother and their baby has been detected in studies that have investigated the breastfeeding and weaning habits of both modern and archaeological populations using stable light isotope analysis (specifically $\delta^{15}N$). Similar studies have corroborated a trend in breastfed children exhibiting higher $\delta^{15}N$ values ($\simeq$+2.4‰) relative to their mothers, which is generally reflected on as a trophic level shift (Fogel et al. 1989; Fuller et al. 2003; Katzenberg 2008). Studies have also noted the depletion in $\delta^{15}N$ values as the children are being weaned, with the values becoming similarly aligned to that of their mothers (Fuller et al. 2003; Fuller et al. 2006; Katzenberg 2008; Burt 2013; Beaumont et al. 2013; Tsutaya and Yoneda 2015).

Reported values of $\delta^{15}N$ have been shown to increase approximately between 3‰ and 3.5‰ with progression up the food chain (increased trophic position) (Schoeninger, 2010). The average $\delta^{15}N$ values for humans consuming mainly within a terrestrial ecosystem are between 6‰ and 12‰, while the average $\delta^{15}N$ values for humans consuming mainly within an aquatic ecosystem are enriched by 3‰ to 4‰ relative to their diet (Ambrose, 1991; Kellner and Schoeninger, 2008). In a marine environment, organisms will generally be enriched in $\delta^{15}N$ relative to terrestrial organisms. The enrichment of $\delta^{15}N$ in marine systems is in part due to the many more trophic levels in marine food chains than in terrestrial ones, and in part due to the fractionation associated with denitrification in oceanic waters (DeNiro and Epstein, 1981; Reitsema, 2012; Schoeninger, 2010; Schoeninger and DeNiro, 1984). There are exceptions, such as organisms living in coral reefs, where the presence of nitrogen-fixing algae occupy the foundation of the food chain (Schoeninger, 2010).

In terrestrial systems, the analysis of $\delta^{15}N$ can reflect the contribution of animal protein to an individual's diet. Limitations include the inability of $\delta^{15}N$ ratios to distinguish between the sources of protein, for example meat versus dairy (from the same animal). As well as $\delta^{15}N$

ratios being unable to distinguish between different cuts of meat of a particular animal (Reitsema, 2012; Steele and Daniel, 1978).

There are non-dietary sources that can contribute to variations in the $\delta^{15}N$ values of animals and plants such as nutritional stress and climate. During periods of nutritional stress, Nitrogen enriched in ^{14}N is excreted, leaving bodily tissues that are enriched in $\delta^{15}N$ (Mekota et al., 2006). Climate has been noted to have an effect on measured $\delta^{13}C$ and $\delta^{15}N$ values by affecting the base of the food chain, the $\delta^{15}N$ in soil and $\delta^{13}C$ in plants (Dotsika and Diamantopoulos, 2019). One study found that reduced precipitation caused $\delta^{15}N$ enrichment when measured in bone collagen (Heaton et al., 1986). Similar studies have linked the same effect to dehydration. Animals who go through periods of dehydration or drought (linked to reduced precipitation), are found to have bodily tissues that are enriched in $\delta^{15}N$ as ^{14}N is preferentially excreted through urea (Ambrose, 1991). While these findings have been discovered primarily through animal studies, it has been theorised that hydration would produce similar effects in humans – elevated $\delta^{15}N$ values (White and Armelagos, 1997).

2.4.4. Stable isotopes and DISH

There are limited studies using stable isotope analysis to investigate DISH in skeletal populations. Three studies that have used isotopic analyses on individuals with DISH (with DISH not necessarily being the main focus of the investigation), are discussed below (Müldner and Richards, 2007; Spencer, 2008; Quintelier et al., 2014).

The first of the studies to be discussed, is one whose primary focus was not one of investigating the relationship between diet and DISH. Investigating the diversity of diet present within a group of burials (n=155) from the archaeological site of the Gilbertine priory at the Church of St. Andrew, Fishergate (York, UK) was the main focus. However, the results revealed a link between diet and DISH. The site of Gilbertine priory at the Church of St. Andrew, Fishergate dates from the Late Medieval period, ranging from the 13th to early 16th century AD (Müldner and Richards, 2007). A total of 155 individuals had collagen extracted for isotopic analysis, but it is unclear from the publication where these samples were taken from. The results showed a statistically significant difference in both the $\delta^{13}C$ and $\delta^{15}N$ values between males and females, with males being more enriched in both carbon and nitrogen relative to their female counterparts. This led to the interpretation that the differences in $\delta^{13}C$ and $\delta^{15}N$ values were purely dietary, representing the different roles males and females played

in English Medieval society. The isotopic data also showed a pattern with individuals diagnosed with DISH. During the investigation, only four of the seven individuals diagnosed with DISH discovered at the site, were analysed for $\delta^{13}C$ and $\delta^{15}N$. The four individuals with DISH had $\delta^{13}C$ and $\delta^{15}N$ values indicating "a diet rich in animal protein, which included a significant proportion of marine foods", the $\delta^{13}C$ values ranged from -18.8‰ to -18.2‰, with a mean of -18.5‰, and the $\delta^{15}N$ values ranged from 15.2‰ to 13.2‰, with a mean of 14.1‰ (Müldner and Richards, 2007, pg 169). Although the sample size was minimal, the results from the isotopic analysis were consistent with the contemporary interpretation/hypothesis of DISH being linked to protein consumption. It could be said that the apparent difference in the $\delta^{13}C$ and $\delta^{15}N$ values seen in the four individuals diagnosed with DISH could be simply linked to dietary variation based on sex, as the four aforementioned individuals were all male. However, the authors mention that all four of the individuals plotted above the mean for both $\delta^{13}C$ and $\delta^{15}N$ values when compared to the rest of the male cohort (Müldner and Richards, 2007).

Another study investigating the link between a high protein diet and DISH, using stable isotope analysis included 47 individuals diagnosed with DISH (including a diagnosis of DISH, probable DISH and possible DISH see Spencer, 2008, pg194) across eight archaeological sites (Late Medieval period) in England, United Kingdom. However, due to C/N ratios that fell outside the accepted values, only 23 measurements ($\delta^{13}C$ and $\delta^{15}N$) were acceptable for interpretation from individuals in the DISH group, and 23 measurements for those without DISH. The reported $\delta^{13}C$ values for individuals with DISH ranged from -20.7‰ to -18.2‰, with a mean of -19.9‰ and ranged from 10.7‰ to 14.4‰ for $\delta^{15}N$, with a mean value of 13.4‰ (Spencer, 2008). A statistically significant difference in $\delta^{15}N$ was found when comparing the isotopic values from the DISH group to that of the non-DISH group, with $\delta^{15}N$ among the DISH samples being more enriched. Unlike the results from the analysis at Fishergate, no statistically significant difference was found between males and females for either element, when comparing DISH and non-DISH individuals, as well as between males and females within the DISH group (Spencer, 2008). The author interprets any statistically significant difference present between the DISH and non-DISH groups as being independent of DISH, as they were expecting to see significant $\delta^{15}N$ enrichment between and among all groups of skeletons when comparing those with DISH and those without, which was not the case. It was suggested that the relatively small sample size could have influenced the results, being too small to pick up differences in the isotope ratios. However, a general trend of $\delta^{15}N$ enrichment was found

among individuals with DISH relative to the non-DISH individuals, indicating that the former consumed foods rich in animal protein (Spencer, 2008).

One of the most recent investigations between diet and DISH using stable isotope analysis, was conducted at the site of the post-Medieval (16th to 18th century AD) Carmelite Friary burial grounds at Aalst, Belgium (Quintelier et al., 2014). The focus of the publication was to determine if individuals with DISH had marked differences in their $\delta^{13}C$ and $\delta^{15}N$ values compared to individuals in the sampled group, unaffected by DISH. In total 39 individuals had samples taken and collagen extracted for $\delta^{13}C$ and $\delta^{15}N$ isotope analysis, 10 of whom were diagnosed with DISH, all of them male. Samples were primarily collected form ribs and when ribs were unavailable for sampling, diaphyseal long bone fragments were used. Interestingly, the authors also tested the difference between pathological and non-pathological bone from individuals diagnosed with DISH, to see if any detectable differences in the stable isotope ratios are a result of physiological differences, independent of diet. The non-pathological samples were acquired from ribs, while samples taken from the new bone formation in affected vertebrae were used to represent pathologically altered tissue. The results showed a difference of no more than 0.2‰ between the pathological and non-pathological bone of individuals diagnosed with DISH, which was noted to be no more than the reported error exhibited by the mass spectrometer (Quintelier et al., 2014). The $\delta^{13}C$ values obtained from the non-pathological bone of individuals diagnosed with DISH ranged from -20.5‰ to -18.5‰, with a mean of -19.6‰ (±0.5‰), while the reported $\delta^{15}N$ values ranged from 9.9‰ to 13.5‰, with a mean of 11.9‰ (±0.9‰). Similar to the results of the analysis at the site of Fishergate (Müldner and Richards, 2007), a statistically significant difference was found between males and females for both carbon and nitrogen stable isotope ratio analysis, most likely representing a difference in dietary habits between the sexes (Quintelier et al., 2014). While males diagnosed with DISH had elevated $\delta^{13}C$ and $\delta^{15}N$ values compared to males without DISH, the difference was not statistically significant. The authors note that while no statistically significant difference was found in the isotope ratios between the groups (males with DISH versus males without DISH), this could be attributed to the relatively small sample size. However, they also note that the $\delta^{13}C$ and $\delta^{15}N$ values obtained from the DISH individuals at the Carmelite Friary of Aalst are similar to the reported values of individuals with DISH from two other studies, conducted by Müldner and Richards (2007) and Spencer (2008) (Quintelier et al., 2014). While the results of the study could not definitively prove a link between a diet rich in animal protein and DISH, it could not definitively disprove the theory either.

It is clear that the results from the discussion of the three studies above, present a complex story between DISH and diet, with no definitive answer on their association. A general trend discovered among all three of the studies was one of $\delta^{15}N$ enrichment among individuals diagnosed with DISH, indicating a diet high in animal protein, including foods from a higher trophic level. It would appear that further investigation may help to illuminate any answers to this issue. Documentary evidence of foods consumed by a population under study is invaluable and relevant to the interpretation of isotope ratios.

3. MATERIALS AND METHODS

In this chapter, all three skeletal collections analysed in the study are described, including a summary of the demographic profile of the skeletal collections, respectively. There are detailed descriptions of the methods used in the identification, recording and analysis of DISH in the human skeletal remains. The presence of images to illustrate details of the dry bone diagnoses, an example of the recording sheet used, and a detailed explanation of all the features scored are also included. Information on the use of micro-CT scanning is also provided, as well as detailed information on the collection and preparation methods used on the human bone samples for isotopic analyses. The information on the isotopic skeletal analysis includes detailed information on the collagen extraction process, and details of the mechanics behind the analytical method. Finally, the chapter then goes on to described the statistical analyses used to assess DISH. The statistical analyses cover three aspects of the study, (1) an investigation into the reliability of the intra-and inter-observer results, (2) the investigation of DISH within the skeletal populations, and (3) an investigation into the isotopic signatures of individuals diagnosed with DISH as well as those within the control group.

3.1. Materials

3.1.1. The skeletal collections

Three human skeletal collections were analysed, namely the Pretoria Bone Collection (PBC), the Raymond A. Dart Collection (RDC) and the Kirsten Collection (KC). Macroscopic and invasive methods were employed. Figure 3.1 shows the location of the three skeletal collections, respectively (where they are housed), on a map of South Africa.

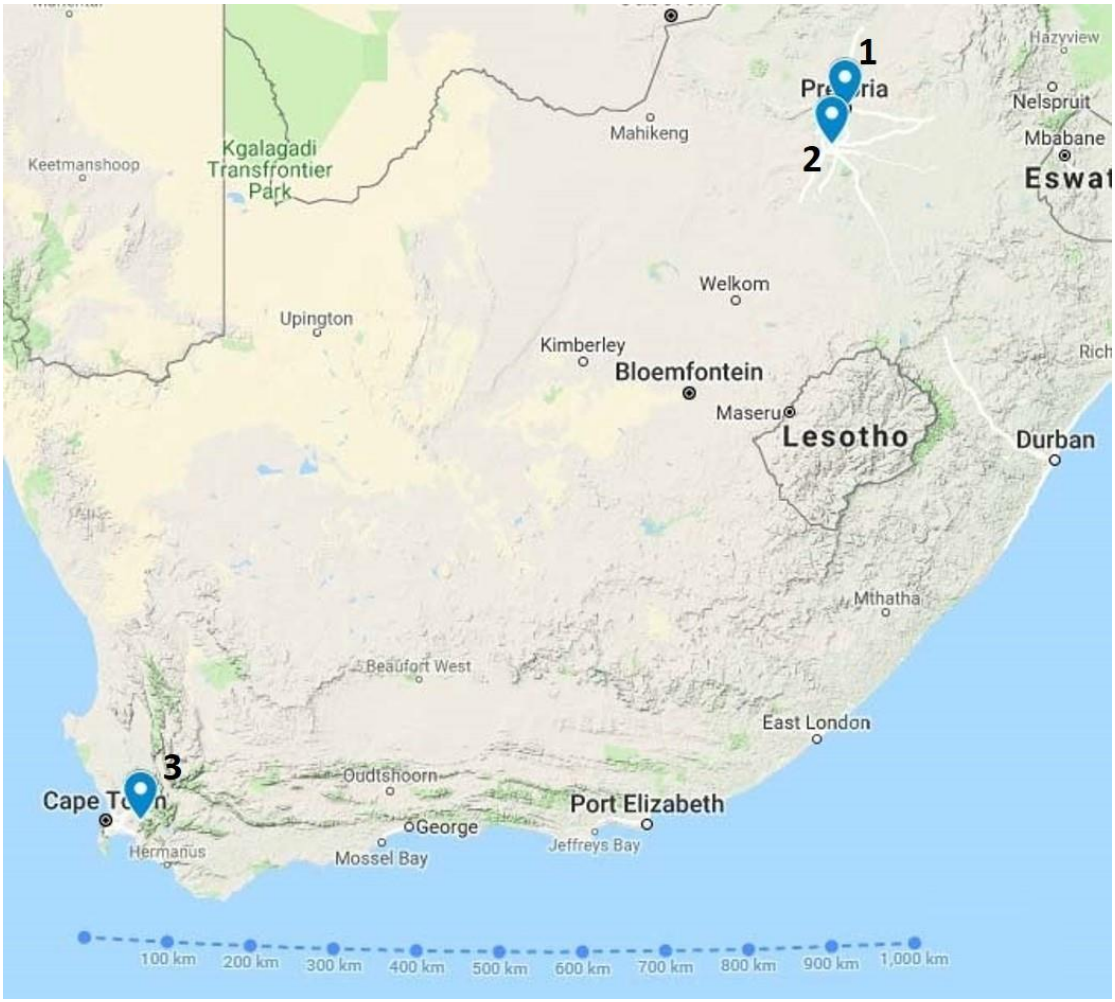

Figure 3.1. Map of South Africa, showing where the Pretoria Bone Collection (1), the Raymond A. Dart Collection of Human Skeletons (2) and the Kirsten Collection (3) are situated geographically. Source: Google Maps (2019).

It may be pertinent to briefly describe the history behind the classifications that exist for ancestry documentation or ancestry estimation within a South African context. South Africans were until 1994 forcibly put into categories that were at the very basic level based on the colour of an individual's skin as a means of keeping certain groups of people oppressed (non-white). The main ancestral classifications and accepted census terms includes black South African, coloured South African, white South African and Asian (Indian and Chinese) South African. After the end of the apartheid regime, many people adopted the ancestral classifications that were previously used and claimed them as their own means of self-identification. It is of the utmost importance to state that while terms like black, and coloured can be used in a derogatory manner in some countries, in South Africa the association is that of self-identification and is a recognised census term (Christopher, 2002).

The PBC is housed at the University of Pretoria and is a cadaveric derived collection used to teach science students, medical students, allied health sciences students, etc. at the University (L'Abbé et al., 2005). There were over 1000 skeletons in the collection at the time of analysis, and the majority are of known age-at-death and sex. With analysis of the Collection Manager's catalogue on the human skeletal remains, the PBC was found to contain roughly 1375 individuals with post cranial remains, however, this grows annually. Of the 1375 human skeletons available at the time of analysis, 1158 individuals were identified to be relevant to this study. This specifically included the post-cranial remains of both males (n=862; 74%) and females (n=296; 26%) whose age-at-death was over 40 years (Figure 3.2). One of the striking features of the collection (evident even before the parameters for inclusion in the study were applied) was the obvious bias between male and female individuals. Of the 1158 individuals, 592 were black South African males; 254 were white South African males; 16 were coloured South African males; 96 were black South African females; 193 were white South African females; and 7 were coloured South African females (Figure 3.3). In total there were 688 black South African individuals (59%), 447 white South African individuals (39%), and 23 coloured South African individuals (2%).

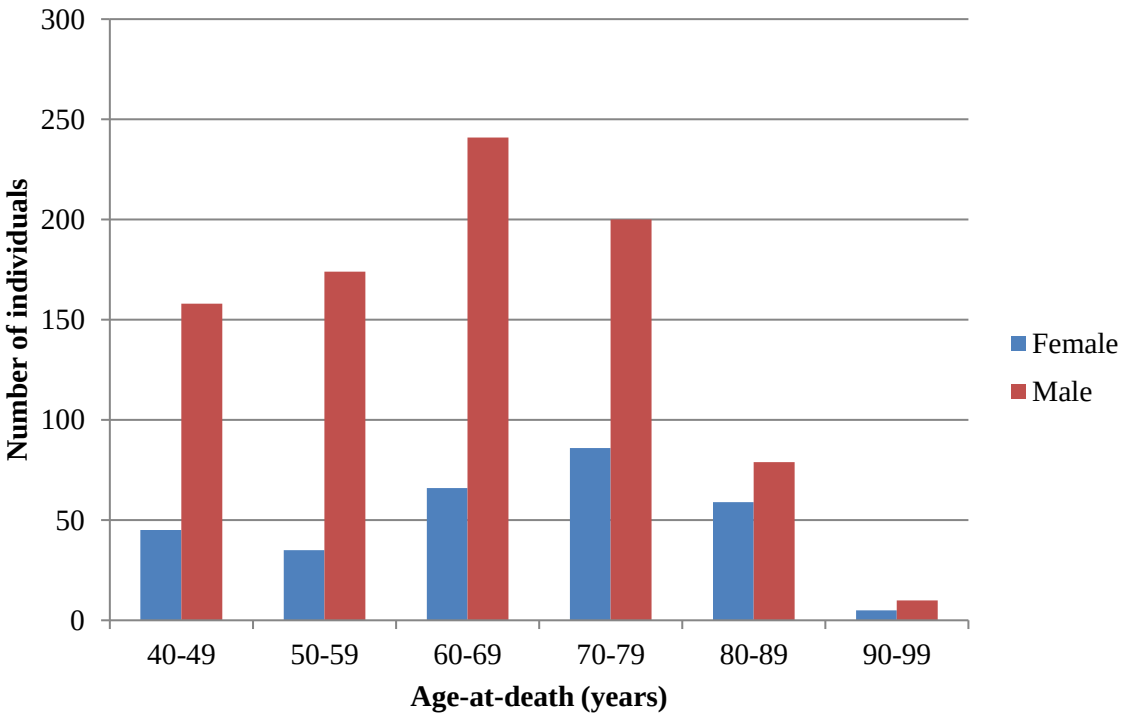

Figure 3.2: Individuals from the Pretoria Bone Collection (n=1158), separated by sex and age-at-death.

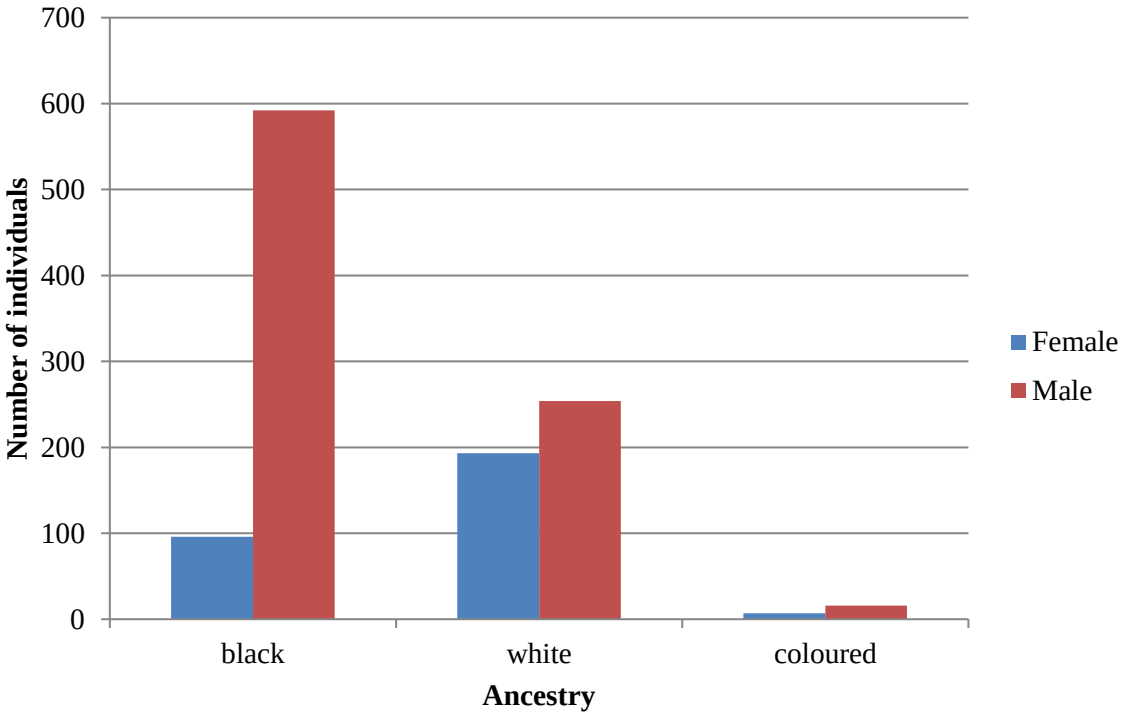

Figure 3.3: Individuals from the Pretoria Bone Collection (n=1158), separated by sex and ancestry.

The RDC is also a cadaver-derived skeletal assemblage, curated at the School of Anatomical Sciences at the University of the Witwatersrand. Originating in the early 1920s, the RDC represents four main ancestral groups, including South African black (76%), white (15%), coloured (4%) and Indian (0.3%) populations, which was noted to be a reasonable reflection of the demographic composition of the Gauteng province (Dayal et al., 2009). Dayal and colleagues (2009) noted that the collection held over 2600 skeletons; however, that number has grown over the years since their publication in 2009 (Dayal et al., 2009). Through analysis of the Collection Manager's catalogue, the RDC was found to contain roughly 2778 human skeletons. Within the 2778 human skeletons, 1932 individuals were identified to be relevant to this study. This number (1932) includes both males (n=1402) and females (n=530) over the age of 40 years at death, with post-cranial remains (Figure 3.4). Similar to the PBC, the part of the assemblage that is relevant to the study has a much larger proportion of male (72%) than female (28%) individuals.

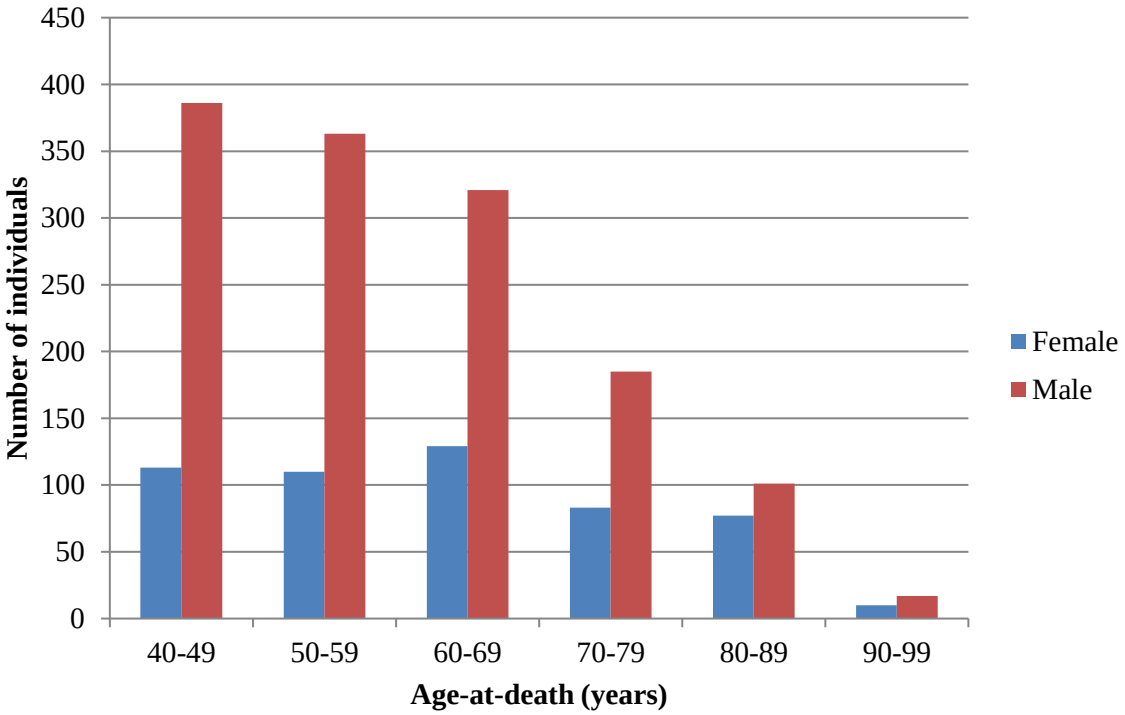

Figure 3.4: Individuals from the Raymond A Dart Collection of Human Skeletons (n=1932), separated by sex and age-at-death.

There were 1020 black South African males, 286 white South African males, 96 coloured South African males, 272 black South African females, 225 white South African females, and 33 coloured South African females (Figure 3.5). In total that was 1292 black South African individuals (67%), 511 white South African individuals (26%), and 129 coloured South African individuals (7%).

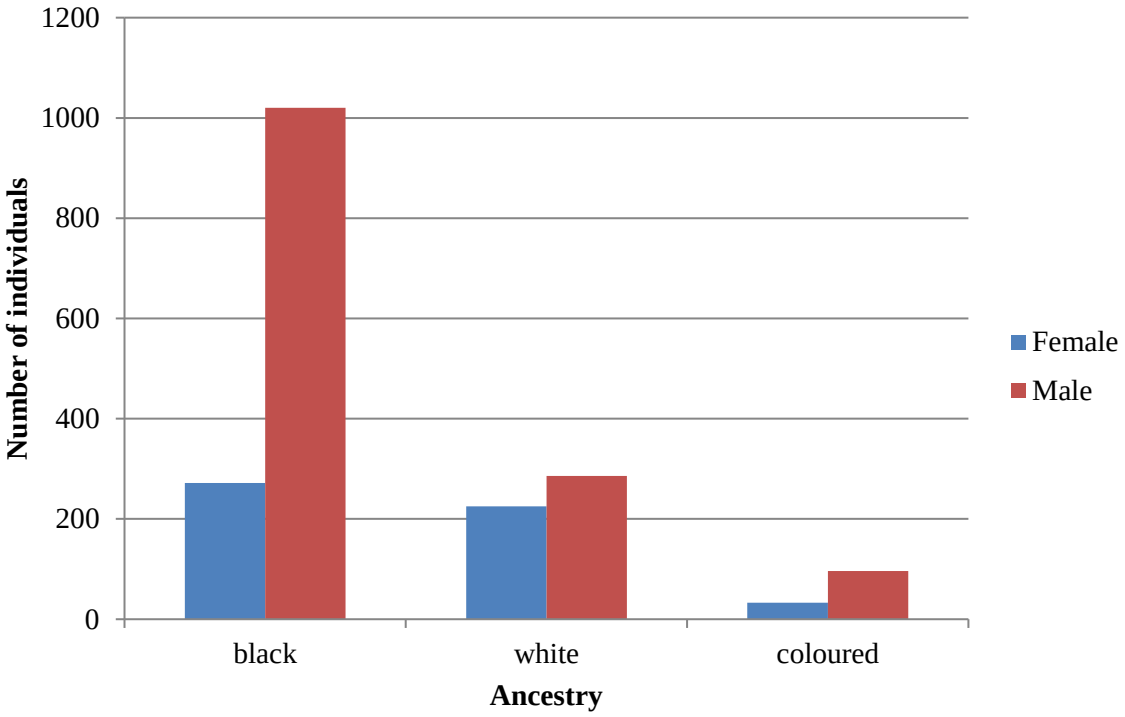

Figure 3.5: Individuals from the Raymond A Dart Collection of Human Skeletons (n=1932), separated by sex and ancestry.

The KC is a modern skeletal assemblage (cadaver-derived) with over 1200 individuals and is curated at the Division of Anatomy and Histology, at Stellenbosch University (Alblas et al., 2018). The KC represents individuals from the northern suburbs of Cape Town and some surrounding towns in the Western Cape. Information is available on most individuals', sex, age-at-death, ancestry, and cause of death; however, some records are incomplete due to the acquisition of unclaimed bodies with little details known (Alblas et al., 2018). Through examination of the curator's catalogue of human skeletons, the KC contained roughly 1161 individuals with post cranial remains and known records, and much like the other two collections, this number is constantly growing (Alblas et al., 2018). Within the 1161 human skeletons, 586 individuals were identified to be relevant to the study for analysis. This number (586) includes both males (n=419) and females (n=167) with post-cranial remains with an age-at-death over 40 years (Figure 3.6). As with the PBC and RDC, there is an inherent bias in the KC towards males, who make up roughly 72% of the number of individuals relevant to the study and females contributing 28%.

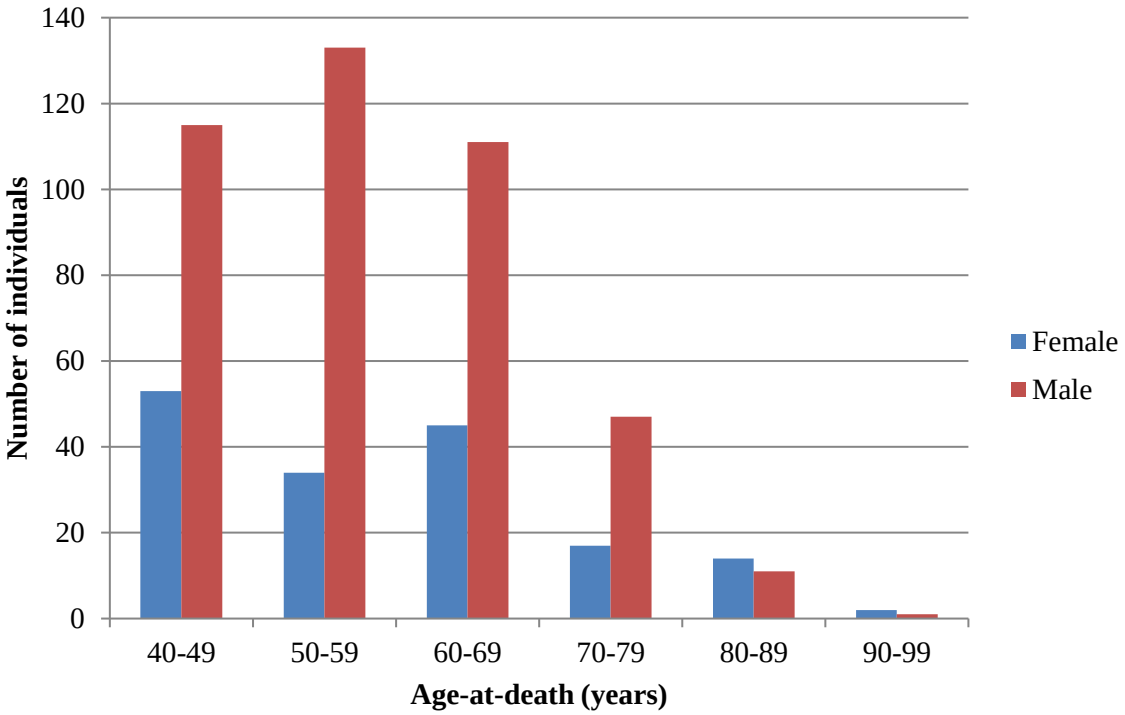

Figure 3.6: Individuals from the Kirsten Collection (n=583), divided by sex and age-at-death.

There were 79 black South African males; 68 white South African males; 272 coloured South African males; 12 black South African females; 45 white South African females; and 110 coloured South African females (Figure 3.7). In total that is 91 black South African individuals (16%), 113 white South African individuals (19%), and 382 coloured South African individuals (65%). As with the disproportionate representation of sexes in this skeletal population, KC also has a particularly high number of coloured South African individuals, especially when compared to the PBC and RDC. This is most likely due to the unique situational circumstances by which individuals are incorporated into the collections. The region in which the KC is housed has a high number of living individuals who self-identify as coloured South African (Department for Statistics South Africa, 2019). Table 3.1. gives a summary of the number of individuals under study separated by collection, sex, ancestry and information around age-at-death.

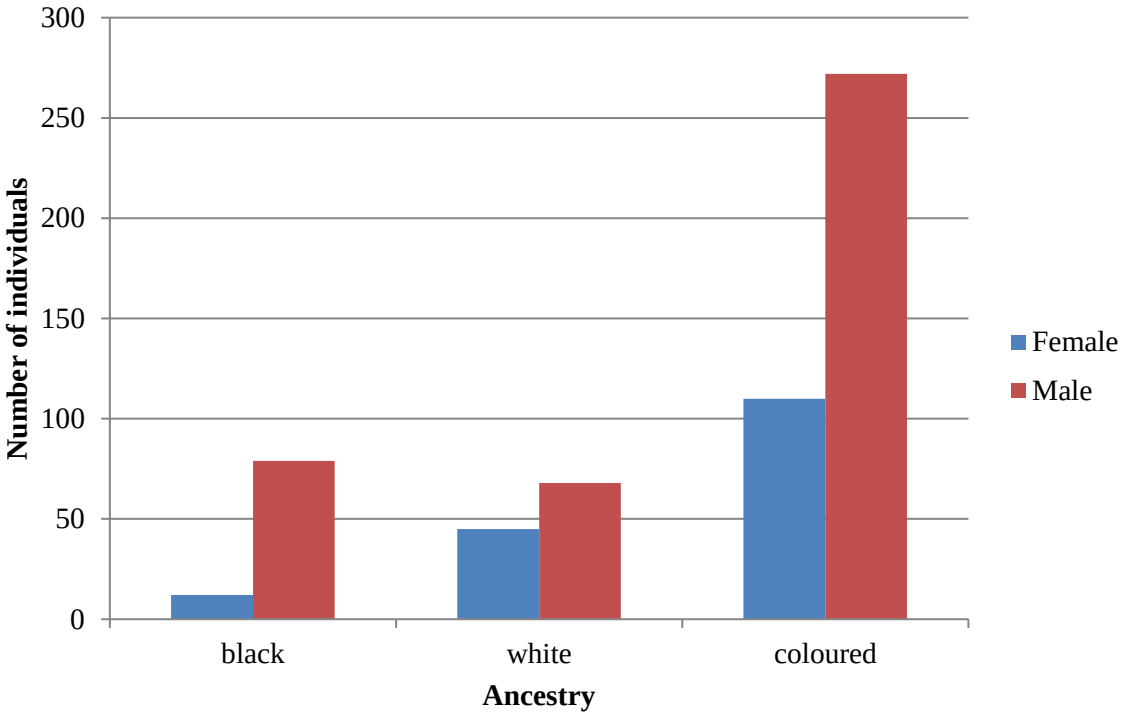

Figure 3.7: Individuals from the Kirsten Collection (n=586), separated by sex and ancestry.

The modern skeletons found in all three of the collections discussed above, come to be in the collections through one of two means: through donation, or they are unclaimed bodies. In South Africa, if an individual dies in a public hospital, with nobody (relative or friend) to collect the body within 24 hours of death, the remains may be given to an institute of higher education for medical research and education (L'Abbé et al., 2005; Dayal et al., 2009; National Health Amendment Act, 2013; Alblas et al., 2018). The institute then embalms the body and keeps it in storage for a year to allow for sufficient time to pass for a family member to claim the body. If the individual is not claimed, then the body is most commonly used for dissection training for students studying for various degrees in the Medical Sciences. After a cadaver has been dissected, the body is then macerated and the skeleton is added to the collection with an accession number with linked information to their biological profile, as well as any relevant information that may be useful to research projects at a later stage, such as cause of death (L'Abbé et al., 2005).

Anybody who wishes to donate their body to research, medical training or for tissue transplantation in South Africa is covered under the National Health Amendment Act, No. 12 of 2013 (L'Abbé et al., 2005; Alblas et al., 2018). Individuals over the age of 65 years at death

and individuals who died of a disease such as cancer or AIDS are rejected for tissue transplantation and would then be automatically given to an institute for medical teaching/research. Many individuals who identify as black South African do not subscribe to the notion of donating their bodies, largely due to cultural and religious reasons (L'Abbé et al., 2005). However, as we have seen from the information on the demographic profiles of the PBC, RDC and KC above, the skeletal populations contain a high proportion of black male individuals except for the KC whose largest demographic group is coloured males. At the University of the Witwatersrand, the vast majority of remains coming into the School of Anatomical Sciences the past few years have been from donated remains (Kramer et al., 2018), following international best practice.

The presence of black males in the PBC and the RDC has been attributed to the history of migrant labour in South Africa, especially in relation to the mining industry in the Gauteng region. Many black males are economic migrants, moving from rural areas of South Africa and neighbouring countries, into urbanised areas where the prospect of a job is much higher (L'Abbé et al., 2005). Often, persons who move to the urbanised areas to find jobs have limited means of communication with their distant families. This complication becomes exacerbated in the event of an individual's death, as long periods of non-communication can be the norm, and it may be some months before a relative will enquire about a loved one. It can then be very difficult for individuals working within the medical profession to contact a family member to inform them of a death. Black women are believed to stay closer to their relatives, therefore in the event of a death, their bodies are often claimed (L'Abbé et al., 2005). This could be the reason why black women have low representation within the collections. While there are more black individuals than white individuals within all three of the skeletal collections, proportionally, there are more whites than blacks relative to the overall South African population. This fundamentally alters the demographics of the collections, reiterating their incomparability to the living population (Komar and Grivas, 2008; Kramer et al., 2018). Whatever an obstacle this may produce in research, useful information can still be uncovered through their analysis.

It is worth reiterating that while the population being investigated is considered modern, the stance of this study is very much a bioarchaeological one, mainly because the primary source of evidence is that of the skeletons themselves. Not only do we have the advantage of (more often than not) perfectly persevered human skeletal remains, but there is also a wealth

of associated knowledge in the form of post-mortem height and weight measurements, accurately (again, more often than not) recorded sex, age-at-death and ancestry information.

Table 3.1. Table presenting the final sample under study, separated by collection, sex, ancestry with information on associated age-at-death.

Collection	Sex	Ancestry	Number of individuals under study	Average age-at-death (years)	Min age-at-death (years)	Max age-at-death (years)
PBC	male	black	592	59	40	98
		white	254	70	40	94
		coloured	16	58	44	73
	female	black	96	55	40	79
		white	193	74	42	94
		coloured	7	52	40	76
	both sexes	black	688	58	40	98
		white	447	71	40	94
		coloured	23	56	40	76
	both sexes	all ancestry	1158	64	40	98
RDC	male	black	1020	55	40	108
		white	286	59	40	95
		coloured	96	62	40	106
	female	black	272	56	40	104
		white	225	72	43	96
		coloured	33	54	40	80
	both sexes	black	1292	55	40	108
		white	511	60	40	106
		coloured	129	70	40	96
	both sexes	all ancestry	1932	60	40	108
KC	male	black	79	57	40	90
		white	68	60	40	80
		coloured	272	54	40	87
	female	black	12	51	40	70
		white	45	61	41	90
		coloured	110	53	40	80
	both sexes	black	91	56	40	90
		white	113	61	40	90
		coloured	382	55	40	103
	both sexes	all ancestry	586	57	40	103
All Collections	male	black	1691	56	40	108
		white	608	68	40	95
		coloured	384	56	40	106
		black	380	55	40	104

	female	white	463	72	41	96
		coloured	150	54	40	80
	both sexes	black	2071	56	40	108
		white	1071	70	40	96
		coloured	534	55	40	106
	both sexes	all ancestry	3676	60	40	108

3.1.1.1. Preservation of the human skeletal remains

Because the majority of the bodies incorporated into the skeletal collections are from medical dissection, the completeness of the skeletal remains was very good. After dissection, the bodies undergo maceration where any adhering tissue that was present on the bones is removed and the bones are cleaned, accessioned with a reference number and incorporated into their respective collection. Overall, while there may be small amounts of damage to some bones with some elements missing from some individuals, the state of preservation was almost perfect in the majority of cases. Where an element used to diagnose DISH was missing, it was marked on the recording form as either element missing post-mortem, or post-mortem damage to element.

3.2. Methods

In order to achieve the aim and objectives of this study, an invasive as well as non-invasive analysis was undertaken. The non-invasive analysis comprised of two types of investigations, the first being the assessment of the skeletons itself and the second was looking at the underlying structures of the vertebral lesions through micro-CT scanning. The macroscopic analysis was used to identify features associated with DISH on a present or absent basis. This binary system for scoring features was chosen to reduce any ambiguity which can arise when using a numerical scoring system, as well as descriptions for scoring each feature. The invasive analysis consisted of taking small samples of bone (from the femur and rib of an individual selected) for isotopic analysis.

3.2.1. Recording macroscopic evidence of DISH amongst human skeletal remains

A full visual assessment of all the individuals within each collection was performed to identify macroscopic signs of DISH, using diagnostic criteria. The only selective process

undertaken was to exclude all individuals whose age-at-death was under 40 years, as we know DISH is a disease that affects individuals over the age of 45/50 years.

The diagnostic criteria used to assess whether an individual had DISH was adapted from Rogers and Waldron (2001) and incorporated bony characteristics that have been highlighted across a number of published studies, as well as incorporating some infrequently observed features to assess their frequency within the individuals highlighted to have DISH. The main features significant to a diagnosis of DISH are:

1. Flowing ossification, present on the right anterolateral surface of a minimum of three contiguous vertebrae (thoracic region); with or without ankylosis (see section 3.2.1.1 below)
2. Retention of the intervertebral disc space (see section 3.2.1.1 below)
3. No superior or inferior apophyseal joint surface ankylosis (see section 3.2.1.1 below)
4. Retention of the SIJ space (see section 3.2.1.2 below)
5. Evidence of bilateral extra-spinal manifestations should be present and include enthesophytes at one or all of the following sites; ulnae (insertion of the *m. triceps brachii*; see section 3.2.1.3), patellae (insertion of the *m. quadriceps femoris*; see section 3.2.1.4), calcanea (insertion of the *m. triceps surae*; see section 3.2.1.5), tibial tuberosities (insertion of the *m. quadriceps femoris*; see section 3.2.1.6) or ossification of the *ligamentum flavum* (see section 3.2.1.7).

If individuals conformed to all of the abovementioned features, then a classification of definite DISH was applied. Where there was insufficient spinal evidence of DISH in an individual (criterion 1 of list above) but the rest of the criteria were met, a classification of probable DISH was applied, i.e. meeting criteria 4 and 5, but has to meet evidence of bilateral extra-spinal manifestations at, at least three of the sites mentioned. When there was clear antero-lateral spinal involvement (on the right side) but no extra-spinal manifestations present, (criterion 5 of the diagnostic list above), then a classification of probable DISH was applied. The classification of probable and definite DISH was useful for diagnosis and identification purposes only. No separation was made between individuals classified as probable and definite DISH during any part of the analyses. Individuals who came under both categories were investigated under the single classification of individuals diagnosed with DISH.

The sites mentioned above for enthesophyte formation were important for diagnostic classification; however, other sites were noted and recorded to assess their frequency within the individuals highlighted to have DISH, namely plantar heel spurring, ossification of the apical ligament and ossification of the supraspinous ligament.

When giving a score based on macroscopic observations, results can differ due to the variability between observers in their experience and comprehension, as well as the recording of some features being objective. However, clear descriptions with photographic examples are helpful. The rest of the chapter provides a description of each of the features scored and provides corresponding photographic examples. Tables 3.1 and 3.2 shows the data scoring sheet with an example individual. Additionally, the same data were collected for inter-observer analysis.

Although each criterion above is needed in order to give a diagnosis of DISH (definite DISH or probable DISH), other features were also incorporated into the analysis and included plantar heel spurring (insertion of the *m. flexor digitorum brevis*), ossification of the apical ligament, and ossification of the supraspinous ligament. As mentioned, these features are not necessary for a diagnosis of DISH, but were included to see how often they occurred as they are mentioned intermittently within the literature as features possibly associated with DISH.

One additional feature that was recorded was a visualisation of the extent of ossification in the vertebrae, using a coding method to denote where the ossification was present and whether there was ankylosis between vertebral units or not (see section named ‘SPINAL’ in Table 8.1 and corresponding key in Table 8.2 in Appendix 1). To portray this, during observational analysis, each vertebra affected by flowing ossification was split into five zones; right lateral (RL), right anterior (RA), centre/anterior (C), left anterior (LA), left lateral (LL). Figure 3.8 provides a visual representation of the aforementioned five zones on an image of the vertebral body. Figure 3.8 also illustrates the codes (^1, ^2, ∨1 or ∨2; see Table 3.2) that were used to denote ossification in the upper and lower portions of the vertebrae (separation in the transverse plane). The division of the vertebrae into upper and lower portions - denoted by codes - indicated if the flowing ossification was with the superior (^) or inferior (∨) articulating vertebrae, as well as whether bony ankylosis (2) had occurred with the contiguous vertebrae or not. In some cases, there was incomplete/no ankylosis of consecutive vertebrae (1), but the flowing ossification was still present.

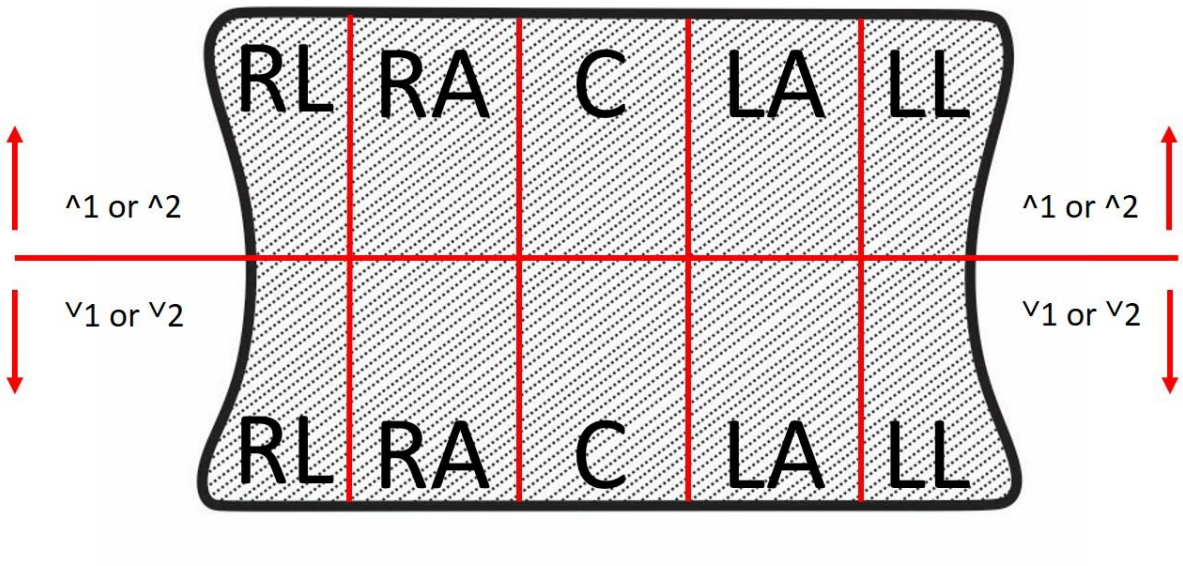

Figure 3.8. A vertebral body (anterior view) depicting codes for recording the extent of flowing ossification as well as where it occurs on the vertebral body. Referring to the different regions of the vertebral body; (RL) right lateral, (RA) right anterior, (C) centre/anterior, (LA) lateral anterior, (LL) left lateral.

3.2.1.1. Flowing ossification, intervertebral disc space retained and apophyseal facets retained

The flowing ossification across at least three contiguous vertebrae, retention of the intervertebral disc space and the apophyseal facet space must be present, and aid in a positive diagnosis of DISH. All three of these features were recorded as either present (1) or absent (0).

The flowing ossification refers to the presence of bone on the right anterolateral side of vertebrae, which in advanced cases simulates a 'dripping candle-wax' appearance (indicated as 1a and 1b on Figure 3.9). The ossification can be continuous/ankylosed (1a on Figure 3.9), touching between vertebrae (1b on Figure 3.9), or developed but not touching. In situations where the flowing ossification was not touching a corresponding ossification, but was present, it needed to be clearly forming from the centre of the anterior aspect of the vertebral body and not from the endplate, as seen with VO or with syndesmophyte formation in AS (see Figure 2.4, page 25).

The retention of the intervertebral disc space is an important diagnostic characteristic of DISH (Resnick et al., 1978; Rogers and Waldron, 2001). Individuals must have retention of the intervertebral disc space in order to rule out other possible diagnoses such as AS, where destruction and ankylosis of the intervertebral disc space is a diagnostic feature. This feature is illustrated in Figure 3.9 (denoted by 2 on the image), showing the retained joint space in both the anterior and left lateral views of the vertebrae.

The retention of the apophyseal facets is another feature that is required for a positive diagnosis of DISH (3 on Figure 3.9). There must be no destruction of the apophyseal facet surface with or without ankylosis. This will help with differential diagnosis as destruction and ankylosis of the apophyseal facets is present in AS. However, the presence of diseases such as degenerative disc disease (DDD) and osteoarthrosis in the apophyseal facets can lead to changes in the joint surface (such as eburnation and degeneration) as well as osteophyte development with subsequent ankylosis. While DDD strictly affects the soft tissue (intervertebral discs), the secondary effects can be seen in the skeleton. Skeletal changes occur as the intervertebral discs dehydrate and lose their height, causing a narrowing, rubbing and eventual ankylosis of articulating apophyseal facets. Therefore, the presence of DDD did not disprove a diagnosis of DISH if flowing ossification, retention of the intervertebral disc space and retention of the sacro-iliac joint were observed.

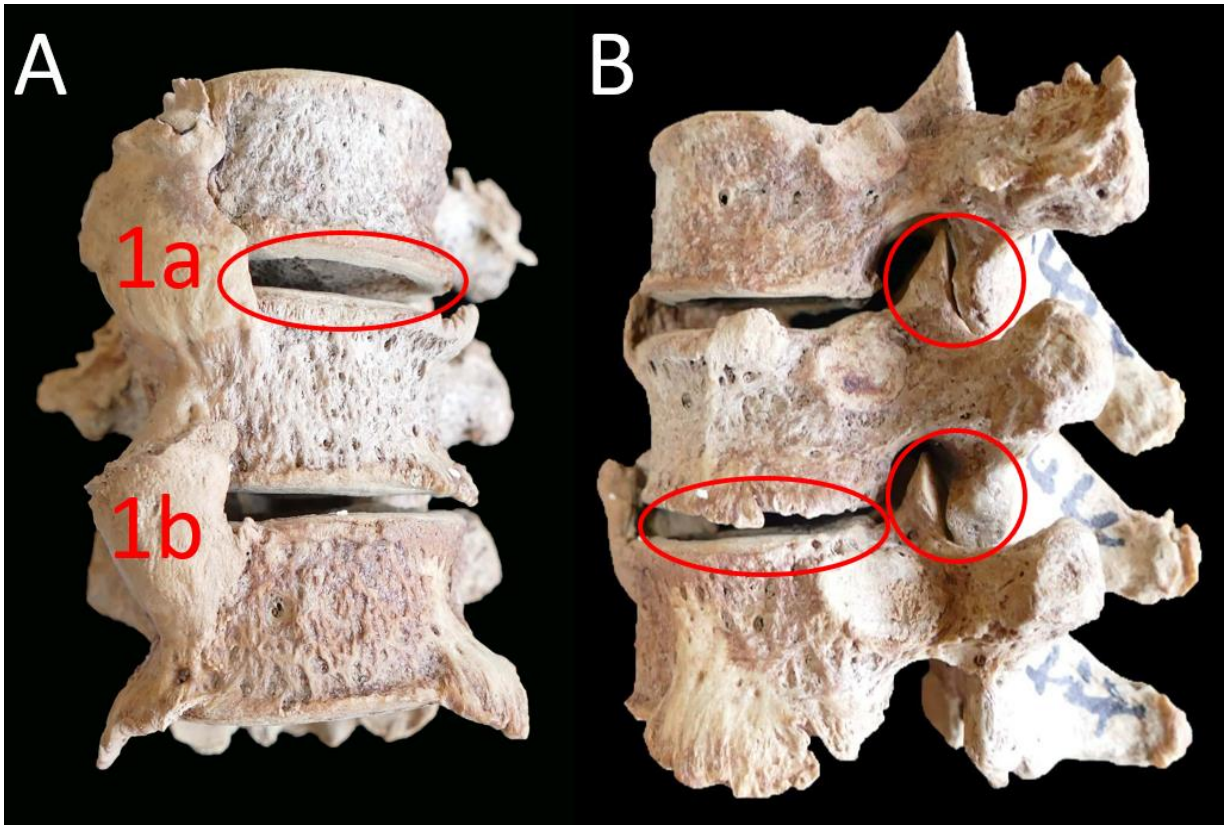

Figure 3.9: Vertebrae T9-T11, affected by DISH. (A) shows an anterior view of the vertebrae, while (B) shows a left lateral view. (1a) shows flowing ossification with ankylosis. (1b) shows touching flowing ossification with no ankylosis. The red ovals illustrate retention of the intervertebral disc space, while the red circles show retention of the apophyseal facets. Individual 5144, from the PBC, housed at the University of Pretoria. Source: Author.

3.2.1.2. Sacro-iliac joint

There are two features that can be seen in the sacro-iliac region of individuals with DISH. In the first instance it is a lack of pathology that helps with a positive diagnosis, as the sacro-iliac joint surface (auricular surface) must be retained. Any pathological damage to the auricular surface would negate a diagnosis of DISH. Secondly, ossification of the anterior sacroiliac ligament (Figure 3.10) is commonly seen. This feature, when associated with DISH, has been reported in the literature as being predominately unilateral. While fusion can occur at the superior portion of the sacro-iliac joint via ossification of the anterior sacro-iliac ligament (Figure 3.10), the auricular surface is not involved. Both features were recorded as either present (1) or absent (0) and the unilaterality or bilaterality was noted.

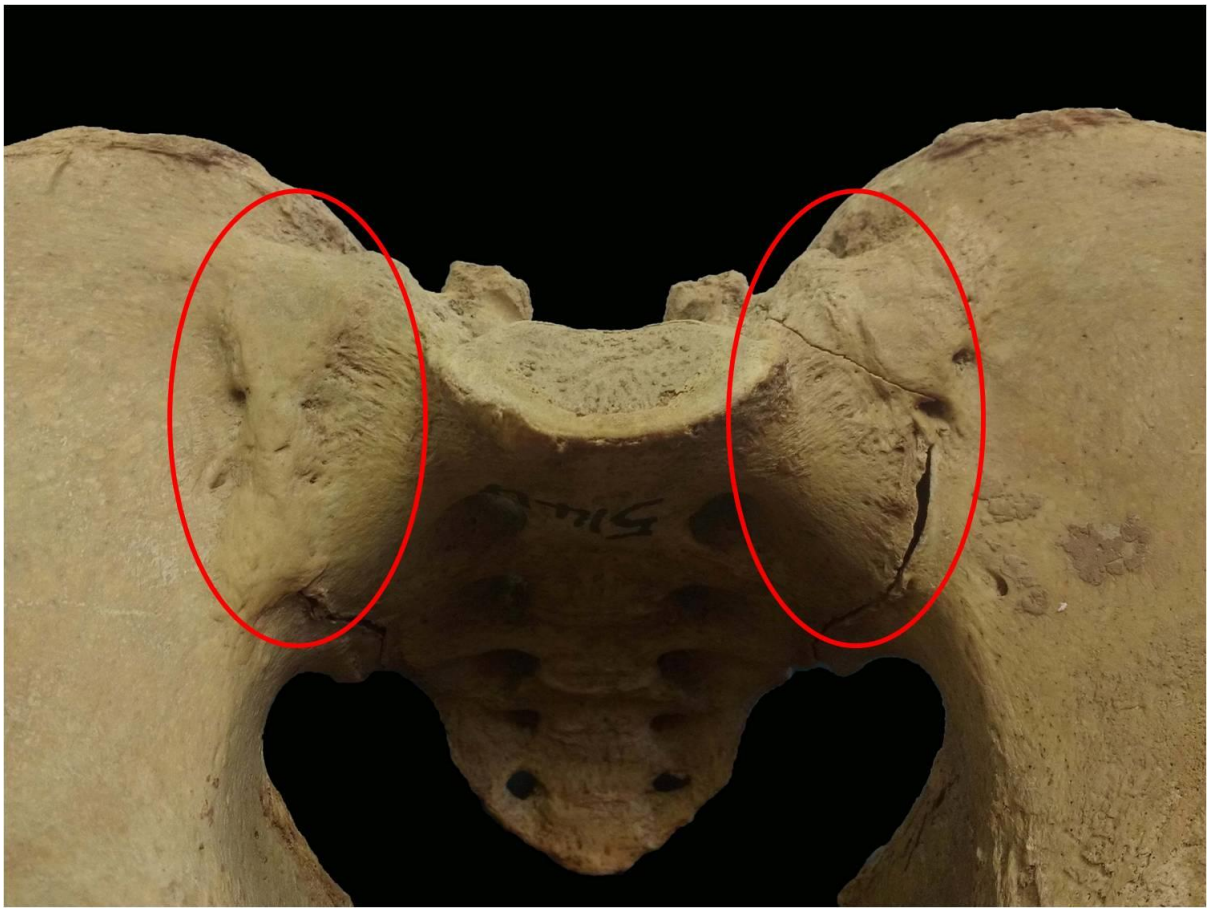

Figure 3.10: Bilateral ossification of the anterior sacroiliac ligament (red ovals). Skeleton 5144, from the PBC. Source: Author.

3.2.1.3. Olecranon tufting - *m. triceps brachii*

Olecranon tufting (Figure 3.11) refers to the presence of an enthesophyte at the site of the insertion of the *m. triceps brachii* on the ulnae. The terms tufting and enthesophyte can be interchangeably used. This feature was scored as present (1) or absent (0) and was scored for both the left and right side to assess unilaterality and bilaterality, as in some cases a positive diagnosis of DISH relied on bilateral involvement.

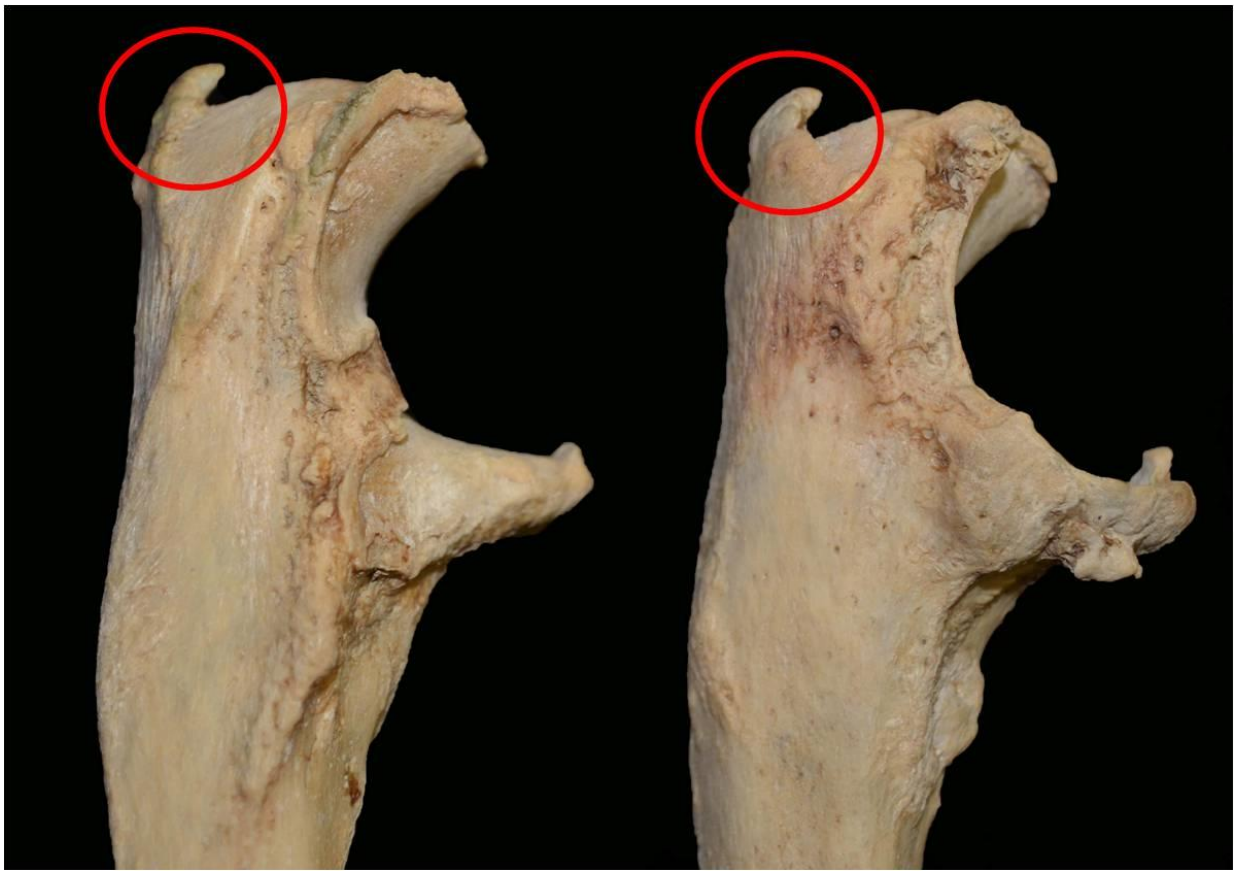

Figure 3.11: Proximal end of the right and left ulnae from indivdual 4969, from the PBC. Note the tufting on the ulnae at the insertion site of the *m. triceps brachii* (red circles). Source: Author.

3.2.1.4. Patellar tufting - *m. quadriceps femoris*

Patellar tufting refers to the presence of an enthesophyte at the site of insertion of the *m. quadriceps femoris* on the patellae (Figure 3.12). Any evidence of patellar tufting was scored as present (1) or absent (0), and its presence on either the right or left patella was recorded to assess unilaterality or bilaterality, aiding in diagnosis of DISH.

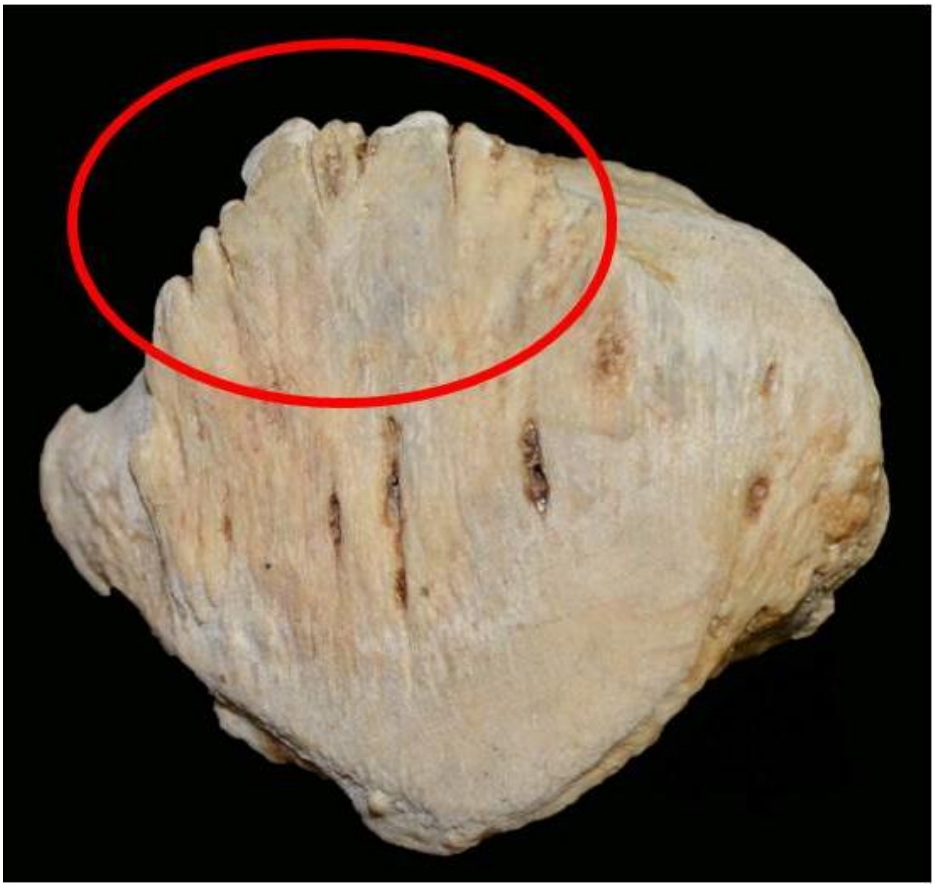

Figure 3.12: Right patella from individual 4969, from the PBC. Note the tufting on the patella at the insertion site of the *m. quadriceps femoris* (red oval). Source: Author.

3.2.1.5. Heel spurring - *m. triceps surae*

Heel spurring (Figure 3.13) describes an enthesophyte at the site of insertion of the *m. triceps surae* on the posterior aspect of the calcanea. This feature was scored as being present (1) or absent (0) and the presence of the heel spurring as unilateral or bilateral within individuals was noted.

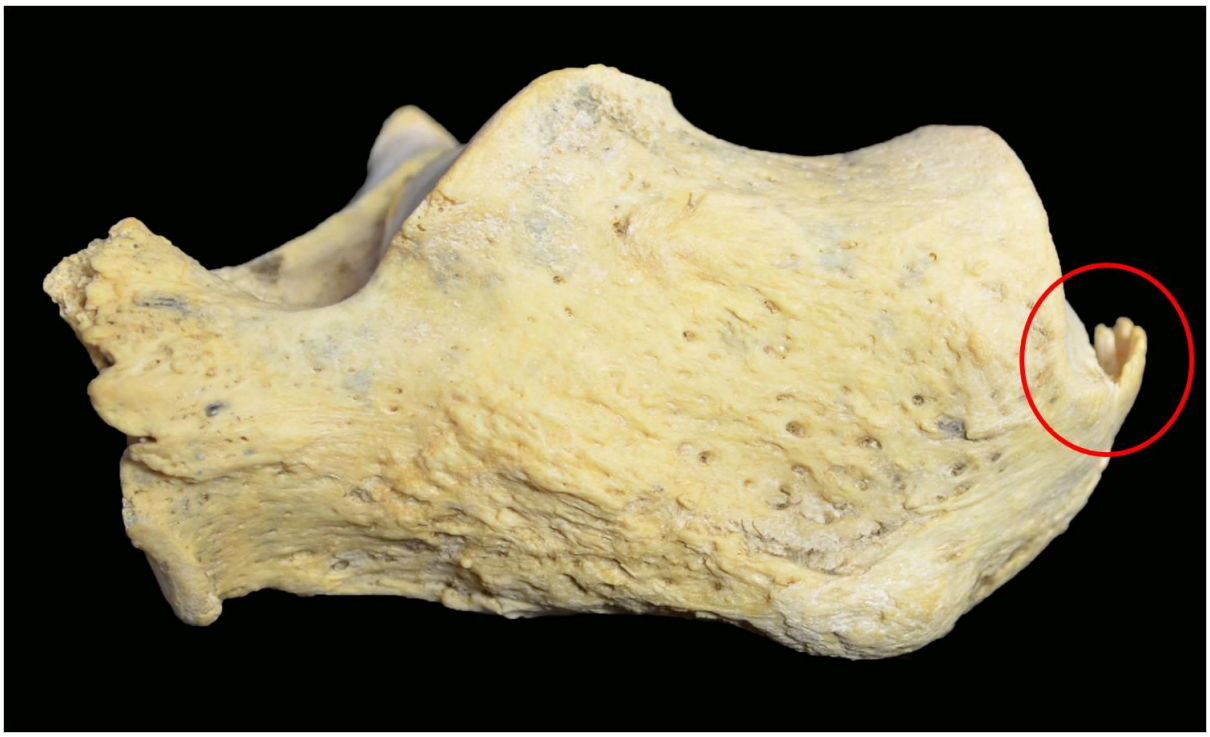

Figure 3.13: Left calcaneus from individual 4969, from the PBC. Note the tufting on the posterior surface at the insertion site of the *m. triceps surae* (red circle). Source: Author.

3.2.1.6. Spurring on tibial tuberosities

The recording of the spurring on the tibial tuberosity (Figure 3.14) refers to the presence of an enthesophyte at the site of insertion of the patellar ligament from the *m. quadriceps femoris* on the tibiae. Spurring on the tibia tuberosity was recorded as either present (1) or absent (0), as well as whether the feature was unilateral or bilateral.

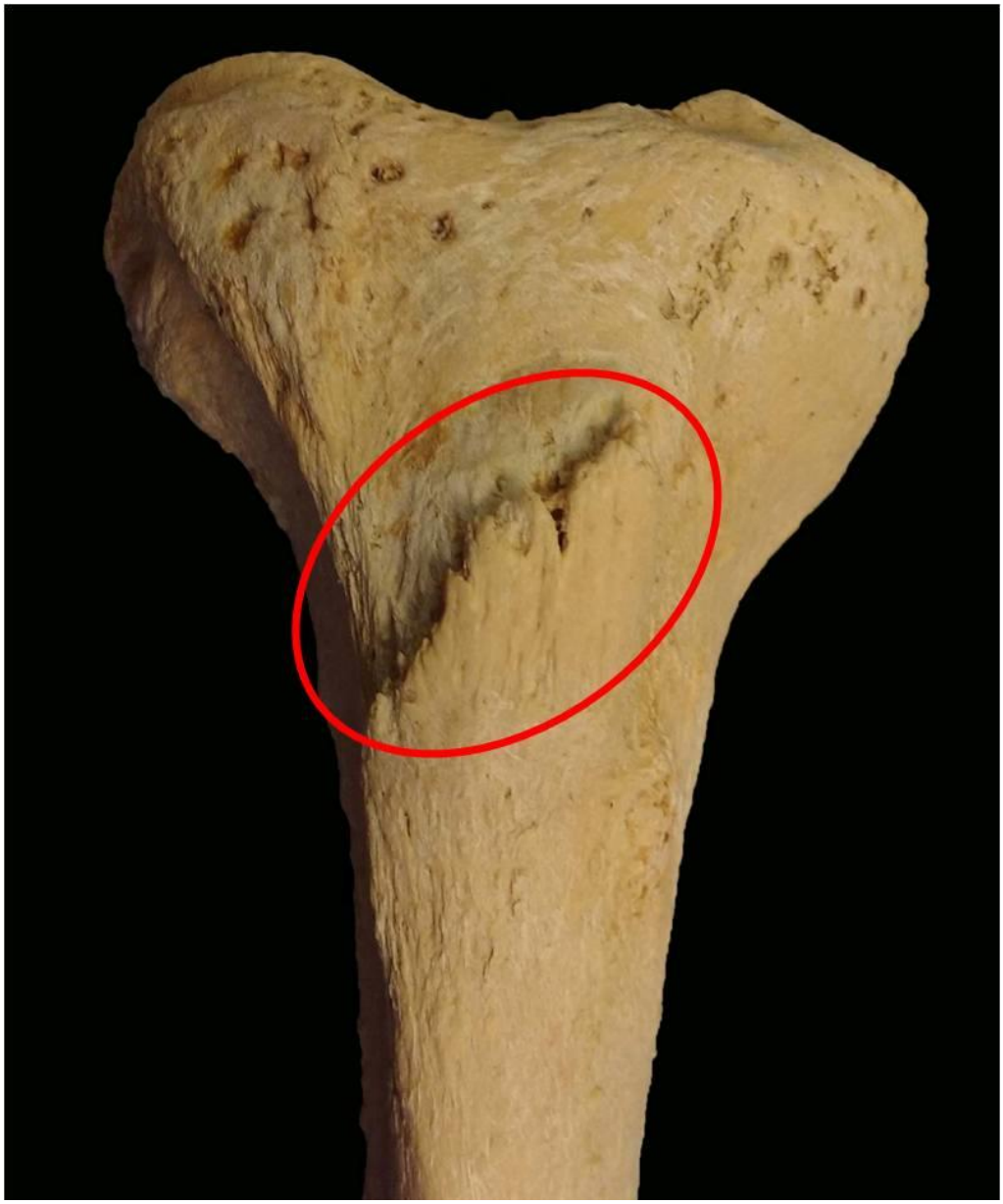

Figure 3.14: Anterior view of the proximal end of the right tibia from individual 6341, showing spurring on the tibial tuberosity (red oval). The individual is from the PBC. Source: Author.

3.2.1.7. Ossification of ligamentum flavum

The ossification of the ligamentum flavum (Figure 3.15) can be seen on the superior or inferior surface of the lamina of vertebrae. The feature was scored as present (1) if there was any evidence of ossification (superiorly or inferiorly) on vertebrae also affected by the flowing ossification. If no evidence was found, the feature was scored as absent (0).

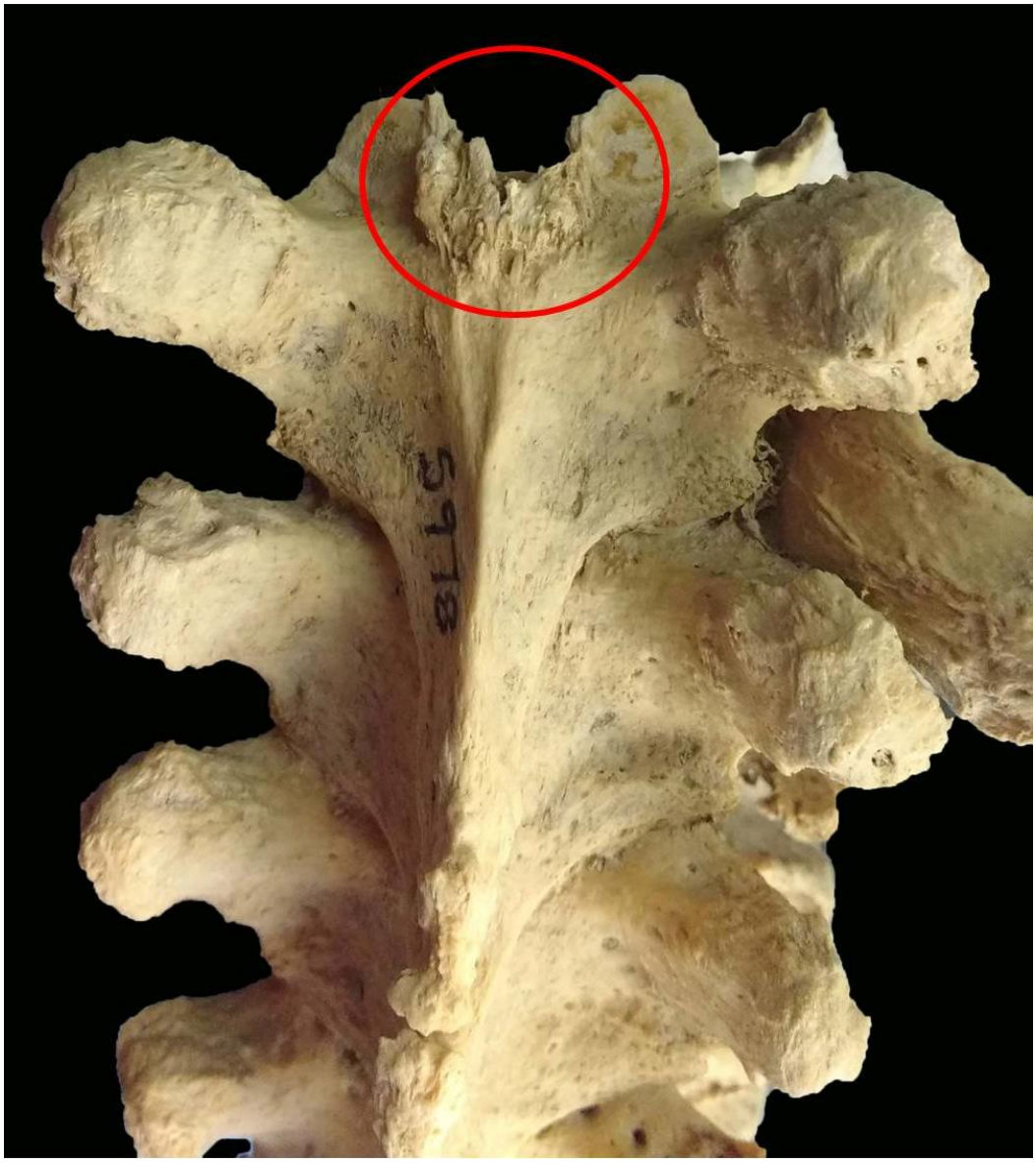

Figure 3.15: Vertebrae from individual 5978, from the PBC. Note the ossification of ligamentum flava on the superior surface of T6 (red circle). Source: Author.

3.2.1.7. Plantar heel spurring

Plantar heel spurring (Figure 3.16) describes the presence of an enthesophyte at the site of insertion of the *m. flexor digitorum brevis* and the plantar fascia on the inferior surface of the calcanea. This feature was not strictly needed for a positive diagnosis of DISH, but was recorded to see the frequency in which it appears in individuals diagnosed with DISH. Plantar heel spurring was recorded as being present (1) or absent (0), as well as whether it was present unilaterally or bilaterally.

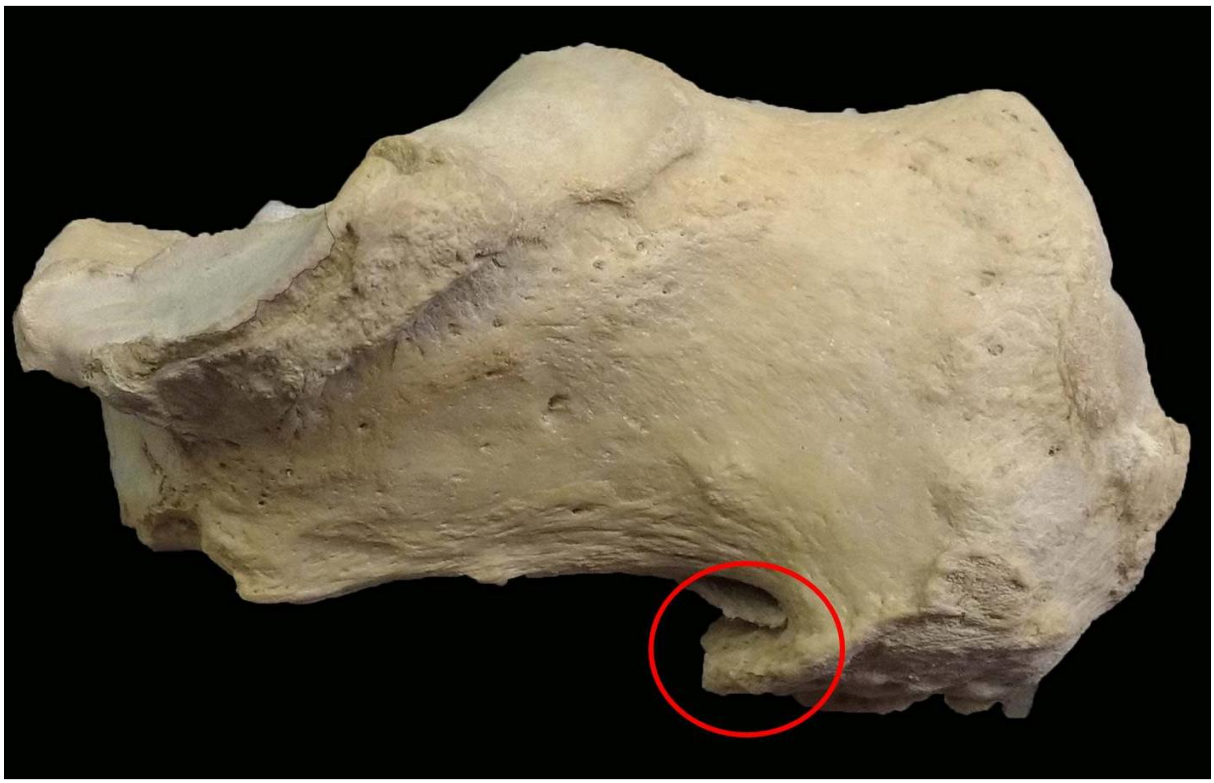

Figure 3.16: Right calcaneus from individual 5908, from the PBC. Note the tufting on the inferior surface of the calcaneus, at the insertion site of the *m. flexor digitorum brevis* and the plantar fascia (red circle). Source: Author.

3.2.1.8. Ossification of apical ligament

The apical ligament is found on the dens of the axis and can ossify (Figure 3.17). The ossification of the apical ligament was recorded as either present (1) or absent (0). This feature was scored as present if the ossification of the apical ligament extended 2 mm or more from the superior point of the dens. This feature was scored as absent if there was evidence of osteoarthritis present on the dens of the axis. This includes but is not limited to the presence of eburnation on the anterior surface of the dens. As with the recording of the plantar heel spurring, this feature was not strictly needed for a positive diagnosis of DISH, but was recorded to see the frequency in which it appears in individuals diagnosed with DISH.

Figure 3.17. Ossification of the apical ligament on the dens of the axis, illustrated by the red circle. Source: adapted from (Suby et al., 2018).

3.2.1.9. Ossification of supraspinous ligament

The supraspinous ligament can be found on the most posterior point (tip) of the spinous process. Figure 3.18 provides an example of the ossification of the supraspinous ligament in an individual. This feature was scored as either present (1) or absent (0). The feature was scored as present (1) if there was any evidence of ossification of the supraspinous ligament on vertebrae also affected by the flowing ossification. While this feature is not included in the diagnostic criteria for DISH, it was recorded to assess its frequency with DISH.

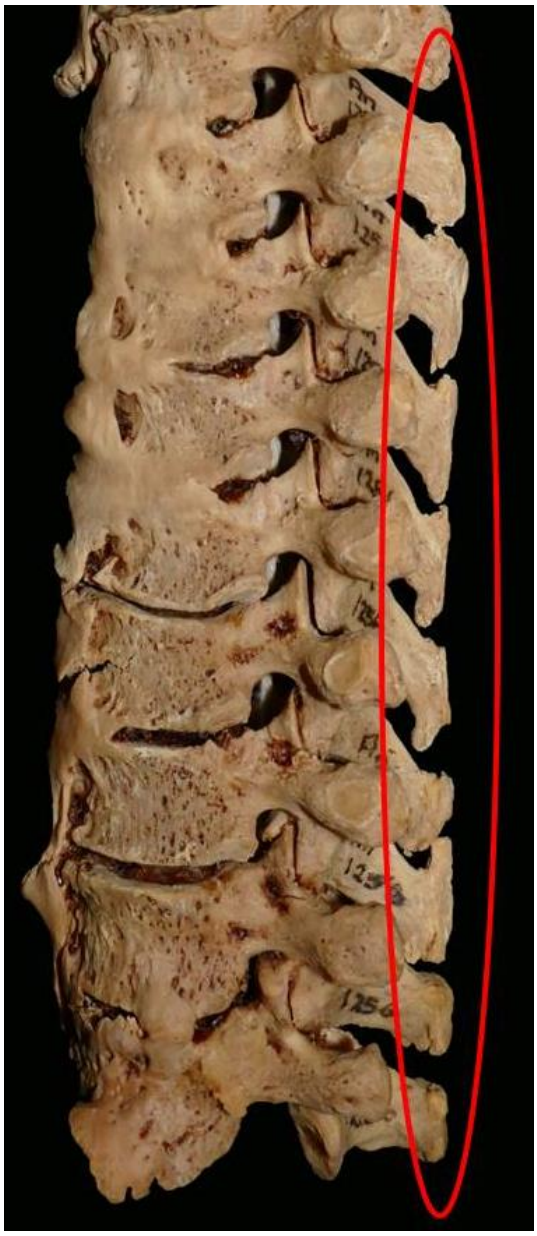

Figure 3.18: Example of an individual with DISH (T3–T11), showing the ossification of the supraspinous ligament (highlighted with a red oval). The individual depicted is AN 1256, from the KC. Source: Author.

3.2.2. Micro-computed tomography

Computed tomography (CT) is a 3D imaging modality, used to create computerised models of objects by reconstructing digital slices (cross sections) into a 3D volume. This method is popular in scientific fields where the object of interest is delicate or irreplaceable as the nature of analysis is non-invasive (Abel et al., 2012). CT is primarily a radiographic imaging technique, however, instead of having a 2D representation of a 3D object, as with traditional radiography, CT scanning creates a 3D volume, giving researchers the tool to further explore the underlying structure of the material. In this research project the micro-focus x-ray tomography facility (MIXRAD) at the Nuclear Energy Corporation of South Africa (NECSA) was utilised to scan the affected vertebrae of some individuals identified with DISH, to ascertain the usefulness of this method in visualising the ossification and the underlying structure of the lesions in the spine. The use of micro-CT scanning on "dry" skeletons provides a great opportunity to examine human remains at a higher resolution than would be possible on living patients, as radiation is too high to use in a clinical setting. Micro-CT scanning provides excellent visualization and has been used to aid in our understanding in academic fields such as dentistry, thus reiterating the relevance these studies have on dry bone in a practical clinical context (Theye et al., 2018).

The scans were created at a tube voltage of 100kV and beam current of 100µA (micro-ampere). After the slices were rendered into volumes, they were viewed using VG Studio Max, at the MIXRAD facility in NECSA, South Africa. The analysis consisted of making observations on the structure of the main spinal manifestations at the antero-lateral surfaces of vertebrae affected by DISH. There was no strict criterion for observations based on the micro-CT scans, as the analysis was qualitative. However, four main features were particularly observed and included (1) noting whether the ossification started at the middle of the vertebral body, when viewed in an anterior aspect; (2) whether there was evidence to suggest retention of the vertebral cortex at the site of ALL ossification; (3) to see if there was any evidence of trabecular bone at the site of the ALL ossification; and (4) to observe and note any abnormal areas of osteosclerosis beyond ossification of the ALL.

All four of the abovementioned features were chosen for analysis to investigate the development of the flowing ossification as much as possible, with what is a static snapshot of DISH. Although research has suggested an inflammatory response being responsible for osteophyte formation in clinical studies (Arad et al., 2017; Mader et al., 2017, 2015), the study

of “dry” human skeletal remains with micro-CT scanning provides exciting possibilities in exploring the effects of inflammation. One feature that may help to highlight possible inflammatory processes occurring in bone (from radiological imaging) is the presence of osteosclerosis; osteosclerosis is seen in radiographic imaging as radiopaque (white). The differential diagnosis for osteosclerosis in radiographic imaging is varied and can include suggestions of metabolic diseases, trauma, vascular disruptions and neoplastic diseases etc. (Ortner, 2003). The location and appearance of osteosclerosis can help in determining its initial cause and will be an interesting feature to note in the micro-CT scans of this study, and to assess whether there appears to be any association with the individuals diagnosed with DISH.

3.2.3. Calculating body mass index (BMI)

With overlapping risk factors, BMI was investigated as the information was available for a large proportion of the sample. Information was collected on post-mortem height and weight of individuals to calculate BMI. However, post-mortem measurements are deemed to be less reliable due to the decompositional changes that can alter the weight and height of an individual after death, as well as recording errors by morgue staff. Nevertheless, this information is to provide general guidance as to whether an individual was considered to be underweight, normal-weight, overweight or obese - modern BMI classifications. As mentioned, it is necessary to know the height and weight of an individual to calculate BMI. BMI can be expressed in the following formulae:

$$\textbf{BMI} = \left(\frac{\text{weight in kilograms}}{\text{height in meters}^2}\right) \text{x } 100$$

Although there are inherent inaccuracies in calculating BMI from post-mortem variables, as discussed in the literature review, the estimated BMI was used to analyse BMI in the context of DISH versus non-DISH individuals. An individual whose BMI could be calculated, was placed into one of the four broad BMI classifications (Table 3.2).

Table 3.2 The range of values for each BMI classification.

BMI Classification	BMI (kg/m^2) Range
Underweight	≤ 18.5
Normal-weight	18.6 – 24.9
Overweight	25.0 – 29.9
Obese	≥30.0

3.2.4. Stable light isotope analysis

3.2.4.1. Collection of samples for isotopic analysis

The process of bone remodelling is primarily fulfilling the function of maintaining bone strength by replacing 'old' (sometimes) damaged bone with new mechanically stronger bone (Clarke, 2008). Often referred to as the 'turn-over rate', bone remodelling differs between different bones (subject to biomechanical structure of the bone) and has been observed to decrease with advancing age (Sealy et al., 1995; Hedges et al., 2007; Lamb et al., 2014). Although the rate of bone turnover is not known precisely, thicker cortical bone (like the femur) has a slower osteon turnover rate than trabecular, or spongy bone, (like a rib) (Lamb et al., 2014). Both a femur and a rib from individuals diagnosed with DISH were selected for sampling for isotopic analysis to represent different lengths of time before death. Isotopic analysis of collagen extracted form femora of adults showed to represent average values from approximately 10 years prior to death (Sealy et al., 1995; Hedges et al., 2007; Lamb et al., 2014). This is because as we eat, the chemical elements are deposited into the bony matrix (organic and non-organic) as it is being formed or while the turnover process is taking place. The femur has dense cortical bone which means the turnover rate takes a relatively longer time than other, smaller bones. In contrast, ribs consist of more trabecular bone, and it has been theorised that regeneration will take 2 – 5 years to take place (Cox and Sealy, 1997; Lamb et al., 2014). As it is unknown how long it takes for the classic characteristics of DISH to develop, the aforementioned sampling strategy of femur versus rib was employed to represent a life record of approximately 10 years prior to death (represented by the femur data) and 2-5 years prior to death (represented by the rib data).

All individuals with DISH were sampled, including those diagnosed as both definite and possible DISH (n=127), as well as those from a control group (n=117). Samples were

extracted using a Dremel® 4000 Multi-Tool (175W) drill with a diamond tipped 10 mm core drill bit. Samples were taken from the popliteal surface, on the posterior surface at the distal end of the right femur. The author was requested by the respective collection managers to take samples from the right side (femur only) in case they were to interfere with the standard measurements taken for stature estimation. When sampling the ribs, it did not matter what side the ribs came from, however, the author was asked to comply with two requests by the collection's committees of the three skeletal populations. Firstly, samples can only be taken from the midshaft, so as to preserve the sternal ends for age estimation analysis. Secondly, fragmented ribs were to be utilised so the impact of the invasive technique was limited to the already damaged bones of an individual. The first request was always adhered to, however, where there were no fragmented ribs to take samples from, an appropriate rib was agreed upon with the respective collection managers and a sample was taken at the midshaft. After each sample was taken, they were placed into 5 ml sterile plastic containers with screw cap lids. Each sample weighed roughly 200-300 mg so that a reasonable amount of collagen could be extracted for isotopic analysis. Tools and equipment were cleaned with acetone between each sample extracted to control for cross contamination and the work station was cleaned with 70% ethanol.

3.2.4.1.1. Preparation of samples for isotopic analysis

All samples were prepared and run in the isotope laboratory (Figure 3.19) in the Mammal Research Institute, at the University of Pretoria, with the assistance of the laboratory manager, Dr Grant Hall, using a Thermo Fisher Delta V Continuous Flow Isotope Ratio Mass Spectrometer (CF-IRMS) with elemental analyser.

The first stage was to isolate the collagen in the samples, which required them to be demineralised. The samples of femur and rib were demineralised using 2% HCl (hydrochloric) acid. The acid for each sample was changed every other day until the samples were a collagenous pseudomorph of the original bone sample. The tubes of bone with HCl acid were put in a centrifuge for 10 minutes so that any collagen floating on the surface of the liquid would be pushed to the bottom of the tube. The tubes were rotated at a rate of approximately 3000 rpm. This meant that the process of replacing the HCl acid could be maximised and little to no collagen would be taken away during the exchange of fresh HCl acid (2%). Separate syringes were used in both the extraction of the 'old' HCl acid and in the addition of 'new/fresh'

HCl acid to control for contamination. The syringe used to take the old HCl acid out of the tubes was cleaned between each extraction with distilled water.

Figure 3.19: Work station in the isotope laboratory in the Mammal Research Institute, at the University of Pretoria, where the processing of all the samples was undertaken. Source: Author.

Samples were weighed using electronic scales. Each sample was measured to weigh between 0.5 mg and 0.65 mg while placed inside a tin capsule. Once the correct weight was reached, the tin capsule was closed off at the top with a pair of tweezers and the capsule was crushed into a rounded shape (with tweezers). The ball shape appears to be the optimal shape for processing the sample in the continuous flow isotope ratio mass spectrometer (CF-IRMS), so that it doesn't get stuck before combustion. The samples were placed in a 12 x 5 plastic tray with a code marked by each sample, *e.g.* A1 – A12, B1 – B12, C1 – C12 etc. Every sample was recorded on paper in the following format; sample code; sample name; weight of sample, running position in mass spectrometer. When weighing the samples, every 11th sample was weighed in duplicate for later quality control analysis. Blanks were also incorporated into the run as another method of quality control. The incorporation of in-lab standards is important for data quality control. Two in-lab standards were used: DL-Valine and Merck Gel. There were

3 sets of 12 samples weighed out for analysis (36 samples in total). Twelve were weighed out at between 0.2 mg and 0.24 mg, another 12 samples were weighed out at between 0.4 mg and 0.44 mg, and the last 12 samples were weighed out at between 0.6 mg and 0.64 mg.

Once samples had been lined up for processing in the CF-IRMS with elemental analyser, the results were given in numerical form on a digitised spreadsheet for processing. One such figure that is required to assess data is the atom to atom ratio. The atom to atom ratio (C/N) for collagen is a widely accepted means of assessing the quality of data produced by stable isotope analysis. It assesses whether or not the quality of the material sampled is affected by diagenesis or not. It has been noted that the accepted C/N range for collagen is between 2.6 and 3.6. If an isotopic result falls outside of this accepted range then the result will be discarded and considered unreliable.

3.2.4.1.2. Continuous flow isotope ratio mass spectrometer with elemental analyser (EA-CF-IRMS)

The analytical processes that produce the isotopic results are important to understand because factors like instrumental drift and instrument isotope fractionation affect the data output and interpretation. Researchers can try and mitigate for them or simply understand what effect they can have on the data and report accordingly. In the current study, to control for these variables where possible, calibrated in-house standards and sample duplicates were used.

The introduction of the elemental analyser (EA) allowed for bulk measurements to be read into the continuous flow isotope ratio mass spectrometer (CF-IRMS), providing an average isotopic value for the whole sample (Bay et al., 2015). Once the sample material is loaded into the EA-CF-IRMS, there are four stages of analysis, namely (1) combustion of the samples, (2) introduction of evolved gases via the interface, (3) ionisation and separation, and (4) raw data evaluation (Carter and Barwick, 2011).

In the first stage, the sample is converted into a simple gas (CO and N_2) using the elemental analyser. This is typically achieved by a two-reactor system: a combustion reactor and a reduction reactor, followed by separation of the evolved gases through an isothermal packed GC column. As carbon and nitrogen are the isotopes of interest, the sample material undergoes combustion in an oxygen atmosphere, reaching temperatures of 1800°C. An interface is needed in the next stage to link the online elemental analyser system to the IRMS. The interface allows working gases to be introduced and the sample gases to be diluted with

helium (a carrier gas) and controls the amount of gas volume entering the ion source. This allows for the measurement of both $\delta^{13}C$ and $\delta^{15}N$ concurrently. Finally, gas molecules that were ionised via interaction with an electron beam in the ion source become accelerated through high voltage, passing through a magnetic field before reaching Faraday cup detectors. The main principle behind the collection of the molecules after acceleration is that the heavier isotopes will interact with the magnet slower than the lighter isotopes. Each faraday cup is connected to its own individual amplifier. The signals from individual amplifiers are digitised and recorded concurrently by the IRMS data system and displayed as a chromatogram, with the peaks indicating the number of ions detected (Carter and Barwick, 2011).

The ratios are normalised using the standards run with the samples and are typically displayed in a spreadsheet for further analysis and interpretation.

3.2.5. Statistical analyses

3.2.5.1. Intra- and inter- observer error

An important aspect of any study is repeatability, as it assesses the reliability of the data collected in a study, the functionality of methods employed, and their validity amongst other researchers. Evaluating rates of error is at the most fundamental level, a method of quality control, and is particularly important when dealing with discrete traits, such as those in the morphological aspects of this study.

Twenty individuals from the PBC were chosen to test intra- and inter-observer reliability of scoring. This analysis was conducted by both the author (intra-observer) as well as by a colleague and fellow PhD researcher in physical anthropology, Ms. Clarisa Sutherland (inter-observer). Both observers recorded features present in Appendix 1 (Tables 8.1 and 8.2), according to explanations as set out in section 3.2.1.

When analysing the inter- and intra-observer error of recorded macroscopic features associated with DISH, a Cohen's kappa coefficient was used to test the level of agreement using the statistical computing programme 'R'. The kappa value gives a degree of agreement between observers, adjusting for the level of agreement due to chance. The reliability of agreement is shown in Table 3.3 below (Landis and Koch, 1977).

Table 3.3. Degree of agreement for kappa values.

Kappa value	Degree of agreement
<=0	Poor
0 – 0.19	Slight
0.2 – 0.39	Fair
0.4 – 0.59	Moderate
0.6 – 0.79	Substantial
0.8 – 1	Almost perfect

3.2.5.2. Investigating DISH

A chi-square analysis was used to compare the expected frequencies and the observed frequencies of DISH within and across all three of the skeletal collections pooled. A chi-square test was also performed to compare DISH: with age-at-death; with sex; among ancestry groups (white, black and coloured); among the skeletal collections (PBC, RDC and KC); and among BMI classifications (underweight, normal-weight, overweight and obese). The tests were performed on the statistical computing programme 'R'. In any analysis including more than two groups (*e.g.* white *versus* black *versus* coloured South Africans) a post hoc test (pairwise test of independence) with Bonferroni correction for multiple comparisons was performed to identify the significant group or groups. By determining whether there were any statistically significant differences in the prevalence of DISH between the abovementioned groups, it could help to assess whether an individuals' sex, ancestry, BMI or geographic origin (represented by their skeletal collection source) could affect their susceptibility to developing DISH.

3.2.5.3. Investigating DISH and isotopes

Due to the nature of isotopic data (*i.e.* the values varying markedly due to the differences between different consumers' diets), it is standard practice to use non-parametric tests to determine statistical significance because it does not assume normality and is able to test parameters such as the difference in means in different groupings from the dataset. As such, Mann-Whitney *U*-test were applied to determine the statistical significance between two groups, categories or classifications (*e.g.* males versus females or DISH group versus the control group). When significance needed to be determined between more than two groups (*e.g.* black *versus* white *versus* coloured South Africans), then the Kruskal-Wallis *H*-test was

used. When a significant result was identified, analysis was followed up with a Bonferroni-adjusted Mann-Whitney post hoc test, so the specific groups could be identified. The tests were performed on the statistical computing programme 'R'. Summaries of the comparisons are detailed below.

The possible presence of statistically significant differences were investigated in the following cases:

- The $\delta^{13}C$ of rib and femur samples (both respectively and combined) between the DISH and control groups.
- The $\delta^{15}N$ of rib and femur samples (both respectively and combined) between the DISH and control groups.

When statistically significant differences were found, comparisons were made between individuals within the DISH and control groups. If no statistically significant differences were found in the above comparisons between the DISH and control groups, then further statistical tests were performed within the DISH and control groups respectively. This was to investigate whether any statistically significant differences found within the DISH group, were also present in the control group. The tests included comparisons to identify any differences in the $\delta^{13}C$ and $\delta^{15}N$ values (respectively) for the rib and femur samples (both respectively, and combined); between the sexes; among ancestry groups; among BMI classification; among the BMI classification and ancestry groups combined; and among the skeletal collections – with this in mind, the comparisons are detailed below:

- The $\delta^{13}C$ between the rib and femur samples.
- The $\delta^{15}N$ between the rib and femur samples.
- The $\delta^{13}C$ of rib and femur samples (both respectively and combined) between males and females.
- The $\delta^{15}N$ of rib and femur samples (both respectively and combined) between males and females.
- The $\delta^{13}C$ of rib and femur samples (both respectively and combined) among the ancestry groups.
- The $\delta^{15}N$ of rib and femur samples (both respectively and combined) among the ancestry groups.

- The $\delta^{13}C$ of rib and femur samples (both respectively and combined) among the BMI classifications.
- The $\delta^{15}N$ of rib and femur samples (both respectively and combined) among the BMI classifications.
- The $\delta^{13}C$ of rib and femur samples (both respectively and combined) among the BMI classifications and ancestry combined.
- The $\delta^{15}N$ of rib and femur samples (both respectively and combined) among the BMI classifications and ancestry combined.
- The $\delta^{13}C$ of rib and femur samples (both respectively and combined) among the skeletal collections.
- The $\delta^{15}N$ of rib and femur samples (both respectively and combined) among the skeletal collections.

4. RESULTS

This chapter starts by exploring the results from the intra-and inter-observer reliability assessment, demonstrating the overall reliability of the initial macroscopic observations. The macroscopic analysis is presented, providing an overview of the frequency of DISH, as well as the results from the appropriate statistical tests used to investigate DISH and its relationship within the skeletal populations and with BMI. For the stable isotopic analyses, information on the calibration of the standards demonstrates the overall reliability and quality of the isotopic data. Stable isotope analyses between the DISH and control groups includes investigating isotopic signatures between sample types (femora and ribs) and the sexes, as well as among ancestry, BMI categories and collection source for both the DISH and control groups. Observations made from the micro-CT scans, from slices of the 3D reconstructions, as well as a summary of the underlying structure of DISH in the spine, is presented.

4.1. Intra- and inter- observer reliability analysis

The inter-observer and intra-observer reliability assessment of the macroscopic features of DISH were performed using a Cohen's Kappa test. Overall, when the data taken from the scoring of the vertebrae (the presence of ossification along the vertebrae) were analysed, there was a moderate level of intra- (κ=0.56) and inter- (κ=0.46) observer agreement. When just the scores from the thoracic vertebrae (where the majority of the ossification occurs) were analysed, the level of intra-observer agreement rose substantially (κ=0.60), while inter-observer agreement remained moderate (κ=0.51). Table 8.1 in Appendix 1 provides the Kappa scores and associated p-values for the intra- and inter-rater vertebral observations.

When the data from the scoring of the extra-spinal manifestations were assessed, it showed a substantial level of agreement for both the intrarater (κ=0.69) and (κ=0.62) interrater agreement. Table 8.2 in Appendix 1 provides the Kappa scores and associated p-values for the intra- and inter-rater extra-spinal observations.

Overall, the level of intra- and inter-rater agreement for the vertebral observations were moderate, while it was substantial for the extra-spinal observations.

4.2. The prevalence of DISH

Among the three skeletal collections, 127 individuals were identified to have characteristics associated with DISH. A summary of the skeletons diagnosed with DISH can be found in Appendix 3. As mentioned in Chapter 3, a diagnosis of 'definite', 'probable' and 'no' DISH was made. The diagnosis of 'definite' DISH and 'probable' DISH was used to aid in diagnosis only, as some cases of early DISH are not necessarily covered by the 'definite' DISH criteria. As such, the two categories were amalgamated (across the PBC, RDC and KC) to represent a combined group, referred to as the DISH group. Due to data from the PBC, RDC and KC being combined and reported on as such, information and chi-square analyses comparing DISH between sexes, among ancestry groups and among age-at-death groups (respectively), separated by the skeletal collection source, is included in Appendix 4.

Of the 127 individuals diagnosed with DISH, 97 (77%) were recorded as male, and 30 (23%) were recorded as female. When the individuals were separated by ancestry, 37 (29%) individuals diagnosed with DISH were recorded as black South Africans, 79 (62%) as white South Africans, 9 (7%) as coloured South Africans, and 2 individuals' (2%) ancestry was not specified (Figure 4.1). Figures 4.2 and 4.3 illustrate the relative frequencies of individuals diagnosed with DISH separated by sex and ancestry respectively. A crude prevalence rate of 3.3% was calculated from all individuals diagnosed with DISH.

No statistically significant difference was found when a chi-square analysis was used to compare the prevalence of DISH within different age-at-death groups ($p>0.05$). No further statistical tests were performed on individuals diagnosed with DISH separated by age-at-death as this is most likely a reflection on the average life expectancy of the population as well as the inherent bias rather than the effects of DISH on the population. However, Figure 4.4 illustrates the distribution of the individuals' age-at-death for reporting purposes only, with Figure 4.5 illustrating the relative frequencies of individuals diagnosed with DISH, separated by age-at-death, while Table 4.1 provides the average age-at-death for each ancestral group. It is important to highlight the inclusion of an individual whose age-at-death was recorded as 38 years. While the author investigated individuals with an age-at-death over 40 years, this particular individual was brought to the authors attention by a fellow researcher as a case of possible interest. It transpired that there was some uncertainty surrounding the age-at-death of this particular individual due to the early acquisition of the skeleton into the collection,

however, after analysis, it was clear the individual had skeletal manifestations of DISH and was included in the study.

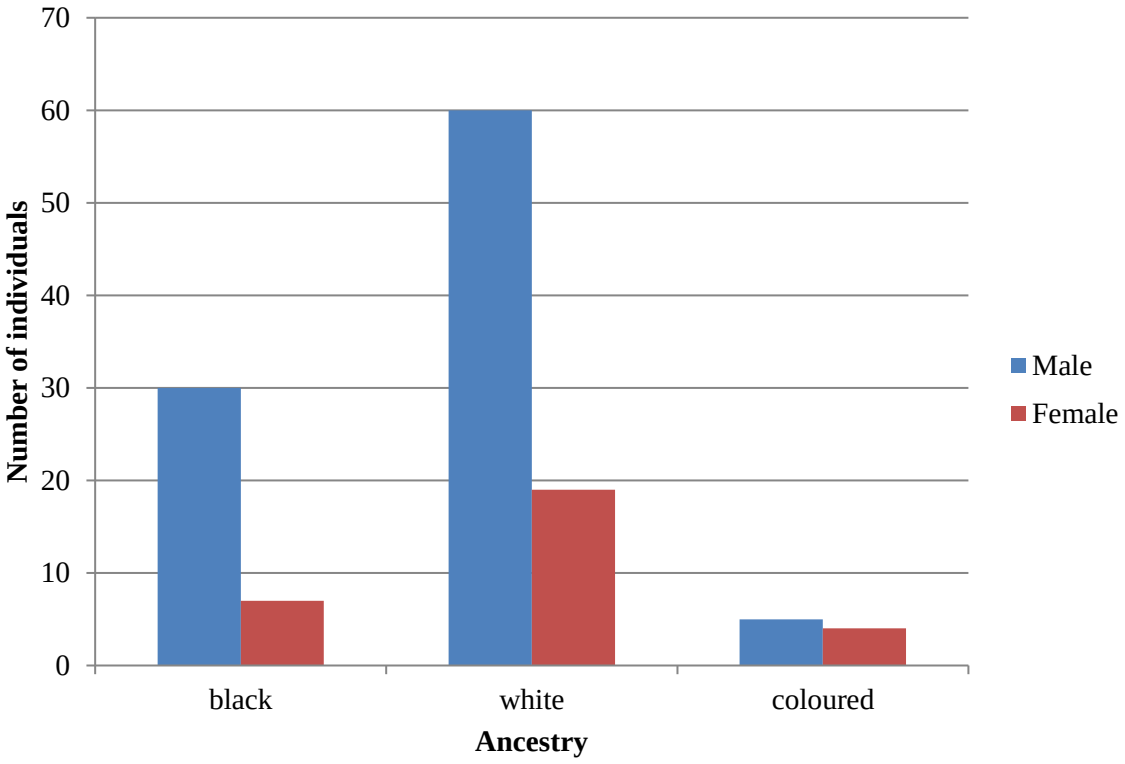

Figure 4.1: All individuals diagnosed with DISH across all three skeletal collections, separated by sex and ancestry (n=125). Two individuals from the Raymond A Dart Collection of Human Skeletal Remains (A2722 and A3223) do not have information on their ancestry recorded.

Table 4.1. The average age-at-death in years for individuals diagnosed with DISH, separated by ancestry and sex.

	Mean age-at-death for males	Mean age-at-death for females	Mean age-at-death (sex combined)
white South African	73	74	73
black South African	65	60	64
coloured South African	69	66	68

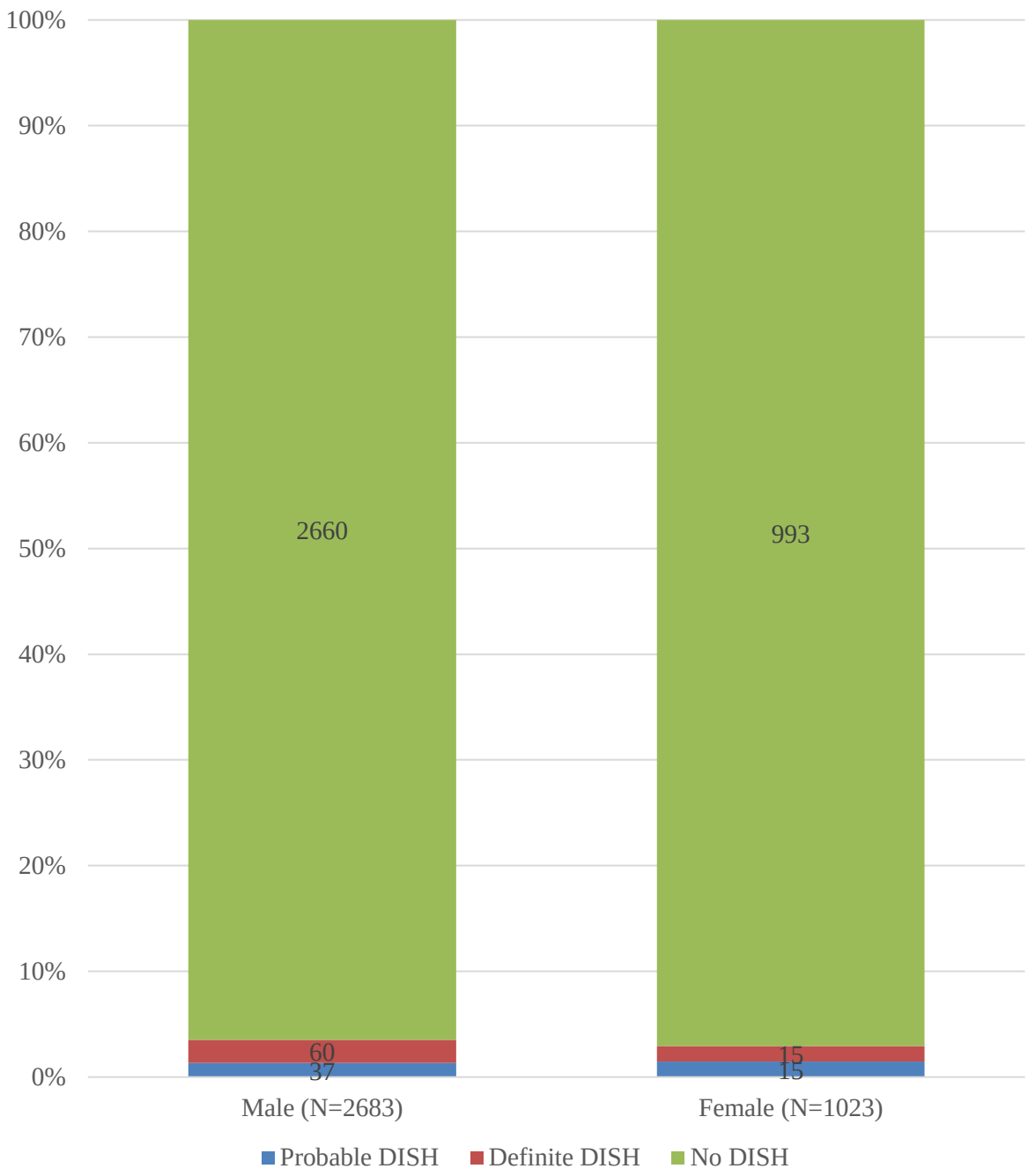

Figure 4.2. Graph illustrating the relative frequencies of males and females diagnosed with DISH across all three of the skeletal collections.

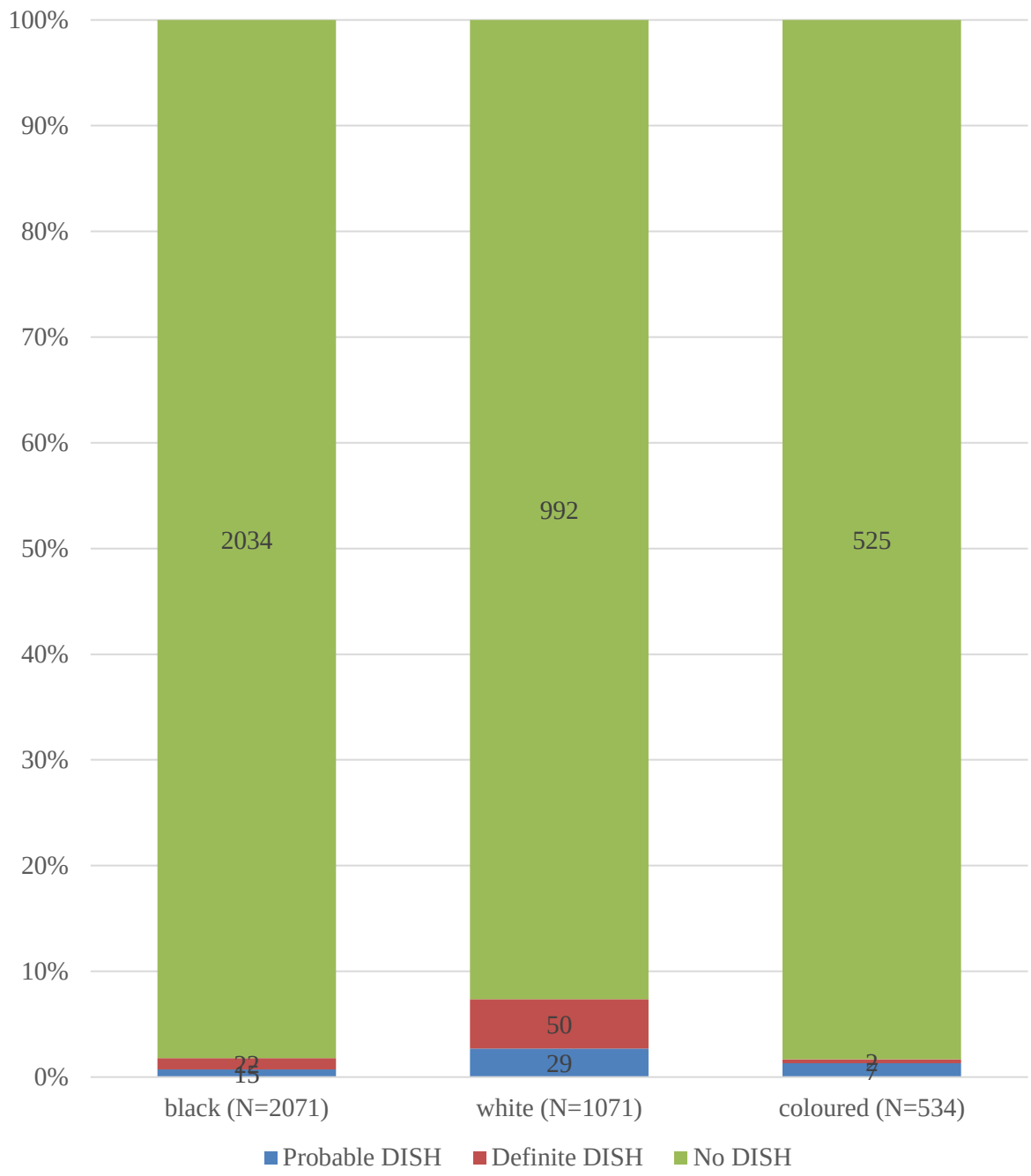

Figure 4.3. Graph illustrating the relative frequencies of black, white and coloured South Africans diagnosed with DISH across all three of the skeletal collections.

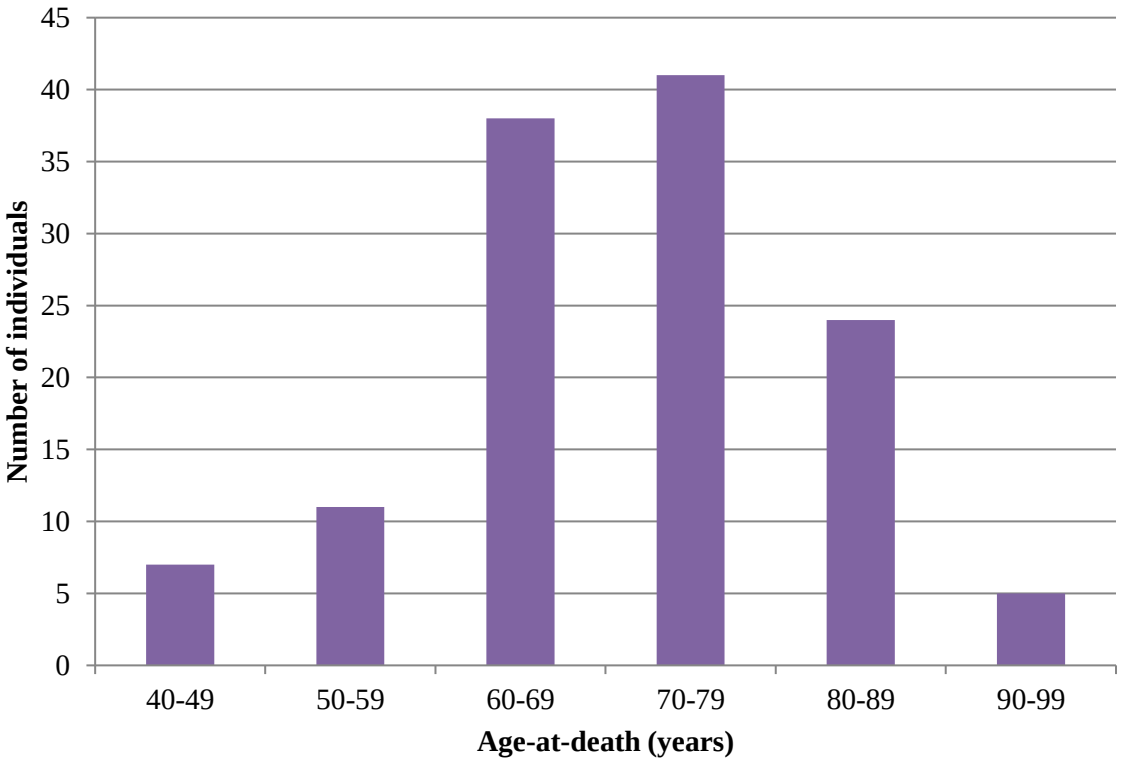

Figure 4.4: All individuals diagnosed with DISH separated by age-at-death categories (n=125). One individual from the RDC's (A4307) age-at-death is recorded as 38 years (with some uncertainty surrounding their age-at-death) and one individual from the KC (AN1256) has no specified age-at-death.

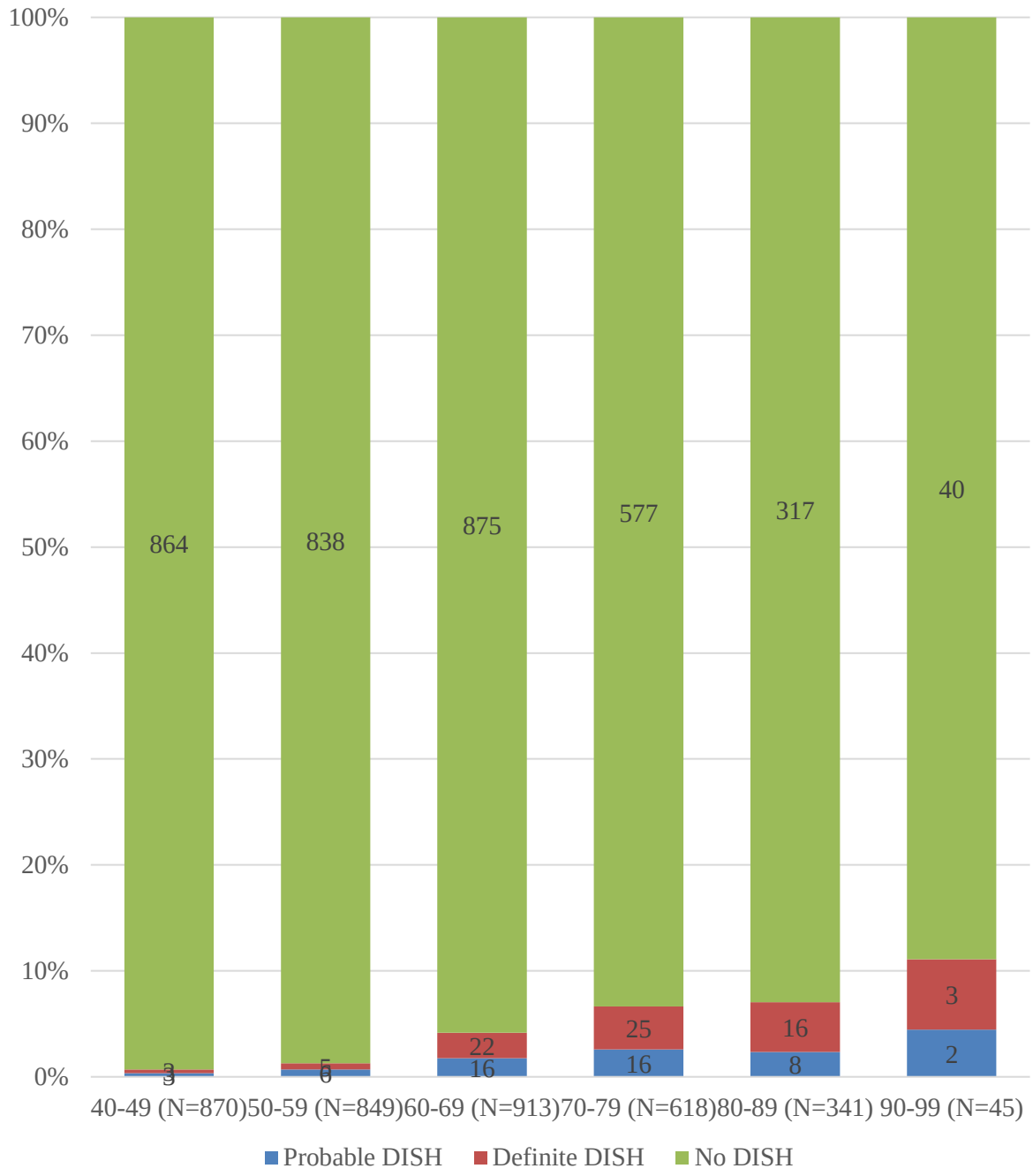

Figure 4.5. Graph illustrating the relative frequencies of individuals diagnosed with DISH, separated by age-at-death.

A chi-square analysis was used to compare the prevalence of DISH according to sex, ancestry and skeletal collection. There was a statistically significant difference among ancestry groups (with sex and skeletal collection pooled). When a post hoc test (pairwise test of independence) with a Bonferroni correction for multiple comparisons was applied, a statistically significant difference was found between white and black South Africans

(p<0.001) as well as between white and coloured South Africans (p< 0.001), with white South Africans exhibiting a higher prevalence of DISH than black or coloured South Africans. No statistically significant difference (p>0.05) was found between males and females diagnosed with DISH (with ancestry and skeletal collection source pooled). When sex and ancestry were analysed together, the chi-square test (with Bonferroni adjusted post hoc) revealed a statistically significant difference between white South African males and black South African females (p<0.001), white South African males and black South African males (p<0.001), white South African males and white South African females (p<0.001), and white South African males and coloured South African males (p<0.001), again indicating the highest rate of DISH among white South Africans, specifically males. A chi-square analysis comparing DISH with collection source showed no statistically significant difference in the prevalence of DISH among the skeletal collections (with ancestry and sex pooled).

4.2.1. The frequency of infrequently observed skeletal manifestations of DISH

Three infrequently observed extra-spinal manifestations of DISH were recorded and the frequency in which they occurred was assessed across all 127 individuals diagnosed with DISH in the current study. The three sites noted and recorded included plantar heel spurring, ossification of the apical ligament and ossification of the supraspinous ligament. Across the three skeletal collections, plantar heel spurring occurred 55%, ossification of the apical ligament occurred 30%, and ossification of the supraspinous ligament occurred 81% (Table 4.2).

Table 4.2. The relative frequency of infrequently observed skeletal manifestations of DISH from the Pretoria Bone Collection, the Raymond A Dart of Human Skeletal Remains and Kirsten Collection combined.

	Present	Absent	Element missing post mortem/post mortem damage	Total	Relative frequency
plantar heel spurring	70	48	9	127	55%
ossification of apical ligament	38	77	12	127	30%
ossification of supraspinous ligament	103	20	4	127	81%

4.3. The relationship between DISH and obesity/BMI

Of the 127 individuals with macroscopic skeletal manifestations of DISH, 96 had information leading to an estimated BMI calculation (weight and height recorded). Fifty-one of the individuals with post mortem height and weight were from the PBC and 45 from the RDC. Unfortunately, none of the individuals diagnosed with DISH from the KC had information on height or weight, so no BMI classification could be estimated. The recorded heights and weights are estimates, but they are useful in assessing broad trends in BMI.

Of the individuals where BMI could be estimated, 76 were male and 20 were female (Figure 4.3). As can be seen in Figure 4.6, the majority of individuals were classified as normal-weight (n=36, 37%), followed by those who were underweight (n=29, 30%), with less classified as overweight (n=18, 19%) or obese (n=13, 14%). In Figure 4.7, the BMI categories according to ancestry for the DISH group are presented. The majority of individuals fell into the underweight or normal-weight categories (37 white South Africans, 23 black South Africans and 2 coloured South Africans) with the majority of overweight and obese groups being attributed to white South Africans (25 white South Africans and 6 black South Africans), for which the majority were males. A chi-square analysis was used to investigate the relationship between DISH and BMI categories. No statistically significant difference was found among the groups ($p>0.05$).

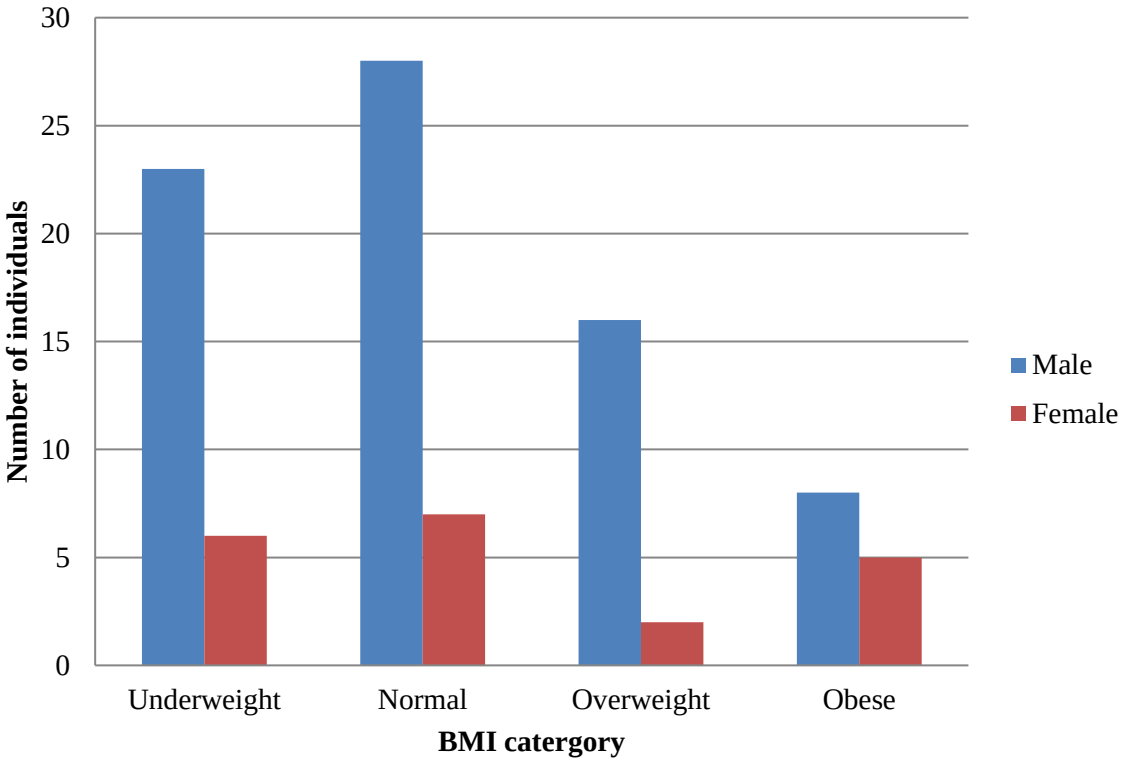

Figure 4.6. BMI classification in individuals with DISH (PBC and RDC combined) (n=96). No individuals from the KC had information on BMI.

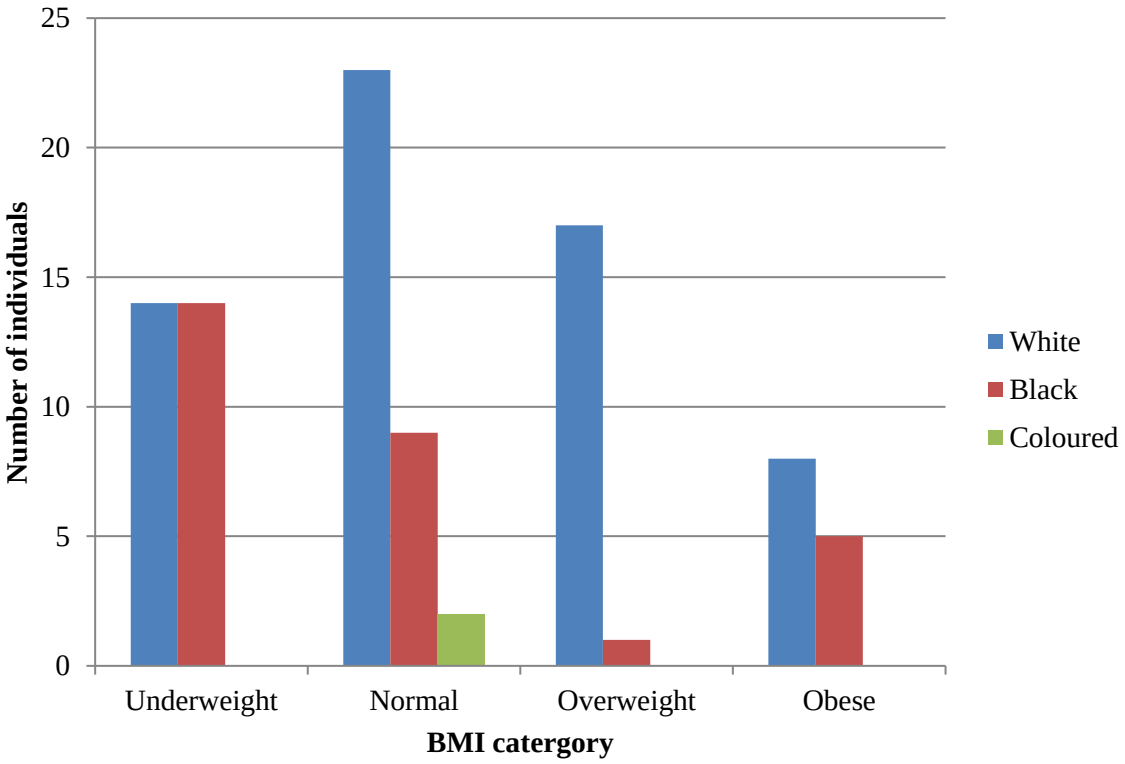

Figure 4.7. The ancestry of individuals in each BMI classification, from the DISH group (PBC and RDC combined) (n=93). No individuals from the KC had information on BMI.

4.4. Stable isotope analyses

4.4.1. Calibration of standards

The raw δ- values for both the in-house standards were normalised using a multi-point linear regression method (Nelson, 2000). Two in-house laboratory standards were used to encompass the expected $\delta^{15}N$ and $\delta^{13}C$ values, namely DL-Valine and Merck Gel. These in-house standards are in turn, calibrated against the National Institute of Standards and Technology (NIST) international standards: NIST 1557b (bovine liver), NIST 2976 (mussel tissue) and NIST 1547 (peach leaves). The certified $\delta^{15}N$ and $\delta^{13}C$ values for both the in-house laboratory standards are shown in Appendix 4. The measured $\delta^{15}N$ and $\delta^{13}C$ values for both standards (DL-Valine and Merck Gel) run alongside the three batches of samples (the ribs and femora of DISH and control groups run in June 2017, November 2017 and December 2017) – to test the validity of each of the datasets - are presented in full in Appendix 4.

The results of the linear regression performed on the measured values of $\delta^{15}N$ and $\delta^{13}C$ (respectively) against the certified values of $\delta^{15}N$ and $\delta^{13}C$ (respectively) for the standards showed a nearly perfect linear relationship across all three of the runs (Table 4.3). This reliability reflects the fact that the measured $\delta^{13}C$ and $\delta^{15}N$ values for the DL-Valine and Merck Gel samples were all very close to the certified values across all three separate analyses.

Table 4.3. Regression values for the measured $\delta^{13}C$ and $\delta^{15}N$ values for inter-laboratory standards DL-Valine and Merck Gel, run in June 2017, November 2017 and December 2017.

Isotope and date of batch run	R^2
$\delta^{13}C$ (‰) June 2017	0.99
$\delta^{15}N$ (‰) June 2017	0.99
$\delta^{13}C$ (‰) Nov 2017	0.99
$\delta^{15}N$ (‰) Nov 2017	0.99
$\delta^{13}C$ (‰) Dec 2017	0.99
$\delta^{15}N$ (‰) Dec 2017	0.99

The results from all the measured standards indicate that the reliability of the data samples (ribs and femora) between runs were high. However, other sources of error such as systematic error or error in sample preparation are a possibility. While the levels of variation between the certified and measured values were very low across all three separate runs of the two in-house standards (<0.07‰ for both $\delta^{15}N$ and $\delta^{13}C$), other precautions and means of quality control of the data output were implemented. Comparisons of the isotopic values from

duplicate samples and the C/N ratio of samples were also used to control for variables of error that can affect the results.

4.4.2. Data quality (C/N)

The atom to atom ratio (C/N) for collagen is a widely accepted means of assessing the quality of data produced by stable isotope analysis. It assesses whether or not the quality of the material sampled is affected by diagenesis. The accepted C/N range for analysis of collagen is between 2.6 and 3.6 (Schoeninger et al., 1983; DeNiro, 1985). If an isotopic result fell outside of this accepted range, then the result was discarded and considered unreliable. From a total of 586 samples that were processed and analysed for isotopic analysis, which included samples from the DISH group, control group, standards, and duplicates, a total of 114 fell outside the accepted C/N range and were discarded from the dataset. Indeed, all 114 of the samples had C/N ratios higher than the accepted range. Samples that fall outside the accepted C/N range can happen for a variety of reasons including poor combustion of the samples during analysis in the mass spectrometer and contamination during sample preparation (see section 5.6 in Chapter 5 for a discussion on the C/N ratios). In this instance, the effect of maceration on the skeletal material may have caused the high C/N ratios (DeNiro et al., 1985).

4.4.3. Procedural duplicates

Procedural duplicates refer to samples that have undergone the same preparation methods, but have been split into two separate samples during the weighing process before analysis by the mass spectrometer. Once a batch of samples had been through the preparation process, every 12th sample weighed was a duplicate of the 11th, and each duplicate was always weighed out in cycles of 12. Figure 4.8 shows the $\delta^{13}C$ and $\delta^{15}N$ values for all the duplicates across all three of the batches run for isotopic analysis.

The samples run in duplicate include both individuals who were diagnosed with DISH and individuals from the control group. The duplicates provide an indication of the reliability and repeatability of the values given by the mass spectrometer and an indication of the homogeneity of the samples. In total, 35 samples were run in duplicate; this excludes the data that were discounted on account of a poor C/N ratio, falling outside of the accepted range (see section 4.4.2. above). For all the duplicates analysed, the minimum difference in $\delta^{13}C$ values between the duplicates was 0.01‰. The largest difference in $\delta^{13}C$ values between the duplicates

was 0.92‰, with an average difference (standard error) of 0.30‰. The minimum difference in $\delta^{15}N$ values for the duplicates was calculated at 0.01‰, while the maximum difference was 1.45‰, which seems fairly high, but only one pair displayed a difference with this value and could be the result of incomplete combustion of the sample. All the other data points were considered consistent, especially as the duplicates displayed an average (standard error) of 0.26‰ difference in $\delta^{15}N$ values between the pairs.

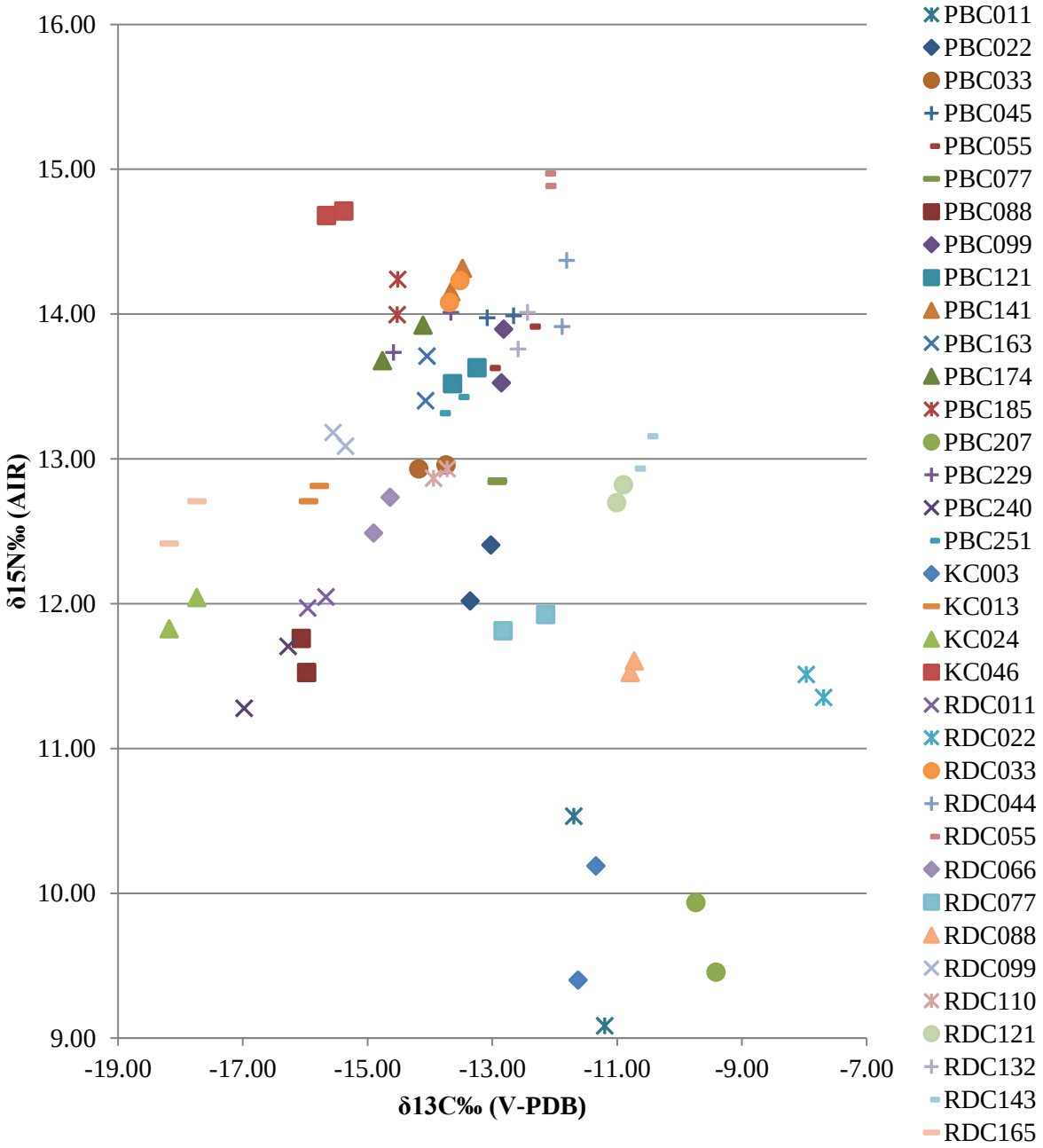

Figure 4.8: The graph shows the distribution of data produced from the duplicated samples, run across all three batches; June 2017; November 2017; and December 2017.

A correlation coefficient (linear regression) was calculated to assess the linear relationship of both the $\delta^{15}N$ and $\delta^{13}C$ values (respectively) of samples against their duplicate (see Appendix 5 for graphic visualisation). Both R^2 values for the $\delta^{13}C$ (R^2=0.98) and $\delta^{15}N$ (R^2=0.93) values show a nearly perfect linear relationship between the paired duplicate samples. Both the linear relationship and the average difference in ‰ for both $\delta^{13}C$ and $\delta^{15}N$ between the paired duplicates demonstrate a good level of reliability and reproducibility for the stable isotopic data, within and between runs. For interpretive purposes it is worth noting that the results from the duplicate analysis would suggest that any difference of less than ±0.3‰ in $\delta^{13}C$ or $\delta^{15}N$ values may indicate analytical error rather than representing a difference in diet.

4.4.4. DISH versus the control group

A total of 415 samples (including both ribs and femora) were run for isotopic analysis from both the DISH and control groups (Figure 4.6). A total of 205 of the samples came from the control group and 210 from the DISH group. The $\delta^{13}C$ for the DISH group ranged from -19.1‰ to -7.5‰, with an average value of -13.3‰. The $\delta^{15}N$ results for the DISH group ranged from 9.2‰ to 16.1‰, with an average value of 13.0‰. For the control group, the minimum value for $\delta^{13}C$ was -18.0‰, the maximum was -7.0‰, with an average $\delta^{13}C$ value of -13.0‰. The $\delta^{15}N$ results for the control group ranged from 9.4‰ to 17.5‰, with an average $\delta^{15}N$ value of 13.0‰.

Little to no separation of the isotopic results exist between the DISH and control groups, as shown in Figure 4.9. Marked overlap is noted with a few outlying samples/individuals. Overall (including both DISH and control samples), there was a negative correlation between the $\delta^{13}C$ and $\delta^{15}N$ values (R^2 0.19). However, visually, two main trends in $\delta^{13}C$ and $\delta^{15}N$ values can be observed in Figure 4.9 (indicated by the two black ovals). The first is where there is a generally positive correlation between $\delta^{15}N$ enrichment and $\delta^{13}C$ enrichment (oval A on Figure 4.6). The second is where a number of individuals are enriched in $\delta^{13}C$ but with no apparent correlation with the $\delta^{15}N$, other than it being generally more depleted (oval B on Figure 4.9). Table 4.4 provides a summary of the values from the isotopic results ($\delta^{13}C$ and $\delta^{15}N$) run for both the DISH and control groups. No statistically significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values of either the rib or femur samples, (separated or combined) between the DISH or control groups were noted.

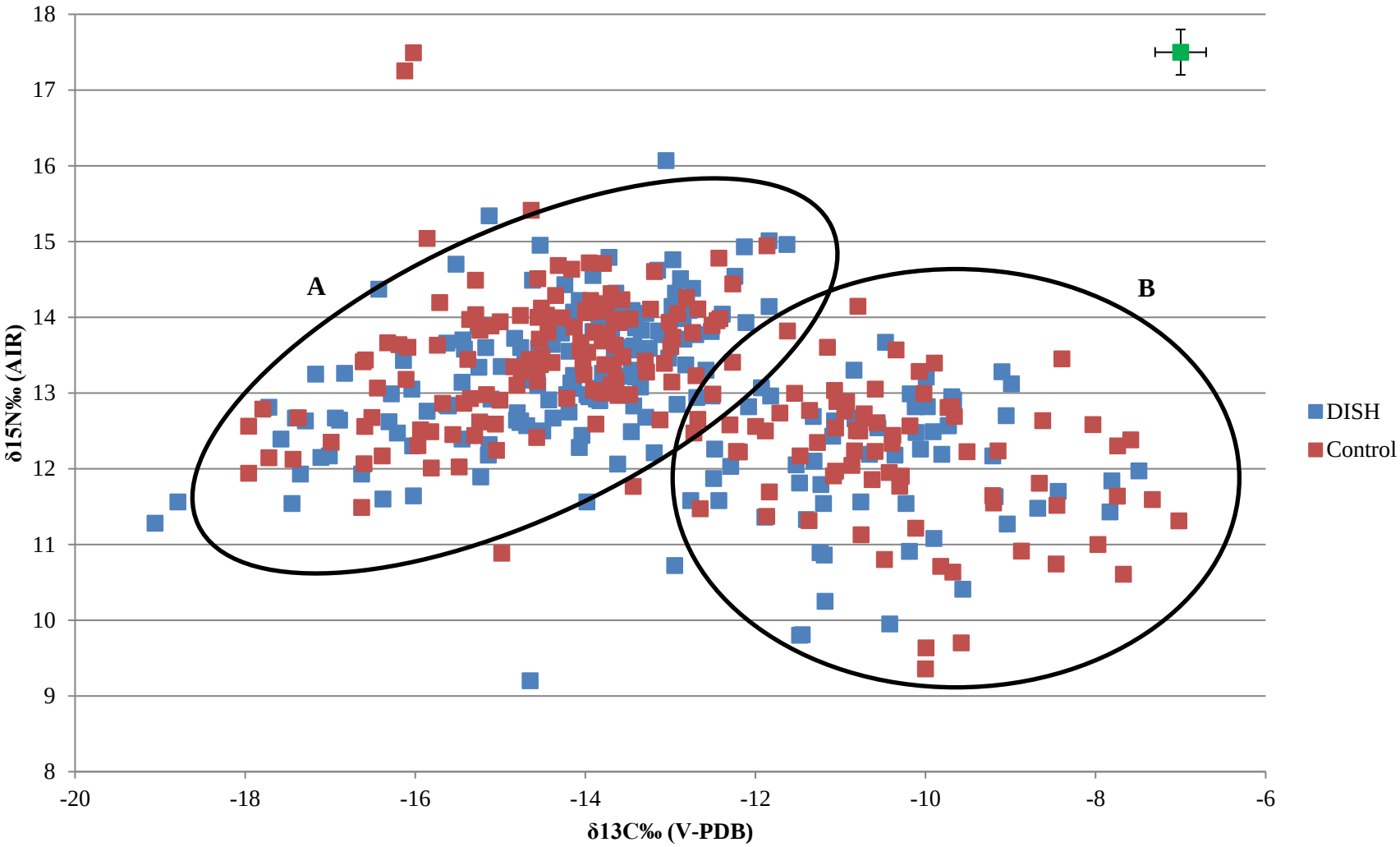

Figure 4.9. Isotopic results for the samples taken from all three skeletal collections (PBC, RDC and KC), comparing both the DISH and the control groups. The green square indicates the error margin of ±0.3‰ for both $\delta^{13}C$ and $\delta^{15}N$.

Table 4.4. The δ¹³C and δ¹⁵N values from both the DISH and control groups, separated by sample type, with associated p-values.

δ¹³C (‰)	Minimum	Maximum	Mean	Median	p-value
$DISH^{Rib}$	-17.5	-7.8	-13.4	-13.7	*p*>0 .05
$control^{Rib}$	-16.6	-7.3	-13.0	-13.7	
$DISH^{Femur}$	-19.1	-7.5	-13.1	-13.4	*p*>0 .05
$control^{Femur}$	-18.0	-7.0	-13.0	-13.2	
$DISH^{Rib+Femur}$	-19.1	-7.5	-13.3	-13.5	*p*>0 .05
$control^{Rib+Femur}$	-18.0	-7.0	-13.0	-13.6	*p*>0 .05
δ¹⁵N (‰)	**Minimum**	**Maximum**	**Mean**	**Median**	**p-value**
$DISH^{Rib}$	10.7	16.1	13.1	13.2	*p*>0 .05
$control^{Rib}$	10.8	14.9	13.1	13.1	
$DISH^{Femur}$	9.2	15.0	12.8	12.9	*p*>0 .05
$control^{Femur}$	9.4	17.5	13.0	13.0	
$DISH^{Rib+Femur}$	9.2	16.1	13.0	13.0	*p*>0 .05
$control^{Rib+Femur}$	9.4	17.5	13.0	13.0	*p*>0 .05

A Mann-Whitney *U*-test was used to evaluate differences within the DISH group according to sex. A summary of the isotopic values can be seen in Table 4.5. No statistically significant difference was noted in the δ¹³C or δ¹⁵N values of either the rib or femur samples between males and females diagnosed with DISH (Table 4.5). When the same statistical test was applied to the control group, no statistically significant difference was found between males and females (Table 4.5). Figure 4.10 shows the distribution of the δ¹³C and δ¹⁵N values of all the individuals diagnosed with DISH, separated by sex.

Table 4.5. The δ¹³C and δ¹⁵N values of individuals from both the DISH and control groups, separated by sex and sample type, with associated p-values.

DISH GROUP					
δ¹³C (‰)	**Minimum**	**Maximum**	**Mean**	**Median**	**p-value**
$male^{Rib}$	-17.5	-9.0	-13.4	-13.7	*p*>0 .05
$female^{Rib}$	-16.1	-7.8	-13.4	-13.6	
$male^{Femur}$	-19.1	-7.5	-13.2	-13.4	*p*>0 .05
$female^{Femur}$	-17.6	-7.8	-13.2	-13.5	
$male^{Rib+Femur}$	-19.1	-7.5	-13.3	-13.5	*p*>0 .05
$female^{Rib+Femur}$	-17.6	-7.8	-13.2	-13.6	
δ¹⁵N (‰)	**Minimum**	**Maximum**	**Mean**	**Median**	**p-value**

male^{Rib}	10.7	16.1	13.1	13.1	*p*>0 .05
female^{Rib}	11.6	15.0	13.2	13.3	
male^{Femur}	9.2	14.8	12.8	13.0	*p*>0 .05
female^{Femur}	10.3	15.0	12.9	12.7	
male$^{Rib+Femur}$	9.2	16.1	12.9	13.0	*p*>0 .05
female$^{Rib+Femur}$	10.3	15.0	13.1	13.1	
CONTROL GROUP					
$\delta^{13}C$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**	**p-value**
male^{Rib}	-16.6	-7.6	-12.8	-13.4	*p*>0 .05
female^{Rib}	-16.6	-7.3	-13.8	-14.0	
male^{Femur}	-18.0	-7.7	-12.9	-13.0	*p*>0 .05
female^{Femur}	-17.7	-7.0	-13.4	-14.1	
male$^{Rib+Femur}$	-18.0	-7.6	-12.8	-13.1	*p*>0 .05
female$^{Rib+Femur}$	-17.7	-7.0	-13.5	-14.0	
$\delta^{15}N$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**	**p-value**
male^{Rib}	10.8	14.9	13.0	13.1	*p*>0 .05
female^{Rib}	11.5	14.7	13.1	12.9	
male^{Femur}	9.4	17.5	12.9	13.0	*p*>0 .05
female^{Femur}	10.6	17.3	13.2	12.9	
male$^{Rib+Femur}$	9.4	17.5	13.0	13.0	*p*>0 .05
female$^{Rib+Femur}$	10.6	17.3	13.1	12.9	

A Kruskal-Wallis *H*-test was used to establish if statistically significant differences existed among ancestry groups diagnosed with DISH. A summary of the isotopic values separated by ancestry are shown in Appendix 7 (Table 14.1). No statistically significant difference was found between the white and coloured South Africans, but a statistically significant difference was found between the black and white South Africans and black and coloured South Africans (Table 4.6). Figure 4.11 shows the distribution of the $\delta^{13}C$ and $\delta^{15}N$ values of all the individuals diagnosed with DISH, separated by ancestry. The main differences that can be seen are between the black and white South Africans, and the black and coloured South Africans (Figure 4.11). Generally black South Africans were more enriched in $\delta^{13}C$ relative to white and coloured South Africans, while the white and coloured South Africans were more enriched in $\delta^{15}N$.

A summary of the $\delta^{13}C$ and $\delta^{15}N$ values obtained from the rib and femur samples in the control group separated by ancestry can also be found in Appendix 7 (Table 14.1). As with the DISH group, a statistically significant difference between black South Africans and both white and coloured South Africans were noted within the control group. No statistically significant difference existed between the white and coloured South Africans (Table 4.6).

Table 4.6. The p-values comparing the $\delta^{13}C$ and $\delta^{15}N$ values among ancestry for both DISH and control groups.

DISH GROUP						
	$\delta^{13}C^{Rib}$	**$\delta^{15}N^{Rib}$**	**$\delta^{13}C^{Femur}$**	**$\delta^{15}N^{Femur}$**	**$\delta^{13}C^{Rib+Femur}$**	**$\delta^{15}N^{Rib+Femur}$**
black and white	***p*< 0.001**	***p*<0 .001**	***p*< 0.001**	***p*<0 .001**	***p*< 0.001**	***p*< 0.001**
black and coloured	***p*<0 .01**	*p*>0 .05	*p*>0 .05	***p*<0 .05**	***p*<0 .01**	***p*<0 .05**
white and coloured	*p*>0.05	*p*>0.05	*p*>0 .05	*p*>0 .05	*p*>0 .05	*p*>0 .05
CONTROL GROUP						
	$\delta^{13}C^{Rib}$	**$\delta^{15}N^{Rib}$**	**$\delta^{13}C^{Femur}$**	**$\delta^{15}N^{Femur}$**	**$\delta^{13}C^{Rib+Femur}$**	**$\delta^{15}N^{Rib+Femur}$**
black and white	***p*<0.001**	***p*<0.001**	***p*<0.001**	***p*<0.001**	***p*<0.001**	***p*<0.001**
black and coloured	***p*<0.001**	***p*<0.001**	***p*<0.001**	***p*<0.001**	***p*<0.001**	***p*<0.001**
white and coloured	*p*>0.05	*p*>0.05	*p*>0.05	*p*>0.05	*p*>0.05	*p*>0.05

A Kruskal-Wallis *H*-test was performed to establish if the $\delta^{13}C$ and $\delta^{15}N$ values of individuals diagnosed with DISH, separated by BMI classification, differed significantly. A summary of the isotopic values separated by BMI classification are shown in Appendix 7 (Table 14.2). No statistically significant differences were found among individuals classified as normal-weight and underweight, normal-weight and overweight, normal-weight and obese or obese and overweight. However, the categories of underweight and overweight did demonstrate a statistically significant difference in their isotopic values (Table 4.7). The majority of underweight persons were generally enriched in $\delta^{13}C$ values relative to the overweight individuals, and the majority of overweight individuals were enriched in $\delta^{15}N$ relative to those individuals in the underweight category. Figure 4.12 illustrates the distribution of the $\delta^{13}C$ and $\delta^{15}N$ values of all the individuals diagnosed with DISH, separated by BMI classification.

A summary of the $\delta^{13}C$ and $\delta^{15}N$ values of individuals from the control group can also be seen in Appendix 7 (Table 14.2). No statistically significant differences in the isotopic values among any of the BMI categories from the control group were noted (Table 4.7).

Table 4.7. The p-values comparing the isotopic values between the different BMI categories of individuals from both the DISH and control groups.

DISH GROUP						
	$\delta^{13}C^{Rib}$	**$\delta^{15}N^{Rib}$**	**$\delta^{13}C^{Femur}$**	**$\delta^{15}N^{Femur}$**	**$\delta^{13}C^{Rib+Femur}$**	**$\delta^{15}N^{Rib+Femur}$**
Normal-weight and underweight	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	***p*< 0.05**
Normal-weight and overweight	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05
Normal-weight and obese	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05
overweight and underweight	*p*> 0.05	***p*< 0.01**	***p*< 0.05**	*p*> 0.05	***p*< 0.01**	***p*< 0.01**
obese and overweight	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05
obese and underweight	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05
CONTROL GROUP						
	$\delta^{13}C^{Rib}$	**$\delta^{15}N^{Rib}$**	**$\delta^{13}C^{Femur}$**	**$\delta^{15}N^{Femur}$**	**$\delta^{13}C^{Rib+Femur}$**	**$\delta^{15}N^{Rib+Femur}$**
Normal-weight and underweight	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05
Normal-weight and overweight	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05
Normal-weight and obese	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05
overweight and underweight	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05
obese and overweight	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05

To further explore possible differences, a Kruskal-Wallis *H*-test was performed on the isotopic values of individuals with DISH, but separated by both BMI and ancestry. A summary of the isotopic values separated by both BMI classification and ancestry can be seen in Appendix 7 (Table 14.3) and Figure 4.13. Appendix 7 (Table 14.4) also provides a full list of comparisons made within the DISH group, and their associated p-values, while Table 4.8 only shows the statistically significant results of the test. These statistically significant differences presented below are likely to be linked directly to ancestry, as seen in the statistical analyses above, as they are directly between the white and black South Africans. As previously discussed, the individuals of white South African ancestry are generally more depleted in their $\delta^{13}C$ values relative to their black South African counterparts, and the individuals of black South African ancestry are on average more depleted in $\delta^{15}N$ than the white South African group.

A comparison between the isotopic values separated by BMI and ancestry (combined) from the control group was undertaken to investigate whether the same differences were present as in the DISH group. Table 4.8 again only gives a summary of the significant p-values from the statistical analyses. As with the DISH group, the statistically significant differences are linked directly to ancestry rather than BMI.

Table 4.8. The significant p-values comparing the isotopic values among the different BMI and ancestry combinations from both the DISH and control groups.

DISH GROUP						
	$\delta^{13}C^{Rib}$	**$\delta^{15}N^{Rib}$**	**$\delta^{13}C^{Femur}$**	**$\delta^{15}N^{Femur}$**	**$\delta^{13}C^{Rib+Femur}$**	**$\delta^{15}N^{Rib+Femur}$**
Normal-weight black and normal-weight white	***p*< 0.05**	*p*> 0.05	***p*< 0.001**	*p*> 0.05	***p*< 0.001**	***p*< 0.05**
Normal-weight black and overweight white	*p*> 0.05	*p*> 0.05	***p*< 0.001**	*p*> 0.05	***p*< 0.001**	*p*> 0.05
Normal-weight black and underweight white	***p*< 0.05**	*p*> 0.05	***p*< 0.01**	*p*> 0.05	***p*< 0.001**	*p*> 0.05
Normal-weight white and obese black	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	***p*< 0.05**
Normal-weight white and underweight black	***p*< 0.001**	***p*< 0.01**	***p*< 0.001**	***p*< 0.001**	***p*< 0.001**	***p*< 0.001**
obese black and overweight white	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	***p*< 0.05**
obese black and underweight white	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	***p*< 0.05**
obese white and underweight black	*p*> 0.05	*p*> 0.05	*p*> 0.05	***p*< 0.05**	*p*> 0.05	***p*< 0.05**
overweight white and underweight black	***p*< 0.05**	***p*< 0.01**	***p*< 0.001**	***p*< 0.01**	***p*< 0.001**	***p*< 0.001**
underweight black and underweight white	***p*< 0.001**	*p*> 0.05	***p*< 0.01**	***p*< 0.001**	***p*< 0.001**	***p*< 0.001**
CONTROL GROUP						
	$\delta^{13}C^{Rib}$	**$\delta^{15}N^{Rib}$**	**$\delta^{13}C^{Femur}$**	**$\delta^{15}N^{Femur}$**	**$\delta^{13}C^{Rib+Femur}$**	**$\delta^{15}N^{Rib+Femur}$**
Normal-weight black and normal-weight white	***p*< 0.001**	*p*> 0.05	***p*< 0.01**	***p*< 0.01**	***p*< 0.001**	***p*< 0.001**
Normal-weight black and obese white	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	***p*< 0.05**	*p*> 0.05

Normal-weight black and overweight white	***p*< 0.05**	*p*> 0.05	***p*< 0.05**	*p*> 0.05	***p*< 0.001**	*p*> 0.05
Normal-weight black and underweight white	***p*< 0.05**	*p*> 0.05	***p*< 0.01**	***p*< 0.01**	***p*< 0.001**	***p*< 0.001**
Normal-weight white and obese black	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	***p*< 0.05**	*p*> 0.05
Normal-weight white and underweight black	***p*< 0.001**	*p*> 0.05	***p*< 0.001**	***p*< 0.01**	***p*< 0.001**	***p*< 0.001**
overweight white and underweight black	*p*> 0.05	*p*> 0.05	***p*< 0.05**	*p*> 0.05	***p*< 0.001**	*p*> 0.05
underweight black and underweight white	***p*< 0.05**	*p*> 0.05	***p*< 0.01**	***p*< 0.05**	***p*< 0.001**	***p*< 0.001**

A Kruskal-Wallis *H*-test was performed to establish the differences of the $\delta^{13}C$ and $\delta^{15}N$ values of the DISH group, separated by geographic location (collection source). A summary of the isotopic values of individuals from the DISH group, separated by their collection source can be found in Appendix 7 (Table 14.5). This separation by collection adds a degree of geographic division among individuals. Statistically significant differences were found between the KC and RDC, and the PBC and the RDC (Table 4.8). This is interesting when one considers the relatively close geographical relationship the PBC (Pretoria, Gauteng) and RDC (Johannesburg, Gauteng) share, when compared to the KC (Stellenbosch, Western Cape). Generally, the KC was more depleted in $\delta^{13}C$ values relative to the RDC, while the RDC was on average more enriched in $\delta^{15}N$ than the PBC. Figure 4.14 shows the distribution of $\delta^{13}C$ and $\delta^{15}N$ values of individuals diagnosed with DISH, when separated by the collection in which they are housed.

Table 14.5 in Appendix 7 also gives a summary of the isotopic values from the control group, separated by collection source. The tests for possible significance of the differences in $\delta^{13}C$ and $\delta^{15}N$ values between the skeletal collections were also performed for individuals in the control group. There were statistically significant differences found between all three of the skeletal collections when compared to one another (Table 4.9). Unlike the DISH group, the control group showed a statistically significant difference in the isotopic values between the PBC and KC.

Table 4.9. The p-values comparing the isotopic values from both DISH and control groups, separated by collection source.

DISH GROUP						
	$\delta^{13}C^{Rib}$	**$\delta^{15}N^{Rib}$**	**$\delta^{13}C^{Femur}$**	**$\delta^{15}N^{Femur}$**	**$\delta^{13}C^{Rib+Femur}$**	**$\delta^{15}N^{Rib+Femur}$**
KC and PBC	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05	*p*> 0.05
KC and RDC	***p*< 0.001**	*p*> 0.05	*p*> 0.05	*p*> 0.05	***p*< 0.01**	*p*> 0.05
PBC and RDC	***p*< 0.01**	***p*< 0.001**	*p*> 0.05	*p*> 0.05	*p*> 0.05	***p*< 0.05**
CONTROL GROUP						
	$\delta^{13}C^{Rib}$	**$\delta^{15}N^{Rib}$**	**$\delta^{13}C^{Femur}$**	**$\delta^{15}N^{Femur}$**	**$\delta^{13}C^{Rib+Femur}$**	**$\delta^{15}N^{Rib+Femur}$**
KC and PBC	*p*> 0.05	***p*< 0.01**	*p*> 0.05	*p*> 0.05	***p*< 0.05**	***p*< 0.001**
KC and RDC	***p*< 0.01**	*p*> 0.05	***p*< 0.01**	***p*< 0.01**	***p*< 0.001**	***p*< 0.001**
PBC and RDC	***p*< 0.01**	*p*> 0.05	*p*> 0.05	*p*> 0.05	***p*< 0.01**	*p*> 0.05

In summary, statistically significant differences were found in the isotopic values among BMI groups ($\delta^{15}N$ values in normal-weight and underweight; $\delta^{13}C$ and $\delta^{15}N$ values between overweight and underweight), among BMI and ancestry combined ($\delta^{15}N$ values between underweight white and obese black South Africans; $\delta^{15}N$ values between normal-weight white and obese black South Africans; $\delta^{15}N$ values between overweight white and obese black South Africans; $\delta^{15}N$ values between obese white and underweight black South Africans), and between collection source ($\delta^{15}N$ values between PBC and RDC), which were specific to the DISH group.

Statistically significant differences in the isotopic values specific to the control group were found among BMI and ancestry combined ($\delta^{13}C$ values between normal-weight black and obese white South Africans, $\delta^{15}N$ values between normal-weight black and underweight white South Africans; $\delta^{13}C$ values between obese black and normal-weight white South Africans), and among collection source ($\delta^{13}C$ and $\delta^{15}N$ values between KC and PBC; $\delta^{15}N$ values between KC and RDC; $\delta^{13}C$ values between PBC and RDC).

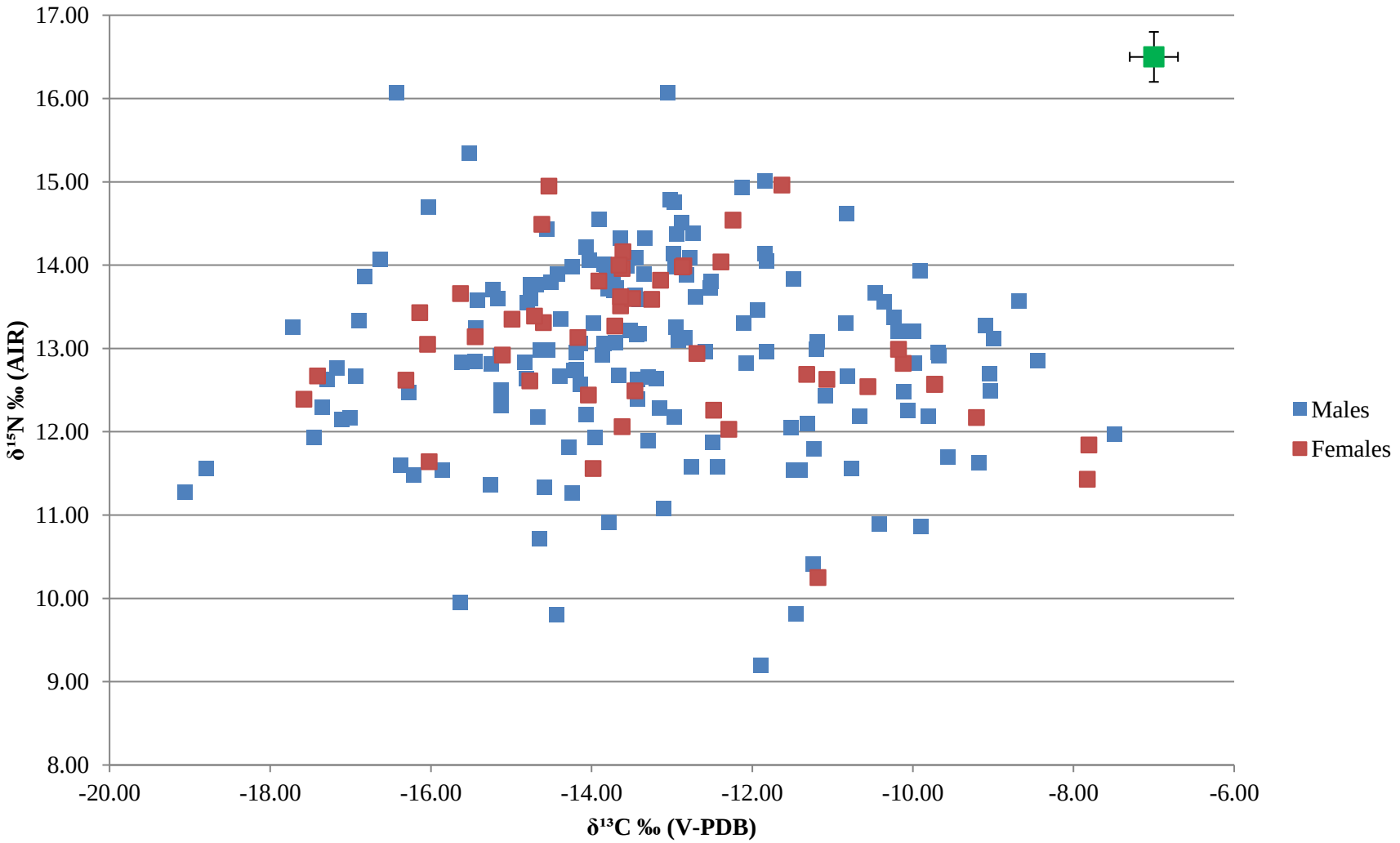

Figure 4.10: Isotopic results from all individuals diagnosed with DISH across all three skeletal collections (PBC, RDC and KC), separated by sex. The green square indicates the error margin of ±0.3‰ for both δ¹³C and δ¹⁵N.

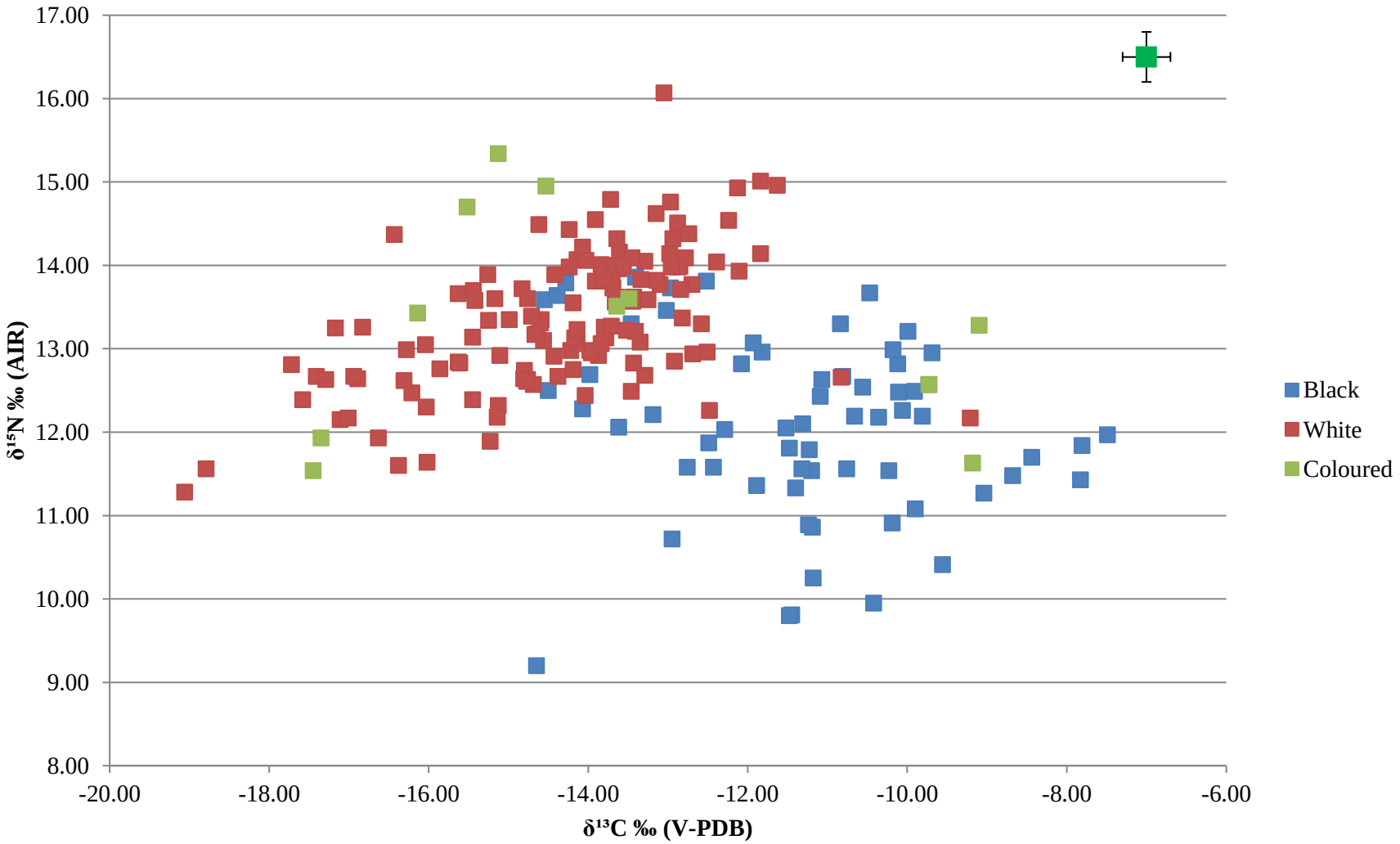

Figure 4.11: Isotopic results from all individuals diagnosed with DISH across all three skeletal collections (PBC, RDC and KC), separated by ancestry. The dark green square (top right) indicates the error margin of ±0.3‰ for both $\delta^{13}C$ and $\delta^{15}N$.

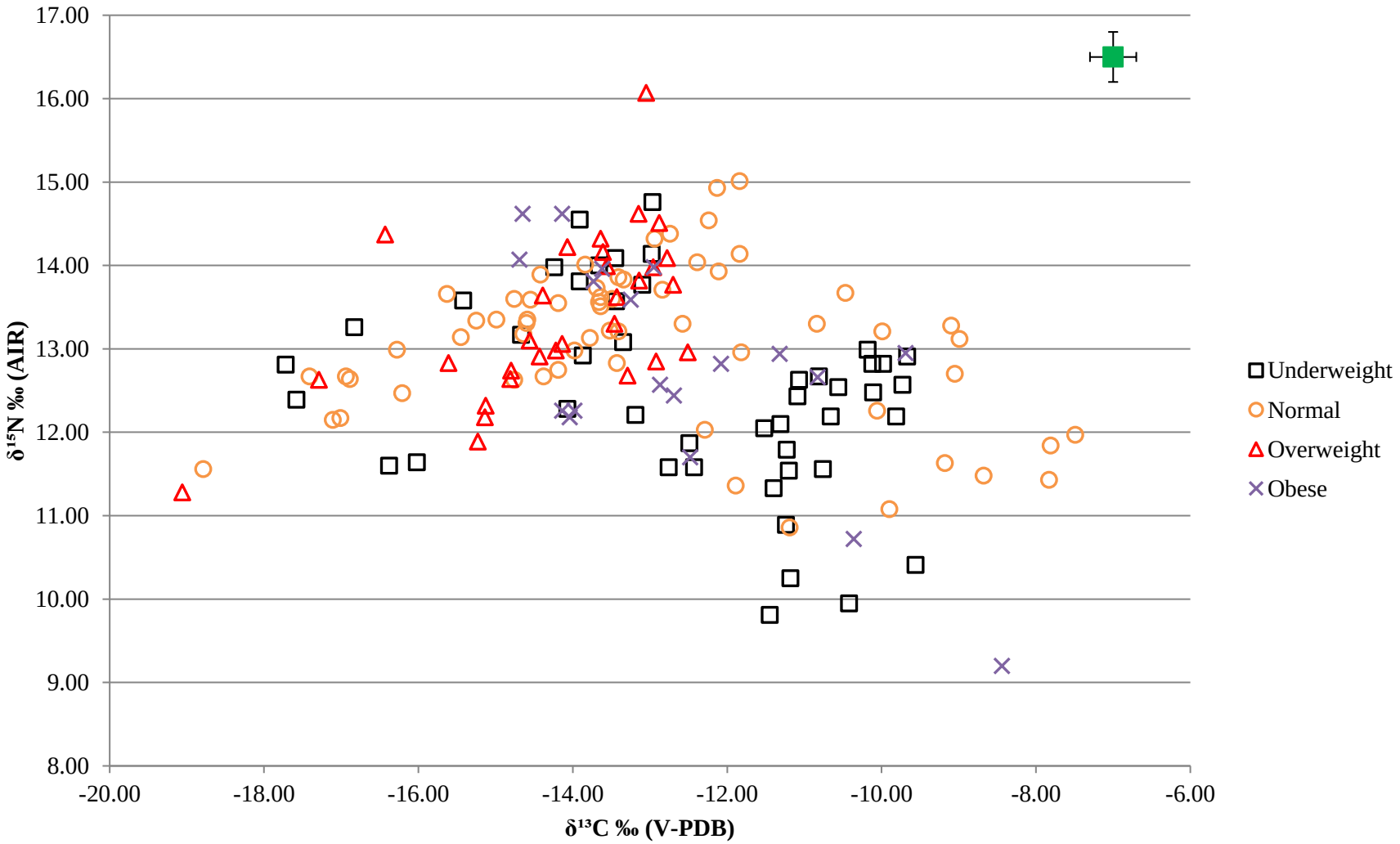

Figure 4.12: Isotopic results from all individuals with information on BMI diagnosed with DISH across all three skeletal collections (PBC, RDC and KC). The green square indicates the error margin of ±0.3‰ for both δ¹³C and δ¹⁵N.

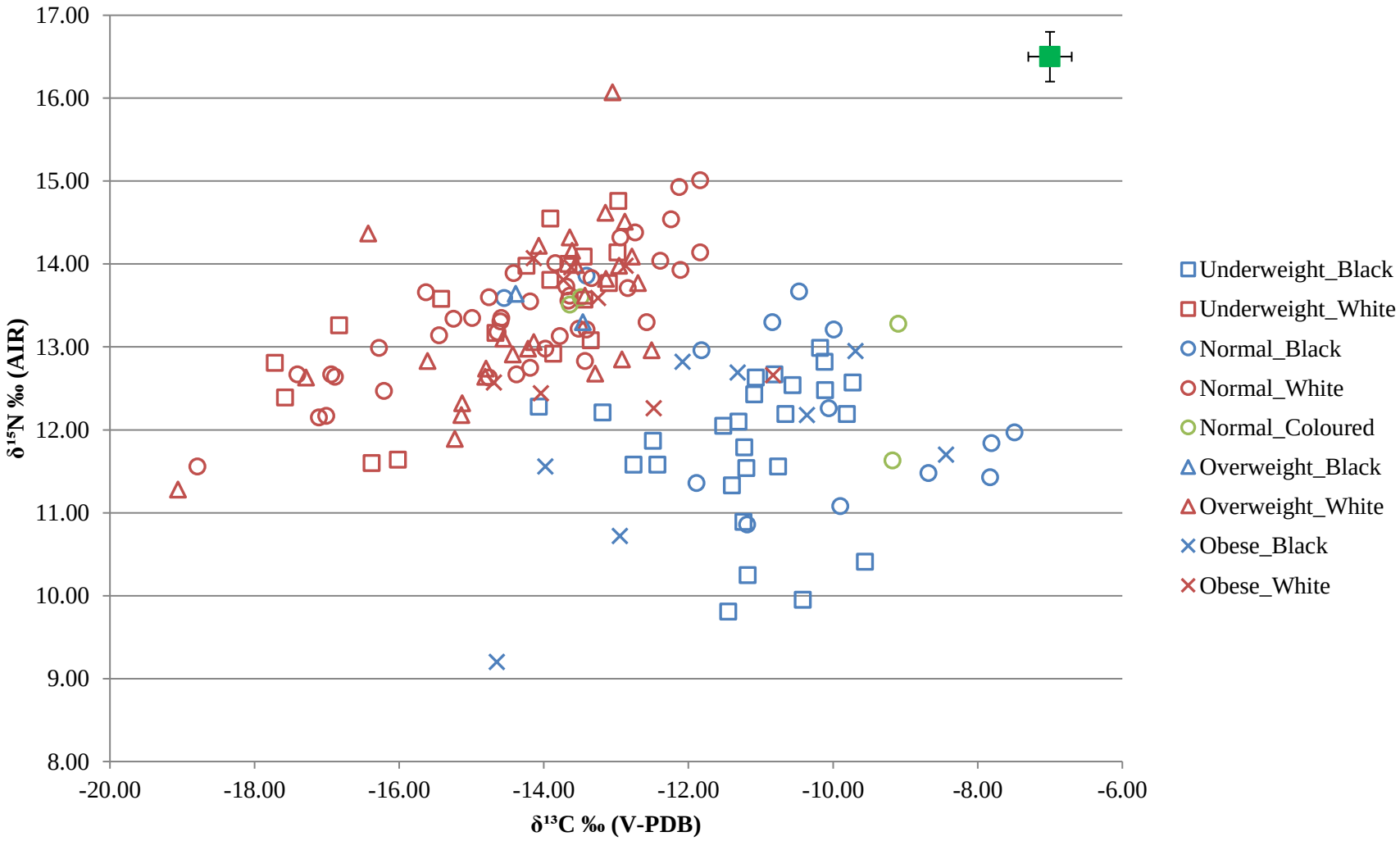

Figure 4.13: Isotopic results from all individuals diagnosed with DISH across all three skeletal collections (PBC, RDC and KC), separated by BMI category and ancestry. The green square indicates the error margin of ±0.3‰ for both δ¹³C and δ¹⁵N.

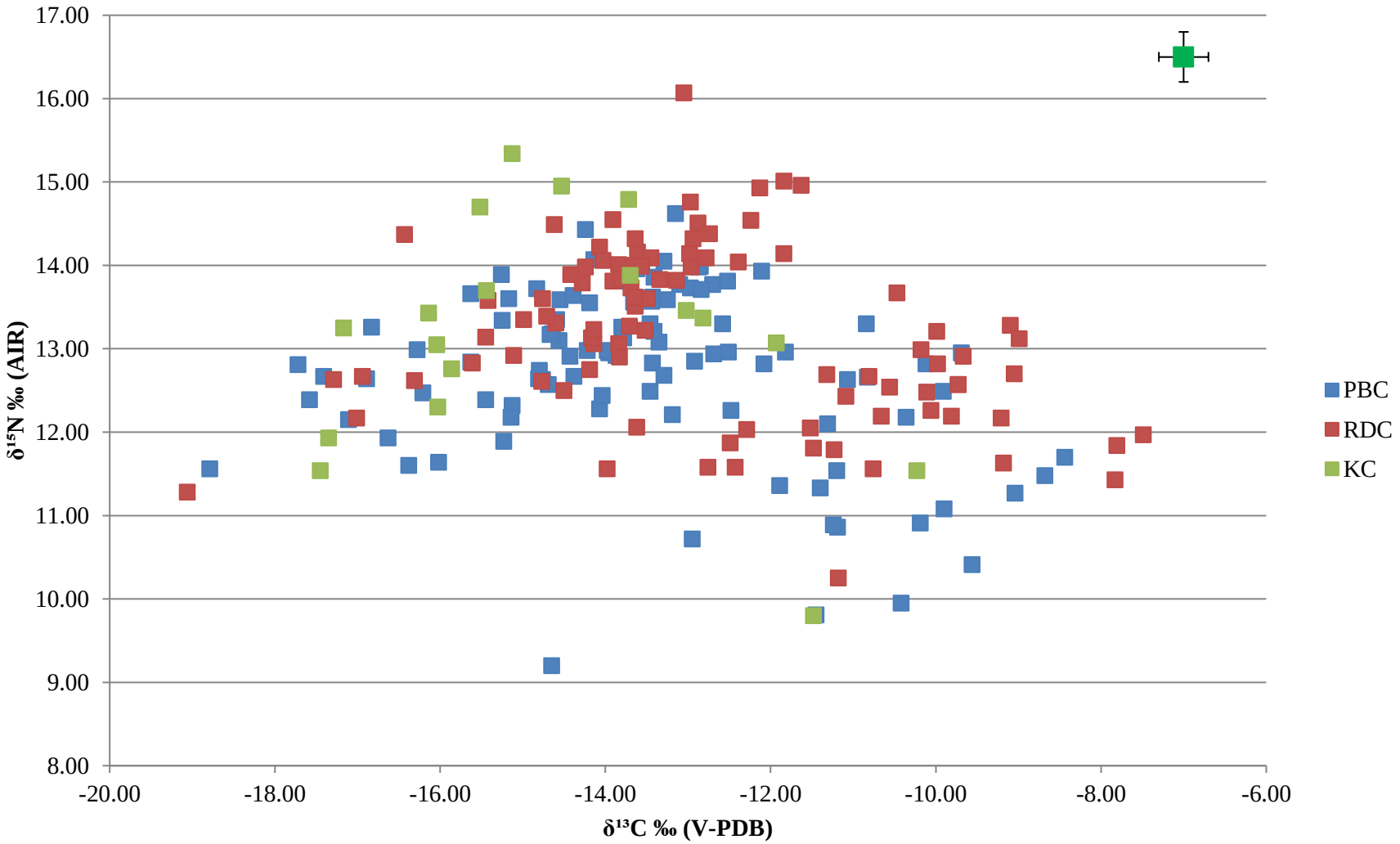

Figure 4.14. Isotopic results from all individuals diagnosed with DISH across all three skeletal collections (PBC, RDC and KC). The darker green square indicates the error margin of ±0.3‰ for both δ¹³C and δ¹⁵N.

4.5. Micro-CT analysis of individuals diagnosed with DISH

Of the 127 individuals diagnosed with DISH across the three skeletal collections, the affected vertebrae of 81 individuals were scanned in the MIXRAD facility at NECSA. Forty-three of the 81 individuals were taken from the PBC and 38 from the RDC. No individuals were taken from the KC for micro-CT analysis due to transportation constraints.

The four main features observed from the micro CT scans are shown in Appendix 8, and includes notes on (1) whether the ossification can be seen to start at the middle of the vertebral body (n=67, 82%); (2) whether the underlying cortex of the vertebral bodies remained intact at the site of the ALL ossification (n=12, 15%); (3) any evidence of trabecular bone at the sight of the ALL ossification (n=77, 94%); and (4) any abnormal areas of osteosclerosis beyond apparent ossification of the ALL (n=16, 20%). Overall, there was a mixture of features found across the individuals who were diagnosed with macroscopic manifestations of DISH. Of note was the fact that the majority of individuals showed development of trabecular bone within the flowing ossification (n=77, 94%), without retention of the vertebral cortex (n=70, 85%). In Figure 4.15, the outline of the vertebral cortex is still visible, while Figure 4.16 provides an example of ossification along the right antero-lateral surface of the vertebral body (a feature synonymous with DISH) but without retention of the cortex. This would suggest a localised erosive/inflammatory process that destroyed the cortex of the vertebral body and resulted in new bone formation.

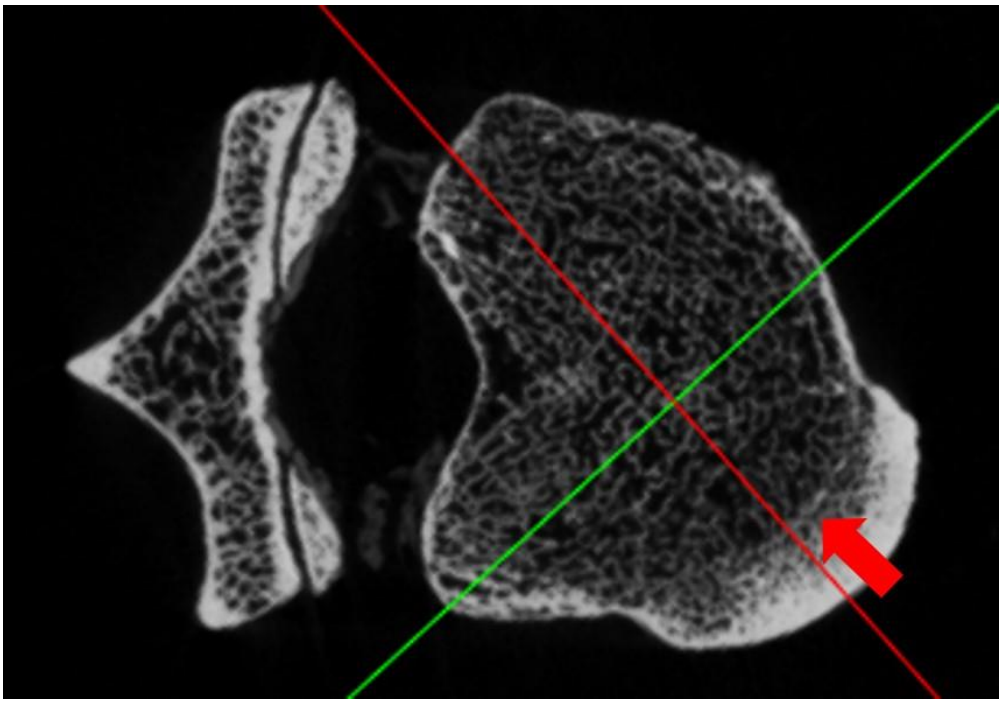

Figure 4.15. Transverse section of T11 of individual Sk5078 from the PBC; note the retention of the original vertebral cortex at the site of the ossification/new bone formation (indicated by the red arrow). Source: Author.

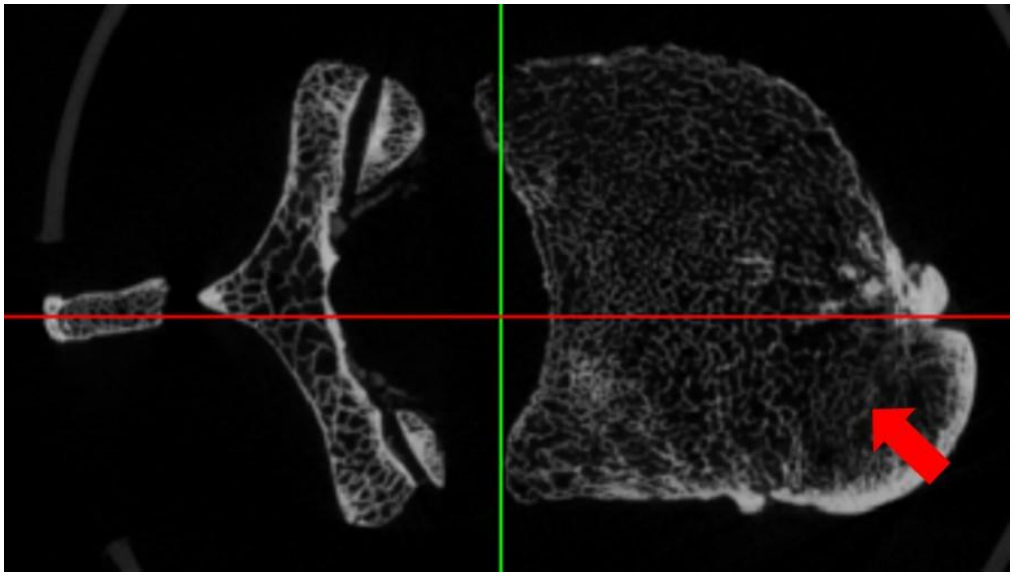

Figure 4.16. Micro-CT slice showing transverse section of flowing ossification on the right anterolateral surface of the vertebral body of T8 of individual 6380, from the PBC. The original cortex at the site of the ossification/new bone formation is no longer visible (indicated by the red arrow). Source: Author.

In the majority of the individuals (n=70, 85%), the extra trabecular bone is continuous with the trabeculae of the vertebral body (no cortex remains at the site of the flowing ossification). This suggests that new bone formed beyond ossification/calcification of the ALL,

and it is not certain if the ALL ossified at all. The development of the ossification /calcification of the ALL (without involvement of the underlying bone) is difficult to evaluate in this sample as the majority of macroscopically diagnosed cases of DISH are likely to have been more advanced cases, making it hard to follow the progression of the changes. However, what is interesting is that lack of ALL involvement has been described in the literature. For example, during a post-mortem examination of an individual, authors noted "The anterior vertebral common ligament is clearly visible. It is drawn to the left by the outgrowth, and the spurs described above are located beneath it. This ligament may easily be detached from the spurs though it is firmly attached at the level of the vertebral bodies as well as at the level of the disks." (Forestier and Rotés-Querol, 1950, page 326). If an erosive element to the development of DISH exists, it is assumed that this process, along with remodelling of the trabeculae, would have already taken place, and that little evidence of the ossification of the ALL would be left to investigate.

In some (n=67), but not all cases, the new bone formation could be seen to originate from the middle of the vertebral bodies on the right anterolateral surface (see Figure 4.17). In many individuals, a thickening of the vertebral wall was noted on the right anterolateral surface of vertebral bodies (osteosclerosis), possibly suggesting involvement of the ALL. In some individuals, this thickening (indicated by the sclerotic area) at the site of the ALL (right anterolaterally) occurred without any extensive trabeculae/new bone formation directly anterolaterally (i.e., on top) (Figure 4.18). This lack of new bone formation could possibly indicate the early stages of DISH. Perhaps as suggested above, the osteosclerosis is indicating the start of a possible inflammatory process. Large amounts of ossification/new bone formation were seen in the majority of individuals and in some, the ossification was shown to be very dense (osteosclerotic) with little evidence of trabecular bone (Figures 4.19 and 4.20). In some cases, extensive flowing ossification was looked at via the micro-CT scanner (in cross section) and found to be so dense that it was just represented by a radiopaque area, with no indication of trabeculae present.

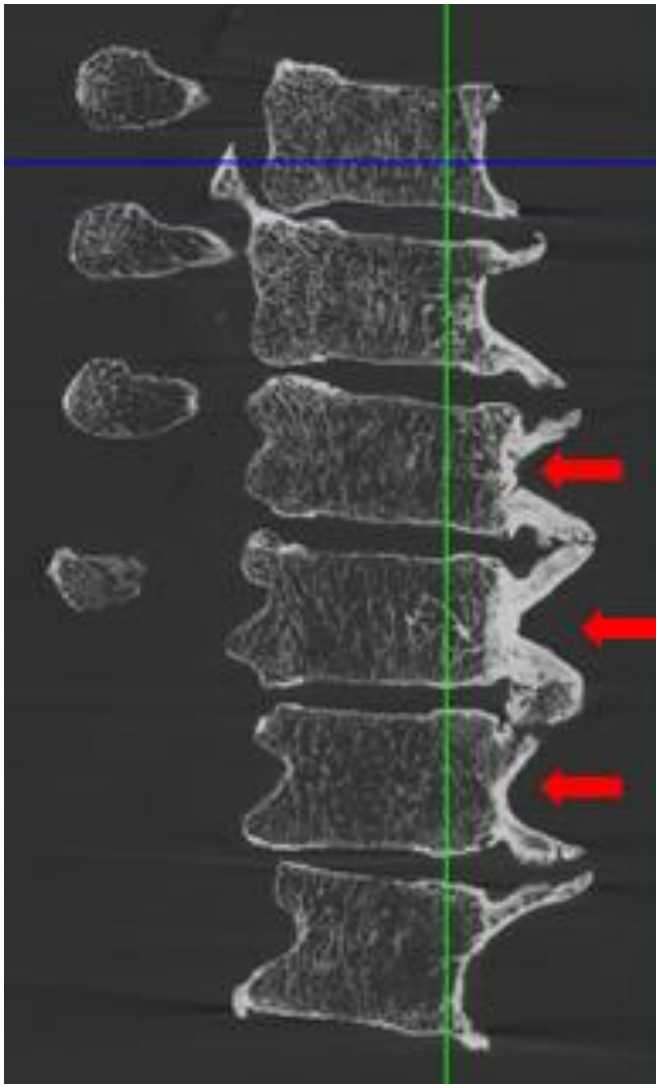

Figure 4.17. Cross section of T5 to T10 through the site of new bone formation associated with DISH. The ossification/new bone formation appears to start in the middle of the vertebral body (indicated by the red arrow), on the anterolateral surface. Note the osteosclerosis associated with the ossification/new bone formation. Skeleton AD3190 from RDC. Source: Author.

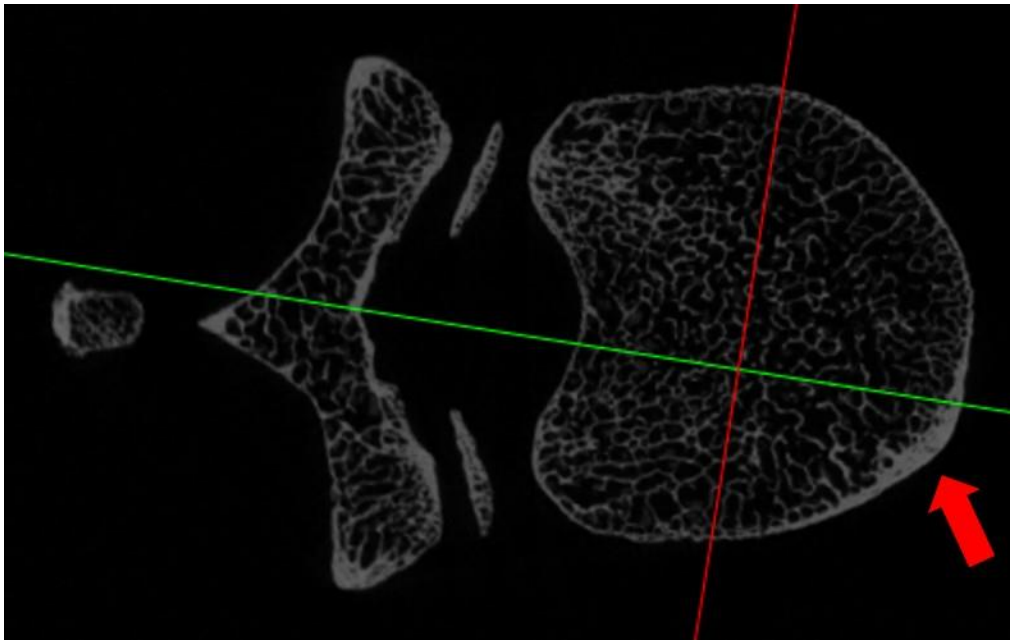

Figure 4.18. Transverse section of T10 of individual Sk3676 from the PBC. Note the thickening of the vertebral wall/cortex on the right anterolateral surface. Source: Author.

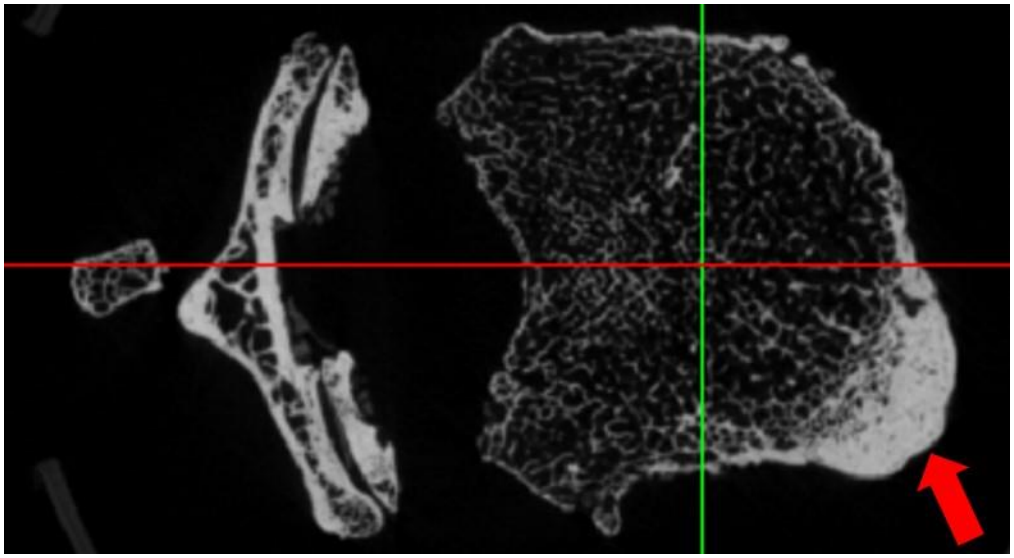

Figure 4.19. Transverse section of T8 showing the dense/sclerotic new bone formation on the right anterolateral surface. Sk6407, from the PBC.

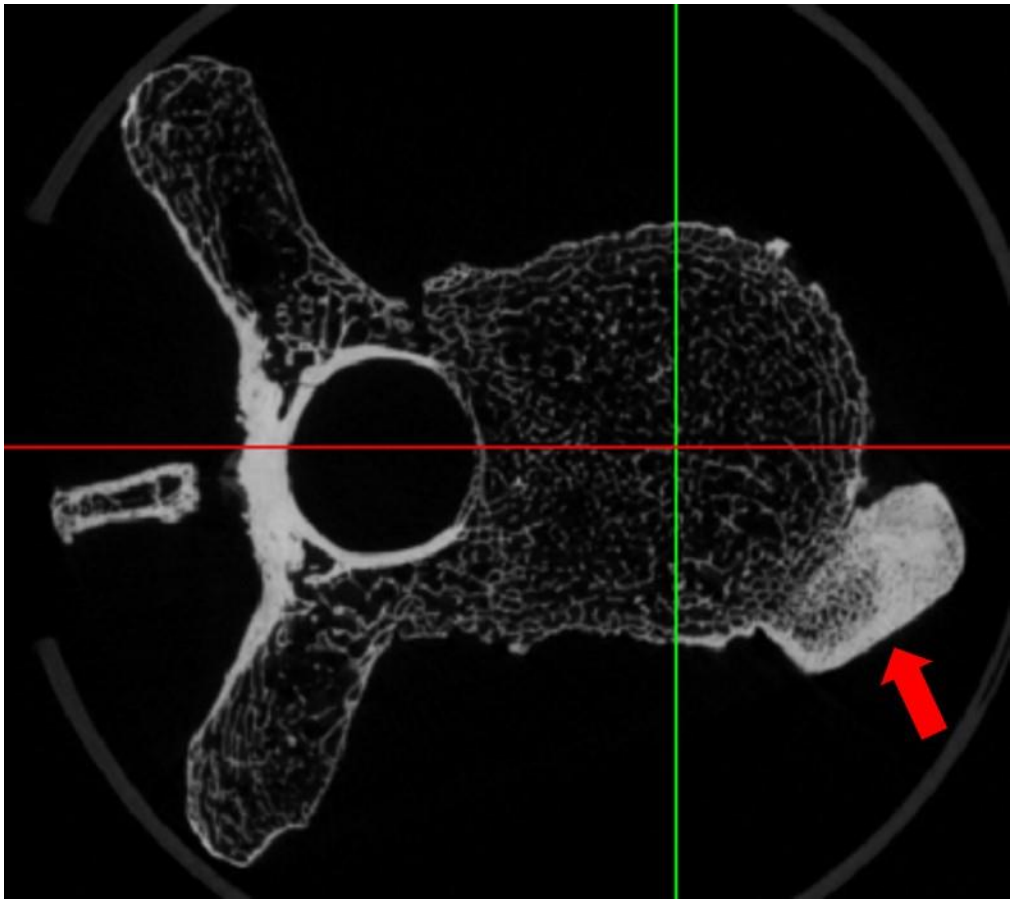

Figure 4.20. Transverse section of T9 showing the new bone formation on the right anterolateral surface as radiopaque, with some trabecular bone visible. Sk6407, from the PBC.

In some of the individuals, flowing ossification with no fusion between articulating vertebrae was noted, while in some, ankylosis was present. The bridging bone (ankylosis) between the vertebrae of individuals diagnosed with DISH mostly had an internal structure that contained trabeculae (Figure 4.21). Some individuals had a bridging structure with large amounts of sclerosis (Figures 4.22 and 4.23), but no clear pattern was identified. There is no visual indication to show that the ALL calcifies between these vertebrae.

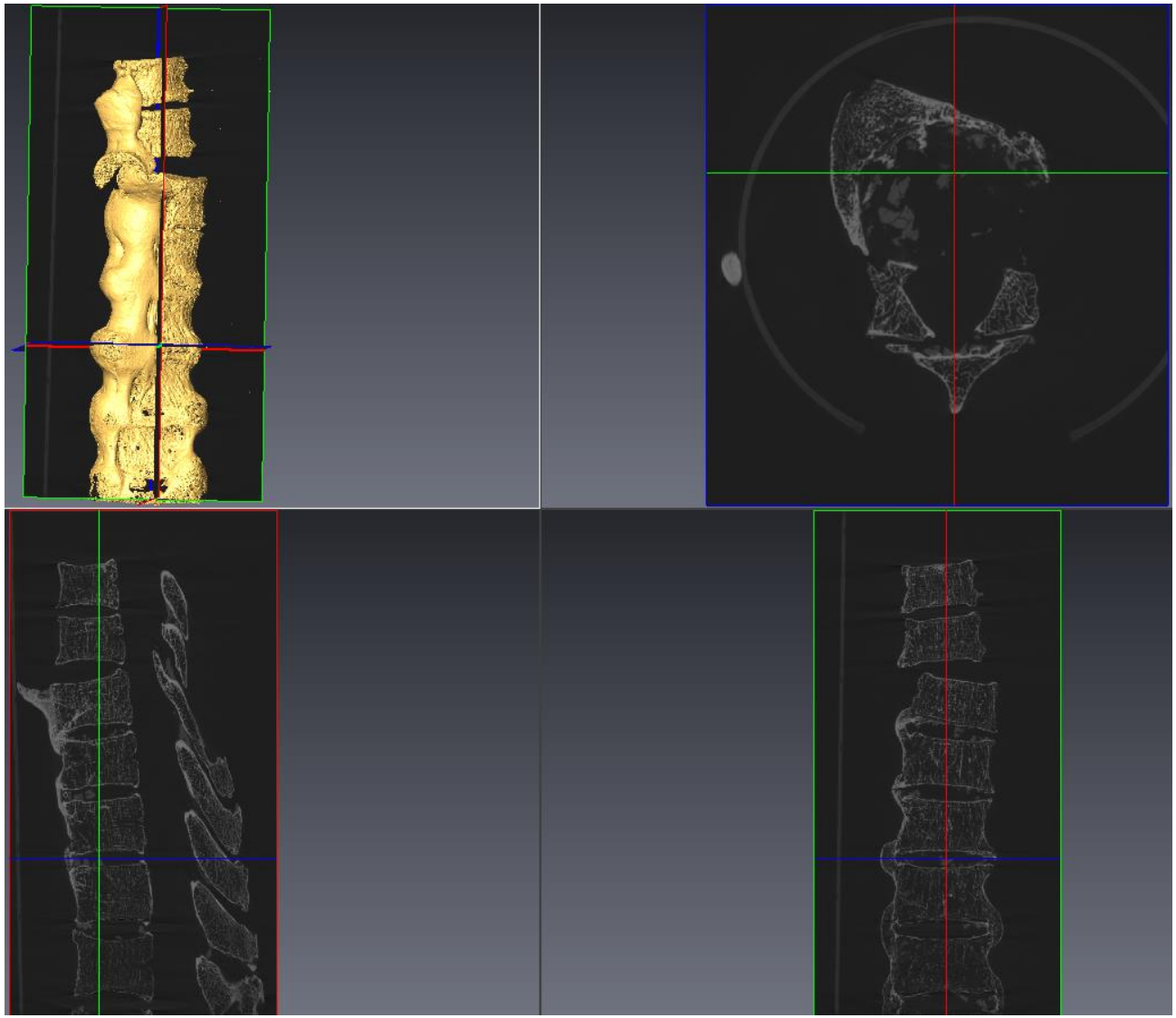

Figure 4.21. Different cross sections of T6-T12, showing the trabeculae within the bony ankylosis between the vertebrae of individual AD6356 from RDC, diagnosed with DISH. Source: Author.

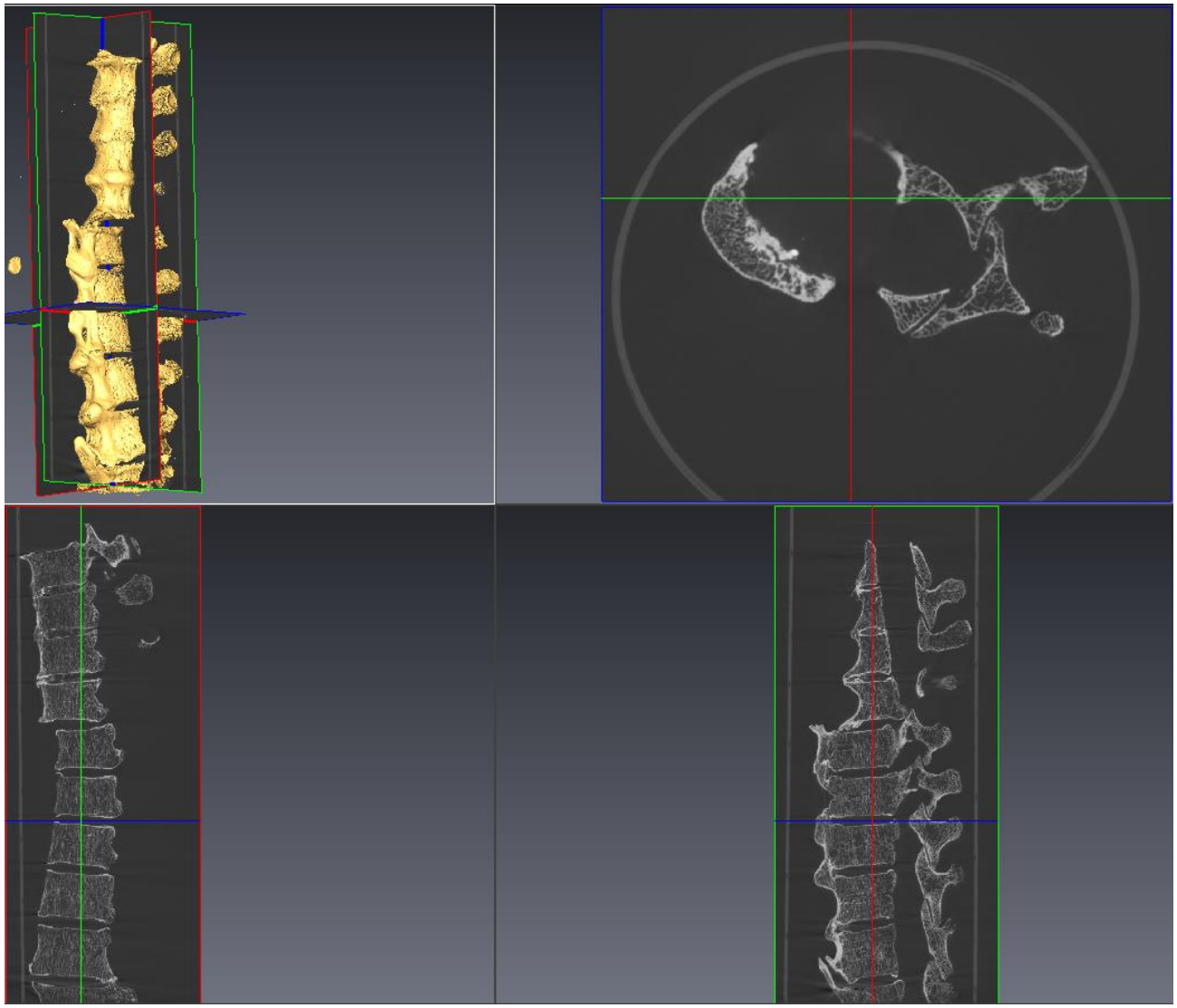

Figure 4.22. Different cross sections of T3-T12, showing the trabeculae within the bony ankylosis between the vertebrae of individual AD4473 from RDC, diagnosed with DISH. Source: Author.

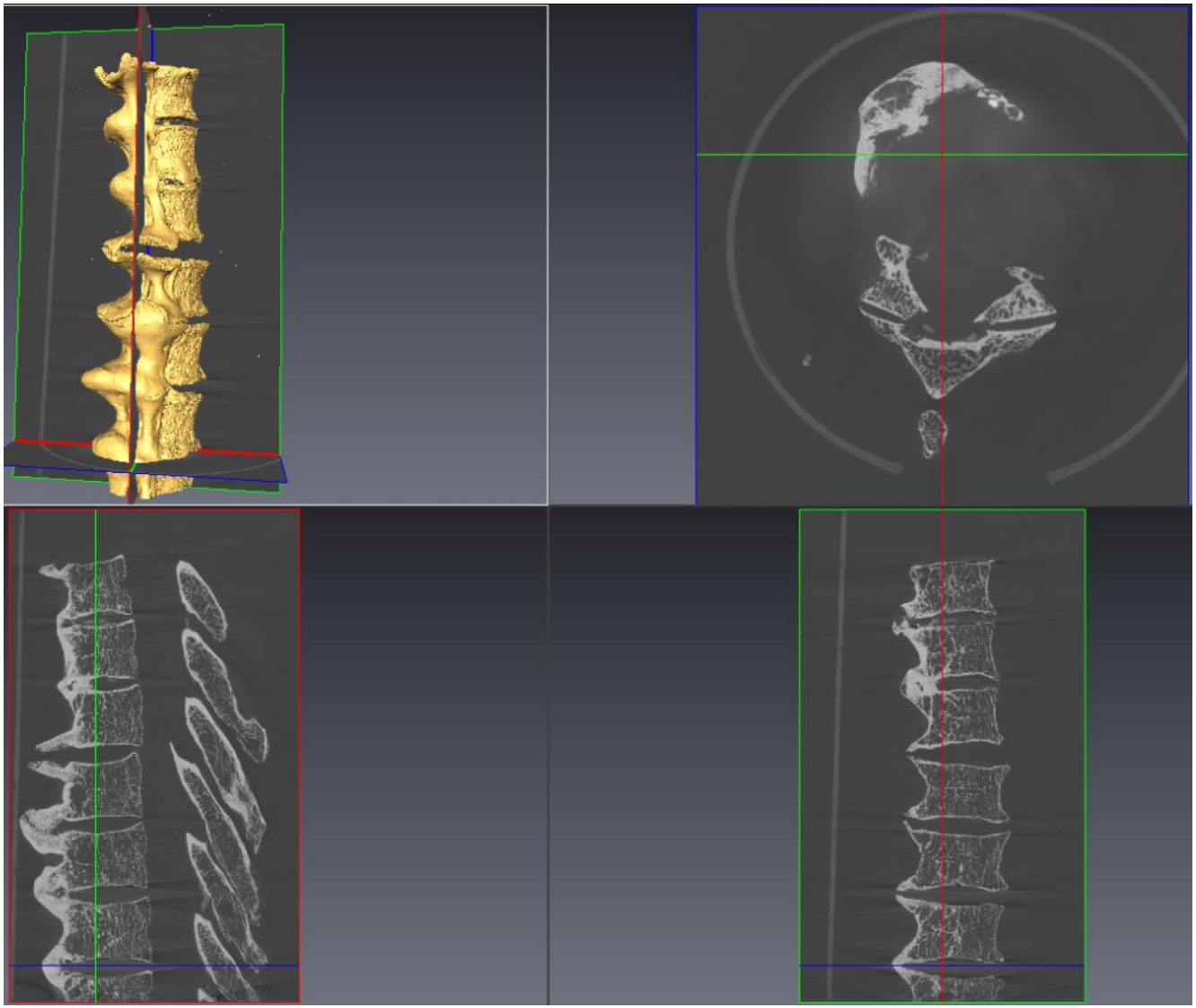

Figure 4.23. Different cross sections of T4-T12, showing the trabeculae within the bony ankylosis between the vertebrae of individual AD6663 from RDC, diagnosed with DISH. Source: Author

Several areas of osteosclerosis were observed which were not located at the site of the ALL. Many examples of the osteosclerosis could be linked to the margins of Schmorl's nodes (Figure 4.24); while in others it was more difficult to pinpoint a possible cause (Figures 4.25 and 4.26). Interestingly, skeleton AD6177 pictured in Figure 4.25 had flowing bone indicative of DISH, but along the left anterolateral surface, suggesting the possibility that the individual had situs inversus.

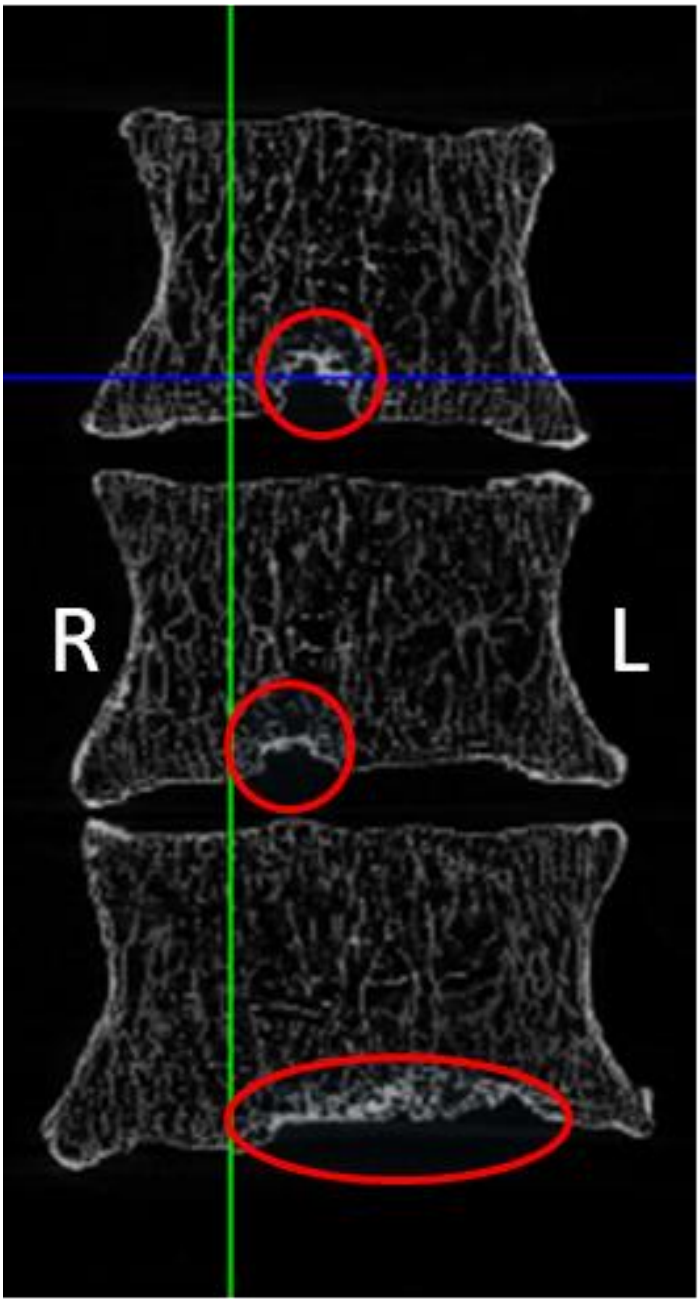

Figure 4.24. Coronal view of T6-T8, showing osteosclerosis around the margins of Schmorl's nodes (indicated by red circles/oval). Sk 7008 from the PBC. Source: Author.

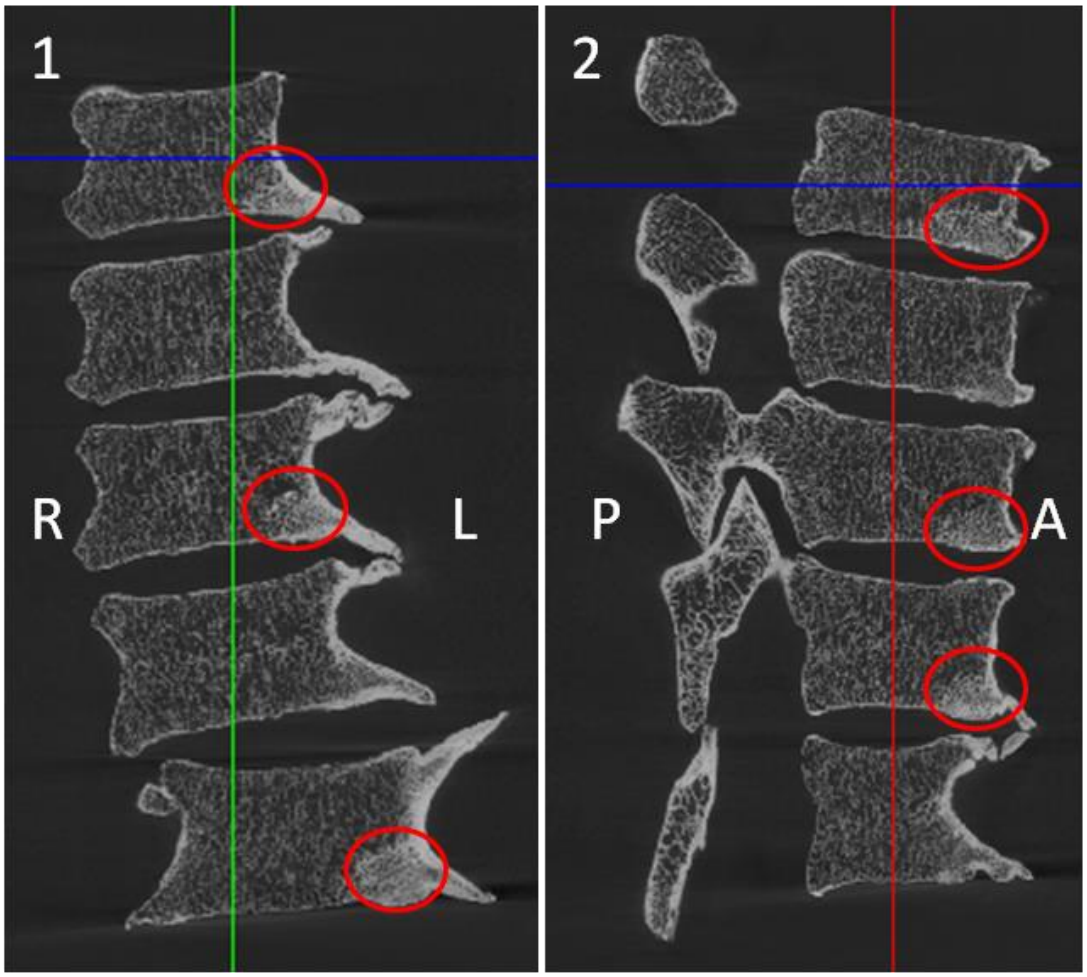

Figure 4.25. View of T7-T11, showing osteosclerosis at the inferio-anterior corner of the vertebral bodies (indicated by red ovals). Image 1 shows the coronal view with indications for right (R) and left (L), while image 2 shows the sagittal view with anterior (A) and posterior (P) indicated. Skeleton AD6177 from the RDC. Source: Author.

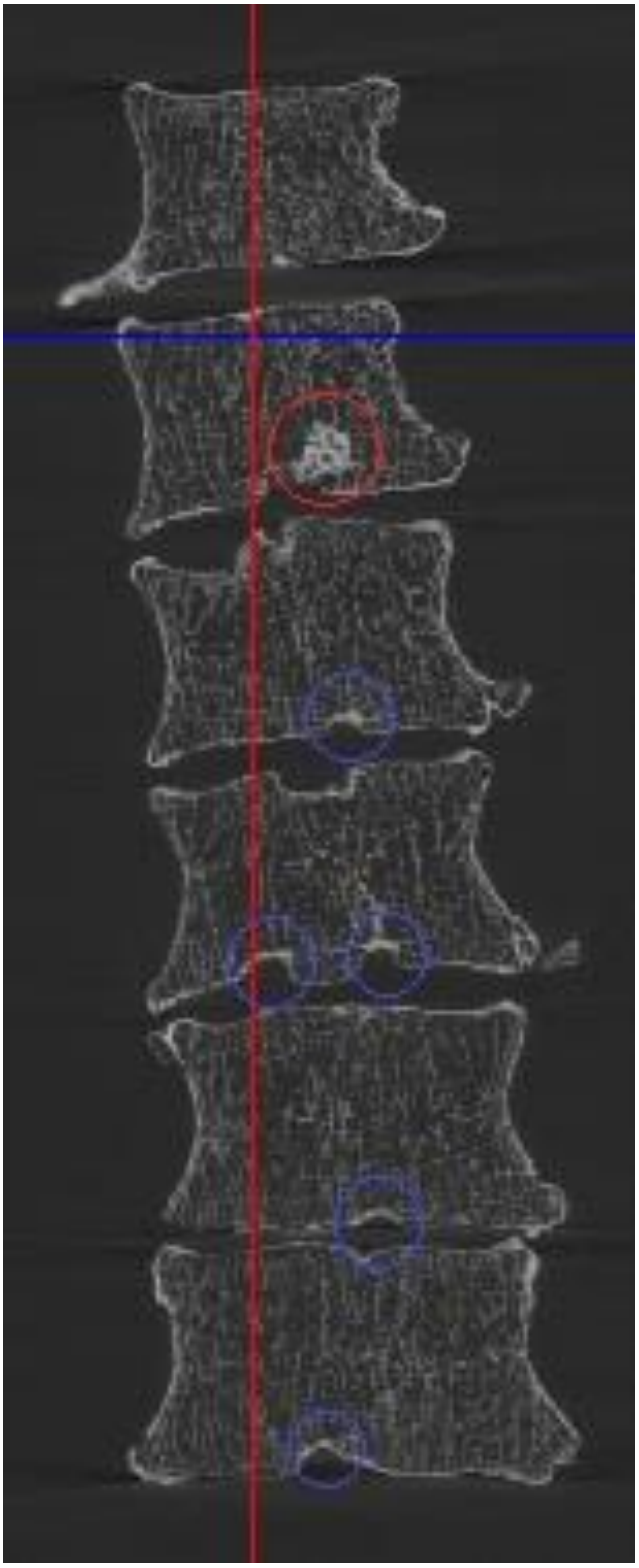

Figure 4.26. Coronal view of T7-T12, showing an area of osteosclerosis around the margins of Schmorl's nodes (blue circles), but also an area of osteosclerosis in T8 with no definitive possible cause (red circle). Skeleton AD7640 from the RDC. Source: Author.

Overall, the micro-CT scans highlighted two patterns of new bone growth: one, sclerotic new bone forms near to the edges of the vertebral walls, along the cortex, and two, a possible erosive process where the cortex of the vertebral wall is eroded and destroyed while a fairly porous structure is formed which becomes continuous with the trabecular bone of the vertebral body. Again, the former could be a precursor to the inflammatory process, or it could just be dense new bone growth. While some of the new bone growth seems to look like osteophytes, the majority do not develop at the margins of the vertebral endplate, but rather juxta-articular.

If these examples of new bone growth are indeed osteophytes, it is unclear as to why their macroscopic appearance looks like “classic” DISH, offering visuals similar to a “candle-wax” appearance. It is also unclear the extent to which the ALL is involved in these micro-CT scans and requires further investigation. It could be that all of the vertebrae start off with sclerosis of the anterior cortex of the vertebral walls at the site of the ALL and is then followed by “beak-like” projections that initially grow horizontally juxta-articular, and then turn vertically to eventually join up with other similar such outgrowths between consecutive vertebrae, eventually causing ankylosis – similar to osteophytes.

5. DISCUSSION

The discussion chapter presents information on whether the observations made in this study are in line with, or contradict, previously established theories and understandings around DISH, when compared to similar studies, or, whether new trends can be observed within the data. At a fundamental level, this includes considerations on the prevalence of DISH, the role obesity plays with DISH, the dietary hypothesis of DISH and the pathogenesis of DISH, all within the context of the South African cohort under study.

5.1. Intra- and inter- observer reliability analysis

Different observers were able to yield acceptable repeatability among the spinal and extra-spinal morphological traits associated with DISH, indicating that the methodology overall, was robust and valid. Yet training in the morphological traits associated with DISH, as well as knowledge and understanding of the bony manifestations of the disease, was necessary for good repeatability in scoring. Clearly the quantification of standard recording can be confusing unless the recorder has prior experience in pathological lesions on bone. For example, the recording of new bone growth/calcification associated with DISH on the vertebral column was necessary for differential diagnosis. However, the method of scoring was fairly complicated as found within the acceptable, but fairly low, κ values. A concise recording system that included information on whether the ossification/growth created ankylosis with articulating vertebrae, as well as providing a comprehensive understanding on its location, was difficult to create and is sorely needed in future research.

Overall, in bone pathology research, a need exists for more rigorous intra-and inter-rater reliability analysis, especially when using diagnostic criteria for DISH. Reliability of trait distinction will ensure robusticity and validity of the data, but will also make different studies on DISH directly comparable (Van der Merwe et al., 2012).

5.2. The frequency of infrequently observed skeletal manifestations of DISH

Three infrequently observed skeletal manifestations of DISH were recorded and assessed to understand the relative frequency in which they occurred during analysis of individuals across the three South African skeletal and death assemblages, the PBC, RDC and KC. The three features included plantar heel spurring (Littlejohn and Hall, 1982; Rogers and

Waldron, 2001; Mader, 2003) which occurred 55% of the time, ossification of apical ligament (Arlet and Mazieres, 1985) which occurred only 30% of the time, and ossification of the supraspinous ligament (Forestier and Lagier, 1971) which occurred 81% of the time. This would suggest that ossification of the supraspinous ligament was the only skeletal manifestation that could be linked to DISH, while the other two features occurred to infrequently for them to be linked with certainty to a manifestation of the disease. Further investigation into the relative frequency of these and other infrequently observed spinal and extra-spinal enthesophytes would be useful for future developments in diagnostic criteria of DISH as well as expanding our understanding of the skeletal manifestations of DISH.

5.3. The prevalence of DISH in South Africa

The crude prevalence rate of DISH in this study (3.3%) is similar to a reported clinical prevalence of DISH (3.9%) using chest radiographs in South Africa, 30 years ago (Cassim et al., 1990). However, the abovementioned clinical prevalence of DISH was only among male and female black South Africans. The research presented in this thesis represented both sexes across three self-identified ancestry groups in South Africa.

DISH was found to occur more frequently among males than females in this study, but not appreciably so. Among various global archaeological populations and clinical studies throughout the 20th century, a sex bias for DISH has been noted with males considerably outnumbering females in prevalence of the disease (Forestier and Rotés-Querol, 1950; Ortner, 2003; Pappone et al., 1996; Rogers and Waldron, 2001; Roberts and Manchester, 2010; Foster et al., 2018; Kim et al., 2018). For archaeological groups, the disparity between the sexes is most likely due to the lack of representation of females in the burial record. Research by Walker (2008) has suggested that this could be due to inaccurate sex estimation of archaeological skeletons as some females appear more robust with an increase in age. However, for clinical studies, the picture is less clear, as both males and females present with skeletal manifestations of the disease. South African clinicians noted a higher prevalence of DISH among females (4.2%) than males (3.8%) (Cassim et al., 1990). Based on clinical studies, variation in manifestations of DISH may not be attributable only to biological sex, but rather to variation in life expectancy, ancestry and social status within a community. Life expectancy at birth for males in South Africa in 2018 was estimated to be 60 years, while life expectancy at birth for females in South Africa in 2018 was 67 (United Nations Population Division, 2019^{a}; United Nations Population Division, 2019^{b}). Theoretically, this would suggest that as women (on

average) are living longer, they would have more time to develop the manifestations of DISH, than that of their male counterparts. However, as this is not the pattern we are seeing in the results of this study, another factor is at play, which is very likely to be the way in which individuals are incorporated into the skeletal collections, and the inherent bias between the sexes, this creates. As previously mentioned, there are two methods by which an individual is incorporated in one of the three studied skeletal collections, namely through donation or they are unclaimed bodies given for medical investigation to institutes of higher education. Globally, women are less likely to donate their bodies to science than men, and within the South African context, this is no different. When it comes to the incorporation of unclaimed individuals into the death assemblage collections, this seems to be dominated by black men as there is a historical movement of these individuals from rural areas to urban areas for economic reasons. Once in the urban environment, their families are unlikely to hear about their passing due to limited communication and with circumstances such as lack of transport and monetary resources, their bodies remain unclaimed. Black women are believed to stay closer to their relatives, therefore in the event of a death, their bodies are often claimed (L'Abbé et al., 2005).

While a larger proportion of males than females were diagnosed with DISH in the current study, white South African males exhibited the greatest prevalence for DISH. Again, we may be seeing a reflection of the inherent bias across the three skeletal and death assemblage collections. In South Africa, elderly men of European descent (white), are more likely to personally donate their bodies to science than any other group. Due to cultural and religious reasons, black South Africans are less likely to donate their bodies for medical research. This could be why we see the greatest prevalence for DISH among white South African males. However, age-at-death could also have played a role in the prevalence of DISH among white South African males.

At archaeological sites, a higher prevalence of DISH has been noted amongst the wealthy, priests and monks when compared to those buried in the lay cemetery (Waldron, 1985; Rogers and Waldron, 2001; Jankauskas, 2003; Verlaan et al., 2007). Based on this observation, researchers concluded that DISH was associated with higher SES and richer diets. The wealthy were more susceptible to becoming obese than the poor, and in turn, to developing DISH. The literature has also shown that the manifestation of the disease is more often noted in those greater than 50 years of age. Considering the above, what if the priests, monks and wealthier groups had lived longer than individuals from the lay population? Perhaps this would have

provided more time for the skeletal manifestations of DISH to develop. While the literature is not clear on the age-at-death of those frequently diagnosed with DISH compared to their non-DISH counterparts, it is certainly a variable to consider. Within this research, a greater number of white South Africans were diagnosed with DISH, when compared to their black and coloured South African counterparts. White South Africans diagnosed with DISH also had a higher mean age-at-death (73 years; 73 years for males; 74 years for females) than their black (64 years; 65 years for males; 60 years for females) and coloured (68 years; 69 years for males; 66 years for females) South Africans counterparts. While a greater mean age-at-death is likely attributed to other factors, such as diet, SES and lifestyle, a longer lifespan may contribute to a greater skeletal manifestation of the disease. It may be pertinent to once again reflect on the fact that in South Africa, elderly white men are more likely to personally donate their bodies for medical science than any other group. This, coupled with the higher age-at-death could mean that the white South African males studied in the current research simply had more time to develop the skeletal manifestations of DISH, with the disease most likely to affect those over the age of 50 years. On the other hand, unknown physiological/genetic factors may also predispose individuals to developing DISH.

5.4. The relationship between DISH and obesity/BMI

Obesity has been considered a risk factor in the development of DISH. This association was noted in both the archaeological and clinical literature (Julkunen et al., 1971; Waldron, 1985; Boachie-Adjei and Bullough, 1987; Maat et al., 1995; Mata et al., 1997; Coacciolo et al., 2000; Rogers and Waldron, 2001; Kiss et al., 2002a; Verlaan et al., 2007; Mader et al., 2009; Giuffra et al., 2010; Zincarelli et al., 2012). In an archaeological context, obesity was interpreted as high-status living, with a focus on cemeteries or populations of higher SES groups. Obesity among high SES groups was interpreted from a variety of sources, such as archaeological material (*e.g.* bones from animals in midden piles) and documentary evidence (*e.g.* contemporary writings) (Waldron, 1985; Rogers and Waldron, 2001; Spencer, 2008). While individuals of higher SES can more readily be identified in a bioarchaeological context through associated rich burial goods and burial location, this is not the case in the three South African skeletal populations examined in this study. It is not enough to use the correlation between obesity and high SES, particularly as obesity within a modern context is often associated with an increased consumption of low quality, inexpensive food which are high in calories, fats and sugars (Kruger et al., 2005). This would suggest that in the analysis of SES

among more modern human skeletal remains, BMI and obesity are no longer the cornerstones of high SES. Suggesting that a patterned relationship among DISH, SES and BMI has become less clear. Another factor to consider is that among the three South African skeletal collections, the majority were likely of low SES, reflected by the way in which the remains are incorporated into the collections.

The current study could not show a relationship between the skeletal manifestations of DISH and BMI, but most skeletons with signs of DISH were most likely to fall within the underweight and normal-weight BMI categories than in the overweight or obese categories. Within the overweight and obese categories, most individuals were male and white South African (n=25, 81%), possibly suggesting a dietary relationship or genetic association with DISH. Some research demonstrates a strong correlation between early onset obesity, and the development of DISH (Mata et al., 1997; Kiss et al., 2002a). So a person could have been obese prior to or around the time they developed DISH and subsequently lost weight, as DISH can take over a decade to develop the characteristic flowing ossification needed for a positive diagnosis (Yaniv et al., 2014).

5.5. Expectations of the dietary analysis

The clinical and archaeological literature not only support a link between DISH and obesity (Julkunen et al., 1971; Waldron, 1985; Boachie-Adjei and Bullough, 1987; Maat et al., 1995; Mata et al., 1997; Coacciolo et al., 2000; Rogers and Waldron, 2001; Kiss et al., 2002a; Verlaan et al., 2007; Mader et al., 2009; Giuffra et al., 2010; Zincarelli et al., 2012), but also suggest a link between the disease and protein intake, represented by $\delta^{15}N$ enrichment among those diagnosed with DISH (Müldner and Richards, 2007; Spencer, 2008; Quintelier et al., 2014). Isotopic analyses were performed in this study to measure the relationship between DISH and protein consumption. Literary sources suggest that the majority of South Africans value meat and seek to include it in their daily dietary habits wherever possible (Viljoen and Gericke, 2001; Puoane et al., 2006). However, access to regular meat consumption may not have been regular or the means to have eaten it in excess for these South African groups. While stable light isotope analysis ($\delta^{15}N$ and $\delta^{13}C$) is useful in assessing the different types of foods eaten, it cannot distinguish between the quantities of foods consumed. Therefore, the question of DISH being linked to an *excess* of protein cannot be established in this study.

Interpretations of the isotopic analyses, diet and DISH has been divided into two sections. Firstly, the dietary hypothesis of DISH, using the results to answer the question of whether a relationship between DISH and diet (protein consumption) exists, is evaluated. Secondly, a broader conversation around the general dietary patterns observed within the DISH group is presented.

5.5.1. The dietary hypothesis of DISH

In this study, no differences were found between isotopic values of the rib and femur among the DISH and control groups. All interpretations on the dietary analysis of individuals has been made on the amalgamated values from the rib and femur samples ($\delta^{13}C^{Rib+Femur}$ and $\delta^{15}N^{Rib+Femur}$).

In similar studies investigating DISH and diet, authors have noted elevated $\delta^{15}N$ values for individuals diagnosed with DISH and reported means of 14.1‰ (Müldner and Richards, 2007; Table 5.1) 13.4‰ (Spencer, 2008; Table 5.1) and 11.9‰ (Quintelier et al., 2014; Table 5.1). The aforementioned investigations displayed a general trend of $\delta^{15}N$ enrichment among individuals with DISH when compared to non-DISH individuals, and this has been interpreted as consumption of foods rich in animal protein (Müldner and Richards, 2007; Spencer, 2008; Quintelier et al., 2014). In the current study, both DISH and non-DISH individuals had generally high $\delta^{15}N$ values, with a mean of 13.0‰, suggesting that there is no clear evidence for elevated levels of $\delta^{15}N$ values being attributed to those with DISH exclusively. The DISH samples across the South African cohort had both the lowest and highest $\delta^{15}N$ values of all three of the above-mentioned studies on DISH and diet (Müldner and Richards, 2007; Spencer, 2008; Quintelier et al., 2014; Table 5.1). The relatively high $\delta^{15}N$ values among the South Africans could indicate a diet rich in animal protein for all groups and both sexes. However, given our understanding of modern 20th century South African diets, a high consumption of animal proteins for all groups and sexes seems unlikely. Some other factor/s (extrinsic or intrinsic) may be responsible, either for the development of DISH, or the elevated $\delta^{15}N$ values seen in both groups, perhaps masking any dietary signals that could be responsible for DISH.

Table 5.1. Summary of similar studies using isotopic analysis to investigate the aetiology of DISH

		$\delta^{15}N$		
Study	**Number of individuals with DISH sampled for isotope analysis**	**Minimum**	**Maximum**	**Mean**
Müldner and Richards, 2007	4	13.2 ‰	15.2 ‰	14.1 ‰
Spencer 2008	47	10.7 ‰	14.4 ‰	13.4 ‰
Quintelier et al., 2014	10	9.9 ‰	13.5 ‰	11.9 ‰
Current study	96	9.2 ‰	16.1 ‰	13.0 ‰

There are other factors that can cause elevated $\delta^{15}N$ values, which may be applicable to the South African cohort and may have served as an influence on the isotopic values of individuals, specifically the $\delta^{15}N$ values.

5.5.1.1. Other factors that may affect $\delta^{15}N$ values

Several factors can influence $\delta^{15}N$ values such as malnutrition, starvation, alcoholism, disease, climate and dehydration (Heaton et al., 1986; Bunout, 1999; Katzenberg and Lovell, 1999; Preedy et al., 1999; Hong-Brown et al., 2001; Mekota et al., 2006; Dotsika and Diamantopoulos, 2019). Each possible contributing factor and how it may have affected modern day South Africans is discussed.

BMI has been shown to be inversely related to $\delta^{15}N$ in a study of patients with anorexia nervosa (Mekota et al., 2006). When patients' weight and BMI decreased, indicating periods of starvation, the $\delta^{15}N$ values (measured from hair samples) increased. The breakdown of bodily protein through catabolism (to access nitrogen that would otherwise be obtained through food intake), has been described as the metabolic process responsible for this nitrogen enrichment in periods of starvation (Hobson et al., 1993; D'Ortenzio et al., 2015). Nitrogen enriched in ^{14}N is excreted, leaving bodily tissue that is enriched in ^{15}N, hence the increase in measurable $\delta^{15}N$ during periods of malnutrition (Mekota et al., 2006). Of the 96 individuals diagnosed with DISH where BMI estimates could be obtained, 29 had been categorized as underweight, but the mean $\delta^{15}N$ value for these individuals from the DISH group was 12.5‰, which is higher than the values from the patients presented in the Mekota and colleagues study

(Mekota et al., 2006). While distinct differences in the $\delta^{15}N$ values from the current study were noted among the underweight and normal-weight groups, and the underweight and overweight groups, the underweight group had the lowest mean value ($\delta^{15}N$) of all four of the BMI categories. Considering this, while some higher $\delta^{15}N$ values may be a result of recycled bodily proteins through a general trend of malnutrition, it does not seem that malnutrition through starvation can alone account for the $\delta^{15}N$ enrichment, especially when we take into consideration the $\delta^{13}C$ values.

Mekota and colleagues' (2006) study also showed that malnutrition could be identified though variation in $\delta^{13}C$ values, with evidence for a trend between $\delta^{13}C$ enrichment and BMI increase. The association between an increase in BMI and enrichment in $\delta^{13}C$ values was attributed to an increase in protein consumption in patient's diets, indicating periods where they were eating (Mekota et al., 2006). Overall, a trend between an increase in patient's BMI (periods when patients ate) and their stable isotope values was observed, namely a decrease in $\delta^{15}N$ values and an increase in $\delta^{13}C$ values (Mekota et al., 2006). In the current study, a general pattern of $\delta^{13}C$ enrichment and $\delta^{15}N$ enrichment was noted in underweight individuals diagnosed with DISH, as well as those with normal-weight and overweight BMI, but not in the obese BMI category. Measured $\delta^{13}C$ values between underweight and overweight individuals diagnosed with DISH showed a marked difference, with those in the underweight group ($\text{underweight}^{\text{Rib+Femur}}$) being, on average enriched in $\delta^{13}C$ (-12.3‰) relative to the $\delta^{13}C$ values of the overweight ($\text{overweight}^{\text{Rib+Femur}}$) individuals (-14.2‰). Considering this, our results do not conform to the expected pattern representing periods of starvation as seen in the abovementioned study by Mekota and colleagues. However, a factor to take into consideration is that the studies identifying periods of severe nutritional stress through stable carbon and nitrogen isotope analysis focus on samples taken from tissues such as hair that grows relatively fast and (if sampled from the root) can reflect dietary changes within days (D'Ortenzio et al., 2015; Hatch et al., 2006; Mekota et al., 2006). Bone was the primary bodily tissue sampled for isotope analysis in this study on South Africans and the dietary signals ($\delta^{13}C$ and $\delta^{15}N$) in bone, unlike hair, is not incorporated in a timely sequential way. No information is available on how long a period of starvation would have to be in order for it to be identified in the isotopic analysis of bone, or indeed if it can be detected at all. Whether malnutrition through starvation can be identified through the resolution of data provided by isotope analysis from bone or not, the interpretation of the results from this study thus far indicate that while general malnutrition

is a possibility, it is difficult to ascertain to what extent this had an effect on the individuals' isotopic values.

Alcoholism can contribute to malnutrition and has been shown to affect a large proportion of the South African population (Olivier et al., 2016; Popova et al., 2017). Excessive consumption of alcohol can result in a loss of skeletal muscle protein and can change protein metabolism in the body (Bunout, 1999; Preedy et al., 1999; Hong-Brown et al., 2001). Alcohol misuse (an excess of) also causes an increase in nitrogen excretion (Bunout, 1999; Preedy et al., 1999). The manifestations of alcoholism in human bone may be similar and cannot be excluded from contributing to the high $\delta^{15}N$ observed in this study. With chronic alcohol consumption, the $\delta^{15}N$ would be more enriched relative to the diet as more ^{14}N would be excreted from the bodily tissues. A possible interpretation of alcoholism could be made from the results of this study which showed elevated $\delta^{15}N$ levels within the population, coupled with the documentary evidence indicating high rates of alcoholism among all groups in South Africa (Olivier et al., 2016). However, while alcohol abuse may have played a role in a subset of the population, it is unlikely to have been the main cause of our isotopic results. Currently no definitive evidence exists for ascertaining alcohol abuse from human skeletal remains, making it difficult to evaluate among the South Africans in this study.

Disease is another factor that can influence $\delta^{15}N$ values (Katzenberg and Lovell, 1999; Katzenberg, 2008; Olsen et al., 2014). In a study analysing the stable isotope chemistry of pathological and non-pathological bones across multiple individuals, variation in $\delta^{15}N$ values was observed among those with pathological changes to their bones (Katzenberg and Lovell, 1999). The individuals that had been sampled included pathological bones that had evidence for post-paralytic atrophy, a healing fracture, active periostitis and healing osteomyelitis, respectively. In all cases, it was stated that "in response to injury or disease, newly formed tissues for repair may have as their source either ingested nitrogen from dietary protein or recycled nitrogen derived from the breakdown of existing proteins in the body" (Katzenberg and Lovell, 1999, page 321). The pathological individuals representing periostitis (active at the time of death) showed very little variation in $\delta^{15}N$ values between the non-pathological and pathological samples, with a difference of 0.1‰ between the pathological (11.0‰) and non-pathological bones (10.9‰), which was within the normal instrumental variation recorded by the authors as 0.2‰. However, the pathological samples taken from a bone that had atrophied (6.6‰, 7.2‰ and 6.9‰) showed significant depletion in the $\delta^{15}N$ values when it was compared to the lowest $\delta^{15}N$ value among non-pathological bone (8.9‰). The sample taken from the

callus of a healing fracture (8.9‰) showed a depletion in $\delta^{15}N$ when compared to the two non-pathological samples (9.7 ‰ and 9.8‰). Finally, the sample taken from the bone displaying osteomyelitis (12.9‰) showed the greatest variation in $\delta^{15}N$ values, when compared to the non-pathological bone (11.3‰) (Katzenberg and Lovell, 1999). From the results of Katzenberg and Lovell's study, one may hypothesise that DISH could reflect similar isotopic variations represented by the individuals with periostitis or a fracture, as it is characterised by new bone formation. Leading on from Katzenberg and Lovell's study (1999), an analysis of stable carbon and nitrogen isotope ratios to investigate DISH was undertaken (Quintelier et al., 2014). In the investigation between DISH and stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$), bones with visual manifestations of the disease (vertebrae) and bones without (ribs) were sampled (from the same individual) to investigate the influence the disease may have on the isotope ratios (Quintelier et al., 2014). As no difference in the $\delta^{13}C$ or $\delta^{15}N$ values between pathological and non-pathological samples was found, the authors concluded that DISH contributes little to no physiological factor for variation in isotope ratios for the sampled individuals (Quintelier et al., 2014). Among the skeletons analysed, from both the control and DISH groups, the disease burden is high, evaluated from the "cause of death" information associated with the individuals at the time of donation into the collections. However, none of the samples taken from the South African individuals had evidence of pathological lesions (associated with DISH or otherwise), active or healed.

Climate has been noted to have an effect on measured $\delta^{13}C$ and $\delta^{15}N$ values by affecting the base of the food chain, namely the $\delta^{15}N$ in soil and $\delta^{13}C$ in plants (Dotsika and Diamantopoulos, 2019). One study found that reduced precipitation caused $\delta^{15}N$ enrichment when measured in bone collagen (Heaton et al., 1986). Similar studies have linked the same effect to dehydration. Animals who go through periods of dehydration or drought (linked to reduced precipitation), are found to have bodily tissues that are enriched in $\delta^{15}N$ as ^{14}N is preferentially excreted through urea (Ambrose, 1991). While these findings have been primarily through animal studies, it has been theorised that dehydration would produce similar effects in humans – elevated $\delta^{15}N$ values (White and Armelagos, 1997). Droughts have affected Southern African countries for decades, mostly affecting communities of low SES. Severe dehydration would most likely affect those living in arid rural environments, where access to infrastructure such as running water is limited or none existent. However, when entered into the skeletal collections, most of the individuals would have been living in an urban environment, perhaps making long periods of dehydration enough to affect isotopic values of

bone collagen unlikely. Climate is certainly a factor that affects all people across the spectrum and is known to have an effect on the nitrogen isotope values, but for the individuals in this study, it seems unlikely to be the cause of variation.

In conclusion, even if South Africans may have experienced malnutrition, periods of starvation or dehydration, disease or alcohol misuse, it seems that these are probably unrelated to DISH and its possible effect on isotope values. We are likely seeing a much more complicated picture than the simple interpretation of elevated $\delta^{15}N$ values (taken to represent a diet rich in animal protein) being linked to DISH. It is possible that other factors among South Africans (such as those mentioned above), may be masking dietary signals associated with DISH. However, this study of diet, taking into account a variety of factors that may affect isotope values, has painted a complicated picture of those individuals diagnosed with DISH, that cannot be fully answered under the scope of this research. While there is a general trend for elevated $\delta^{15}N$ values across the South African cohort, there is no clear evidence for any dietary signals specific to individuals diagnosed with DISH. Indeed, the $\delta^{13}C$ and $\delta^{15}N$ values within the DISH group suggests a large amount of dietary diversity, and required further discussion.

5.5.2. DISH and general dietary trends

While no differences were found in the isotope values of individuals with DISH and those without, some general statements can be made about the dietary patterns of the South Africans studied. Before doing so, it may be pertinent to reiterate that while foods consumed can be compared between groups using stable isotope chemistry, the main limitation is that the quantity of foods eaten cannot be estimated. The reported $\delta^{13}C$ and $\delta^{15}N$ values vary considerably among all South African groups, indicating a large amount of dietary diversity.

Two major visual separations were noted in the $\delta^{13}C$ and $\delta^{15}N$ values. Firstly, in a large number of individuals a generally positive correlation between $\delta^{15}N$ enrichment and $\delta^{13}C$ enrichment was noted, while another group of individuals are defined by $\delta^{13}C$ enrichment with relatively depleted nitrogen ratios (see Figure 4.7). Some broad interpretations from the group with $\delta^{13}C$ and $\delta^{15}N$ enrichment suggest the consumption of foods from a higher trophic level such as animal protein and marine fish. Individuals that conform to a pattern of enriched $\delta^{13}C$ and depleted $\delta^{15}N$, indicate a diet predominantly based on the consumption of C_4 foods, for example, corn, maize, sugarcane, millet and sorghum. Both patterns presented across the sexes

but was differentiated among populations. For example, the majority of black South Africans were enriched in $\delta^{13}C$, but relatively depleted in $\delta^{15}N$, while the white and coloured South Africans were enriched in both $\delta^{15}N$ and $\delta^{13}C$.

The recognised carbon isotopic signatures range from between -9‰ and -14‰ for C_4 plants, and range from between -20‰ to -35‰ for C_3 plants (Ballentine et al., 1998; Hoefs, 2015; Katzenberg, 2008). If we consider the approximate +5‰ shift in $\delta^{13}C$ values from diet to collagen, the majority of individuals showed $\delta^{13}C$ values indicating a mixture of both C_4 and C_3 contributions to their diets, with individuals more depleted (more negative) in $\delta^{13}C$ being more likely to have had a C_3 based diet, and those individuals more enriched (more positive) in $\delta^{13}C$ being more likely to have had a predominantly C_4 based diet - whether this be from plant based foods or from animals subsisting on C_3, C_4 or a mixed diet. Examples of C_3 plants include rice, wheat, rye, barley, peanuts, potatoes, soybean and most vegetables, while examples of C_4 plants include maize, sorghum, millet, sugar cane and other tropical grasses (Smith and Epstein, 1971; Bender et al., 1981; Katzenberg, 2008; Reitsema, 2012).

The isotopic analysis indicated a large variation in diet among populations in this study. Generally speaking, $\delta^{13}C$ enrichment indicates predominantly C_4 based diets, while $\delta^{13}C$ depletion is seen in terrestrial diets dominated by C_3 plants (Carter et al., 2019). The results from the study indicates that the coloured and white South Africans had predominantly C_3 based diets, while the data provided by the black South Africans indicates a diet based in C_4 foods. The types of food traditionally eaten by South Africans stated in the literature, namely maize, lentils, pap and other cereals/grains, is consistent with the reported $\delta^{13}C$ values - C_4 based foods. This could indicate a strong reliance on these types of foods among black South Africans. However, while enriched $\delta^{13}C$ values are considered to be consistent with diets based in C_4 based foods, when seen with elevated $\delta^{13}N$ values, it can also be an indication of consumption from a marine environment (Carter et al., 2019).

An enrichment in $\delta^{13}N$ values may indicate subsistence within a marine environment. In similar studies, the subsistence of foods from a marine environment has been reported as a positive correlation between $\delta^{15}N$ and $\delta^{13}C$ values (Müldner and Richards, 2007). As mentioned, the $\delta^{15}N$ values of all the individuals within the current study are high, ranging from 9.20‰ to 17.49‰. The $\delta^{15}N$ values from mammals subsisting in a terrestrial environment range from between 1.9‰ and 10.0‰, whereas secondary carnivores (whales, dolphins and porpoises) in a marine environment have $\delta^{15}N$ values ranging from between approximately

11.7‰ and 17.8‰ (Schoeninger and DeNiro, 1984). Humans who gain their dietary protein from a marine environment have been reported to display $\delta^{15}N$ values ranging from approximately 12.0‰ to 22.0‰, while those subsisting on terrestrial protein sources can display ranges from 5.0‰ to 12.0‰ (Schoeninger and DeNiro, 1984; Lubell et al., 1994; Dotsika and Diamantopoulos, 2019). The positive correlation of the $\delta^{13}C$ and $\delta^{15}N$ values seen in the majority of white and coloured South Africans may indicate that the consumption of foods from a marine environment is a possibility. For the individuals from the KC, having access to marine based foods is likely, as individuals within the collections are assumed to come from the immediate geographical region, which is coastal. However, it is more difficult to assess the accessibility of marine produce for individuals from the Pretoria and Johannesburg region (PBC and RDC). There is little documentary evidence on the consumption of fish in South Africa and it does not compare to the documentary evidence on the consumption of meat.

Documentary evidence on the presumed general dietary habits of South Africans, broadly corroborates the isotopic results, and provides additional information on sources of protein. In a study analysing the dietary diversity of adults (16 years and older) in South Africa, white individuals were shown to have more diverse diets than other ancestral groups studied (black, mixed ancestry and Indian) (Labadarios et al., 2011). Another study which looked at calcium (Ca) intake among other micronutrients (sodium - Na and Potassium - K) across black and white South Africans, and South Africans of mixed (not specified) ancestry (all adult), showed that white South Africans had a higher mean dietary intake of Ca when compared to black South Africans or those of mixed ancestry (Charlton et al., 2005). This source of Ca was shown to mainly come from foodstuffs such as full cream milk and cheddar cheese (Charlton et al., 2005). As the source of protein from the same animal (*e.g.* meat versus dairy) cannot be distinguished from the $\delta^{15}N$ ratios, it is possible (as indicated in the documentary evidence above), that some of the protein came from dairy sources rather than directly from meat.

Since the industrial revolution, the burning of fossil fuels has markedly changed atmospheric $\delta^{13}C$ values, giving rise to differences in $\delta^{13}C$ values between human bone analysed from archaeological and modern populations (Sealy and van der Merwe 1991; Hedges et al., 2007). Additionally, previous studies on the investigation of DISH using stable isotope analysis have all been from Europe, particularly England. The $\delta^{13}C$ values presented in this study are not directly comparable to European archaeological research, primarily because of the variation in $\delta^{13}C$ values (Müldner and Richards, 2007; Spencer, 2008; Quintelier et al., 2014). Although $\delta^{15}N$ values were the main focus of this research, giving direct evidence for

the consumption of animal protein, the $\delta^{13}C$ data aids in an overall dietary analysis, and therefore, cannot be used directly. However, there is no evidence in the literature to suggest a marked change in $\delta^{15}N$ values over time, which suggests this data can still be compared.

Table 5.1 provides a summary of the $\delta^{15}N$ values for individuals diagnosed with DISH from four studies, including the current study. It is clear from the data presented (Table 5.1) that reported values of $\delta^{15}N$ from the South African cohort are consistent with skeletal remains diagnosed with DISH from the other archaeological sites in Europe. These studies include the Gilbertine priory at the Church of St. Andrew, Fishergate, UK, across eight late Medieval archaeological sites in England, and the post-Medieval (16^{th} to 18^{th} century AD) Carmelite Friary burial grounds at Aalst, Belgium (Müldner and Richards, 2007; Spencer, 2008; Quintelier et al., 2014). Overall, the researchers interpreted $\delta^{15}N$ values among the DISH individuals from archaeological sites to suggest "a diet rich in animal protein, which included a significant proportion of marine foods" (Müldner and Richards, 2007: pg 169). In many of these studies, obesity was often related to high $\delta^{15}N$ values and DISH, making it seem to be a disease of the upper class. Some elements of the dietary interpretations made from the studies above can be applied to the DISH group in this study, such as there being evidence for animal protein consumption and the subsistence from a marine environment, although this seems unlikely for the majority of the individuals in the study. However, the elevated $\delta^{15}N$ values from the individuals in this study, indicating the consumption of marine and terrestrial protein is not exclusive to the individuals diagnosed with DISH.

It has already been discussed that the high values of $\delta^{15}N$, as seen in non-DISH and DISH individuals in the South African cohort, may be linked to undernutrition, starvation and alcoholism, and all of this was not associated to obesity. The link of dietary provisions to possible social status and health, or poor health in this case, is a complex situation, particularly among persons who lived within a previously unjust socio-political system (*apartheid*) and had experienced abuse of manual labour in the mines (mostly black South Africans). Overweight South Africans were generally more enriched in $\delta^{15}N$ and depleted in $\delta^{13}C$, while the underweight were more enriched in $\delta^{13}C$ and depleted in $\delta^{15}N$, relatively speaking. This demonstrates that while broadly speaking the dietary differences can be linked to cultural and religious differences, it is very likely that other economic and social factors played a role in dietary choice, such as salary and underlying health conditions.

This study highlights that diet may play a role in the development of DISH, but that socio-cultural and socio-political circumstances of a country are paramount for interpretation of the diet of its previous inhabitants. Dietary differences are likely linked to economics, religion, culture and access to food stuffs. Variation between and among population groups for $\delta^{13}C$ and $\delta^{15}N$ values likely represents acquisition of dietary resources due to cultural and economic differences, rather than a direct association with DISH. Further investigation into the effects of disease on stable isotopes are needed so as to better address complex questions on the association between diet and disease. Throughout this study, DISH has been found among all South African groups and has not been correlated with socio-economic status, diet or obesity. This study therefore lends no support to the theory that the presence of DISH is alone, associated with a diet high in protein.

5.6. The pathogenesis of DISH

Most studies have described DISH as a non-inflammatory disease, with the assumption of ossification of the ALL with no damage to the underlying bone (Smythe and Littlejohn, 1998; Ortner, 2003; Vigorita, 2008). However, a few recent studies (Yaniv et al., 2014; Mader et al., 2015; Arad et al., 2017) implied that a local inflammatory process predates enthesophyte formation at the extra-spinal locations associated with DISH and that a local inflammatory process could be associated with the new bone growth in the spine (Yaniv et al., 2014; Arad et al., 2017). This study supports these findings.

As seen on the micro-CT scans, many individuals had a possible local inflammatory process on their bones where the cortex of the vertebral wall was eroded and destroyed while a porous structure formed and became continuous with the trabeculae of the vertebral bodies. Retention of the cortex did not seem to correlate with (what could be considered) earlier stages of DISH as most of the individuals possessed clear macroscopic manifestations of DISH, such as flowing new bone growth across contiguous vertebrae, which has been noted to develop over approximately 10 years (Yaniv et al., 2014). A possibility for the suggested inflammatory response in most, but not all of the vertebral columns assessed, could be as a response to trauma.

Spinal manifestations associated with DISH can cause ankylosis of the spine, leading to a susceptibility of fractures in the vertebral column (Yaniv et al., 2014; Mader et al., 2017). The new bone formation observed on the vertebral columns in this study could have, in part been a response to trauma, which would have reinforced the structural integrity of the vertebrae,

resulting from inflammation at the site. Inflammation is the body's immune response to tissue damage from a variety of causes, including trauma (Weston, 2012). In a study evaluating the relation between DISH (specifically men) and vertebral fractures, the results indicated that vertebral fractures were more frequent in individuals with DISH than those without (Diederichs et al., 2011). Individuals who had both (exuberant) new bone formation without retention of the vertebral wall/cortex had undergone structural change after an inflammatory response to repeated trauma. It is understood that DISH is a systemic disease, while trauma is very often localised, which would lead to the interpretation that while DISH is largely a non-inflammatory disease, it may have inflammatory elements due to fragility of bony structures.

The implications of an inflammatory response in the development of DISH may aid in our understanding of the mechanisms behind the pathogenesis of DISH and further our understanding of the perceived spinal characteristics of DISH, *i.e.* whether some of the structural changes are caused by trauma secondary to DISH, in addition to the initial lesions, rather than being a primary result of the disease. However, one must be careful as there are many causes of inflammation beyond trauma, and there are many inflammatory diseases that affect the joints and the skeletal system, which are not secondary to trauma. While trauma may play a role, DISH in itself may be an inflammatory condition, such as what is seen in auto-immune diseases.

5.7. Future direction in the investigation of DISH

The most promising investigatory avenue to aid in our understanding of DISH would (in the author's opinion) be to undertake a longitudinal study of individuals with DISH. The aim of the longitudinal study would be to observe the natural progression of the spinal manifestations of DISH using medical imaging modalities such as radiography, CT scanning and MRI to assess the progression occurring in the development of the spinal and extra-spinal manifestations of DISH. There would of course be limitations to such a study as it would be needed on living patients, and there are ethical and financial constraints. The analysis of histological appearance along with bone density analysis of the vertebrae of individuals diagnosed with DISH as well as cases of individuals suffering from vertebral osteophytes would be useful but would of course only be possible in cadaveric cases. A wealth of this kind of data would aid in our understanding of the mechanisms behind the pathogenesis of DISH, and may lend a hand into the investigation of the aetiology of DISH, and its prevention in patients who would be identified to be at risk of developing the disease.

6. CONCLUSIONS

The possible contributing factors and pathogenesis of DISH have been studied through the analysis of stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) and micro-CT scanning of affected spinal columns in modern human skeletons, meeting the aims and objectives, as set out at the beginning of the research. In conclusion, it was found that:

- A significant difference in $\delta^{13}C$ and $\delta^{15}N$ values between black South Africans and their white and coloured South African counterparts were found in both the DISH and non-DISH groups, suggesting that a dietary difference based on cultural and economic constraints is detectable, but unlikely to be related to DISH.
- A significant difference in $\delta^{13}C$ and $\delta^{15}N$ values was found between the overweight and underweight individuals diagnosed with DISH. This presented a more complex pattern of dietary differences based on cultural and economic constraints. The variation in stable isotope values among South African groups was seen as dietary and unrelated to DISH. However, the possibility that a disease burden, such as malnutrition, starvation, and alcohol abuse, present in the population is masking any dietary signals indicative of DISH between the groups cannot be excluded.
- A significant difference in $\delta^{15}N$ values was found in the DISH group between individuals in the PBC and RDC, again indicating differing dietary habits constrained by cultural and economic factors.
- DISH may have an inflammatory component preceding new bone formation, which corroborates some of the clinical research within the literature.

Overall, the data from this study presents a complex picture of DISH among South Africans, with no clear evidence for individuals diagnosed with DISH being linked to a diet enriched in animal protein exclusively, even though these individuals did present with generally high $\delta^{15}N$ values. The results from the micro-CT analysis presented the possibility of an inflammatory component preceding new bone formation, which is an exciting new development which needs further investigation and corroboration. This study is a small step in the direction of trying to understand the relationship between diet and DISH and the pathogenesis of the spinal manifestation of DISH. While there is nothing definitive that can be declared from the results, some intriguing discoveries have been made about DISH, and it should provide fuel for future research.

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APPENDIX 1

Table 8.1. Data collection sheet, with example individual from the Pretoria Bone Collection, University of Pretoria.

	Box Number	1066				
	Skeleton Number	6906				
	R&W DISH Classification	2				
	Sex	male				
	Age	77				
	Ancestry	white				
		RL	**RA**	**C**	**LA**	**LL**
SPINAL	**Atlas**					
	Axis					
	C3					
	C4					
	C5					
	C6					
	C7					
	T1					
	T2	∨2 ^1	∨2 ^1	∨2		
	T3	∨2 ^2	∨2 ^2	∨2 ^2		
	T4	∨2 ^2	∨2 ^2	∨2 ^2		
	T5	∨2 ^2	∨2 ^2	∨2 ^2		
	T6	∨2 ^2	∨2 ^2	∨2 ^2		
	T7	∨2 ^2	∨2 ^2	∨2 ^2		
	T8	∨2 ^2	∨2 ^2	∨2 ^2		
	T9	∨1 ^2	∨1 ^2	∨1 ^2		
	T10					
	T11					
	T12					
	L1					
	L2					
	L3					
	L4					
	L5					
	Flowing ossification	1				
	Intervertebral disc space retained	1				
	Apophyseal facets retained	1				
		R			**L**	
EX TRA-SPINAL	***Sacro-iliac joint***	1			1	
	Olecranon tufting - *m. triceps brachii*	0			0	

	Patellar tufting - *m. quadriceps femoris*	0	0
	Heel spurring - *m. triceps surae*	1	0
	Spurring on tibial tuberosities	0	0
	Ossification of *ligamentum flavum*	0	
	Plantar heel spurring	1	1
	Ossification of *apical ligament*	0	
	Ossification of *Supraspinous ligament*	1	

Table 8.2. Key for table 8.1.

Abbreviations for documenting spinal manifestation	
∧1	incomplete/no ankylosis with vertebra above
∨1	incomplete/no ankylosis with vertebra below
∧2	bony ankylosis with vertebra above
∨2	bony ankylosis with vertebra below
Abbreviations for documenting extra spinal manifestation	
0	absent / no
1	present / yes
R&W (Rogers and Waldron) DISH classification	
0	no DISH (suitable for control group)
1	probable DISH
2	definite DISH
Additional abbreviations	
R	right
L	left
RL	right lateral
RA	right anterior
C	centre/anterior
LA	left anterior
LL	left lateral
PM	post mortem damage
EP	element missing post mortem

APPENDIX 2

Table 9.1. Intra- and inter-observer agreement results for the veterbal observations.

Vertebral unit observed	intra-observer (κ)	p-value	inter-observer (κ)	p-value
C3_right	1.000	0.000	-0.053	0.632
C3_centre	0.649	0.000	0.341	0.042
C4_right	0.649	0.000	0.259	0.104
C4_centre	0.643	0.002	0.630	0.000
C7_centre	-0.053	0.747	0.780	0.000
T1_right	0.459	0.015	0.483	0.028
T1_centre	0.444	0.047	0.231	0.264
T2_right	0.484	0.016	0.771	0.000
T2_centre	0.238	0.209	0.160	0.413
T3_right	0.540	0.002	0.670	0.000
T3_centre	0.467	0.004	0.242	0.013
T4_right	0.727	0.000	0.426	0.009
T4_centre	0.310	0.002	0.286	0.064
T5_right	1.000	0.000	0.624	0.000
T5_centre	0.467	0.004	0.348	0.019
T7_right	0.917	0.000	0.911	0.000
T7_left	1.000	0.000	0.474	0.000
T8_right	0.813	0.000	0.724	0.000
T8_left	1.000	0.000	0.835	0.000
T9_right	0.780	0.000	1.000	0.000
T9_left	1.000	0.000	0.286	0.025
T10_right	0.767	0.000	0.527	0.005
T10_centre	0.459	0.015	0.273	0.076
T11_right	0.916	0.000	0.916	0.000
T11_centre	0.773	0.000	0.615	0.003
T12_right	0.924	0.000	0.692	0.000
T12_centre	-0.053	0.747	-0.067	0.680
L1_right	0.749	0.000	0.685	0.000

L1_left	0.610	0.000	0.735	0.000
L2_right	0.805	0.000	0.658	0.000
L2_centre	0.341	0.042	0.238	0.209
L2_left	0.548	0.005	0.528	0.007
L3_right	0.500	0.010	0.529	0.015
L3_centre	0.459	0.015	0.385	0.071
L3_left	0.178	0.162	0.223	0.279
L4_right	0.255	0.158	0.394	0.019
L4_centre	0.643	0.002	0.167	0.389
L4_left	0.273	0.076	0.368	0.069
L5_right	0.219	0.061	0.328	0.026
L5_centre	0.643	0.002	0.115	0.308
L5_left	0.273	0.037	0.468	0.004

Table 9.2. Intra- and inter-observer agreement results for the extra-spinal observations.

Extra-spinal locations and side	intra-observer (κ)	p-value	inter-observer (κ)	p-value
Olecranon tufting_right	1.000	0.000	0.615	0.003
Olecranon tufting_left	0.886	0.000	0.417	0.043
Patellar tufting_right	0.889	0.000	0.881	0.000
Patellar tufting_left	0.881	0.000	0.857	0.001
Plantar heel spurring_right	0.689	0.003	0.092	0.682
Plantar heel spurring_left	1.000	0.000	0.198	0.160
Heel spurring_right	0.894	0.000	0.320	0.072
Heel spurring_left	0.898	0.000	0.556	0.009
Ligamentum flavum	0.200	0.628	0.352	0.089
Spurring on tibial tuberosities_right	0.857	0.001	0.579	0.004
Spurring on tibial tuberosities_left	1.000	0.000	0.565	0.010
Sacro-iliac joint_right	0.900	0.000	0.895	0.000
Sacro-iliac joint_left	0.798	0.001	0.787	0.000
Supraspinous ligament	0.044	1.000	0.026	0.608
Posterior iliac crest_right	0.500	0.033	1.000	0.000
Posterior iliac crest_left	0.400	0.087	1.000	0.000
Soleal line_right	0.348	0.161	1.000	0.000
Soleal line_left	0.205	0.613	1.000	0.000

APPENDIX 3

Table 10.1. The biological profile of individuals diagnosed with DISH. U = underweight, N = normal-weight, Ov = overweight, Ob = obese, N/S = not specified, PD = possible DISH, DD = definite DISH.

Collection	Box No_Sk No	Sex	Age	Ancestry	BMI category	DISH Diagnosis
The Pretoria Bone Collection, University of Pretoria (N=62)	PBC 1026_6024	F	65	B	U	PD
	PBC 1049_5682	F	78	W	Ob	PD
	PBC 1064_7018	M	80	W	U	PD
	PBC 1066_6906	M	77	W	N	DD
	PBC 1070_6238	M	80	W	Ov	DD
	PBC 1078_4006	M	94	W	N/S	DD
	PBC 1101_5908	M	69	W	Ob	DD
	PBC 1117_5144	M	60	B	U	PD
	PBC 1119_5561	M	62	B	Ob	DD
	PBC 1127_4611	F	84	W	N/S	PD
	PBC 1137_5333	M	60	B	N	DD
	PBC 1138_6446	M	83	W	N/S	PD
	PBC 1149_5684	M	80	W	Ob	DD
	PBC 1169_5864	M	72	W	Ov	DD
	PBC 1171_5209	M	60	B	U	PD
	PBC 1186_6435	M	85	W	N	DD
	PBC 1214_4953	M	64	W	U	PD
	PBC 1217_5474	M	59	B	N	PD
	PBC 1218_6437	M	78	W	Ov	DD
	PBC 1220_6356	M	60	W	N	PD
	PBC 1241_6472	M	70	W	N/S	DD
	PBC 1242_5978	M	72	W	Ob	PD
	PBC 1243_6341	M	89	W	U	DD
	PBC 1276_5174	M	76	B	N	PD
	PBC 1301_5304	M	90	W	U	DD
	PBC 1317_6484	M	72	W	N/S	PD
	PBC 1324_5499	F	64	W	Ob	PD
	PBC 1335_5346	M	75	B	N	DD
	PBC 1358_5078	M	60	B	Ob	DD
	PBC 1377_4601	M	62	W	N/S	DD
	PBC 1394_6380	M	71	W	N	DD
	PBC 1414_6406	M	69	W	Ov	DD
	PBC 1417_6497	M	66	W	N/S	PD
	PBC 1420_5145	M	75	B	U	DD
	PBC 1439_5287	M	65	B	U	DD
	PBC 1464_5665	M	60	B	Ob	DD
	PBC 1526_6407	M	71	W	Ov	PD
	PBC 1533_5814	M	72	W	N/S	PD

	PBC 1537_2053	M	70	B	N/S	DD
	PBC 1546_6386	M	72	W	N	PD
	PBC 1567_6248	M	49	W	Ov	PD
	PBC 1569_6295	F	85	W	U	PD
	PBC 1594_6320	F	86	W	N	DD
	PBC 1600_3676	M	58	B	N/S	PD
	PBC 1609_4969	M	64	B	Ob	DD
	PBC 1616_6313	M	91	W	N	DD
	PBC 1690_7008	M	53	W	Ov	PD
	PBC 1701_6979	M	56	W	N	DD
	PBC 1706_2978	M	60	B	N/S	DD
	PBC 1721_6198	M	71	W	N	DD
	PBC 1743_6241	M	87	W	N	DD
	PBC 1735_6104	M	75	B	Ov	PD
	PBC 1739_6264	M	45	W	N	DD
	PBC 1766_5914	M	61	B	U	DD
	PBC 1774_6904	M	79	W	N	DD
	PBC 1782_6399	F	86	W	Ob	PD
	PBC 1790_6018	M	65	B	N	DD
	PBC 1819_6294	F	70	W	Ob	DD
	PBC 1845_6205	M	84	W	U	PD
	PBC 1891_6987	M	81	W	Ov	DD
	PBC 1897_6489	M	76	W	Ov	DD
The Kirsten Collection, Stellebosch University (N=13)	AN352	F	54	C	N/S	PD
	AN405	M	66	B	N/S	PD
	AN427	M	54	W	N/S	DD
	AN504	M	60	B	N/S	DD
	AN570	M	65	W	N/S	DD
	AN936	F	95	C	N/S	PD
	AN1425	M	83	W	N/S	DD
	AN1428	F	58	W	N/S	PD
	AN1355	M	69	C	N/S	DD
	AN1305	M	72	C	N/S	PD
	AN1256	M	N/S	C	N/S	PD
	AN1259	M	83	W	N/S	DD
	AN261	M	76	C	N/S	DD
The Raymond A Dart Human Skeletal Collection, The University of Witwatersrand (N=53)	A449_AD483	M	74	B	N/S	PD
	A531_AD487	F	45	C	N/S	PD
	A1524_AD2033	M	60	B	N/S	PD
	A1799_AD2445	F	80	B	U	DD
	A1808_AD2430	F	69	C	N	PD
	A1998_AD2654	F	45	B	N	PD
	A2029_AD2653	M	83	W	N	DD
	A2091_AD2612	M	85	W	Ov	DD

	A2182_AD2923	M	60	C	N	PD
	A2197_AD2895	M	74	W	N	PD
	A2286_AD3298	F	69	W	Ov	DD
	A2346_AD3190	M	86	W	Ov	PD
	A2361_AD3188	M	48	W	Ov	DD
	A2397_AD3420	F	75	W	N/S	DD
	A2478_AD3437	M	70	W	Ov	DD
	A2518_AD3449	M	79	W	Ov	PD
	A2607_AD3605	M	80	W	N	DD
	A2722_AD3625	M	78	N/S	N	DD
	A2731_AD3579	M	70	W	U	PD
	A2740_AD3594	M	78	W	N	DD
	A2765_AD3641	M	72	W	U	PD
	A2921_AD4207	M	65	B	U	PD
	A3048_AD4515	F	75	W	N	DD
	A3066_AD4473	F	54	W	N/S	DD
	A3103_AD4583	M	70	B	N	DD
	A3223_AD4938	M	50	N/S	U	PD
	A3325_AD5375	M	80	B	U	DD
	A3408_AD5448	M	65	B	N	PD
	A3517_AD5874	F	75	B	U	DD
	A3522_AD5755	F	82	W	N/S	PD
	A3574_AD6049	M	70	B	U	DD
	A3589_AD6177	M	66	B	U	DD
	A3613_AD6168	F	67	W	U	DD
	A3614_AD6195	F	60	B	N	PD
	A3636_AD6726	M	76	W	N	PD
	A3644_AD6356	M	60	B	U	PD
	A3647_AD6663	F	95	W	N	PD
	A3694_AD6509	M	55	B	U	DD
	A3956_AD7352	M	76	W	N	PD
	A3962_AD7287	M	60	W	U	PD
	A3965_AD7378	F	89	W	N	DD
	A3971_AD7485	F	76	W	N/S	DD
	A3989_AD7634	M	72	W	Ov	DD
	A4006_AD7640	M	65	W	N	DD
	A4017_AD7769	M	83	W	U	DD
	A4061_AD7927	M	72	W	N	DD
	A4091_AD7964	M	66	W	U	DD
	A4298_AD8826	F	63	W	Ov	DD
	A4307_AD8831	F	38	B	N/S	DD
	A4311_AD8723	F	46	W	U	DD
	A4347_AD8755	M	78	W	Ob	DD
	A4359_AD8677	F	57	B	Ob	DD

	A4368_AD9125	M	61	W	U	DD

APPENDIX 4

Of the 1158 human skeletons housed within the PBC, 61 individuals were identified to conform to the criteria for having DISH when analysed by the principal investigator. This is a combination of those diagnosed as definite and probable DISH. Of these 61 individuals diagnosed with DISH, 53 were male and 8 were female. A chi-square test comparing DISH between the sexes showed a statistically significant difference ($p<0.05$), indicating a higher prevalence of DISH in males. Eighteen individuals diagnosed with DISH were black South Africans and 42 were recorded as white South African (Figure 11.1). A chi-square analysis (with Bonferroni adjusted post hoc) showed a statistically significant difference ($p<0.01$) in the prevalence of DISH between black South Africans and white South Africans, with a higher rate of DISH in white South Africans. Figure 11.2 illustrates the age-at-death of individuals diagnosed with DISH in the PBC. A crude prevalence rate was calculated at 5.35 for the PBC.

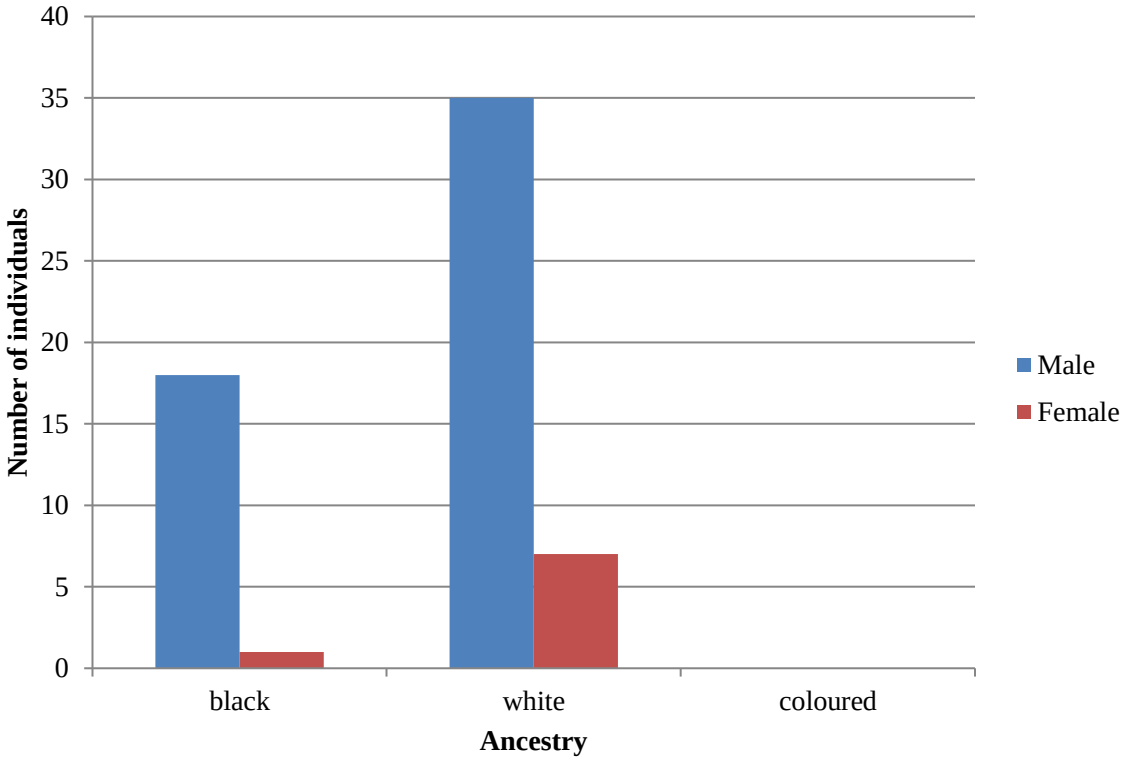

Figure 11.1. Individuals from the Pretoria Bone Collection diagnosed with DISH, separated by sex and ancestry (n=61).

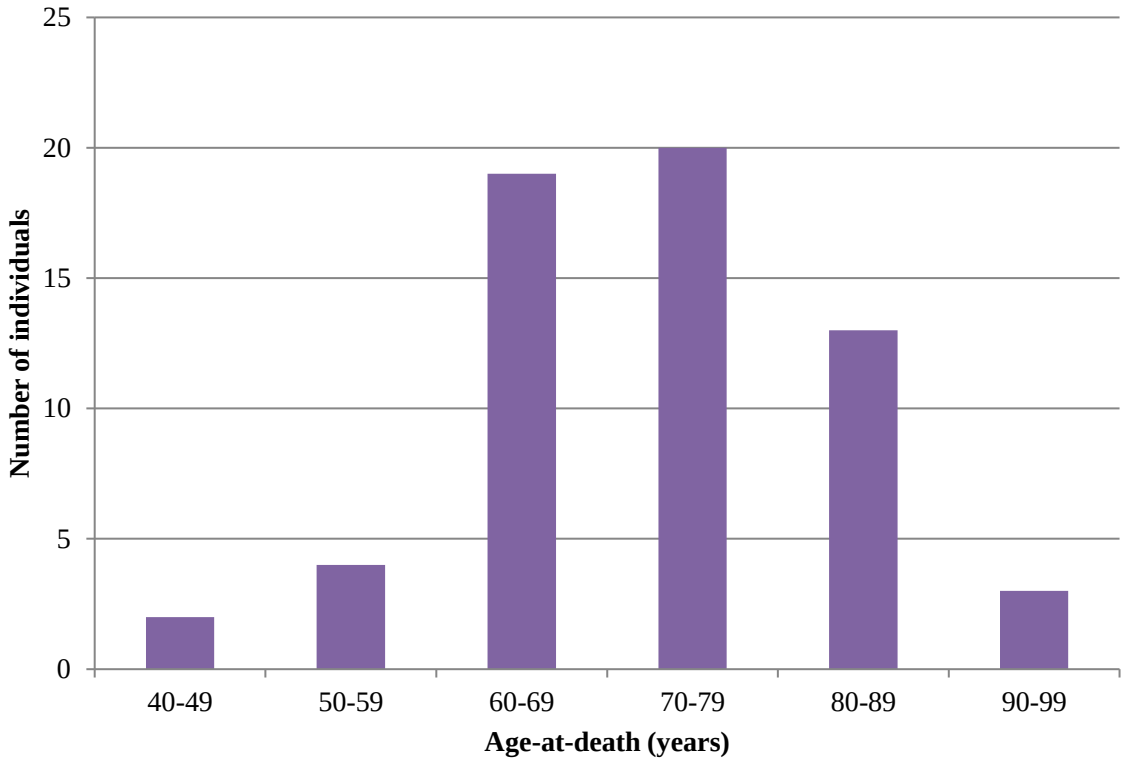

Figure 11.2. Individuals from the Pretoria Bone Collection (males and females) diagnosed with DISH, separated by age-at-death categories (n=61).

When the human remains were analysed at the RDC, 53 individuals were identified to conform to the diagnostic criteria for DISH (definite and probable DISH pooled), from the 1932 analysed. Of these 53 individuals diagnosed with DISH, 34 were recorded as male and 19 were recorded as female. Figure 11.3 illustrates the number of males and females diagnosed with DISH, also separated by ancestry. A chi-square analysis comparing DISH between the sexes showed no statistically significant difference ($p>0.05$) in the rate of DISH between males and females. Among the individuals diagnosed with DISH at the RDC, 16 were black South African, 32 were white South African and 3 were classified as coloured South African (Figure 11.3). A chi-square analysis (with Bonferroni adjusted post hoc) showed a statistically significant difference ($p<0.01$) in the prevalence of DISH between black South Africans and white South Africans, with a higher rate of DISH in white South Africans. This trend was also observed in the PBC. Figure 11.4 illustrates the age-at-death of individuals diagnosed with DISH in the RDC. A crude prevalence rate was calculated at 2.74 for the RDC.

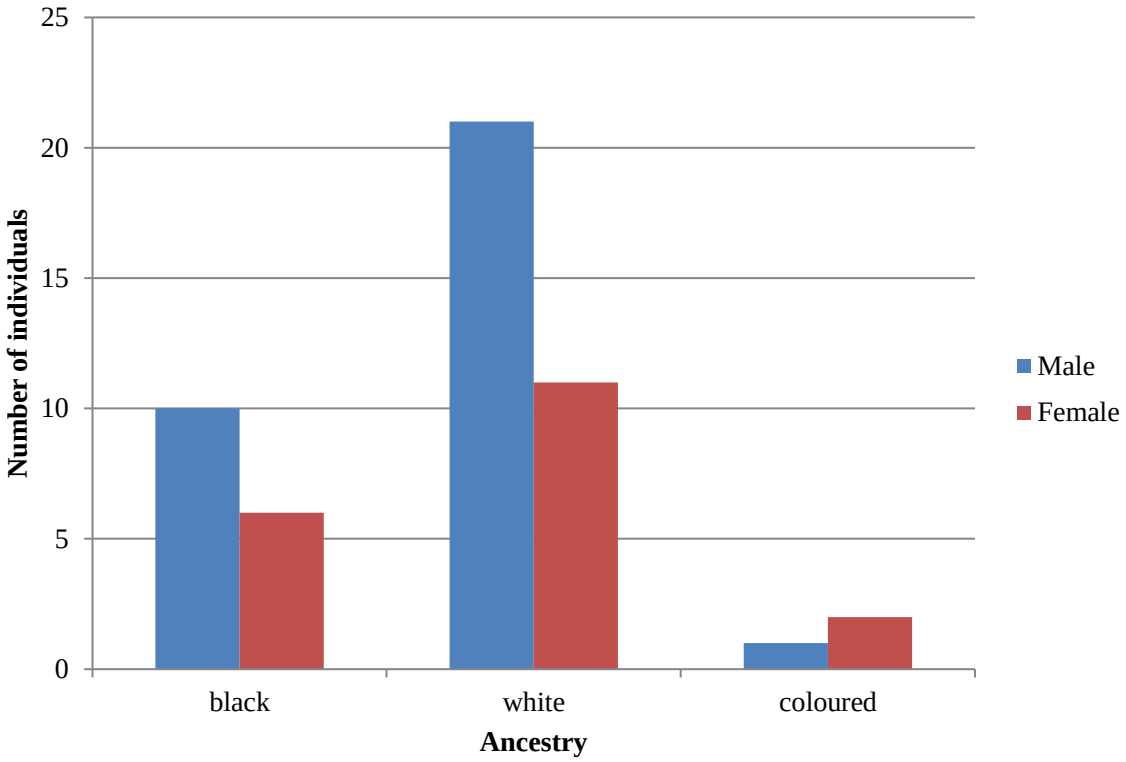

Figure 11.3. Individuals from the Raymond A Dart Collection of Human Skeletons diagnosed with DISH, separated by sex and ancestry (n=53).

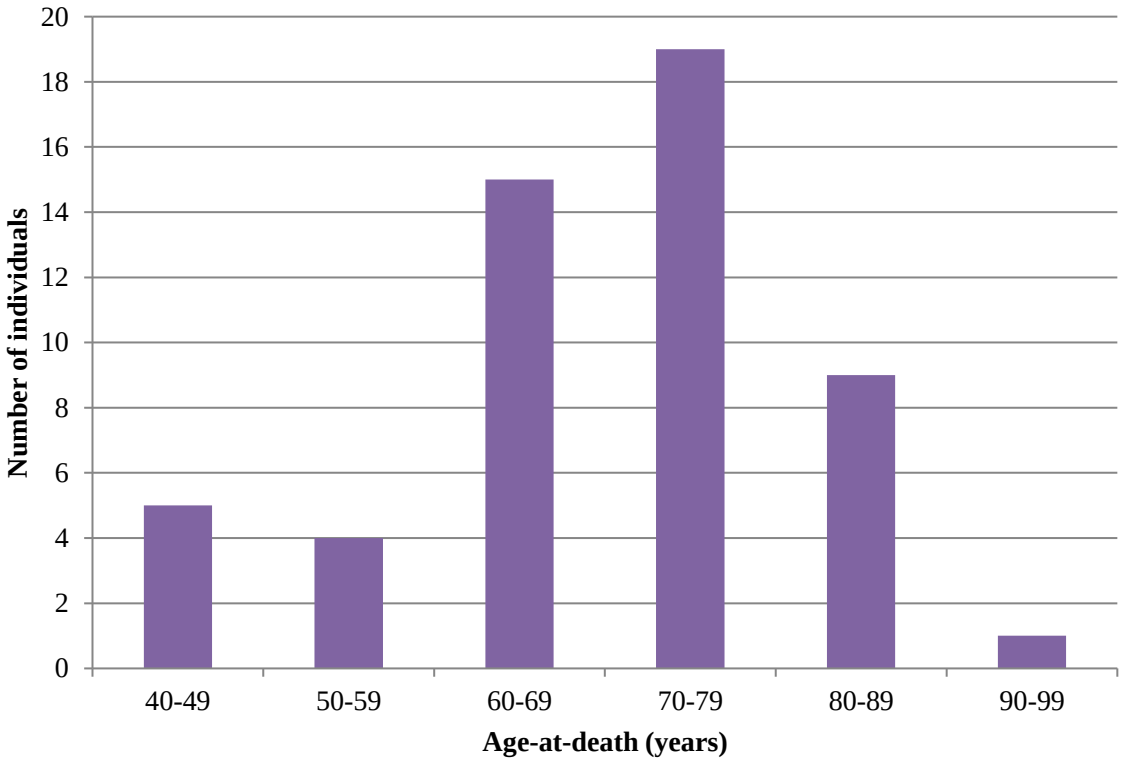

Figure 11.4. Individuals from the Raymond A Dart Collection of Human Skeletons diagnosed with DISH, separated by age-at-death categories (n=52).

Thirteen individuals from the originally analysed 784 individuals housed at the KC were found to have skeletal manifestations of DISH. Of the 13 individuals diagnosed with DISH, 10 were recorded as male and 3 were recorded as female (Figure 11.5). A chi-square analysis showed no statistically significant difference ($p>0.05$) in the rate of DISH between males and females. When separated by ancestry, 2 individuals were recorded as black South African, 5 were recorded as white South African and 6 were recorded as coloured South African (Figure 11.5). Figure 11.6 illustrates the age-at-death of individuals diagnosed with DISH in the PBC. A crude prevalence rate was calculated at 1.65 for the KC.

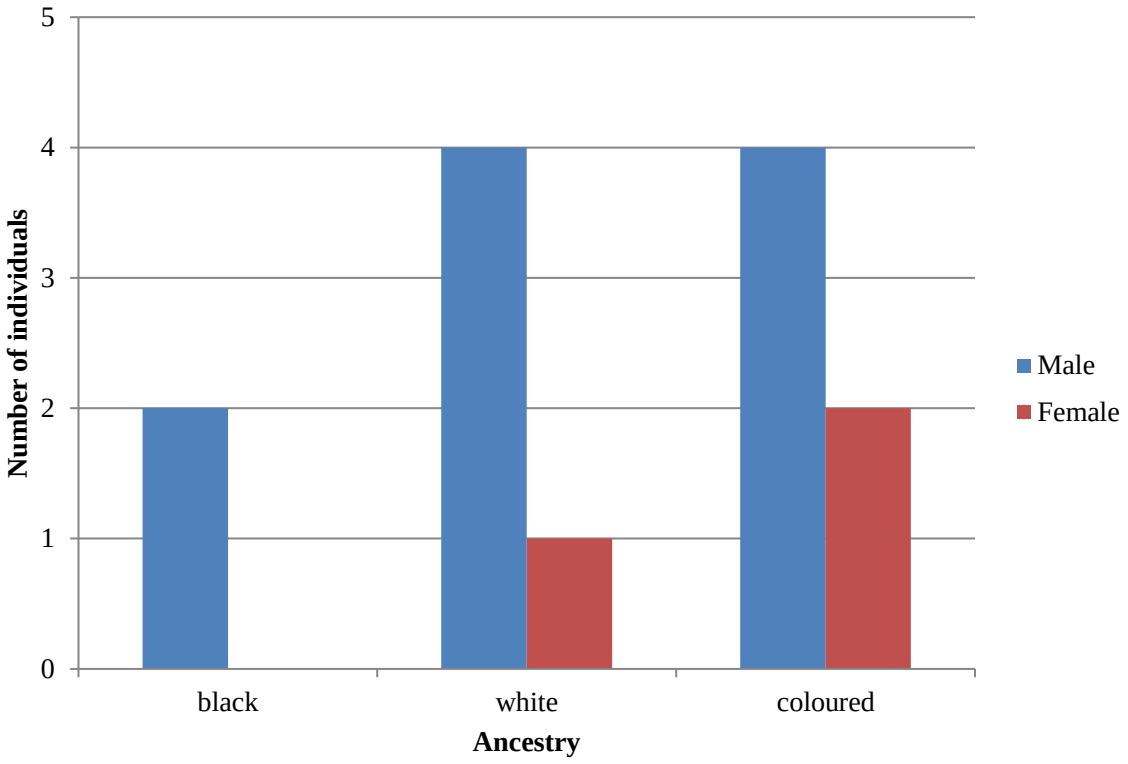

Figure 11.5. Individuals from the Kirsten Collection diagnosed with DISH, separated by sex and ancestry (n=13).

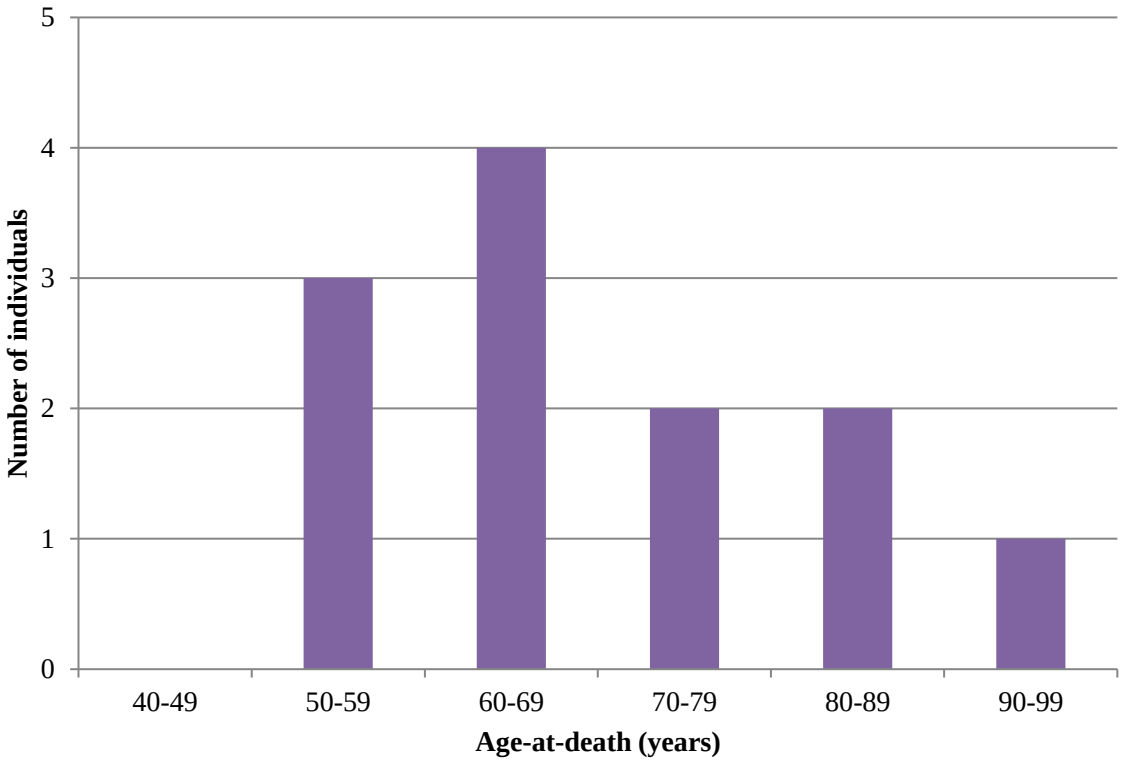

Figure 11.6. Individuals from the Kirsten Collection diagnosed with DISH, separated by age-at-death categories (n=12). One individual's (AN1256) age-at-death information was not specified.

APPENDIX 5

The raw δ- values for both the in-house standards were normalised using a multi-point linear regression method (Nelson, 2000). Two in-house laboratory standards were used to encompass the expected $\delta^{15}N$ and $\delta^{13}C$ values, namely DL-Valine and Merck Gel. These in-house standards are in turn, calibrated against the National Institute of Standards and Technology (NIST) international standards: NIST 1557b (bovine liver), NIST 2976 (mussel tissue) and NIST 1547 (peach leaves). The certified values for both in-house laboratory standards are shown in Table 12.1. As the samples were run in three batches over the course of 2017 (June 2017; November 2017; and December 2017), the three batches of standards run are presented here separately to analyse the validity of each of the data sets. Tables 12.2 and 12.3 give the measured $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) values for the first batch run (June 2017) of DL-Valine and Merck Gel.

Table 12.1. The certified values of $\delta^{13}C$ and $\delta^{15}N$ (‰ V-PDB and AIR) for the inter-laboratory standards.

Standard	Certified $\delta^{13}C$‰ (V-PDB)	SD	Certified $\delta^{15}N$‰ (AIR)	SD
DL-Valine	-10.57	0.1	-6.15	0.1
Merck Gel	-20.26	0.1	7.89	0.1

Table 12.2. The measured values of $\delta^{13}C$ and $\delta^{15}N$ (‰ V-PDB and AIR) for the inter-laboratory standard DL-Valine. First batch run in June 2017.

Standard	Measured $\delta^{13}C$ (‰)	Measured $\delta^{15}N$ (‰)
DL-Valine	-10.50	-6.05
DL-Valine	-10.69	-6.12
DL-Valine	-10.57	-6.28
DL-Valine	-10.52	-6.10
DL-Valine	-10.58	-6.30
DL-Valine	-10.53	-6.13
DL-Valine	-10.54	-6.07
DL-Valine	-10.61	-6.13
DL-Valine	-10.55	-6.22
DL-Valine	-10.61	-6.16
DL-Valine	-10.50	-6.08
DL-Valine	-10.56	-6.13
DL-Valine	-10.65	-6.13
DL-Valine	-10.62	-6.06
DL-Valine	-10.55	-6.22
DL-Valine	-10.53	-6.20
DL-Valine	-10.51	-6.08
DL-Valine	-10.63	-6.18
DL-Valine	-10.65	-6.22
DL-Valine	-10.58	-6.14
DL-Valine	-10.50	-6.16
DL-Valine	-10.53	-6.24
DL-Valine	-10.57	-6.05
DL-Valine	-10.59	-6.09
DL-Valine	-10.57	-6.19
Mean	**-10.57**	**-6.15**
Standard deviation	**0.05**	**0.07**

Table 12.3. The measured values of $\delta^{13}C$ and $\delta^{15}N$ (‰ V-PDB and AIR) for the inter-laboratory standard Merck Gel. First batch run in June 2017.

Standard	Measured $\delta^{13}C$ (‰)	Measured $\delta^{15}N$ (‰)
Merck	-20.35	7.92
Merck	-20.26	7.92
Merck	-20.22	7.91
Merck	-20.20	7.89
Merck	-20.11	7.73
Merck	-20.31	7.79
Merck	-20.24	7.98
Merck	-20.39	7.98
Merck	-20.15	7.89
Merck	-20.41	7.95
Merck	-20.32	7.85
Merck	-20.25	7.98
Merck	-20.15	7.78
Merck	-20.26	7.81
Merck	-20.22	7.89
Merck	-20.33	7.95
Merck	-20.25	7.90
Merck	-20.23	7.89
Merck	-20.36	7.90
Merck	-20.26	7.95
Merck	-20.20	7.83
Merck	-20.28	7.86
Merck	-20.15	7.91
Merck	-20.29	7.84
Merck	-20.30	7.95
Merck	-20.28	7.89
Mean	**-20.26**	**7.89**
Standard deviation	**0.07**	**0.06**

A linear regression was performed on the measured values of $\delta^{13}C$ (‰) against the certified values of $\delta^{13}C$ (‰) for the standards (Table 12.4 & Figure 12.1). The correlation coefficient of the certified and measured values of $\delta^{13}C$ (‰ V-PDB) showed a nearly perfect linear relationship (very close to 1).

Table 12.4. Regression values for the measured $\delta^{13}C$ (‰) for inter-laboratory standards DL-Valine and Merck Gel, run in June 2017.

Intercept	1.77
Slope	1.00
R^2	0.99

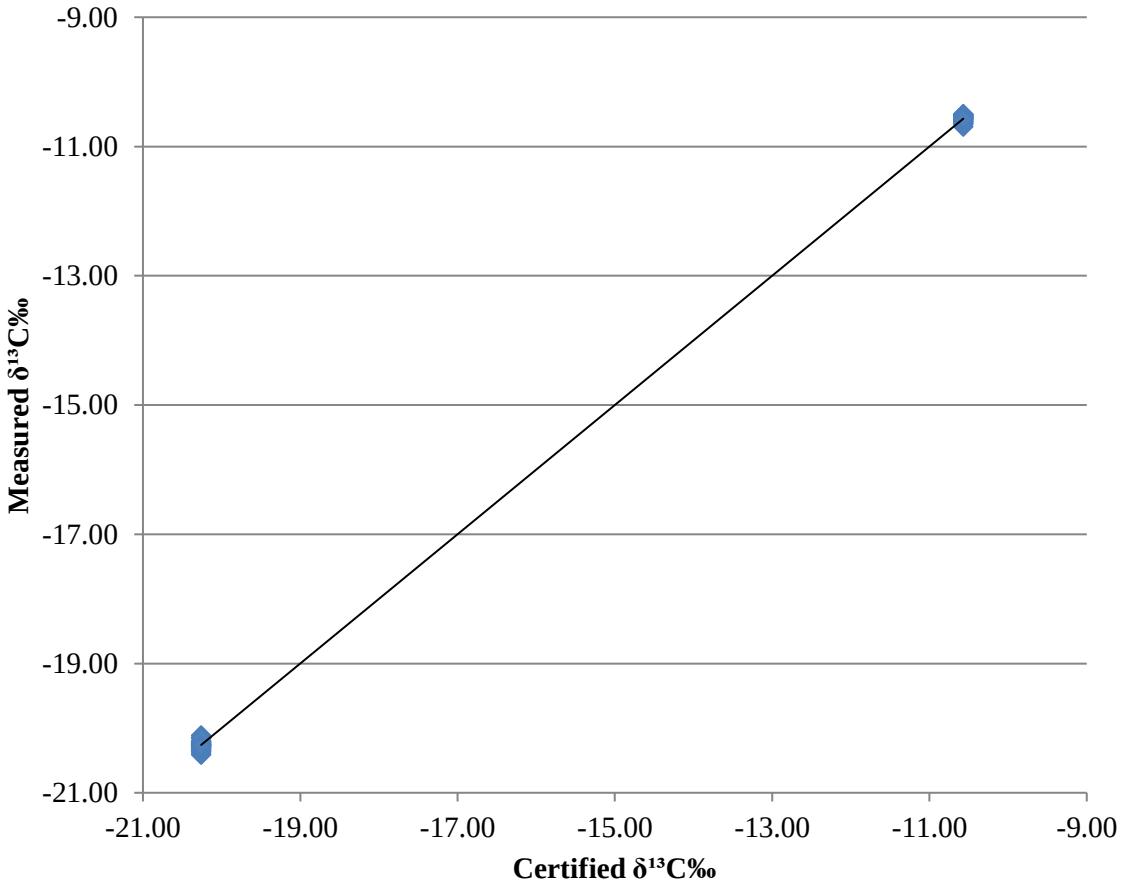

Figure 12.1. The graph shows the nearly perfect linear relationship (R^2) between the certified and measured values of $\delta^{13}C$ for the inter-laboratory standards (‰ V-PDB). First batch run in June 2017.

A linear regression was performed on the measured values of $\delta^{15}N$ (‰) against the certified values of $\delta^{15}N$ (‰) for the standards (Table 12.5 & Figure 12.2). The correlation coefficient of the certified and measured values of $\delta^{15}N$ (AIR) showed a nearly perfect linear relationship (very close to 1).

Table 12.5. Regression values for the measured $\delta^{15}N$ (‰) for inter-laboratory standards DL-Valine and Merck Gel, run in June 2017.

Intercept	2.22
Slope	1.00
R^2	0.99

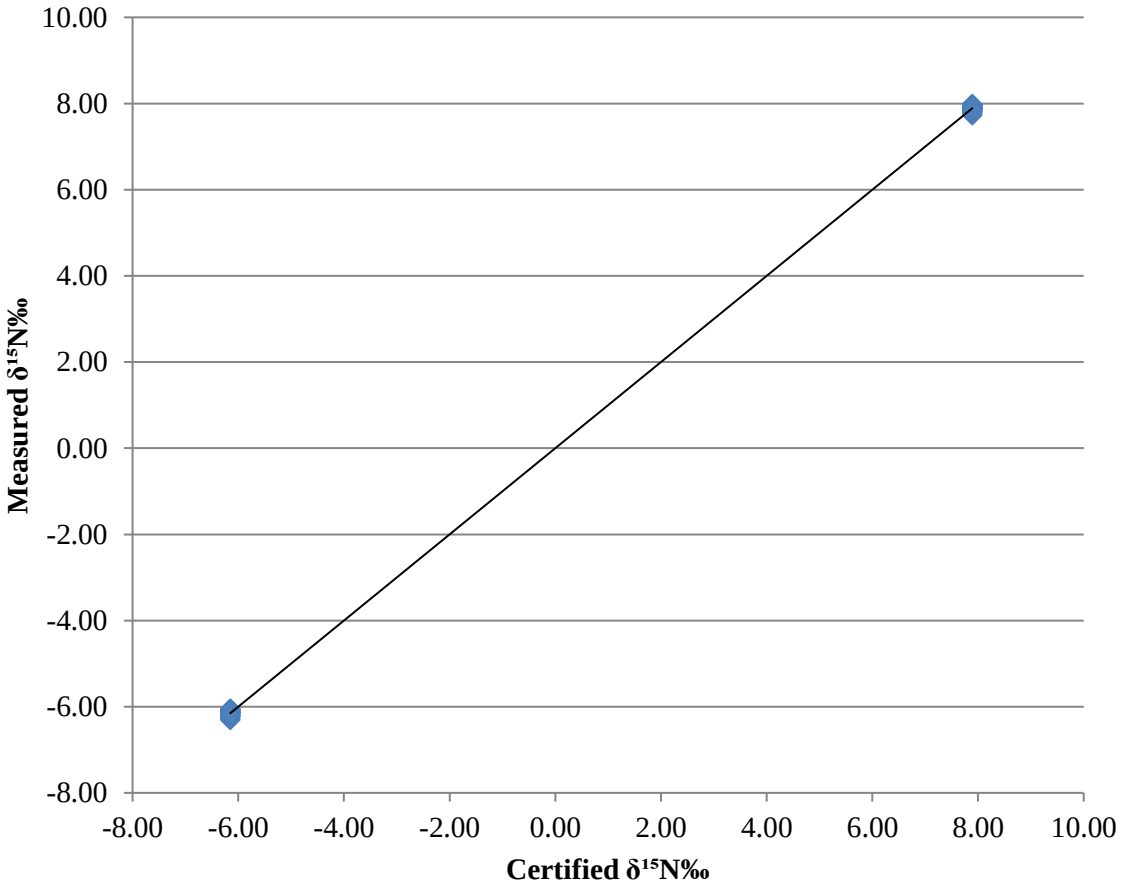

Figure 12.2. The graph shows the nearly perfect linear relationship (R^2) between the certified and measured values of $\delta^{15}N$ for the inter-laboratory standards (‰ AIR). First batch run in June 2017.

The second batch of samples and standards run for isotopic analysis was conducted in November 2017. Tables 12.6 and 12.7 give the measured $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) values for the second batch (November 2017) of DL-Valine and Merck Gel.

Table 12.6. The measured values of $\delta^{13}C$ and $\delta^{15}N$ (‰ V-PDB and AIR) for the inter-laboratory standard DL-Valine. Second batch run in November 2017.

Standard	Measured $\delta^{13}C$ (‰)	Measured $\delta^{15}N$ (‰)
DL-Valine	-10.53	-6.17
DL-Valine	-10.57	-6.14
DL-Valine	-10.70	-6.09
DL-Valine	-10.48	-6.19
DL-Valine	-10.64	-6.16
DL-Valine	-10.42	-6.15
DL-Valine	-10.67	-6.12
DL-Valine	-10.56	-6.20
DL-Valine	-10.52	-6.03
DL-Valine	-10.61	-6.23
DL-Valine	-10.62	-6.17
DL-Valine	-10.52	-6.13
DL-Valine	-10.51	-6.12
DL-Valine	-10.63	-6.18
DL-Valine	-10.61	-6.22
DL-Valine	-10.52	-6.05
DL-Valine	-10.59	-6.18
DL-Valine	-10.58	-6.10
DL-Valine	-10.47	-6.21
DL-Valine	-10.65	-6.14
DL-Valine	-10.63	-6.18
DL-Valine	-10.53	-6.12
DL-Valine	-10.47	-6.12
DL-Valine	-10.65	-6.18
DL-Valine	-10.62	-6.13
DL-Valine	-10.58	-6.13

DL-Valine	-10.45	-6.23
DL-Valine	-10.58	-6.10
DL-Valine	-10.65	-6.26
DL-Valine	-10.55	-6.06
Mean	**-10.57**	**-6.15**
Standard deviation	**0.07**	**0.06**

Table 12.7. The measured values of $\delta^{13}C$ and $\delta^{15}N$ (‰ V-PDB and AIR) for the inter-laboratory standard Merck Gel. Second batch run in November 2017.

Standard	Measured $\delta^{13}C$ (‰)	Measured $\delta^{15}N$ (‰)
Merck	-20.29	7.85
Merck	-20.21	7.91
Merck	-20.28	7.98
Merck	-20.27	7.83
Merck	-20.31	7.92
Merck	-20.21	7.97
Merck	-20.29	7.79
Merck	-20.19	7.75
Merck	-20.27	7.94
Merck	-20.30	7.96
Merck	-20.29	7.88
Merck	-20.21	7.90
Merck	-20.28	7.92
Merck	-20.26	7.87
Merck	-20.34	7.88
Merck	-20.20	7.89
Merck	-20.21	7.94
Merck	-20.26	7.82
Merck	-20.24	7.91
Merck	-20.31	7.89
Merck	-20.27	7.96
Merck	-20.27	7.78
Merck	-20.20	7.89
Merck	-20.30	7.92
Merck	-20.32	7.93
Merck	-20.26	7.95
Merck	-20.20	7.73
Merck	-20.21	7.91
Merck	-20.24	7.91
Merck	-20.34	7.91

Mean	**-20.26**	**7.89**
Standard deviation	**0.04**	**0.06**

A linear regression was performed on the measured values of $\delta^{13}C$ (‰) against the certified values of $\delta^{13}C$ (‰) for the standards (Table 12.8 & Figure 12.3). The correlation coefficient of the certified and measured values of $\delta^{13}C$ (‰ V-PDB) showed a nealry perfect linear relationship (very close to 1).

Table 12.8. Regression values for the measured $\delta^{13}C$ (‰) for inter-laboratory standards DL-Valine and Merck Gel, run in November 2017.

Intercept	-3.55
Slope	1.00
R^2	0.99

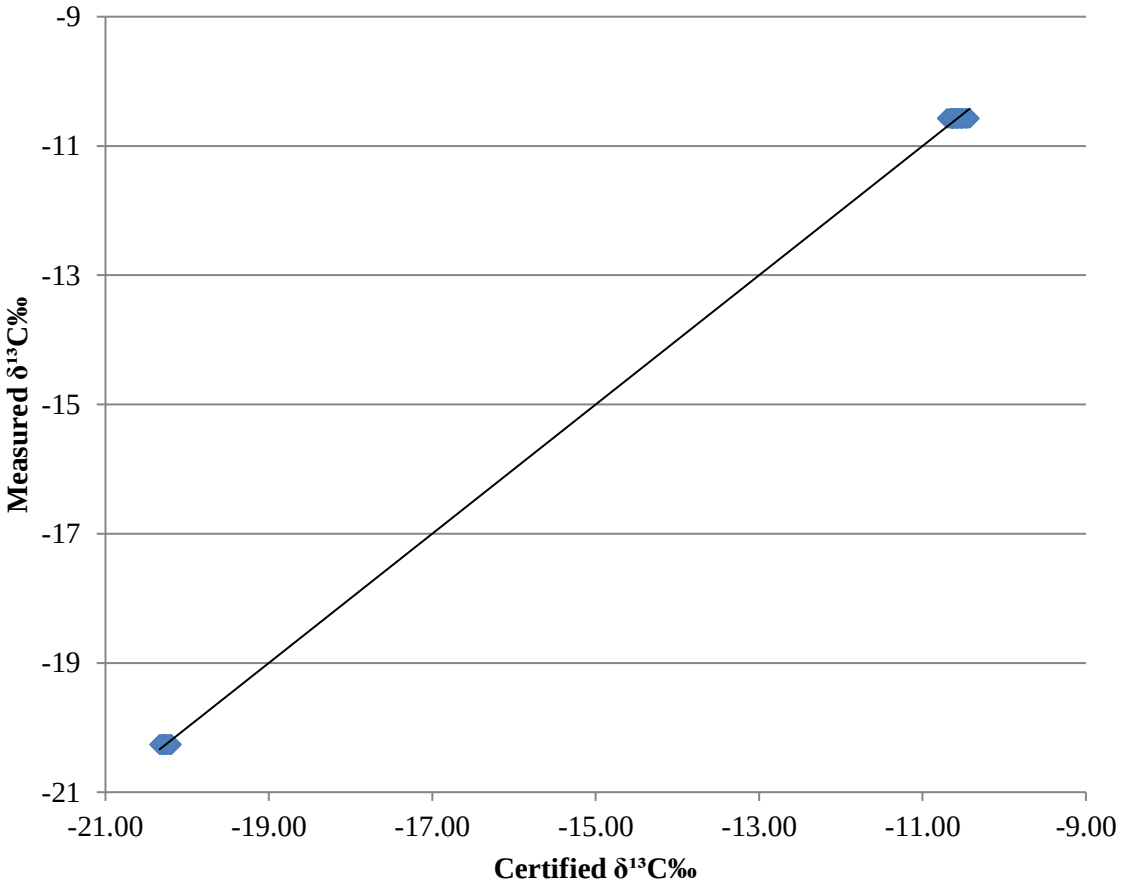

Figure 12.3. The graph shows the nearly perfect linear relationship (R^2) between the certified and measured values of $\delta^{13}C$ for the second batch of inter-laboratory standards (‰ V-PDB), run in November 2017.

A linear regression was performed on the measured values of $\delta^{15}N$ (‰) against the certified values of $\delta^{15}N$ (‰) for the standards (Table 12.9 & Figure 12.4). The correlation coefficient of the certified and measured values of $\delta^{15}N$ (AIR) showed a nearly perfect linear relationship (very close to 1).

Table 12.9. Regression values for the measured $\delta^{15}N$ (‰) for inter-laboratory standards DL-Valine and Merck Gel, run in November 2017.

Intercept	3.22
Slope	1.00
R^2	0.99

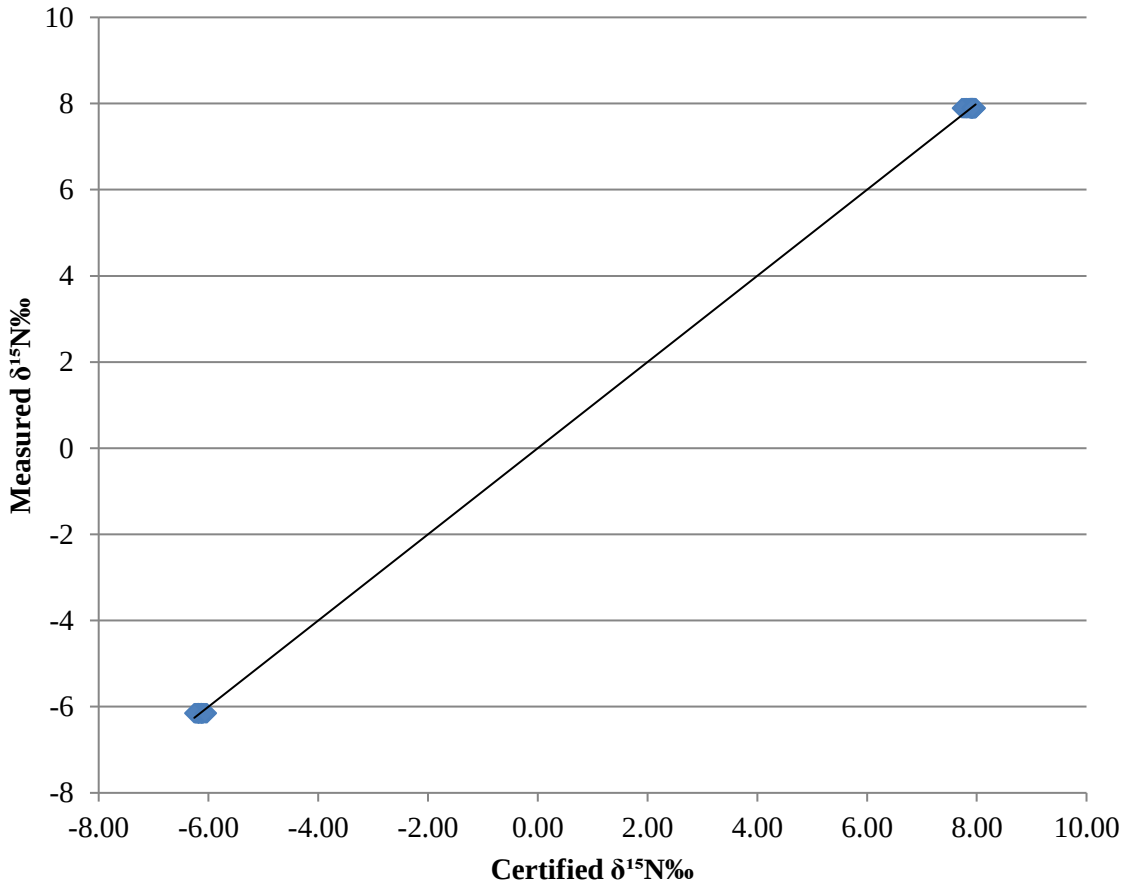

Figure 12.4. The graph shows the nearly perfect linear relationship (R^2) between the certified and measured values of $\delta^{15}N$ for the second batch of inter-laboratory standards (‰ AIR), run in November 2017.

The third batch of samples and standards run for isotopic analysis was conducted in December 2017. Tables 12.10 and 12.11 give the measured $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) values for the third and final batch (December 2017) of DL-Valine and Merck Gel run.

Table 12.10. The measured values of $\delta^{13}C$ and $\delta^{15}N$ (‰ V-PDB and AIR) for the inter-laboratory standard DL-Valine. Third batch run in December 2017.

Standard	Measured δ^{13C} (‰)	Measured $\delta^{15}N$ (‰)
DL-Valine	-10.49	-6.14
DL-Valine	-10.62	-6.21
DL-Valine	-10.61	-6.15
DL-Valine	-10.62	-6.14
DL-Valine	-10.56	-6.08
DL-Valine	-10.47	-6.18
DL-Valine	-10.59	-6.17
DL-Valine	-10.63	-6.13
DL-Valine	-10.58	-6.10
DL-Valine	-10.53	-6.21
DL-Valine	-10.56	-6.15
DL-Valine	-10.59	-6.15
DL-Valine	-10.56	-6.15
DL-Valine	-10.61	-6.18
DL-Valine	-10.61	-6.18
DL-Valine	-10.44	-6.09
DL-Valine	-10.56	-6.12
DL-Valine	-10.63	-6.13
DL-Valine	-10.53	-6.16
DL-Valine	-10.60	-6.19
DL-Valine	-10.53	-6.15
DL-Valine	-10.54	-6.08
DL-Valine	-10.62	-6.15
DL-Valine	-10.60	-6.24
DL-Valine	-10.57	-6.17
DL-Valine	-10.53	-6.14

DL-Valine	-10.69	-6.13
DL-Valine	-10.51	-6.11
DL-Valine	-10.58	-6.18
DL-Valine	-10.53	-6.14
Mean	**-10.57**	**-6.15**
Standard deviation	**0.05**	**0.04**

Table 12.11. The measured values of $\delta^{13}C$ and $\delta^{15}N$ (‰ V-PDB and AIR) for the inter-laboratory standard Merck Gel. Third batch run in December 2017.

Standard	Measured $\delta^{15}N$ (‰)	Measured δ^{13C} (‰)
Merck	7.88	-20.20
Merck	7.92	-20.29
Merck	7.89	-20.27
Merck	7.83	-20.29
Merck	7.90	-20.25
Merck	7.93	-20.22
Merck	7.82	-20.25
Merck	7.97	-20.36
Merck	7.93	-20.34
Merck	7.83	-20.13
Merck	7.87	-20.28
Merck	7.94	-20.22
Merck	7.87	-20.28
Merck	7.83	-20.32
Merck	7.88	-20.27
Merck	7.95	-20.19
Merck	7.88	-20.26
Merck	7.98	-20.19
Merck	7.92	-20.27
Merck	7.78	-20.32
Merck	7.79	-20.22
Merck	7.88	-20.26
Merck	7.96	-20.31
Merck	7.92	-20.30
Merck	7.90	-20.24
Merck	7.94	-20.20
Merck	7.94	-20.28
Merck	7.87	-20.29
Merck	7.89	-20.27
Merck	7.81	-20.23

Mean	**7.89**	**-20.26**
Standard deviation	**0.05**	**0.05**

A linear regression was performed on the measured values of $\delta^{13}C$ (‰) against the certified values of $\delta^{13}C$ (‰) for the standards (Table 12.12 & Figure 12.5). The correlation coefficient of the certified and measured values of $\delta^{13}C$ (‰ V-PDB) showed a nealry perfect linear relationship (very close to 1).

Table 12.12. Regression values for the measured $\delta^{13}C$ (‰) for inter-laboratory standards DL-Valine and Merck Gel, run in December 2017.

Intercept	0.00
Slope	1.00
R^2	0.99

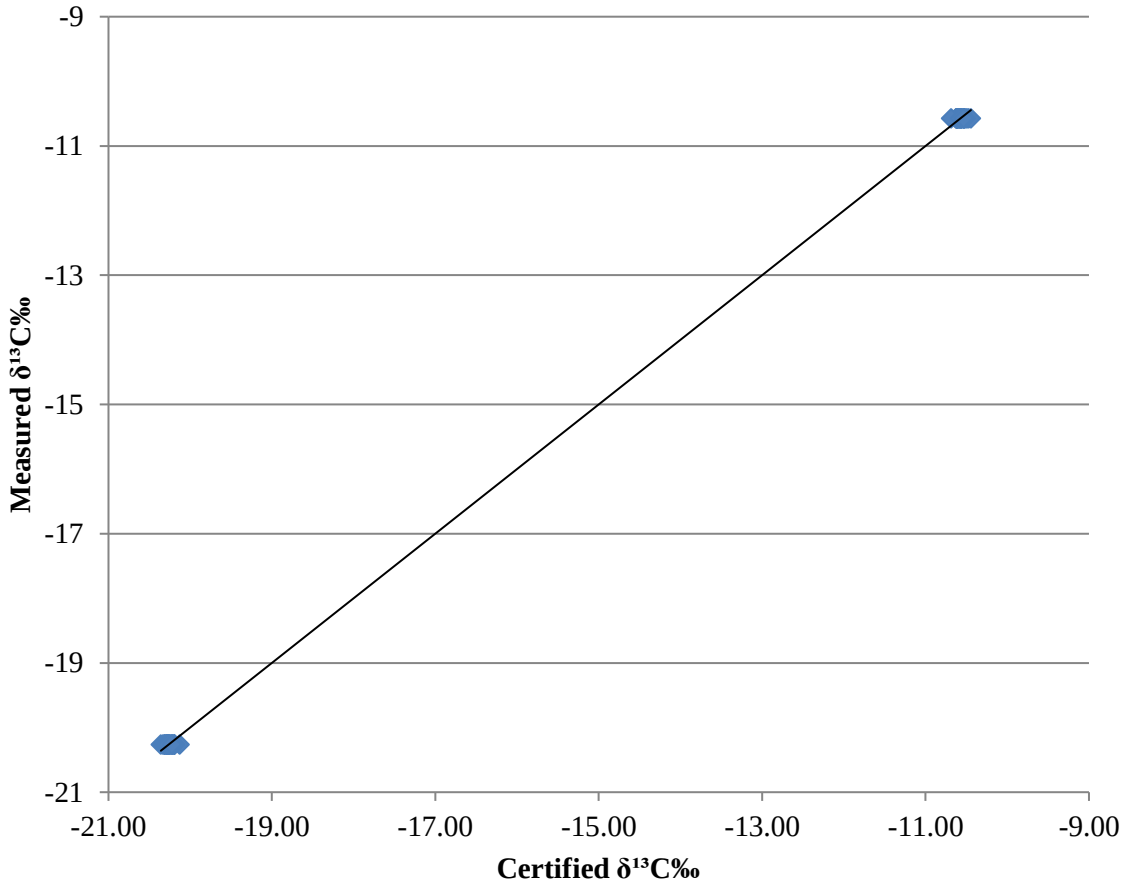

Figure 12.5. The graph shows the nearly perfect linear relationship (R^2) between the certified and measured values of $\delta^{13}C$ for the inter-laboratory standards (‰ V-PDB). Third batch run in December 2017.

A linear regression was performed on the measured values of $\delta^{15}N$ (‰) against the certified values of $\delta^{15}N$ (‰) for the standards (Table 12.13 & Figure 12.6). The correlation coefficient of the certified and measured values of $\delta^{15}N$ (AIR) showed a nearly perfect linear relationship (very close to 1).

Table 12.13. Regression values for the measured $\delta^{15}N$ (‰) for inter-laboratory standards DL-Valine and Merck Gel, run in December 2017.

Intercept	2.55
Slope	1.00
R^2	0.99

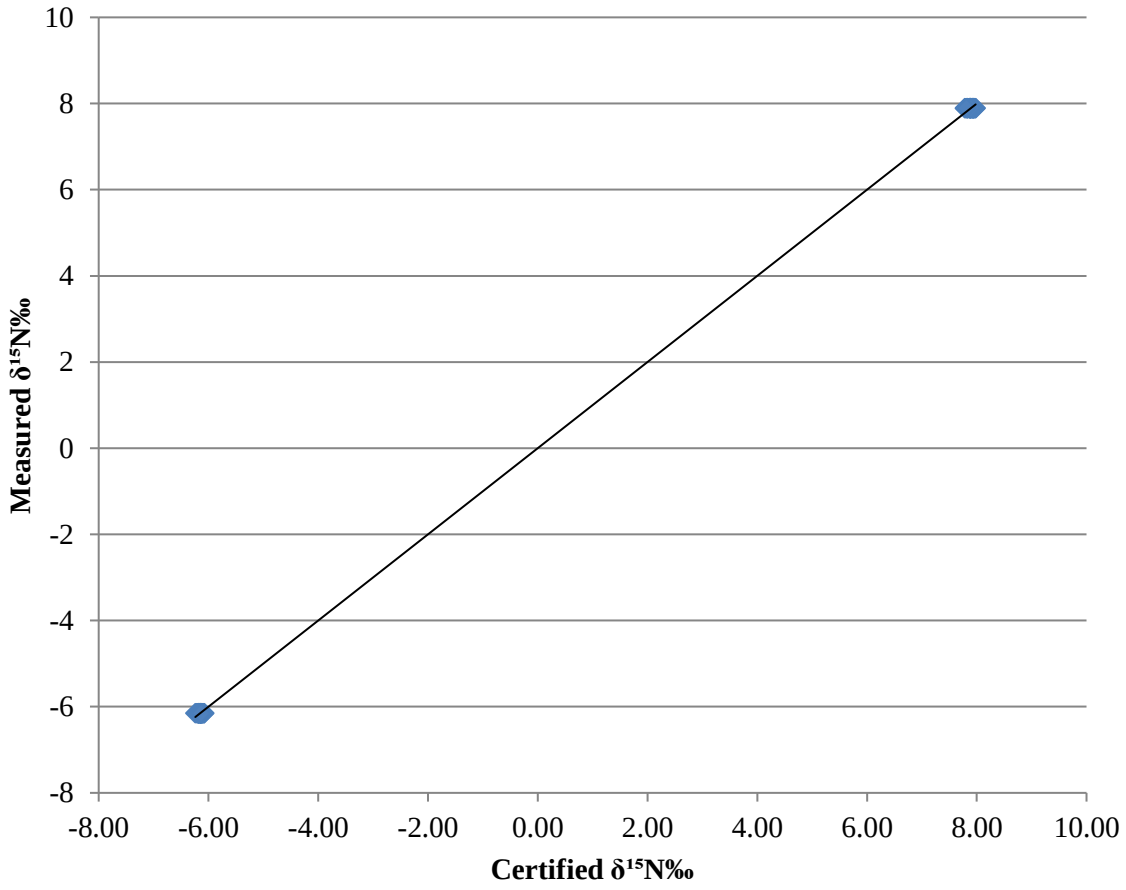

Figure 12.6. The graph shows the nearly perfect linear relationship (R^2) between the certified and measured values of $\delta^{15}N$ for the inter-laboratory standards (‰ AIR). Third batch run in December 2017.

APPENDIX 6

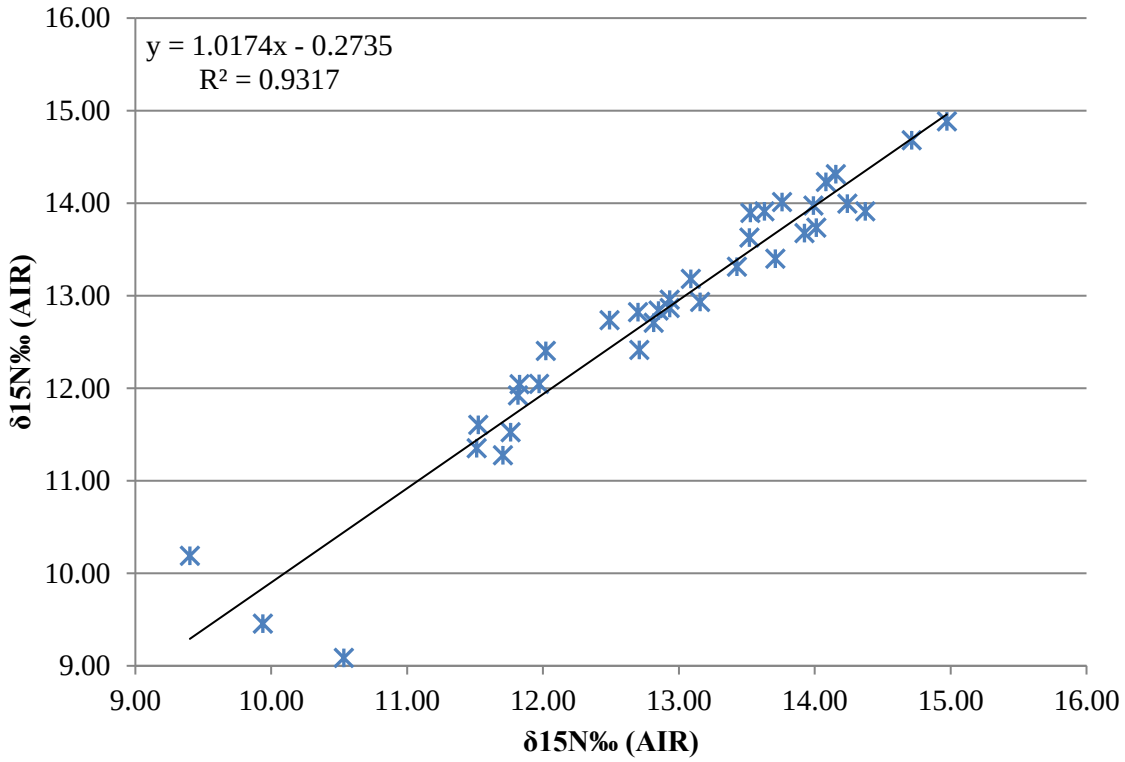

Figure 13.1. The graph shows the nearly perfect linear relationship (R^2) between the $\delta^{15}N$ values of the duplicate samples (‰ AIR).

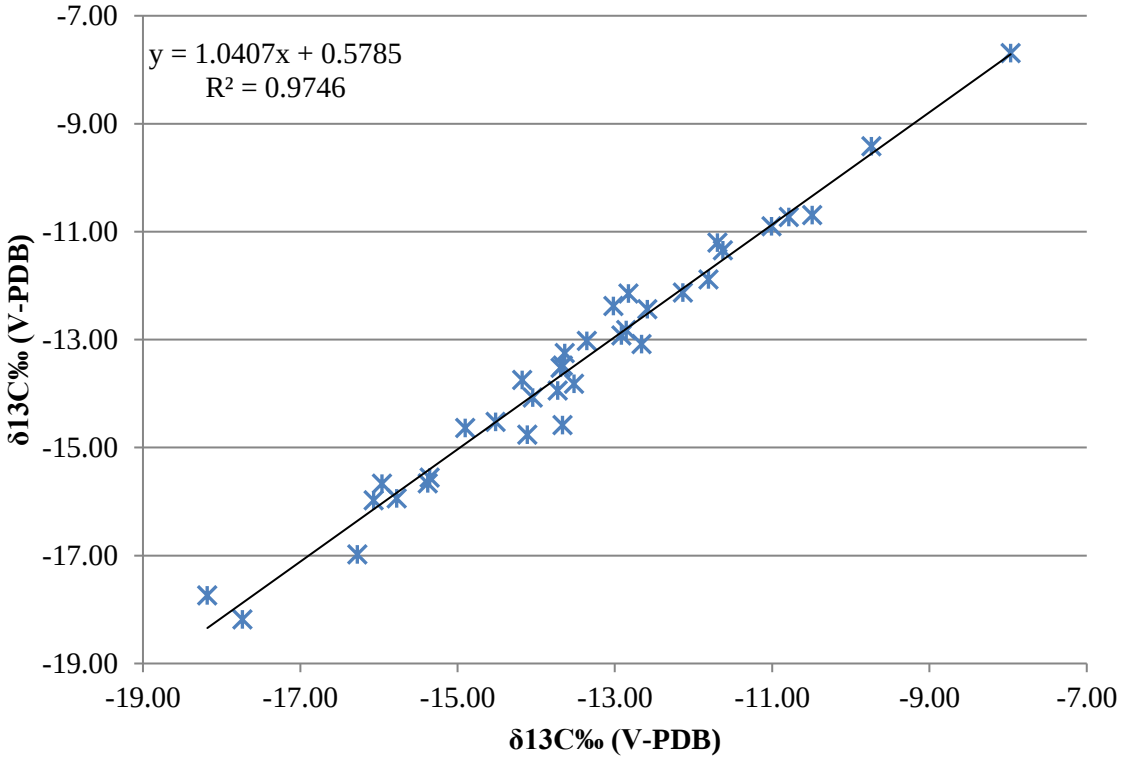

Figure 13.2. The graph shows the nearly perfect linear relationship (R^2) between the $\delta^{13}C$ values of the duplicate samples (‰ V-PDB).

APPENDIX 7

Table 14.1. The $\delta^{13}C$ and $\delta^{15}N$ values of individuals from both the DISH and control groups, separated by ancestry and sample.

DISH GROUP				
$\delta^{13}C$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
black^{Rib}	-14.6	-7.8	-11.7	-11.2
white^{Rib}	-17.1	-10.8	-14.3	-14.2
coloured^{Rib}	-17.5	-9.1	-14.8	-15.6
black^{Femur}	-14.7	-7.5	-11.1	-11.1
white^{Femur}	-19.1	-9.2	-14.3	-13.9
coloured^{Femur}	-15.5	-9.2	-12.5	-13.6
black$^{Rib+Femur}$	-14.7	-7.5	-11.3	-11.2
white$^{Rib+Femur}$	-19.1	-9.2	-14.3	-14.0
coloured$^{Rib+Femur}$	-17.5	-9.1	-13.8	-14.5
$\delta^{15}N$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
black^{Rib}	10.7	13.8	12.4	12.5
white^{Rib}	11.6	16.1	13.5	13.5
coloured^{Rib}	11.5	15.3	13.2	13.4
black^{Femur}	9.2	13.9	11.8	12.0
white^{Femur}	11.3	14.8	13.3	13.2
coloured^{Femur}	11.6	15.0	13.5	13.5
black$^{Rib+Femur}$	9.2	13.9	12.1	12.1
white$^{Rib+Femur}$	11.3	16.1	13.4	13.3
coloured$^{Rib+Femur}$	11.5	15.3	13.3	13.4
CONTROL GROUP				
$\delta^{13}C$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
black^{Rib}	-15.0	-7.3	-10.5	-10.6
white^{Rib}	-16.6	-16.6	-14.6	-14.6
coloured^{Rib}	-16.6	-13.8	-14.9	-14.2
black^{Femur}	-15.3	-7.0	-10.3	-10.4
white^{Femur}	-18.0	-11.6	-14.4	-14.0
coloured^{Femur}	-15.9	-9.7	-13.6	-14.3
black$^{Rib+Femur}$	-15.3	-7.0	-10.4	-10.6
white$^{Rib+Femur}$	-18.0	-11.6	-14.5	-14.3
coloured$^{Rib+Femur}$	-16.6	-9.7	-14.1	-14.3
$\delta^{15}N$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**

black^{Rib}	10.8	14.1	12.5	12.6
white^{Rib}	11.5	14.9	13.4	13.4
coloured^{Rib}	12.0	14.7	13.9	14.2
black^{Femur}	9.4	14.0	11.8	12.0
white^{Femur}	11.9	17.5	13.5	13.5
coloured^{Femur}	10.6	15.4	13.5	13.5
black$^{Rib+Femur}$	9.4	14.1	12.2	12.3
white$^{Rib+Femur}$	11.5	17.5	13.5	13.5
coloured$^{Rib+Femur}$	10.6	15.4	13.7	14.2

Table 14.2. The $\delta^{13}C$ and $\delta^{15}N$ values of individuals from both the DISH and control groups, separated by BMI classification and sample type.

DISH GROUP				
$\delta^{13}C$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
$underweight^{Rib}$	-16.8	-9.7	-12.6	-11.9
$normal^{Rib}$	-17.1	-7.8	-13.1	-13.7
$overweight^{Rib}$	-15.2	-12.8	-13.9	-13.6
$obese^{Rib}$	-14.7	-10.8	-13.2	-13.7
$underweight^{Femur}$	-17.7	-9.2	- 12.40	-12.4
$normal^{Femur}$	-18.8	-7.5	-13.0	-13.4
$overweight^{Femur}$	-19.1	-12.5	-14.5	-14.2
$obese^{Femur}$	-14.7	-8.4	-12.2	-12.7
$underweight^{Rib+Femur}$	-17.7	-9.2	-12.3	-12.0
$normal^{Rib+Femur}$	-18.8	-7.5	-13.1	-13.5
$overweight^{Rib+Femur}$	-19.1	-12.5	-14.2	-14.1
$obese^{Rib+Femur}$	-14.7	-8.4	-12.6	-13.0
$\delta^{15}N$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
$underweight^{Rib}$	10.9	14.8	12.5	12.5
$normal^{Rib}$	10.9	15.0	13.3	13.3
$overweight^{Rib}$	11.9	16.1	13.8	14.0
$obese^{Rib}$	10.7	14.0	12. 57	12.6
$underweight^{Femur}$	9.8	14.1	12.4	12.6
$normal^{Femur}$	11.1	14.4	12.9	13.0
$overweight^{Femur}$	11.3	14.6	13.1	13.0
$obese^{Femur}$	9.2	14.1	12.6	12.9
$underweight^{Rib+Femur}$	9.8	14.8	12.5	12.6
$normal^{Rib+Femur}$	10.9	15.0	13.1	13.2
$overweight^{Rib+Femur}$	11.3	16.1	13.4	13.3
$obese^{Rib+Femur}$	9.2	14.1	12.6	12.7
CONTROL GROUP				
$\delta^{13}C$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
$underweight^{Rib}$	-15.4	-8.4	-12.6	-12.8
$normal^{Rib}$	-16.6	-8.6	-13.6	-14.2
$overweight^{Rib}$	-16.6	-10.4	-13.7	-13.8
$obese^{Rib}$	-14.8	-10.8	-13.0	-13.5
$underweight^{Femur}$	-17.4	-8.5	-12.6	-12.7
$normal^{Femur}$	-18.0	-7.7	-13.4	-13.6
$overweight^{Femur}$	-16.5	-10.1	-13.8	-13.8

obese^{Femur}	-14.1	-8.7	-11.7	-12.7
underweight$^{Rib+Femur}$	-17.4	-8.4	-12.6	-12.7
normal$^{Rib+Femur}$	-18.0	-7.7	-13.5	-13.8
overweight$^{Rib+Femur}$	-16.6	-10.1	-13.7	-13.8
obese$^{Rib+Femur}$	-14.8	-8.7	-12.4	-13.5
$\delta^{15}N$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
underweight^{Rib}	10.8	14.6	12.8	12.8
normal^{Rib}	11.5	14.9	13.0	12.8
overweight^{Rib}	12.1	14.0	13.1	13.4
obese^{Rib}	11.9	14.1	13.1	13.2
underweight^{Femur}	9.6	14.5	12.8	12.8
normal^{Femur}	9.4	17.5	13.2	13.4
overweight^{Femur}	11.2	17.3	13.3	13.1
obese^{Femur}	11.8	13.6	12.5	12.5
underweight$^{Rib+Femur}$	9.6	14.6	12.8	12.8
normal$^{Rib+Femur}$	9.4	17.5	13.1	13.1
overweight$^{Rib+Femur}$	11.2	17.3	13.2	13.2
obese$^{Rib+Femur}$	11.8	14.1	12.8	12.6

Table 14.3. The $\delta^{13}C$ and $\delta^{15}N$ values of individuals from both the DISH and control groups, separated by ancestry, BMI and sample type.

DISH GROUP				
$\delta^{13}C$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
black_underweight^{Rib}	-13.2	-10.1	-11.4	-11.2
black_normal^{Rib}	-14.6	-7.8	-11.0	-10.8
black_overweight^{Rib}	-14.4	-14.4	-14.4	-14.4
black_obese^{Rib}	-14.0	-12.1	-13.0	-13.0
white_underweight^{Rib}	-16.8	-13.0	-14.9	-14.5
white_normal^{Rib}	-17.1	-11.8	-14.2	-14.4
white_overweight^{Rib}	-15.2	-12.8	-13.9	-13.6
white_obese^{Rib}	-14.7	-10.8	-13.4	-13.7
coloured_normal^{Rib}	-13.5	-9.1	-11.3	-11.3
black_underweight^{Femur}	-14.1	-9.6	-11.0	-10.9
black_normal^{Femur}	-13.4	-7.5	-10.6	-10.5
black_overweight^{Femur}	-13.5	-13.5	-13.5	-13.5
black_obese^{Femur}	-14.7	-8.4	-10.9	-10.4
white_underweight^{Femur}	-17.7	-9.2	-14.0	-13.8
white_normal^{Femur}	-18.8	-11.8	-14.5	-13.8
white_overweight^{Femur}	-19.1	-12.5	-14.5	-14.2
white_obese^{Femur}	-14.1	-12.5	-13.3	-13.1
coloured_normal^{Femur}	-13.6	-9.2	-11.4	-11.4
black_underweight$^{Rib+Femur}$	-14.1	-9.6	-11.2	-11.1
black_normal$^{Rib+Femur}$	-14.6	-7.5	-10.6	-10.5
black_overweight$^{Rib+Femur}$	-14.4	-13.5	-13.9	-13.9
black_obese$^{Rib+Femur}$	-14.7	-8.4	-11.7	-11.7
white_underweight$^{Rib+Femur}$	-17.7	-9.2	-14.3	-13.9
white_normal$^{Rib+Femur}$	-18.8	-11.8	-14.3	-14.1
white_overweight$^{Rib+Femur}$	-19.1	-12.5	-14.2	-14.1
white_obese$^{Rib+Femur}$	-14.7	-10.8	-13.3	-13.6
coloured_normal$^{Rib+Femur}$	-13.6	-9.1	-11.4	-11.3
$\delta^{15}N$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
black_underweight^{Rib}	10.9	13.0	12.1	12.2
black_normal^{Rib}	10.9	13.7	12.7	13.1
black_overweight^{Rib}	13.6	13.6	13.6	13.6
black_obese^{Rib}	10.7	12.8	11.7	11.6
white_underweight^{Rib}	11.6	14.8	13.4	13.5
white_normal^{Rib}	12.2	15.0	13.5	13.4

white_overweight^{Rib}	11.9	16.1	13.8	14.0
white_obese^{Rib}	12.4	14.0	13.1	12.7
coloured_normal^{Rib}	13.3	13.6	13.4	13.4
black_underweight^{Femur}	9.8	12.8	11.5	11.8
black_normal^{Femur}	11.1	13.9	12.2	12.0
black_overweight^{Femur}	13.3	13.3	13.3	13.3
black_obese^{Femur}	9.2	13.0	11.7	12.2
white_underweight^{Femur}	12.2	14.1	13.3	13.3
white_normal^{Femur}	11.6	14.4	13.3	13.3
white_overweight^{Femur}	11.3	14.6	13.1	13.0
white_obese^{Femur}	12.3	14.1	13.4	13.4
coloured_normal^{Femur}	11.6	13.5	12.6	12.6
black_underweight$^{Rib+Femur}$	9.8	13.0	11.8	12.1
black_normal$^{Rib+Femur}$	10.9	13.9	12.3	12.0
black_overweight$^{Rib+Femur}$	13.3	13.6	13.5	13.5
black_obese$^{Rib+Femur}$	9.2	13.0	11.7	11.9
white_underweight$^{Rib+Femur}$	11.6	14.8	13.3	13.4
white_normal$^{Rib+Femur}$	11.6	15.0	13.4	13.4
white_overweight$^{Rib+Femur}$	11.3	16.1	13.4	13.3
white_obese$^{Rib+Femur}$	12.3	14.1	13.2	13.2
coloured_normal$^{Rib+Femur}$	11.6	13.6	13.0	13.4
CONTROL GROUP				
$\delta^{13}C$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
black_underweight^{Rib}	-15.0	-8.4	-11.1	-11.1
black_normal^{Rib}	-11.0	-8.6	-10.2	-10.4
black_overweight^{Rib}	-11.5	-10.4	-10.9	-10.9
black_obese^{Rib}	-13.5	-10.8	-11.8	-11.1
white_underweight^{Rib}	-15.4	-13.2	-14.4	-14.6
white_normal^{Rib}	-16.6	-11.9	-14.7	-14.9
white_overweight^{Rib}	-16.6	-12.7	-14.6	-14.7
white_obese^{Rib}	-14.8	-13.6	-14.2	-14.0
black_underweight^{Femur}	-15.3	-8.5	-10.8	-10.7
black_normal^{Femur}	-11.4	-7.7	-10.0	-10.2
black_overweight^{Femur}	-13.8	-10.1	-12.0	-12.1
black_obese^{Femur}	-12.7	-8.7	-10.2	-9.2
white_underweight^{Femur}	-17.4	-11.6	-14.1	-14.1
white_normal^{Femur}	-18.0	-12.4	-14.3	-13.9
white_overweight^{Femur}	-16.5	-12.7	-14.6	-13.8

white_obese^{Femur}	-14.1	-13.9	-14.0	-14.0
*coloured_underweight^{Femur}	-10.3	-10.3	-10.3	-10.3
black_underweight$^{Rib+Femur}$	-15.3	-8.4	-11.0	-10.9
black_normal$^{Rib+Femur}$	-11.4	-7.7	-10.1	-10.4
black_overweight$^{Rib+Femur}$	-11.5	-10.1	-10.9	-10.9
black_obese$^{Rib+Femur}$	-13.5	-8.7	-11.0	-11.0
white_underweight$^{Rib+Femur}$	-17.4	-11.6	-14.2	-14.3
white_normal$^{Rib+Femur}$	-18.0	-11.9	-14.5	-14.1
white_overweight$^{Rib+Femur}$	-16.6	-12.7	-14.6	-14.5
white_obese$^{Rib+Femur}$	-14.8	-13.6	-14.1	-14.0
$\delta^{15}N$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
black_underweight^{Rib}	10.8	13.5	12.3	12.6
black_normal^{Rib}	11.8	13.4	12.5	12.6
black_overweight^{Rib}	12.2	13.6	12.9	12.9
black_obese^{Rib}	11.9	13.0	12.4	12.2
white_underweight^{Rib}	11.8	14.6	13.3	13.3
white_normal^{Rib}	11.5	14.9	13.2	13.1
white_overweight^{Rib}	12.1	14.0	13.2	13.4
white_obese^{Rib}	13.3	14.1	13.8	13.9
black_underweight^{Femur}	9.6	14.0	11.9	12.5
black_normal^{Femur}	9.4	12.3	11.1	11.6
black_overweight^{Femur}	11.2	13.2	12.6	13.0
black_obese^{Femur}	11.8	12.5	12.2	12.2
white_underweight^{Femur}	12.1	14.5	13.5	13.6
white_normal^{Femur}	12.6	17.5	13.7	13.5
white_overweight^{Femur}	12.2	17.3	13.7	13.2
white_obese^{Femur}	12.6	13.6	13.1	13.1
*coloured_underweight^{Femur}	11.9	11.9	11.9	11.9
black_underweight$^{Rib+Femur}$	9.6	14.0	12.1	12.5
black_normal$^{Rib+Femur}$	9.4	13.4	11.8	12.1
black_overweight$^{Rib+Femur}$	11.2	13.6	12.5	12.6
black_obese$^{Rib+Femur}$	11.8	13.0	12.3	12.2
white_underweight$^{Rib+Femur}$	11.8	14.6	13.4	13.3
white_normal$^{Rib+Femur}$	11.5	17.5	13.5	13.4
white_overweight$^{Rib+Femur}$	12.1	17.3	13.4	13.4
white_obese$^{Rib+Femur}$	12.6	14.1	13.5	13.6

***There was only one individual who fell under the coloured underweight category and only their femur sample fell within the accepted C/N ratio range.**

Table 14.4. The significant p-values comparing the isotopic values among the different BMI and ancestry combinations from both the DISH and control groups.

DISH GROUP						
	$\delta^{13}C^{Rib}$	$\delta^{15}N^{Rib}$	$\delta^{13}C^{Femur}$	$\delta^{15}N^{Femur}$	$\delta^{13}C^{Rib+Femur}$	$\delta^{15}N^{Rib+Femur}$
normal black and normal coloured	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal black and normal white	**p< 0.05**	p> 0.05	**p< 0.001**	p> 0.05	**p< 0.001**	**p< 0.05**
normal black and obese black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal black and obese white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal black and overweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal black and overweight white	p> 0.05	p> 0.05	**p< 0.001**	p> 0.05	**p< 0.001**	p> 0.05
normal black and underweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal black and underweight white	**p< 0.05**	p> 0.05	**p< 0.01**	p> 0.05	**p< 0.001**	p> 0.05
normal coloured and normal white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal coloured and obese black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal coloured and obese white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal coloured and overweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal coloured and overweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05

normal coloured and underweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal coloured and underweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal white and obese black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	**p< 0.05**
normal white and obese white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal white and overweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal white and overweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal white and underweight black	**p< 0.001**	**p< 0.01**	**p< 0.001**	**p< 0.001**	**p< 0.001**	**p< 0.001**
normal white and underweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese black and obese white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese black and overweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese black and overweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	**p< 0.05**
obese black and underweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese black and underweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	**p< 0.05**

obese white and overweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese white and overweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese white and underweight black	p> 0.05	p> 0.05	p> 0.05	**p< 0.05**	p> 0.05	**p< 0.05**
obese white and underweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
overweight black and overweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
overweight black and underweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
overweight black and underweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
overweight white and underweight black	**p< 0.05**	**p< 0.01**	**p< 0.001**	**p< 0.01**	**p< 0.001**	**p< 0.001**
overweight white and underweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
underweight black and underweight white	**p< 0.001**	p> 0.05	**p< 0.01**	**p< 0.001**	**p< 0.001**	**p< 0.001**
CONTROL GROUP						
	$\delta^{13}C^{Rib}$	**$\delta^{15}N^{Rib}$**	**$\delta^{13}C^{Femur}$**	**$\delta^{15}N^{Femur}$**	**$\delta^{13}C^{Rib+Femur}$**	**$\delta^{15}N^{Rib+Femur}$**
normal black and normal white	**p< 0.001**	p> 0.05	**p< 0.01**	**p< 0.01**	**p< 0.001**	**p< 0.001**
normal black and obese black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05

normal black and obese white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	**p< 0.05**	p> 0.05
normal black and overweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal black and overweight white	**p< 0.05**	p> 0.05	**p< 0.05**	p> 0.05	**p< 0.001**	p> 0.05
normal black and underweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal black and *underweight coloured	-	-	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal black and underweight white	**p< 0.05**	p> 0.05	**p< 0.01**	**p< 0.01**	**p< 0.001**	**p< 0.001**
normal white and obese black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	**p< 0.05**	p> 0.05
normal white and obese white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal white and overweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal white and overweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal white and underweight black	**p< 0.001**	p> 0.05	**p< 0.001**	**p< 0.01**	**p< 0.001**	**p< 0.001**
normal white and *underweight coloured	-	-	p> 0.05	p> 0.05	p> 0.05	p> 0.05
normal white and underweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05

obese black and obese white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese black and overweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese black and overweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese black and underweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese black and *underweight coloured	-	-	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese black and underweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese white and overweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese white and overweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese white and underweight black	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese white and *underweight coloured	-	-	p> 0.05	p> 0.05	p> 0.05	p> 0.05
obese white and underweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
overweight black and overweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
overweight black and	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05

underweight black						
overweight black and underweight coloured	-	-	p> 0.05	p> 0.05	p> 0.05	p> 0.05
overweight black and underweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
overweight white and underweight black	p> 0.05	p> 0.05	**p< 0.05**	p> 0.05	**p< 0.001**	p> 0.05
overweight white and underweight coloured	-	-	p> 0.05	p> 0.05	p> 0.05	p> 0.05
overweight white and underweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05
underweight black and *underweight coloured	-	-	p> 0.05	p> 0.05	p> 0.05	p> 0.05
underweight black and underweight white	**p< 0.05**	p> 0.05	**p< 0.01**	**p< 0.05**	**p< 0.001**	**p< 0.001**
*underweight coloured and underweight white	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05	p> 0.05

***There was only one underweight coloured individual, and only the femur sample fell within the accepted C/N ratio, and was therefore eligible for analysis.**

Table 14.5. The $\delta^{13}C$ and $\delta^{15}N$ values for DISH and control groups, separated by collection source and sample type.

DISH GROUP				
$\delta^{13}C$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
PBC^{Rib}	-17.1	-9.9	-13.9	-14.5
RDC^{Rib}	-15.5	-7.8	-12.5	-13.0
KC^{Rib}	-17.5	-10.2	-15.1	-15.9
PBC^{Femur}	-18.8	-8.4	-13.2	-13.4
RDC^{Femur}	-19.1	-7.5	-12.9	-13.5
KC^{Femur}	-17.2	-11.5	-13.9	-13.7
$PBC^{Rib+Femur}$	-18.8	-8.4	-13.5	-13.5
$RDC^{Rib+Femur}$	-19.1	-7.5	-12.8	-13.1
$KC^{Rib+Femur}$	-17.5	-10.2	-14.6	-15.3
$\delta^{15}N$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
PBC^{Rib}	10.7	14.0	12.7	12.8
RDC^{Rib}	11.6	16.1	13.6	13.7
KC^{Rib}	11.5	15.3	13.0	13.1
PBC^{Femur}	9.2	14.6	12.7	12.9
RDC^{Femur}	10.3	14.5	12.9	12.8
KC^{Femur}	9.8	15.0	13.4	13.4
$PBC^{Rib+Femur}$	9.2	14.6	12.7	12.9
$RDC^{Rib+Femur}$	10.3	16.1	13.2	13.2
$KC^{Rib+Femur}$	9.8	15.3	13.2	13.3
CONTROL GROUP				
$\delta^{13}C$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
PBC^{Rib}	-16.6	-8.6	-13.4	-13.8
RDC^{Rib}	-16.6	-7.3	-11.7	-11.1
KC^{Rib}	-16.6	-10.8	-14.5	-14.2
PBC^{Femur}	-17.8	-7.7	-13.0	-13.2
RDC^{Femur}	-18.0	-7.0	-12.3	-12.4
KC^{Femur}	-18.0	-11.4	-14.6	-14.4
$PBC^{Rib+Femur}$	-17.8	-7.7	-13.2	-13.6
$RDC^{Rib+Femur}$	-18.0	-7.0	-12.0	-11.7
$KC^{Rib+Femur}$	-18.0	-10.8	-14.6	-14.3
$\delta^{15}N$ (‰)	**Minimum**	**Maximum**	**Mean**	**Median**
PBC^{Rib}	10.8	14.6	12.9	13.0
RDC^{Rib}	11.0	14.9	13.1	13.0
KC^{Rib}	12.0	14.7	13.8	14.2

PBC^{Femur}	9.4	17.3	13.0	13.1
RDC^{Femur}	10.6	17.5	12.7	12.6
KC^{Femur}	11.9	15.4	13.9	14.1
$PBC^{Rib+Femur}$	9.4	17.3	12.9	13.0
$RDC^{Rib+Femur}$	10.6	17.5	12.9	12.8
$KC^{Rib+Femur}$	11.9	15.4	13.8	14.1

APPENDIX 8

Table 15.1. Results from the four observations made from the micro-CT scans. '1' indicates yes/present, '0' indicates no/absent.

Skeleton Number	Ossification starts at middle of vertebral body	Evidence for retention of vertebral wall at sight of ALL ossification	Evidence of trabecular bone at sight of ALL ossification	Abnormal areas of osteosclerosis beyond ossification of ALL
Sk6906	1	1	1	1
Sk6238	1	0	1	1
Sk4006	1	0	1	0
Sk5908	1	1	1	0
Sk5144	1	0	1	0
Sk5561	1	0	1	0
Sk4611	1	0	1	0
Sk5333	1	0	1	0
Sk6435	1	0	1	1
Sk6437	1	0	1	0
Sk6356	1	0	1	0
Sk6472	1	0	1	0
Sk5978	1	0	1	0
SK6341	1	0	1	0
SK5174	1	0	1	0
Sk5384	1	0	1	0
Sk6527	1	0	1	0
Sk6484	1	0	1	0
Sk5666	0	0	1	0
Sk5499	1	0	1	0
Sk5346	1	0	1	0
Sk5078	1	1	1	0
Sk4601	1	0	1	1
Sk6380	1	1	1	0
Sk6406	1	0	1	0
Sk5287	1	1	1	0
Sk5665	1	1	1	0
Sk6421	1	0	1	1
Sk5840	0	0	1	0
Sk6444	0	1	1	0
Sk6407	0	0	0	0
Sk5816	1	0	1	0
Sk2053	1	0	1	0
Sk6248	1	0	0	0
Sk6295	0	0	1	0
Sk6320	1	0	1	1

Sk4564	1	0	1	0
Sk3676	0	1	1	0
Sk4969	1	0	1	0
Sk6313	0	0	1	1
Sk6202	0	0	1	0
Sk7008	1	0	1	1
Sk6976	1	0	1	1
Sk6198	1	0	1	0
A1799	1	0	1	0
A1808	1	0	1	0
A2029	1	0	1	0
A2091	0	0	1	0
A2197	1	0	1	1
A2286	0	0	1	0
A2346	1	1	1	0
A2361	1	0	1	0
A2607	0	0	1	1
A2722	1	0	0	0
A2765	1	0	1	1
A2921	0	0	0	0
A3066	1	0	1	0
A3103	1	0	1	0
A3325	1	0	1	0
A3574	0	0	1	0
A3589	1	0	1	1
A3613	1	0	1	1
A3614	1	0	1	1
A3636	1	0	1	0
A3644	1	0	1	0
A3647	1	0	0	0
A3694	1	1	1	0
A3956	1	0	1	0
A3962	1	0	1	0
A3965	1	0	1	0
A3971	1	0	1	0
A3989	1	1	1	0
A4006	0	0	1	1
A4017	0	0	1	0
A4061	1	1	1	0
A4091	1	0	1	0
A4298	1	0	1	0
A4307	1	0	1	0
A4311	1	0	1	0
A4347	1	0	1	0

A4359	1	0	1	0
A4368	1	0	1	0