The Impact of Diet and Health on Bone Stable Isotope Ratios:

A Comparative Study

By

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Declaration

This is to certify that:

1. The thesis contains only my original work towards the fulfilment of the degree of Doctor of Philosophy at the University of Kent, except where stated otherwise.

2. Acknowledgement has been made in the text to all other material used.

All photographs have been taken by the author, if not stated otherwise.

Ana Curto, September 2018

Abstract

There is a bidirectional interaction across nutrition, infection and immunity. While good nutrition increases the immune system's response, immune deficits following malnutrition early in life have been shown to persist for weeks and even years. This study combines osteological and archaeometric analysis providing significant novel perspectives on the synergy between diet and health exploring stable isotope analysis. It is intended to assess whether stable isotope analysis can be used as a tool to study the impact of diet on the individuals' susceptibility to pathogens. This study will help understanding the physiological mechanisms of stress and disease prior to the advent of modern medicine and antibiotics, as well as improve dietary isotope data interpretation.

The samples under study were recovered from Santa Maria do Olival, Tomar, Portugal (11^{th} – 17^{th} centuries). Stable isotope analyses were performed to 66 skeletons, 33 individuals without macroscopic indicators of physiological stress or skeletal lesions and 33 individuals with skeletal lesions of possible infectious origin (n=23) or healed fracture calluses (n=10). Stable isotope analyses were also preformed to fauna remains (n=13) to estimate the baseline diet. The individuals with lesions were divided into active (n=6) and healed lesions (n=7), a combination of active and healed lesions (n=10) and fracture calluses (n=10) and the data compared with sites within the same bone without lesions. In total 134 samples (94 from long bones, 27 from ribs, 13 from faunal remains) were used for stable isotope analyses. Carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$) and sulphur ($\delta^{34}S$) stable isotopes were used to estimate the diet at Tomar. $\delta^{13}C$ and $\delta^{15}N$ values were compared between individuals with and without lesions and non-lesion and lesion sites were also compared within the same bone.

The diet at Tomar was complex ($\delta^{13}C$=-18.6±0.5‰, $\delta^{15}N$=10.8±0.8‰, $\delta^{34}S$=13.1±1.5‰), low in terrestrial animal protein and high in aquatic protein intake, despite its inland location. No statistically significant differences (p>0.05) were found between sexes or socio-economic status. $\delta^{15}N$ differed significantly between skeletons with non-specific generalised infections ($\delta^{13}C$=-18.7±0.8‰, $\delta^{15}N$=9.9±0.4‰) and those with only healed tibial periosteal reactions (p<0.003, $\delta^{13}C$=-18.0±1.1‰, $\delta^{15}N$=10.9±0.7‰) or without lesions (p<0.004, $\delta^{13}C$=-18.6±0.5‰, $\delta^{15}N$=10.8±0.8‰). No significant differences were noticed between sexes. Bone segments with active lesions ($\delta^{15}N$=11.1±0.9‰) had higher $\delta^{15}N$ than those without lesions ($\delta^{15}N$=10.7±0.7‰), a statistically significant increase of 0.4‰, t(13)=-2.58, p=0.02.

Individuals with unspecific generalised infections potentially had less access to animal protein than those without lesions or only healed periostitis. Still, no signs of protein catabolism were observed in the bones without lesions but the same was not true to bone growths that grew during or after the disease. The increase in $\delta^{15}N$ seen in active lesions, when compared with $\delta^{15}N$ from non-lesion regions on the same long bone, may be a consequence of altered protein metabolism. These results suggest that different diets may be linked to an individual's susceptibility to pathogens and that intra-bone stable isotope variation may be related to different diets and/or metabolism during or after the disease. Stable isotopes can help better understanding diseases in the past and the individuals' response to the diseases in the absence of modern medicine and antibiotics.

Keywords: paleodiets, infectious diseases, stable isotopes, anabolism, catabolism

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Chapter 1

Introduction

Stable isotope analysis is frequently used to reconstruct diet from past populations. However, stable isotope values can be compromised by other factors, still not well known, such as physiological stress or pathologies. The associations between diet, immune function and infectious disease are of great importance to public health as well as having evolutionary significance.

This project combines osteological and archaeometric analysis in order to investigate the relationship between diet and health, testing if this synergy can be assessed from stable isotope analysis to bone collagen. The main goal of this project is to determine if there is a link between diet and health assessed by carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) ratios from bone collagen of skeletons that retain evidence of disease. This knowledge will improve dietary interpretations and test the possibility of using stable isotope analysis as a tool to better understand health in past populations. It will be particularly illuminating to investigate this in a population for which the absence of antibiotics and modern medicine provides an overview free of these potentially confounding morbidity reduction interventions.

There is a bidirectional relationship between diet and health. Malnutrition can lead to a rapid death or result in physiological stress and impair the immune function (e.g. Woodward 2001), increasing the individual's susceptibility to infectious diseases. Infections can also lead to a rapid death but the individual can also recover from the disease or survive long enough for it to leave lesions on the individual's bones (Wood et al. 1992, Ortner 2003). Infectious diseases, on the other hand, increase resting energy expenditure, decrease

dietary intake and are usually associated with nutrient malabsorption (Calder 2013, Mitra et al. 1997, Murray and Murray 1979) resulting in malnutrition.

It is intended to assess whether stable isotope ($\delta^{13}C$ and $\delta^{15}N$) analysis can be used as a tool to study the impact of diet on the individuals' immune system and their susceptibility to pathogens. This information will provide answers on the physiological mechanisms of stress and disease prior to the advent of modern medicine as well as improve dietary isotope data interpretation.

Studying ancient populations gives an important contribution, not only for the study of health in the past, but also as a predictor of how future environmental conditions may affect health at a time when the continued supply of effective antibiotics is under threat.

1.1. Aims and predictions

1.1.1. Osteological sample and demographics of Tomar skeletal collection

There are a wide variety of factors that can affect stable isotope ratios and various studies suggest dietary differences between sex, age groups and social status (e.g. Adamson 2004, Kjellström et al. 2009, Linderholm et al. 2008, Polet and Katzenberg 2003, Schutkowski et al. 1999, Reitsema et al. 2010, Reitsema and Vercellotti 2012). For these reasons it is important to have background information about the samples under study. This is particularly important for Tomar skeletal collection. By being controlled by religious military orders the social status gap between males and females, for example, could have been larger than in other populations.

Socio-economic status affects not only the access to food resources but it also has an important impact on the health status (Goodman and Leatherman 1998) as it influences access to nutritious food resources and health care, exposure to pathogens, strenuous

biomechanical effort, settlement density, sanitation and hygiene. The distribution of individuals from different sex and age within Tomar's necropolis will give a better understanding on how these different groups were viewed as proximity to church and the type of grave can reflect socio-economic status (Binski 1996, Daniell 1998, Graves 1989, Ottaway 1992, Platt 1981, Swanson 1989). I expect that in the areas closer to the church the amount of males will be larger than the females, more structures graves and fewer non-adults than the areas further away from the church.

Stature is frequently used as an indicator of physiological stress (e.g. DeWitte and Hughes-Morey 2012, Barker et al. 1990) but it is also related with socio-economic status (e.g. Bogin 1999, Johnson and Padez 1999, Larsen 1987). I expect that the individuals buried closer to the church will have higher statures than those buried further away from the church. Since stature is related with physiological stress, shorter individuals usually reach reproductive maturity earlier stages and die at younger ages (Metcalf and Monaghan 2001, Walker et al. 2006, Kuzawa 2007, Stock and Migliano 2009) I expect that, also in Tomar, the elderly individuals will have higher stature than young adults. It is also expected that the individuals buried in structured graves will be taller than those buried in graves excavated on the soil.

Given the importance of correctly estimating sex and stature and their high population specification (e.g. Bidmos and Dayal, 2004) one of this project's aims is to develop specific equations for Tomar collection.

1.1.2. Estimating the diet at Tomar

This work will provide insight into the medieval diet in Portugal, being the first stable isotope analysis to reconstruct diet during this historical period. Various studies suggest dietary differences between sex, age groups and social status in Medieval times (e.g. Adamson 2004, Kjellström et al. 2009, Linderholm et al. 2008, Polet and Katzenberg 2003, Schutkowski et al. 1999, Reitsema et al. 2010, Reitsema and Vercellotti 2012). The diet at Tomar will be estimated using carbon, nitrogen and sulphur stable isotopes. By reconstructing diet at Tomar it will also be possible to distinguish dietary patterns between sex, age groups and socio-economic status that may have been influenced by the presence of the religious military orders. This study will also provide a dietary control group which will aid understanding the relationship between diet, health and metabolism.

Historical literature indicates that the amount of meat consumption among Templars was lower than in individuals with similar social status (Barber and Bate 2002). In medieval times religious military orders had total control of towns and hunting and fishing rights (Vicente 2013), but their influence on the general population diet remains unknown. I expect that religious dietary restrictions may have had a higher impact in towns governed by religious military orders. Therefore, their influence probably led to a higher intake of aquatic or vegetal protein when meat consumption was not allowed than in other towns from similar chronologies. I also expect that there will be a larger dietary difference between sexes than in other populations. At Tomar men would actively be part of the local army alongside the order's knights (Conde 1996) which could have given them access to different food sources than the females.

This town's rich history and people movement make it very interesting for dietary reconstruction and comparison with other European medieval collections. Tomar was a

Templar town located in the main Portuguese route and one of its functions was to receive and protect refugees in case of invasion (Conde 1996). I expect to see a few outliers that may not have lived all of their life in Tomar. This study will also gather some information about new food resources, such as sugar cane, taken to Portugal during the Age of Exploration (15^{th} to 17^{th} centuries), which would only be available for the people in the higher socio-economic ranks. It may be possible to see these new food sources intakes among the individuals from Tomar.

Estimating stable isotope ratios for terrestrial fauna will aid understanding the biological distribution of stable isotopes geographically, as well as provide information on domestication and animal husbandry.

1.1.3. Diet and Health

Health is a complex state that can be reflected through skeletal indicators of physiological stress (Temple et al. 2014). Most of the modern developing countries still struggle to decrease malnutrition and infectious diseases (WHO 2009, Doak et al. 2005). One of the aims of this project is to infer the effect of diet on health by exploring the correspondence between stable isotope ratios and indicators of non-specific (periostitis and/or osteomyelitis) and specific (venereal syphilis) disease. It is also intended to examine stable isotope ratios between individuals at different disease stages.

There is a bidirectional relationship between diet and health. Protein malnutrition over a long period of time impairs the immune system and increases the likelihood of an individual contracting an infectious disease (e.g. Woodward 2001, Woodward 1998). Individuals with skeletal signs of infectious diseases might have had different diets than those without skeletal lesions.

Woven bone is produced during rapid bone formation and when it is observed in adults it is considered of pathological origin (Ortner 2003, Ortner and Putschard 1985). Since in chronic or healing stages the woven bone is rapidly remodelled into compact bone, woven bone is considered a lesion which was active *peri-mortem*, while compact bone is considered a lesion which was healed *peri-mortem* (Ortner 2003, Ortner and Putschard 1985). Chronic infections can also have various acute phases and be very informative about the nutritional adequacy of the diet in a specific community (Goodman and Martin 2002).

Since protein malnutrition impairs the immune system (e.g. Woodward 2001, Woodward 1998, Scrimshaw and SanGiovanni 1997, Calder 1991), I predict that skeletons without lesions may have had diets richer in animal protein than those with lesions, with those with only active lesions having the lowest animal protein intake. The presence of skeletal lesions can also represent an adaptation to a pathological condition (Ortner 2003, Wood et al. 1992) indicating that the individual survived long enough to the pathology for it to leave evidence in the skeletal tissues (Wood et al. 1992). The individuals with only healed lesions are expected to have had similar diets to those without lesions as they survived the disease long enough for the bone to remodel into compact bone (Ortner 2003, Wood et al. 1992, Ortner and Putschard 1985).

However, the absence of osteological stress markers does not necessarily mean low level of physiological stress (Wood et al. 1992). Acute infections can lead to a rapid death, without leaving any signs in the skeleton. The absence of skeletal lesions is ambiguous; it can indicate either a good health status or a fast death as result of an acute disease (DeWitte and Stojanowski 2015, Siek 2013, Wood et al. 1992).

The complex relationship between nutrition and immunity to pathogens has received increasing attention in modern populations. In ancient pre-antibiotic populations

this context has been little considered. Still, archaeological collections are pre-antibiotic allowing a more direct study of human-pathogen co-evolution and may helderly important lessons at a time when the continued supply of effective antibiotics is under threat. For these reasons bioarchaeological collections are a good model to study diet and health without the confounding factor of modern medicine.

1.1.4. Effect of different healing stages on stable isotope ratios in skeletal lesions

Physiological stress is one of the factors that can affect stable isotope ratios (e.g. D'Ortenzio et al. 2015, Fuller et al. 2005, Gaye-Siesseger et al. 2004, Hobson and Clark 1992, Hobson et al. 1993, Oelbermann and Scheu 2001, Steele and Daniel 1978). However, it is not clear how bone may reflect different metabolic stages during periods of physiological stress or its recovery.

This study compares bone collagen stable isotope ratios between sites that retained evidence of disease with those that did not retain evidence of disease. The main aim of this study is to infer if skeletal lesions at different healing stages (active or healed) show different stable isotope ratios between themselves and when compared with non-lesion sites from the same bone. This thesis represents the first study to differentiate between healed and active legions, which may highlight different metabolic stages. Previous research showed higher values of $\delta^{15}N$ in lesions when compared with non-lesion sites (Olsen et al. 2014, Katzenberg and Lovell 1999) but the different healing stages were not taken into consideration.

Infectious diseases can decrease nutrient availability due to malabsorption (e.g. Mitra et al. 1997) and increase resting energy expenditure, altering the metabolism and

redistribution of nutrients (Calder 2013) within the body's tissues. Therefore, differences between healed and active diseases may represent different metabolic stages. Chronic physiological stress resulting from infectious diseases may affect the isotopic composition of the individual's bone collagen. In prolonged cases of disease, nutritional or physiological stress, dietary protein cannot adequately replace nitrogen losses (Welle 1999, Powanda 1977, Grossman et al. 1945). Consequently, the body proteins are recycled resulting in enriched ^{15}N and consequently high $\delta^{15}N$ values (e.g. D'Ortenzio et al. 2015, Deschner et al. 2012, Hobson et al. 1993, Steele and Daniel 1978).

Bone collagen from non-lesion sites may represent an average dietary signal of up to ten years prior to the individual's death (Hedges et al. 2007). However, bone lesions are the result of repair mechanisms initiated before the cessation of the disease state or the clearance of a pathogen from the organism (Klaus 2014, Ragsdale and Lehmer 2012, Neve et al. 2011). As mentioned above, skeletons with active lesions represent individuals whose disease was active *peri-mortem* while those with healed lesions may represent individuals who were healed from the disease. Consequently, while non-lesion sites represent long-term isotopic signals, stable isotope ratios from the lesions represent short-term changes that may represent fluctuations on the metabolic balance of the individual. Therefore, I expect to observe a $\delta^{15}N$ increase in active lesions and a $\delta^{15}N$ decrease in healed lesions when compared to non-lesion sites within the same bone.

1.2. Thesis organization

This dissertation includes three independent research articles submitted for publication in peer-reviewed journals (Chapters 5, 6 and 7). Each one of these chapters has specific objectives, introduction, methodology, results, discussion and conclusion. Additionally to these data chapters there is an introductory chapter to the thesis (Chapter 1), a chapter with the research background (Chapter 2), another one with general methodology (Chapter 3) and one introducing the necropolis. At the end of the thesis there is a general conclusion (Chapter 8).

Chapter 1 describes the thesis organization and highlights the general aims of this research, as well as main questions and predictions.

Chapter 2 provides a general research background, introducing the historical background of Tomar, an introduction to bone remodelling and stable isotope analysis.

Chapter 3 gives a general overview of the samples, sampling strategy and methodologies used in this thesis.

Chapter 4 analyses the raw data from the excavation at Santa Maria do Olival, Tomar. Understanding the historic and demographic context of Tomar osteological collection will give a better insight on social status and its relationship with indicators of health. This insight is key to posterior diet estimations within Tomar's collection.

Chapter 5 analyses bone collagen stable isotope data (carbon, nitrogen and sulphur) from 33 human adults without skeletal lesions (15 females, 18 males) and 13 faunal remains in order to understand the baseline diet at Tomar.

Chapter 6 explores the correspondence between stable isotope ratios (carbon and nitrogen) and indicators of non-specific (periostitis and/or osteomyelitis) and specific (venereal syphilis) disease as well as different disease stages.

Chapter 7 aims to assess if pathological bone growth, active or healed, has a measurable effect on stable isotope ratios (carbon and nitrogen). Isotope values of lesions are compared with tissues without lesions to better understand the use of carbon and nitrogen (ingested or recycled in the body) in bone tissue repair.

In Chapter 8 the results from the different chapters are discussed together highlighting the contributions of this research. This chapter summarizes the conclusions of the preceding chapters and directs towards possible future directions.

Chapter 2

General background

2.1. Historical context

Tomar is a Portuguese city crossed by the Nabão River and located approximately 10km from the right margin of the Zêzere River and 50km from the Atlantic coast (Figure 2.1.). The city of Tomar is part of Santarém District in the centre of Portugal.

The construction of the Convent of Christ (Figure 2.2), a Templar stronghold, started in 1160 and it was most likely about this time that the Church of Santa Maria do Olival (Figure 2.3) was constructed (Conde 1996). The church was built over the Monastery of Santa Maria de Selho, likely from the 7th century (Dias 1979). The Church of Santa Maria do Olival would later become the pantheon of the Grand Masters of the Temple.

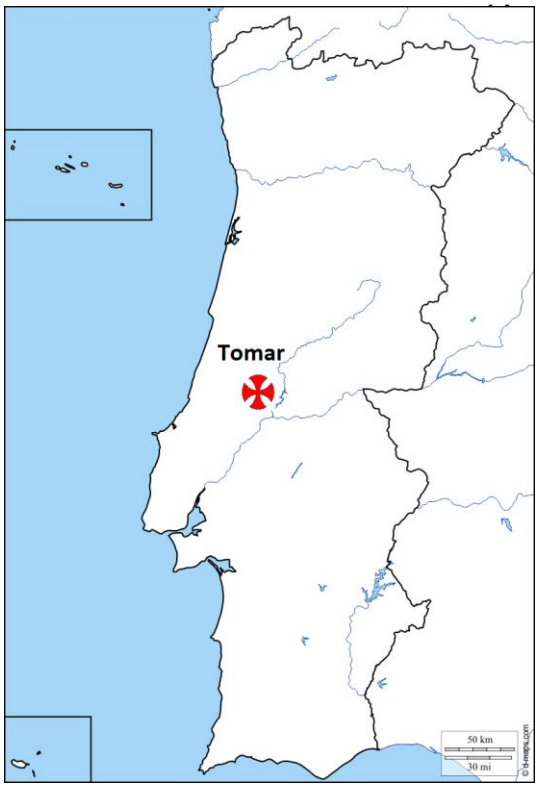


Figure 2.1. Map of Portugal showing the location of Tomar. Adapted from d-maps.com.

Figure 2.2. Convent of Christ. Taken in April 2015.

Figure 2.3. Church of Santa Maria do Olival main entrance and bell tower. Taken in April 2015.

Tomar had a very important military role consolidating the Kingdom of Portugal by resisting the advances of the last Moroccan king of Hispania, Iacub ben Iuçuf Almançor (França 1994). Catholic religious military orders were formed with the purpose of opposing against Islamic conquests in the Holy Land and later in the Iberian Peninsula. The Knights Templar was the largest and most influential of the military orders. The Templars were closely related to the Crusades but up to 90% of the order's members could be non-combatants managing large economic infrastructure (Barber 2012). The Order of the Temple, and later the Order of Christ, had an extensive estate between the rivers Zêzere and Tagus where they applied their own law, being described as "a little kingdom inside the kingdom" (Vicente 2013). The local church had no power over the Order of the Temple as the military order only answered to the Pope (França 1994, Pereira 2006). The control that the military orders had on Tomar may have had an impact on the general population,

including its diet due to strict religious dietary restrictions (e.g. Müldner et al. 2009, Vicente 2013) and their control over hunting and angling rights (Vicente 2013).

As a Templar town, one of its functions was to receive and protect refugees from other Portuguese towns in case of invasion (Conde 1996), so it is possible that some of the individuals buried at Tomar did not live there all of their life. Tomar was located at *strada maiore*, the main Portuguese route connecting the North of the country to the limits of the *Reconquista* (Conde 1996), having a mixture of goods, people and diseases. The advances of the Portuguese frontier towards the south encouraged migration from the north of the country. This may have led to integration problems but was important for the demographic balance of the country (Mattoso 2009). In the beginning of the 11th century, due to the reorganization of the Christians and the disruption of the caliphate, religious intolerance increased, ending the long period (8th to 10th centuries) of familiarity and acceptance between Christians and Muslims (Mattoso 2009).

In 1317 Pope Clement issued the bull *Pastoralis Praeeminentiae*, instructing all Christian monarchs in Europe to arrest the Templars and seize their assets (Barber 2012). Portugal successfully lobbied the papacy and the Templars did not face a trial, instead the Order's assets and personnel were transferred to the newly-established Order of Christ, a continuation of the Templar Order in Portugal (Valente 1998). Tomar became a centre of Portuguese overseas expansion under Henry the Navigator, the Grand Master of the Order of Christ (Conde 1996).The black plague reached Portugal in 1348 and its impact on the demographic recession was visible until the 15th century (Conde 1996). The impact of leprosy, on the other hand, decreased significantly during the 14th century as in almost all Occidental Europe (Sournia and Ruffié 1986). During the 14th century there was also political

instability due to the civil war between King Dinis and Prince Afonso, who later became King Afonso IV, as well as Castilian invasions (Conde 1996).

The only known document with demographic information about Tomar dates from 1527, documenting a total of 2,253 people from which 737 lived inside the town walls (Freire 1908). The houses in medieval Tomar were made of stone, wood, clay and pug. With an average area of 60m^2 (higher than in most Portuguese towns, which varied between 36 and 42m^2), the houses had dirt floor and tile or thatched roof (Conde 1996). Frequently the houses had small yards (usually less than 50m^2) with vegetables and fruits, and animals such as pigs and poultry often moving freely in the street (Conde 1996). Outside the town's limits there were plots of cultivated land and orchards with olive trees, vineyards and cereals (Conde 1996).

2.1.1. The excavation of the necropolis of Santa Maria do Olival

Santa Maria do Olival graveyard was used from the 11th to the 18th century. This wide chronology adds to the complexity of studying Tomar's collection. Over this period of time there were new food sources introduced in Europe from America, which may be visible in stable isotope data from Tomar's collection. Structures from the roman city *Sellium* and its *Forum* were found near the Church of Santa Maria do Olival in previous excavations (Batata 1997, Batata and Ponte 1983, Ponte 1982, 1985, 1989, Ponte and Batata 1987, Ponte and Silva 1982). The Islamic presence in Tomar is not very clear, so far only a silver coin was found in the area of the church (Ponte and Miranda 1994).

The excavation occurred in two phases, the first in 2007 and the second in 2008, in an area of approximately 6,500m^2. The first phase of the excavation was divided into 11 areas (two of them without human remains) and the second phase was divided from area

13 to 20 (Figure 2.4). However, these divisions were made by the archaeologists to organize the excavation and do not represent different chronologies or graveyard arrangements. The masters of the Templar Order would be inhumed inside the church (Barroca 1987), however, Templar graves (Figure 2.5) were found in area 18. These graves were not excavated, as they were not going to be affected by the construction works.

Tomar's archaeological collection is made up of 3,675 primary inhumations and 1,456 ossuaries with a total of at least 6,792 individuals, 4,991 adults and 1,801 non-adults. The skeletons were positioned in accordance with Christian funerary rituals, in dorsal decubitus and oriented from west to east (Barroca 1987). The upper members were frequently flexed at the lumbar or pelvic region and the lower limbs were mostly straight or with ankles crossed. The graves were mostly excavated into the soil with anthropomorphic, oval or sub-rectangular morphology. In addition to the 3,675 primary inhumations, 1,456 ossuaries were excavated, from which 1,233 were associated to primary inhumations. The minimum number of individuals in each ossuary varied from 1 to 14 with both sexes and all ages represented. Six ditches were also excavated, varying between 2 and 32m^2, the smallest one with 2 primary inhumations and 1 ossuary and the largest one with 35 primary inhumations and 9 ossuaries.

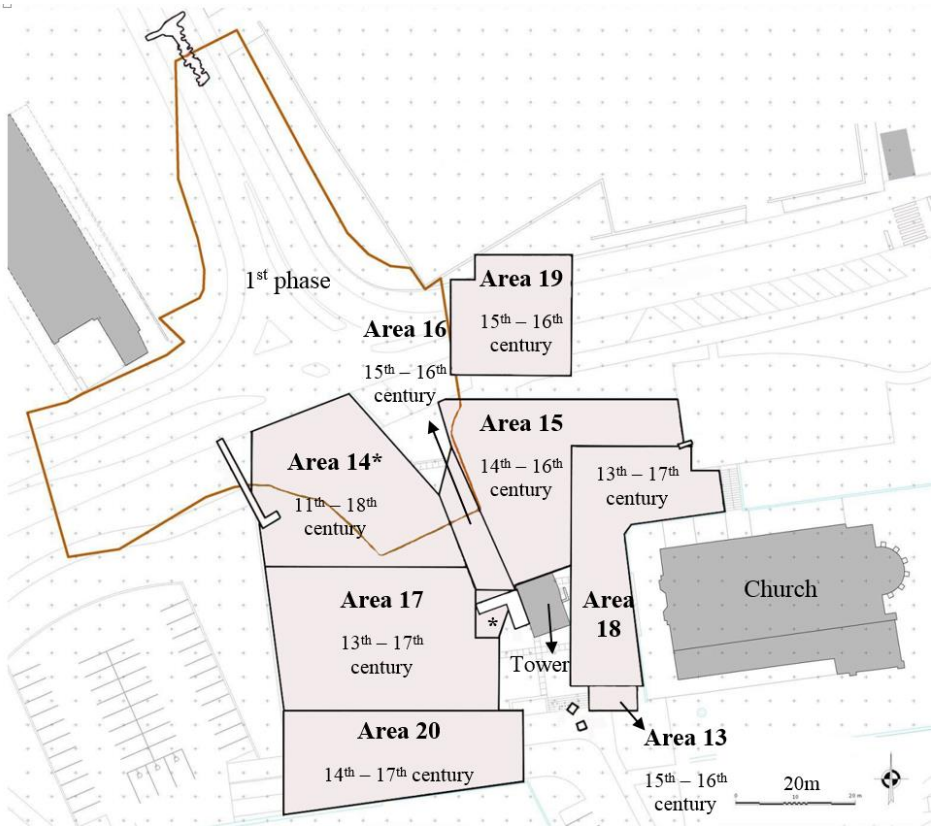


Figure 2.4. Map of the excavation. Area excavated during the 1st phase and areas 13 to 20, excavated during the 2nd phase. Adapted from the maps provided by Sérgio Pereira and Ricardo Ribeiro.

Figure 2.5. Examples of possible Templar graves in area 18. Photography taken by Sónia Ferro during the excavation.

2.1.2. Medieval diet in Portugal

To date, there are no published dietary studies based on stable isotope data for the medieval times in Portugal. There are various stable isotope studies from medieval Mediterranean collections (e.g. Alexander et al. 2015, Bourbou et al. 2011, Fuller et al. 2012, Reitsema and Vercellotti 2012, Salamon et al. 2008) but none from Portugal for this historical period. According to historical data, the base of the medieval diet in Portugal was bread, wine, olives and olive oil (Vicente 2013). A significant part of the agriculture was focused on cereals, indispensable to make bread, the dietary staple. However, a large percentage of the harvest would have been used to pay tribute to the lords and the church (Vicente 2013). When necessary, chestnuts and sweet acorns could substitut the bread (Vicente 2013) and some legumes could have been reduced to flour when there was a lack of cereals (Gonçalves 2004).

Meat was considered the food of the powerful, who consumed it in abundance (Gonçalves 2004). Cattle were not abundant, in contrast to sheep and goat, and only pigs were purposely raised for meat production (Gonçalves 2004). Acorns were frequently used to feed the livestock, especially the swine (Vicente 2013). Other sources of meat were chicken, duck, goose, pigeon, turtledove and pheasant as well as a variety of game (Gonçalves 2004). While hunting was a ludic activity for the lords, for the peasants it could represent the only access to meat and a way to get money from its sale (Vicente 2013). As the economic system started relying more on agriculture based around cereals the access to meat became more difficult, especially combined with hunting limitation imposed by the lords. In addition to hunting there was also some foraging from which the peasants could collect honey before it was produced in resting fields in the 1400's (Vicente 2013).

Eggs and cheese were part of the diet of people from all social statuses (Gonçalves 2004). Salmon, flounder, hake, shad and lamprey were expensive food items, while sardines were more abundant and easy to preserve by either salting or smoking them (Gonçalves 2004). Fish was indispensable during the numerous fast days imposed by the medieval religious calendar (Vicente 2013). Still, fish was consumed mostly in the littoral despite its presence in the Portuguese rivers (Gonçalves 2004). Fishing in rivers was limited, due to angling right restricitons, reducing access to fish for individuals from low socio-economic status (Vicente 2013). Molluscs and crustaceans were part of the diet of people from all social status but were considered a "food of the poor" due to their abundance (Gonçalves 2004).

Fruits and legumes, fresh or dry, were consumed by both rural and urban populations. A variety of figs, olives, pears, apples, plums, peaches, cherries, pomegranates, walnuts, almonds, hazelnuts and chestnuts as well as lupin beans, beans, peas, chickpeas, lentils, cabbage, turnips, lettuce, spinach, carrots, eggplants, onions and garlic (Gonçalves 2004, Vicente 2013) were very important sources of vitamins and minerals. Wine was accessible to all social statuses, from childhood to elderly age, watered down and sweetened with honey and spices (Gonçalves 2004).

Tomar's collection is particularly interesting as it represents a Templar town, with its own rules. The historical bibliography (Barber and Bate 2002) suggests that the amount of meat consumption in Templars was lower than in individuals with similar social status and the vegetables intake higher, which Franceschi et al. (2014) associate with their longevity. Müldner et al. (2009) observed different isotope ratios between bishops and the general population in Scotland, suggesting that the bishops had higher fish intake than the rest of the population, possibly related to religious fasting. It is possible that these dietary

restrictions could reflect in the population as angling and warren rights belonged to the Military Orders, which also had large extensions of land from which they received tribute (Vicente 2013). In Tomar, merchants, crafters and farmers participated actively in the local army alongside with knights, raising their status (Conde 1996) and likely having access to similar food resources to the Templars.

Tomar has a complex history marked by the presence of religious military orders and probably frequent movement of people and goods. Since there are no published stable isotope data from Portugal close to this chronology, the available dietary information rely on historical data. Historical data suggest that the Portuguese diet was diverse, but bread was the base of people diet despite their social status. Still, there may be large dietary differences between individuals from different socio-economic groups, or as a result of the large chronology of this collection (11^{th} – 17^{th} centuries).

2.2. Stable isotope analysis (SIA)

Isotopes are atoms of the same element with the same number of protons but different number of neutrons. As the atomic mass is determined by the number of protons and neutrons, an extra neutron makes the nucleus heavier but does not affect most chemistry that is related to reactions in the electron shell. Fractionation is the basis for stable isotope variation in biological and geochemical systems (Fry 2006). In kinetic reactions the light isotopes usually react faster, while the heavy isotopes concentrate where bonds are strongest and the sources recombine completing the isotope cycle (Fry 2006). The stable isotope ratios compare a sample to a set standard, positive values indicate that a sample has more of the heavy isotope than the standard and negative values indicate that a sample has fewer heavy isotopes than the standard (Katzanberg 2008). Since the alterations

resulting from fractionation are so small the isotopic composition is represented as *per mil* (‰) and presented with the relative difference between R's isotopic ratio through the expression:

$$\delta\ (‰) = [R_{(sample)} / R_{(standard)} - 1] \times 1000,$$

where

$$R = {}^{H}F / {}^{L}F \text{ and}$$

F = fractional abundance of the heavy (^{H}F) or light (^{L}F) isotope.

To determine the isotope values for the carbon, the international reference standard (Hoefs 1997, Coplen 1994) is the PeeDee Belemnite (PDB) from the marine fossil *Belemnitella americana*, to the nitrogen the standard is the atmospheric air or Ambient Inhalable Reservoir (AIR), so:

$$\delta^{13}C\ (‰) = [(^{13}C/^{12}C)_{sample} / (^{13}C/^{12}C)_{PDB} - 1] \times 1000$$

$$\delta^{15}N\ (‰) = [(^{15}N/^{14}N)_{sample} / (^{15}N/^{14}N)_{AIR} - 1] \times 1000$$

It is possible to use a wide variety of tissues in stable isotope analysis, from bone and teeth to nails, hair and blood, reflecting different time periods in an individual life. The first one to be used in an archaeological context was the bone collagen. Although bone collagen degrade over time it is often preserved long after burial, depending on the burial environment (Schwarcz and Schoeninger 1991, Stafford *et al*. 1988) and it has been found collagen fibre remains in fossils dating at least to the Cretaceous (Schweitzer *et al*. 2009, Bertazzo *et al*. 2015). Carbonate, present in the mineral portion of bone, is another source of carbon in both bones and teeth (Katzenberg 2008), especially when collagen is not well preserved (Lee-Thorp 1989, Lee-Thorp and Sponheimer 2006, Sponheimer and Lee-Thorp

1999). However, while collagen stable isotope ratios reflect mainly ingested protein, stable isotope ratios in biological apatite reflect whole diet (Ambrose and Norr 1993, Krueger and Sullivan 1984).

Stable isotopic analysis is a way to directly follow and trace details of element cycling of organic matter (Fry 2006) and it use as a way to infer diet started in the late 70's, with the pioneer works from Vogel and van der MerI (1977), van der MerI and Vogel (1978) and DeNiro and Epstein (1978) focusing on carbon ratios. Later, DeNiro and Epstein (1978, 1981), Schoeninger et al. (1983), Minagawa and Wada 1984, Schoeninger and DeNiro (1984) and Ambrose and DeNiro (1987) applied the stable isotopic analysis to the nitrogen enrichment within food webs.

Analysis of stable isotope ratios from mineralized tissue has been widely used for dietary reconstruction. This technique is based on the assumption that "you are what you eat (plus a few ‰)" (DeNiro and Epstein 1976), as a consumer`s tissues reflect the isotopic signature of the ingested foods. However, stable isotope ratios can be compromised by other factors, such as physiological stress (D'Ortenzio *et al*. 2015, Fuller *et al*. 2005, Gaye-Siesseger *et al.* 2004, Hobson and Clark 1992, Hobson *et al*. 1993, Oelbermann and Scheu 2001, Steele and Daniel 1978) or pathological conditions (Katzenberg and Lovell 1999, Olsen *et al*. 2014). Habitat, body size, metabolism and digestive physiology are other factors that may confound diet/tissue relationships (e.g.Metges et al. 1990, Schoeninger et al. 1997, 1998, 1999, Cerling and Harris 1999, Passey et al. 2005). Another one of the limitations on using bone collagen for diet estimation is that bone collagen do not reflect whole diet but only protein intake (e.g. Froehle et al. 2010).

Stable isotopic analysis can address questions in human biology about diet, mobility and nutritional stress. Although initially applications of stable isotope analysis were mostly directed to diet reconstruction thereafter it has been addressed to questions that include determining the duration of breastfeeding, effects of disease processes and determination of migration patterns. Lately, SIA has expanded from paleoanthropology and bioarchaeology to applications among living humans, becoming a tool even more important for understanding dietary habits and physiology.

2.2.1. Carbon stable isotopes

The carbon cycle involves active exchanges of CO_2 among the atmosphere, terrestrial ecosystems and the surface ocean. Most of the active carbon in the cycle is in the bicarbonate form dissolved in the ocean (about 1‰) and withdraw -8‰ during fractionation to the atmospheric CO_2 (Fry 2006). During photosynthesis the plants use the atmospheric CO_2 as a carbon source. This process results in isotopic fractionation, with a decrease in the heavier carbon isotope (^{13}C), once the lighter isotope (^{12}C) is preferentially incorporated in the plant's tissues (Schoninger and DeNiro 1984). However, the fractionation during photosynthesis varies depending on the photosynthetic carbon fixation by plants: C_3 pathway (Calvin-Benso cycle), C_4 pathway (Hatch-Slack cycle) or CAM pathway (crassulacean acid metabolism) (Chisholm 1989). Most CAM plants are succulents, while C_3 plants include most vegetables and fruits, wheat, rice and nuts. C_4 plants have as examples sugar cane, maize, sorghum, African millets and tropical pasture grasses (Smith 1972). The $\delta^{13}C$ values are very important to understand the domestication process of both plants and animals.

C_4 plants discriminate against the isotopically heavier ^{13}C isotope when using CO_2, as a result C_4 plants have a less negative $\delta^{13}C$ values (averaging – 12,5 ‰, Vogel 1978, Chisholm 1989) than C_3 plants (averaging – 26,5 ‰, Vogel 1978, Chisholm 1989) and CAM plants. The CAM plants have $\delta^{13}C$ values between C_3 and C_4 plants (Katzenberg 1992) but in hot arid regions their isotope values are similar to the ones found in the C_4 plants (Schoeninger 1995). These isotopes ratios have an impact on the food webs isotope values due to the correlation between the animal's tissues $\delta^{13}C$ values and their diet (DeNiro and Epstein 1978, Teeri and Schoeller 1979). There is enrichment in ^{13}C on the animal's body tissues relatively to their diet caused by the fractionation that occurs during the tissue's formation (van der MerI and Vogel 1978). The consumers have a fractionation factor (enrichment in ^{13}C) of approximately 5‰ on their bone collagen relatively to their diet (van der MerI and Vogel 1978, Ambrose and Norr 1993) and an enrichment of 1‰ between trophic levels (DeNiro and Epstein 1978, Tieszen et al. 1983), although the trophic level enrichment is not yet completely understood (van Klinken et al. 2000, Bocherens and Drucker 2003).

In marine plants the main carbon source is dissolved carbonate (0‰), instead of atmospheric CO_2 (-7‰), therefore, this difference reflects on the $\delta^{13}C$ values in mammal's tissues feeding from these two different ecosystems (Tauber 1981, Chisholm et al. 1982, 1983). Marine plants have $\delta^{13}C$ values between the extremes of the terrestrial C_3 and C_4 plants (Katzenberg 1992, Schoeninger 1995). The average $\delta^{13}C$ value for phytoplankton is approximately -19‰ (Chisholm et al. 1982, Chisholm 1989). Like in the terrestrial plants there is an enrichment of 5‰ in the phytoplankton consumers and approximately 1‰ between trophic levels (Chisholm 1989). Although it is possible to analyse shifts in dietary habits and exploration of coastal areas (e.g. Chisholm et al. 1982, Schoeninger *et al*. 1983,

Tauber 1981) there are some limitations when estimating the importance of marine foods in the diet using only $\delta^{13}C$ values: it is only valid in the absence of C_4 plants (Schoeninger and DeNiro 1984).

The $\delta^{13}C$ values on bone collagen can also help identifying freshwater food sources. Katzenberg and Weber (1999) observed a range of - 14,2 to - 24,6 ‰ in fish bones. Freshwater fish exhibit variation in $\delta^{13}C$ values depending on the ecosystem as freshwater plants have numerous sources of carbon, unlike terrestrial plants (Zohary et al. 1994, Dufour et al. 1999).

2.2.2. Nitrogen isotopes

Most nitrogen in the biosphere is present as N_2 gas in the atmosphere (with an isotope composition near 0‰) and the $\delta^{15}N$ values present on the organisms vary depending on how the nitrogen enters the biological domain of the ecosystems (Fry 2006). The nitrogen cycle has various microbially-driven processes responsible by the chemical reactions that change the nitrogen components. During the nitrogen fixation, N_2 is converted into biologically available nitrogen by nitrogen-fixing organisms (free-living or symbiotic). Nitrification consists in oxidation of ammonium compounds in organic material (ammonia) into nitrates by soil bacteria making the nitrogen available to plants. Anammox, which was only recently described (Strous et al. 1999) is a type of ammonia oxidation but occurs under anoxic conditions. Ammonification consists in the decomposition of organic material by fungi and prokaryotes releasing inorganic nitrogen back into the ecosystem as ammonia. Finally, during denitrification the nitrates are converted into nitrogen gas which is then released into the atmosphere.

Legumes have a symbiotic relationship with bacteria of the genus *Rhizobium* (nitrogen-fixing organisms) forming nitrogen-fixing nodules on the plants roots. This more direct way to access nitrogen results in $\delta^{15}N$ values for the legumes closer to that of atmospheric nitrogen. Non leguminous plants on the other hand are more enriched in ^{15}N, once they receive the nitrogen from decomposed organic matter (nitrification or ammonification). Their $\delta^{15}N$ values are closer to the ones found in the soil (10‰, Shearer and Kohl 1989). In terrestrial ecosystems there is an increment shift by 3 to 5‰ between trophic levels when compared with the consumers' diet (Schoeninger et al. 1983, Minagawa and Wada 1984, Schoeninger and DeNiro 1984, Bocherens and Drucker 2003). This fractionation allows using stable nitrogen isotopes to infer trophic level and high $\delta^{15}N$ values usually indicate high-protein diets (Sponheimer *et al*. 2003a, 2003b).

Nitrogen isotope ratios can also be used to differentiate between terrestrial and marine food resources (e.g. DeNiro and Epstein 1981, Schoeninger et al. 1983, Richards and Hedges 1999), especially when combined with carbon stable isotope values. Marine plants have $\delta^{15}N$ values approximately 4‰ higher than terrestrial plants (Ambrose et al. 1997). Animals feeding exclusively from terrestrials resources have $\delta^{15}N$ values for bone collagen lower than 9‰ while the ones feeding exclusively from marine sources have $\delta^{15}N$ values higher than 15‰ (Schoeninger et al. 1983).The stable isotopes fractionation also occurs in marine ecosystems with $\delta^{15}N$ values of approximately 3‰ alongside the trophic webs (Minagawa and Wada 1984, Schoeninger and DeNiro 1984).

The $\delta^{15}N$ values can also be used to analyse access to fresh water resources, once organisms in these ecosystems have similar $\delta^{15}N$ values to the ones found in marine ecosystems (van Klinken et al. 2000). However, lakes are more variable in isotope composition than the ocean (Fry 2006). Bonsall et al. (2000) observed $\delta^{15}N$ values of

approximately 11‰ in fresh water fishes. Other studies recorded values from 10 to 14‰ in fishes from lakes and values between 7 and 9‰ in fishes from rivers (Katzenberg 1989, Hobson 1990, Hesslein et al. 1991).

There are other factors that can raise the $\delta^{15}N$ values, such as water (Virginia and Delwiche 1982, Shearer *et al*. 1983, Ambrose and DeNiro 1986, Heaton *et al*. 1986, Heaton 1987, Sealy *et al*. 1987), physiological (Katzenberg and Lovell 1999, Oelbermann and Scheu 2001, Gaye-Siesseger et al. 2004, Vogel et al. 2012, Deschner et al. 2012, D'Ortenzio et al. 2015) and protein stress (Steele and Daniel 1978, Hobson and Clark 1992, Hobson et al. 1993). In arid ecosystems more urea is excreted relative to the total volume of urine, so there is more ^{14}N loss while ^{15}N is retained in body tissues resulting in $\delta^{15}N$ values increase (Ambrose and DeNiro 1986). The $\delta^{15}N$ values can also detect changes in nutrition without starvation, as observed in primates to whom food was always available but digestible energy content varied (Vogel et al. 2012, Deschner et al. 2012). During insufficient protein intake, new proteins are synthesized from the products of catabolism of existing protein which leads to a ^{15}N enrichment due to the ^{14}N excretion (Hobson and Clark 1992, Hobson et al. 1993). It is important to keep in mind that there is a wide variety of factors responsible for high $\delta^{15}N$ values that can lead to incorrect dietary interpretations (Katzenberg 2008).

2.2.3. Sulphur stable isotopes

One of the largest sulphur reservoir found on earth is the ocean, in the sulphate form, which fixation by phytoplankton occurs with a small isotope effect (1 to 2‰) while sulphate reduction in marine sediment occurs with a larger effect (30 to 70‰, Fry 2006). Although sulphur is primarily found in sedimentary rocks or ocean water, it is particularly important for living organisms, playing an important role in protein structure (Ingenbleek 2006). There

are two important types of bacteria for the sulphur cycle in both the terrestrial and the aquatic part of the cycle: sulphur-oxidizing bacteria (SOBs) and sulphur-reducing bacteria (SBRs). SRBs convert sulphate into reduced sulphur, such as hydrogen sulphide (Fry 2006). SOBs oxidize sulphides into sulphate, which can be absorbed by the plants, as well as sulphur dioxide, which is taken through photosynthesis (Fry 2006).

Recent advances in mass spectrometry and methodology development (Giesemann et al. 1994, Morrison et al. 2000, Richards et al. 2001) following the work of Leach et al. (1996) allow an easier and more frequent analysis of $\delta^{34}S$ values. These values can be useful in discriminating between C_3 and C_4 plants, Richards et al. (2003) observed a decrease of 1‰ in the consumers' tissues when compared with the C_3 plant used in their diet and an increase of 4‰ in the tissues' $\delta^{34}S$ values from the C_4 plant diet. Since $\delta^{34}S$ values are very variable depending on the geology of different places, the use of sulphur isotopes analysis can not only give some information about the diet but also the origin of food sources, to an extend (Bol and Pflieger 2002, Nehlich 2015).

Sulphur isotope analysis can shed some light on the use of fresh water and marine resources (Nehlich 2015), especially when combined with the carbon and nitrogen stable isotope analysis. The mean $\delta^{34}S$ values in the ocean is 20‰ (Ault and Kulp 1959, Thode et al. 1961, Rees et al. 1978, Böttcher et al. 2007) while the $\delta^{34}S$ values in fresh water vary between 0 and 10‰ (Nriagu et al. 1991) and riverine ecosystems have $\delta^{34}S$ values between -5 and 15‰ (Ivanov 1983, Hoefs 2006). Sea spray sulphates have $\delta^{34}S$ values of 20‰ (Nielsen 1974, Norman et al. 2006) affecting the soil $\delta^{34}S$ values, which varies from 10 to 18‰ within a distance of 30km (Wakshal and Nielsen 1982, Mizota and Sasaki 1996). Sea spray also affects riverine ecosystems between that same distance (Cortecci et al. 2002). Marine ecosystems do not have a high $\delta^{34}S$ variation (Fry 1988, Krouse and Herbert 1988,

Thomas and Cahoon 1993, Kwak and Zedler 1997, Hoekstra et al. 2002, Nehlich et al. 2003, Beavan-Athfield et al. 2008). However, $\delta^{34}S$ values in fresh water ecosystems are highly dependent from the local geology and source of water sulphates (Nehlich 2015). Hesslein et al. (1991) observed a range of $\delta^{34}S$ values between less than 5 to 35‰ in rivers fish. The fresh water ecosystems have an important impact also on terrestrial $\delta^{34}S$ values, especially if the fauna fed on the floodplains of a river (Nehlich et al. 2011).

The isotopic fractionation of sulphur between trophic levels is highly variable (Peterson et al. 1985, 1986, Katzenberg and Krouse 1989, Gonzalez-Martin et al. 2001, McCutchan et al. 2003, Richards et al. 2003, Barnes and Jennings 2007, Tanz and Schmidt 2010) with a $\delta^{34}S$ average of 0,5 ± 2,4‰ on the consumers tissues when compared with their diet (Nehlich 2015).

2.3. Health

2.3.1. Bone turnover

Bone is a physiologically active tissue, repairing itself when damaged at a microscopic and macroscopic scale via the constant remodelling and replacement of bone. During growth, bone is remodelled to meet its mechanical demands by combining mechanical resistance with a minimum use of material (Wolff et al. 1986). Bone mass and its architecture are continuously being adapted to the prevailing mechanical loads. Parfitt (1994) estimated that in humans 3% of cortical bone and 25% of trabecular bone are re-absorbed and replaced each year. The bone turnover rate also varies between the different bones and age of the individual (e.g. Fahy et al. 2017, Skedros et al. 2013, Hedges et al. 2007, Hollinger 2005, Seibel 2005, Parfitt 2002).

Bone remodelling is initiated by the appearance of osteoclasts that start re-absorbing bone in response to signals that may relate with local damage (Burr et al. 1996) or osteocyte death (Tapscott 2005, Gabetet al. 2004, Bronckerset al. 1996). Osteoclasts are responsible for the re-absorption of bone tissue, through mineral dissolution and organic matrix degradation (Bab and Sela 2012). Osteoblasts are then recruited, produce and regulate bone matrix and mineralization during the development, remodelling and regeneration. The intercellular communication between osteoblasts and osteoclasts is crucial to bone homeostasis (Xu et al. 2005). The osteoblastic cells control the growth and function of osteoclastic cells by expressing specialised molecules that bind to osteoclasts (Teitelbaum and Ross 2003, Teitelbaum 2000). However, the mechanism in which osteoblasts and osteoclasts communicate and coordinate bone remodelling is still not fully understood. The osteoblasts and osteocytes (mature form of the osteoblast) connect with the embedded cells and mineralization of the matrix completing the osteocytic maturation, which is responsible for function and metabolism of bone tissue.

Bone formation occurs in two distinct stages: bone matrix formation and bone matrix mineralization (Li and Jee 2005). During the bone matrix formation the osteoblasts synthesize and secrete type I collagen fibres orderly parallel oriented to form multilayer lamellar sheets. As the layers of lamellar sheets accumulate some osteoblasts are housed in lacunae and differentiate into osteocytes establishing a three dimensional osteocytic network through the entire bone. The mineralization process occurs then by hydroxyapatite deposition in the gap regions of the mature bone matrix, advancing from the mineralization front to the upper lamellar layer. During growth the remodelling process is responsible for converting woven bone in lamellar bone. In adulthood the remodelling process is the

physiological mechanism responsible for replacing aged or damaged bones balancing bone formation and re-absorption.

Although the bone is a relatively elastic tissue and resistant to external forces a repetitive mechanical stress or a force strong enough to exceed the natural bone structure might cause a fracture (Aufderheide and Rodríguez-Martín 1998, Rodríguez-Martín 2006). Skeletal injuries initiate a multifaceted healing process and according with Boer et al (2015) 4 to 7 days after a bone fracture it is microscopically visible the lesion margins eroded by osteoclasts, smoothening them. The phases of bone healing are similar to the ones observed in osseous growth and development (Caplan 1987, Vortkamp et al. 1998, Schneider and Helms 1998, Shapiro 2008, Marzona and Pavolini 2009). The healing process of a fracture consists is three different phases: cellular, metabolic and mechanical. Ingle et al (1999) as well as Veitch et al (2006) registered bone loss at both proximal and distal sites from the fracture, however, the bone loss was greater distal to the fracture than proximal to it (Ingle et al. 1999). The re-absorption markers increase later than the formation markers, indicating that the early increase in bone markers reflects the callus formation and the later changes represent the callus remodelling (Ingle et al. 1999).

Depending where and at what speed the bone tissue develops, its microarchitecture varies. In woven bone the collagen fibrils are randomly organized (e.g. Chappard et al. 2011). Woven bone has a very rapid growth and in adults is indicative of an aggressive abnormal bone formation active at the time of death (Rana et al. 2009, Roberts and Manchester 2007). The presence of woven bone in adults is the result of abnormal bone tissue growth that may be related with bone fractures healing, neoplasms, response do infection or simply inflammation of the tissues near the bone (Ortner 2003). Although infections are among the most common causes of the inflammatory response, not all such

responses are caused by infection (Bush 1989). In chronic conditions woven bone tends to be replaced by compact bone (Rana et al. 2009, Turner-Walker 2008), a mature, highly organized tissue characterized by regular parallel bands of collagen (Young at al. 2006).

Infection occurs when the body encounters pathogenic organisms while inflammation is the body's vascular response to tissue damage (Weston 2008). Osteomyelitis is an inflammation of bone and bone marrow caused by pus-producing bacteria and clinically may show three different stages: acute, sub-acute and chronic (Aufderheide and Rodríguez-Martín1998). Bone damage triggers the remodelling process, increasing the bone turnover rates (Cho and Stout, 2001). Un-remodelled woven bone indicates active abnormal bone formation at the time of death since in chronic conditions woven bone formed during an acute phase tends to be remodelled into compact bone (Turner-Walker 2008). Although the inflammation can be a response to infection it is not always caused by it.

Non-pathological bone turnover also varies depending on the type of bone, biomechanical load applied on the bone, age and sex of the individuals (e.g. Fahy et al. 2017, Skedros et al. 2013, Hedges et al. 2007, Hollinger 2005, Seibel 2005, Parfitt 2002). While, for example, trabecular bone may be completely renewed between three to four years, the complete skeletal turnover for cortical bone may take over 25 years (e.g. Hedges et al. 2007, Hollinger 2005). Weight-bearing bones are expected to have high turnover rates as the biomechanical load triggers the remodelling process (Cho and Stout 2011). The biomechanical load is also variable between individuals, depending on their physical exercise. Bone turnover rate is higher in non-adults than in adults (Seibel 2005, Mora et al. 1998) also varying depending on the sex of the individuals (Hedges et al. 2007, Seibel 2005).

These factors that affect bone turnover, even in the absence of skeletal pathological conditions.

Bone turnover is also a factor that may have an impact on stable isotope analysis in a way that is still not completely understood. While most studies in human bones did not find significant differences in stable isotope analysis from different bones of the same individual (e.g. Olsen et al. 2014, DeNiro and Schoeniger 1983). Fahy et al (2017) observed a change between -1.6‰ and -0.4‰ in carbon and 1.0‰ and 1.9‰ in nitrogen stable isotope ratios. However, the samples size was small (5 males and 5 females; Fahy et al. 2017). In animal bones various studies have reported significant differences in stable isotope ratios of different bones from the same individual (e.g. Brady et al. 2008, Larson and Longstaffe 2007, Balasse et al. 1999).

2.3.2. Indicators of disease and physiological stress

Health is a complex state that can be reflected through skeletal indicators of physiological stress (Temple et al. 2014). Physiological stress is the result of the disequilibrium in an individual physiology, which can be related with a wide variety of factors such as disease, nutritional deficiencies and strenuous biomechanical effort (Zuckerman and Armelagos 2011, Armelagos 2003, Goodman and Martin 2002, Huss-Ashmore et al. 1992). The interpretation of stress indicators is very important when reconstructing the health status of past populations, as well as their adaptation and physiological response to those situations (Goodman and Martin 2002, Larsen 1997). Chronic diseases or long periods of physiological stress have a higher impact on longevity rather than isolated incidents, which can be overcome during growth (Watts 2015, Palubeckaite et al. 2002, Stodder 1997).

Nutritional stress may result in either greater susceptibility to physiological stress or greater resilience to stress later in life (Bogin et al. 2007). Even though systemic physiological stress is not directly observable in the skeleton its consequences, in some cases, are (Klaus 2014). Skeletal indicators of physiological stress, such as low stature and periostitis, have also been related with long-term effects on health throughout reduced lifespan (Watts 2013) and increased risk of death during epidemics (DeWitte and Wood 2008).

Disease and periods of physiological stress during childhood can result in an increased risk of early mortality and disease in adult life (Barker and Osmond 1986, Barker et al. 1989, Power and Peckham 1990, Blackwell et al. 2001). The cessation or slowing of long-bone growth, a consequence of physiological stress, can result in a decrease in the potential stature of the individual, known as stunting (Saunders and Hoppa 1993, WHO 2013). The adult stature of an individual results from the accumulation of different frequency and amount of growth during each life event (Lampl 2012), which may cause permanent small stature, particularly in females (Bose, 2018). Stature has been shown to be sensitive to both environmental conditions (such as nutrition and disease) and physiological factors (Jantz and Owsley 1984) and stunted growth is one of the main complications that can result from chronic inflammation and infection in juvenile individuals (Pinhasi et al. 2005). Poverty may exacerbate stunting, perpetuating the vicious cycle as vulnerability to malnutrition and disease grows. Nutrients such as vitamin A, zinc, protein and total calories ingested have a very important role in growth maintenance, as well as other factors intervening between diet and nutritional status like work and disease loads (Allen 1984). Stature reveals developmental trends, environmental stress such as nutritional deficits and evolutionary relationships (Moore and Ross 2013), being an important indicator of relative

nutritional health, as poor childhood health and nutrition reflect in adult stature. There is evidence that nutritional interventions in girls are associated with substantial increases in the growth of their offspring (Behrman et al. 2009). Shorter individuals usually reach reproductive maturity earlier stages and die at younger ages (Metcalf and Monaghan 2001, Walker et al. 2006, Kuzawa 2007, Stock and Migliano 2009).

Enamel hypoplasias are also a consequence of systemic physiological stress, such as malnutrition or disease, which disturbs the enamel formation during growth (Hillson 1996). Once formed, the enamel hypoplasias become permanent. These stress indicators are frequently associated with reduced longevity (Goodman and Armelagos 1988, DeWitte and Wood 2008). Still, Watts (2015) found no evidence of the impact of enamel hypoplasias on long term survival. Some researchers (e.g. Palubeckaite et al. 2002, Stodder 1997) suggest that it is the number of defects per tooth, and not only its presence, that has a strong association with age-at-death. These studies indicate that chronic diseases or long periods of physiological stress have a larger impact on longevity than isolated incidents that can be overcome during growth.

Porotic hyperostosis and cribra orbitalia are indicators of physiological stress frequently considered to be indicative of iron-deficiency anaemia (Facchini et al. 2004, Goodman and Martin 2002, McIlvaine 2013) but their aetiology still is not completely understood. These stress indicators can also be related to sickle cell anaemia, vitamin deficiencies, parasites or infectious pathologies (Brickley and Ives2008, Facchini et al. 2004, Goodman and Martin 2002, Larsen 1997, Oxenham and Cavill 2010, Suby 2014, Walker et al. 2009, Wapler et al. 2004).

Tibial periostitis is frequently used as an indicator of physiological stress (e.g. Robb et al. 2001). Woven bone formation can also be considered an indicator of physiological

stress and is especially associated to communities with lower socio-economic status (Goodman and Martin 2002, Peck 2013), systematic infections (Goodman and Martin 2002, Ortner 2003) and malnutrition (Weston 2012). However, it has been argued that individuals with healed periostitis are of lower frailty as they survived the stressor and may be more resistance to disease (e.g. DeWitte 2010, Ortner 2003, Wood et al. 1992).

The presence of skeletal lesions can represent an adaptation to a pathological condition indicating that the individual survived long enough to the pathology for it to leave evidence in the skeletal tissues (Ortner 2003, Wood et al. 1992). Therefore, the individuals with skeletal pathological indicators had an efficient immunity system which allowed them to survive at least for some time to the disease. The absence of osteological stress markers does not necessarily mean low level of physiological stress. The absence of skeletal lesions is ambiguous and can indicate either a good health status or a fast death as consequence of an acute disease (DeWitte and Stojanowski 2015, Siek 2013, Wood et al. 1992). Frail individuals would have died before registering skeletal lesions and therefore without evidence of disease, while skeletal lesions are the result of the struggle to adjust to the stressor (Wood et al. 1992).

The use of multiple indicators, such as biocultural and biosocial aspects, helps discerning patterns of health (Agarwal and Glencross 2011, DeWitte and Stojanowski 2015, Goodman and Martin 2002, Wright and Yoder 2003). Identifying and controlling potential sources of heterogeneity, like sex or age, also reduce the limitations of using aggregate skeletal data (DeWitte and Stojanowski 2015).

2.3.3. Synergy between diet and health

Infectious pathologies, especially when linked with undernutrition, are the largest contributor to morbidity and mortality worldwide (WHO 2009). The study of nutrition-infection interactions is important to understand the complexity of the relationships of these factors with immunological status, co-morbidity and mortality (Ulijaszek et al. 2012). The complex relationship between nutrition and immunity to pathogens has received increasing attention in modern populations. In ancient pre-antibiotic populations this context has been little considered. Still, archaeological collections are pre-antibiotic allowing a more direct study of human-pathogen co-evolution and may helderly important lessons at a time when the continued supply of effective antibiotics is under threat. For these reasons bioarchaeological collections are a good model to study diet and health without the confounding factor of modern medicine.

Health and good nutrition shape human populations and are key for human happiness and wellbeing. Good nutrition increases the immune system's response to pathogens (e.g. Woodward 2001, Calder 2013), while immune deficits following malnutrition early in life have been shown to persist for years (e.g. MacDade 2005, Reitsema et al. 2016). Most of the modern developing countries still struggle to decrease malnutrition and infectious diseases (WHO 2009, Doak et al. 2005). The richness and prevalence of human pathogens in the environment are related to climate and animal species diversity that can be pathogens' hosts (Dunn et al. 2010). The susceptibility to infection, on the other hand, relies on genetics (Cooke and Hill 2001), environmental factors and nutritional state (e.g. Woodward 2001).

Malnutrition impairs the immune system (e.g. Calder 1991, Scrimshaw and SanGiovanni 1997). Individuals with poorer nutrition are less resistant to infectious diseases,

and infectious disease decreases nutrient availability (e.g. Martorell 1980, Mata et al. 1971). The effect of protein-energy malnutrition on aspects of immune function and susceptibility to infection (e.g. Kuvibidila et al. 1993, Scrimshaw and SanGiovanni 1997, Woodward 1998, Woodward 2001) affects practically all forms of immunity, in particular cell mediated immunity (Kuvibidila et al. 1993, Woodward 1998, 2001), immune barrier function (Deitch et al. 1990, Sherman et al. 1985) and the functioning of lymphoid organs (Lee and Woodward 1996). On the other hand, infections can decrease nutrient availability due to malabsorption (e.g. Mitra et al. 1997) and increase resting energy expenditure, altering the metabolism and redistribution of nutrients (Calder 2013). However, if nutrition is adequate, diseases like tuberculosis may have a less severe infection, instead of an exacerbated one, resulting in prolonged chronic infections with a higher probability to affect the skeleton (Ulijaszek et al. 2012).

Infections can also inhibit physical growth by negatively affecting nutritional status, through decreased food intake, impaired nutrient absorption, direct nutrient losses, increased metabolic requirements, catabolic losses of nutrients, and impaired transport of nutrients to specific tissues (Ulijaszek et al. 2012).

2.3.4. Stable isotope analysis and physiological stress

There are three possible balance conditions in stable isotope values of nitrogen and carbon: tissue maintenance in healthy adults, positive balance associated with tissue gain during growth and negative balance related with tissue loss during stress (e.g. Jim et al. 2006, Fuller et al. 2004).

During tissue maintenance, the same amount of nitrogen ingested is excreted and bone collagen reflects the ingested protein from diet (e.g. Champe et al. 2008). D'Ortenzio

et al. (2015) suggested that short-term fluctuations of $\delta^{15}N$ values may be the result of changes in the metabolic balance of an individual.

During prolonged periods of disease, nutritional or physiological stress, dietary protein cannot adequately replace nitrogen losses (Powanda 1977, Grossman et al. 1945). When in physiological stress body tissues are recycled and used as a protein source, resulting in enriched in ^{15}N and consequently high $\delta^{15}N$ values. An increase in $\delta^{15}N$ may be an indicator of lean mass loss during nutrient deprivation (Lee et al. 2012). Diseased patients may lose more than 20% of body protein (Wolfe et al. 1983), increasing morbidity and mortality while delaying the recovery from the illness (Petterson et al. 1993). The loss of body protein mobilizes the body's amino acids to support crucial metabolic functions (Papet et al. 2002, Mansoor et al. 1996, Breuillé et al. 1994). Nutritional stress reduces the production and the maturation of immune cells impairing the immune system (Papet et al. 2002, Walrand et al. 2001, Walrand et al. 2000, Chandra 1997). The hypermetabolic response to infection, such as inflammation, fever and immune activation, changes the body protein homeostasis leading to a negative nitrogen balance and a redistribution of body proteins (Biolo et al. 1997). Additionally, infectious diseases are also associated with the development of anorexia. The fasting metabolic state, caused by the anorexia, acts as an infection inhibitor in different species, from insects to humans (Wang et al. 2016), but results in a high catabolic state (Englert and Rogers 2016). Therefore, changes in stable isotope ratios in individuals with infectious diseases are a result of both nutritional stress and the infection itself.

In humans ^{15}N enrichment has been observed in osteoporotic bones (White and Armelagos 1997), in skeletal lesions of infectious origin (Olsen et al. 2014, Katzenberg and Lovell 1999), in a probable paleopathological case of celiac disease (Scorrano et al. 2014)

and in the hair of patients with anorexia (Neuberger et al. 2013, Mekota et al. 2006), all related with fasting and wasting.

While increases in $\delta^{15}N$ in tissues are frequently associated with fasting or nutritional stress (e.g. Alamaru et al. 2009, Boag et al. 2006, Fuller et al. 2005, Scrimgeour et al. 1995, Hobson et al. 1993), this pattern has not always been registered (e.g. Mayor et al. 2011, McFarlane et al. 2010, McCue and Pollock 2008, Kempster et al. 2007, Castillo and Hatch 2007). It is still not clear how the different tissues, particularly bone tissue, are affected by the body's net loss of light nitrogen or the mechanisms underlying changes in $\delta^{15}N$ during physiological stress. Recycled carbon from fat deposits, on the other hand, results ^{12}C enrichment leading to a decrease in $\delta^{13}C$ values (Neuberger et al. 2013). $\delta^{13}C$ decrease was observed in dentin of children who went through the Irish famine (Beaumont and Montgomery 2013).

Despite during fasting, catabolism and anabolism becoming unbalanced this does not occur in the same manner across the different tissues of an individual. In muscle catabolism exceeds anabolism during fasting, and protein synthesis ceases (Waterlow 2006, Hasselgren 2000, Cherel et al. 1991). However, in the liver protein synthesis continues, even if at levels below normal, or increases with catabolism, increasing the demand for amino acids (Breuillé et al. 1994, Waterlow 2006, Cherel et al. 1991, Garlick et al. 1975). Additionally, the liver contribution to the whole-body protein synthesis doubles during infections (Breuillé et al. 1994). During catabolism a decrease in ^{14}N-containing amino acids during protein breakdown leads to an increase in the $\delta^{15}N$ values of the tissue undergoing catabolism (Martínez del Rio and Wolf 2005, Gaye-Siessegger et al. 2007, Lohuis et al. 2007, McCue and Pollock 2008, McFarlane et al. 2010, Hobson et al. 1993). When in catabolism $\delta^{15}N$ values are expected to increase in tissues in proportion to their use as catabolic protein

stores. However, not all animals in nutritional stress reuse amino acids from catabolism (Lee et al. 2012, Kempster et al. 2007, Williams et al. 2007, Sears et al. 2009), only if the stressor is present long enough for the tissues to be broken down (Hobson et al. 1993). In non-adult humans Waters-Rist and Katzemberg (2010) did not find $\delta^{15}N$ increase in epiphyses, metaphyses and diaphysis of growing long bones, despite the bone turnover being faster during growth.

Studies in hibernating animals contradict the catabolic model (Lee et al. 2012) which can be related with a "preparation for fasting". Before hibernation animals build up large fat stores to support metabolic costs while an unpredicted decrease in food availability or period of disease increases metabolic costs which may rely entirely in tissue catabolism. Sepsis leads to breakdown of carbohydrate and fat reserves and protein is degraded in several organs (Van Wyngene et al. 2018).

During periods of positive nitrogen balance there is more nitrogen ingested than excreted (Champe et al. 2008) which can lead to less enriched ^{15}N or even ^{14}N increase within the body's tissues resulting in increased $\delta^{15}N$ values. Signs of positive nitrogen balance have been registered during pregnancy or recovery after periods of disease or starvation (e.g. Fuller et al. 2004, 2005, Mekota et al. 2006, Harvey and Ferrier 2011). Increased $\delta^{13}C$ values have been observed in patients recovering from starvation (Mekota et al. 2006, Neuberger et al. 2013), which may be related with an increase of meat and fat intake after the nutritional stress period (Van der Merwe 1982, Chisholm et al. 1982).

Chapter 3

Sample and methodology

3.1. Sample

Tomar's collection is one of the largest osteological samples in Europe with almost 7,000 skeletons. As these skeletons had not been studied before, I consulted the fieldwork form of each primary inhumation (n=2,412) excavated during the 2nd phase of the excavation. An example of one of these forms can be seen it the Appendix. Unfortunately, it was not possible for me to find the forms from the 1st phase of Tomar's excavation. The access to the forms from the 2nd phase of the excavation allowed me to transfer the information collected during the fieldwork to a spreadsheet in a quantifiable manner. For a better understanding of Tomar's population I analysed the data collected by the archaeologists and anthropologists during the excavation.

By looking at the excavation map (Figure 2.1) I analysed the individuals' distribution within the graveyard to check if it would be possible to differentiate between different socioeconomic statuses. To better understand the population distribution within the graveyard I calculated the number of inhumations *per* square meter, the percentage of each age group, the ratio between adults and non-adults and the ratio between males and females in each excavated area. Proximity to church was used as a proxy for socio-economic status (Binski 1996, Daniell 1998, Graves 1989, Ottaway 1992, Platt 1981, Swanson 1989). To confirm this I also compared the percentage of structured and excavated graves within the different areas and the percentage of structured and excavated graves for each age

group. Since structured graves require more work, time and materials, they are also related with socio-economic status.

Sex can also have an impact on the individuals' socio-economic status, particularly in some societies. Tomar could have been remarkably susceptive to sex-biased socio-economic status, not only due to its chronology but also by being controlled by male religious military orders. Besides calculating the ratio between males and females within the different excavated areas, I also calculated the percentage of individuals from each age group by sex.

Skeletal evidence gives an incomplete chronological record of health. Acute conditions are unlikely to register directly in bone material as they do not last long. The individuals quickly recover or die. Since frail individuals would have died without skeletal evidence of the disease (Wood et al. 1992), age at death distribution in a population can be indicative of health and survivorship as the skeletons represent the individuals who died. Populations with a large number of non-adults may be the result of environmental constrains, such as physiological stress and acute infections. While those with a large number of elderly individuals may represent populations with few environmental constrains or high resistance to stress and disease, particularly if they have physiological stress indicators. I studied these patterns within Tomar's graveyard in an attempt to better understand the health status of the population in general. Survivorship can help differentiating between different social statuses. Children from low socio-economic status are more susceptible to disease and/or malnutrition which can lead to premature death so the number of adults and non-adults were compared between the different excavated areas.

I considered the maximum length of the skeleton, measured *in situ* from close to the bregma to the distal point of the talus following the procedure proposed by Boldsen (1984)

as a proxy for stature. Using the measurements taken during the excavation I calculated the mean stature, by sex, for each excavated area, for the different adult age groups and the type of grave. Stature mean was also compared between the different areas, the type of grave and age intervals. Since stature is linked with physiological stress during childhood I wished to better understand if the individuals with higher socio-economic status, inferred by proximity to church and type of grave, have higher stature than those from lower socio-economic status. Individuals from higher socio-economic groups may have had more access to nutritious food and less periods of disease than those from lower socio-economic status, resulting in less growth disruptions and consequently higher stature. Comparing stature between age groups can be very helpful in understanding the impact of stunting on survivorship. Elderly individuals may have higher stature than young individuals meaning that those who had less growth disruptions survived longer than those who had more growth disruptions, possibly associated with disease or other physiological stress. However, elderly individuals may also have lower stature than young individuals suggesting that they survived the stressor and may be more resistant, while the individuals who died at younger ages experienced less physiological stress while growing but were frailer (Wood et al. 1992).

The total number of individuals with different skeletal lesions and other signs of physiological stress (e.g. *cribra orbitalia*, porotic hyperostosis and enamel hypoplasias), and their spatial and demographic distribution, would be very important to better understand health in the population from which Tomar's collection derived. This analysis was not possible as the data were not always recorded in the same manner and often did not reflect what I observed in the skeletons. The large size of this collection, in both area and number of individuals, required many anthropologists and archaeologist who may not have been familiar with paleopathology. Still, I used skeletal metric measurements taken during the

excavation as their record is more straightforward and require less experience than paleopathological differential diagnosis.

Still using the data collected during the excavation, I used regression equations to estimate sex (1,144 adult skeletons: 535 females, 609 males) and stature (543 adults: 256 females, 287 males) using long bone length. The maximum length of the bones was recorded during the excavation avoiding bones with fractures or other lesions. Sex was estimated using morphological characteristics of the pelvis (Phenice 1969, Buikstra and Ubelaker 1994) and cranium (Buikstra and Ubelaker 1994). I also calculated the sexual dimorphism index for each bone (Tarli and Repetto 1986). I used the length of the skeleton, measured *in situ* as a proxy for the individual's stature.

More details about the methodology used to study Tomar's demography can be found in Chapter 4.

3.1.1. Selection of skeletons for stable isotope analysis

Using the data recorded during the excavation I made a first selection of individuals with and without indicators of physiological stress. I only selected adult individuals as they may have different diets from the non-adults. Additionally, adults represent individuals who survived childhood into adulthood.

Finding the selected skeletons revealed to be a very challenging task due to the large number of boxes (a few thousand) with skeletons from Tomar and other collections, stored without any organization or sequence (Figure 3.1). The number of skeletons analysed in this thesis was limited by the difficulty in finding the skeletons that I pre-selected from the field information.

Figure 3.1. Example of one of the rooms at the University of Évora where Tomar's collection is stored.

I dry cleaned the skeletons I found with brushes, reconstructed their biological profile and paleopathology. I estimated the sex of the selected individuals using morphological features of the pelvis (Phenice 1969, Buikstra and Ubelaker 1994) and cranium (Buikstra and Ubelaker 1994). For age at death estimations I used methods based on degenerative processes (Brooks and Suchey 1990, Buikstra and Ubelaker 1994, Lovejoy et al. 1985) as only adults were selected. It was not necessary to use the equations I developed for sex and stature estimation for these individuals as the completeness of the skeletons allowed the use of more reliable methods.

Skeletons without lesions were only selected if, in addition to not having skeletal lesions, the individuals had a stature equal or above the mean calculated for Tomar's collection (Chapter 4) and no signs of physiological stress (*cribra orbitalia*, porotic hyperostosis and enamel hypoplasias). It was possible to find 33 individuals with these characteristics. The absence of skeletal lesions does not necessarily mean the individual was healthy. A frail individual could die before the disease could leave a skeletal marker (Wood et al. 1992). Low stature and enamel hypoplasias are related with physiological stress during childhood and adolescence (e.g. WHO 2013). Since physiological stress during growth increased the risk of disease (e.g. Barker and Osmond 1986, Barker et al. 1989, Power and Peckham 1990, Blackwell et al. 2001), controlling these stress indicators increases the probability of selecting individuals who were not diseased. The aetiology for *cribra orbitalia* and porotic hyperostosis is still not completely understood but has been related with sickle cell anaemia, vitamin deficiencies, parasites or infectious pathologies (Brickley and Ives 2008, Facchini et al. 2004, Oxenham and Cavill 2010, Stuart-Macadam 1989, Suby 2014, Ulijaszek et al. 2012, Walker et al. 2009, Wapler et al. 2004, Larsen 1997). Even though selecting skeletons without any indicator of physiological stress still does not guarantee that the individuals were healthy, this is the best way to potentially identify "healthy" individuals.

In order to find individuals with skeletal lesions, I also searched for indications recorded in the field that would suggest the presence of disease. I started by searching the skeletons said to have traumas, periostitis, osteomyelitis or any signs of infectious diseases. However, not all the forms had this information, referred it in the same manner or actually corresponded to what I observed in the skeletons. Still, it was possible to find 33 individuals with skeletal lesions of possible infectious origin (n=23) and healed fractures (n=10).

I considered the lesions as being possibly caused by infection if abnormal bone formation or bone formation and destruction, compatible with periostitis or osteomyelitis (Ortner and Putschard 1985, Buikstra and Ubelaker 1994, Aufderheide and Rodríguez-Martín 1998, Ortner 2003), were present and not associated with trauma. I considered lesions scored 2 (markedly accentuated longitudinal striations on the surface of cortical bone (Figure 3.2, Steckel et al. 2006) to 5 (extensive periosteal reaction involving over half of the diaphysis, with cortical expansion, pronounced deformation, Steckel et al. 2006) as periostitis. Lesions scored as 6 (involving most of the diaphysis with cloacae, Steckel et al., 2006) were taken as osteomyelitis (Figure 3.3). Unremodelled lesions (Figure 3.4) were thought as active disease *perimortem* and remodelled lesions (Figure 3.5) as healed disease *perimortem* while a combination of active and healed lesions were considered as chronic disease (Ortner and Putschard 1985, Ortner 2003). Individuals with *caries sicca* (Figure 3.6) were diagnosed as having venereal syphilis (Ortner and Putschard 1985, Ortner 2003). Syphilis is a sexually transmitted long-term infection that only affects the bone tissue in 20 to 50% of non-treated patients (Aufderheide and Rodríguez-Martín 1998).

Figure 3.2. Example of healed tibial periostitis (skeleton 15.96).

Figure 3.3. Example of healed osteomyelitis in a femur (skeleton 20.240).

Figure 3.4. Example of a chronic active lesion in an ulna (skeleton 16.225).

Figure 3.5. Example of a healed lesion in a tibia (skeleton 18.158).

Figure 3.6. Example of *caries sicca,* a crater-like lesion with a central destructive focus and reactive, compact bone formation on the margins of the lesion (skeleton 16.225).

The individuals selected (Table 3.1) for stable isotope analyses were grouped in different ways:

a) Skeletons without lesions (n=33) and skeletons with lesions compatible with infectious diseases (n=23),
b) Skeletons without lesions (n=33), skeletons with active lesions (n=6), skeletons with healed lesions (n=7), skeletons with active and healed lesions (n=10),
c) Skeletons without lesions (n=33), skeletons with generalised infection (n=7), skeletons with localised infection (n=9) (individuals who did not fit into these groups were not considered for this comparison, n=7),
d) Skeletons without lesions (n=33), skeletons with generalised specific infectious disease (n=2), skeletons with generalised non-specific infectious disease (n=5), skeletons with only healed tibial periostitis (n=7) (individuals who did not fit into these groups were not considered for this comparison, n=9).

The selection factors and difficulty of finding the skeletons limited the amount of individuals used for stable isotope analysis (Table 3.1). A more detailed description of how the skeletons were selected can be observed in Chapter 5 for those without lesions and in Chapter 6 for the individuals with lesions. A list of the individuals analysed can be found in the Appendix (Tables A2, A3, A4 and A5).

I also identified the genus of the fauna remains recovered from the excavation and selected the possible samples from the different genus found (2 wild *Sus*, 2 domestic *Sus*, 1 juvenile *Sus*, 1 *Canidae*, 3 *Bos*, 1 *Equus*, 3 *Ovicapridae*).

Table 3.1. Number of individuals selected for each group.

			Females				Males				Undetermined	Total	
			Young	Mature	Elderly	Undetermined	Young	Mature	Elderly	Undetermined			
Without lesions			3	4	5	3	5	9	1	3	-	33	
With lesions	Unspecific generalized infections	Active	1	-	-	-	1	-	-	-	-	2	5
		Healed	-	-	-	-	-	-	-	-	-	0	
		Active and healed	-	-	2	-	1	-	-	-	-	3	
	Specific generalized infections	Active	-	-	-	-	-	-	-	-	-	0	2
		Healed	-	-	-	-	-	-	-	-	-	0	
		Active and healed	-	-	-	-	1	1	-	-	-	2	
	Localised lesions	Active	-	-	-	1	-	2	-	-	-	3	9
		Healed	-	-	-	-	-	-	1	-	-	1	
		Active and healed	1	-	-	1	1	-	1	1	-	5	
	Only healed periostitis		-	1	-	1	-	3	-	1	1	7	
	Healed fracture calluses			1		1		3	1	2	2	10	
Total			5	6	7	7	9	18	4	7	3	66	
			25				38						

3.1.2. Selection of bones for stable isotope analysis

From the skeletons without lesions or indicators of physiological stress only the tibia was sampled for stable isotope analysis. For the individuals with lesions a combination of long bones (mostly tibiae) and ribs were sampled. Additionally to the skeletons with lesions compatible with lesions compatible with infectious diseases a new group of samples with healed fractures were added to the study. The objective of adding samples of fracture callus are to use them as a control group, as they represent bone remodelling but in the absence of pathogens. Only bones with healed fractures but without periostitis or osteomielytis were sampled. The bones were cut at the Laboratory of Biological Anthropology at the University of Évora using a diamond saw. These sections were then sent to the University of Kent for the collagen extraction.

The lesions were identified and differentiated by macroscopic observations and grouped as:

a) active lesions (14 long bones, 4 ribs),

b) healed lesions (10 long bones, 9 ribs),

c) healed fractures (9 long bones, 3 ribs).

In total stable isotope analysis was performed for 134 samples (Table 3.2).

The difference between samples from the lesions and from distant to the lesions was compared with intra-bone variations estimated by Olsen et al. (2014) for ribs and by Katzenberg and Lovell (1999) for long bones.

The selection of skeletons and bone samples required a few visits to the University of Évora in a total of about 9 months. More details on how bones were sampled can be found in Chapter 7.

Table 3.2. Number of samples used for stable isotope analysis.

Samples			Long bones	Ribs
Humans	Skeletons without lesions		33	-
	Skeletons with lesions	Bone without lesions	28	11
		Bone with lesions	33	16
	Total		94	27
			121	
Fauna			13	
Total			134	

3.2. Collagen extraction and analysis

3.2.1. Collagen extraction

At the University of Kent I cleaned the bones again (human and fauna) using water and a brush and I cut the bone samples in small pieces using a drill. Before using the drill I put it in the sonicator, in a beaker with hydrochloric acid (HCL), to clean it. The drill was cleaned between samples using an air-spray duster. The new bone formations were removed by scraping the lesion or removing the top layer affected, carefully avoiding sampling the compact bone underneath or trabecular bone (particularly in the ribs), as it remodels more quickly than cortical bone (Sealy et al., 1995). On the ribs this process was more difficult due to the smaller size of the lesions and the bones.

Bone collagen was extracted using a modified Longin method (Longin 1971, Brown et al. 1988, Richards and Hedges 1999). For the demineralization I put the sampled pieces of bone in a test-tube, filled it with 0.5M HCL and put it in the fridge at 5ºC. I changed the HCL every two days for two weeks. After two weeks in the fridge I centrifuged the samples for

three minutes at 2,500 revolutions *per* minute (RPM), pipetted off the HCL and added demineralised water (dH_2O) and centrifuged again. I continued changing the dH_2O and centrifuging until the pH of the solution was neutral (pH=7). To start the gelatinization the pH has to be acidic again so I added a few drops of HCL to the solution until its pH was equal to two or three. The samples were then put in the heating block where they stayed for 48 hours at 75ºC. After being in the heating block a 5µm EZEE© filter was used to filter the insoluble fractions in the solution. The samples were then frozen for 48 hours followed by a process of freeze-drying for another 48 hours. After this process the collagen was weighted to make sure that there was enough collagen for stable isotope analysis.

3.2.2. Collagen analysis

The collagen from the fauna and the skeletons without lesions was analysed at NERC Isotope Geosciences Facility (Nottingham, UK). I stayed at NERC facilities for a week weighing the collagen samples and standards into tin capsules. These capsules were then combusted into CO_2 and N_2 using an Elemental analyzer (Flash/EA) coupled to a Thermo Finnigan Delta^{Plus} XL isotope ratio mass spectrometer via a ConFlo III interface. $\delta^{13}C$ and $\delta^{15}N$ values were calibrated using an in-house reference material M1360p (powdered gelatine from British Drug Houses) with expected δ values of –20.32‰ (calibrated against CH_7, IAEA) and +8.12‰ (calibrated against N-1 and N-2, IAEA) for carbon and nitrogen respectively. Samples were run in duplicate and the 1σ reproducibility for mass spectrometry controls for these analyses were $\delta^{15}N = \pm 0.08$‰ and $\delta^{13}C = \pm 0.07$‰.

The remaining samples were analysed at HERCULES Laboratory at the University of Évora where I stayed in total about four weeks weighing the collagen and standards into tin capsules. These capsules were then combusted into CO_2 and N_2 using an Elemental analyzer

(Flash/EA) coupled to a Thermo Delta V^{TM} Advantage Isotope Ratio Mass Spectrometer. $\delta^{13}C$ and $\delta^{15}N$ values were calibrated using IAEA-CH-6 (sucrose, −10.449‰), IAEA-CH-7 (polyethylene, −32.151‰), IAEA-N-1 (ammonium sulphate, +0.4‰) and IAEA-N-2 (ammonium sulphate, +20.3‰). Measurement errors were less than ±0.1‰ for $\delta^{13}C$ and ±0.2‰ for $\delta^{15}N$.

The sulphur isotope analysis was done at SIIAF at the University of Lisbon, combusting the collagen with additional V_2O_5 and a pulse of oxygen. $\delta^{34}S$ values were calibrated using the inorganic international standards NBS127 (+20.3‰), IAEA S1 (-0.3‰) and casein protein (+4.0‰). Mass spectrometry control for these analyses was $\delta^{34}S$ = ± 0.08‰.

The C/N ratios of the analysed samples were within 2.9 and 3.6, the acceptable range in C/N ratios for archaeological bone determined by De Niro (1985). Sample information and stable isotopic data are provided in the Appendix (Tables A2, A3 and A4).

Carbon and nitrogen stable isotope ratios were analysed in all samples while sulphur stable isotope ratios were only measured in the fauna remains and in the individuals without lesions. Tomar is crossed by a river so analysing $\delta^{34}S$ values was very important to estimate the intake of aquatic protein. $\delta^{34}S$ analysis would also be helpful to better understand if intra-bone variations between sites with and without skeletal lesions were related with dietary shifts or metabolic changes associated with physiological stress or its recuperation. Funding limitations did not allow analysing $\delta^{34}S$ values for all the samples.

3.2.3. Statistical analysis

Assumptions such as normal distribution of the data, when this is not true, make it impossible to draw accurate and reliable conclusions (e.g. Field 2009). To test whether the analysed data is normally distributed the Shapiro-Wilk test was performed, which compares the sample distribution to a normal distribution (Shapiro and Wilk 1965). Shapiro-Wilk test has shown to have the best statistical power when compared to other normality tests (Razali and Wah 2011, Peat and Barton 2005, Thode 2002). As statistical power increases, the probability of failing to reject a false null hypothesis (type II error) decreases.

I considered the null hypothesis to be that the population was normally distributed. For *p*-values ≤0.05 the null hypothesis would be rejected and the data considered as not being normally distributed. Still, while for *p*-values ≤0.05 the data significantly deviate from a normal distribution, *p*-values ≥0.05 do not necessarily mean that the data is normally distributed, particularly for small sample sizes such as those analysed in this dissertation. Hence, alongside with the results from the Shapiro-Wilk test (Appendix, Table A1) I also analysed the graphics of the stable isotope ratios distribution. Figure 3.6 shows a example of a data set with *p*-values ≥0.05 and by their graphic representation it is possible to observed that the data set is too small to understand if the data is follows a normal distribution or not. Additionally, not all data sets have *p*-values ≥0.05 so in order to appropriately compare them parametric tests will not be used.

Since the data sets are too small to understand if the data is normally distributed, non-parametric methods were used to test the samples as they do not assume a normal distribution of the data. Mann-Whitney U non-parametric tests test the equality of the means in two independent groups from the same population while Kruskal-Wallis non-parametric tests do the same but in more than two independent groups. Mann-Whitney U

non-parametric tests (Mann and Whitney 1947) were used for pair-wise comparisons considering the null hypothesis to be that the distribution of both groups is equal. Kruskal-Wallis non-parametric tests were used to compare more than two groups. Similarly to the Mann-Whitney U non-parametric tests, the null hypothesis is that all groups have identical distribution. The alternative hypothesis states that at least one group stochastically dominates the other group. The null hypothesis were rejected if the *p*-values ≤0.05. Statistical analysis was computed in SPSS 24 for Windows *p*-values ≤0.05 were considered statistically significant.

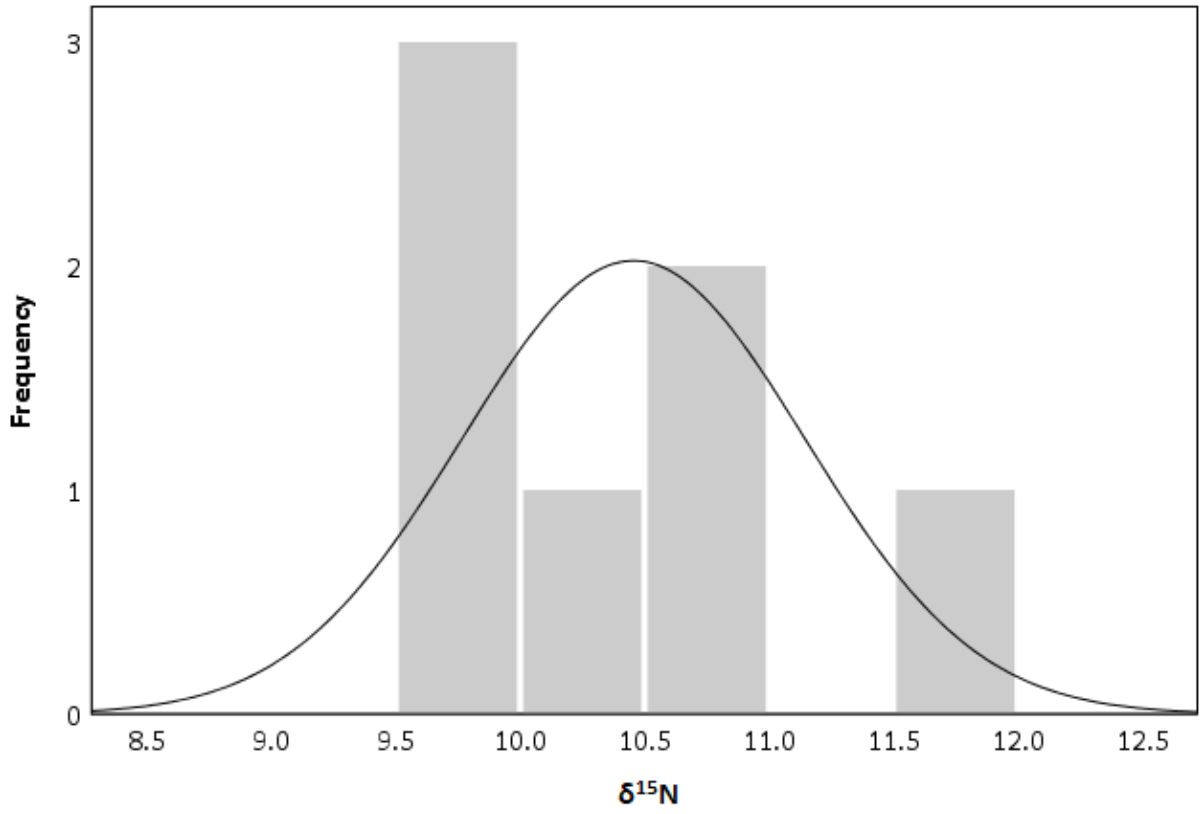


Figure 3.7. Example of a histogram which variable ($\delta^{15}N$ values distribution in individuals with active lesions) would be considered to fallow a normal distribution based on the *p*-values =0.34.

1

Chapter 4

Osteological sample and demographics of Tomar skeletal collection

Abstract

This study relies on raw data collected during the excavation to better understand Tomar's collection. A better understanding of the demographics of Tomar osteological collection will improve diet estimations as well as better understand the possible relationship between diet and health in this sample. The distribution of the skeletons, of all ages and both sexes, within the necropolis suggests that Santa Maria do Olival collection represents the general population of Tomar and not only the individuals from the religious military orders. The proximity to the church and a higher percentage of structured graves suggest that the individuals buried at areas 15, 18 and 19 could have had higher social status. The uniform sex spatial distribution within the graveyard and the use of structured graves for both males and females implies that, at least in death, social status was not dependent on sex. However, social status seems to increase with age as older individuals were more frequently buried in structured graves. In general, females may have been more exposed to chronic physiological stress, as those buried in structured graves (higher status) and older individuals have higher stature.

4.1. Introduction

This chapter presents raw data collected during the excavation of Tomar's necropolis to assist the understanding of the context of the collection understudy. The distribution of individuals from different sex and age is presented and compared to the proximity of the church and type of graves to study the possibility of identifying different socio-economic statuses.

Health, disease and mortality are conditions related not only with biological factors but also the socio-economic context of the individuals (Cockerham 2007). Socio-economic status can influence access to nutritious food resources and health care, exposure to pathogens, strenuous biomechanical effort, settlement density, sanitation and hygiene. Cultural systems can operate both as buffering system, mitigating environmental constrains and stressors, or as a producer of new stressors and constrains (Goodman and Armelagos 1989). According with the osteological paradox (Wood et al 1992), heterogeneous frailty and selective mortality affect paleopathological research, thus, identifying and controlling potential sources of heterogeneity, such as sex, age or socio-economic status reduce the limitations of using collective skeletal data (DeWitte and Stojanowski 2015).

Christian burial rituals usually do not have associated material culture (Barroca 1987), being also the case at Tomar having few artefacts, which hampers the dating of the site and the graves as well as estimations of socio-economic status. In the absence of artefacts, social status can also be inferred by proximity to the church (Binski 1996, Daniell 1998, Graves 1989, Ottaway 1992, Platt 1981, Swanson 1989) and the amount of structured graves.

A comparison between age at death with proximity to church will help understanding if social status has an impact on age at death. People from higher socio-economic status might have survived longer due to overall better living conditions. A better understanding of the distribution of age groups within the graveyard will improve the knowledge about Tomar's population. In most populations females live longer than males but often suffer from poorer health later in life (Crimmins et al. 2002, Oksuzyan et al. 2008). This paradox might be explained, at least in part, by higher male mortality which results in males facing higher selective pressure than females such that those who survive to later ages are healthier in general than females of the same age (Crimmins et al. 2002). Additionally, females often have a different social status from males in general, which can lead to less access to nutritious food and health care.

Estimating human sex is one of the first steps in reconstructing a biological profile of skeletonised remains. Sexual dimorphism develops just before sexual maturity and is related to faster growth in females and an extension of the pre-pubertal growth phase in males (e.g. Willner & Martin, 1985, Tanner, 1990, Roche, 1992, Gasser et al., 2000). Routinely, bioarchaeologists incorporate morphological traits of the pelvis (Phenice 1969) and cranium, into their sex estimations (e.g. Sauter and Privat 1955, Ferembach et al. 1980, Buikstra and Mielke 1985, Buikstra and Ubelaker 1994, Bruzek 2002, Bruzek and Murail 2006). When the pelvis and cranium are not available, due to for example post-mortem taphonomic processes (e.g. Willey et al. 1997), alternative methods for estimating sex rely on anthropometric analyses of other bones (e.g. Pons 1955, Đscan and Shihai 1995, Silva 1995, Steyn and Đscan 1997, Mall et al. 2000, Wasterlain 2000, Mall et al. 2001, Bašić et al. 2013). These metric methods are population specific and their accuracy can vary both regionally and chronologically (e.g. Bidmos and Dayal 2004). Bone length reveals developmental

trends, environmental stress such as nutritional deficits and evolutionary relationships (e.g. Bogin 1999, Padez and Johnston 1999, Padez 2003, 2007, Moore and Ross 2013), being largely affected by secular changes in stature (e.g. Jantz 1992, Moore and Ross 2013).

The adult stature of an individual is the accumulation of different frequency and amount of growth during their life event (Lampl 2012). The cessation or slowing of long-bone growth can result in a decrease in the potential stature of the individual, known as stunting (Saunders and Hoppa 1993, WHO 2013). This could result in a permanent small stature, particularly in females (Bose 2018), or lead to "catch-up growth" if conditions improve. Stature has been shown to be sensitive to both environmental and physiological factors (Jantz and Owsley 1984). Stunted growth can result from chronic inflammation and infection in juvenile individuals (Pinhasi et al. 2005). Poverty may exacerbate stunting, perpetuating the vicious cycle as vulnerability to malnutrition and disease increases. Since stature is related with physiological stress, shorter individuals usually reach reproductive maturity earlier stages and die at younger ages (Metcalf and Monaghan 2001, Walker et al. 2006, Kuzawa 2007, Stock and Migliano 2009).

4.1.1. Demography

Tomar's archaeological collection has 3,675 primary inhumations and 1,456 ossuaries in a total of 6,792 individuals (4,991 adults and 1,801 non-adults). However this collection has not been continually studied yet. This chapter presents the demographic analysis of 2,412 individuals from primary inhumations recovered from areas 13 to 20 (2^{nd} phase of the excavation). I was given access to the field excavation forms (example of one of these forms can be found in the Appendix, Figure A1) from the second phase of the excavation which had information registered during the excavation such as degree of fragmentation, sex, age,

bone measurements and length of the skeleton. I compiled the information on these forms and analysed the data to better understand the population from which Tomar's collection derived.

Non-adult age was estimated with a combination of skeleton maturation indicators (Scheuer and Black 2000) and adult age at death estimates employed a combination of skeleton maturation (Scheuer and Black 2000), pubic symphysis degeneration (Brooks and Suchey 1990, Buikstra and Ubelaker 1994) and auricular surface degeneration (Lovejoy et al. 1985) during the excavation. Non-adult individuals were divided into: children (less than 12 years old) and adolescents (12 to 18 years old). The adults were divided into: young adults (19 to 29 years), mature adults (30 to 60 years) and elderly adults (more than 60 years). Age at death is very important to understand the individual's health, diet and the relationship between the two of them.

Sex was estimated during the excavation, only for adult skeletons, based on pelvic (Phenice 1969, Buikstra and Ubelaker 1994) and cranial features (Buikstra and Ubelaker 1994). It was not possible to estimate sex for almost one third of individuals. This issue highlights the need of alternative methods for estimating sex relying on other bone types.

4.1.2. Sex estimation equations

Using the raw data from the excavation I used a logistic regression to develop equations for sex estimation based on long bone length:

$$\log\left(\frac{\mu}{1-\mu}\right) = \beta_0 + \sum_i \beta_i x_i$$

where,

μ - estimated mean for the population

β_0 - intercept term

β_i - slope (expected increment in the response per unit change in *x*)

x_i- full length of the bone

Data was collected from 1,144 adult skeletons (535 females, 609 males) from the 2nd phase of the excavation, but it was not possible to measure all long bones for all the individuals. Age was not taken into consideration given the difficulty of accurately estimate age in adult individuals (see Merrit 2017). The sex estimations relied upon standard morphological characteristics of the pelvis (Phenice 1969, Buikstra and Ubelaker 1994) and cranium (Buikstra and Ubelaker 1994). Maximum length of each long bone (humerus, femur, radius, tibia) was measured during the excavation avoiding bones with healed fractures and other lesions or damage that could interfere with maximum length measurements. The sexual dimorphism index was also calculated for each bone following the methodology developed by Tarli and Repetto (1986).

4.1.3. Stature equations

I used the skeleton length measurements collected during the excavation to develop regression equations to estimate stature from long bone length. Skeleton length was measured from close to the bregma to the distal point of the talus following the procedure proposed by Boldsen (1984) and used as a proxy for stature. Measurements were taken during the excavation while the skeleton was still articulated, *in situ*, in extended supine position. A folding ruler was placed on the sagittal midline of the skeleton to allow following any eventual curvature of the skeleton. Skeletal length *in situ* was considered equivalent to living stature. The maximum length of the skeleton was measured for 543 adults (256 females, 287 males). The long bone length was measured as described before (section 4.2.2).

To measure the spread of the variability relative to the mean, the coefficient of variation was calculated for the skeleton and long bone length (humerus, radius, femur and tibia). The strength of the linear relationship between skeleton and long bone length was measured by calculating the Pearson's correlation coefficient. Following this, logistic regression was used to develop an equation for stature estimation:

$$y = (\beta_0 + \beta_1 x) \pm \varepsilon$$

where,

y - estimated stature
β_0 - intercept term
β_1 - slope (expected increment in the response per unit change in x)
x - full length of the bone
ε - error term

4.2. Resulting age distribution

By analysing the data from the excavation, I observed that during the 2nd phase of the excavation 2,412 primary inhumations were recovered from areas 13 to 20. From these inhumations 771 of the individuals were non-adults and 1,645 were adults. It was possible to estimate age for 648 non-adults and 717 adults.

The children (n=545, Figure 4.1) represent the group with the highest number of skeletons for which it was possible to estimate age. The children were found mostly in areas 13, 16 and 17, comprising about 50% of the skeletons found in these areas (Figure 4.1). The high percentage of non-adults, particularly the children (Figure 4.1), can be over-represented due to greater ease in estimating age-at-death for these skeletons than for adults. Areas 15 and 19 (left side of the graveyard, Figure 2.4) have the highest ratio

between adult and non-adult skeletons, while areas 13 and 20 (right side of the graveyard, Figure 2.4) have the lowest ratios (Table 4.1). It is possible that specific places within the necropolis were preferred to bury non-adults, as reported in other medieval graveyards (e.g. Ariès 1962, Cunha 1994, Oliveira 2007). The mature adults (n=494) are the second age group with more individuals and comprise about 50% of the skeletons excavated from areas 15 and 18 and over 60% of the skeletons from area 19 (Figure 4.1).

Comparing the data from the 1st and 2nd phase of the excavations could highlight any possible differences between groups of different socio-economic status as the 2nd phase of the excavation represents areas closer to the church than the areas excavated during the 1st phase of the excavation (Figure 2.4).

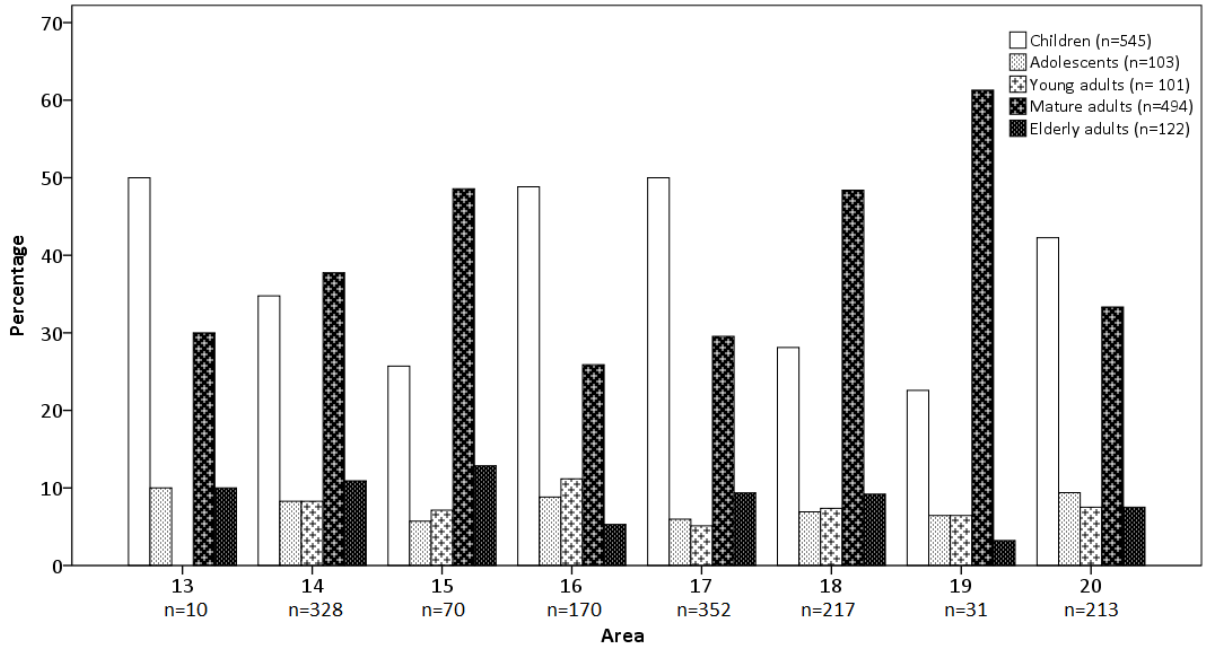


Figure 4.1. Percentage of each age group, by area.

Table 4.1. Number of primary inhumations *per* m^2, ratio between the number of adults and non-adults and between males and females from primary inhumations

Area	Inhumations/m^2	Adults/Non-adults	Males/Females
13	0.5	1.5	1.0
14	0.6	2.1	1.6
15	0.4	7.1	1.3
16	2.2	1.5	0.8
17	0.7	1.6	1.0
18	0.6	2.9	0.7
19	0.2	6.3	1.4
20	0.6	1.6	1.4
2nd phase	**0.6**	2.1	**1.1**

4.3. Resulting sex distribution

Tomar's collection is made out of individuals of all ages and both sexes, indicating that the graveyard was used by the general population of Tomar and not, or at least not only, the knights of the military order. Out of the 1,645 adults it was possible to estimate 535 females and 609 males, while 501 individuals were of unknown sex.

Area 14 has the highest ratio between males and females and areas 16 and 18 are the only ones with more skeletons estimated as female than male (Table 4.1). These results can be related with different social status between sexes or the artificial division of the areas. I observed that the mature adults have the highest percentages for both females and males (Figure 4.2) but this group also has a large age interval (31 to 60 years old) which can explain the high number of individual. Both mature and elderly adults have higher percentage of males, while the group of the young adults have more females (Figure 4.2). However there are no statistically significant differences (*p-value*>0.05) between age group.

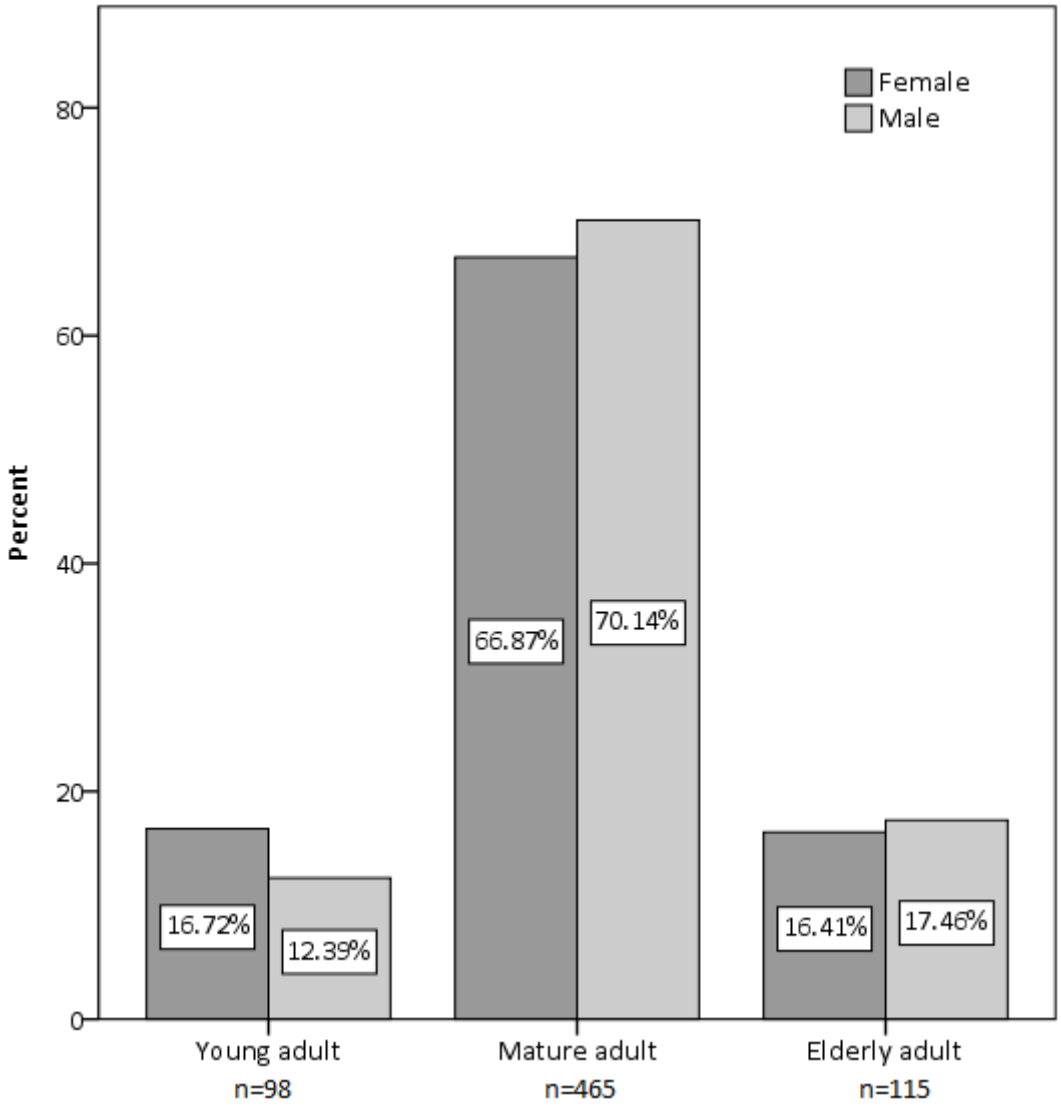


Figure 4.2. Percentage of individuals from each age group, by sex.

4.4. Inferring social status

The graves directly excavated in the soil are the most common at the 2nd phase of the excavation, comprising 2,192 of the primary inhumations of both adults and non-adults, while only 168 graves are structured. The structured graves are mostly present in areas 13, 15, 18 and 19 (Figure 4.3) suggesting that individuals with higher social status would be preferably buried in these areas. Areas 15, 18 and 19 also have the highest ratio between adults and non-adult skeletons (Table 4.1) and structured graves were more commonly used for older individuals (Figure 4.4). Since high childhood mortality may be related with low

socio-economic status, these results suggest that the individuals buried in these areas may have had higher social statuses than the individuals in other areas.

Areas 15 and 18 are the closest areas to the church (Figure 2.4) and Templar graves were found at area 18, reinforcing the idea that these areas represent the individuals with higher social status (Binski 1996, Daniell 1998, Graves 1989, Ottaway 1992, Platt 1981, Swanson 1989), despite these areas being artificial divisions made with the purpose of organizing the excavation.

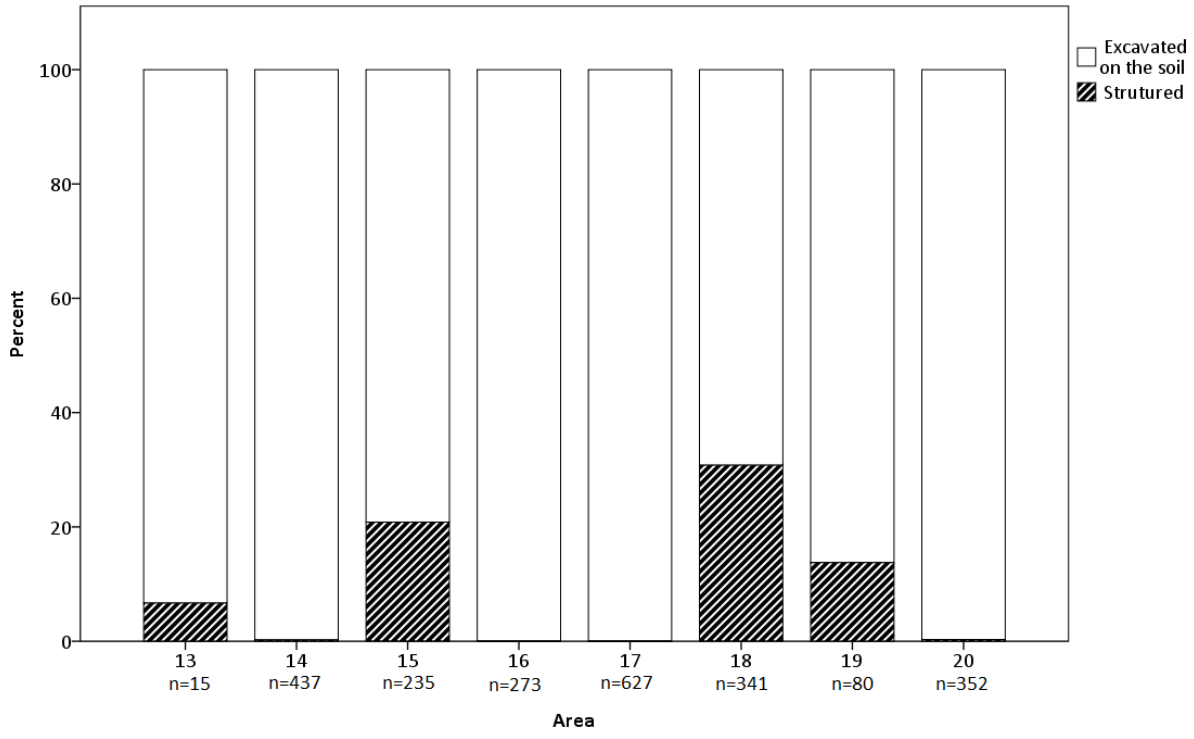


Figure 4.3. Percentage of graves excavated in the soil and structured graves, by area.

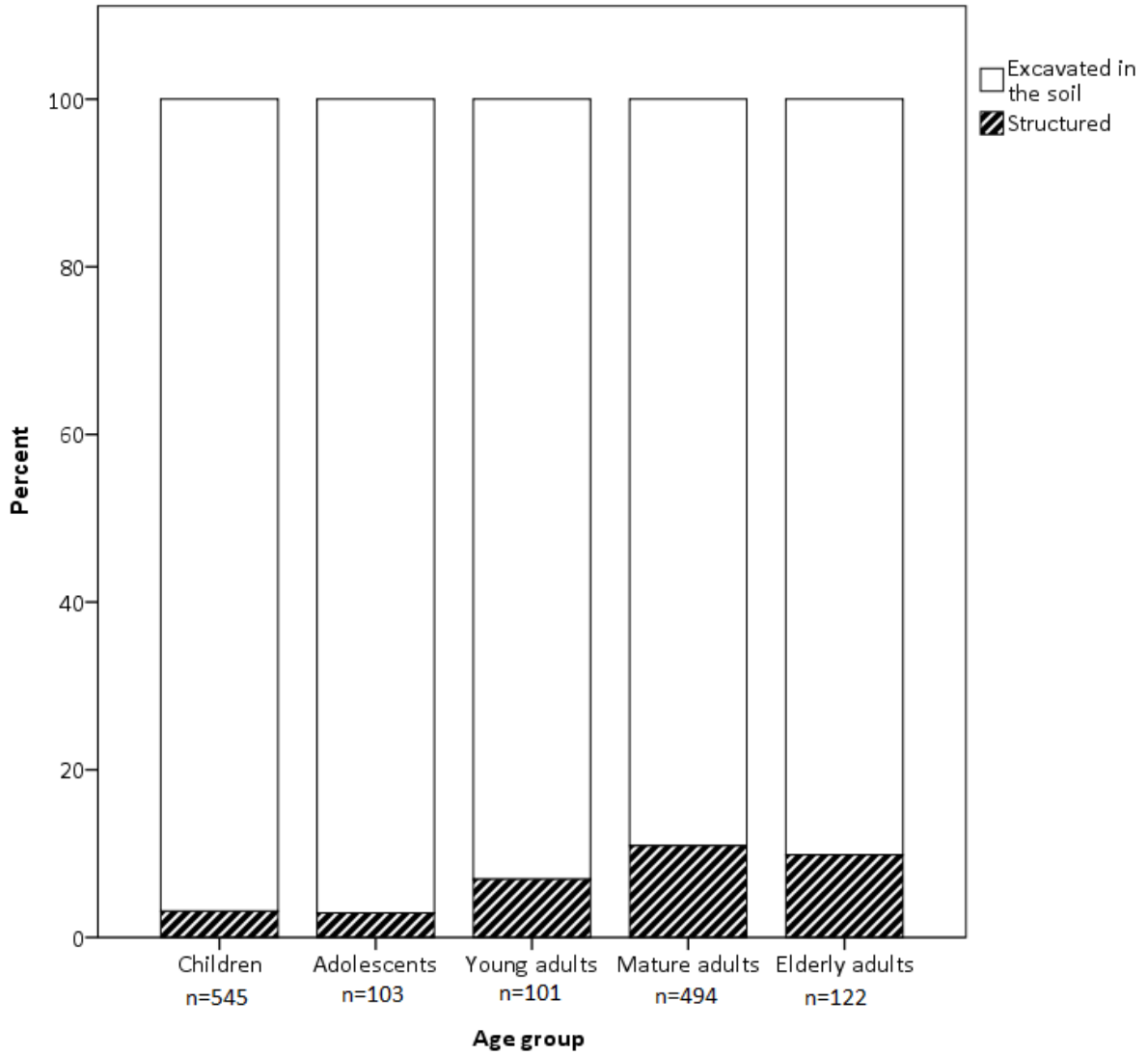


Figure 4.4. Percentage of graves excavated in the soil and structured graves, by age group.

Area 16 has the highest density of inhumations (2.2 inhumations/m^2, Table 4.1) while area 19 has the lowest density (0.2 inhumations/m^2, Table 4.1). Area 16 is close to the bell tower (Figure 2.4), which might have made it more desirable as a burial site than other areas. Area 19 is further away towards the left side of the graveyard (Figure 2.4), which could have made it less appealing as a burial site. The remaining areas have similar density of inhumations.

About 9% of the males (n=593) and 12% of the females (n=527) have structured graves, suggesting that there was no social status difference between sexes, at least at the

place where they were buried. Area 14 has the highest ratio between males and females and areas 16 and 18 are the only ones with more skeletons estimated as female than male (Table 4.1). Since area 18 is the one closest to the church, situated between the church and the tower bell, it is unexpected to find more females than males, as males usually have higher socio-economic status than females. This unexpected ratio reinforces tha there were not large status difference between both sexes, despite the presence of the military orders. At the 2nd phase of the excavation there are 0.6 inhumations *per* m^2, the number of adults doubles the number of non-adults and there is similar number of males and females. The sex distribution among the excavated areas is uniform (Table 4.1) suggesting that the skeletons buried outside Santa Maria do Olival church represent mostly the general population of Tomar and not the individuals from the Military Orders.

4.5. Social status and stature

Area 19 is one of the areas further away from the church (Figure 2.4), which can mean that the individuals buried there had lower socio-economic status then those from areas such as 18, 13 or 15. Area 19 has the largest stature difference between sexes (Figure 4.5) with the lowest mean for the females (147.0 cm) and the second highest mean for the males (164.6 cm). While area 15 has the smallest sexual dimorphism in stature out of the areas analysed being also one of the areas closest to the church and with more structural graves. Sexual dimorphism can also be related with diet (e.g. Steyn and Işcan 1999), Gray and Wolfe (1980) suggest that within a population, those who consume either excessive or deficient amount of protein, exhibit the least sexual dimorphism.

The mean stature is similar between individuals buried in structured graves and those in graves excavated in the soil, for both males and females (Figure 4.6). However, the

skeletons from structured graves have a slightly higher stature than the collection's mean, particularly the females. The higher stature for females buried in structured graves suggests that females from lower social status could have been more exposed to physiological stress affecting growth than males.

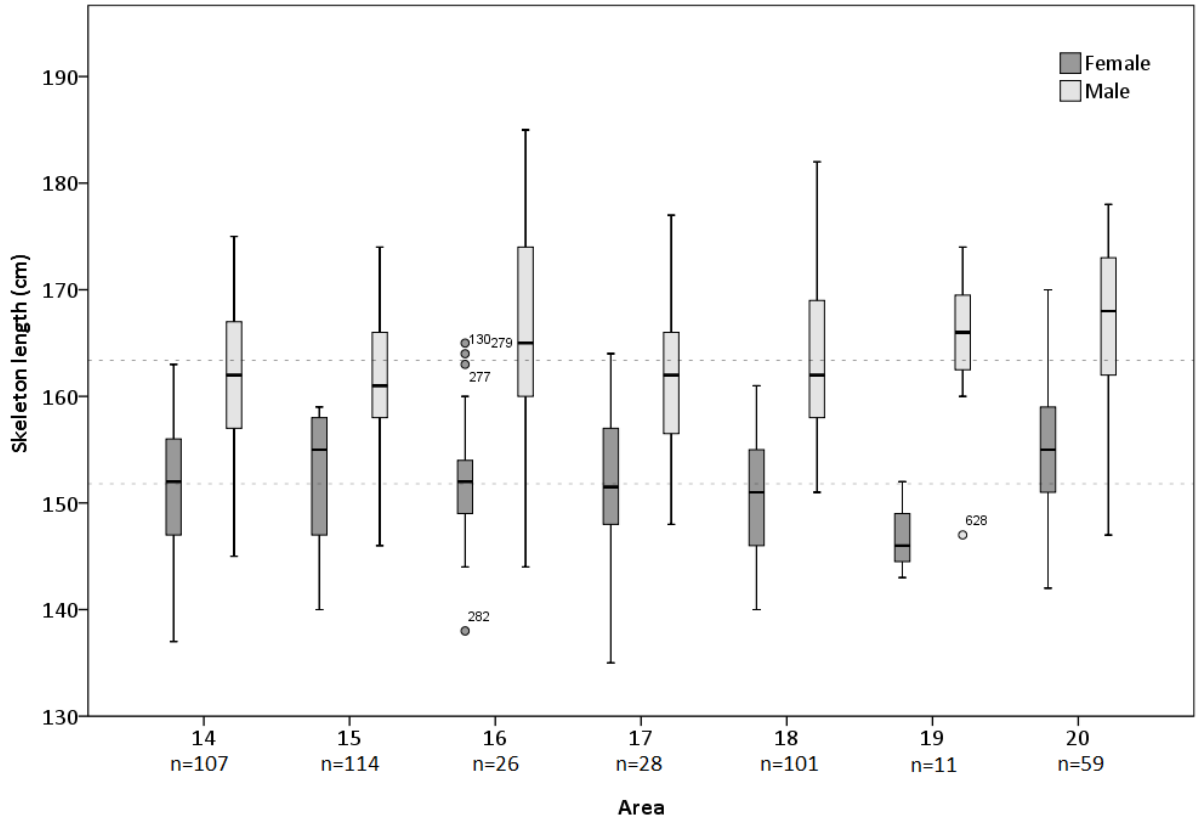


Figure 4.5. Boxplot for the maximum length of the skeleton (cm) registered for each area, by sex. Horizontal lines represent the overall mean for the females (151.8 cm) and males (163.4 cm).

At the areas of the 2nd phase of the excavation there are not significant differences in stature between the different age groups. Still, the mean female stature is higher for elderly adults (Figure 4.7) than the other age groups. Since stature is related with physiological stress (e.g. Bogin, 1999, Padez and Johnston, 1999, Padez, 2003, 2007, Moore and Ross, 2013) this result reinforces the idea that females may be more exposed to chronic stress than males, at least in Tomar, and that those who suffered less physiological stress may have lived longer. The association between stature and increased risk of mortality can

reflect the exposure to chronic stress during development (Haviland 1967, Roberts and Manchester 2007). Shorter individuals usually reach reproductive maturity at earlier stages and die at younger ages (e.g. Metcalf and Monaghan 2001, Walker et al. 2006, Stock and Migliano 2009). It is therefore important to consider stature when studying the diet and/or health.

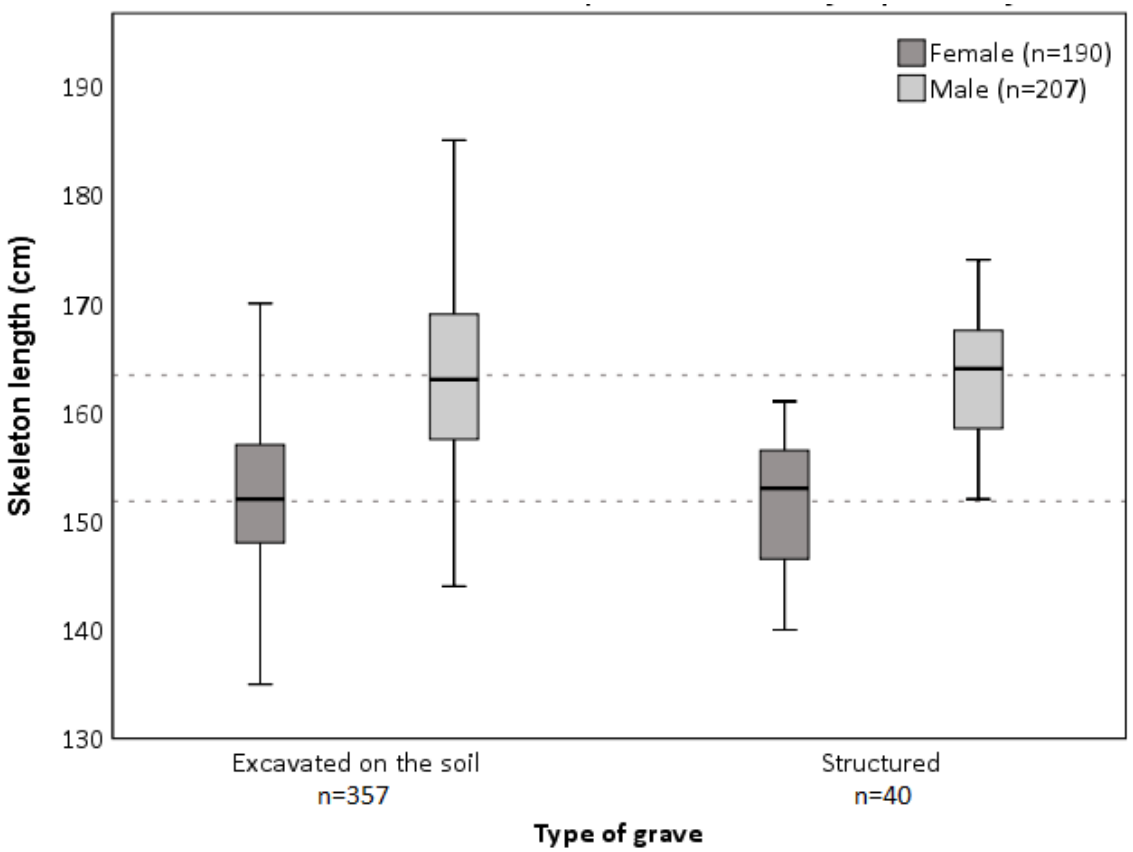


Figure 4.6. Boxplot for the maximum length of the skeleton (cm) registered for graves excavated on the soil and structured graves, by sex. Horizontal lines represent the overall mean for the females (151.8 cm) and males (163.4 cm).

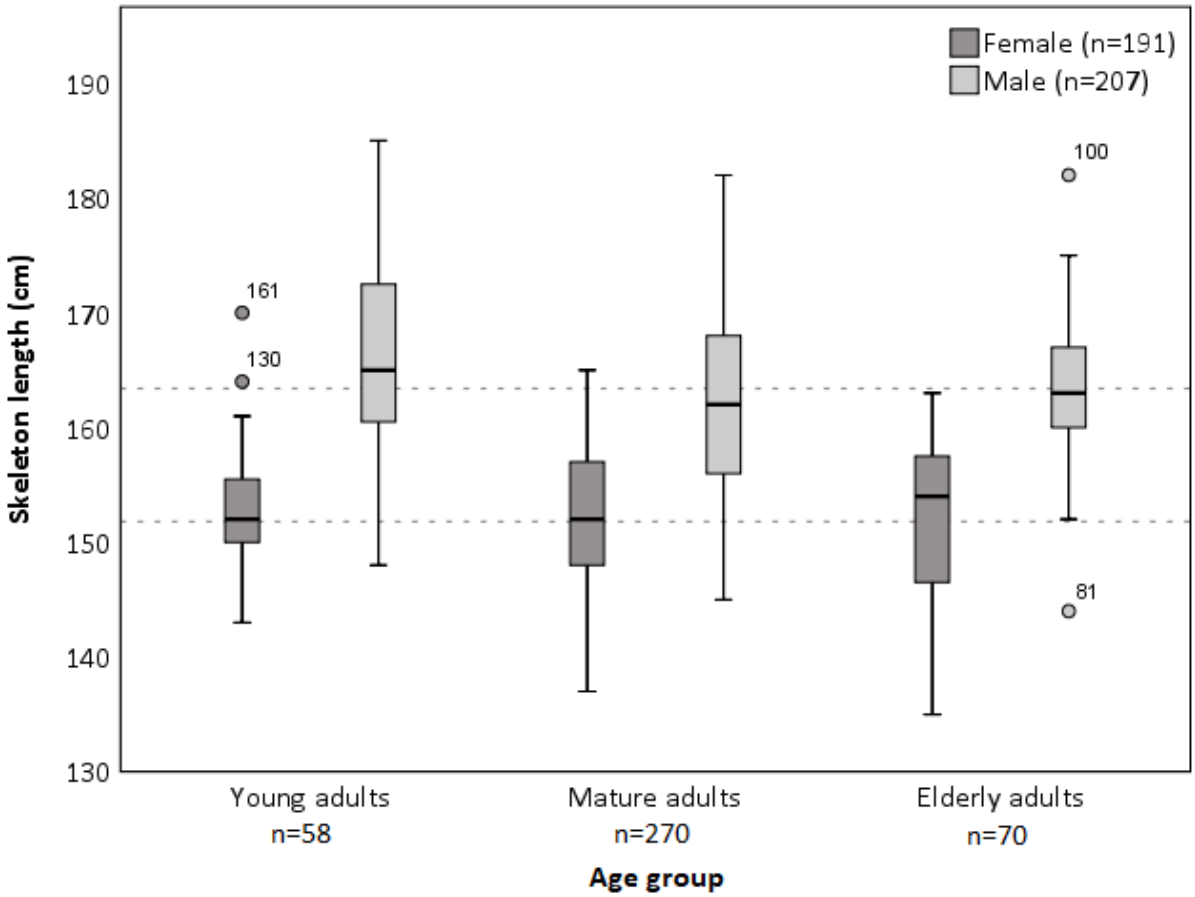


Figure 4.7. Boxplot for the maximum length of the skeleton (cm) registered for age groups, by sex. Horizontal lines represent the overall mean for the females (151.8 cm) and males (163.4 cm).

4.6. Estimating sex

The mean length of each long bone is significantly shorter in females, when compared to males (Table 4.2). The result of the logistic regression analysis can be found in Table 4.3 and the classification accuracy for original and cross-validation in Table 4.4. The combination of measurements from the humerus, radius, femur and tibia gives the best estimation of sex, correctly classifying 89.7% of the skeletons analysed (Table 4.4). However, just by using the radius and the tibia (89.4%) similar results can be obtained. The upper long bones (humerus and radius) are a better option to estimate sex with 88.2% of individuals correctly classified, compared to the lower long bones (femur and tibia) with 85.6% of individuals correctly

classified. Out of the individual bones, the radius (84.7%) and the humerus (84.4%) have the highest percentage of correct estimations while the femur has the lowest (80.0%).

The sexual dimorphism index (SDI) calculated for each bone (Table 4.2) is comparable with the predicted cross-validated percentage (Table 4.4) indicating that bones with high SDI (Tarli and Repetto 1986) also have high percentages of correct sex estimations. The radius has the highest SDI and the femur the lowest, which can also be observed in a higher predicted cross-validated percentage for the radius (84.7%) and a lower percentage of correct estimations for the femur (80%). Similar to previous research (e.g. Mall et al., 2001), when only one of the long bones is present, the most accurate results derived from the radius, even though the humerus and tibia can also be good predictors for some collections. When the upper and lower limbs are combined, the number of individuals correctly classified as either male or female is similar to that achieved when only two long bones are entered into the regression (Table 4.4).

These results support previously published findings that report sexual dimorphism of long bones as being highly population specific (e.g. Bidmos and Dayal, 2004). The length of the radius is the most sexually dimorphic and can contribute to sex estimations when the pelvis is not present.

To develop the equations for sex estimation using Tomar's collection the sex of these individuals was estimated through morphological methods based on the pelvis and cranium. Therefore, the sex of some individuals may have not been correctly estimated to begin with. In this study it was not possible to test other measurements besides bone length as these data were not available. While ephiphyseal dimensions may be better discriminators between sexes (Charisi et al. 2011, Işcan et al. 1998, Frutos 2005) they can also be more prone to change as a response to intense physical activity (Safont et al. 2000, Ruff, 1987,

Carlson et al. 2007). Bone length depends on genetic factors, physiological stress, nutrition and secular trends (Cowgill & Hager, 2007, Gustafsson et al. 2007, Holden & Mace, 1999, Stein & Işcan, 1999). Therefore, sexual dimorphism in bone length may be even more population specific than in other measurements.

Table 4.2. Sample size (n), mean (x), standard deviation (sd) and sexual dimorphism index (SDI) for maximum length of long bones in females and males (SMOL.B).

Maximum length	Females			Males			t	df	*p*	SDI
	n	x (cm)	sd (cm)	n	x (cm)	sd (cm)				
Humerus	336	286.76	14.54	386	317.76	16.561	-26.787	720	0.000	1.108
Radius	111	212.59	12.07	154	240.29	12.353	-18.175	263	0.000	1.130
Femur	383	402.19	19.81	447	440.23	22.055	-26.168	826	0.000	1.095
Tibia	345	330.62	17.13	424	366.11	19.904	-26.163	767	0.000	1.107

Table 4.3. Estimated coefficients for the sexual diagnosis binary models (β_0– intercept term, β_i– slope). A – humerus+radius+femur+tibia, B – humerus+femur+tibia, C – humerus+radius, D – femur+tibia, E – humerus+tibia, F – radius+tibia, G – radius+femur, H – humerus, I – radius, J – femur, K – tibia.

		A	B	C	D	E	F	G	H	I	J	K
N		173	524	240	700	541	199	223	722	265	830	769
β_i	Humerus	0.033	0.082	0.087		0.090			0.132			
	Radius	0.117		0.129			0.144	0.156		0.190		
	Femur	0.023	0.023		0.059			0.076			0.095	
	Tibia	0.064	0.046		0.060	0.057	0.086					0.109
β_0		-67.967	-50.165	-55.23	-45.437	-46.689	-62.079	-66.535	-39.749	-42.559	-39.651	-37.547

Table 4.4. Classification accuracy for original and cross-validation for females, males and pooled sex. Sectioning point is set to zero in all cases.

Long bone length	N			Predicted Group Membership					
				Original group (%)			Cross-validated (%)		
	Females	Males	Total	Females	Males	Total	Females	Males	Total
humerus+radius+femur+tibia	52	84	136	94.2	90.5	91.9	90.4	89.3	**89.7**
humerus+femur+tibia	168	208	376	89.3	86.5	87.8	89.3	86.5	87.8
humerus+radius	78	108	186	88.5	88.0	88.2	88.5	**88.0**	88.2
femur+tibia	210	249	459	89.0	82.7	85.6	89.0	82.7	85.6
humerus+tibia	173	215	388	89.6	87.0	88.1	89.6	87.0	88.1
radius+tibia	57	94	151	93.0	87.2	89.4	**93.0**	87.2	**89.4**
radius+femur	66	105	171	90.9	86.7	88.3	90.9	86.7	88.3
Humerus	242	266	508	88.8	80.5	84.4	88.8	80.5	84.4
Radius	82	117	199	87.7	82.6	80.9	87.7	82.6	84.7
Femur	252	287	539	84.1	76.3	80.0	84.1	76.3	80.0
Tibia	217	263	480	87.1	80.6	83.5	87.1	80.6	83.5

4.7. Estimating stature

It was possible to measure the maximum length of the skeleton, during the excavation, for 543 individuals (256 females, 287 males). The mean skeleton length is 151.8cm (sd=6.2cm) for the females and 163.4cm (sd=7.5cm) for the males. The coefficient of variation (CV) is close to 5% (Table 4.5) for all the analysed bones, for both females and males, suggesting a small variability in bone length within individuals of the same sex. Coefficient of variation is negatively correlated with Pearson's correlation coefficient (PCC) suggesting that less variable bones have stronger correlation with skeleton length. There is a strong correlation between long bone and skeleton length (PCC>0.5) for both females and males, except the female radius (PCC=0.45), with all results being statistically significant ($p<0.001$). For the females the bone that correlates better with stature is the tibia (PCC=0.57), followed by the femur (PCC=0.56). For the males the radius (PCC=0.62) and femur (PCC=0.57) correlates better with stature. Higher correlation (CV, Table 4.5) between long bone and skeleton length also reflects lower coefficient of variation (PCC, Table 4.5), suggesting that the methods are more precise for less diverse groups. The equations for stature estimation based on the different long bones can be observed in Table 4.6.

Stature estimations based on the femur and tibia have smaller 95% confidence intervals than the ones observed for the humerus and particularly the radius (Figure 4.8), suggesting that bones from the lower member give better predictions for stature. For all the long bones analysed the 95% confidence interval increases at both ends of the regression line, suggesting that both shorter and taller individuals have a lower probability of their stature being correctly estimated.

The combination of measurements from the humerus, radius, femur and tibia gives the best stature estimation for both males and females. The coefficient of determination

(R^2) is lower than 0.52 for all equations (Table 4.6), but since the objective is to predict a stature interval and not a precise measurement, lower R^2 values are not as problematic. In Figure 4.8 it is possible to see that the estimated stature is close to the skeleton length. Even though R^2 is lower than 0.50 for most methods they are statistically relevant (p<0.001) and correctly estimated more than 70% of both female and male stature.

Using more variables to estimate stature, and relying in larger stature intervals, increase the probability of more precise stature estimation. If the long bones are not all present, the femur or tibia length give higher percentages of correct estimations. Body size is highly population specific and it is important to compare the samples under study with the ones from which the methods were developed in order to choose the ones that better adapt to the study subject.

Table 4.5. Sample size (n), mean (x), standard deviation (sd), coefficient of variation (CV) and Pearson's correlation coefficient (PCC) for long bones length.

	Maximum length	n	x (cm)	sd (cm)	CV (%)	PCC
Females	Humerus	202	286.5	15.49	5.41	0.52
	Radius	62	213.8	11.85	5.54	0.45
	Femur	240	402.2	20.11	5.00	0.56
	Tibia	220	330.8	17.22	5.21	0.57
Males	Humerus	237	317.0	16.34	5.15	0.54
	Radius	93	239.5	11.55	4.82	0.62
	Femur	270	440.9	22.51	5.11	0.57
	Tibia	262	365.6	20.27	5.54	0.52

Table 4.6. Equations to estimate stature for females and males using long bone length. Coefficient of determination (R^2) indicates how close the data are to the fitted regression line. H – full length of the humerus, R – full length of the radius, F – full length of the femur, T – full length of the tibia.

Method	Regression equation	R^2	df	*F*	*p*
Females	Stature = (92.356+0.207H) ± 5.33	.27	200	72.991	.000
	Stature = (100.952+0.236R) ± 5.67	.20	60	14.877	.000
	Stature = (82.646+0.172F) ± 5.15	.31	238	107.269	.000
	Stature = (84.625+0.203T) ± 5.10	.32	218	102.495	.000
	Stature = (78.999+0.231H+0.028R) ± 5.13	.33	57	13.734	.000
	Stature = (67.441+0.115F+0.115T) ± 4.91	.38	212	64.146	.000
	Stature = (62.636+0.099H+0.074F+0.093T) ± 4.9	.40	175	39.049	.000
	Stature = (45.557+0.057R+0.140F+0.113T) ± 4.64	.50	53	17.311	.000
	Stature = (49.107+0.077H+0.008R+0.14F+0.067T) ± 4.5	.51	50	13.042	.000
Males	Stature = (85.762+0.245H) ± 6.32	.29	235	94.477	.000
	Stature = (70.791+0.390R) ± 5.79	.38	91	55.600	.000
	Stature = (80.829+0.187F) ± 6.08	.32	268	128.795	.000
	Stature = (92.646+0.194T) ± 6.45	.27	260	96.933	.000
	Stature = (50.815+0.174H+0.244R) ± 5.43	.47	84	37.786	.000
	Stature = (70.009+0.074T+0.15F) ± 5.81	.38	247	76.475	.000
	Stature = (60.056+0.101H+0.114F+0.057T) ± 5.78	.41	211	49.675	.000
	Stature = (56.846+0.268R+0.051F+0.057T) ± 5.49	.44	80	20.662	.000
	Stature = (49.811+0.102H+0.232R+0.02F+0.049T) ± 5.37	.48	75	16.945	.000

Cardoso and Gomes (2009) analysed approximately 7000 years of mean stature in Portugal and observed a slow increase in it from prehistory to middle ages, followed by a negative trend to the late 19th century and a rapid increase during the second half of the 20th century. This has also been recorded in other studies across Europe (Roberts and Manchester 2007, Arcini et al. 2014). However, some populations might have not reached the upper limit of human height potential (Stulp and Barrett 2016). Particularly in Portugal, a significant secular increase in stature was only observed after the 1960's and 1970's (Padez 2003), revealing the importance of using methods to estimate stature from an appropriate time period.

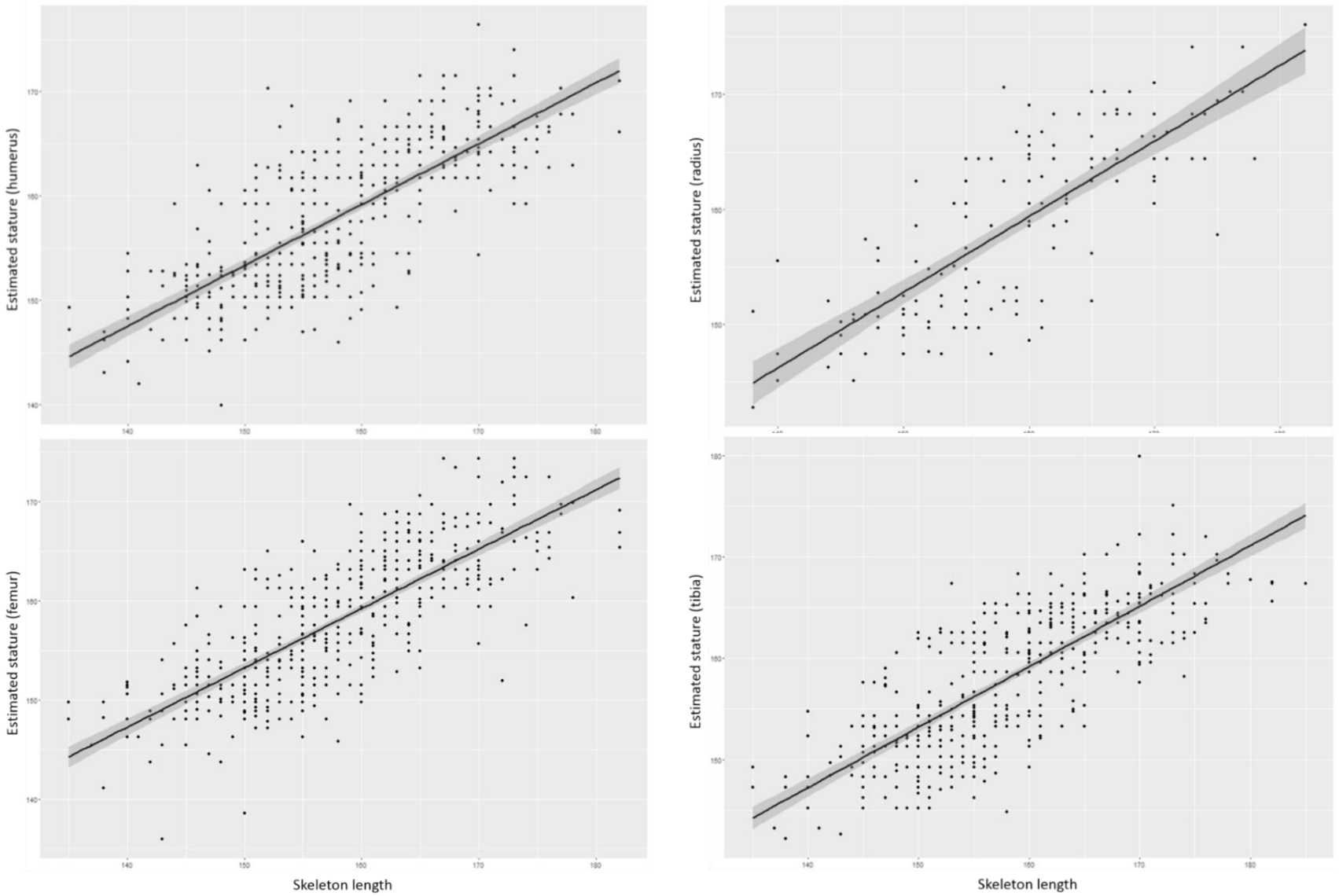


Figure 4.8. Comparison between skeleton length and estimated stature by the long bones, for both females and males. Blue line represents the linear regression line slope and the grey area represents the 95% confidence interval.

4.8. Conclusion

Tomar's collection represents the general population that lived in Tomar at the time and not, or at least not only, the knights of the military order. The areas closer to the church represent individuals from higher socio-economic status than areas further away from the church. However, it is possible that the individuals from the highest and the lowest socio-economic statuses are not represented among the sample excavated during the 2nd phase of the excavation. The data suggest that there were no differences, at least in burial, in socio-economic status between males and females. Still, females may have been more exposed to physiological stress than males.

Sex estimations based on the maximum length of long bones are population specific and using various long bone measurements to estimate sex increases the probability of accurately classifying a skeleton as either male or female. If not all of the bones are present the radius can still be used to estimate sex with a relatively high degree of precision.

The artificial division of the excavated areas (Figure 2.4) makes it difficult to fully understand the distribution of the individuals within the graveyard. Some individuals from area 14, for example, may actually have been buried closer to the church than some skeletons in area 15 (Figure 2.4). The individuals with the highest socio-economic status would be buried inside the church and those with the lowest socio-economic status were probably buried in the areas excavated during the 1st phase of the excavation or even further from the church. This would mean that it was not possible to analyse the individuals from the highest and the lowest socio-economic status in Tomar. A detailed study of Tomar's collection, the individuals from both the 1st and 2nd phase of the excavation, is necessary for a more comprehensive context of the population that was buried at Santa Maria do Olival, Tomar.

1

Chapter 5

Did military orders influence the general population diet? Stable isotopes analysis from Medieval Tomar, Portugal

Abstract

This study integrates bone collagen stable isotope data (carbon, nitrogen and sulphur) from 33 human adult tibiae (15 females, 18 males) and 13 faunal remains from Tomar, while it was under the Military Orders domain (11^{th} – 17^{th} centuries). Historical literature indicates that the amount of meat consumption among Templars was lower than in individuals with similar social status. In medieval times these Military Orders had total control of towns and angling and fishing rights, but their influence on the general population diet remains unknown. While no statistically significant differences (p>0.05) were found between sexes, social status, or for bone collagen $\delta^{13}C$ and $\delta^{34}S$ values between age groups, $\delta^{15}N$ did differ significantly with age, which may be related to tooth loss in elderly individuals. Additionally, the human samples have higher stable isotope differences, in comparison to faunal samples, than would be expected within the food web, particularly for $\delta^{13}C$ values. This human bone collagen $\delta^{13}C$ values increase may reflect a diet rich in aquatic protein intake, which is also supported by $\delta^{34}S$ values archived in human and faunal samples, and the presence of oysters and cockles shells at the excavation. The religious diet restrictions might have led to a higher intake of aquatic protein when meat consumption was not allowed.

5.1. Introduction

This study investigates diet in a town ruled by religious military orders and how the general population diet may have adapted to religious dietary restrictions. This study is the first of its kind to analyse carbon, nitrogen and sulphur stable isotope data from skeletons of the 11^{th} – 17^{th} centuries in Portugal.

5.1.1. Stable isotope analysis

Analysis of stable isotope ratios from mineralized tissue has been widely used for dietary reconstruction. This technique is based on the assumption that "you are what you eat (plus a few ‰)" (DeNiro and Epstein 1976), as a consumer's tissues reflect the isotopic array of the ingested foods. Food webs have an impact on carbon isotope values due to the correlation between animal tissues carbon values ($\delta^{13}C$) and their diet (DeNiro and Epstein 1978, Teeri and Schoeller 1979). There is an increase in $\delta^{13}C$ values in an animal's body tissues relative to its diet due to the fractionation that occurs during the formation of tissues (van der Merwe and Vogel 1978). Primary consumers have a fractionation factor (increase in $\delta^{13}C$ values) of approximately 5‰ in their bone collagen relative to their diet (van der Merwe and Vogel 1978, Ambrose and Norr 1993) and an increase of 1‰ between trophic levels (DeNiro and Epstein 1978, Tieszen et al. 1983). In marine plants the main carbon source is dissolved carbonate (0‰), instead of atmospheric CO_2 (-7‰), therefore, this difference is reflected in the $\delta^{13}C$ values in tissues of mammals feeding from these two different ecosystems (Tauber 1981, Chisholm et al. 1982, 1983). $\delta^{13}C$ values in bone collagen can also help identifying freshwater resources. Katzenberg and Weber (1999) observed a range of – 14.2 to – 24.6‰ in fish bones in Siberia with higher $\delta^{13}C$ values in species inhabiting shallow waters and lower $\delta^{13}C$ values for fish inhabiting deeper open waters on the lake. Freshwater fish exhibit variation in $\delta^{13}C$ values depending on the ecosystem as

freshwater plants have numerous sources of carbon, unlike terrestrial plants (Zohary et al. 1994, Dufour et al. 1999).

In terrestrial ecosystems there is an increment of 3‰ to 5‰ in stable nitrogen isotopes values ($\delta^{15}N$) between trophic levels when compared with consumer diet (Schoeninger et al. 1983, Minagawa and Wada 1984, Schoeninger and DeNiro 1984, Bocherens and Drucker 2003). This fractionation enables the use of $\delta^{15}N$ values to infer trophic level and high $\delta^{15}N$ values recorded in bone collagen usually indicates high-protein diets (Sponheimer et al. 2003). $\delta^{15}N$ values can also be used to differentiate between terrestrial and marine food sources (DeNiro and Epstein 1981, Schoeninger et al. 1983, Walker and DeNiro 1986, Richards and Hedges 1999), especially when combined with carbon isotope data. Bone collagen $\delta^{15}N$ values can also be used to analyse access to fresh water resources, as organisms in these ecosystems exhibit higher $\delta^{15}N$ values than those in terrestrial ecosystems (van Klinken et al. 2000).

Advances in mass spectrometry and methodology development, following the work of Leach et al. (1996) allow an easier and more frequent analysis of sulphur isotope data ($\delta^{34}S$). Sulphur isotope analysis can shed some light on the use of freshwater or marine resources (Nehlich and Richards 2009, Nehlich et al. 2010, Nehlich 2015), especially when combined with the analysis of carbon and nitrogen stable isotopes. A freshwater ecosystem, which is highly depending on the geological conditions and source of water sulphates (Nehlich 2015), has an impact on terrestrial $\delta^{34}S$ values, especially if the fauna fed on the floodplains of the river (Fry 2002, Nehlich et al. 2011). $\delta^{34}S$ values at riverine ecosystems fall between -5‰ and +15‰, but the values can be outside this range in relatively small geographical scale due to specific environmental conditions (Nehlich 2015).

5.1.2. Historic background

The city of Tomar had a very important military role consolidating the Kingdom of Portugal by resisting the advances of the last Moroccan king of Hispania, Iacub ben Iuçuf Almançor (França 1994). The construction of the Convent of Christ, a Templar stronghold, began in 1160 and was also likely around that time that the Church of Santa Maria do Olival was constructed (Conde 1996). In 1317 Pope Clement issued the Papal Bull *Pastoralis Praeeminentiae*, which instructed all Christian monarchs in Europe to arrest all Templars and seize their assets (Barber 2012). Portugal successfully lobbied the papacy and the Templars did not face a trial, instead the Order's assets and personnel were transferred to the newly established Order of Christ, a continuation of the Templars in Portugal (Valente 1998). Tomar then became a centre of Portuguese overseas expansion under Henry the Navigator, the Grand Master of the Order of Christ (Conde 1996).

Trade in Europe began to increase in the 11th century (Malgosa 2011), since Tomar was located at the main Portuguese road connecting the North of the country to the limits of the *Reconquista* (Conde 1996). Given Tomar's location it would have frequent movement of goods but also people and one of its functions was to receive and protect refugees in case of invasion (Conde 1996).

According to historical data, the staple medieval diet in Portugal was bread accompanied by wine, olives and olive oil (Vicente 2013). A significant part of agriculture was focused on cereals but a large percentage of the harvest was inaccessible to peasants after paying tributes to lords and the church (Vicente 2013). Chestnuts and sweet acorns could sometimes substitute the bread (Vicente 2013) and some legumes could be reduced to flour when there was a lack of cereals (Gonçalves 2004). The acorns were frequently used

to feed the livestock, especially swine that also fed from various roots and mushrooms (Vicente 2013).

Cattle were not abundant, compared to sheep and goats, and only the pigs were purposely raised for meat production (Gonçalves 2004). Other sources of meat were chicken, duck and goose as well as a variety of game (Gonçalves 2004). For the peasants, hunting could represent the only access to meat. However, in Tomar angling and warren rights were reserved for the military orders. Among medieval Iberian faunal assemblages the domestic animals predominate (Grau-Sologestoa 2017), which can be a result of hunt restrictions. Fish was an expensive food, with the exception of sardines which were more abundant and easy to preserve salted or smoked (Gonçalves 2004). Fish was indispensable during the numerous fast days that the medieval religious calendar imposed (Vicente 2013) but it was consumed more in the littoral despite the availability of Portuguese rivers (Gonçalves 2004). Molluscs and crustaceans were also part of the diet of all social status but were considered a "food of the poor" due to their abundance (Gonçalves 2004).

Various studies suggest dietary differences between sex, age groups and social status in medieval times (e.g. Adamson 2004, Kjellström et al. 2009, Linderholm et al. 2008, Polet and Katzenberg 2003, Schutkowski et al. 1999, Reitsema et al. 2010, Reitsema and Vercellotti 2012). I expect that in Tomar there will be large dietary differences between sexes as a consequence of the presence of the religious military orders. Since fish was expensive but necessary for religious fasts and the military orders had angling and warren rights, I expect that the diet in Tomar may also reflect social status. The historical literature (Barber and Bate 2002) implies that the amount of meat consumption among Templars was lower than in individuals with similar social status, and the intake of vegetables was higher. In Tomar, merchants, crafters and farmers participated actively at the local army alongside

with knights, raising their status (Conde 1996) and probably having access to similar food resources to the Templars. Therefore general diet in Tomar is expected to be rich in aquatic protein.

5.2. Materials and sampling

This study analyses bone collagen stable isotope data from 33 human adult tibiae (15 females, 18 males) and 13 fauna remains (2 wild *Sus*, 2 domestic *Sus*, 1 juvenile *Sus*, 1 *Canidae*, 3 *Bos*, 1 *Equus*, 3 *Ovicapridae*) from Santa Maria do Olival graveyard (11^{th} – 17^{th} centuries), in Tomar, Portugal. Only individuals from areas 13 to 20 (2^{nd} phase of the excavation, Figure 2.4) were analysed. Areas 13, 15, 18 and 19 were considered to be a place of burial for individuals with higher social status, not only due to the proximity to the church (Binski 1996, Daniell 1998, Graves 1989, Ottaway 1992, Platt 1981, Swanson 1989) but also because of the higher frequency of structured graves. Faunal remains were collected from areas 14, 17 and 20 (Figure 2.4). The faunal remains from area 20 were mixed with human remains in an ossuary with at least 14 human adults. The faunal remains recovered from areas 14 and 17 were in grave fill material.

The skeletons (all ages and both sexes) distribution within the necropolis suggest that Santa Maria do Olival collection represents the general population of Tomar and not, or at least not only, the individuals from the military orders. The uniform spatial distribution between sexes within the graveyard and the use of structured graves for both males and females suggest that, at least at death, social status was not dependent on sex. However, social status seems to increase with age as older individuals were more frequently buried in structured graves.

Only individuals without signs of physiological stress were sampled in an attemptto estimate the diet of the general population and avoid isotopic data that may represent differing metabolism during disease and/or malnutrition (Steele and Daniel 1978, Hobson and Clark 1992, Hobson et al. 1993, Gaye-Siessegger et al. 2004, Fuller et al. 2005, D'Ortenzio et al. 2015). To avoid sampling individuals with physiological stress only individuals without skeletal markers of stress, such as *cribra orbitalia* or obvious enamel hypoplasias, were selected. Since low stature can also be associated with physiological stress (e.g. Haviland 1967, Morris and McAlpin 1979, Allen and Uauy 1994, Roberts and Manchester 2007, Moore and Ross 2013), only individuals with maximum length of the skeleton (measurement was taken during the excavation while the skeleton was still articulated, in situ, in extended supine position and used as a proxy for stature) equal or above the mean for this population (151.8±6.1cm for the females, *n*=256, 163.4±7.5cm for the males, *n*=287) were sampled.

5.3. Methods

Sex was estimated based on pelvic (Phenice 1969, Buikstraand Ubelaker 1994) and cranial features (Buikstravand Ubelaker 1994). Adult age at death estimates employed a combination of skeleton maturation (Scheuer and Black 2000), pubic symphysis degeneration (Brooks and Suchey 1990, Buikstraand Ubelaker 1994) and auricular surface degeneration (Lovejoy et al. 1985). The skeletons analysed were classified as young (18 to 29 years), mature (30 to 60 years) and elderly (more than 60 years) adults.

Collagen extraction was done following Longin (1971), Brown et al. (1988) and Richards and Hedges (1999). The collagen samples were weighed into tin capsules and combusted into CO_2 and N_2using an Elemental analyzer (Flash/EA) coupled to a Thermo

Finnigan Delta^{Plus} XL isotope ratio mass spectrometer via a ConFlo III interfaceat NERC Isotope Geosciences Facility (Nottingham, UK). Sulphur stable isotopes were analysed at the Faculdade de Ciências da Universidade de Lisboa (Lisbon, Portugal). $\delta^{13}C$ values and $\delta^{15}N$ values were calibrated using an in-house reference material M1360p (powdered gelatine from British Drug Houses) with expected δ values of –20.32‰ (calibrated against CH_7, IAEA) and +8.12‰ (calibrated against N-1 and N-2, IAEA) for carbon and nitrogen respectively. Samples were run in duplicate and the 1σ reproducibility for mass spectrometry controls for these analyses were $\delta^{15}N$ = ± 0.08‰ and $\delta^{13}C$ = ± 0.07‰. The sulphur isotope analysis was done at SIIAF (University of Lisbon), using an IsoPrime mass spectrometer. The collagen was combusted with additional V_2O_5 and a pulse of oxygen. $\delta^{34}S$ values were calibrated using the inorganic international standards NBS127 (+20.3‰), IAEA S1 (-0.3‰) and casein protein (+4.0‰). Mass spectrometry control for these analyses was $\delta^{34}S$ = ± 0.08‰

Mann-Whitney U non-parametric tests were used for pair-wise comparisons and Kruskal-Wallis non-parametric tests were used to compare more than two groups. All statistics were computed in SPSS 24 for Windows and $p \leq 0.05$ were considered statistically significant.

5.4. Results

The bones from all individuals in the present study had acceptable C:N ratios (2.9 to 3.6, DeNiro 1985) and S% (0.15% to 0.35%, Nehlich andRichards 2009) (Appendix, Tables A1 and A2). Herbivores, with the exception of *Equus*, have similar values for bone collagen $\delta^{13}C$ values (-21.2‰ to -20.9‰), while bone collagen $\delta^{15}N$ (4.8‰ to 7.8‰) and $\delta^{34}S$ (13.1‰ to 18.5‰) values are more variable (Figures 5.1 and 5.2). The domestic *Sus* and the only carnivore analysed (*Canidae*) have similar $\delta^{13}C$ and $\delta^{15}N$ values to the herbivores (Figure

5.1). The faunal remains have higher bone collagen $\delta^{34}S$ values than the human remains (Figure 5.2), only the *Equus* displays bone collagen $\delta^{34}S$ values expected for an exclusive terrestrial diet.

Among the humans sampled there is an outlier, a male young adult. While his bone collagen $\delta^{15}N$ values (12.3‰) are amongst the highest, his bone collagen $\delta^{13}C$ values (-15.4‰) is high (Figure 5.1) and he displays low bone collagen $\delta^{34}S$ values (9.3%) compared to the other individuals (Figure 5.2). Overall, bone collagen $\delta^{15}N$ and $\delta^{34}S$ values recorded in the humans are more variable than their bone collagen $\delta^{13}C$ values (Table 5.1). The females show higher variance in their bone collagen $\delta^{15}N$ values, while the males display a higher variance in their bone collagen $\delta^{34}S$ values (Table 5.1). There are no statistically significant differences (p>0.05) in stable isotope values between sexes, social status, or for $\delta^{13}C$ and $\delta^{34}S$ values between age groups. However, bone collagen $\delta^{15}N$ values recorded in the human skeletons display significant differences (p=0.05) with age groups (Table 5.2).

Table 5.1. Descriptive statistics for the stable isotope ratios analysed ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) by sex and grouped sexes.

	Female			Male			Female & Male		
Isotope	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$
Mean	-18.6	10.7	13.4	-18.5	10.9	12.9	-18.6	10.8	13.1
sd	0.4	0.9	0.9	0.6	0.8	1.8	0.5	0.8	1.5
variance	0.1	0.8	0.8	0.35	0.62	3.2	0.2	0.7	2.0
max	-17.7	12.5	14.8	-17.3	12.1	15.6	-17.3	12.5	15.6
min	-19.1	9.0	11.5	-19.4	9.4	9.3	-19.4	9.0	9.3
N	15	15	10	17	17	10	32	32	20

Table 5.2. Non-parametric statistics tests of stable isotope analysis ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) comparing groups by sex, age and social status (without outlier).

		$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$
Sex	Mann-Whitney U	114.00	109.00	44.50
	p-value	0.61	0.48	0.68
Age	Kruskal-Wallis H	1.29	5.84	2.76
	p-value	0.53	**0.05**	0.25
Social status	Mann-Whitney U	114.00	108.00	41.50
	p-value	0.97	0.78	0.97

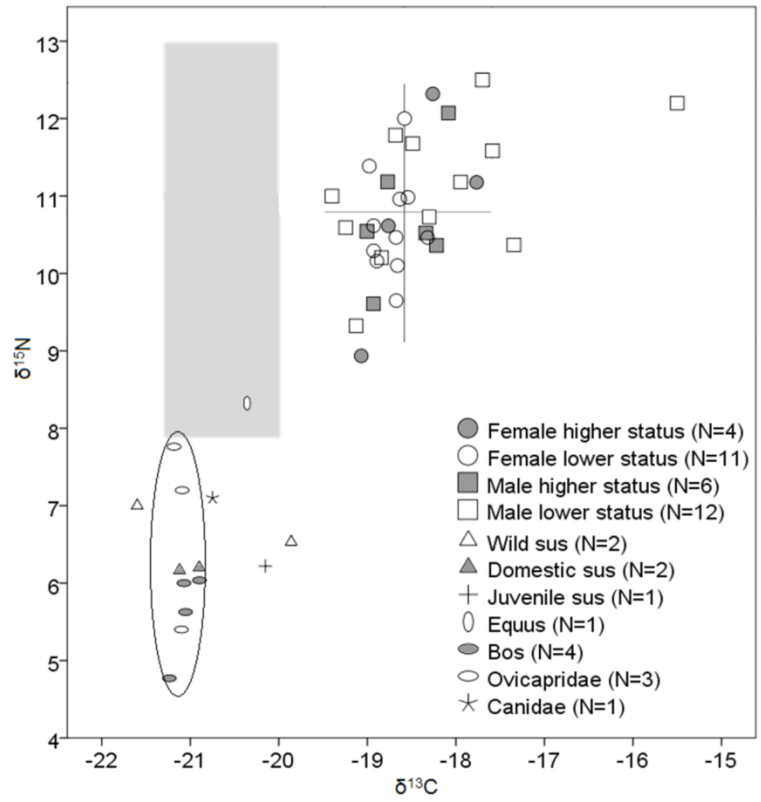


Figure 5.1. Stable isotope values of fauna and human (from different social status) bone collagen. Lines indicate the mean without the outlier ($\delta^{13}C$ = -18.6‰, $\delta^{15}N$ = 10.8‰) and two standard deviations (μ ± 2σ). Grey area indicates the expected values for the trophic level increase from the analysed fauna.

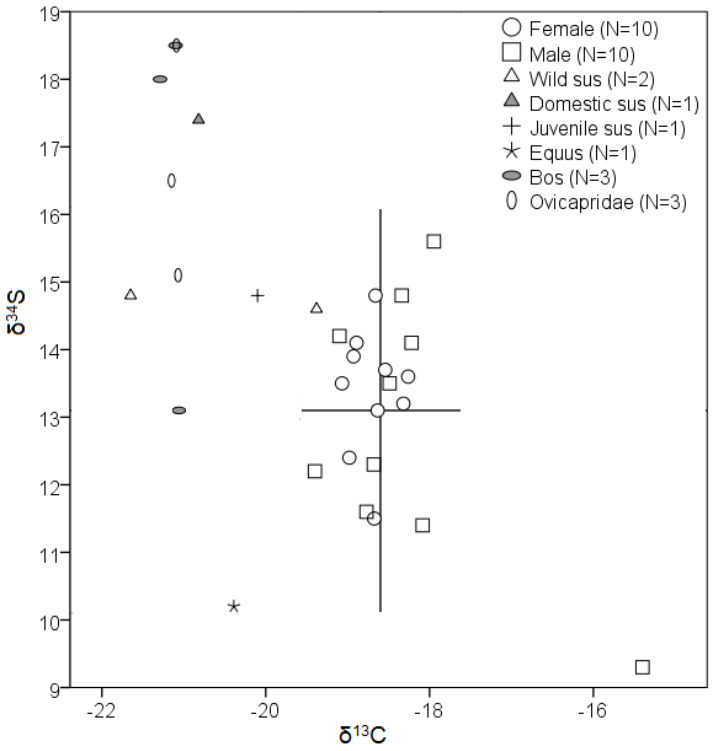


Figure 5.2. Stable isotope values of fauna and human bone collagen. Lines indicate themean without the outlier ($\delta^{13}C$ = -18.6‰, $\delta^{34}S$ = 13.1‰) and two standard deviations (μ ± 2σ).

The individuals for which it was possible to estimate sex and age are represented in Figure 5.3 illustrating $\delta^{13}C$ and $\delta^{15}N$ values differences in bone collagen between age groups. While the young adults are above or close to the mean values for $\delta^{13}C$ (-18.6‰) and $\delta^{15}N$ (10.8‰) values, the elderly adults are all under the mean values for both $\delta^{13}C$ and $\delta^{15}N$ values (with the exception of one), but most fall within two standard deviations from the mean. There are no differences in bone collagen $\delta^{34}S$ values between the age groups (Figure 5.4).

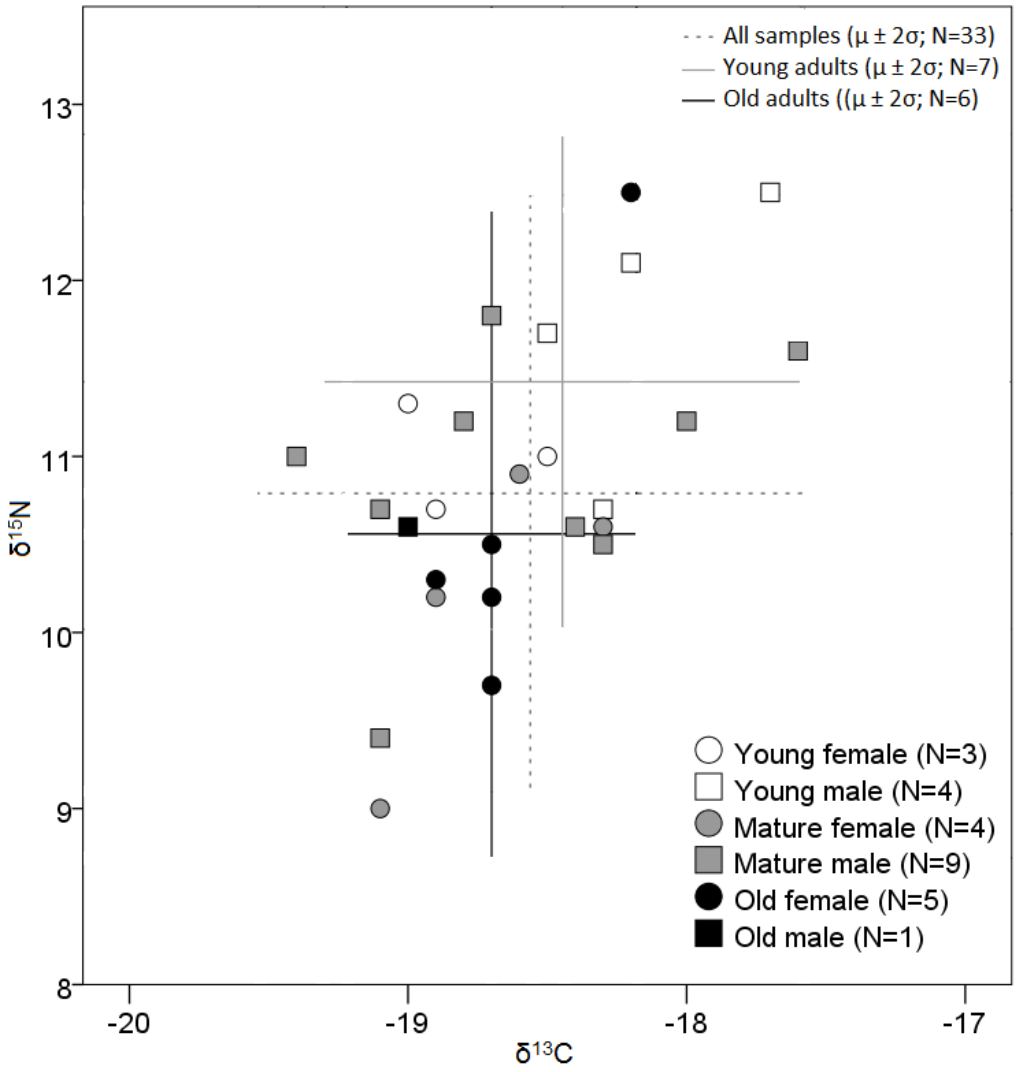


Figure 5.3. Stable isotope values of individuals with estimated sex and age. Lines indicate the mean and two standard deviations (μ ± 2σ) for all the samples except the outlier ($\delta^{13}C$ = -18.6 ± 1.0‰, $\delta^{15}N$ = 10.8 ± 1.7‰), the young ($\delta^{13}C$ = -18.4 ± 0.9‰, $\delta^{15}N$ = 11.4 ± 1.4‰) and the elderly ($\delta^{13}C$ = -18.7 ± 0.5‰, $\delta^{15}N$ = 10.6 ± 1.8‰) adults.

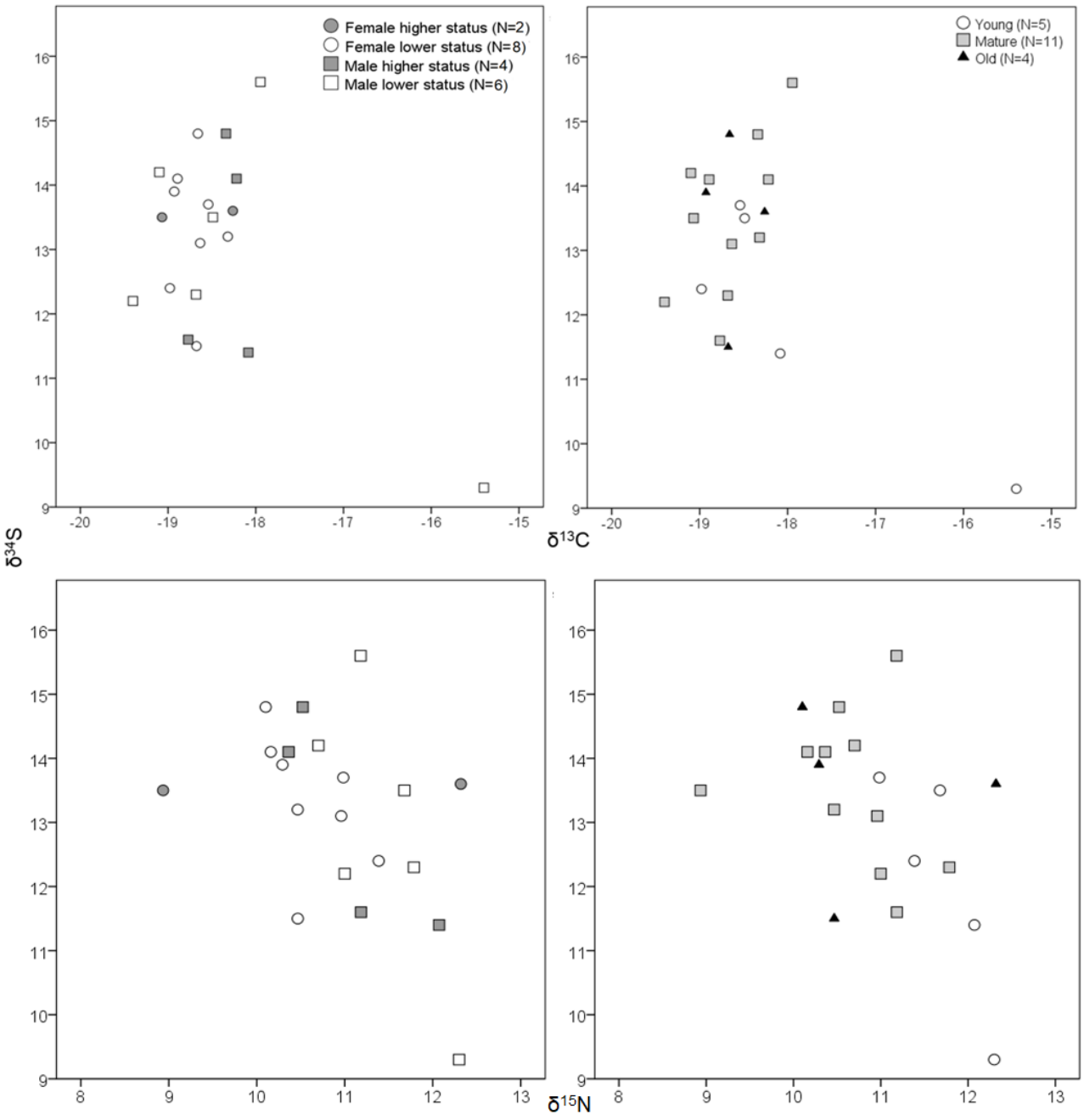


Figure 5.4. Stable isotope values ($\delta^{34}S$, $\delta^{15}N$ and $\delta^{13}C$) of individuals from different social status and with estimated age (young, mature and elderly adults).

5.5. Discussion

5.5.1. General diet at Tomar

There might be dietary differences between different chronologies, especially after the 16th century with the introduction of new food sources, like C_4 plants from America, but unfortunately it was not possible to decrease the chronological interval estimated for Tomar (11th – 17th centuries). The high density of burials and the fact that Christian burials usually do not have associated artefacts did not allow reliable dating.

Herbivores' $\delta^{13}C$ values (-21.3‰ to -20.1‰, Figure 5.1, Appendix: Table A2) suggest a diet based on C_3 plants (Vogel 1978, Schoeninger and DeNiro 1984, Chisholm 1989). Despite the wide range of the estimated chronology for Tomar's necropolis (11th to 17th centuries) and the possibility that the analysed fauna represents different times (areas 14, 17 and 20), the herbivores' $\delta^{13}C$ values are similar, arguing against the introduction of new food sources like maize (C_4 plants). In contrast, bone collagen $\delta^{15}N$ values recorded in herbivores are more variable (4.8‰ to 7.9‰, Figure 5.1) and with some enrichment, particularly observed for the *Ovicapridae*. Enrichment in faunal bone collagen $\delta^{15}N$ values may be related to variable animal husbandry practices and land management. Manured soils raise $\delta^{15}N$ values in soil and plants (van Klinken et al. 2000, Bogaard et al. 2007), having an impact on the local food web. High $\delta^{15}N$ values are particularly evident between the *Ovicapridae*, which may be related to different food sources for sheep (grass, hay) and goats (bushes, tree leaves/bark). The *Ovicapridae* have higher $\delta^{15}N$ values than *Bos*. Higher $\delta^{15}N$ values in *Ovicapridae* compared to *Bos* are also observed in faunal remains (Appendix, Figure A2) from Koksijde (Polet and Katzenberg 2003) but not from Benipeixcar (Alexander et al. 2015). The domestic and wild *Sus* have similar $\delta^{13}C$ and $\delta^{15}N$ values to the herbivores, as well as the only carnivore analysed (*Canidae*), suggesting a diet poor in animal protein.

While pigs are frequently kept as herbivores (e.g. Quirós Castillo 2013), dog isotopic ratios usually cluster with the humans (e.g. Haffman and Velemínský 2015, Quirós Castillo 2013, Lubritto et al. 2013). As dogs frequently eat food scraps, their isotope values can be indicative of a human diet poor in animal protein.

The mean increase from faunal (except the *Equus* and *Canidae*) to human remains is 2.3‰ (in some individuals more than 3‰, Figure 5.1) for $\delta^{13}C$ values and 4.9‰ for the $\delta^{15}N$ values. Some individuals have higher enrichment than would be expected within the food web: up to 1‰ for $\delta^{13}C$ (Schoeninger et al. 1983, Minagawa and Wada 1984, Schoeninger and DeNiro 1984) and 3‰ to 5.7‰ for $\delta^{15}N$ (van der MerI and Vogel 1978, Ambrose and Norr 1993). The trophic level increase expected based on the faunal isotope values would be between 7.8‰ and 13.6‰ for $\delta^{15}N$ values and from -21.3‰ to -19.9‰ for $\delta^{13}C$ values (grey area at Figure 5.1). While human $\delta^{15}N$ values at Tomar range between 9.0‰ and 12.5‰ and can be explained by the trophic increment, their $\delta^{13}C$ values vary between -19.4‰ and -17.3‰, being clearly higher when compared to the faunal remains recovered in Tomar. These high $\delta^{13}C$ values observed in the human remains can reflect a diet complemented by some aquatic (Tauber 1981, Chisholm et al. 1982, 1983) or C_4 plants (Vogel et al. 1978, Chisholm 1989) intake. Due to the presence of oysters and cockles shells at the excavation (areas 14 and 17) and that the analysed fauna were feeding on C_3 plants it is more probable that the $\delta^{13}C$ values in the human bone collagen are related with aquatic protein than with C_4 plants intake.

To better understand diet at Tomar, $\delta^{34}S$ values were also analysed for some faunal and human remains. Surprisingly, the fauna $\delta^{34}S$ values are higher than would be expected for terrestrial animals ($\delta^{34}S$>12‰, Nehlich 2015) and correspond to values expected from coastal fauna influenced by marine sea spray (Nehlich 2015). However, Tomar is located at

approximately 70km from the coast and sea spray sulphates only reach up to 30km inland (Wakshal and Nielsen 1982). Tide floods from the Atlantic Ocean increase the Tagus River flow and its salinity, reaching the floodplains near Santarém (Figure 5.5) and increasing the sea spray reach but not enough to justify the $\delta^{34}S$ values registered for the fauna on its own.

Riverine sulphates can also be found on the riverbanks and floodplains, influencing the isotopic composition of the surrounding landscape (Fry 2002, Nehlich et al. 2011) and the values observed for Tomar's fauna may be related to the livestock feeding on the floodplains. However, floodplains tend to have lower $\delta^{34}S$ values than areas further away from freshwater ecosystems (Nehlich et al. 2011). Therefore it is possible that the use of algae as a fertilizer may have increased the $\delta^{34}S$ values in the food web as fresh seaweed can also be used to feed livestock, mostly ruminants and pigs (Chapman and Chapman 1980). In Portugal, algae has been used in agriculture previously to the 14th century (Veiga de Oliveira et al. 1975, Vieira and Santos 1995) but it would likely be restricted to coastal areas as algae are heavy and usually not carried very far inland (McHugh 2003). Even though the seaweed could be sundried and stored to be used as winter feedstuff for sheep and cattle (Evans and Critchley 2014) it would probably not be taken so far inland.

$\delta^{34}S$ values vary not only by dietary behaviour (Richards et al. 2001) but also by location (Hobson, 1999), ranging from -40‰ to +40‰ in terrestrial rocks (Nielsen et al. 1991) and between -20‰ and +20‰ in terrestrial organic matter (Peterson and Fry 1987). The oxidation of sulphides and organic sulphur by microorganisms in the soils can also result in high $\delta^{34}S$ values and therefore influence the food web (Böttcher et al. 1998, Nehlich et al. 2011). Therefore it is possible that the higher $\delta^{34}S$ values observed in the terrestrial fauna from Tomar may be related to the geochemistry of that area and not with agricultural or husbandry practices. Tomar is located at an area with evaporites, gypsum and marl (yellow

area at Figure 5.5) that would increase the $\delta^{34}S$ values in the food webs of this region. The *Equus,* with the lowest $\delta^{34}S$ (10.2‰, Figure 5.2), supports this hypothesis, as it was more mobile than the other domestic animals. The wild *Sus* also have lower $\delta^{34}S$ values (14.6‰ and 14.8‰), which can also be related with a higher mobility. Interestingly, the human collagen from Tomar has lower $\delta^{34}S$ values (9.3‰ to 15.6‰) than the faunal remains (13.1‰ to 18.5‰), suggesting that those terrestrial animals were not frequently consumed by the local population, who could have relied on other food sources from another geographical area with lower $\delta^{34}S$ values in its geo-ecosystem.

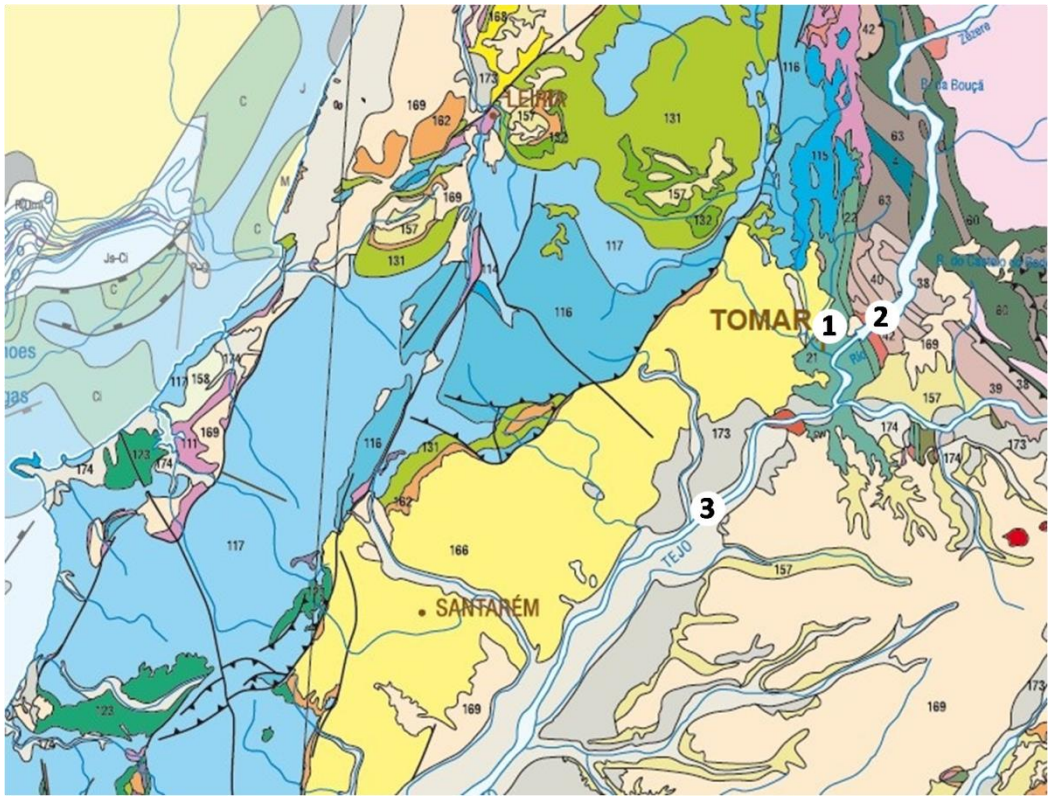


Figure 5.5. Geological map of Tomar's region. Yellow area represents the evaporites, gypsum and marl. 1– Nabão River, 2 – Zêzere River, 3 – Tagus River.

$\delta^{34}S$ values at riverine ecosystems usually fall between -5‰ and +15‰ (Nehlich 2015), but values can fall outside this range, depending on the geological surroundings of the river basin, ultimately influencing the $\delta^{34}S$ values of the river fauna (Nehlich 2010, 2011). Unfortunately, fish bones were not recovered from Tomar's excavation to confirm the

values of the fish consumed by this population. If fresh water protein intake was important and with low $\delta^{34}S$ values, it could decrease the high $\delta^{34}S$ values within the surroundings of Tomar, related to its particular geological context. However, Nabão River, that crosses Tomar, is also located within the same geological substrate and thus, $\delta^{34}S$ values within its food webs are probably high. Zêzere River is located at approximately 10km from Tomar rising at Serra da Estrela (a granitic and metamorphic mountain range) and meeting the Tagus River (an international river) at about 15km from Tomar (Figure 5.5). Since Zêzere and Tagus rivers do not pass through an area with evaporites and gypsum, $\delta^{34}S$ values of their food webs are probably lower than those at Nabão River. Human bone collagen $\delta^{34}S$ values suggest that if they were eating fresh water protein it was probably coming from Zêzere and/or Tagus. Besides, their larger dimensions could offer more food sources than Nabão River. The presence of shells at the excavation suggests also some marine protein intake. Nazaré is the closest coastal town where today fish and octopus are still sundried at the beach, this way of preserving the fish might have allowed its consumption further inland, in towns like Tomar, alongside with fish from the surrounding rivers.

The lower $\delta^{34}S$ values registered in human bone collagen can also be related with terrestrial intake from a geographical location with lower $\delta^{34}S$ values in its food webs. Since the staple medieval diet in Portugal was bread (Vicente 2013) it is possible that it was being made with flour from cereals grown in a location different from Tomar's surroundings. If bread, made with cereals with low $\delta^{34}S$ values, was consumed in high quantities, it could also have lowered the $\delta^{34}S$ values of individuals, independently of the geological substrate in the surroundings of Tomar. The possibility of C_4 plants being consumed only by humans cannot be excluded. It could have been entering their diet in the form of maize flour, for example, and if the maize was not cultivated in Tomar, it could explain both the lower $\delta^{34}S$

values and the higher $\delta^{13}C$ values recorded in human bone collagen. However, the negative relation between $\delta^{34}S$ and $\delta^{15}N$ values (Figure 5.4) likely indicates that the higher $\delta^{15}N$ values are related with protein with lower $\delta^{34}S$ values and therefore the high $\delta^{15}N$ values represent protein from fresh water rather than from terrestrial fauna.

As Tomar was ruled by the Order of the Temple and later the Order of Christ (Vicente 2013) it is possible that religious dietary restrictions would be reflected in Tomar's population. Also, in Tomar, merchants, crafters and farmers participated actively at local army levels alongside with knights, raising their status (Conde 1996) and probably giving them access to similar food resources. Müldner et al (2009) found isotopically distinct diets between bishops and the general population in Scotland, the latter having higher fish intake, related to religious fasting. As predicted, these dietary restrictions may have led to a higher intake of aquatic protein when meat consumption was not allowed with towns controlled by military orders likely being under increased pressure to follow religious dietary restrictions. More isotopic data from different places with similar chronologies is necessary to understand if the high intake of aquatic protein is due to the presence of the military orders at Tomar or if it was a generalised religious phenomenon.

Human diet at medieval Tomar was complex and likely included food sources from outside Tomar. The general diet was poor in terrestrial protein and rich in fresh water protein with possible terrestrial protein from other geographical locations.

5.5.2. Dietary differences within Tomar

Even though some historical (e.g. Adamson 2004) and anthropological (e.g. Kjellström et al. 2009, Linderholm et al. 2008, Polet and Katzenberg 2003, Schutkowski et al. 1999, Reitsema et al. 2010, Reitsema and Vercellotti 2012) sources suggest different food access based on age, sex and status in medieval times, that was not observed in Tomar. When the skeletons sampled were grouped by sex, age or inferred social status only $\delta^{15}N$ values in the age groups was statistically different ($p<0.05$, Table 5.2).

The bone collagen of young adults display higher $\delta^{15}N$ values than the elderly adults (Figure 5.4) suggesting a higher animal protein intake for the young individuals. Since only skeletons without signs of physiological stress were sampled the higher $\delta^{15}N$ values for the young adults is not related to chronic stress (Steele and Daniel 1978, Hobson et al. 1993, Gaye-Siessegger et al. 2004, Fuller et al. 2005, Deschner et al. 2012, D'Ortenzio et al. 2015) that might have resulted in premature death, due to ill health (Wood et al. 1992). These isotopic differences between young and elderly adults may be related to severe tooth loss that was observed in elderly individuals who therefore may have had increased difficulty ingesting some foods along with changes associated with metabolism in the aging, such as reductions in taste, smell and hunger, and delayed rate of absorption (Roberts and Rosenberg 2006). The amount of fresh water fish is variable and not related to sex, social status or age (Figure 5.4). Overall, the skeletons analysed had similar diets with smaller $\delta^{34}S$ differences compared to other European samples (e.g. Nehlich et al. 2011), despite the wide chronology estimated for Tomar's necropolis (11^{th} – 17^{th} century). There are no $\delta^{34}S$ values differences between age, sex or social status but there could be dietary differences between chronologies. Unfortunately it was not possible do date the faunal or human remains.

The absence of statistically significant isotopic differences between sexes (Table 5.2) suggests that males and females had similar protein intakes at Tomar, in contrast to what was expected. However, sample sizes may be too small and dispersed (sex, age, social status and chronology) to detect significant differences and the wide chronology of this collection could have also biased the results. The uniform spatial distribution for males and females within the graveyard and the use of structured graves for both sexes (Chapter 4) also suggest that, at least at death, social status was not dependent on sex though medieval society was male-dominant.

The only outlier analysed, a young adult male, has higher values of both $\delta^{15}N$ (12.3‰) and $\delta^{13}C$ (-15.4‰) values and low $\delta^{34}S$ (9.3‰) values. The low $\delta^{34}S$ values suggest that this individual might be an outsider, coming from a place with lower $\delta^{34}S$ values in its ecosystem, but the possibility of these isotopes values being the result of a high fresh water protein intake from low $\delta^{34}S$ values cannot be excluded. The high $\delta^{13}C$ and $\delta^{15}N$ values can also represent a terrestrial diet rich in C_4 plants, directly or fed to the livestock, particularly if this individual was from a different geographical location.

Food can reflect social status and define social, cultural and religious boundaries (e.g. Thomas 2007, Curet and Pestle 2010), however this was not observed within the samples analysed. The $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values distribution is uniform when comparing individuals with higher and lower social status, opposite to what was predicted. Individuals from lower social status may be more susceptible to physiological stress, particularly due to malnutrition (e.g. Weston 2012). Since only individuals without skeletal signs of physiological stress (low stature, *cribra orbitalia*, porotic hyperostosis) were sampled, the ones with lower social status could have been avoided. Adult stature is determined by genetics but also has an environmental determinant (e.g. Haviland 1967, Larsen 1997, Bogin

1999, Cardoso and Gomes 2009). The areas further away from the church (1st phase of the excavation, Figure 2.4) better represent the individuals from the lower social status and the ones buried inside the church would be a better example of the people from higher social status. Therefore the individuals analysed probably represent the average population and neither of the social extremes. More isotopic data from different social and health status would help understand if the diet at Tomar was uniform or if our results were biased by selecting only apparently healthy individuals.

5.5.3. Other European studies

Comparing the data with other late medieval European samples (Figure 5.6), those with similar $\delta^{13}C$ and $\delta^{15}N$ average values are Koksijde (from a coastal Belgian monastery, Poletand Katzenberg 2003) and Rome (from an Italian mass grave, Salamon et al. 2008). Contrary to what would be expected the stable isotope values from Tomar are closer to the Belgian sample (Poletand Katzenberg 2003) than to the other Iberian samples (Lubritto et al. 2013, Alexander et al. 2015), which may be related to religious dietary requirements, particularly low meat consumption, as the Belgian sample represents a monastic community (Poletand Katzenberg 2003). The similar faunal values for Tomar and Koksijde (Appendix, Figure A2) allow a comparison between the two locations, despite their geographical and social differences. The impact of religious directives of the Catholic Church on the diet has been registered before (Salamon et al. 2008). This was facilitated by industrial-scale fishing in the Atlantic (Barret et al. 2004) and improvement of food preservation methods (Heinrich 1986, Robinson 2000). Müldner and Richards (2007) also associated the increased intake of aquatic protein (mostly marine fish with some freshwater fish or molluscs) at St. Andrew (Figure 5.6) with religious dietary habits in Later Middle Ages. Agricultural and husbandry

practices used during the Middle Ages may also explain the different isotopic values between the medieval skeletons buried in Tomar and the prehistoric ones, alongside with a higher aquatic protein intake (Figure 5.6).

Out of the Iberian samples compared, Tomar has the highest $\delta^{15}N$ mean, particularly when compared to Zaballa (Lubrito et al. 2013), Treviño (Quirós Castillo 2013) and Zornoztegi (Quirós Castillo 2013). The high $\delta^{15}N$ mean can represent high animal protein intake, however, $\delta^{34}S$ values suggests a high aquatic protein intake which can also be related with high $\delta^{15}N$ values. The faunal remains recovered from Zaballa (Lubrito et al. 2013), Treviño (Quirós Castillo 2013) and Zornoztegi (Quirós Castill, 2013) also have lower $\delta^{15}N$ values when compared with the ones from Tomar. Colegiata St. Maria (Alexander et al. 2015) and Benipeixcar (Alexander et al. 2015) have similar $\delta^{15}N$ mean to Tomar's but higher $\delta^{13}C$ values, which the authors relate to C_4 plants consumption (directly or fed to domestic animals) or marine fish intake. It is also important to note the different locations of the Spanish collections. While Zaballa, Treviño and Zornoztegi are located at the Basque Country, Northeast of Spain, at approximately 90km to the North Atlantic Ocean, the collections from Colegiata St. Maria and Benipeixcar are from Catalonia, South East of Spain, and at approximately 5km to the Mediterranean Sea. The different locations of these collections may explain why Colegiata St. Maria and Benipeixcar may have higher aquatic protein intake than Zaballa, Treviño and Zornoztegi. Tomar's population has closer mean $\delta^{15}N$ to the ones closer to the Mediterranean Sea, suggesting also a higher intake of aquatic protein, while the different $\delta^{13}C$ values may be related to C_4 plants consumption at Colegiata St. Maria and Benipeixcar (Salamon et al. 2008).

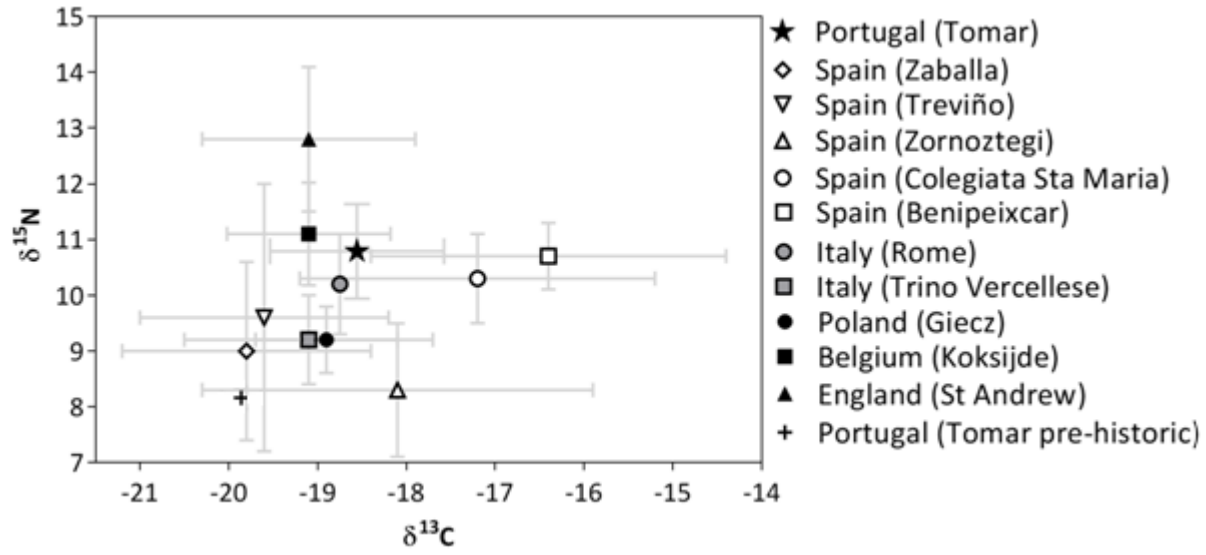


Figure 5.6. Carbon and nitrogen stable isotope comparison between pre-historic and late medieval Tomar and other late medieval European samples. Portugal: Tomar (this study), Tomar prehistoric (n=2, Abrigo do Morgado Superior, unpublished data). Spain: Zaballa (n=14, 10^{th} – 15^{th} century, Lubritto et al., 2013), Treviño (n=15, 12^{th} – 14^{th} century, Quirós Castillo, 2013), Zornoztegi(n=7, 12^{th} – 14^{th} century, Quirós Castillo, 2013), Colegiata St. Maria (n=24, 13^{th} – 16^{th} century, Alexander et al., 2015),Benipeixcar (n=20, 15^{th} – 16^{th} century, Alexander et al., 2015). Italy: Rome (n=29 15^{th} century Salamon et al., 2008), TrinoVercellese (n=30, 8^{th} – 13^{th} century, Reitsema et al., 2012). Poland: Giecz (n= 24, 11^{th} – 12^{th} century, Reitsema et al., 2010).Belgium: Koksijde (n=19, 12^{th} – 15^{th} century, Polet& Katzenberg, 2003). England: St. Andrew (n=155, 13^{th} – 16^{th} century, Müldner& Richards, 2007).

5.6. Conclusion

This study is part of a larger project comparing stable isotopic data from individuals without skeletal lesions compatible with diseases and/or physiological stress (presented here) and those with signs of infectious diseases. Since skeletons with lesions were not analysed, this study might better represent the diet at Tomar, instead of metabolic changes during physiological stress. The bone collagen stable isotope values ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values) suggest that individuals in Tomar had a complex diet, low in terrestrial animal protein and high in aquatic protein intake, despite its inland location, which could be related to the presence of the military orders in the town and more strict religious dietary restrictions. Dietary differences between sex or social status were not observed for the population of Tomar, but the quantity of aquatic protein intake is variable, with $\delta^{34}S$ values ranging from 11.4‰ to 15.6‰ (excluding the outlier). Diet appears to be very diverse in Medieval Iberia. Isotopic data from more archaeological sites are necessary to better understand how diet represents social, religious and economic factors, as well as increase our knowledge of trade, agricultural and husbandry practices in medieval times. Data from archaeological sites near Tomar would also help understanding the impact of the presence of religious orders on a town's general population.

Chapter 6

Diet and disease in Tomar, Portugal: comparing stable carbon and nitrogen isotope ratios between skeletons with and without signs of infectious disease

Abstract

This study explored the correspondence between stable isotope ratios and indicators of non-specific (periostitis and/or osteomyelitis) and specific (venereal syphilis) disease in a sample of human skeletons from a Portuguese archaeological collection. Additionally, this study examined stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios between individuals at different disease stages. $\delta^{13}C$ and $\delta^{15}N$ data from previously analysed skeletons without signs of infectious disease or physiological stress (n=32) were compared to new data from skeletons with active (n=6), healed (n=7) or a combination of both lesions (n=10). Skeletons with lesions (n=23) were also grouped as having only healed tibial periostitis (n=7), generalised non-specific (n=5) and generalised specific infections (n=2). The skeletons with lesions that did not fit into these groups (n=9) were not used in this analysis. The $\delta^{15}N$ values from skeletons with non-specific generalised infections in several bones differed significantly when compared to skeletons that had either only healed tibial periostitis or were without lesions. Skeletons with venereal syphilis had similar mean $\delta^{13}C$ and $\delta^{15}N$ values to either skeletons without signs of disease or those with only healed tibial periostitis. These results suggest different diets may be linked into an individual's susceptibility to these pathogens. Diet influences resistance to infectious disease, while

infections decrease nutrient availability, increase malabsorption and resting energy expenditure. Potentially therefore, combining isotopic evidence of diet with pathology may contribute to a new understanding of health and lifestyle in the past.

6.1. Introduction

The main objective of this study is to determine if there is a link between diet and health assessed by $\delta^{13}C$ and $\delta^{15}N$ ratios from bone collagen in skeletons that retain evidence of non-specific disease. The stable isotope ratios from long bones' collagen are a long-term measure of dietary protein consumed by an individual over a period of about 10 years of life (Hedges et al. 2007). Thus, I seek to determine if longer term diet corresponds with disease at the point of death. Our predictions are as follows:

Protein malnutrition over a long period of time impairs the immune system and increases the likelihood of an individual contracting an infectious disease (e.g. Calder 2013, Scrimshaw and SanGiovanni 1997, Woodward 1998, Woodward 2001). Therefore, individuals with skeletal signs of infectious diseases might have had different diets than those without skeletal lesions. Skeletons with signs of infection might have had a diet poorer in animal protein, than the individuals without lesions, which might have lowered their resistance to disease (e.g. Calder 2013, Kuvibidila et al. 1993, Scrimshaw and SanGiovanni 1997, Woodward 1998, Woodward 2001, Ulijaszek et al. 2012, Weston 2012).

$\delta^{15}N$ values in particular are very informative of trophic level and high $\delta^{15}N$ values usually indicate high-protein diets (Schoeninger et al. 1983, Minagawa and Wada 1984, Schoeninger and DeNiro 1984, Bocherens and Drucker 2003). Therefore I predict that skeletons without signs of infectious disease have higher $\delta^{15}N$ values than the ones with skeletal lesions. However, there are other factors that can raise the $\delta^{15}N$ values including

physiological (Katzenberg and Lovell 1999, Oelbermann and Scheu 2001, Gaye-Siessegger et al. 2004, Deschner et al. 2012, D'Ortenzio et al. 2015) and/or nutritional stress (Steele and Daniel 1978, Hobson et al. 1993), which have been associated with $\delta^{15}N$ increase due to protein catabolism. In prolonged cases of disease, nutritional or physiological stress, dietary protein cannot adequately replace nitrogen losses (Grossman et al. 1945, Powanda 1977, Welle 1999). Consequently, the body proteins are recycled resulting in high $\delta^{15}N$ values (e.g. Steele and Daniel 1978, Hobson et al. 1993, Deschner et al, 2012, D'Ortenzio et al. 2015).

Periostitis generally reflects a reaction to pathologic changes of the underlying bone, or part of it, but can also result from trauma and/or inflammation of the surrounding tissues (Ortner and Putschard 1985, Ortner 2003). Generalised infections (various bones with periostitis and/or osteomyelitis), on the other hand, might represent severe infections which spread across the body (Ortner and Putschard 1985, Ortner 2003). However, the presence of skeletal lesions can also represent good physiological state, allowing these individuals to survive long enough to the disease for it to be visible on their bones (Wood et al. 1992). Periostitis reflects physiological stress and morbidity but frequently represents later phases of the inflammation and succeeding recovery from the stress incident (Klaus 2014). For this reason bone collagen $\delta^{15}N$ and $\delta^{13}C$ values from skeletons without lesions (and other skeletal markers of physiological stress, Chapter 5, Curto et al. 2018) will be compared with bone collagen $\delta^{15}N$ and $\delta^{13}C$ values from 1) skeletons with only healed tibial periostitis, 2) skeletons with non-specific generalised infections and 3) skeletons with venereal syphilis.

Woven bone is produced during rapid bone formation and when it is observed in adults it is considered of pathological origin (Ortner and Putschard 1985, Ortner 2003). Since in chronic or healing stages the woven bone is rapidly remodelled into compact bone,

woven bone is considered a lesion which was active *perimortem*, while compact bone is considered a lesion which was healed *perimortem* (Ortner and Putschard 1985, Ortner 2003). Chronic infectious diseases can also have various acute phases and be very informative about the nutritional adequacy of the diet in a specific community (Goodman and Martin 2002). Therefore, bone collagen $\delta^{15}N$ and $\delta^{13}C$ from skeletons without lesions (and other skeletal markers of physiological stress) will be compared with bone collagen $\delta^{15}N$ and $\delta^{13}C$ values from 1) skeletons with only active lesions, 2) skeletons with only healed lesions and 3) skeletons with both healed and active lesions. Since Protein malnutrition impairs the immune system (e.g. Calder 2013, Scrimshaw and SanGiovanni 1997, Woodward 1998, Woodward 2001), I predict that skeletons without lesions have higher $\delta^{15}N$ values than those with lesions, with the ones with only active lesions having the lowest $\delta^{15}N$ values. The skeletons with only healed lesions are expected to have $\delta^{15}N$ values similar to the skeletons without lesions as they survived the disease long enough for the bone to remodel into compact bone (Ortner and Putschard 1985, Ortner 2003, Wood et al. 1992).

6.1.1. Effect of diet on health

Nutritional stress may result in either greater susceptibility to physiological stress or greater resilience to stress later in life (Bogin et al. 2007). Malnutrition impairs the immune system (e.g. Calder 1991, Scrimshaw and SanGiovanni 1997). Individuals with poorer nutrition are less resistant to infectious diseases, and infectious disease decreases nutrient availability (e.g. Martorell 1980, Mata et al. 1971). The effect of protein-energy malnutrition on aspects of immune function and susceptibility to infection (e.g. Kuvibidila et al. 1993, Scrimshaw and SanGiovanni 1997, Woodward, 1998, Woodward 2001) affects practically all forms of immunity, in particular cell mediated immunity (Kuvibidila et al. 1993, Woodward 1998,

2001), immune barrier function (Deitch et al. 1990, Sherman et al. 1985) and the functioning of lymphoid organs (Lee and Woodward 1996). On the other hand, infections can decrease nutrient availability due to malabsorption (e.g. Mitra et al. 1997) and increase resting energy expenditure, altering the metabolism and redistribution of nutrients (Calder 2013). However, if nutrition is adequate, diseases like tuberculosis may have a less severe infection, instead of an exacerbated infection, resulting in prolonged chronic infections with a higher probability to affect the skeleton (Ulijaszek et al. 2012).

6.1.2. Skeletal lesions as health indicators

Health is a complex state that can be reflected through skeletal indicators of physiological stress (Temple and Goodman 2014). Physiological stress can be related to a wide variety of factors such as disease and nutritional deficiencies (Armelagos 2003, Goodman and Martin 2002, Huss-Ashmore et al. 1992, Zuckerman and Armelagos 2011). Even though systemic physiological stress is not directly observable in the skeleton their consequences, in some cases, are (Klaus 2014).

Infectious diseases were a significant cause of death in past populations, particularly prior to the antibiotic era (Ortner and Putschard 1985). Pathogens can reach the skeleton by direct infection through wounds, extensions from adjacent soft tissue infections or spread by the blood from the site of a remote infection (Ortner and Putschard 1985, Ortner 2003). The body reacts to infection through an inflammatory response which aims to neutralize the pathogen and repair the resultant damage (Weston 2012). Infection damages the normal cells and accelerates the cell turnover (inflammatory process) (Ragsdale and Lehmer 2012). Inflammation affects the bone tissue at some level through the production of pathological skeletal phenotypes (e.g. Ragsdale and Lehmer 2012, Redlich and Smolen 2012). However,

inflammation can be caused by other factors (e.g. Larsen 1987, Ortner 2003, Ortner and Putschard 1985). Bone reacts in a limited number of ways (production or destruction of bone, or a combination of production and destruction of bone) for either infection or other causes such as trauma (e.g. Ragsdale and Lehmer 2012, Weston 2008, 2009). However, by analysing the skeleton as a whole and taking into account other bone-forming disorders, systemic non-specific infection remains a contextually plausible diagnostic option (Klaus 2014).

The bone changes associated with periostitis, an inflammation of the periosteum resulting in deposition of new bone (Bush 1989), vary from one or more layers of woven or compact bone to spiculae perpendicular to the surface of the bone (Ortner 2003). Periostitis not associated with a specific skeletal syndrome, particularly on the tibiae, can be linked to pathogens such as *Staphylococcus* or *Streptococcus* (Goodman and Martin 2002). However, the periosteum responds in a similar way regardless of the etiology (Weston 2008, Weston 2009). Tibial periostitis is the most commonly reported skeletal lesions in archaeological samples (e.g. DeWitte 2010, Weston 2012), being frequently considered an indicator of non-specific physiological stress (e.g. DeWitte 2010, Robb et al. 2001).

In case of infection leading to pathological new bone formation, inflammation-derived pathological periosteal new bone formation is rooted in biological stress (Klaus 2014). Osteomyelitis is the result of the introduction of infectious agents into bone, affecting the medullar cavity (Ortner and Putschard 1985, Ortner 2003). Bones with osteomyelitis can present a combination of cloacae, sequestrated bone and involucrum or only reactive bone formation in the marrow and outer cortex that can result in smooth or lumpy compact bone (Ortner and Putschard 1985, Ortner 2003, Pinhasi 2008). The

expression of osteomyelitis can vary depending on age, nature of the initial infection and immunity of the individual (Pinhasi and Mays 2008).

Acute infections are usually associated with rapid death rarely affecting the skeleton but it may also stimulate new bone formation (Ortner and Putschard 1985, Ortner 2003). Rapid bone formation produces woven bone (active lesions) that typically is the initial stage in many abnormal bone forming lesions caused by infection (Ortner and Putschard 1985, Ortner 2003). In chronic or healing stages (healed lesions) the woven bone is remodelled into compact bone (Ortner and Putschard 1985, Ortner 2003). However, chronic infectious diseases often have various acute phases. Chronic infections are very informative about the nutritional adequacy of the diet, the state of waste disposal and hygiene in a specific community (Goodman and Martin 2002). Infectious pathologies, especially when linked with malnutrition, are the largest contributor to morbidity and mortality worldwide (Keusch and Farthing 1986). The study of nutrition-infection interactions is important to understand the complexity of the relationships of these factors with immunological status, co-morbidity and mortality (Ulijaszek et al. 2012), especially in pre-antibiotic societies.

New bone formation can also be considered an indicator of physiological stress and has been associated with lower socio-economic status (e.g. Goodman and Martin 2002, Peck 2013, Robb et al. 2001), systematic infections (e.g. Goodman and Martin 2002, Larsen 2002, Ortner 2003), malnutrition (e.g. Weston 2012) and niacin deficiency (Paine and Brenton 2006), which can leave the individuals more susceptible to pathogens. Deposits of new bone may also be associated with elevated risks of mortality and are therefore informative about ill health (e.g. DeWitte and Wood 2008).

6.1.3. Stable isotope analysis

Analysis of stable isotope ratios from mineralized tissue has been widely used for dietary reconstruction. This technique is based on the assumption that "you are what you eat (plus a few ‰)" (DeNiro and Epstein 1976), as a consumer's tissues reflect the isotopic array of the ingested foods.

There is enrichment in $\delta^{13}C$ values in an animal's body tissues relative to its diet due to the fractionation that occurs during the tissue's formation (van der Merwe and Vogel 1978). Consumers have a carbon fractionation factor (enrichment in $\delta^{13}C$) of approximately 5‰ in their bone collagen relative to their diet (Ambrose and Norr 1993, van der Merwe and Vogel 1978) and an enrichment of 1‰ between trophic levels (DeNiro and Epstein 1978, Tieszen et al. 1983). There is an increase in $\delta^{15}N$ values of 3‰ to 5‰ between trophic levels when compared with consumer's diet (Bocherens and Drucker 2003, Minagawa and Wada 1984, Schoeninger and DeNiro 1984, Schoeninger et al. 1983). This fractionation enables the use of $\delta^{15}N$ values to infer trophic level and high $\delta^{15}N$ values recorded in bone collagen usually indicates high-protein diets (Sponheimer et al. 2003). There are other factors that can raise bone $\delta^{15}N$ values, such as aridity (Ambrose and DeNiro 1986, Heaton 1987, Heaton et al. 1986, Sealy et al. 1987), physiological (Deschner et al. 2012, D'Ortenzio et al. 2015, Gaye-Siesseger et al. 2004, Katzenberg and Lovell 1999, Oelbermann and Scheu 2001) or protein stress (Hobson et al. 1993, Steele and Daniel 1978).

Previous research on archaeological samples with and without lesions indicative of leprosy showed no significant differences in $\delta^{13}C$ or $\delta^{15}N$ values, suggesting that there were not dietary differences between the two groups (Bayliss et al. 2004, Linderholm and Kjellström 2011). However, other studies showed marked differences between individuals who survived childhood and those who did not (Beaumont et al. 2015, Reitsema et al.

2016), with the ones who survived having higher animal protein in their post-weaning diets (Reitsema et al. 2016) suggesting that investigation of dietary protein, using stable isotopic analysis, might be used to better understand disease and physiological stress in past populations. Skeletal indicators of physiological stress, such as low stature and *cribra orbitalia*, have also been related to long-term effects on health throughout reduced lifespan (Watts 2013) and increased risk of death during epidemics (DeWitte and Hughes-Morey 2012, DeWitte and Wood 2008).

6.1.4. Diet at Tomar

People living in Tomar had a complex diet, low in terrestrial animal protein and high in aquatic protein intake, despite its inland location (Chapter 5, Curto et al. 2018). Being controlled by religious military orders (Conde 1996, Valente 1998), it is possible that their presence in the town would have an impact on the general population particularly on their diet (Chapter 5, Curto et al., 2018), due to religious fasting (Barber and Bate 2002, Müldner et al. 2009, Müldner and Richards 2007, Salamon et al. 2008). Fish was an expensive food source, particularly further away from the coast (Gonçalves 2004, Vicente 2013), therefore higher amounts of fish consumption may reflect higher socio-economic status (Chapter 5, Curto et al. 2018).

There were no significant differences found between sexes or age groups for bone collagen $\delta^{13}C$ and $\delta^{34}S$ values, however $\delta^{15}N$ values did differ significantly with age (lower $\delta^{15}N$ in older individuals), which may be related to tooth loss in elderly individuals (Chapter 5, Curto et al. 2018). There was one outlier, a young adult male, with higher values of both $\delta^{15}N$ and $\delta^{13}C$ values and lower $\delta^{34}S$ values than the other skeletons analysed, suggesting he may be an outsider (Chapter 5, Curto et al. 2018). There were no differences between

inferred social status, estimated through burial type and proximity to the church (Chapter 5, Curto et al. 2018).

6.2. Materials and Methods

Santa Maria do Olival necropolis, at Tomar, is one of the largest in Europe (6,792 individuals recovered: 4,991 adults and 1,801 non-adults) but has not been continuously studied yet. Even though Tomar was a Templar town the distribution of the skeletons, of all ages and both sexes, within the necropolis suggests that Santa Maria do Olival collection represents the general population of Tomar and not, or at least not only, the individuals from the military orders (Chapter 5, Curto et al. 2018).

Bone collagen stable isotope data (carbon, nitrogen and sulphur) from 32 human adult tibiae (15 females, 18 males) and 13 faunal remains (2 wild *Sus*, 2 domestic *Sus*, 1 juvenile *Sus*, 1 *Canidae*, 3 *Bos*, 1 *Equus*, 3 *Ovicapridae*) from Tomar (11^{th} – 17^{th} century) were previously analysed to reconstruct the general diet of the population (Chapter 5, Curto et al. 2018). These are reused here and compared to new isotope data from skeletons with signs of disease (Table 6.1). These data are compared to new isotope ratios from 23 adult individuals (8 females, 14 males, 1 undetermined) with skeletal lesions compatible with non-specific (n=21) and specific (venereal syphilis, n=2) infectious diseases.

All samples are from Santa Maria do Olival graveyard (areas 13 to 20, 11^{th} to 17^{th} centuries) in Tomar. The individuals without lesions (n=32), previously analysed (Chapter 5, Curto et al. 2018), were used to estimate the baseline diet at Tomar and were selected based on the absence of skeletal lesions or skeletal stress markers (see Chapter 5, Curto et al. 2018 for more detail, the outlier was not considered for this study). There were no significant differences found between sexes or inferred social status, estimated through burial type and proximity to the church (Chapter 5, Curto et al. 2018).

6.2.1. Estimating age and sex

Sex was estimated based on pelvic (Phenice 1969, Buikstra and Ubelaker 1994) and cranial features (Buikstra and Ubelaker 1994). Adult age at death estimates employed a combination of skeleton maturation (Scheuer and Black 2000), pubic symphysis degeneration (Brooks and Suchey 1990, Buikstra and Ubelaker 1994) and auricular surface degeneration (Lovejoy et al. 1985). The skeletons analysed were grouped as young (18 to 30 years, n=5), mature (31 to 60 years, n=8) and elderly (60+ years, n=4) adults, for six skeletons it was not possible to estimate age.

6.2.2. Signs of infection

From the 23 skeletons with lesions (Table 6.1), 21 have signs of non-specific infectious diseases and 2 have lesions compatible with specific infections (venereal syphilis). The 23 individuals were grouped in two different ways: a) active (n=6), healed (n=7) and a combination of both active and healed lesions (n=10), b) Skeletons with only healed tibial perostitis (n=7), those with non-specific (n=5) and specific (n=2) infectious diseases, while individuals who did not fit into these groups (n=9) were not considered for this analysis.

Skeletal lesions were considered to be from possible infectious causes if abnormal bone formation or bone formation and destruction, compatible with periostitis or osteomyelitis (Ortner and Putschard 1985, Buikstra and Ubelaker 1994, Aufderheide and Rodríguez-Martín 1998, Ortner 2003), were present and not associated with trauma. Periostitis usually represents pathologic changes resulting in new bone growth, which is remodelled into lamellar bone during the healing process, but it can also result from inflammation of the surrounding tissues following a trauma (Ortner and Putschard 1985, Ortner 2003).

For this study, lesions scored 2 (markedly accentuated longitudinal striations on the surface of cortical bone, Steckel et al. 2006) to 5 (extensive periosteal reaction involving over half of the diaphysis, with cortical expansion, pronounced deformation, Steckel et al. 2006) were considered periostitis. Lesions that were scored as 6 (involving most of the diaphysis with cloacae, Steckel et al. 2006) were taken as evidence of osteomyelitis. Periostitis or osteomyelitis associated with fractures was not considered for this study.

Lesions with unremodelled woven bone (Figure 3.4) were considered active at the time of death (Ortner and Putschard 1985, Ortner 2003). Rapidly formed woven bone is poorly organized and has a porous appearance due to the loose organization of the mineralized osteoid fibres (Ortner and Putschard 1985, Ortner 2003). Markedly accentuated longitudinal striations (Figure 3.2) and compact bone growth (Figure 3.5), without the presence of woven bone, were considered healed lesions (Ortner and Putschard 1985, Ortner 2003). The presence of both compact bony growth and woven bone was considered a combination of both healed and active lesions. The skeletons with only active lesions represent infectious diseases active *perimortem* and the ones with only healed lesions represent healed individuals. Skeletons with a combination of both types of lesions represent chronic infections, to which the individuals survived long enough to the disease for the bone to heal but with the disease still present. The skeletons with the different lesions (healed, active and both) were combined and compared with the individuals without lesions, by age group: young without lesions (n=8), young with lesions (n=5), mature without lesions (n=13), mature with lesions (n=8), elderly without lesions (n=4) and elderly with lesions (n=4).

Since tibial periostitis is frequently used as an indicator of physiological stress (e.g. DeWitte 2010, Robb et al. 2001) and can be caused by a variety of factors, including trauma,

only individuals with bilateral healed periostitis on the tibiae were selected (markedly accentuated longitudinal striations, score 2, Steckel et al. 2006). The cases of venereal syphilis were diagnosed due to the presence of *caries sicca*, a sign specifically characteristic of venereal syphilis (Ortner and Putschard 1985, Aufderheide and Rodriguez-Martin 1998, Ortner 2003). These groups with signs of infections where then compared with the skeletons without lesions (n=32, Chapter 5, Curto et al. 2018).

The skeletons were grouped in different ways to better understand how diet may affect the susceptibility to generalised infections (by grouping non-specific generalised infections, specific generalised infections and individuals with only healed tibial periostitis) or the ability to recover from infectious diseases (by grouping the skeletons as having active, healed or a combination of both active and healed lesions).

Only tibiae collagen was analysed in an attempt to estimate the average long term diet of the individuals and avoid stable isotopes data that may represent different diet and/or metabolism during the disease. Following the attempt to avoid stable isotope values related to faster bone remodelling and therefore more recent diet, samples were only collected at areas of the bone without any sign of lesions.

6.2.3. Collagen extraction and analysis

Collagen extraction was done following Longin (1971), Brown et al. (1988) and Richards and Hedges (1999). The collagen samples were weighed into tin capsules and combusted into CO_2 and N_2 in an isotope-ratio mass spectrometer at NERC Isotope Geosciences Facility and HERCULES laboratory. At NERC, $\delta^{13}C$ and $\delta^{15}N$ values were calibrated using an in-house reference material M1360p (powdered gelatine from British Drug Houses) with expected δ values of –20.32‰ (calibrated against CH_7, IAEA) and +8.12‰ (calibrated against N-1 and N-

2, IAEA) for carbon and nitrogen respectively. Samples were run in duplicate and the 1σ reproducibility for mass spectrometry controls for these analyses were $\delta^{15}N$ = ±0.08‰ and $\delta^{13}C$ = ±0.07‰. At HERCULES Laboratory, $\delta^{13}C$ and $\delta^{15}N$ values were calibrated using IAEA-CH-6 (sucrose, −10.449‰), IAEA-CH-7 (polyethylene, −32.151‰), IAEA-N-1 (ammonium sulphate, +0.4‰) and IAEA-N-2 (ammonium sulphate, +20.3‰). Measurement errors were less than ±0.1‰ for $\delta^{13}C$ values and ±0.2‰ for $\delta^{15}N$ values.

Mann-Whitney U non-parametric tests were used for pair-wise comparisons and Kruskal-Wallis non-parametric tests were used to compare more than two groups. All statistics were computed in SPSS 24 for Windows and *p*-values ≤0.05 were considered statistically significant.

6.3. Results

Individual isotopic data and collagen integrity for lesion and non-lesion sites can be found in the Appendix (Table A4).

6.3.1. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ values of skeletons with generalised infections or healed tibial periostitis compared to skeletons without lesions

Osteomyelitits was only observed in the skeletons with venereal syphilis (skeletons 16.225 and 18.158) and skeleton 16.255 ($\delta^{13}C$=-18.7‰, $\delta^{15}N$=10.0‰), a mature male with osteomyelitis on the right tibia. Therefore, the results from this study are focused mainly on lesions within the scope of periostitis.

Figure 6.1 illustrates the $\delta^{13}C$ and $\delta^{15}N$ values for skeletons without lesions (n=32, Chapter 5, Curto et al. 2018), with only healed tibial periostitis (n=7) and those with generalised specific (n=2) and non-specific (n=5) infections. There is one outlier with healed tibial periostitis ($\delta^{13}C$=-15.6‰, $\delta^{15}N$=11.5‰) that seems to have very different diet from the

general population and therefore was not considered for the statistical analysis. Among the individuals with skeletal lesions, the ones with healed tibial periostitis (n=6, one is an outlier) have the highest mean values for both $\delta^{13}C$ (-18.0±1.1‰, Table 6.1) and $\delta^{15}N$ (10.9±0.7‰, Table 6.1) values, while those with non-specific generalised infections (n=5) have the lowest mean for $\delta^{13}C$ (-18.7±0.8‰, Table 6.1) and $\delta^{15}N$ (9.9±0.4‰, Table 6.1). The skeletons with venereal syphilis (n=2) have similar mean values ($\delta^{13}C$=-18.5±0.2‰, $\delta^{15}N$=11.2±0.3‰) to the skeletons without lesions (n=32, $\delta^{13}C$=-18.6±0.5‰, $\delta^{15}N$=10.8±0.8‰) and those with only healed tibial periostitis (n=6), however the sample size is too small for an appropriate statistical analysis. The difference in $\delta^{15}N$ between skeletons with non-specific generalised infection ($\delta^{13}C$=-18.7±0.8‰, $\delta^{15}N$=9.9±0.4‰) and healed periostits ($\delta^{13}C$=-18.1±1.2‰, $\delta^{15}N$=11.2±0.4‰) is highly significant ($p<0.003$, Table 6.2) as is the difference between skeletons with non-specific generalised infection and those without lesions ($\delta^{13}C$=-18.5±0.7‰, $\delta^{15}N$=10.9±0.9‰) ($p<0.004$, Table 6.1). There are no statistically significant differences for $\delta^{13}C$ ($p>0.53$, Table 6.1) or between skeletons without lesions and skeletons with only healed tibial periostitis for both $\delta^{13}C$ and $\delta^{15}N$ ($p>0.20$).

Table 6.1. Mean, standard deviation and non parametric tests for $\delta^{13}C$ and $\delta^{15}N$ (‰) of individuals without lesions, with healed periostitis and with generalized infections (without outliers). Data from skeletons without lesions previously analysed in Chapter 5 (Curto et al. 2018).

	N	Mean ± sd		Non parametric test		
		$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	
Without lesions	32	-18.5 ± 0.7	10.9 ± 0.9	95.00	80.00	Mann-Whitney U
Healed periostitis	6	-18.0 ± 1.1	10.9 ± 0.6	0.49	0.21	sig
Without lesions	32	-18.5 ± 0.7	10.9 ± 0.9	74.00	19.00	Mann-Whitney U
Non-specific generalized infection	5	-18.7 ± 0.8	9.9 ± 0.4	0.74	**0.00**	sig
Healed periostitis	6	-18.0 ± 1.1	10.9 ± 0.6	20.00	7.00	Mann-Whitney U
Non-specific generalized infection	5	-18.7 ± 0.8	9.9 ± 0.4	0.53	**0.00**	sig

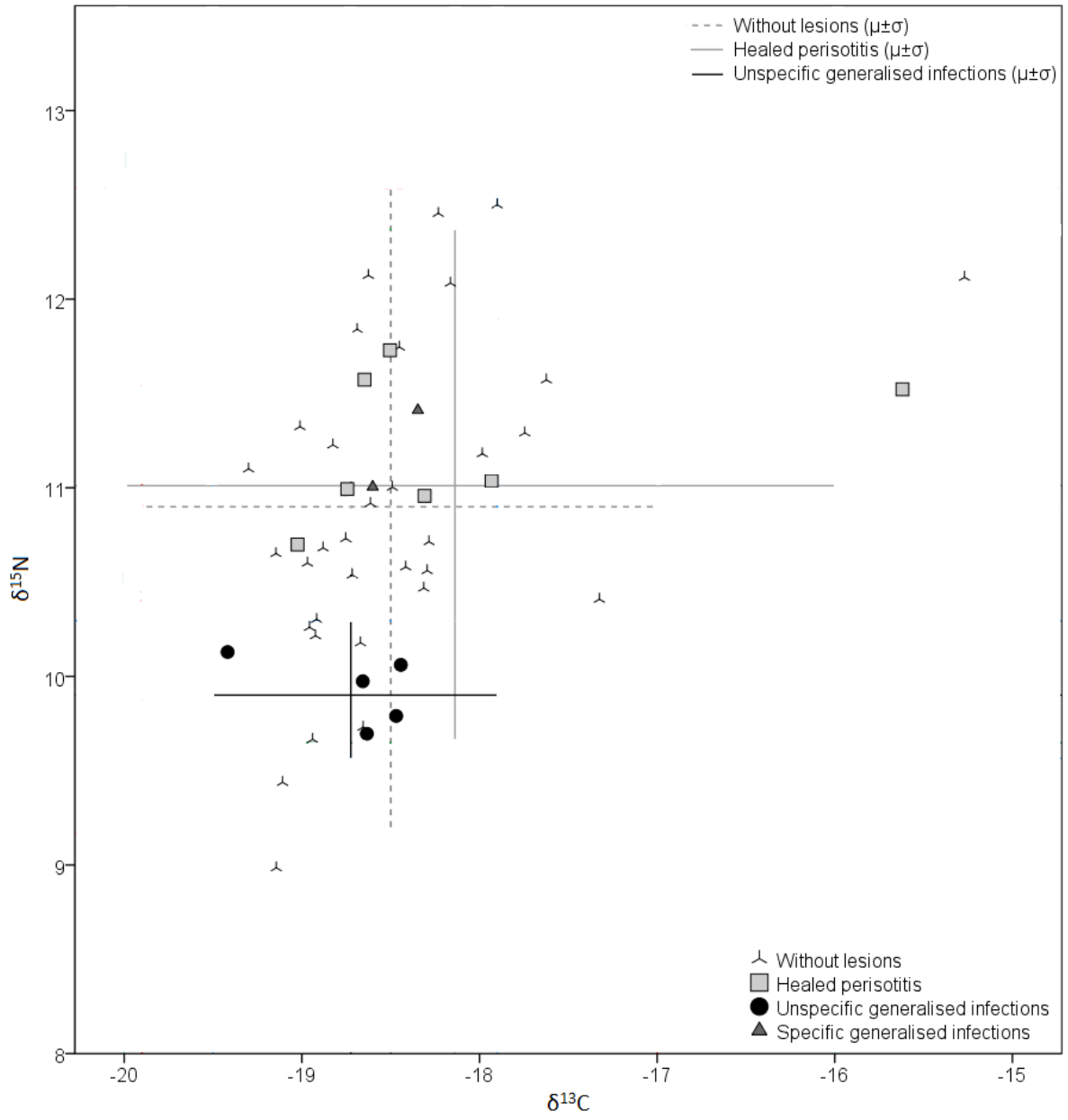


Figure 6.1. $\delta^{13}C$ and $\delta^{15}N$ values (‰) for individuals without lesions, with healed periostitis and with generalized infections. Data from skeletons without lesions previously analysed in Chapter 5 (Curto et al. 2018).

Table 6.2. Mean, standard deviation and non parametric tests for $\delta^{13}C$ and $\delta^{15}N$ values (‰) of individuals with and without lesions, by age group (without outliers). Data from skeletons without lesions previously analysed in Chapter 5 (Curto et al. 2018).

		N	Mean ± sd		Non parametric test		
			$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	
Young	Without lesions	7	-18.5 ± 0.4	11.4 ± 0.7	7.00	7.00	Mann-Whitney U
	With lesions	5	-18.8 ± 0.4	10.5 ± 0.8	0.09	0.09	sig
Mature	Without lesions	13	-18.6 ± 0.6	10.5 ± 0.7	60.00	59.00	Mann-Whitney U
	With lesions	8	-18.5 ± 0.5	10.7 ± 0.7	0.51	0.49	sig
Old	Without lesions	4	-18.6 ± 0.3	10.7 ± 1.2	5.00	6.00	Mann-Whitney U
	With lesions	4	-18.4 ± 0.3	10.3 ± 0.4	0.39	0.56	sig

6.3.2. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ values of skeletons with lesions compared to skeletons without lesions, by age groups

Figure 6.5 illustrates $\delta^{13}C$ and $\delta^{15}N$ values for individuals with (including healed, active or a combination of both lesions) and without lesions by age group (Table 6.3). Young adults without lesions (n=8) have higher $\delta^{13}C$ (-18.5±0.4‰) and $\delta^{15}N$ (11.4±0.7‰) than the ones with lesions (n=5, $\delta^{13}C$=-18.8±0.4‰, $\delta^{15}N$=10.5±0.8‰) but still falling within the two standard deviations of each other and the general sample without lesions. There is no statistically significant differences in $\delta^{13}C$ or $\delta^{15}N$ values for the mature (without lesions: n=13, $\delta^{13}C$=-18.6±0.6‰, $\delta^{15}N$=10.5±0.7‰, with lesions: n=8, $\delta^{13}C$=-18.5±0.5‰, $\delta^{15}N$= 10.7±0.7‰) and elderly adults (without lesions: n=4, $\delta^{13}C$=-18.6±0.3‰, $\delta^{15}N$=10.7±1.2‰, with lesions: n=4, $\delta^{13}C$=-18.4±0.3‰, $\delta^{15}N$=10.3±0.4‰) ($p>0.38$, Table 6.3).

Table 6.3. Mean, standard deviation and non parametric tests for $\delta^{13}C$ and $\delta^{15}N$ values (‰) of individuals with different types of lesions and without lesions (without outliers). Data from skeletons without lesions previously analysed in Chapter 2 (Curto et al. 2018).

	N	Mean ± sd		Non parametric test		
		$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	
Without lesions	32	-18.6 ± 0.5	10.8 ± 0.8	66.00	77.00	Mann-Whitney U
Healed lesions	6	-18.4 ± 0.4	10.8 ± 0.7	0.53	0.89	sig
Without lesions	32	-18.6 ± 0.5	10.8 ± 0.8	87.00	73.00	Mann-Whitney U
Active lesions	6	-18.5 ±0.7	10.5 ± 0.7	0.72	0.36	sig
Without lesions	32	-18.6 ± 0.5	10.8 ± 0.8	120.00	134.00	Mann-Whitney U
Both lesions	10	-18.4 ± 0.2	10.7 ± 0.8	0.24	0.44	sig

6.3.3. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ values of skeletons with active, healed or a combination of both lesions compared to skeletons without lesions

The only healed lesions were found within the mature adults group (Figure 6.5). Results show there is no statistically significant difference in $\delta^{13}C$ or $\delta^{15}N$ values when the skeletons without visible lesions (n=32, $\delta^{13}C$=-18.6±0.5‰, $\delta^{15}N$=10.8±0.8‰, Table 6.3) were compared with the skeletons with healed (n=6, $\delta^{13}C$=-18.4±0.4‰, $\delta^{15}N$=10.8±0.7‰, p=0.53, Table 6.3), active (n=6, $\delta^{13}C$=-18.5±0.7‰, $\delta^{15}N$=10.5±0.7‰, p=0.72, Table 6.3) or a combination of both lesions (n=10, $\delta^{13}C$=-18.4±0.2‰, $\delta^{15}N$=10.7±0.8‰, p=0.24, Table 6.3).

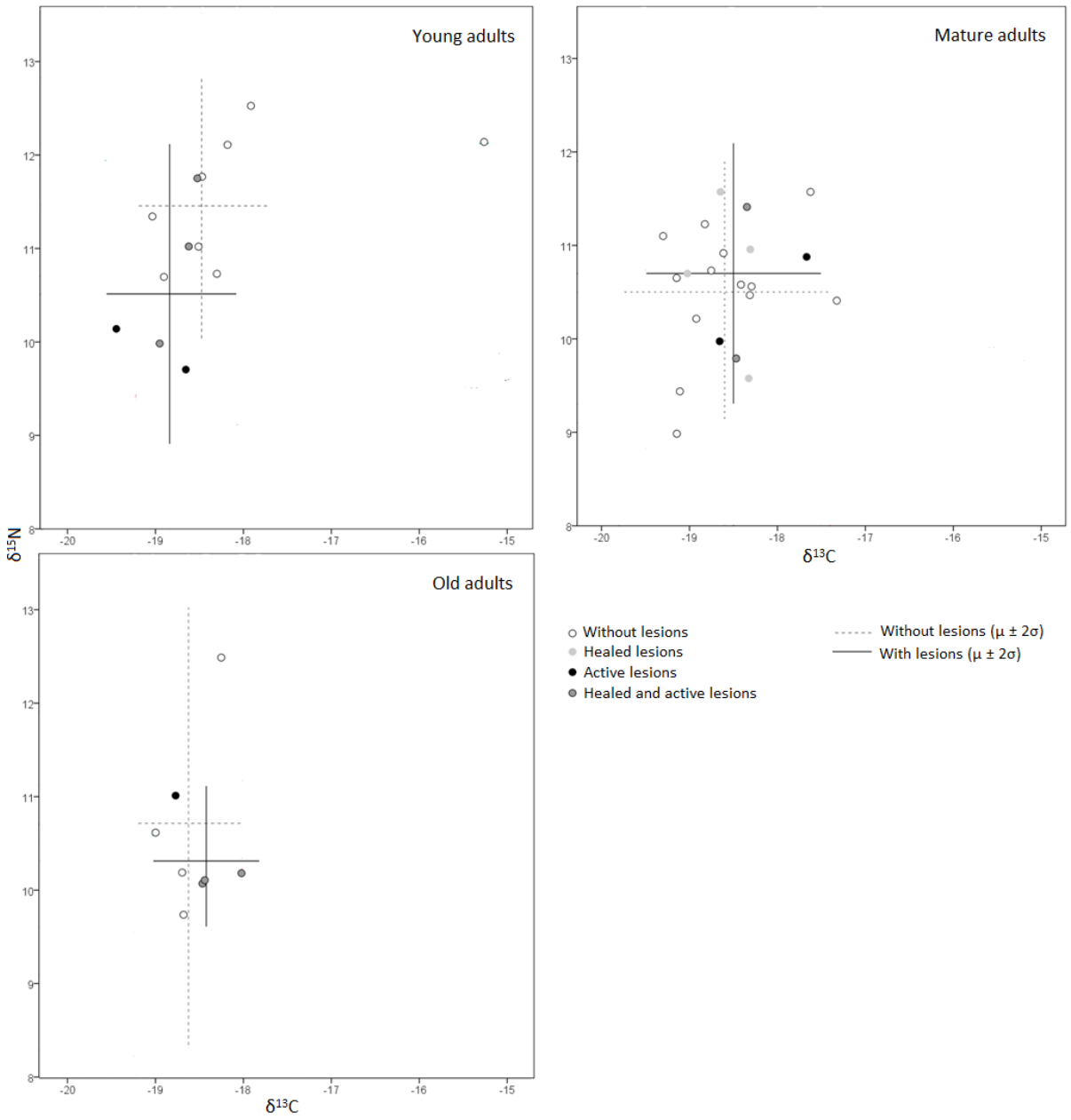


Figure 6.2. $\delta^{13}C$ and $\delta^{15}N$ values (‰) for individuals with and without lesions, by age group (means calculated without outliers). Data from skeletons without lesions previously analysed in Chapter 5 (Curto et al. 2018).

6.4. Discussion

6.4.1. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ values of skeletons with generalised infections or healed tibial periostitis compared to skeletons without lesions

The $\delta^{15}N$ enrichment observed in skeletons with only healed tibial periostitis (N=6, without the outlier), when compared to those with non-specific generalised infections (n=5), may represent evidence of chronic physiological stress (Steele and Daniel 1978, Hobson et al. 1993, Gaye-Siessegger et al. 2004, Fuller et al. 2005, Deschner et al. 2012, D'Ortenzio et al. 2015, Scorrano et al. 2014). However, the individuals with non-specific generalised infections (n=5) were also exposed to chronic physiological stress and survived long enough for it to be observable in their bones (Wood et al. 1992), yet they display lower $\delta^{15}N$ (9.9±0.4‰) than the individuals without lesions (n=32, $\delta^{15}N$=10.8±0.8‰), those with only healed tibial periostitis (n=6, $\delta^{15}N$=10.9±0.7‰) and the ones with venereal syphilis (n=2, $\delta^{15}N$=10.5±0.6‰).

The only skeleton with osteomyelitis (16.255), besides the ones with venereal syphilis, has similar $\delta^{13}C$ (-18.7‰) and $\delta^{15}N$ (10.0‰) to the individuals with non-specific generalised infections ($\delta^{13}C$=-18.7±0.8‰, $\delta^{15}N$=9.9±0.4‰, Table 6.2), suggesting that a diet lower in animal protein might have made him more susceptible to infectious disease (e.g. Kuvibidila et al. 1993, Scrimshaw and SanGiovanni 1997, Woodward 1998, Woodward 2001). Venereal syphilis is a sexually transmitted disease and human hosts have no natural immunity to pathogenic treponemes (Kiple 1993). Therefore, the immune system of the individuals before the disease is not as relevant to the individuals' susceptibility to these infections. However, good health prior to venereal syphilis infection may prolong the individual's survival (not only to the treponeme but also to other infections trough skin

ulcers which increase the exposure to other pathogens) and increase the amount and severity of the lesions (Wood et al. 1992).

The skeletons without lesions were also carefully chosen not only based on the absence of infectious lesions (including tibial periostitis) but also other physiological stress indicators such as cribra orbitalia, porotic hyperostosis, enamel hypoplasias and stature above the average for the population under study (Chapter 5, Curto et al. 2018). Even so, the skeletons with only healed tibial periostitis have similar $\delta^{13}C$ and $\delta^{15}N$ to those without any sign of physiological stress (Figure 6.4).

The osteological paradox (Wood et al. 1999) may explain the higher $\delta^{13}C$ and $\delta^{15}N$ for the skeletons with only healed tibial periostitis when compared to the ones with non-specific generalised infections (Figure 6.1 and Table 6.2). It is possible that the skeletons with only healed tibial periostitis had a diet richer in animal protein and therefore were more resistant to diseases (e.g. Calder 2013, Kuvibidila et al. 1993, Scrimshaw and SanGiovanni 1997, Woodward 1998, Woodward 2001, Ulijaszek et al. 2012, Weston 2012) than those who had non-specific generalised infections. It has been argued that individuals with healed periostitis are of lower frailty, having a lower risk of death (e.g. DeWitte 2010, Ortner 2003, Wood et al. 1992).

The diet of the population under study was complex and likely included food sources from outside Tomar (Chapter 5, Curto et al. 2018). The diet of these individuals was poor in terrestrial protein and rich in aquatic protein ($\delta^{13}C$=-18.6‰, $\delta^{15}N$=10.8‰, $\delta^{34}S$=13.1‰, Chapter 5, Curto et al. 2018). Stable isotope values are similar for males and females but the young adults have higher $\delta^{15}N$ (11.4±0.6‰) than the elderly adults (10.6±0.8‰), suggesting a higher animal protein intake for the young individuals (Chapter 5, Curto et al. 2018). The high $\delta^{15}N$ from skeletons without lesions seem to be related with higher aquatic protein

intake (Chapter 5, Curto et al. 2018), which may be related with these individuals having better health than those with signs of infection. Since fish was expensive (Gonçalves 2004) and the military orders had angling rights (Vicente 2013) it is also possible that the individuals without skeletal stress markers, or only healed tibial periostitis, had a higher socio-economic status. Socio-economic status may also have an impact on an individual's diet, not only directly on their diet but also the type of pathogens they would be exposed to.

The effect of protein malnutrition on the immune system is well known (Calder 2013, Kuvibidila et al. 1993, Scrimshaw and SanGiovanni 1997, Woodward 1998, Woodward 2001) and the possibility of dietary differences being present before the disease cannot be excluded. $\delta^{15}N$ values were significantly different between skeletons with non-specific generalised infections and those without lesions ($p<0.004$) or with only healed tibial periostitis ($p<0.003$). The higher $\delta^{15}N$ values values observed in the two individuals with venereal syphilis, may not be related to physiological stress but may be due to the nature of the disease instead (sexually transmitted infection) and the $\delta^{15}N$ values might suggest a richer diet that could have allowed survival despite the disease and susceptibility to other pathogens. The possibility of these $\delta^{15}N$ differences being related with social status cannot be excluded. Various studies suggest dietary differences between sex and social status in Medieval times (e.g. Adamson 2004, Kjellström et al. 2009, Linderholm et al. 2008, Polet and Katzenberg 2003, Schutkowski et al. 1999, Reitsema et al. 2010, Reitsema and Vercellotti 2012). However, a previous study showed no significant stable isotope data between individuals of different sex or social status in Tomar (Chapter 5, Curto et al. 2018).

There are two outliers among the skeletons sampled for isotopic analysis (Figure 6.1), one without lesions and another one with healed tibial periostitis. The skeleton without lesions, a young adult male, might be an outsider as his sulphur isotopes ratios

(9.3‰) differ from the other individuals without lesions (mean $\delta^{34}S$=13.1‰, Chapter 5, Curto et al., 2018). This skeleton was not considered for the statistical analysis. There are no sulphur isotopes values for the outlier with healed tibial periostitis but $\delta^{13}C$ (-15.6‰) and $\delta^{15}N$ (11.5‰) values are similar to those of the outlier without lesions ($\delta^{13}C$=-15.4‰, $\delta^{15}N$=12.3‰).

6.4.2. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ values of skeletons with lesions compared to skeletons without lesions

The values for the young adults show a statistical trend towards a significance ($p<0.09$, Table 6.3) difference in both $\delta^{13}C$ and $\delta^{15}N$ values between skeletons with (n=5) and without (n=8) lesions. Young individuals without lesions have higher $\delta^{13}C$ (-18.5±0.4‰) and $\delta^{15}N$ (11.4±0.7‰) than those with lesions ($\delta^{13}C$=-18.8±0.4‰, $\delta^{15}N$=10.5±0.8‰), which may suggest that the individuals with lesions may have had a diet with lower animal protein (Figure 6.2). There is no difference for mature ($p>0.49$, Table 6.3) and elderly ($p>0.39$, Table 6.3) individuals with or without lesions. Previous research on archaeological samples showed marked differences between individuals who survived childhood and those who did not (Beaumont et al. 2015, Reitsema et al. 2016), with the ones who survived having higher animal protein in their post-weaning diets (Reitsema et al. 2016) suggesting that diet at younger ages can have a high impact on the health status of an individual. The impact of diet on an individual's health might be prolonged throughout adult life as well. The young adult skeletons analysed do not have healed lesions, only active or a combination of both active and healed lesions, meaning that they died during acute phases of the disease (Ortner and Putschard 1985, Ortner 2003, Turner-Walker 2008).

6.4.3. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ values of skeletons with active, healed or a combination of both lesions compared to skeletons without lesions

The absence of significant differences in $\delta^{13}C$ or $\delta^{15}N$ between individuals without lesions (n=32, $\delta^{13}C$=-18.6±0.5‰, $\delta^{15}N$=10.8±0.8‰, Table 4) and those with healed (n=6, $\delta^{13}C$=-18.4±0.4‰, $\delta^{15}N$=10.8±0.7‰, Table 4), active (n=6, $\delta^{13}C$=-18.5±0.7‰, $\delta^{15}N$=10.5±0.7‰, Table 4) or a combination of both lesions (n=10, $\delta^{13}C$=-18.4±0.2‰, $\delta^{15}N$=10.7±0.8‰, p=0.24, Table 4) suggests that diet may have a higher impact on the susceptibility to chronic generalised infections than to infectious disease in general. It is therefore important to take into account the severity and stage of the disease. The $\delta^{15}N$ average is slightly higher for the individuals without lesions (10.8‰, n=32) than for the one ones with active lesions (10.5‰, n=6, Table 4). This slight difference may indicate that the individuals without lesions might have a diet richer in animal protein than those with active lesions, however the sample size is too small to make conclusions.

6.5. Conclusion

This study explored the dietary differences between individuals with and without skeletal lesions compatible with infectious diseases to better understand the impact of diet on individuals' health status and their susceptibility to infectious disease. There is a highly significant difference in $\delta^{15}N$ values between skeletons with healed tibial periostitis and non-specific generalised infection, as well as a difference at the margin of statistical significance between skeletons without lesions and those with generalised infections. These results suggest that the individuals with non-specific generalised infections had diets lower in animal protein than those without lesions or with only healed tibial periostitis. Poorer diets may increase susceptibility to pathogens leading more frequently to generalised infections while richer diets might increase the survivorship and ability to heal from infectious diseases. However, the possibility of these isotope ratios being a result of the disease cannot be excluded and more data from different periods of time within the individual's' life is necessary to understand when these differences started to manifest. These results indicate that diet has a higher impact on the health status of young people than mature or elderly individuals, being linked to selective mortality. Our results suggest that while non-specific generalised infections are a sign of ill health and poor diet, only healed tibial periostitis may indicate a state of comparatively good overall health and diet.

1

Chapter 7

Effect of different healing stages on stable isotope ratios in skeletal lesions

Abstract

This study compared stable isotope ratios from cortical bone (long bones and ribs) sites that retained evidence of healed or active disease, or healed fracture to isotope ratios from regions on the same bone that did not retain evidence of disease or fractures. Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios were assessed in 33 skeletons that retained evidence of infectious disease and healed fractures. Samples were taken from active lesions (long bones n=14, ribs n=4), healed lesions (long bones n=10, ribs n=9) or a fracture callus (long bones n=9, ribs n=3). Results were compared to stable isotope ratios calculated for regions on these bones that did not retain evidence of disease or fracture. Long bones with active lesions had a significantly larger $\delta^{15}N$ values ($\delta^{15}N$=11.1±0.9‰) compared to those without lesions ($\delta^{15}N$=10.7±0.7‰, p=0.02). There were no significant differences in stable isotope ratios when compared between non-lesion and lesion sites in the ribs. The increase in $\delta^{15}N$ seen in active lesions, when compared with $\delta^{15}N$ from non-lesion regions on the same long bone, may be a consequence of altered protein metabolism. This study suggests that stable isotope data can contribute information about diseases in the past, as well as an individuals' response to diseases in the absence of modern medicine and antibiotics.

7.1. Introduction

Comparing stable isotope ratios within an individual, from apparently healthy bone to bone that formed from an injury or disease can potentially reveal slight variations in diet or metabolism during the period in which the disease was active or the injury healed. Here, I assess variation in stable isotope ratios within an individual, by assessing stable isotope ratios in bone with a disease that is active, has healed, or retains evidence of a fracture. This study builds on previous research into the relationship between skeletal pathology, metabolism and stable isotope ratios (Katzenberg and Lovell 1999, Olsen et al. 2014). Here I present the first study to assess skeletal lesions of long bones and ribs at different healing stages against stable isotope ratios calculated for these lesions, and for regions of the same bones where there is no evidence of disease. Differentiating between healed and active diseases may highlight different metabolic stages as the stable isotope ratios may represent tissue anabolism or catabolism.

It is still not clear how different tissues, particularly bone, are affected by the body's net loss of light nitrogen or the mechanisms underlying changes in $\delta^{15}N$ values during periods of physiological stress. Most studies of metabolism using isotope ratios rely on data recorded during hibernation or fasting in birds, reptiles or small mammals (e.g. Lee et al. 2012, McCue and Pollock 2008, Hatch et al. 2006). Others have examined fast-growing tissues in humans such as hair (e.g. Eerkens et al. 2017, D'Ortenzio et al. 2015, Neuberger et al. 2013, Mekota et al. 2006). During fasting, catabolism and anabolism become unbalanced to an extent that differs among tissues. Increases in $\delta^{15}N$ values in tissues have been associated with fasting or physiological stress (e.g. Alamaru et al. 2009, Boag et al. 2006, Fuller et al. 2005, Hobson et al. 1993), though an increase in $\delta^{15}N$ values has not always

been registered in individuals suffering from physiological stress (e.g. Mayor et al. 2011, McFarlane Tranquilla et al. 2010, McCue and Pollock 2008, Castillo and Hatch 2007).

The presence of woven bone in a skeleton indicates new bone formation, or active bone growth at the time of death (Roberts and Manchester 2007). Woven bone can sometimes be present with some disease processes, and can be remodelled into compact bone (healed lesions) as healing progresses (Turner-Walker 2008). Katzenberg and Lovell (1999) observed that new bone deposition as consequence of infection showed higher $\delta^{15}N$ values than the unaffected segments of bone, and suggested that variation in $\delta^{13}C$ values only occur in response to dietary intake. However, the four pathological specimens in their study had different types of lesions. One individual had osteomyelitis, one had active periostitis, one had a fracture and another one had post-paralytic atrophy (Katzenberg and Lovell, 1999). Olsen et al (2014) observed different values of both $\delta^{15}N$ and $\delta^{13}C$ in bones with osteomyelitis (n=6), healed fractures (n=11) or periostitis (n=18), but for their study the healing stage of the lesions were not taken into consideration.

The lesions were grouped as 1) active lesions where woven bone is present, 2) healed lesions where compact bone is present and 3) healed fracture calluses. I expect to see a negative nitrogen balance (compatible with tissue catabolism) in active lesions and a positive nitrogen balance (compatible with tissue anabolism) in healed lesions and fracture calluses of the individuals. Anabolism should lead to an increase in $\delta^{15}N$ values as a result of protein synthesis and not protein breakdown (e.g. Habran et al. 2010, Wolf et al. 2009, Fuller et al. 2005). Catabolism is based on a disproportionate loss of ^{14}N-containing amino acids during protein breakdown which results in residual $\delta^{15}N$ values in any tissue undergoing catabolism (e.g. McFarlane Tranquilla et al. 2010, Gaye-Siessegger et al. 2007, Martínez del Rio and Wolf 2005, Hobson et al. 1993). During prolonged periods of disease,

or nutritional or physiological stress, dietary protein cannot adequately replace nitrogen losses (Powanda 1977, Grossman et al. 1945). Consequently, the body's proteins can be recycled resulting in enriched ^{15}N (e.g. D'Ortenzio et al. 2015, Neuberger et al. 2013, Deschner et al. 2012, Mekota et al. 2006, Hobson et al. 1993, Steele and Daniel 1978). Studies of isotope ratios in hibernating animals contradict the catabolic model, which predicts that tissues broken down during fasting should have an increase in $\delta^{15}N$ values (e.g. Lee et al. 2012). However, before hibernation animals anticipate fasting by building up large fat stores to support the increased metabolic costs. I expect to see $\delta^{13}C$ values decrease in active lesions and $\delta^{13}C$ values increase in healed lesions and fracture calluses when compared to non-lesion sites within the same bone (e.g. Neuberger et al. 2013, Mekota et al. 2006).

The main objective of this study is to compare stable isotope ratios from bones that retain evidence of lesions in the form of disease, or healed fractures, to ratios from cortical bone in the same individual that do not retain these skeletal lesions. By comparing $\delta^{13}C$ and $\delta^{15}N$ values between bone collagen from lesion and non-lesion sites from the same bone I sought to determine if stable isotope ratios during or after a disease (indicated by the lesions) correspond with the longer term record of diet that is represented by stable isotope ratios from non-lesion sites. Isotopically, the slower turnover of long bone collagen reflects a longer-term and average dietary signal, which may be more or less than ten years prior to death (Hedges et al. 2007). In contrast, ribs have faster turnover rates and may represent diet from a more recent period prior to death (e.g. Cox and Sealy 1997).

7.1.1. The Tomar skeletal collection

Tomar was a Templar town and had an important military role consolidating the Kingdom of Portugal by resisting the Medieval Muslim Conquest (França 1994). After the Knights Templar dissolution, the Order's assets and personnel were transferred to the newly established Order of Christ, a continuation of the Order of the Temple of Solomon in Portugal (Valente 1998). In Tomar, merchants, crafters and farmers participated actively in the local army alongside knights, raising their status (Conde 1996) and probably having access to similar food resources as the Templars.

Tomar's necropolis was excavated in an area of approximately 6,500m^2, from where 6,792 individuals (4,991 adults and 1,801 non-adults) were recovered. Despite being a Templar town the necropolis represents the general population and not, or at least not only knights. In a previous study (Chapter 5, Curto et al. 2018) the diet of this population was estimated through stable isotope analysis of 13 faunal remains and 32 human adults without skeletal lesions compatible with infections or physiological stress. People living in Tomar had a complex diet, low in terrestrial animal protein and high in aquatic protein intake, despite its inland location (Chapter 5, Curto et al. 2018). Fish was an expensive food source, particularly further away from the coast (Gonçalves 2004, Vicente 2013). Therefore higher amounts of fish consumption may reflect higher socio-economic status. There were no significant different bone collagen $\delta^{13}C$ or $\delta^{34}S$ values between sexes or age groups. However $\delta^{15}N$ values did differ significantly with age (lower $\delta^{15}N$ values in older individuals, Chapter 5, Curto et al. 2018). The diet of 23 adult individuals with skeletal lesions was also estimated and compared with the diet of those without lesions (Chapter 6, Curto et al. 2019).

7.1.2. Intra-skeletal isotopic variation

During tissue maintenance, the same amount of nitrogen ingested is excreted and bone collagen will largely reflect ingested protein from diet, averaging over several years (e.g. Champe et al. 2008). Fahy et al. (2017) found intra-skeletal stable isotopes variation between -1.6‰ to -0.5‰ in $\delta^{13}C$ values and between 1.0‰ to 3.1‰ in $\delta^{15}N$ values. Katzenberg and Lovell (1999) registered intra-bone stable isotope variation from 0.2‰ to 0.7‰ for $\delta^{13}C$ values and from 0.3‰ to 0.4‰ for $\delta^{15}N$ values. Olsen et al. (2014) recorded intra-rib isotopic ratios among non-pathological sites from -0.1‰to 0.1‰ for $\delta^{13}C$ and 0.0‰ to 0.5‰ for $\delta^{15}N$ values, while the intra-skeleton ratios varied between -0.2‰ to 0.4‰ for $\delta^{13}C$ values and -0.9‰ to 0.7‰ for $\delta^{15}N$ values.

The body reacts to infection through an inflammatory response that aims to neutralize the pathogen and repair the resultant damage (Weston 2012). There is a limited number of ways in which bone reacts to inflammation, it either produces or destroys bone, or a combination of both (Ragsdale and Lehmer 2012, Weston 2008, 2009). The intercellular communication between osteoblasts and osteoclasts is crucial to bone homeostasis (Xu et al. 2005). Osteoclasia and osteoblastic repair are always coupled but one or the other may predominate in a given disease state at a given time period (Ragsdale and Lehmer 2012).

In new bone depositions Katzenberg and Lovell (1999) observed higher $\delta^{15}N$ values than bone segments without lesions for osteomyelitis (+1.6‰), active periostitis (+0.1‰) and fracture callus (+0.3‰). $\delta^{13}C$ values did not differ. This study (Katzenberg and Lovell, 1999) suggests that $\delta^{13}C$ values are expected to only vary due to dietary intake. However, Olsen et al. (2014) observed different values for both $\delta^{15}N$ and $\delta^{13}C$ values in bones with osteomyelitis ($\bar{x}$ $\delta^{15}N$= +1.2‰, $\bar{x}$ $\delta^{13}C$= +0.3‰), healed fractures ($\bar{x}$ $\delta^{15}N$= +0.5‰, $\bar{x}$ $\delta^{13}C$= +0.1‰) and periostitis ($\bar{x}$ $\delta^{15}N$= -0.1‰, $\bar{x}$ $\delta^{13}C$= 0‰). Olsen et al. (2014) relate the different

$\delta^{15}N$ and $\delta^{13}C$ values with a net loss of protein from the body, which may have been intensified by low food intake. In extreme cases, reduced appetite or anorexia is part of the normal physiological response to infection (Murray and Murray, 1979, Exton, 1997).

7.1.3. Nitrogen physiological balance

Apart from tissue maintenance, the body can also be in negative or positive nitrogen balance. When protein intake is insufficient, tissues catabolism results in body tissues enriched in ^{15}N and body wastes (urea) enriched in ^{14}N relative to the diet (Steele and Daniel 1978). In these situations the proteins in the body will be recycled resulting in enriched $\delta^{15}N$ (Steele and Daniel 1978, Hobson and Clark 1992, Hobson et al. 1993). ^{15}N enrichment was also observed in osteoporotic bones (White and Armelagos 1997) and in a probable case of celiac disease (Scorrano et al. 2014). Chronic malnutrition resulting from the severe malabsorption of essential nutrients may have affected the isotopic composition of the individual's bone collagen (Scorrano et al. 2014). Negative nitrogen balance is associated with tissue loss during stress (Katzenberg and Lovell 1999).

When in positive nitrogen balance there is more nitrogen ingested than it is excreted (Champe et al. 2008) and some trophic enrichment relative to diet is expected, as dietary amino acids are less enriched in ^{15}N (Fuller et al. 2004). ^{14}N can also increase in the metabolic pool due to urea salvage (Fuller et al. 2004). Positive nitrogen balance is associated with tissue gain during growth (Fuller et al. 2004). Examples of positive nitrogen balance could be during pregnancy or recovering from starvation or diseases, when the body assimilates more dietary amino acids less enriched in ^{15}N (Fuller et al. 2004, 2005, Mekota et al. 2006, Harvey and Ferrier 2011, Neuberger et al. 2013). Beaumont and Montgomery (2016) found a starvation pattern ($\delta^{15}N$ values increase) in dentine of children

from the Irish famine followed by a $\delta^{15}N$ values decrease after a dietary shift, which may also be related to recovery from nutritional stress.

7.1.4. Carbon physiological balance

Similar to nitrogen, the carbon balance in body can also be in equilibrium or in negative or positive balance. When dietary protein is inadequate, the body synthesizes the nonessential amino acids normally routed directly from diet (e.g. Jim et al. 2006) and may rely more heavily on other macronutrients like carbohydrates (Ambrose and Norr 1993). Carbohydrate metabolism also changes during periods of infection (Long 1977, Mizock 1995), due to the higher demand for glucose energy, increasing the use of metabolic pathways that preserve and recycle carbon affecting fat and protein reserves (Wolfe 1981, Mizock 1995). Carbon recycled from fat deposits results in more ^{12}C into the new tissues, reducing the $\delta^{13}C$ values (Neuberger et al. 2013). $\delta^{13}C$ decrease is indicative of a severe reduction of energy intake through nutrition (Mekota et al. 2006, Neuberger et al. 2013) and was also observed in dentin of children from the Irish famine (Beaumont and Montgomery 2013).

$\delta^{13}C$ increase was observed in patients recovering from starvation (Mekota et al. 2006, Neuberger et al. 2013) and might be related to higher meat and fat intake after the nutritional stress period (Van der MerI 1982, Chisholm et al. 1982). Higher $\delta^{13}C$ values can also be related to changes in diet due to goods availability (e.g. C_3 to C_4 plants, Beaumont and Montgomery 2016) or even medicines containing carbohydrates (Eerkens et al. 2017).

7.2. Materials and Methods

Tomar was a Templar town but the distribution of the skeletons of all ages and both sexes within the necropolis suggests that this collection represents the general population of Tomar. There are no apparent differences in diet (Chapter 5, Curto et al. 2018), place or type of inhumation (Chapter 4) between sexes.

Bone collagen stable isotope data (carbon, nitrogen) from 49 skeletal lesions in long bones (n=33) and ribs (n=16) were analysed in 33 adult skeletons (22 males, 8 females, 3 undetermined). Out of these skeletons, 23 individuals (8 females, 14 males, 1 undetermined) had skeletal lesions compatible with infectious diseases (2 venereal syphilis, 21 non-specific infections, from which 5 were generalised) and 10 individuals had healed bone fractures. Venereal syphilis was diagnosed by the presence of *caries sicca* (Ortner and Putschar 1985, Aufderheide and Rodríguez-Martín 1998, Ortner 2003) and skeletons with lesions in various bones but without pathognomonic lesions or patterns of lesions were considered to have generalised non-specific infections.

Skeletal lesions were considered to result from possible infectious causes if abnormal bone formation or bone formation and destruction, compatible with periostitis or osteomyelitis (Ortner and Putschar 1985, Buikstra and Ubelaker 1994, Aufderheide and Rodríguez-Martín 1998, Ortner 2003), were present and not associated with trauma. Lesions with unremodelled woven bone were considered active at the time of death (Ortner and Putschar 1985, Ortner 2003). Rapidly formed woven bone is poorly organized and has a porous appearance due to the loose organization of the mineralized osteoid fibres (Ortner and Putschar 1985, Ortner 2003). New bone growth tends to remodel into compact bone during the healing process. Compact bony growths, without the presence of woven bone, were considered healed lesions (Ortner and Putschar 1985, Ortner 2003). The skeletons

with active lesions (Figure 3.4) represent infectious diseases which were active *perimortem*, while the ones with only healed lesions (Figure 3.2) represent diseases overcome by the individuals.

Intra-bone pathological variation was analysed in long bones and ribs. Due to the different turnover rate between long bones and ribs, I expect larger differences in $\delta^{13}C$ and $\delta^{15}N$ values between lesion and non-lesion sites in long bones than in ribs, due to probable differences in bone formation.Non-lesion sites from long bones of individuals with active and/or healed lesions were previously analysed to compare their long term diets with those ones without lesions (Chapter 5, Curto et al. 2018, Chapter 6, Curto et al. 2019). Skeletons with healed traumatic fractures were added to the present study and analysed to compare stable isotope ratios between bone growth as a result of infection or trauma. Bone collagen data, from non-lesion cortical bone (Chapter 6, Curto et al. 2019), are reused here and compared to new isotope data from new bone formation of potential pathological origin (skeletal lesions). Lesion samples include: active lesions (woven bone: long bones n=14, ribs n=4), healed lesions (healed periostitis/osteomyelitis: long bones n=10, ribs n=9) and fracture callus (long bones n=9, ribs n=3).

7.2.1. Sampling lesions

Skeletal lesions were considered to be from possible infectious cause if abnormal bone formation or bone formation and destruction, compatible with periostitis or osteomyelitis (Ortner and Putschard 1985, Buikstra and Ubelaker 1994, Aufderheide and Rodríguez-Martín 1998, Ortner 2003), was present and not associated with trauma. New bone formations usually represent pathologic changes resulting in new bone growth, which is

remodelled into lamellar bone during the healing process (Ortner and Putschard 1985, Ortner 2003).

Lesions were divided into three groups: a) active lesions (14 long bones, 4 ribs), b) healed lesions (10 long bones, 9 ribs) and c) fractures (9 long bones, 3 ribs). Lesions with unremodelled woven bone (Figure 3.4) were considered active at the time of death and lesions with lamellar bone (Figure 3.3) were considered healed or healing lesions (Ortner and Putschard 1985, Ortner 2003). Active and healed lesions were differentiated by macroscopic observations. Rapidly formed woven bone is poorly organized and has a porous appearance due to the loose organization of the mineralized osteoid fibres (Ortner and Putschard 1985, Ortner 2003). These type of lesions were considered active *perimortem*. Markedly accentuated longitudinal striations and compact bony growth, without the presence of woven bone, were considered healed lesions (Ortner and Putschard 1985, Ortner 2003). Fracture calluses were considered healed bone traumas as the bridging callus connecting the bone fragments provides the externally visible evidence of healed fracture in an archaeological specimen (Ortner and Putschard 1985).

The new bone formations were removed by scraping the lesion or removing the top layer affected, carefully avoiding sampling the compact bone underneath or trabecular bone (particularly in the ribs), as it remodels more quickly than cortical bone (Sealy et al. 1995). On the ribs this process was more difficult due to the smaller size of the lesions and the bones. The skeletons with only active lesions represent infectious diseases active *perimortem* and the ones with healed lesions represent healed individuals. *Perimortem* fractures were not considered for this study, only healed calluses.

7.2.2. Collagen extraction and analysis

Collagen extraction was done following Longin (1971), Brown et al. (1988) and Richards and Hedges (1999). The collagen samples were weighed into tin capsules and combusted into CO_2 and N_2 in an isotope-ratio mass spectrometer at HERCULES Laboratory. $\delta^{13}C$ and $\delta^{15}N$ values were calibrated using IAEA-CH-6 (sucrose, −10.449‰), IAEA-CH-7 (polyethylene, −32.151‰), IAEA-N-1 (ammonium sulphate, +0.4‰) and IAEA-N-2 (ammonium sulphate, +20.3‰). Measurement errors were less than ±0.1‰ for $\delta^{13}C$ and ±0.2‰ for $\delta^{15}N$.

Mann-Whitney U non-parametric tests were used for pair-wise comparisons and Kruskal-Wallis non-parametric tests were used to compare more than two groups. All statistics were computed in SPSS 24 for Windows and *p*-values ≤0.05 were considered statistically significant.

7.3. Results

Individual isotopic data and collagen integrity for long bones and ribs can be found in the Appendix (Tables A3 and A4 respectively and Figure A3). Figures 7.1 and 7.2 illustrate the $\delta^{13}C$ and $\delta^{15}N$ difference between non-lesion and lesion (healed fractures, healed infectious lesions and active lesions) sites within the same bone. Values below zero point to an increase of $\delta^{13}C$ and $\delta^{15}N$ values, at the lesion, when compared to the non-lesion site while values above zero imply a decrease of $\delta^{13}C$ and $\delta^{15}N$ values. The grey areas across the boxplots represent the expected normal intra-bone range (Katzenberg and Lovell 1999, Olsen et al. 2014). Scatter plots of the lesion and non-lesion sites for each individual can be found in the Appendix (Figure A3).

7.3.1. Intra-bone collagen $\delta^{13}C$ and $\delta^{15}N$ values comparison between lesions and areas without lesions – long bones

A paired-samples t-test was conducted to compare collagen $\delta^{13}C$ and $\delta^{15}N$ values in bone segments with and without lesions. Bone segments with active lesions ($\bar{x}$ $\delta^{15}N$=11.1±0.9‰) had higher $\delta^{15}N$ values than those without lesions ($\bar{x}$ $\delta^{15}N$=10.7±0.7‰), a statistically significant increase of 0.4 (95%CI:-0.7 to -0.1‰, *t*(13)=-2.58, p=0.02, Table 1). Additionally, median $\delta^{15}N$ increases and $\delta^{13}C$ decreases in active lesions, while in healed lesions $\delta^{15}N$ values decreases and $\delta^{13}C$ slightly increases.

Figure 7.1 shows a larger difference between $\delta^{15}N$ and $\delta^{13}C$ values from non-lesion and lesion sites than would be expected for normal intra-bone variation in long bones (grey area at Figure 7.1, Katzenberg and Lovell 1999).

The $\delta^{15}N$ median for active lesions (x̃=-0.5‰, n=14) and both quartiles are lower than zero (Figure 7.1) and the expected $\delta^{15}N$ values intra-bone variation (+0.3 to +0.4‰, Katzenberg and Lovell, 1999). The active lesions boxplot is “negatively skewed” and the two outliers with the highest $\delta^{15}N$ values increase have lesions in various bones (skeletons 16.169 and 18.158). These results indicate that intra-bone $\delta^{15}N$ values increased in active lesions and decreased in healed lesions. The active lesions $\delta^{13}C$ median is higher than zero (x̃=+0.3‰, n=14) and falls within the expected intra-bone $\delta^{13}C$ range (+0.2 to +0.7‰, Katzenberg and Lovell 1999), however, the lower quartile is bellow the expected intra-bone $\delta^{13}C$ range (Figure 7.1).

The $\delta^{15}N$ median for healed lesions (x̃=+0.5‰, n=10) is higher than zero (Figure 7.1) and slightly higher than the expected normal $\delta^{15}N$ values intra-bone range (+0.3 to +0.4‰, Katzenberg and Lovell 1999). The healed lesions $\delta^{13}C$ median is lower than zero (x̃=-0.1‰, n=10) and the expected intra-bone $\delta^{13}C$ range. The healed lesions boxplot is “positively

skewed" and while the outlier with the highest $\delta^{15}N$ values decrease only has healed tibial lesions (skeleton 18.250), the one the highest $\delta^{15}N$ values increase has a generalised infection (skeleton 14.72).

Table 7.1. Intra-bone differences in collagen $\delta^{13}C$ and $\delta^{15}N$ values between non-lesion (distant from lesion) and lesion (active, healed or fractured) sites at long bones. Mean values are reported as relative rather than absolute values in order to preserve the directionality of the difference (positive or negative) between different bone sites.

		Paired Differences								
		N	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
						Lower	Upper			
$\delta^{15}N$	Non-lesion - active	14	**-0.4**	0.6	0.2	-0.7	-0.1	-2.58	13	**0.02**
	Non-lesion - healed	10	0.2	0.9	0.3	-0.4	0.8	0.68	9	0.51
	Non-lesion - fracture	9	0.1	0.8	0.3	-0.5	0.8	0.54	8	0.61
$\delta^{13}C$	Non-lesion - active	14	0.2	0.5	0.1	-0.1	0.6	1.63	13	0.13
	Non-lesion - healed	10	0.0	0.6	0.2	-0.4	0.4	0.17	9	0.87
	Non-lesion - fracture	9	0.2	1.1	0.4	-0.6	1.1	0.62	8	0.55

The $\delta^{15}N$ values of facture calluses have the highest variability, with both the highest maximum and lowest minimum values. The $\delta^{15}N$ median for fracture callus ($\tilde{x}$=-0.1‰, n=9) is close to zero (Figure 7.1) but lower than the expected $\delta^{15}N$ intra-bone variation (+0.3 to +0.4‰, Katzenberg and Lovell 1999). Similar to what was observed for $\delta^{15}N$ values, facture callus have both the higher maximum and lower minimum $\delta^{13}C$ (Figure 7.1). The $\delta^{13}C$ median for fracture callus ($\tilde{x}$=+0.4‰, n=9) falls within the expected intra-bone $\delta^{13}C$ range (+0.2 to +0.7‰, Katzenberg and Lovell 1999).

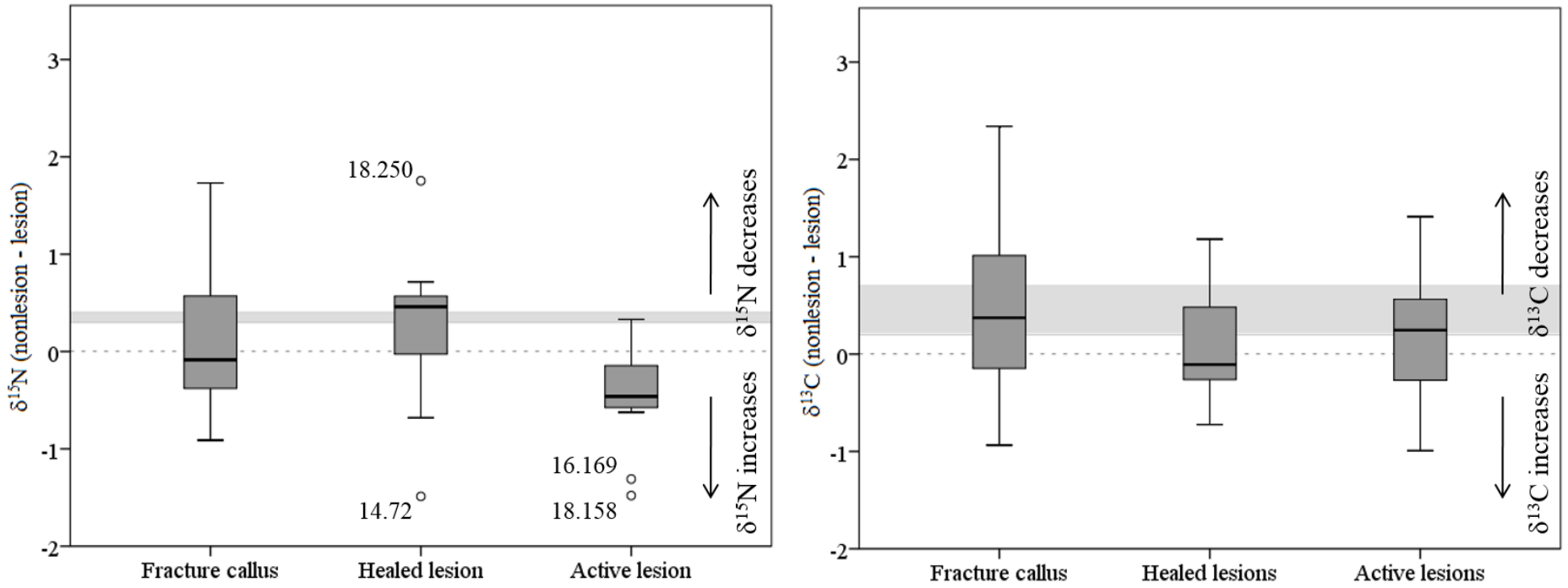


Figure 7.1. Intra-bone differences in collagen $\delta^{13}C$ and $\delta^{15}N$ values between non-lesion (distant from lesion) and lesion (active, healed or fractured) sites at long bones. Values are reported as relative rather than absolute values in order to preserve the directionality of the difference (positive or negative) between different bone sites. Grey area represents the expected intra-bone variation (Katzenberg and Lovell 1999).

7.3.2. Intra-bone collagen $\delta^{13}C$ and $\delta^{15}N$ values comparison between lesions and areas without lesions – ribs

A paired-samples t-test was conducted to compare collagen $\delta^{13}C$ and $\delta^{15}N$ in bone segments with and without lesions but the results were not statistically significant (Table 7.2).

Figure 7.2 shows a larger difference between $\delta^{15}N$ and $\delta^{13}C$ values from non-lesion and lesion sites than would be expected for normal intra-bone range in ribs (grey area at Figure 4.2, Olsen et al. 2014). The ribs boxplots have a similar pattern to the long bone boxplots.

For the active lesions both the median ($\tilde{x}$=-0.3‰, n=4) and the lower quartile have lower values than the expected normal intra-rib $\delta^{15}N$ range (0.0 to +0.5‰, Olsen et al. 2014). The $\delta^{13}C$ median for active lesions ($\tilde{x}$=-0.1‰, n=4) and lower quartile are lower than the expected normal intra-bone $\delta^{13}C$ range (-0.1 to +0.1‰, Olsen et al. 2014). The healed lesions $\delta^{15}N$ ($\tilde{x}$=+0.2‰, n=9) and $\delta^{13}C$ medians ($\tilde{x}$=-0.1‰, n=9) falls within the expected normal intra-bone variation in ribs ($\delta^{15}N$: 0.0 to +0.5‰, $\delta^{13}C$: -0.1 to +0.1‰, Olsen et al. 2014). However, the $\delta^{13}C$ values lower quartile is lower than the normal intra-rib $\delta^{13}C$ range. The fracture callus $\delta^{15}N$ median ($\tilde{x}$=0.0‰, n=3) falls within the expected normal intra-bone $\delta^{15}N$ variation in ribs (0.0 to +0.5‰, Olsen et al. 2014). The fracture callus $\delta^{13}C$ median ($\tilde{x}$=-0.3‰, n=3) and lower quartile (Figure 7.2) are lower than the expected intra-bone $\delta^{13}C$ (-0.1 to +0.1‰, Olsen et al. 2014). The fracture calluses have the most variable differences, while active lesions have the lowest difference. The two outliers with healed lesions have generalised infections (various bones affected). However, while skeleton 18.158 has the highest $\delta^{15}N$ values decrease, skeleton 14.72 has the highest $\delta^{15}N$ values increase (Figure 7.2).

Table 7.2. Intra-bone differences in collagen $\delta^{13}C$ and $\delta^{15}N$ values between non-lesion (distant from lesion) and lesion (active, healed or fractured) sites at ribs. Mean values are reported as relative rather than absolute values in order to preserve the directionality of the difference (positive or negative) between different bone sites.

		Paired Differences								
		N	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
						Lower	Upper			
$\delta^{15}N$	Non-lesion - active	4	-0.1	0.2	0.1	-0.4	0.1	-1.44	3	0.25
	Non-lesion - healed	9	-0.2	0.4	0.1	-0.5	0.1	-1.83	8	0.10
	Non-lesion - fracture	3	-0.2	0.5	0.3	-1.3	1.0	-0.56	2	0.63
$\delta^{13}C$	Non-lesion - active	4	**-0.4**	0.9	0.4	-1.8	1.0	-0.92	3	0.43
	Non-lesion - healed	9	0.1	0.9	0.3	-0.7	0.8	0.18	8	0.86
	Non-lesion - fracture	3	0.2	0.5	0.3	-0.9	1.4	0.84	2	0.49

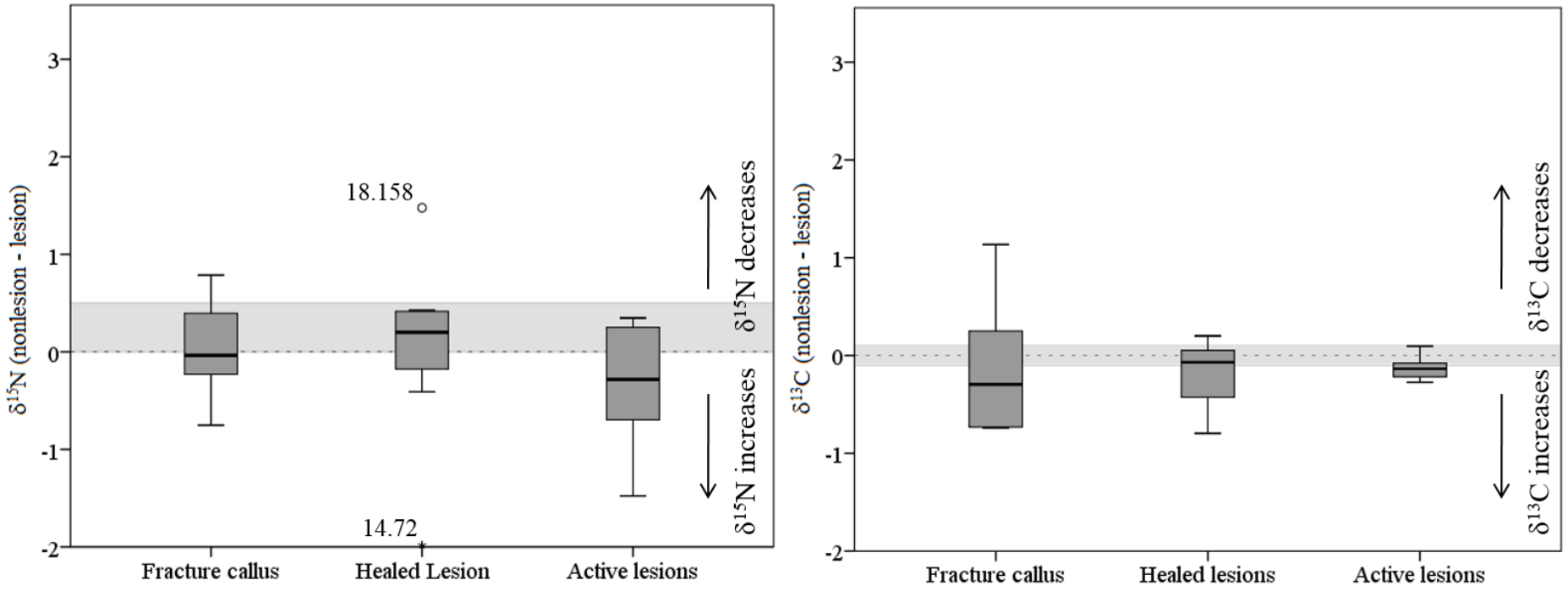


Figure 7.2. Intra-bone differences in collagen $\delta^{13}C$ and $\delta^{15}N$ values between non-lesion (distant from lesion) and lesion (active, healed or fractured) sites at ribs. Values are reported as relative rather than absolute values in order to preserve the directionality of the difference (positive or negative) between different bone sites. Grey area represents the expected intra-bone variation (Olsen et al. 2014).

7.4. Discussion

7.4.1. Intra-bone collagen $\delta^{13}C$ and $\delta^{15}N$ values comparison between lesions and areas without lesions – long bones

The intra-bone $\delta^{13}C$ and $\delta^{15}N$ values variability observed in this study is larger than expected (Figure 7.1, Katzenberg and Lovell 1999) and may be related to differences in metabolism or diet during the disease and/or recovery from the disease. Bone formation and remodelling can occur during some disease processes (e.g. Ragsdale and Lehmer 2012, McQueen et al. 2011, Wlodarski 1989). The repair mechanism is initiated before the cessation of the disease state or the clearance of a pathogen from the organism (Klau, 2014, Ragsdale and Lehmer 2012, Neve et al. 2011). In a diseased state, bone formation does not stop, instead, it is active in fewer locations, where new woven bone may be formed (Lian et al. 2011). D'Ortenzio et al. (2015) suggested that short-term fluctuations of $\delta^{15}N$ values might be the result of changes in the metabolic balance of an individual. Recycled body tissues used as a protein resource are enriched in ^{15}N, increasing the $\delta^{15}N$ values within the individual's tissues (Steele and Daniel 1978, Hobson and Clark 1992, Hobson et al. 1993, Oelbermann and Scheu 2002, Gaye-Siesseger et al. 2004, Deschner et al. 2012, D'Ortenzio et al. 2015). Carbon recycled from fat deposits decreases the $\delta^{13}C$ in the body (Neuberger et al. 2013).

Active lesions

The results indicate an increase in $\delta^{15}N$ ($\bar{x}$=0.4‰, Table 7.1) and a decrease in $\delta^{13}C$ values ($\bar{x}$=0.2‰, Table 7.1) in active lesions when compared with $\delta^{13}C$ and $\delta^{15}N$ values from non-lesion sites of the same bone (Figure 7.1, Table 7.1). Since woven bone at the lesions was formed during or after the disease (e.g. Klaus 2014, Lian et al. 2011, McQueen et al. 2011,

Ragsdale and Lehmer 2012, Wlodarski 1989), these stable isotope results represent physiological or nutritional differences between different periods of the individual's life.

While malnutrition impairs the immune system (e.g. Scrimshaw and SanGiovanni 1997), infections can decrease nutrient availability due to malabsorption (e.g. Mitra et al. 1997) and increase resting energy expenditure (Calder 2013). During physiological or nutritional stress the proteins in the body will be recycled resulting in higher $\delta^{15}N$ values within the individual's tissues (Steele and Daniel 1978, Hobson and Clark 1992, Hobson et al. 1993, Katzenberg and Lovell 1999, Oelbermann and Scheu 2002, Gaye-Siesseger et al. 2004, Mekota et al. 2006, Deschner et al. 2012, D'Ortenzio et al. 2015), as dietary protein cannot adequately replace nitrogen losses during these situations (Grossman et al. 1945, Powanda 1977, Welle 1999).

^{15}N enrichment has also been observed in a probable case of celiac disease (Scorrano et al. 2014) in which the chronic malnutrition resulting from the severe malabsorption of essential nutrients may have affected the isotopic composition of the bone collagen. Beaumont and Montegomery (2016) observed raised $\delta^{15}N$ and lower $\delta^{13}C$ values in the dentine profiles of children from the Irish famine. Eerkens et al. (2017) registered stable isotope ratios changes across a series of hair samples of a mummified girl consistent with undernourishment. The $\delta^{15}N$ values slowly increased about 7 months prior to death followed by acceleration in ^{15}N enrichment about 3 months before death suggesting a final phase which can be related to a cessation or significant reduction of protein intake (Eerkens et al. 2017).

Reduced appetite or anorexia is part of the normal response to infection, in extreme cases (Murray and Murray 1979, Exton 1997). Starvation is associated with increases in hair keratin $\delta^{15}N$ values (Eerkens et al. 2017, Fuller et al. 2005, Hatch et al. 2006, Mekota et al.

2006, Neuberger et al. 2013), and collagen from bones with signs of infection have higher $\delta^{15}N$ values than unaffected areas in the same individuals (Katzenberg and Lovell 1999). Even though nutritional restriction usually results in negative nitrogen balance (e.g. Hobson and Clark 1992, Hobson et al. 1993, Robertson et al. 2014), that is not always the case. Williams et al. (2007) observed lower $\delta^{15}N$ values in blood cells of moderately nutritional restricted puffin chicks, than those fed *ad libitum*. Hatch et al. (2006) did not observe ^{15}N enrichment in hair samples of bulimic patients and the authors suggest that the nutritional stress might not be as severe as in anorexic patients. The varying $\delta^{15}N$ values difference between lesions and non-lesion sites can be related with the degree of nutritional or physiological stress and if it is severe enough to trigger protein catabolism in the individuals' tissues. Despite these studies relevance a direct comparison to, and among, the various human tissues is not yet possible. Still, ^{15}N enrichment possibly related with prolonged nutritional stress was reported in Napoleonic soldiers (Holder 2013, Holder et al. 2015). In migrants from the Great Irish Famine elevated $\delta^{15}N$ values possibly related to nutritional stress were only observed in bones of infants, which can be related with their faster bone turnover (Beaumont et al., 2013).

$\delta^{15}N$ values from an individual with osteomyelitis, who had AIDS, showed an increase of 1.6‰ at active lesions (12.9‰) and a decrease of 0.3‰ at healed lesions (11.3‰) when compared with non-lesion sites (11.3‰, Katzenberg and Lovell 1999). Following the work from Steele and Daniel (1978) and Hobson et al. (1993), Katzenberg and Lovell (1999) connected this phenomenon to negative nitrogen balances. The wasting syndrome characteristic of AIDS might have led, to physiological stress and consequently higher $\delta^{15}N$ values. The higher ^{15}N enrichment observed by Katzenberg and Lovell (1999) than in this study can be the result of more severe stress than Tomar sample or modern care giving and

medication, as these authors studied a forensic sample. Olsen et al. (2014) also observed a $\delta^{15}N$ increase in osteomyelitic lesions (+0.5 to +2.5‰) when compared to distant to lesion sites. In periosteal lesions the difference from non-lesion sites to lesion sites varied between -2.1 and +1.2‰ (Olsen et al. 2014). This wide interval might be explained by the cluster of both active and healed lesions.

I observed a statistically significant difference in $\delta^{15}N$ values between non-lesion and active lesion sites even though bone should be one of the last tissues affected by short-term dietary or metabolic changes. The possibility of ^{15}N enrichment being associated with nitrogen catabolism due to faster bone growth (Steele and Daniel 1978, Hobson and Clark 1992, Hobson et al. 1993, Katzenberg and Lovell 1999, Oelbermann and Scheu 2002, Gaye-Siesseger et al. 2004, Mekota et al. 2006, Deschner et al. 2012, D'Ortenzio et al. 2015) at the lesions cannot be excluded. However, Waters-Rist and Katzenberg (2010) did not find a detectable $\delta^{15}N$ growth effect when analysing epiphyses, metaphyses and diaphysis of growing long bones.

Even though the difference between $\delta^{13}C$ values in non-lesion and active lesion sites is not statistically significant, there is a trend for $\delta^{13}C$ values to decrease. ^{13}C depletion is compatible with what was observed in starving patients and can be indicative of a severe reduction of energy intake through nutrition (Hatch et al. 2006, Mekota et al. 2006, Neuberger et al. 2013). Katzenberg and Lovell (1999) did not find differences in $\delta^{13}C$ values between bone sites with and without lesions. Olsen et al. (2014), on the other hand, observed a $\delta^{13}C$ values increase in osteomyelic lesions (+0.1 to +0.5‰) and some periosteal lesions (-0.3 to +0.2‰). Eerkens et al. (2017) observed a slight increase in $\delta^{13}C$ values in hair segments of a mummified girl from the late 19^{th} century, which the authors relate to possible introduction of foods and/or medicines containing oils or carbohydrates.

Healed lesions

The increase in $\delta^{13}C$ values and decrease $\delta^{15}N$ values at healed lesion sites, when compared with non-lesion sites, may be related with the metabolism during the recovery from the disease, similarly to what has been observed in hair keratin during recovery from starvation (Mekota et al. 2006, Neuberger et al. 2013). Compact bone at healed lesions was formed after the disease representing a period when the individual was recovering from the disease (e.g. Ragsdale and Lehmer 2012, McQueen et al. 2011, Wlodarski 1989). These results may stand for physiological or nutritional differences between before and after the disease, which can be observable in skeletonised human remains. However these results cannot be directly compared with those from the studies mentioned (Mekota et al. 2006, Neuberger et al. 2013) as these studies do not refer bone tissues but hair keratin.

Both quartiles of $\delta^{15}N$ values for healed lesions have values higher than zero, indicating that $\delta^{15}N$ values decreased in healed lesions when compared to non-lesions (positive nitrogen balance). These differences are not statistically significant, which may be related to the small sample size. Positive nitrogen balances occur when more nitrogen is consumed than excreted, being linked with recovery from disease and/or starvation observed in hair keratin (e.g. Fuller et al. 2004, 2005, Hatch et al. 2006, Mekota et al. 2006, Neuberger et al. 2013). While recovering from physiological stress the body assimilates more dietary amino acids less enriched in ^{15}N resulting in a $\delta^{15}N$ values decrease in tissues formed during this period (Fuller et al. 2004, 2005, Harvey and Ferrier 2011, Mekota et al. 2006, Neuberger et al. 2013).

In a study with patients with anorexia, $\delta^{13}C$ values in hair keratin increased with increasing BMI in patients recovering from starvation (Mekota et al. 2006). This increase can be largely due to an increase in meat and fat intake (Van der MerI 1982, Chisholm et al.

1982) or medicines containing carbohydrates (Eerkens et al. 2017). However, a significant $\delta^{13}C$ values increase was not observable in this study when comparing healed lesions with non-lesion sites.

Fracture calluses

The difference between non-lesion sites and facture calluses is the most variable for both $\delta^{13}C$ and $\delta^{15}N$ values, out of the types of lesions analysed. In fractures re-absorption usually precedes formation but it has been reported that the re-absorption biochemical markers increased later than the formation markers of bone turnover (Ingle et al. 1999). This suggests that the early increase in bone markers reflects the callus formation and the later changes represent callus remodelling and increased turnover in bone around the lesion (Ingle et al. 1999).

Trauma disrupts normal metabolism by increasing muscle protein catabolism and nitrogen excretion, resulting in a net protein loss or negative nitrogen balance within days (Long et al. 1981, Yuet al. 2017). An increase in $\delta^{15}N$ values was expected alongside the increased turnover rate at the fracture site (Ingle et al. 1999, Olsen et al. 2014, Veitch et al. 2006) as a result of protein catabolism (e.g. Hobson et al. 1993, Katzenberg and Lovell 1999, Steele and Daniel 1978). However, this was not always the case in our samples. Another study shows that well-healed fractures registered lower $\delta^{15}N$ values than areas without lesions but the sample size was also small (Katzenberg and Lovell 1999). These results suggest that the isotopic composition of fracture calluses may represent positive or negative nitrogen imbalances given that healing stages may vary from callus to callus depending of factors such as healing stage and time after the trauma.

The $\delta^{13}C$ values decrease observed in fracture calluses suggests either a change in dietary protein sources (Beaumont and Montgomery 2016) or nutritional stress (Mekota et al. 2006, Neuberger et al. 2013). $\delta^{13}C$ values increase can be associated with starvation recovery (Mekota et al. 2006, Neuberger et al. 2013), apart from representing dietary changes in either quantity (Van der MerI 1982, Chisholm et al. 1982) or source (Beaumont and Montgomery 2016).

Outliers

The outliers with active generalised infections (Figure 7.1, skeletons 16.169 and 18.158) have similar $\delta^{15}N$ values increase to that observed in an individual with AIDS, a wasting disease, described by Katzenberg and Lovell (1999). The individuals with generalised infections survived long enough with the disease for its consequences to be visible on the skeleton (Wood et al. 1992), representing chronic infections and may have been in a wasting stage. If in a wasting stage, this may have led to protein catabolism due to lower nutrient intake (Murray and Murray 1979, Exton 1997), malabsorption (e.g. Mitra et al. 1997) and increased resting expenditure (Calder 2013). However, the possibility of these individuals having a dietary shift duringthe disease which may have resulted in high $\delta^{15}N$ values cannot be excluded. The outlier with the highest $\delta^{15}N$ increase among the healed lesions (Figure 7.1, skeleton 14.72) also has a generalised infection and similar values to the ones with active generalised infections. These results suggest that ^{15}N enrichment may be variable depending on the aetiology or severity of the disease and how it affects the nutritional status and metabolism of the individual.

The only outlier with a high $\delta^{15}N$ values decrease (Figure 7.1, skeleton 18.250), in contrast with the others outliers, only has healed tibial periostitis suggesting that this individual might have recovered from physiological stress. Periostitis may reflect stress and

morbidity but may also often represent later phases of the inflammation and subsequent recovery from disruption of normal physiology (Klaus 2014).

The diets of the individuals, prior to the disease, suggest that the ones with non-specific generalised infections had diets lower in animal protein than those without lesions or with only healed tibial periostitis (Chapter 6, Curto et al. 2019). While non-specific generalised infections can be an indication of poor health and protein intake, healed tibial periostitis may indicate a state of comparatively good overall health and diet (Chapter 6, Curto et al. 2019).

7.4.2. Intra-bone collagen $\delta^{13}C$ and $\delta^{15}N$ values comparison between lesions and areas without lesions – ribs

The intra-rib $\delta^{13}C$ and $\delta^{15}N$ values variability observed in this study is larger than expected (Figure 7.2, Olsen et al. 2014), similarly to what was observed for long bones (Figure 7.1). As mentioned before recycled body tissues are enriched in ^{15}N and depleted in ^{13}C (Deschneret al. 2012, D'Ortenzio et al. 2015, Hobson and Clark 1992, Hobson et al. 1993, Gaye-Siesseger et al. 2004, Neuberger et al. 2013, Oelbermann and Scheu 2002, Steele and Daniel 1978).

Active lesions

The results suggest an increase in both $\delta^{15}N$ and $\delta^{13}C$ values from active lesions when compared with $\delta^{13}C$ and $\delta^{15}N$ values from non-lesion rib sites (Figure 7.2, Table 7.2). The increase in $\delta^{15}N$ ($\bar{x}$=+0.4‰) observed for the active lesions of ribs is similar to what was registered in long bones ($\bar{x}$=+0.4‰, Table 7.1) but not statistically significant for the ribs (p=0.25, Table 7.2). Opposite to what was observed in long bones, a slight increase in $\delta^{13}C$ values was registered in active rib lesions ($\bar{x}$=+0.1‰, Table 7.2). However the $\delta^{13}C$ values difference is not statistically significant and both quartiles are very close to zero.

This difference in $\delta^{15}N$ and $\delta^{13}C$ values registered in active rib and long bone lesions can be related to the smaller ribs sample size (n=4) but also to the different bone turnover rates between long bones and ribs (Cox and Sealy 1997, Hedges et al. 2007). Cortical bone in ribs represent a smaller time frame interval, than in long bones, when compared to the woven bone formed during or after the disease (e.g. Ragsdale and Lehmer 2012, McQueen et al. 2011, Wlodarski 1989). At the ribs it was also more difficult to separate abnormal new bone from underlying cortical bone, which can be related with the similarity between lesions and non-lesion sites.

Healed lesions

The $\delta^{15}N$ values quartiles, except part of the lower quartile, fit within the expected intra-rib $\delta^{15}N$ range (Figure 7.2). Still, the $\delta^{15}N$ values boxplot is positively skewed suggesting a tendency for $\delta^{15}N$ values to decrease in healed rib lesions when compared with non-lesion sites. Even though the $\delta^{13}C$ values difference median is close to zero, both quartiles are lower than zero (Figure 7.2), suggesting $\delta^{13}C$ values increase in healed rib lesions when compared to non-lesion sites. However the difference between lesion and non-lesion sites is not statistically significant for either $\delta^{15}N$ or $\delta^{13}C$ values.

Similar results have been described in hair of patients recovering from starvation (e.g. Mekota et al. 2006, Neuberger et al. 2013) but while hair lacks turnover bone is constantly remodelled. Still, these results suggest that physiological or nutritional differences between before and after the disease can also be observable in human skeletons. Since ribs have a faster turnover than long bones (Cox and Sealy 1997, Hedges et al. 20017), if the individual lived long enough with the disease (stressor) it is possible that

the collagen from healed rib lesions represents not only the average stable isotope ratios before the disease but also ratios from periods during and after the disease.

Fracture calluses

Fracture calluses have the closest to zero $\delta^{15}N$ median out of all the types of lesions analysed (Figure 7.2), similarly to what was observed in long bones (Figure 7.1). Depending on the healing stage and time after the trauma, fracture calluses can represent either positive or negative nitrogen balances or even tissue maintenance. Opposite to what was observed for the long bones, the ribs fracture calluses show an $\delta^{13}C$ values increase when compared to non-lesion sites. ^{13}C enrichment has been observed in starvation recoveries (Mekota et al. 2006, Neuberger et al. 2013). Food intake after a period of nutritional stress (Van der MerI 1982, Chisholm et al. 1982) or changes in diet due to food availability (e.g. Beaumont and Montgomery 2016) or even medicines (Eerkens et al. 2017) can affect stable isotope ratios. However the small sample size (n=3) does not allow conclusions to be drawn.

Outliers

Both outliers (skeletons 18.158 and 14.72, Figure 7.2) have generalised infections (various bones affected) with both healed and active lesions. The lesion site of skeleton 14.72, an elderly female with an unspecific generalised infection, shows $\delta^{15}N$ values increase when compared with non-lesion site of the same bone. The lesion of skeleton 18.158, a mature male diagnosed with syphilis, shows $\delta^{15}N$ values decrease at the lesion site. These results suggest that the individual with the unspecific generalised infection (skeleton 14.72) could have been in a state of physiological stress more severe than the individual with venereal syphilis.

Skeleton 18.158 has larger ^{15}N depletion in healed rib lesions (Figure 7.2) than what was observed for healed long bone lesions (Figure 7.1), when compared to non-lesion sites. ^{15}N depletion, compatible with recovery from the stressor (Mekota et al. 2006, Neuberger et al. 2013), was expected for healed lesions. The lesions in the long bones of this individual are also compatible to what was expected: ^{15}N enrichment in active lesions and ^{15}N depletion in healed lesions. However, the difference in healed rib lesions is larger than in healed long bone lesions, which can be related with different healing stages and therefore different nitrogen balance. ^{15}N enrichment is expected in active lesions, since the individual would be in physiological stress (e.g. Fuller et al. 2004,2005, Hatch et al. 2006, Mekota et al. 2006, Neuberger et al. 2013).

7.5. Conclusion

This study compared stable isotope ratios from cortical bone that retained evidence of lesions in the form of disease, or healed fractures, to ratios from cortical bone in the same individual that do not retain these skeletal lesions. Results indicate a $\delta^{15}N$ values increase and a $\delta^{13}C$ values decrease in active skeletal lesions and the opposite in healed skeletal lesion but only the increase in $\delta^{15}N$ values on active lesions is statistically significant. Fracture callus $\delta^{15}N$ and $\delta^{13}C$ values are more variable than both active and healed infectious lesions. The difference in stable isotope ratios between non-lesion sites and those with active lesions may be directly related to the disease and/or due to diet shifts related to the disease, such as inappropriate ingestion of nutrients or malabsorption leading to starvation and wasting.

These results suggest that stable isotope analysis can be applied to archaeological samples to increase our understanding of the relationship between diet, metabolism, and

diseases. Future developments on bone formation and tissue repair, allied with stable isotope analysis from different human tissues, will improve our understanding of pathological processes in past populations by assessing their nitrogen balance and skeletal lesions.

Chapter 8

General discussion, study limitations, conclusion and future directions

The main aim of this doctoral dissertation was to grasp the potential of stable isotope analysis for the study of paleopathology, improving our understanding of the synergy between diet and health in the absence of modern medicine and antibiotics. In this general discussion I analyse the results of my dissertation and summarize the impact of these results in paleopathology, specifically the relationship between diet and health in past populations.

Chapter 1 outlined this project aims and predictions, presenting testable hypothesis to answer the research questions. In this chapter the complex relationship between diet, health and physiology was presented justifying the rationality of how the research was designed and conducted. The thesis organization was also described to facilitate the reading through the different data chapters.

Chapter 2 set a general background for this dissertation by illustrating the historical context of Tomar, the excavation of the necropolis and the historical information about the medieval diet in Tomar. Background information on the stable isotope analysis, bone turnover, indicators of physiological stress and the synergy between diet and health were also given in this chapter.

Chapter 3 portrayed the materials and methods used for this study. The selection of skeletons and bones for stable isotope analysis was described in this chapter, as well as

details about the collagen extraction and analysis. The information in this chapter in complemented and specified in each data chapter.

Chapter 4 explored Tomar's osteological collection enabling an understanding of who was buried at Tomar. This graveyard represents the general population and not, or at least not only, the individuals from the military orders. This chapter also provided some input on how the necropolis was organized, providing information about the individuals' socio-economic status, which can influence both their diet (e.g. Kjellström et al. 2009, Linderholm et al. 2008, Polet and Katzenberg 2003, Schutkowski et al. 1999, Reitsema et al. 2010, Reitsema and Vercellotti 2012) and their exposure to pathogens. Population specific equations were developed for Tomar's collection, improving sex and stature estimations for the analysed samples.

Chapter 5 explored the adult baseline diet at Tomar, suggesting that the presence of military orders in the town may have had an impact on the general population's diet. People living in Tomar had a complex diet and likely included food sources from outside Tomar. The general diet was poor in terrestrial protein and with variable amounts of aquatic protein, which may be related to religious dietary restrictions to meat consumption. Previous studies suggest different food access based on sex and status in medieval times (e.g. Kjellström et al. 2009, Linderholm et al. 2008, Polet and Katzenberg 2003, Schutkowski et al. 1999, Reitsema et al. 2010, Reitsema and Vercellotti 2012) however that was not observed in Tomar. The fauna's stable isotope ratios suggest that the introduction of new food sources such as maize was not relevant, despite the necropolis's wide chronology (11^{th} to 17^{th} centuries).

Chapter 6 investigated the impact of diet on disease susceptibility by comparing stable isotope ratios between skeletons with and without lesions. This study highlights the

significant difference in $\delta^{15}N$ values between skeletons with non-specific generalised infections and those with either only healed tibial periostitis or no apparent lesions. Individuals without lesions and those who recovered from physiological stress and/or infectious disease (healed tibial periostitis) may have had diets richer in protein intake than the individuals with generalised infections. These results imply that stable isotope analysis has the potential to better understand how diet influences the susceptibility of certain individuals or populations, to pathogens prior to the use of antibiotics.

Chapter 7 examined if diet and/or metabolism during the disease has a measurable effect on skeletal human remains. This study showed a significant $\delta^{15}N$ increase in active lesions when compared with non-lesion $\delta^{15}N$ in the same bone. The $\delta^{15}N$ increase in active lesions and $\delta^{15}N$ decrease in healed lesions suggest that stable isotopes have the potential to be measured before, during and after the disease. These results reinforce the value of stable isotope analysis for the study of health in past populations.

8.1. General discussion

This study shows a potential link between diet, physiology and disease in human bone, despite the synergy between diet and health being very complex (Figure 8.1). Therefore, future studies can consider the implications of this link when reconstructing diet and life style of past populations.

Diet in Iberia is still not well documented using stable isotope analysis. This study (Chapter 5, Curto et al. 2018) suggests that diet at Tomar was complex with variable amount of aquatic protein intake and probably relying on food sources from outside Tomar's region. Despite the large chronological interval of Tomar's sample (11^{th} – 17^{th} centuries), stable isotope analysis did not indicate the presence of new food sources such as maize and sugar

cane. These new food sources would be expected to be first identified in Iberian populations within Europe but the time when they became widely used by the general population is not known yet.

Even though stable isotope ratios were variable between the analysed skeletons there are no significant dietary differences between sexes or social status (Table 5.2). These results contradict other studies suggesting that individuals with different sex or socio-economic status had different diets (e.g. Adamson 2004, Kjellström et al. 2009, Linderholm et al. 2008, Polet and Katzenberg 2003, Schutkowski et al. 1999, Reitsema et al. 2010, Reitsema and Vercellotti 2012). This is particularly unexpected due to the presence of Military Orders, which could have increased socio-economic status gaps. However, the areas studied do not represent the individuals from the highest (who would be buried inside the church) or the lowest socio-economic status (buried in the areas corresponding to the 2nd phase of the excavation or even further away from the church, Figure 2.4) (Chapter 4).

Tomar's dietary study increased our knowledge about European medieval times, particularly in Iberia, where Religious Military Orders had an important role. Religious dietary restrictions during medieval times may have had a larger impact in people's life than previously thought. Religious dietary restrictions had been reported before (Müldner et al. 2009, Salamon et al. 2008). Müldner et al (2009) observed a higher intake of aquatic protein in bishops that in the general population. Salamon et al. (2008) observed a dramatic increase of fish consumption in medieval Mediterranean people, which can be a consequence of trying to meet religious dietary directions. Stable isotope analysis from Tomar (Figure 5.6) shows values closer those observed in a coastal monastery in Belgium (Polet and Katzenberg 2003) than in other Iberian collections (Lubritto et al. 2013, Alexander et al. 2015). Diet in medieval Europe, and particularly in Iberia, seems to be very diverse.

Future studies will improve not only our understanding about Iberian diet but also trade, agricultural and husbandry practices, exogenous new food sources implementation and religious dietary restrictions.

There are no significant dietary differences between sexes or social status that could influence the stable isotope analysis of individuals with and without lesions (Chapter 5, Curto et al. 2018). Therefore it is expected that stable isotope differences between these two groups would be related to the stable isotope analysis being affected by physiological stress or different individual diets, which may have different outcomes on the individuals' health.

The possibility of the high $\delta^{15}N$ values observed in skeletons without lesions or indicators of physiological stress (Chapter 5, Curto et al. 2018) being related with protein catabolism cannot be excluded. However, these values increase directly proportional to $\delta^{13}C$ values (Figure 5.2) suggesting dietary differences, as during protein catabolism usually only $\delta^{15}N$ values increase significantly (e.g. Hobson and Clark 1992, Hobson et al. 1993, Martínez del Rio and Wolf 2005, Gaye-Siessegger et al. 2007, Lohuis et al. 2007, McCue 2008, McFarlane Tranquilla et al. 2010). The $\delta^{34}S$ values (Figure 5.4, Table 5.1) also suggest that the high $\delta^{15}N$ values observed in these individuals reflect diets rich in aquatic protein. The individuals selected to estimate the general diet at Tomar were selected based on not having skeletal lesions and indicators of physiological stress, so it was not expected to observe protein catabolism in these individuals. Still, the absence of skeletal lesions does not necessarily mean that the individuals were healthy; they may have died before skeletal lesions developed (Wood et al. 1992). Stable isotope analysis from bones reflect average values resulting from the diet and possible periods of protein catabolism within the time that bone was remodelling (up to 25 years, Hedges et al. 2007). If the individual did not

survive long enough with the disease (for it to affect the bone in the form of skeletal lesions), then it is also probable that the disease would have an impact on the values of stable isotopes in bone collagen. However, not all diseases affect the skeleton and prolonged chronic malnutrition during adulthood may not leave indicators of physiological stress such as enamel hypoplasias and low stature as they are a consequence of growth stopping or slowing down (e.g. Saunders and Hoppa 1993, Hillson 1996, Lampl 2012, WHO 2013).

Tibial periostitis is frequently considered an indicator of physiological stress (e.g. DeWitte 2010, Robb et al. 2001) but it can also be an indicator of relatively good health, particularly healed periostitis (Wood et al. 1992). This study did not find any differences between the diet of individuals with and without tibial periostitis (Chapter 6), despites physiological stress being frequently associated with nutritional stress (e.g. MacDade 2005, Weston 2012, Reitsema et al. 2016).

The skeletons with only healed tibial periostitis may have had a diet rich in animal protein and therefore could have been more resistant to infectious diseases (e.g. Kuvibidila et al. 1993, Scrimshaw and SanGiovanni 1997, Woodward 1998, Woodward 2001, Ulijaszek et al. 2012, Weston 2012). The $\delta^{15}N$ values registered in the skeletons with only tibial periotitis can also be related with high aquatic protein intake, as the amount of its consumption is variable in Tomar (Chapter 5, Curto et al. 2018). Since fish was an expensive protein source (Gonçalves 2004) a diet rich in aquatic protein may also represent high socio-economic status. Socio-economic status also have an important role in people's health as it can influence access to nutritious food resources and health care, exposure to pathogens, strenuous biomechanical effort, settlement density, sanitation and hygiene (e.g. Goodman and Armelagos 1989, Cockerham 2007). It has been argued that individuals with healed

periostitis are of lower frailty, having a lower risk of death (e.g. DeWitte 2010, Ortner 2003, Wood et al. 1992). This idea is reinforced by the present study. The individuals with healed periostitis survived long enough to the stressor for it to be registered in their skeleton and may have even overcome it, as the lesions are healed.

The non-survival of extremely malnourished individuals (Wood et al. 1992) can be the reason why protein catabolism was not observed in skeletons with generalised infections besides the diets being poorer in animal protein (Chapter 6). Malnutrition, if extreme, can lead to a rapid death without leaving any skeletal indicator or actual changes in the stable isotopes of bone collagen. Bone is the last tissue in which dietary shifts and protein catabolism is observable, as it has slow turnover (Cox and Sealy 1997, Hedges et al. 2007). However, a less severe chronic malnutrition can allow the individual to survive long enough for it to impair their immune system (e.g. Calder 1991, Scrimshaw and SanGiovanni 1997, Woodward 1998, Woodward 2001). The physiological stress and impaired immune system due to malnutrition may increase the individual's susceptibility to infectious diseases (Figure 8.1). If an individual dies soon after the infection it is not possible to observe skeletal lesions, meaning that those who have them (skeletons analysed) survived some time with the disease (Wood et al. 1992).

The osteological paradox (Wood et al. 1992) can also explain the similarity in the diets of individuals without signs of infection or physiological stress and those with syphilis. The skeletons with *caries sicca* (pathognomonic of syphilis) were in the tertiary stage of the disease (Ortner and Putschard 1985, Ortner 2003), meaning that these individuals survived a long time with the disease. Good health prior to venereal syphilis infection may prolong the individual's survival (not only to the treponeme but also to other infections trough skin

ulcers which increase the exposition to other pathogens) and increase the amount and severity of the lesions (Wood et al. 1992).

While malnutrition impairs the immune system, diseases also have an impact in an individual's diet and metabolism (Figure 8.1). Infections lead to physiological stress by increasing resting expenditure (Calder 2013), decreasing appetite (Murray and Murray 1979), and causing malabsorption (e.g. Mitra et al. 1997).

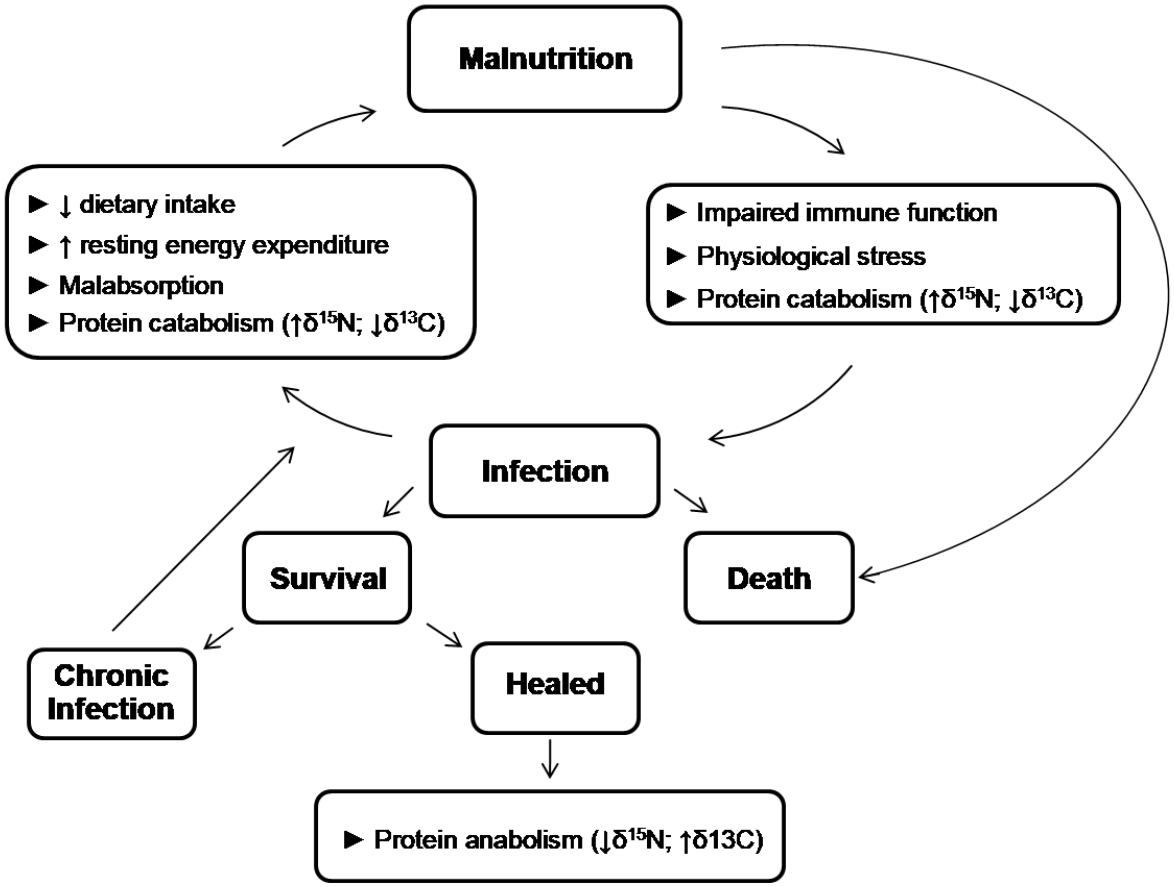


Figure 5.1. Diagram representing the nutrition-infection relationship and its possible relevance for stable isotopes analysis (e.g. Armelagos 2003, Calder 1991, Calder 2013, Deschner et al. 2012, D'Ortenzio et al. 2015, Hobson et al. 1993, Gaye-Siessegeret al. 2004, Goodman and Martin 2002, Huss-Ashmore et al. 1992, Katzenberg and Lovell 1999, Mekota et al. 2006, Mitra et al. 1997, Murray and Murray 1979, Neuberger et al. 2013, Oelbermann and Scheu 2001, Ortner and Putschard 1985, Ortner 2003, Scrimshaw and SanGiovanni 1997, Steele and Daniel 1978, Vogel et al. 2012, Wood et al., 1992, Woodward 1998, Woodward 2001, Zuckerman and Armelagos 2011).

Acute infections are usually associated with rapid death rarely affecting the skeleton but it may also stimulate new bone formation (Ortner and Putschard 1985, Ortner 2003). The different timing of these new bone formations explain why signs of protein catabolism is found in the lesions (Chapter 7, Curto et al. *in revised submission*) but not in the bones without lesions of diseased individuals (Chapter 8). Some authors (e.g. Weston 2012) suggest that during physiological stress the body cannot produce bone and therefore periosteal reactions should not be considered stress indicators. Indeed, physiological stress inhibits osteoblast differentiation and function, reducing potential bone formation in response to infection (Matzelle et al. 2012, Redlich and Smolen 2012). However, bone formation and remodelling had been demonstrated to occur during active and stressful disease states (e.g. Ragsdale and Lehmer 2012, McQueen et al. 2011, Wlodarski 1989). The repair mechanism is initiated before the cessation of the disease state or the clearance of a pathogen from the organism (Klaus 2014, Ragsdale and Lehmer 2012, Neve et al. 2011). In a diseased state, bone formation does not stop, it is active in fewer locations, where new woven bone may be formed (Lian et al. 2011). Bone formation being active at the lesion sites can explain the results observed in active lesions, described in chapter 7 (Curto et al. *in revised submission*).

Increases in $\delta^{15}N$ in tissues are frequently associated with fasting or nutritional stress (e.g. Alamaru et al. 2009, Boag et al. 2006, Fuller et al. 2005, Scrimgeour et al. 1995, Hobson et al. 1993). Nevertheless, this pattern has not always been registered (e.g. Mayor et al. 2011, McFarlane Tranquilla et al. 2010, McCue and Pollock 2008, Kempster et al. 2007, Castillo and Hatch 2007) and it is still not clear how bone is affected by the body's net loss of ^{14}N or the mechanisms underlying changes in $\delta^{15}N$ values during physiological stress. The present study brought new knowledge on this topic by comparing $\delta^{15}N$ and $\delta^{13}C$ values

between lesion and non-lesion sites within the same bone (Chapter 7, Curto et al. *in revised submission*).

An increase in $\delta^{15}N$ values and decrease in $\delta^{13}C$ values, compatible with protein catabolism (e.g. Hobson et al. 1993, Katzenberg and Lovell 1999, Steele and Daniel 1978), was observed in active lesions (Chapter 7, Curto et al. *in revised submission*) by comparing two timeframes of an individual's life (before and during or after the stressor). Not all physiological stress leads to protein catabolism (e.g. Kempster et al. 2007, Williams et al. 2007, Sears et al. 2009), which may only happen if the stress is sufficient to demand breaking down and reincorporating endogenous protein stores (Hobson et al. 1993). Since woven bone at the lesions was formed during or after the disease (e.g. Klaus, 2014, Lian et al., 2011, McQueen et al., 2011, Ragsdale and Lehmer, 2012, Wlodarski, 1989), the stressor may have also been strong enough to trigger protein catabolism.

In healed tibial periostitis $\delta^{15}N$ values decreased and $\delta^{13}C$ values increased, when compared to non-lesion sites (Chapter 7, Curto et al. *in revised submission*), similarly to what was observed in patients recovering from starvation (Mekota et al. 2006, Neuberger et al. 2013) and compatible with protein anabolism (Figure 8.1). The results of this dissertation provide additional support to the hypothesis that healed tibial periostitis, despite being associated with physiological stress, can be considered a sign of relative good health (Chapters 6 and 7, Curto et al. 2019, DeWitte 2010, Ortner 2003, Wood et al. 1992).

In Chapter 7, it was demonstrated that dietary shifts and/or metabolism during a disease prone to bone formation can be measured through stable isotope analysis. Bone formed during the disease represents a timeframe during which the individual had different metabolism and/or diet than their long term diet (non-lesion sites). This can be particularly problematic when estimating diet of non-adults, especially weaning patterns. Not only non-

adults have faster bone turnover, but they can have also have been submitted to physiological stress for long periods of time before their death (e.g. Eerkens et al. 2017). Therefore, stable isotope analysis of their remains may have ratios related with protein catabolism and not only their diet. Future studies can consider the implications of this dissertation when reconstructing diet and life style of past populations.

Among other factors (such as diet, aridity and mobility), stable isotope ratios can also represent signals from the individual's metabolism during or after diseases. This can be particularly problematic when studying non-adults as their bones have faster turnover than adults. The premature death of non-adults may be the result of diseases or physiological stress even if there are no signs in their skeleton. It has been argued that bone collagen is unreliable to estimate childhood diet, particularly weaning timings and patterns (Beaumont et al. 2018, Beaumont et al. 2015, DeWitte and Stojanowski 2015). Since physiological stress may be measured in bone collagen, particularly that formed closer to the time of death (e.g. skeletal lesions, Chapter 7, Curto et al. *in revised submission*), it can also be measured in dentine collagen. This dissertation suggests that caution must be taken when estimating childhood diet, particularly from individuals who did not survive into adulthood. Stable isotopes ratios associated with weaning patters estimations might be biased by isotopic signals from physiological stress.

This thesis reinforces the need for reconstructing a complete biological profile, not only the individual's sex, age and stature but also any lesions observable in the skeleton, their severity and stage of the disease. The health status of the individuals may have a high impact when estimating the diet of past populations.

8.2. Study limitations

The major limitation of this study is the impossibility of knowing the cause of death for the individuals analysed, alongside it not being possible to know which diseases caused most of the lesions and how long the individuals survived with the disease. The challenges of the osteological paradox (Wood et al. 1992) also hamper the data interpretation. The presence of skeletal lesions can represent an adaptation to a pathological condition (Ortner 2003) indicating that the individual survived long enough for evidence to manifest in the skeletal tissues (Wood et al. 1992). Another limitation is that, while individuals with poorer nutrition are less resistant to infectious diseases, infectious disease further lowers nutritional status (e.g. Calder 2013, Mata et al. 1971, Martorell et al. 1980, Scrimshaw and SanGiovanni 1997). The absence of skeletal lesions is ambiguous and can result from either good health, or a fast death as result of an acute disease (DeWitte and Stojanowski 2015, Siek 2013, Ortner 2003, Wood et al. 1992). A way to avoid this could be using collections of identified skeletons, individuals from who their age, sex and sometimes their medical record is known. Although these collections do exist, it is difficult to have permission to perform destructive analysis to these skeletons. Additionally, collections of identified skeletons are usually from the 20th century, meaning that there would be even more confounding factors affecting stable isotope, mainly modern medicine. Medicine could affect stable isotope ratios through direct intake of medication but it can also bias the physiological stress and the effect of diet on health.

Ideally, more samples would have been taken from each individual to estimate intra-bone and intra-skeleton variation in skeletons with and without lesions. From the individuals without lesions at least two samples from the same tibia and two samples from the same rib should have been analysed for each individual. In the skeletons with lesions it

would be desirable to collect two samples without new bone growth (distant from the lesion), at least one sample at the lesion and another one near the lesion, in both long bones and ribs. Due to funding limitations it was not possible to collect and analyse the necessary samples to estimate intra-bone and intra-skeleton variation. The small sample size did also not allow multivariable analysis as the already small sample sizes would be even smaller if divided by other factors such as excavated area, sex and age.

Another limitation of this study was the impossibility of choosing sample sizes due to constrains of time, access to the material and funding. The number of sampled skeletons available for analyses was limited by various factors, despite the large size of Tomar's collection. The difficulty of finding skeletons that would fit into the different groups, resulted in small sample sizes that are also a limitation for interpreting the results from this study. This undermined the use of power analysis and the sample size was limited by the number of skeletons that were possible to find and access to funding for stable isotope analysis independently of the smallest sample size suitable for this study. Therefore, other researchers may find different results when replicating this study. Future research could explore a multivariate approach to the analyses of isotopic data related to age, sex, excavated area, and burial type. Such an approach may reveal the different ways multiple lines of evidence can interact to predict isotope data.

There are various factors that can have an effect on stable isotope ratios observed in human tissues, such as habitat, environment, agricultural and husbandry practices, bone turnover rate and physiological stress (e.g. Metges et al. 1990, Schoeninger et al. 1997, 1998, 1999, Cerling and Harris 1999, van Klinken et al. 2000, Passey et al. 2005, Bogaard et al. 2007, Fahy et al. 2017). The origin of isotopic signals is very complex, namely the nitrogen cycle as it is also affected by different routes of nitrogen movement between trophic and

source amino acids through the metabolic nitrogen pool (these factors are reviewed by O'Connell 2017). The complexity of the nitrogen cycle is particularly important for this dissertation as $\delta^{15}N$ values gave the most significant results in this thesis. Funding limitations did not allow analysing $\delta^{34}S$ values for all samples, which would be useful to better understand if the $\delta^{15}N$ values observed were reflecting diet or physiological stress. Stable isotope analysis to bone collagen represents the average dietary and metabolic signature from the years during which the bone was formed, which can be up to the last 25 years of the individual's life (Hedges et al. 2007). Moreover, collagen reflects only protein intake and not whole diet (e.g. Froehle et al. 2010). These factors make it challenging to state if stable isotope values variation reflects diet, disease, physiological stress or something else.

The artificial division of the areas within the excavated area also restricted the data interpretation as well as information about the 1st phase of the excavation which may represent the individuals with low socio-economic status. Not knowing each individual socio-economic status and chronology was also an important limitation for this study. The large chronology of the samples (11th - 17th centuries) also limits the interpretation of the data. During this period there were large historical and social changes, alongside new food sources brought from other two continents: America and Asia. The large chronological spam may have biased the results presented in this dissertation.

8.3. Conclusion

This dissertation builds on the existing knowledge about European diet, in particular in Iberian Peninsula, and represents the first paleodietary study from this chronology in Portugal. It also provides additional knowledge on how indicators of physiological stress may help studying disease in the past and on how disease and physiological stress affects bone tissues. This thesis studied the diet-health synergy and advocates the use of new tools to study health in archaeological samples.

Individuals in Tomar had a complex diet, low in terrestrial animal protein and high in aquatic protein intake, despite its inland location (Chapter 5, Curto et al. 2018). The high intake in aquatic protein may be related to the presence of the military orders in the town and consequently stricter religious dietary restrictions. Dietary differences between sex or social status were not observed for the population of Tomar, but the quantity of aquatic protein intake is variable (Chapter 5, Curto et al. 2018).

This study suggests that the individuals with non-specific generalised infections had diets lower in animal protein than those without lesions or with only healed tibial periostitis. Diets poorer in animal protein may increase susceptibility to pathogens leading more frequently to generalised infections while richer diets might increase the survivorship and ability to heal from infectious diseases. Hence, while non-specific generalised infections are a sign of ill health and poor diet, the presence of only healed tibial periostitis may indicate a state of comparatively good overall health and diet.

$\delta^{15}N$ and $\delta^{13}C$ values were not significantly different between long term diet of skeletons without lesions or those with lesions. In contrast, significant differences were found when the lesions were grouped by healing stages (active, healed or both). $\delta^{15}N$ values

increased and $\delta^{13}C$ values decrease in active skeletal lesions, alike to what was observed in previous studies of patients suffering from starvation or wasting diseases. The difference in stable isotope ratios between non-lesion sites and those with active lesions may be related with protein catabolism. In healed lesions $\delta^{15}N$ values decreased and $\delta^{13}C$ values increased, similarly to what have been observed in patients recovering from starvation, and may be related with protein anabolism.

Stable isotope ratios are complex and can be influenced by a variety of different factors. Still, their analysis can often be relevant to better understand health and disease in the past. With the increasing knowledge and technologies available, stable isotopes analysis can become an important tool for paleopathology and the nutrition-immunity synergy, particularly in the absence of antibiotics. A better understanding of bone formation and tissue repair, allied with stable isotope analysis, can become an important tool for the study of paleopathology. Stable isotope analysis has the potential to improve the understanding of pathological processes in past populations by assessing their nitrogen balance and skeletal lesions. The biological profile and any lesions or diseases observable in the individuals selected for paleodietary studies can have an impact on a population's diet estimations. Stable isotope ratios can represent signals from the individual's metabolism during or after diseases and not only food intake. This can be particularly problematic for non-adults as their bones have faster turnover than adults and might have died of chronic diseases or physiological stress even if there are no signs in their skeleton. Therefore, stable isotopes ratios associated with weaning patters estimations might be masked by isotopic signals from physiological stress.

8.4. Future directions

Diet appears to be very diverse in Medieval Iberia and this study is the first one in Portugal from this chronology (11th to 17th century). It is important to increase the amount of Portuguese paleodietary studies in order to understand if high intake of aquatic protein was general within the country or if it is related with the influence of religious military orders (Chapter 2). A good comprehension of a population's diet is essential to better understand how diet may affect the individual's health.

Post-natal nutritional deficits have been shown to increase the risk of early death, the majority related to infectious diseases (Moore et al. 1999). Previous research on archaeological samples also showed marked isotopes differences between individuals who survived childhood and those who died (Beaumont et al. 2015, Reitsema et al. 2016). Not only breastfeeding and weaning patterns had a significant impact on morbidity and mortality (Beaumont et al. 2015), but also individuals who survived childhood had higher animal protein intake in their post-weaning diets (Reitsema et al. 2016) suggesting that diet at younger ages have a high impact on the health status of the individuals. Significant stable isotope differences were found between apparently healthy adult skeletons and those with generalised unspecific infections, suggesting different diets before the disease, which might have affected their susceptibility to pathogens (Chapter 3). These stable isotope differences in adults were more marked comparing healthy and diseased young adults. Various studies documented the immediate immunosuppressive effects of protein-energy malnutrition in infancy and childhood (e.g. Woodward 2001). However, malnutrition implications for immune function beyond childhood are still not well documented and it would be important to better understand how childhood diet, particularly weaning patterns and timing, affected the individuals understudy.

Adult stature is determined by genetics but also has an environmental determinant (e.g. Haviland 1967, Larsen 1997, Cardoso and Gomes 2009). Nutrients, total calories ingested, work and disease loads affect growth and resulting adult stature (Allen and Uauy 1994, Tanner et al. 1982, Takahashi 1984, 1994). Stature reveals developmental trends, environmental stress such as nutritional deficits and evolutionary relationships (Moore and Ross 2013), being an important indicator of relative nutritional health, as poor childhood health and nutrition reflect in adult stature. Since low stature can be an indicator of physiological stress (e.g. Haviland 1967, Morris and McAlpin 1979, Allen and Uauy 1994, Roberts and Manchester 2007, Moore and Ross 2013) only individuals with stature equal or above the mean for this population were used to estimate the general diet (Chapter 2). It would be interesting to compare both adult and childhood diet of tall and short individuals from Tomar.

Stable isotopes have been used as diagnostic tools in biomedicine (e.g. Albarede et al. 2016, Costas-Rodriguez et al. 2016, Heuser 2016, Larner 2016), however, their potential to study health in past populations is still not well understood. Even if not studied in dept yet, mineralized tissues are likely to record isotopic signals of disease. This new line of study for paleopathology has the potential to better understand the synergy between diet, disease and metabolism in the absence of antibiotics and modern medicine. Stable isotopes analysis will be even more relevant the more I know about bone turnover (e.g. Hedges et al. 2007, Fahy et al. 2017) allowing a better understanding of the meaning of stable isotope ratios in lesions at different healing stages (Chapter 4).

Zinc homeostasis and its importance in various pathologies have been multiply reviewed (e.g. Boaventura et al. 2015, Fukada et al. 2011, Maret 2013, Maret and Krzel 2007, Lichten and Cousisns 2009). Zinc and copper isotopes are expected to be significant

influenced by protein sources (Van Heghe et al. 2012, Jaouen et al. 2013b) and can improve diet estimations. Red meat, some shellfish and legumes are some of the foods with the highest zinc concentration (Otten et al. 2006) and plant-based diets increase the risk of developing zinc deficiency (King et al. 2016). Zinc deficiency is easily and rapidly produced, leading to worse response to infection (Gammoh and Rink 2017). Physiological stress can also enhance copper and zinc isotopic fractionation by accelerating their turnover (Jaouen et al. 2013a,b). Altered zinc and copper homeostasis is associated with several conditions, like for example neurodegenerative disorders (Mezzetti et al. 1998, Kozlowski et al. 2012). There is also an age and sex effect on zinc and copper stable isotopes. Stable isotope zinc ratios increase with age, while stable isotopes copper ratios decrease (Jaouen et al. 2013a). The copper/zinc ratio may be used as a mortality biomarker in elderly (Malavolta et al. 2010), particularly when associated with other stable isotopes, like calcium, for example. Sex effect on zinc and copper have been observed in both blood and bone (Albarede et al. 2011, Jaouen et al. 2012, Van Heghe et al. 2014) and can be important to understand the evolution of menarche, menstruation and menopause patterns and how they can relate to a population's general health. Research on metal stable isotope compositions in humans are still at early stages and most studies focus on young healthy living individuals (e.g. Alberede et al. 2011, Stenberg et al. 2005, Ohno et al. 2004, 2005, Van Heghe et al. 2012, Walczyk and von Blanckenburg 2002).

Calcium isotopes for example may be important in the evolution of menopause patterns. With age an imbalance between the amount of bone reabsorbed and deposited occurs (Teitelbaum 2000). Calcium isotopes fractionation have been observed in different tissues (Heuser et al. 2016) and revealed changes in bone mineral balances more rapidly than the currently used biomarkers and X-ray densiometry (Morgan et al. 2012). Iron

isotopes on the other hand can be an important measure of health, as it is expected to be unrelated to age (Jaouen et al. 2013). While low iron isotope ratios coincide with high iron status, high iron isotope ratios correspond with low iron status (Van Heghe et al. 2013). The transfer of dietary iron to the foetus is regulated in response to maternal iron status (O'Brien et al. 1999) and postnatal feeding practices and growth rate also affects iron balances (Chaparro 2008). Absorption of dietary iron in breast-fed infants undergoes developmental changes between 6 and 9 months old, enhancing their ability to adapt to low-iron diet and avoid iron deficiency (Domellöf et al. 2002). Therefore, analysing iron isotopes alongside weaning patterns of individuals who died at young ages or survived to adulthood can give us a more complete insight on weaning and health.

Tomar's osteological collection has the potential to be used for a large health project combining different tools accessible for the study of human remains. The large size of this collection (6,792 individuals), with individuals of all ages (4,991 adults, 1,801 non-adults), allows comparing groups of individuals belonging to the same population. A continuous study of this osteological collection could be very highlighting about the relationship between diet, health and socio-economic status in pre-antibiotic populations. Data from individuals who died at young ages can be compared to those who survived into adulthood but also those who show signals of physiological stress and/or diseases and the ones that do not show these indicators. Combining different stable isotopes can improve diet estimations but also improve our understanding on the relationship between diet, health and metabolism. The rapid improvement of techniques, particularly for stable isotope analysis and bone turnover can give us new tools to study health in past population, creating a new line of research in paleopathology. Tomar's collection also has the potential to be used to test modern methodologies in medieval populations. An example of this are equations for

both sex and stature estimation developed specifically for Tomar's collection (briefly mentioned in Chapter 4) that can be compared with equations developed from forensic cases. These tests will not only allow better sex and age estimation for Tomar's skeletons but also a better understanding on the efficiency of modern equations in past populations.

Bibliography

Adamson MW (2004) *Food in medieval times*. Greenwood Publishing Group.

Agarwal SC, Glencross BA (2011). *Social bioarchaeology*. John Wiley & Sons.

Alamaru A, Yam R, Shemesh A, Loya Y (2009) Trophic biology of Stylophora pistillata larvae: evidence from stable isotope analysis. *Marine Ecology Progress Series* 383:85-94.

Albarede F, Télouk P, Balter V, Bondanese VP, Albalat E, Oger P, Bonaventura P, Miossec P, Fujii T (2016) Medical applications of Cu, Zn, and S isotope effects. *Metallomics* 8(10):1056-70.

Albarede F, Telouk P, Lamboux A, Jaouen K, Balter V (2011) Isotopic evidence of unaccounted for Fe and Cu erythropoietic pathways. *Metallomics* 3(9):926-33.

Alexander MM, Gerrard CM, Gutiérrez A, Millard AR (2015) Diet, society, and economy in late medieval Spain: Stable isotope evidence from Muslims and Christians from Gandía, Valencia. *American Journal of Physical Anthropology* 156(2):263-273.

Allen VG (1984) Influence of dietary aluminum on nutrient utilization in ruminants. *Journal of Animal Science* 59(3):836-44.

Allen LH, Uauy (1994) Guidelines for the study of mechanisms involved in the prevention or reversal of linear growth retardation in developing countries. *European Journal of Clinical Nutrition* 48(Suppl 1):S212–S216.

Ambrose SH, DeNiro MJ (1986) Reconstruction of African human diet using bone collagen carbon and nitrogen isotope ratios. *Nature* 319:321–324.

Ambrose SH, Norr L (1993) Experimental evidence for the relationship of the carbon isotope ratios of whole diet and dietary protein to those of bone collagen and carbonate. In *Prehistoric human bone* (pp. 1-37). Springer, Berlin, Heidelberg.

Armelagos GJ (2003) Bioarchaeology as anthropology. *Archaeological Papers of the American Anthropological Association* 13(1):27–40.

Aufderheide AC, Rodriguez-Martin C (1998) *Human paleopathology*. Cambridge University Press.

Ault WU, Kulp JL (1959) Isotopic geochemistry of sulphur. *Geochimica et Cosmochimica Acta* 16(4):201-35.

Bab IA, Sela JJ (2012) *Cellular and molecular aspects of bone repair. Principles of bone regeneration,* Springer, Boston, MA.

Balasse M, Bocherens H, Mariotti A 1999 Intra-bone variability of collagen and apatite isotopic composition used as evidence of a change of diet. *Journal of Archaeological Science* 26(6):593-8.

Barber M (2012) *The new knighthood: A history of the order of the temple* Cambridge University Press.

Barber M, Bate K (2002) *The Templars: Selected sources*. Manchester University Press.

Barker DJ, Osmond C (1986) Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *The Lancet* 327(8489):1077-81.

Barker DJ, Osmond C, Winter PD, Margetts B, Simmonds SJ (1989) Weight in infancy and death from ischaemic heart disease. *The Lancet* 334(8663):577-80.

Barnes C, Jennings S (2007) Effect of temperature, ration, body size and age on sulphur isotope fractionation in fish. *Rapid Communications in Mass Spectrometry* 21(8):1461-7.

Bayliss A, Popescu ES, Beavan-Athfield N, Ramsey CB, Cook GT, Locker A (2004) The potential significance of dietary offsets for the interpretation of radiocarbon dates: an archaeologically significant example from medieval Norwich. *Journal of Archaeological Science* 31(5):563–575.

Beaumont J, Geber J, Powers N, Wilson A, Lee-Thorp J, Montgomery J (2013) Victims and survivors: stable isotopes used to identify migrants from the Great Irish Famine to 19th century London. *American Journal of Physical Anthropology*, 150(1):87-98.

Beaumont J, Montgomery J (2016) The great Irish famine: Identifying starvation in the tissues of victims using stable isotope analysis of bone and incremental dentine collagen. *PLoS One*, 11(8), e0160065.

Beaumont J, Montgomery J, Buckberry J, Jay M (2015) Infant mortality and isotopic complexity: New approaches to stress, maternal health, and weaning. *American Journal of Physical Anthropology* 157(3):441–457.

Beavan-Athfield N, Green RC, Craig J, McFadgen B, Bickler S (2008) Influence of marine sources on ^{14}C ages: isotopic data from Watom Island, Papua New Guinea inhumations and pig teeth in light of new dietary standards. *Journal of the Royal Society of New Zealand* 38:1–23.

Behrman JR (2009) Early life nutrition and subsequent education, health, wage, and intergenerational effects. *Health and growth* 6:167-83.

Binski P (1996) In O'Connor M (Ed.) *Medieval death: Ritual and representation*. London: British Museum Press.

Biolo G, Toigo G, Ciocchi B, Situlin R, Iscra F, Gullo A, Guarnieri G (1997) Metabolic response to injury and sepsis: changes in protein metabolism. *Nutrition* 13(9):52-7.

Blackwell DL, Hayward MD, Crimmins EM (2001) Does childhood health affect chronic morbidity in later life? *Social science & medicine52*(8):1269-84.

Boag B, Neilson R, Scrimgeour CM (2006) The effect of starvation on the planarian Arthurdendyus triangulatus (Tricladida: Terricola) as measured by stable isotopes. *Biology and Fertility of Soils*, 43, 267-270.

Bocherens H, Drucker D (2003) Trophic level isotopic enrichment of carbon and nitrogen in bone collagen: case studies from recent and ancient terrestrial ecosystems. *International Journal of Osteoarchaeology* 13(1-2):46-53.

Bogaard A, Heaton TH, Poulton P, Merbach I (2007) The impact of manuring on nitrogen isotope ratios in cereals: Archaeological implications for reconstruction of diet and crop management practices. *Journal of Archaeological Science* 34(3):335-343.

Bogin B, Silva MIV, Rios L (2007) Life history trade-offs in human growth: Adaptation or pathology?. *American Journal of Human Biology*, 19(5):631–642.

Bol R, Pflieger C (2002) Stable isotope (^{13}C, ^{15}N and ^{34}S) analysis of the hair of modern humans and their domestic animals. *Rapid Communications in Mass Spectrometry* 16(23):2195-200.

Boldsen J (1984) A statistical evaluation of the basis for predicting stature from lengths of long bones in european populations. *American Journal of Physical Anthropology* 65(3):305-311.

Bonaventura P, Benedetti G, Albarède F, Miossec P (2015) Zinc and its role in immunity and inflammation. *Autoimmunity reviews* 14(4):277-285.

Bose A. 2018. Let Us Talk about Stunting. *Journal of Tropical Pediatrics* 64(3):174–175

Böttcher ME, Oelschläger B, Höpner T, Brumsack, HJ, Rullkötter J (1998).Sulfate reduction related to the early diagenetic degradation of organic matter and “black spot” formation in tidal sandflats of the German Wadden Sea (southern North Sea): stable isotope (13C, 34S, 18O) and other geochemical results. *Organic Geochemistry* 29(5):1517-1530.

Böttcher ME, Brumsack HJ, Dürselen CD (2007) The isotopic composition of modern seawater sulfate: I. Coastal waters with special regard to the North Sea. *Journal of Marine Systems* 67(1-2):73-82.

Brady AL, White CD, Longstaffe FJ, Southam G (2008) Investigating intra-bone isotopic variations in bioapatite using IR-laser ablation and micromilling: Implications for identifying diagenesis?. *Palaeogeography, Palaeoclimatology, Palaeoecology* 266(3-4):190-9.

Breuillé D, Rose F, Arnal M, Melin C, Obled C (1994) Sepsis modifies the contribution of different organs to whole-body protein synthesis in rats. *Clinical Science* 86(6):663-9.

Brickley M, Ives R (2008) The Bioarchaeology of Metabolic Disease. Academic Press.

Bronckers AL, Goei W, Luo G, Karsenty G, D'souza RN, Lyaruu DM, Burger EH (1996) DNA fragmentation during bone formation in neonatal rodents assessed by transferase-mediated end labeling. *Journal of Bone and Mineral Research* 11(9):1281-91.

Brooks S, Suchey JM (1990) Skeletal age determination based on the os pubis: A comparison of the acsádi-nemeskéri and suchey-brooks methods. *Human Evolution* 5(3):227–238.

Brown TA, Nelson DE, Vogel JS, Southon JR (1988) Improved collagen extraction by modified longin method. *Radiocarbon* 30(2):171–177.

Buikstra JE, Ubelaker DH (1994) *Standards for data collection from human skeletal remains: Proceedings of a seminar at the Field Museum of Natural History*. Arkansas Archaeology Research Series, 44. Fayetteville Arkansas Archaeological Survey.

Burr DB, Milgrom C, Fyhrie D, Forwood M, Nyska M, Finestone A, Hoshaw S, Saiag E, Simkin A (1996) In vivo measurement of human tibial strains during vigorous activity. *Bone* 18(5):405-10.

Bush HM (1989) *The Recognition of Physiological Stress in Human Skeletal Material: A Critique of Method and Theory with Specific Reference to the Vertebral Column*. Ph.D. Dissertation, University of Sheffield.

Calder PC (2013) Feeding the immune system. *Proceedings of the Nutrition Society* 72(3):299–309.

Calder PC, Jackson AA (2000) Malnutrition, infection and immune function. *Nutrition research reviews* 13(1):3–29.

Caplan A (1987) The cellular and molecular embryology of bone formation. *Journal of Bone and Mineral Research*, 5:117-183.

Cardoso H, Gomes J (2009) Trends in adult stature of peoples who inhabited the modern Portuguese territory from the mesolithic to the late 20th century. *International Journal of Osteoarchaeology* 19(6):711-725.

Castillo LP, Hatch KA (2007) Fasting increases δ^{15}N-values in the uric acid of Anolis carolinensis and Uta stansburiana as measured by non-destructive sampling. *Rapid Communications in Mass Spectrometry*, 21:4125-4128.

Cerling TE, Harris JM (1999) Carbon isotope fractionation between diet and bioapatite in ungulate mammals and implications for ecological and paleoecological studies. Oecologia 120(3):347-63.

Champe P, Harvey R, Ferrier D (2008) *Complex lipid metabolism*. Lippincott's illustrated reviews: Biochemistry, Lippincott Williams and Wilkins, Philadelphia.

Chappard D, Baslé MF, Legrand E, Audran M (2011) New laboratory tools in the assessment of bone quality. *Osteoporosis International* 22(8):2225-40.

Chaparro CM (2008) Setting the stage for child health and development: prevention of iron deficiency in early infancy. *The Journal of nutrition* 138(12):2529-33.

Chapman VJ, Chapman DJ (1980) *Seaweeds and their uses*. Chapmanand Hall, London.

Chandra RK (1997) Nutrition and the immune system: an introduction. *The American journal of clinical nutrition* 66(2):460S-3S.

Cherel Y, Attaix D, Rosolowska-Huszcz D, Belkhou R, Robin JP, Arnal M, Le Maho Y (1991) Whole-body and tissue protein synthesis during brief and prolonged fasting in the rat. *Clinical science* 81(5):611-9.

Chisholm BS, Nelson DE,Schwarcz HP (1982) Stable-carbon isotope ratios as a measure of marine versus terrestrial protein in ancient diets. *Science* (New York, N.Y.), 216(4550): 1131-1132.

Stout RW, Cho DY, Gaunt SD, Taylor HW, Baker DG 2001 Transcutaneous blood gas monitoring in the rat. *Comparative medicine* 51(6):524-33.

Conde MAS (1996) *Tomar Medieval. O espaço e os homens*, Cascais.

Cooke GS, Hill AV (2001) Genetics of susceptibitlity to human infectious disease. *Nature Reviews Genetics* 2(12):967.

Cortecci G, Dinelli E, Bencini A, Adorni-Braccesi A, La Ruffa G (2002) Natural and anthropogenic SO4 sources in the Arno river catchment, northern Tuscany, Italy: a chemical and isotopic reconnaissance. *Applied Geochemistry* 17(2):79-92.

Costas-Rodriguez M, Delanghe J, Vanhaecke F (2016) High-precision isotopic analysis of essential mineral elements in biomedicine: natural isotope ratio variations as potential diagnostic and/or prognostic markers. *TrAC Trends in Analytical Chemistry* 76:182-93.

Cox G, Sealy J (1997) Investigating identity and life histories: Isotopic analysis and historical documentation of slave skeletons found on the cape town foreshore, south africa. *International Journal of Historical Archaeology* 1(3):207-224.

Curet LA, Pestle WJ (2010) Identifying high-status foods in the archeological record. Journal of *Anthropological Archaeology* 29(4):413-431.

Curto A, Maurer AF, Barrocas-Dias C, Mahoney P, Fernandes T, Fahy GE (2018) Did military orders influence the general population diet? Stable isotope analysis from Medieval Tomar, Portugal. *Archaeological and Anthropological Sciences* 1–13.

Curto A, Mahoney P, Maurer AF, Barrocas-Dias C, Fernandes T, Fahy GE (2019) Diet and disease in Tomar, Portugal: Comparing stable carbon and nitrogen isotope ratios between skeletons with and without signs of infectious disease. *Journal of Archaeological Science* 105:59-69.

Curto A, Mahoney P, Maurer A-F, Barrocas-Dias C, Fernandes T, Fahy G.Effect of different healing stages on stable isotope ratios in skeletal lesions. In revised submission (*American Journal of Physical Anthropology*).

Daniell C (1998) In Dawsonera (Ed.), *Death and burial in medieval England, 1066-1550*. London: Routledge.

De Boer H, Van der Merwe AE, Hammer S, Steyn M, Maat G (2015) Assessing post-traumatic time interval in human dry bone. *International Journal of Osteoarchaeology* 25(1):98-109.

Deitch EA (1990) The role of intestinal barrier failure and bacterial translocation in the development of systemic infection and multiple organ failure. *Archives of Surgery* 125(3):403–404.

DeNiro MJ (1985) Postmortem preservation and alteration of in vivo bone collagen isotope ratios in relation to palaeodietary reconstruction. *Nature* 317(6040):806-809.

DeNiro MJ, Epstein, S (1978) Influence of diet on the distribution of carbon isotopes in animals. *Geochimica et cosmochimica acta* 42(5):495-506.

DeNiro MJ, Epstein S (1976) You are what you eat (plus a few‰): the carbon isotope cycle in food chains. *Geological Society of America* 6:834.

DeNiro MJ, Epstein S (1981) Influence of diet on the distribution of nitrogen isotopes in animals. GeochimicaEtCosmochimicaActa 45(3): 341-351.

Deschner T, Fuller BT, Oelze VM, Boesch C, Hublin J, Mundry R, Hohmann G (2012) Identification of energy consumption and nutritional stress by isotopic and elemental analysis of urine in bonobos (*Pan paniscus*). *Rapid Communications in Mass Spectrometry* 26(1):69–77.

DeWitte SN (2010) Sex differentials in frailty in medieval England. *American Journal of Physical Anthropology* 143(2):285-297.

DeWitte SN, Hughes-Morey G (2012) Stature and frailty during the Black Death: the effect of stature on risks of epidemic mortality in London, AD 1348–1350. *Journal of Archaeological Science* 39(5):1412-1419.

DeWitte SN, Stojanowski CM (2015) The osteological paradox 20 years later: Past perspectives, future directions. *Journal of Archaeological Research* 23(4):397–450.

DeWitte SN, Wood JW (2008) Selectivity of Black Death mortality with respect to pre-existing health. *Proceedings of the National Academy of Sciences* 105(5):1436–1441.

Doak CM, Adair LS, Bentley M, Monteiro C, Popkin BM (2005) The dual burden household and the nutrition transition paradox. *International journal of obesity* 29(1):129.

Domellöf M, Lönnerdal B, Abrams SA, Hernell O (2002) Iron absorption in breast-fed infants: effects of age, iron status, iron supplements, and complementary foods. *The American journal of clinical nutrition* 76(1):198-204.

D'Ortenzio L, Brickley M, Schwarcz H, Prowse T (2015) You are not what you eat during physiological stress: Isotopic evaluation of human hair. *American Journal of Physical Anthropology* 157(3):374–388.

Dufour E, Bocherens H,Mariotti A (1999) Palaeodietary implications of isotopic variability in Eurasian lacustrine fish. *Journal of Archaeological Science* 26(6):617-627.

Dunn RR, Davies TJ, Harris NC, Gavin MC (2010) Global drivers of human pathogen richness and prevalence. Proceedings of the Royal Society B: Biological Sciences 277: 2587–2595.

Eerkens JW, Hull B, Goodman J, Evoy A, Kapp JD, Hussain S, Green RE (2017) Stable C and N isotope analysis of hair suggest undernourishment as a factor in the death of a mummified girl from late 19th century San Francisco, CA. *PloS One* 12(9):e0184921.

Englert JA, Rogers AJ (2016) Metabolism, metabolomics, and nutritional support of patients with sepsis. *Clinics in chest medicine* 37(2):321-31.

Eshed, V., Gopher, A., Pinhasi, R., & Hershkovitz, I. (2010). Paleopathology and the origin of agriculture in the Levant. *American Journal of Physical Anthropology*, 143(1), 121–133.

Evans FD, Critchley AT (2014) Seaweeds for animal production use. *Journal of applied phycology* 26(2):891-899.

Exton MS (1997) Infection-induced anorexia: Active host defence strategy. *Appetite* 29(3):369-383.

Facchini F, Rastelli E, Brasili P (2004) Cribraorbitalia and cribracranii in roman skeletal remains from the Ravenna area and Rimini (I–IV century AD). *International Journal of Osteoarchaeology* 14(2):126-136.

Fahy G, Deter C, Pitfield R, Miszkiewicz J, Mahoney P (2017) Bone deep: Variation in stable isotope ratios and histomorphometric measurements of bone remodelling within adult humans. *Journal of Archaeological Science* 87:10-16.

Field A. (2009) Discovering statistics using SPSS. 3 ed. London: SAGE publications Ltd.

França JA (1994) *Cidades e Vilas de Portugal: Tomar*, Vol. 18. Lisboa, Editorial Presença.

Fry B. (2002) Conservative mixing of stable isotopes across estuarine salinity gradients: a conceptual framework for monitoring watershed influences on downstream fisheries production. *Estuaries and Coasts* 25(2):264-271.

Froehle AW, Kellner CM, Schoeninger MJ (2010) FOCUS: effect of diet and protein source on carbon stable isotope ratios in collagen: follow up to. *Journal of Archaeological Science* 37(10):2662-70.

Fukada T, Yamasaki S, Nishida K, Murakami M, Hirano T (2011) Zinc homeostasis and signaling in health and diseases. *Journal of Biological Inorganic Chemistry* 16(7):1123-34.

Fuller BT, Fuller JL, Sage NE, Harris DA, O'Connell TC, Hedges RE (2004). Nitrogen balance and $\delta^{15}N$: Why you're not what you eat during pregnancy. *Rapid Communications in Mass Spectrometry* 18(23):2889-2896.

Fuller BT, Fuller JL, Sage NE, Harris DA, O'Connell TC, Hedges RE (2005) Nitrogen balance and $\delta^{15}N$: Why you're not what you eat during nutritional stress. *Rapid Communications in Mass Spectrometry*, 19(18), 2497–2506.

Gabet Y, Muller R, Regev E, Sela J, Shteyer A, Salisbury K, Chorev M, Bab I (2004) Osteogenic growth peptide modulates fracture callus structural and mechanical properties. *Bone* 35:65–73.

Gammoh NZ, Rink L (2017) Zinc in infection and inflammation. *Nutrients* 9(6):624.

Garlick PJ, Millward DJ, James WP, Waterlow JC (1975) The effect of protein deprivation and starvation on the rate of protein synthesis in tissues of the rat. Biochimica *et Biophysica Acta (BBA)-Nucleic Acids and Protein Synthesis* 414(1):71-84.

Gaye-Siessegger J, Focken U, Abel H, Becker K (2004) Individual protein balance strongly influences $\delta^{15}N$ and $\delta^{13}C$ values in Nile tilapia, *Oreochromis niloticus*. *Naturwissenschaften* 91(2):90–93.

Giesemann A, Jäger HJ, Norman AL, Krouse HR, Brand WA (1994) Online sulfur-isotope determination using an elemental analyzer coupled to a mass spectrometer. *Analytical Chemistry* 66(18):2816-9.

Gonçalves I (2004) *Entre a abundância e a miséria: as práticas alimentares da Idade Média portuguesa*. Estudos medievais. Quotidiano Medieval: imaginário, representação e práticas. Lisboa: Livros Horizonte.

González-Martín I, Pérez CG, Méndez JH, González CS (2001) Differentiation of dietary regimene of Iberian swine by means of isotopic analysis of carbon and sulphur in hepatic tissue. *Meat Science* 58(1):25-30.

Goodman AH, Martin DL (2002) *Reconstructing health profiles from skeletal remains*. The Backbone of History.Cambridge University Press, Cambridge, UK.

Goodman AH, Armelagos GJ (1989) Infant and childhood morbidity and mortality risks in archaeological populations. *World Archaeology*, 21(2), 225–243.

Goodman AH, Martin DL, Armelagos GJ, Clark G (1984) Indications of stress from bones and teeth. In: Cohen MN, Armelagos, GJ (eds.) *Paleopathology at the origins of agriculture*. Orlando: Academic Press. p 13–49.

Grau-Sologestoa I (2017) Socio-economic status and religious identity in medieval Iberia: The zooarchaeological evidence. *Environmental Archaeology* 22(2):189-199.

Graves, C. (1989). Social space in the English medieval parish-church. *Economy and Society* 18(3):297-322.

Grossman CM, Sappington TS, Burrows BA, Lavietes PH, Peters JP (1945) Nitrogen metabolism in acute infections. *The Journal of clinical investigation*, 24(4), 523-531.

Grumett D,Muers R (2010) *Theology on the menu: asceticism, meat and Christian diet*. Routledge.

Habran S, Debier C, Crocker DE, Houser DS, Lepoint G, Bouquegneau J-M, Das K (2010) Assessment of gestation, lactation and fasting on stable isotope ratios in northern elephant seals (Mirounga angustirostris). *Marine Mammal Science* 26:880-895.

Hasselgren PO (2000) Catabolic response to stress and injury: implications for regulation. *World journal of surgery* 24(12):1452-9.

Hatch KA, Crawford MA, Kunz AW, Thomsen SR, Eggett DL, Nelson ST, Roeder BL (2006) An objective means of diagnosing anorexia nervosa and bulimia nervosa using $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios in hair. *Rapid Communications in Mass Spectrometry* 20(22):3367-3373.

Haviland WA (1967) Stature at Tikal, Guatemala: Implications for ancient Maya demography and social organization. *American Antiquity* 316-325.

Heaton THE (1987) The $^{15}N/^{14}N$ ratios of plants in South Africa and Namibia: relationship to climate and coastal/saline environments. *Oecologia* 74, 236–246.

Heaton THE, Vogel JC, von la Chevallerie G, Collett G (1986) Climatic influence on the isotopic composition of bone nitrogen. *Nature* 322, 822–823.

Hedges RE, Clement JG, Thomas CDL, O'Connell TC (2007) Collagen turnover in the adult femoral mid-shaft: Modeled from anthropogenic radiocarbon tracer measurements. *American Journal of Physical Anthropology* 133(2):808–816.

Hedges RE, Reynard LM (2007) Nitrogen isotopes and the trophic level of humans in archaeology. *Journal of Archaeological Science* 34(8):1240-1251.

Heuser A (2016) Biomedical application of Ca stable isotopes. In *Calcium stable isotope geochemistry* (pp. 247-260). Springer, Berlin, Heidelberg.

Heuser A, Eisenhauer A, Scholz-Ahrens KE, Schrezenmeir J (2016) Biological fractionation of stable Ca isotopes in Göttingen minipigs as a physiological model for Ca homeostasis in humans. *Isotopes in environmental and health studies* 52(6):633-48.

Hillson S (1996) Dental anthropology. Cambridge University Press.

Hobson KA (1999) Tracing origins and migration of wildlife using stable isotopes: a review. *Oecologia* 120(3):314-326.

Hobson KA, Alisauskas RT, Clark RG (1993) Stable-nitrogen isotope enrichment in avian tissues due to fasting and nutritional stress: Implications for isotopic analyses of diet. *Condor* 388–394.

Hobson KA, Clark RG (1992) Assessing avian diets using stable isotopes I: Turnover of ^{13}C in tissues. *Condor* 181-188.

Hoefs J (2006) *Stable Isotope Geochemistry*. Springer, Berlin.

Hoekstra PF, Dehn LA, George JC, Solomon KR, Muir DC, O'Hara TM (2002) Trophic ecology of bowhead whales (Balaena mysticetus) compared with that of other arctic marine biota as interpreted from carbon-, nitrogen-, and sulfur-isotope signatures. Canadian Journal of Zoology 80(2):223-31.

Holder S (2013) *Interpreting Diet And Nutritional Stress In Napoleon's Grand Army Using Stable Carbon And Nitrogen Isotope Analysis*. Electronic Theses and Dissertations. 2752.

Holder S, Dupras TL, Jankauskas R, Williams L, Schultz J (2017) Reconstructing diet in Napoleon's Grand Army using stable carbon and nitrogen isotope analysis. *American journal of physical anthropology* 163(1):53-63.

Hollinger JO (2005) Bone dynamics. In *Bone Regeneration and Repair* (pp. 1-19). Humana Press.

Howard JE (1945) Protein metabolism during convalescence after trauma: Recent studies. *Archives of Surgery* 50(3):166-170.

Huss-Ashmore R, Schall J, Hediger M (1992) *Health and lifestyle change*. UPenn Museum of Archaeology.

Ingle B, Hay S, Bottjer H, Eastell R (1999) Changes in bone mass and bone turnover following distal forearm fracture. *Osteoporosis International* 10(5):399-407.

Ivanov MV (1983) The sulfur cycle in continental reservoirs. In: Ivanov MV, Freney JR (Eds.) *The Global Biogeochemical Sulfur Cycle-Scope*. Scientific Committee on Problems of the Environment pp. 297–356.

Jantz RL, Owsley DW (1984) Long bone growth variation among Arikara skeletal populations. American Journal of Physical Anthropology 63(1):13-20.

Jaouen K, Balter V, Herrscher E, Lamboux A, Telouk P, Albarède F (2012) Fe and Cu stable isotopes in archeological human bones and their relationship to sex. *American journal of physical anthropology* 148(3):334-40.

Jaouen K, Gibert M, Lamboux A, Telouk P, Fourel F, Albarede F, Alekseev AN, Crubézy E, Balter V (2013a) Is aging recorded in blood Cu and Zn isotope compositions?. *Metallomics* 5(8):1016-24.

Jaouen K, Pons ML, Balter V (2013b) Iron, copper and zinc isotopic fractionation up mammal trophic chains. *Earth and Planetary Science Letters* 374:164-72.

Jim S, Jones V, Ambrose SH, Evershed RP (2006) Quantifying dietary macronutrient sources of carbon for bone collagen biosynthesis using natural abundance stable carbon isotope analysis. *British Journal of Nutrition* 95(6):1055-1062.

Jørkov MLS, Heinemeier J, Lynnerup N (2007) Evaluating bone collagen extraction methods for stable isotope analysis in dietary studies. *Journal of Archaeological Science* 34(11):1824-1829.

Katsimbri P (2017) The biology of normal bone remodelling. European Journal of *Cancer Care* 26(6):e12740.

Katzenberg MA, Lovell NC (1999) Stable isotope variation in pathological bone. *International Journal of Osteoarchaeology* 9(5):316-324.

Katzenberg MA, Krouse HR (1989) Application of stable isotope variation in human tissues to problems in identification. *Canadian Society of Forensic Science Journal* 22(1):7-19.

Kempster B, Zanette L, Longstaffe FJ, MacDougall-Shackleton SA, Wingfield JC, Clinchy M (2007) Do stable isotopes reflect nutritional stress? Results from a laboratory experiment on song sparrows. *Oecologia* 151(3):365-71.

Keusch GT, Farthing MJ (1986) Nutrition and infection. *Annual review of nutrition* 6(1):131–154.

King JC, Brown KH, Gibson RS, Krebs NF, Lol NM, Siekmann JH, Raiten DJ (2016) Biomarkers of Nutrition for Development (BOND)—Zinc Review–5. *The Journal of nutrition* 146:858S–885S.

Kiple KF (1993) The Treponematoses. In *The Cambridge World History of Human Disease*. Kiple KF (ed.), 1053–1055. Cambridge: Cambridge University Press.

Kjellström A, Storå J, Possnert G, Linderholm A (2009) Dietary patterns and social structures in medieval Sigtuna, Sweden, as reflected in stable isotope values in human skeletal remains. *Journal of Archaeological Science* 36(12):2689-2699.

Klaus HD (2014) Frontiers in the bioarchaeology of stress and disease: Cross-disciplinary perspectives from pathophysiology, human biology, and epidemiology. *American Journal of Physical Anthropology* 155(2):294-308. Kozlowski H, Luczkowski M, Remelli M, Valensin D (2012) Copper, zinc and iron in neurodegenerative diseases (Alzheimer's, Parkinson's and prion diseases). *Coordination Chemistry Reviews* 256(19-20):2129-41.

Krouse HR, Herbert HK (1988) Sulphur and carbon isotope studies of food webs. Diet and subsistence: *Current archaeological perspectives* 315-22.

Kuvibidila S, Yu L, Ode D, Warrier RP (1993) The immune response in protein-energy malnutrition and single nutrient deficiencies. In *Nutrition and immunology* 121–155. Springer US.

Kuzawa CW (2007) Developmental origins of life history: growth, productivity, and reproduction. *American Journal of Human Biology* 19(5):654-61.

Kwak TJ, Zedler JB (1997) Food web analysis of southern California coastal wetlands using multiple stable isotopes. *Oecologia* 110(2):262-77.

Lampl M (2012) Perspectives on modelling human growth: Mathematical models and growth biology. *Annals of human biology* 39(5):342-51.

Larner F. (2016) Can I use high precision metal isotope analysis to improve our understanding of cancer?. *Analytical and bioanalytical chemistry* 408(2):345-349.

Larsen C (1997) Bioarchaeology: Interpreting behavior from the human skeleton. New York: Cambridge University Press.

Larson TE, Longstaffe FJ (2007) Deciphering seasonal variations in the diet and drinking water of modern White-Tailed deer by in situ analysis of osteons in cortical bone. *Journal of Geophysical Research: Biogeosciences* 112(G4).

Leach BF, Quinn CJ, Lyon GL (1996) A stochastic approach to the reconstruction of prehistoric human diet in the pacific region from bone isotope signatures. *Tuhinga* 1-54.

Lee TN, Buck CL, Barnes BM, O'Brien DM (2012) A test of alternative models for increased tissue nitrogen isotope ratios during fasting in hibernating arctic ground squirrels. *Journal of Experimental Biology* 215(19):3354-61.

Lee WH, Woodward BD (1996) The CD4/CD8 ratio in the blood does not reflect the response of this index in secondary lymphoid organs of weanling mice e in models of protein–energy malnutrition known to depress thymus-dependent immunity. *The Journal of nutrition*, 126(4), 849–859.

Li XZ, Jee SS (2005) Integrated bone tissue anatomy and physiology, Chapter 2 In: Deng H, Liu Y, Guo C, Chen D. (eds.). *Current Topics in Bone Biology* 11-56. World Scientific.

Lian JB, Gravallese EM, Stein GS (2011) Osteoblasts and their signaling pathways: New frontiers for linkage to the immune system. In *Osteoimmunology* (pp. 101-140) Elsevier.

Lichten LA, Cousins RJ (2009) Mammalian zinc transporters: nutritional and physiologic regulation. *Annual review of nutrition* 29:153-176.

Linderholm A, Jonson CH, Svensk O, Liden K (2008). Diet and status in Birka: stable isotopes and grave goods compared. Antiquity 82:446–461.

Linderholm A, Kjellström A (2011) Stable isotope analysis of a medieval skeletal sample indicative of systemic disease from Sigtuna Sweden. *Journal of Archaeological Science* 38(4):925–933.

Lohuis TD, Harlow HJ, Beck TD (2007) Hibernating black bears (Ursus americanus) experience skeletal muscle protein balance during winter anorexia. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 147(1):20-8.

Long C (1977) Energy balance and carbohydrate metabolism in infection and sepsis. *The American Journal of Clinical Nutrition* 30(8):1301-1310.

Long C, Birkhahn R, Geiger J, Blakemore W (1981) Contribution of skeletal muscle protein in elevated rates of whole body protein catabolism in trauma patients. *The American Journal of Clinical Nutrition* 34(6):1087-1093.

Longin R (1971) New method of collagen extraction for radiocarbon dating. *Nature* 230 (5291):241–242.

Lovejoy CO, Meindl RS, Pryzbeck TR, Mensforth RP (1985) Chronological metamorphosis of the auricular surface of the ilium: A new method for the determination of adult skeletal age at death. *American Journal of Physical Anthropology* 68(1):15–28.

Lubritto C, Sirignano C, Ricci P, Passariello I, Castillo JQ (2013) Radiocarbon chronology and paleodiet studies on the medieval rural site of Zaballa (Spain): Preliminary insights into the social archaeology of the site. *Radiocarbon* 55(3):1222-1232.

Malavolta M, Giacconi R, Piacenza F, Santarelli L, Cipriano C, Costarelli L, Tesei S, Pierpaoli S, Basso A, Galeazzi R, Lattanzio F (2010) Plasma copper/zinc ratio: an inflammatory/nutritional biomarker as predictor of all-cause mortality in elderly population. *Biogerontology* 11(3):309-19.

Mann HB, Whitney DR (1947) On a test of whether one of two random variables is stochastically larger than the other. *The annals of mathematical statistics* 50-60.

Maret W (2013) Zinc biochemistry: from a single zinc enzyme to a key element of life. Advances in nutrition 4(1):82-91.

Maret W, Krężel A (2007) Cellular zinc and redox buffering capacity of metallothionein/thionein in health and disease. *Molecular medicine* 13(7-8):371.

Martínez del Rio C, Wolf BO (2005) Mass-balance models for animal isotopic ecology. In *Physiological and Ecological Adaptations to Feeding in Vertebrates* (ed. JM Starck, T Wang), pp. 141-174. Enfield, NH: Science Publishers.

Macko SA, Estep MLF, Engel MH, Hare P (1986) Kinetic fractionation of stable nitrogen isotopes during amino acid transamination. *Geochimica Et Cosmochimica Acta* 50(10):2143-2146.

Macko SA, Fogel ML, Hare PE, Hoering T (1987) Isotopic fractionation of nitrogen and carbon in the synthesis of amino acids by microorganisms. *Chemical Geology* 65(1):79-92.

Martin DL, Armelagos GJ (1979) Morphometrics of compact bone: An example from sudanese nubia. *American Journal of Physical Anthropology* 51(4):571-577.

Martorell R, Yarbrough C, Yarbrough S, Klein RE (1980) The impact of ordinary illnesses on the dietary intakes of malnourished children. *The American Journal of Clinical Nutrition* 33(2):345–350.

Marzona L, Pavolini B (2009) Play and players in bone fracture healing match. *Clinical Cases in Mineral and Bone Metabolism* 6(2):159-162.

Mata LJ, Urrutia JJ, Lechtig A (1971) Infection and nutrition of children of a low socio-economic rural community. *American Journal of Clinical Nutrition* 24(2):249–259.

Matzelle MM, Gallant MA, Condon KW, Walsh NC, Manning CA, Stein GS, Lian JB, Burr DB, Gravallese EM (2012) Resolution of inflammation induces osteoblast function and regulates the wnt signaling pathway. *Arthritis and Rheumatism* 64(5):1540-1550.

Mayor, D. J., Cook, K., Thornton, B., Walsham, P., Witte, U. F. M., Zuur, A. F. and Anderson, T. R. (2011). Absorption efficiencies and basal turnover of C, N and fatty acids in a marine Calanoid copepod. *Functional Ecology*, 25, 509-518.

McCue MD, Pollock ED (2008) Stable isotopes may provide evidence for starvation in reptiles. Rapid Commun. *Rapid Communications in Mass Spectrometry* 22:2307-2314.

McCutchan Jr JH, Lewis Jr WM, Kendall C, McGrath CC. 2003. Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. *Oikos* 102(2):378-90.

McFarlane Tranquilla LM, Hedd A, Burke C, Montevecchi WA, Regular PM, Robertson GJ, Stapleton LA, Wilhelm SI, Fifield DA, Buren AD (2010) High Arctic sea ice conditions influence marine birds wintering in Low Arctic regions. *Estuarine, Coastal and Shelf Science* 89:97-106.

McHugh DJ (2003) *A guide to the seaweed industry. Rome: Food and Agriculture.* Organization of the United Nations.

McQueen P, Ghaffar S, Guo Y, Rubin EM, Zi X, Hoang BH (2011) The wnt signaling pathway: Implications for therapy in osteosarcoma. *Expert Review of Anticancer Therapy* 11(8):1223-1232.

Mekota A, Grupe G, Ufer S, Cuntz U (2006) Serial analysis of stable nitrogen and carbon isotopes in hair: Monitoring starvation and recovery phases of patients suffering from anorexia nervosa. *Rapid Communications in Mass Spectrometry* 20(10):1604-1610.

Metcalf NB, Monaghan P (2001) Compensation for a bad start: grow now, pay later? Trends in Ecology and Evolution 16:254–260.

Metges C, Kempe K, Schmidt HL (1990) Dependence of the carbon-isotope contents of breath carbon dioxide, milk, serum and rumen fermentation products on the δ 13 C value of food in dairy cows. *British Journal of Nutrition* 63(2):187-96.

Mezzetti A, Pierdomenico SD, Costantini F, Romano F, De Cesare D, Cuccurullo F, Imbastaro T, Riario-Sforza G, Di Giacomo F, Zuliani G, Fellin R (1998) Copper/zinc ratio and systemic oxidant load: effect of aging and aging-related degenerative diseases. *Free Radical Biology and Medicine* 25(6):676-81.

Minagawa M, Wada E. (1984) Stepwise enrichment of 15N along food chains: further evidence and the relation between $\delta^{15}N$ and animal age. *Geochimica et cosmochimica acta* 48(5):1135-1140.

Mitra AK, Akramuzzaman SM, Fuchs GJ, Rahman MM, Mahalanabis D (1997) Long-term oral supplementation with iron is not harmful for young children in a poor community of Bangladesh. *The Journal of nutrition* 127(8):1451–1455.

Mizock BA (1995) Alterations in carbohydrate metabolism during stress: A review of the literature. *The American Journal of Medicine* 98(1):75-84.

Mizota C, Sasaki A (1996) Sulfur isotope composition of soils and fertilizers: differences between Northern and Southern Hemispheres. *Geoderma* 71(1-2):77-93.

Moore SE, Cole TJ, Collinson AC, Poskitt EM, McGregor IA, Prentice AM (1999) Prenatal or early postnatal events predict infectious deaths in young adulthood in rural Africa. International journal of epidemiology 28(6):1088-1095.

Moore MK, Ross AH (2013) Stature estimation. *Research methods in human skeletal biology*. Waltham: Elsevier.

Mora S, Barera G, Ricotti A, Weber G, Bianchi C, Chiumello G (1998) Reversal of low bone density with a gluten-free diet in children and adolescents with celiac disease. *The American journal of clinical nutrition* 67(3):477-81.

Morgan JL, Skulan JL, Gordon GW, Romaniello SJ, Smith SM, Anbar AD (2012) Rapidly assessing changes in bone mineral balance using natural stable calcium isotopes. *Proceedings of the National Academy of Sciences* 109(25):9989-94.

Morris D, McAlpin M (1979) *Measuring the condition of the world's poor*. Pergamons Press.

Morrison J, Fourel F, Churchman D (2000) Isotopic sulphur analysis by continuous flow Isotope ratio mass spectrometry (CF-IRMS). Application Note 509

Müldner G, Montgomery J, Cook G, Ellam R, Gledhill A, Lowe C (2009) Isotopes and individuals: diet and mobility among the medieval Bishops of Whithorn. *Antiquity* 83(322):1119–1133.

Müldner G, Richards MP (2007) Stable isotope evidence for 1500 years of human diet at the city of York, UK. *American Journal of Physical Anthropology* 133(1):682–697.

Murray M, Murray A (1979) Anorexia of infection as a mechanism of host defense. *The American Journal of Clinical Nutrition* 32(3):593-596.

Nehlich O (2015) The application of sulphur isotope analyses in archaeological research: A review. *Earth-Science Reviews* 142:1-17.

Nehlich O, Barrett JH, Richards MP (2013) Spatial variability in sulfur isotope values of rchaeological and modern cod (Gadus morhua). *Rapid Communication in Mass Spectrometry* 27:2255–2262.

Nehlich O, Borić D, Stefanović S, Richards MP (2010) Sulphur isotope evidence for freshwater fish consumption: A case study from the Danube Gorges, SE Europe. *Journal of Archaeological Science* 37(5):1131-1139.

Nehlich O, Fuller BT, Jay M, Mora A, Nicholson RA, Smith CI, Richards MP (2011) Application of sulphur isotope ratios to examine weaning patterns and freshwater fish consumption in roman Oxfordshire, UK. *Geochimica Et Cosmochimica Acta* 75(17):4963-4977.

Nehlich O, Richards MP (2009) Establishing collagen quality criteria for sulphur isotope analysis of archaeological bone collagen. *Archaeological and Anthropological Sciences* 1(1):59-75.

Neuberger FM, Jopp E, Graw M, Püschel K, Grupe G (2013) Signs of malnutrition and starvation—Reconstruction of nutritional life histories by serial isotopic analyses of hair. *Forensic Science International* 226(1-3):22-32.

Neve A, Corrado A, Cantatore FP (2011) Osteoblast physiology in normal and pathological conditions. *Cell and Tissue Research* 343(2):289-302.

Nielsen H (1974) Isotopic composition of the major contributors to atmospheric sulfur. *Tellus* 26(1-2):213-21.

Nielsen H., Pilot J, Grinenko LN, Grinenko VA, Lein AY, Smith JW, Pankina RG (1991) Lithospheric sources of sulphur. In *Stable isotopes: Natural and Anthropogenic sulphur in the environment*.

Norman A, Anlauf K, Hayden K, Thompson B, Brook JR, Li S, Bottenheim J (2006) Aerosol sulphate and its oxidation on the Pacific NW coast: S and O isotopes in PM2.5. Atmospheric Environment 40:2676–2689.

Nriagu JO, Coker RD, Barrie LA (1991) Origin of sulphur in Canadian Arctic haze from isotope measurements. *Nature* 349(6305):142.

O'Brien KO, Zavaleta N, Caulfield LE, Yang DX, Abrams SA (1999) Influence of prenatal iron and zinc supplements on supplemental iron absorption, red blood cell iron

incorporation, and iron status in pregnant Peruvian women. *The American journal of clinical nutrition* 69(3):509-15.

O'Connell TC (2017) 'Trophic'and 'source'amino acids in trophic estimation: a likely metabolic explanation. Oecologia 184(2):317-26.

Oelbermann K, Langel R, Scheu S (2008) Utilization of prey from the decomposer system by generalist predators of grassland. *Oecologia* 155(3):605–617.

Oelbermann K, Scheu S (2001) Stable isotope enrichment ($\delta^{15}N$ and the $\delta^{13}C$) in a generalist predator (Pardosalugubris, Araneae: Lycosidae): effects of prey quality. *Oecologia* 130:337–344.

Ohno T, Shinohara A, Kohge I, Chiba M, Hirata T (2004) Isotopic analysis of Fe in human red blood cells by multiple collector-ICP-mass spectrometry. *Analytical sciences* 20(4):617-21.

Ohno T, Shinohara A, Chiba M, Hirata T (2005) Precise Zn isotopic ratio measurements of human red blood cell and hair samples by multiple collector-ICP-mass spectrometry. *Analytical sciences* 21(4):425-8.

Olsen KC, White CD, Longstaffe FJ, Heyking K, McGlynn G, Grupe G, Rühli FJ (2014) Intraskeletal isotopic compositions ($\delta^{13}C$, $\delta^{15}N$) of bone collagen: Nonpathological and pathological variation. *American Journal of Physical Anthropology* 153(4):598-604.

Ortner DJ (2003) I*dentification of pathological conditions in human skeletal remains*. Academic Press.

Ortner DJ, Putschar WGJ (1985) *Identification of Pathological Conditions in Human Skeletal Remains*. Smithsonian Institution Press. Washington, London.

Ottaway P (1992) *Archaeology in British towns: From the emperor Claudius to the Black Death*. London: Routledge.

Otten JJ, Hellwig JP, Meyers LD (Eds.) (2006) *Dietary reference intakes: the essential guide to nutrient requirements*. National Academies Press.

Oxenham MF, Cavill I (2010) Porotic hyperostosis and cribra orbitalia: the erythropoietic response to iron-deficiency anaemia. *Anthropological Science* 118(3):199-200.

Paine RR, Brenton BP (2006) The paleopathology of pellagra: Investigating the impact of prehistoric and historical dietary transitions to maize. *Journal of Anthropological Sciences* 84:125–135.

Palubeckaitė Ž, Jankauskas R, Boldsen J (2002) Enamel hypoplasia in Danish and Lithuanian late medieval/early modern samples: a possible reflection of child morbidity and mortality patterns. *International Journal of Osteoarchaeology* 12(3):189-201.

Papet I, Ruot B, Breuille D, Walrand S, Farges MC, Vasson MP, Obled C (2002) Bacterial infection affects protein synthesis in primary lymphoid tissues and circulating lymphocytes of rats. *The Journal of nutrition* 132(7):2028-32.

Parfitt AM (1983) Dietary risk factors for age-related bone loss and fractures. *The Lancet* 322(8360):1181–1185.

Parfitt AM (2002) Life history of osteocytes: relationship to bone age, bone remodeling, and bone fragility. Jour*nal of Musculoskeletal and Neuronal Interactions* 2(6):499-500.

Passey BH, Robinson TF, Ayliffe LK, Cerling TE, Sponheimer M, Dearing MD, Roeder BL, Ehleringer JR (2005) Carbon isotope fractionation between diet, breath CO_2, and bioapatite in different mammals. Journal of Archaeological Science 32(10):1459-70.

Peat J, Barton B (2005) Medical Statistics: A Guide to Data Analysis and Critical Appraisal. 2005. Malden, Massachusetts: Blackwell.

Peck JJ (2013) Status, health, and lifestyle in Middle Iron Age Britain: A bioarcheological study of elites and non-elites from East Yorkshire, Northern England. *International Journal of Paleopathology* 3(2):83–94.

Peterson BJ, Fry B (1987) Stable isotopes in ecosystem studies. *Annual review of ecology and systematics* 18(1):293-320.

Peterson BJ, Howarth RW, Garritt RH (1985) Multiple stable isotopes used to trace the flow of organic matter in estuarine food webs. *Science* 227:1361–1363.

Peterson BJ, Howarth RW, Garritt RH (1986) Sulfur and carbon isotopes as tracers of salt–marsh organic matter flow. *Ecology* 67:865–874.

Pfeiffer SK, Lazenby RA (1994) Low bone mass in past and present aboriginal populations. *Nutrition and osteoporosis* (pp. 35-51). Springer, Boston, MA.

Phenice TW (1969) A newly developed visual method of sexing the os pubis. *American Journal of Physical Anthropology* 30(2):297–301.

Pinhasi R, Mays S (Eds.) (2008). *Advances in human palaeopathology*. John Wiley & Sons.

Platt C (1981) *The parish churches of medieval England*. London: Secker & Warburg.

Polet C, Katzenberg MA (2003) Reconstruction of the diet in a mediaeval monastic community from the coast of Belgium. Journal of Archaeological Science 30(5):525-533.

Powanda MC (1977) Changes in body balances of nitrogen and other key nutrients: description and underlying mechanisms. *The American journal of clinical nutrition* 30(8):1254-1268.

Power C, Peckham C (1990) Childhood morbidity and adulthood ill health. *Journal of Epidemiology & Community Health* 44(1):69-74.

Quirós Castillo JÁ (2013) Los comportamientos alimentarios del campesinado medieval en el País Vasco y suentorno (siglos VIII–XIV). *Historia Agraria* 59:13–41.

Ragsdale BD, Lehmer LM (2012) A knowledge of bone at the cellular (histological) level is essential to paleopathology. *In*: Grauer AL (ed.). *A companion to paleopathology*. Chichester, UK: Wiley-Blackwell. 227–249.

Rana R, Wu J, Eisenberg R (2009) Periosteal reaction. *American Journal of Roentgenology* 193:w259–w272

Razali NM, Wah YB (2011) Power comparisons of shapiro-wilk, kolmogorov-smirnov, lilliefors and anderson-darling tests. *Journal of statistical modeling and analytics* 2(1):21-33.

Redlich K, Smolen JS (2012) Inflammatory bone loss: pathogenesis and therapeutic intervention. *Nature Reviews Drug Discovery* 11:234–250.

Rees CE, Jenkins WJ, Monster J (1978) The sulphur isotopic composition of ocean water sulphate. *Geochimica et Cosmochimica Acta* 42(4):377-81.

Reitsema LJ, Crews DE, Polcyn M (2010) Preliminary evidence for medieval Polish diet from carbon and nitrogen stable isotopes. *Journal of Archaeological Science* 37(7):1413-1423.

Reitsema LJ, Vercellotti G (2012) Stable isotope evidence for sex-and status-based variations in diet and life history at medieval TrinoVercellese, Italy. *American Journal of Physical Anthropology* 148(4): 589-600.

Reitsema LJ, Vercellotti G, Boano R (2016) Subadult dietary variation at Trino Vercellese, Italy, and its relationship to adult diet and mortality. *American journal of physical anthropology* 160(4):653–664.

Richards MP, Fuller BT, Hedges RE (2001) Sulphur isotopic variation in ancient bone collagen from Europe: implications for human palaeodiet, residence mobility, and modern pollutant studies. *Earth and Planetary Science Letters* 191(3):185-190.

Richards MP, Fuller BT, Sponheimer M, Robinson T, Ayliffe L (2003) Sulfur isotopes in palaeodietary studies: a review and results from a controlled feeding experiment. *International Journal of Osteoarchaeology* 13:37–45.

Richards MP, Hedges RE (1999) Stable isotope evidence for similarities in the types of marine foods used by Late Mesolithic humans at sites along the Atlantic coast of Europe. *Journal of Archaeological Science* 26(6):717–722.

Robb J, Bigazzi R, Lazzarini L, Scarsini C, Sonego F (2001) Social "status" and biological "status": A comparison of grave goods and skeletal indicators from pontecagnano. *American Journal of Physical Anthropology* 115(3):213–222.

Roberts C, Manchester K (2007) The *archaeology of disease*. Cornell University Press.

Roberts SB, Rosenberg I (2006) Nutrition and aging: changes in the regulation of energy metabolism with aging. *Physiological reviews* 86(2):651-667.

Robertson KL, Rowland NE, Krigbaum J (2014) Effects of caloric restriction on nitrogen and carbon stable isotope ratios in adult rat bone. *Rapid Communications in Mass Spectrometry* 28(19):2065-2074.

Salamon M, Coppa A, McCormick M, Rubini M, Vargiu R, Tuross N (2008) The consilience of historical and isotopic approaches in reconstructing the medieval Mediterranean diet. *Journal of Archaeological Science* 35(6):1667–1672.

Saunders SR, Hoppa RD (1993) Growth deficit in survivors and non-survivors: biological mortality bias in subadult skeletal samples. *American journal of physical anthropology* 36(S17):127-51.

Scheuer L, Black S (2000) Development and ageing of the juvenile skeleton. *Human Osteology in Archaeology and Forensic Science* 9-21.

Schlecht SH, Pinto DC, Agnew AM, Stout SD (2012) Brief communication: The effects of disuse on the mechanical properties of bone: What unloading tells us about the adaptive nature of skeletal tissue. *American Journal of Physical Anthropology* 149(4):599-605.

Schneider RA, Helms JA (1998) Development and regeneration of the musculoskeletal system. *Current Opinion in Orthopaedics* 9(6):20-24.

Schoeninger MJ, DeNiro MJ (1984) Nitrogen and carbon isotopic composition of bone collagen from marine and terrestrial animals. *Geochimica et Cosmochimica Acta* 48(4):625-639.

Schoeninger MJ, DeNiro MJ, Tauber H (1983) Stable nitrogen isotope ratios of bone collagen reflect marine and terrestrial components of prehistoric human diet. *Science* 220(4604):1381-1383.

Schutkowski H, Herrmann B, Wiedemann F, Bocherens H,Grupe G (1999) Diet, status and decomposition at Weingarten: trace element and isotope analyses on early mediaeval skeletal material. *Journal of Archaeological Science* 26(6):675-685.

Scorrano G, Brilli M, Martínez-Labarga C, Giustini F, Pacciani E, Chilleri F, Scaldaferri F, Gasbarrini A, Gasbarrini G, Rickards O (2014) Palaeodiet reconstruction in a woman with probable celiac disease: A stable isotope analysis of bone remains from the archaeological site of Cosa (Italy). *American Journal of Physical Anthropology* 154(3):349–356.

Scheuer JL, Black S (2000) Development and ageing of the juvenile skeleton. In M Cox, S Mays (Eds.). *Human osteology in archaeology and forensic science*, 9–21. London: Greenwich Medical Media.

Scrimshaw NS, SanGiovanni JP (1997). Synergism of nutrition, infection, and immunity: an overview. *The American journal of clinical nutrition* 66(2):464S–477S.

Sealy JC, van der Merwe NJ, Thorp JA, Lanham JL (1987) Nitrogen isotopic ecology in southern Africa: implications for environmental and dietary tracing. *Geochim Cosmochim Acta* 51:2707–2717.

Sears J, Hatch SA, O'Brien DM (2009) Disentangling effects of growth and nutritional status on seabird stable isotope ratios. Oecologia 159(1):41-8.

Seibel MJ (2005) Clinical use of markers of bone turnover in metastatic bone disease. *Nature Reviews Clinical Oncology* 2(10):504.

Shapiro F (2008) Bone development and its relation to fracture repair. The role of mesenchymalosteoblasts and surface osteoblasts. *European Cells and Materials* 15:53–76, Review.

Siek T (2013) The osteological paradox and issues of interpretation in paleopathology. *Vis-à-Vis: Explorations in Anthropology* 13:92–101.

Sfeir C, Ho L, Doll BA, Azari K, Hollinger JO (2005) Fracture repair. In *Bone regeneration and repair* (pp. 21-44). Humana Press.

Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). *Biometrika* 52(3/4):591-611.

Sherman P, Forstner J, Roomi N, Khatri I, Forstner G (1985) Mucin depletion in the intestine of malnourished rats. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 248(4):G418–G423.

Skedros JG, Knight AN, Clark GC, Crowder CM, Dominguez VM, Qiu S, Mulhern DM, Donahue SW, Busse B, Hulsey BI, Zedda M (2013) Scaling of Haversian canal surface area to

secondary osteon bone volume in ribs and limb bones. *American journal of physical anthropology*151(2):230-44.

Sponheimer M, Robinson T, Ayliffe L, Roeder B, Hammer J, Passey B, ... Ehleringer J (2003) Nitrogen isotopes in mammalian herbivores: hair $\delta^{15}N$ values from a controlled feeding study. *International Journal of Osteoarchaeology* 13(1-2):80-87.

Steckel RH, Larsen CS, Sciulli PW, Walker PL (2006) Data collection codebook. *The Global History of Health Project* 1–41.

Steele K, Daniel RM (1978) Fractionation of nitrogen isotopes by animals: A further complication to the use of variations in the natural abundance of 15 N for tracer studies. *The Journal of Agricultural Science* 90(1):7–9.

Stenberg A, Malinovsky D, Öhlander B, Andrén H, Forsling W, Engström LM, Wahlin A, Engström E, Rodushkin I, Baxter DC (2005) Measurement of iron and zinc isotopes in human whole blood: preliminary application to the study of HFE genotypes. *Journal of Trace Elements in Medicine and Biology* 19(1):55-60.

Stock JT, Migliano AB (2009) Stature, mortality, and life history among indigenous populations of the Andaman Islands, 1871–1986. *Current Anthropology* 50(5):713-25.

Stodder AL (1997) Subadult stress, morbidity, and longevity in Latte Period populations on Guam, Mariana Islands. *American Journal of Physical Anthropology* 104(3):363-80.

Stuart-Macadam P (1989) Porotic hyperostosis: relationship between orbital and vault lesions. American Journal of Physical Anthropology 80(2):187-93.

Swanson RN (1989) *Church and society in late medieval England*. Oxford: Blackwell.

Suby JA (2014) Porotic hyperostosis and cribra orbitalia in human remains from southern Patagonia. *Anthropological Science* 140430.

Takahashi E (1984) Secular trend in milk consumption and growth in Japan. Human Biology 56:427–437.

Takahashi E (1994) Secular changes in growth of Japanese children. Journal of Pediatric Endocrinology 7:163–173.

Tanner JM, Hayashi T, Preece MA, Cameron N (1982) Increase in length of leg relative to trunk in Japanese children and adults from 1957 to 1977: comparison with British and with Japanese Americans. Annals of Human Biology 9:411–423.

Tanz N, Schmidt HL (2010) δ^{34}S-value measurements in food origin assignments and sulfur isotope fractionations in plants and animals. *Journal of agricultural and food chemistry* 58(5):3139-46.

Tapscott SJ (2005) The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. *Development* 132:2685–2695.

Tarli SB, Repetto E (1986) Methodological considerations on the study of sexual dimorphism in past human populations. Human Evolution 1(1):51-66.

Tauber H (1981) ^{13}C evidence for dietary habits of prehistoric man in Denmark. *Nature* 292(5821):332-333.

Teeri J, Schoeller D (1979) δ^{13}C values of an herbivore and the ratio of C_3 to C_4 plant carbon in its diet. *Oecologia* 39(2):197-200.

Teitelbaum SL (2000) Bone resorption by osteoclasts. *Science* 289(5484):1504-1508.

Teitelbaum SL, Ross FP (2003) Genetic regulation of osteoclast development and function. *Nature Reviews Genetics* 4(8):638.

Temple DH, Goodman AH (2014) Bioarcheology has a "health" problem: Conceptualizing "stress" and "health" in bioarcheological research. *American Journal of Physical Anthropology* 155(2):186–191.

Thode HC (2002) Testing for normality. CRC press.

Thode HG, Monster J, Dunford HB (1961) Sulphur isotope geochemistry. *Geochimica et Cosmochimica Acta* 25(3):159-74.

Thomas RM (2007) Food and the maintenance of social boundaries in medieval England. *The Archaeology of Food, and Identity, Occasional Papers* 34:130-151.

Thomas CJ, Cahoon LB (1993) Stable isotope analyses differentiate between different trophic pathways supporting rocky-reef fishes. *Marine Ecology-Progress Series* 95:19.

Tieszen LL, Boutton TW, Tesdahl KG, Slade NA (1983) Fractionation and turnover of stable carbon isotopes in animal tissues: implications for δ^{13}C analysis of diet. *Oecologia* 57(1-2):32-37.

Turner-Walker G (2008) The chemical and microbial degradation of bones and teeth. *Advances in human palaeopathology* 592.

Ulijaszek SJ, Mann N, Elton S (2012) *Evolving human nutrition: Implications for public health*. Cambridge University Press.

Valente J (1998) The new frontier: the role of the knights templar in the establishment of Portugal as an independent kingdom. *Mediterranean Studies* 7:49–65.

Van der Merwe NJ (1982) Carbon isotopes, photosynthesis, and archaeology: Different pathways of photosynthesis cause characteristic changes in carbon isotope ratios that make possible the study of prehistoric human diets. *American Scientist* 70(6):596-606.

Van der Merwe NJ, Vogel JC (1978) 13C content of human collagen as a measure of prehistoric diet in woodland North America. *Nature* 276(5690):815.

Van Heghe L, Engström E, Rodushkin I, Cloquet C, Vanhaecke F (2012) Isotopic analysis of the metabolically relevant transition metals Cu, Fe and Zn in human blood from vegetarians and omnivores using multi-collector ICP-mass spectrometry. *Journal of Analytical Atomic Spectrometry* 27(8):1327-1334.

Van Heghe L, Delanghe J, Van Vlierberghe H, Vanhaecke F (2013) The relationship between the iron isotopic composition of human whole blood and iron status parameters. *Metallomics* 5(11):1503-9.

Van Heghe L, Deltombe O, Delanghe J, Depypere H, Vanhaecke F (2014) The influence of menstrual blood loss and age on the isotopic composition of Cu, Fe and Zn in human whole blood. *Journal of Analytical Atomic Spectrometry* 29(3):478-82.

van Klinken GJ, Richards MP, Hedges REM (2000) An overview of causes for stable isotopic variations in past European human populations: environmental, ecophysiological, and cultural effects. In *Biogeochemical Approaches to Paleodietary Analysis*, Ambrose SH, Katzenberg MA (eds.). Kluwer Academic: New York; 39–63.

Van Klinken GJ, Richards MP, Hedges BE (2002) An overview of causes for stable isotopic variations in past European human populations: Environmental, ecophysiological, and cultural effects. *Biogeochemical approaches to paleodietary analysis* pp. 39-63 Springer.

Van Wyngene L, Vandewalle J, Libert C (2018) Reprogramming of basic metabolic pathways in microbial sepsis: therapeutic targets at last?. *EMBO molecular medicine* 10(8).

Veiga de Oliveira E, Galhano F, Pereira B (1975) *Actividades agro-marítimas em Portugal*. Instituto de Alta Cultura, Lisboa.

Veitch S, Findlay S, Hamer A, Blumsohn A, Eastell R, Ingle B (2006) Changes in bone mass and bone turnover following tibial shaft fracture. *Osteoporosis International* 17(3):364-372.

Vicente M (2013) *Entre Zêzere e Tejo: Propriedade e Povoamento*. Doutoramento em História Medieval. Universidade de Lisboa [Unpublished].

Vieira VV, Santos M (1995) *Directório de aquacultura e biotecnologia marinha*. Escola Superior de Biotecnologia da UniversidadeCatólica Portuguesa, Porto.

Vogel JC (1978) Isotopic assessment of the dietary habits of ungulates. *South African Journal of Science* 74(8):298-301.

Vogel JC (2012) Variability of Carbon Isotope Fractionation during. Stable isotopes and plant carbon-water relations 29.

Vortkamp A, Pathi S, Peretti GM, Caruso EM, Zaleske DJ, Tabin CJ (1998) Recapitulation of signals regulating embryonic bone formation during postnatal growth and in fracture repair. *Mechanisms of Development* 71(1-2):65-76.

Xu J, Phan TCA, Zheng MH (2005) Intercellular Comunication of Osteoblast and Osteoclast in Bone Diseases, Chapter 5 In: Deng H, Liu Y, Guo C, Chen D. (eds.). Current *Topics in Bone Biology* 11-56. World Scientific.

Wakshal E, Nielsen H (1982) Variations of $\delta^{34}S$ (SO_4), $\delta^{18}O$ (H_2O) and Cl/SO_4 ratio in rainwater over northern Israel, from the Mediterranean Coast to Jordan Rift Valley and Golan Heights. *Earth and Planetary Science Letters* 61(2):272-282.

Walczyk T, von Blanckenburg F (2002) Natural iron isotope variations in human blood. Science. 295(5562):2065-6.

Walker PL, DeNiro MJ (1986) Stable nitrogen and carbon isotope ratios in bone collagen as indices of prehistoric dietary dependence on marine and terrestrial resources in southern California. *American Journal of Physical Anthropology* 71(1):51-61.

Walker PL, Bathurst RR, Richman R, Gjerdrum T, Andrushko VA (2009) The causes of porotic hyperostosis and cribraorbitalia: A reappraisal of the iron-deficiency-anemia hypothesis. *American Journal of Physical Anthropology* 139(2):109-125.

Walker RS, Gurven M, Hill K, Migliano A, Chagnon N, De Souza R, Djurovic G, Hurtado AM, Kaplan H, Kramer K,Oliver WJ, Vallegia C, Yamauchi Y (2006) Growth rates and life histories in twenty-two small-scale societies. *American Journal of Physical Anthropology* 18:295–311.

Walrand S, Chambon-Savanovitch C, Felgines C, Chassagne J, Raul F, Normand B, Farges MC, Beaufrère B, Vasson MP, Cynober L (2000) Aging: a barrier to renutrition? Nutritional and immunologic evidence in rats. *The American journal of clinical nutrition* 72(3):816-24.

Walrand S, Moreau K, Caldefie F, Tridon A, Chassagne J, Portefaix G, Cynober L, Beaufrère B, Vasson MP, Boirie Y (2001) Specific and nonspecific immune responses to fasting and refeeding differ in healthy young adult and elderly persons. *The American Journal of Clinical Nutrition* 74(5):670-8.

Wang A, Huen SC, Luan HH, Yu S, Zhang C, Gallezot JD, Booth CJ, Medzhitov R (2016) Opposing effects of fasting metabolism on tissue tolerance in bacterial and viral inflammation. *Cell* 166(6):1512-25.

Wapler U, Crubezy E, Schultz M (2004) Is cribra orbitalia synonymous with anemia? Analysis and interpretation of cranial pathology in Sudan. *American Journal of Physical Anthropology* 123(4):333-9.

Waterlow JC (2006) Protein turnover. Cambridge, MA 2139.

Waters-Rist AL, Katzenberg MA (2010) The effect of growth on stable nitrogen isotope ratios in subadult bone collagen. *International Journal of Osteoarchaeology*, 20(2), 172-191.

Watts R (2013) Childhood development and adult longevity in an archaeological population from Barton-upon-Humber, Lincolnshire, England. *International Journal of Paleopathology* 3(2):95-104.

Watts R (2015) The long-term impact of developmental stress. Evidence from later medieval and post-medieval London (AD1117–1853). *American journal of physical anthropology* 158(4):569-80.

Welle S (1999) *Human protein metabolism*. Springer, Berlin Heidelberg New York

Weston DA (2008) Investigating the specificity of periosteal reactions in pathology museum specimens. *American Journal of Physical Anthropology* 137(1):48-59.

Weston DA (2009) Brief communication: paleohistopathological analysis of pathology museum specimens: can periosteal reaction microstructure explain lesion etiology?. *American Journal of Physical Anthropology* 140(1):186-193.

Weston DA (2012) Nonspecific infection in paleopathology: interpreting periosteal reactions. In: Grauer AL (Eds.) *A companion to paleopathology*. Chichester, UK: Wiley-Blackwell, 492–512.

White CD, Armelagos GJ (1997) Osteopenia and stable isotope ratios in bone collagen of nubian female mummies. *American Journal of Physical Anthropology* 103(2) 185-199.

Williams CT, Buck CL, Sears J, Kitaysky AS (2007) Effects of nutritional restriction on nitrogen and carbon stable isotopes in growing seabirds. *Oecologia* 153(1):11-18.

Wlodarski KH (1989) Normal and heterotopic periosteum. *Clinical Orthopaedics and Related Research* (241):265-277.

Wolfe R (1981) Glucose metabolism in sepsis and endotoxicosis. *Infection: The Physiologic and Metabolic Responses of the Host*. New York: Elsevier 213-243.

Wolff J, Maquet P, Furlong R (1986) The Law of Bone Remodeling. Springer, Berlin, Germany.

Wood J, Milner G, Harpending H, Weiss K (1992) The osteological paradox - problems of inferring prehistoric health from skeletal samples. *Current Anthropology* 33(4): 343–370.

Woodward B (1998) Protein, calories, and immune defences. *Nutrition Reviews* 56(1):S84–S92.

Woodward B (2001) The effect of protein-energy malnutrition on immune competence. In Suskind R M, Tontisirin KT (Eds.) *Nestle Nutrition Workshop Series*, 45,89–120. Philadelphia, Lippincott-Raven.

World Health Organization (2009) World health statistics 2009. World Health Organization.

World Health Organization (2013) Global tuberculosis report 2013. World Health Organization.

Wright LE, Yoder CJ (2003) Recent progress in bioarchaeology: Approaches to the osteological paradox. *Journal of Archaeological Research* 11(1):43–70.

Young B, Lowe J, Stevens A, et al. (2006) *Wheater's Functional Histology: A Text and Colour Atlas*. Philadelphia: Elsevier

Yu Y, Ryan CM, Cai W (2017) Studying amino acid and protein metabolism in burn and other trauma patients. In: El-Khoury AE (ed). *Methods for Investigation of Amino Acid and Protein Metabolism*. Boca Raton, FL: CRC Press. p 211–230.

Zohary T, Erez J, Gophen M, Berman-Frank I, Stiller M (1994) Seasonality of stable carbon isotopes within the pelagic food web of Lake Kinneret. *Limnology and Oceanography* 39(5):1030-1043.

Zuckerman MK, Armelagos GJ (2011) The origins of biocultural dimensions in bioarchaeology. *Social bioarchaeology* 13-43.

Appendix

Ficha Antropológica de Campo

Número/UE : 388 / [141355] **Sector/Área:** Area14 **Data de Exumação :** 17-12-2008

Registo Gráfico e Fotográfico:

Sepultura : Desenhado **Esqueleto :** Desenhado **Fotografia :** Realizada

Dados do Genéricos

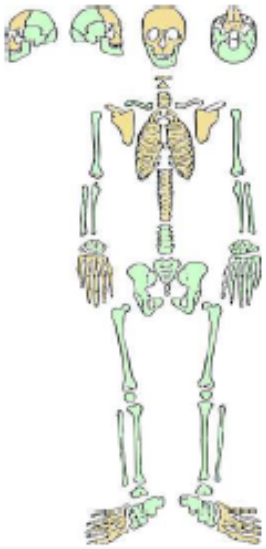
Idade à morte/Nível Etário : ± / Adulto Jovem

Método : Desgaste dentário

Sexo : M **Método :** Morfologia da bacia
Morfologia do crânio

Tafonomia - Inventário do Esqueleto

Notas :



Antropologia Funerária

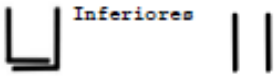
Tipo de Sepultura : Covacho Simples
Orientação : SO-NE

Deposição do Esqueleto :

Crânio : Sobre Occipital, sobre ombro esquerdo

Esqueleto Pós-Craniano : Decúbito Dorsal

Posição dos Membros : **Superiores** **Inferiores**

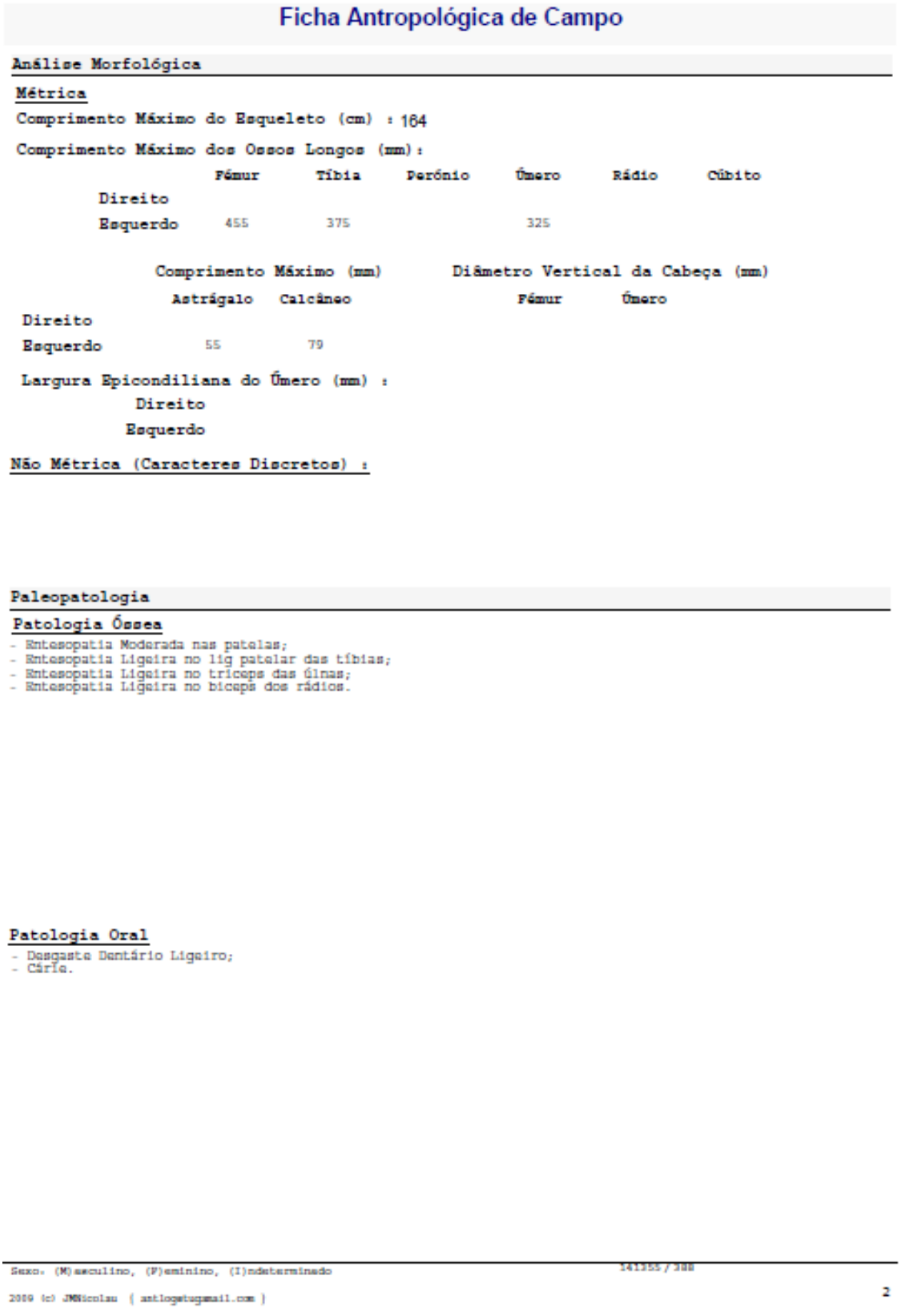


Observações Gerais

Sexo: (M)asculino, (F)eminino, (I)ndeterminado 141355 / 388

2009 (c) JMNicolau (antlogetu@mail.com) 1

Figure A1. Example of an excavation form filled during the excavation at Tomar,



Ficha Antropológica de Campo

Análise Morfológica

Métrica

Comprimento Máximo do Esqueleto (cm) : 164

Comprimento Máximo dos Ossos Longos (mm) :

	Fémur	Tíbia	Perónio	Úmero	Rádio	Cúbito
Direito						
Esquerdo	455	375		325		

	Comprimento Máximo (mm)		Diâmetro Vertical da Cabeça (mm)	
	Astrágalo	Calcâneo	Fémur	Úmero
Direito				
Esquerdo	55	79		

Largura Epicondiliana do Úmero (mm) :

Direito

Esquerdo

Não Métrica (Caracteres Discretos) :

Paleopatologia

Patologia Óssea

- Entesopatia Moderada nas patelas;
- Entesopatia Ligeira no lig patelar das tíbias;
- Entesopatia Ligeira no tricops das úlnas;
- Entesopatia Ligeira no bicaps dos rádios.

Patologia Oral

- Desgaste Dentário Ligeiro;
- Cárie.

Sexo. (M)asculino, (F)eminino, (I)ndeterminado 141355/388

2009 (c) JMNicolau (antlogetu@gmail.com) 2

Figure A1. (continuation) Example of an excavation form filled during the excavation at Tomar,

Table A1. Skewness, kurtosis and normality tests.

							Shapiro-Wilk		
			Mean±SD	Mean±SEM	Skewness	Kurtosis	Statistics	df	sig
Individuals without lesions	$\delta^{13}C$	Female	-18.7±0.3	-18.7±0.1	0.307	-0.95	0.942	10	0.58
		Male	-18.2±1.1	-18.2±0.3	2.172	5.918	0.769	10	0.01
		Young	-17.9±1.4	-17.9±0.6	1.966	4.069	0.755	5	0.03
		Mature	-18.7±0.4	-18.7±0.1	0.01	-0.66	0.98	11	0.97
		Elderly	-18.6±0.3	-18.6±0.1	0.77	1.82	0.93	4	0.61
	$\delta^{15}N$	Female	10.6±0.9	10.6±0.3	0.13	1.44	0.96	10	0.74
		Male	11.3±0.7	11.3±0.2	0.16	-1.22	0.96	10	0.73
		Young	11.7±0.5	11.7±0.2	-0.22	-1.26	0.98	5	0.91
		Mature	10.7±0.7	10.7±0.2	-1.08	2.58	0.92	11	0.33
		Elderly	10.8±1.0	10.8±0.5	1.88	3.59	0.77	4	0.05
	$\delta^{34}S$	Female	13.4±0.9	13.4±0.3	-0.75	1.16	0.96	10	0.72
		Male	12.9±1.9	12.9±0.6	-0.46	-0.10	0.97	10	0.85
		Young	12.1±1.8	12.1±0.8	-1.00	0.34	0.91	5	0.47
		Mature	13.5±1.2	13.5±0.4	0.04	-0.51	0.98	11	0.95
		Elderly	13.5±1.4	13.5±0.7	-1.19	2.12	0.92	4	0.53
Individuals with and without lesions	$\delta^{13}C$	Non-lesion	-18.6±0.5	-18.6±0.1	0.92	0.72	0.94	31	0.10
		Lesion	-18.3±0.7	-18.3±0.1	2.54	9.72	0.76	23	0.00
		Active	-18.4±0.6	-18.4±0.2	-0.272	-0.507	0.94	7	0.64
		Healed	-18.0±1.2	-18.0±0.5	1.951	4.246	0.78	6	0.04
		Both	-18.4±0.2	-18.4±0.1	-0.311	2.715	0.91	10	0.30
		Generalised	-18.7±0.4	-18.7±0.1	-2.092	4.923	0.75	7	0.01
		Healed periostitis	-18.1±1.2	-18.1±0.4	2.177	5.092	0.73	7	0.01
	$\delta^{15}N$	Non-lesion	10.8±0.9	10.8±0.2	0.146	-0.102	0.98	31	0.66
		Lesion	10.7±0.7	10.7±0.2	0.137	-1.381	0.93	23	0.09
		Active	10.5±0.7	10.5±0.3	0.625	-1.119	0.90	7	0.34
		Healed	10.9±0.7	10.9±0.3	-1.342	2.156	0.87	6	0.24
		Both	10.7±0.8	10.7±0.2	0.505	-1.488	0.89	10	0.15
		Generalised	10.3±0.7	10.3±0.2	1.140	-0.195	0.84	7	0.09
		Healed periostitis	11.2±0.4	11.2±0.1	0.152	-1.801	0.91	7	0.37
Intras-keleton variation (long bones)	$\delta^{13}C$	Active	0.2±0.7	0.2±0.2	0.122	-0.470	0.99	13	1.00
		Healed	0.5±1.1	0.5±0.4	0.445	0.149	0.98	7	0.93
		Both	0.1±0.6	0.1±0.2	0.751	-0.607	0.886	11	0.124
	$\delta^{15}N$	Active	-0.5±0.5	-0.5±0.1	-0.742	0.684	0.93	13	0.58
		Healed	0.2±0.9	0.2±0.3	0.891	0.138	0.93	7	0.42
		Both	0.2±0.8	0.2±0.3	-0.478	1.582	0.927	11	0.307
Intras-keleton variation (ribs)	$\delta^{13}C$	Active	-0.1±0.1	-0.1±0.1	0.845	0.540	0.96	5	0.78
		Healed	-0.1±0.7	-0.1±0.3	1.094	0.597	0.875	6	0.248
		Both	-0.2±0.4	-0.2±0.1	-0.572	-1.161	0.914	9	0.346
	$\delta^{15}N$	Active	-0.4±0.7	-0.4±0.3	-0.767	-0.341	0.927	5	0.576
		Healed	0.0±0.5	0.0±0.2	0.035	0.082	0.989	6	0.987
		Both	0.1±0.9	0.1±0.3	-1.123	3.239	0.874	9	0.576

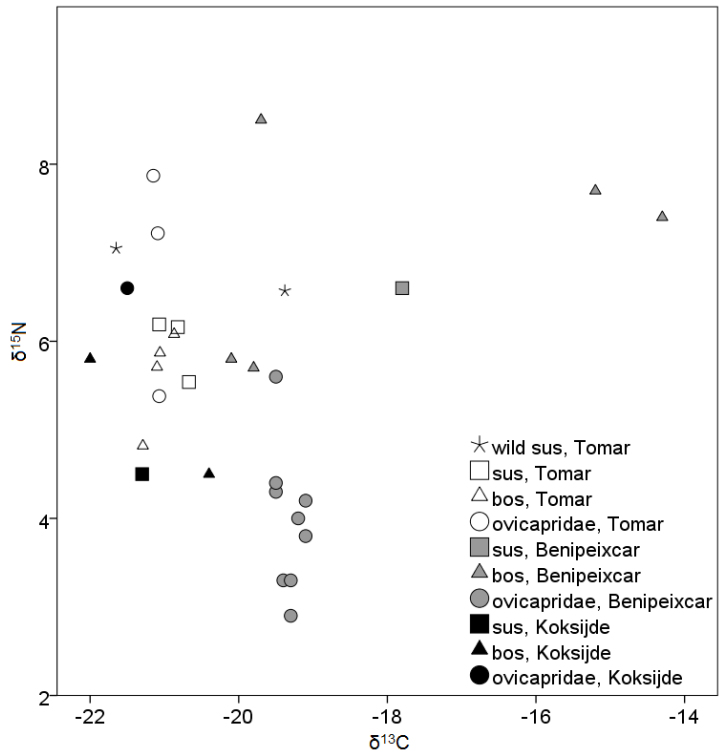


Figure A2. Carbon and nitrogen stable isotope comparison between faunal remains from Tomar, Benipeixcar (15^{th} – 16^{th} century Alexander et al. 2015) and Koksijde (12^{th} – 15^{th} century, Polet and Katzenberg 2003).

Table A2. Stable isotope data ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) of bone collagen from fauna remains.

	Genus	Area	**$\delta^{13}C$**	**%C**	**$\delta^{15}N$**	**%N**	**C/N**	**$\delta^{34}S$**	**%S**	**C/S**	**N/S**
Fauna	Bos	20	-21.3	42.5	4.8	14.9	3.3	18.0	0.2	212.6	74.3
	Bos	20	-21.1	29.9	5.9	11.1	3.3	13.1	0.1	299.1	110.6
	Bos	20	-20.9	38.2	6.1	13.4	3.3	-	-	-	-
	Bos	20	-21.1	42.2	5.7	14.8	3.3	18.5	0.2	211.1	74.1
	Canidae	20	-21.1	27.5	7.2	9.0	3.4	-	-	-	-
	Domestic sus	14	-21.1	36.3	6.2	12.4	3.4	-	-	-	-
	Domestic sus	14	-20.7	38.8	5.5	13.4	3.4	-	-	-	-
	Domestic sus	14	-20.8	15.9	6.2	5.4	3.4	17.4	0.1	158.5	54.4
	Equus	20	-20.4	41.0	8.5	14.4	3.3	10.2	0.2	204.8	72.0
	Juvenile sus	14	-20.1	41.0	6.3	14.2	3.4	14.8	0.2	205.0	71.1
	Ovicapridae	17	-21.1	10.1	5.4	3.5	3.4	15.1	0.1	101.0	35.0
	Ovicapridae	20	-21.2	22.6	7.9	7.4	3.4	16.5	0.1	225.6	74.0
	Ovicapridae	20	-21.1	42.7	7.2	15.1	3.3	18.5	0.2	213.5	75.5
	Wild sus	20	-21.7	17.8	7.1	6.1	3.4	14.8	0.1	178.0	61.0
	Wild sus	17	-19.4	27.3	6.6	9.5	3.4	14.6	0.1	273.4	94.7

Table A3. Stable isotope data ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) of human bone collagen (without lesions or indicators of physiological stress) .

	Sex	Age	Status	Area	$\delta^{13}C$	%C	$\delta^{15}N$	%N	C/N	$\delta^{34}S$	%S	C/S	N/S
Humans	Female	-	Higher	13	-18.8	32.1	10.6	11.2	3.4	-	-	-	-
	Female	-	Lower	14	-18.6	31.3	12.0	10.4	3.4	-	-	-	-
	Female	Mature	Lower	16	-18.3	41.8	10.5	14.8	3.3	13.2	0.2	208.8	74.0
	Female	Mature	Lower	14	-18.9	41.3	10.2	14.6	3.3	14.1	0.2	206.5	73.2
	Female	Old	Lower	14	-18.9	21.9	10.3	7.5	3.4	13.9	0.1	218.9	75.0
	Female	Old	Lower	14	-18.7	41.0	10.5	14.5	3.3	11.5	0.2	204.8	72.6
	Female	Mature	Higher	18	-19.1	40.8	8.9	14.4	3.3	13.5	0.2	204.0	71.9
	Female	Old	Lower	20	-18.7	36.7	9.6	12.9	3.3	-	-	-	-
	Female	Young	Lower	20	-18.5	23.2	11.0	7.7	3.4	13.7	0.1	232.2	76.8
	Female	Old	Higher	18	-18.3	34.5	12.3	11.9	3.4	13.6	0.2	172.4	59.7
	Female	Mature	Lower	20	-18.6	23.0	11.0	7.9	3.4	13.1	0.1	229.9	78.7
	Female	Young	Lower	14	-19.0	15.5	11.4	5.2	3.4	12.4	0.1	154.8	52.0
	Female	Old	Lower	14	-18.7	41.7	10.1	14.3	3.4	14.8	0.2	208.4	71.5
	Female	Young	Lower	16	-18.9	18.5	10.6	6.1	3.4	-	-	-	-
	Female	-	Higher	19	-17.8	32.7	11.2	11.5	3.3	-	-	-	-
	Male	-	Lower	20	-18.8	39.1	10.2	13.7	3.3	-	-	-	-
	Male	-	Higher	19	-18.9	41.0	9.6	14.1	3.4	-	-	-	-
	Male	Mature	Higher	18	-18.8	36.6	11.2	12.8	3.3	11.6	0.2	183.0	64.1
	Male	Mature	Higher	19	-18.2	35.3	10.4	12.3	3.4	14.1	0.2	176.5	61.5
	Male	Old	Higher	18	-19.0	37.9	10.5	12.5	3.4	-	-	-	-
	Male	Mature	Lower	14	-19.4	14.8	11.0	5.2	3.3	12.2	0.1	148.0	52.0
	Male	-	Lower	17	-17.3	43.0	10.4	14.8	3.4	-	-	-	-
	Male	Young	Higher	18	-18.1	24.1	12.1	8.4	3.4	11.4	0.2	120.5	41.8
	Male	Mature	Lower	14	-19.1	39.0	9.3	13.3	3.4	-	-	-	-
	Male	Young	Lower	14	-17.8	39.2	12.5	14.4	3.2	-	-	-	-
	Male	Young	Lower	20	-18.5	40.8	11.7	14.1	3.4	13.5	0.2	203.8	70.3
	Male	Young	Lower	16	-18.3	36.1	10.7	12.6	3.4	-	-	-	-
	Male	Young	Lower	14	-15.4	25.7	12.3	9.0	3.3	9.3	0.2	128.5	45.1
	Male	Mature	Lower	14	-17.9	42.1	11.2	14.9	3.3	15.6	0.2	210.3	74.4
	Male	Mature	Higher	15	-18.3	33.7	10.5	11.7	3.4	14.8	0.2	168.5	58.4
	Male	Mature	Lower	14	-17.6	32.6	11.6	10.9	3.4	-	-	-	-
	Male	Mature	Lower	14	-18.7	41.4	11.8	14.3	3.4	12.3	0.2	207.0	71.4
	Male	Mature	Lower	17	-19.1	22.8	10.7	7.6	3.4	14.2	0.1	227.7	76.0

Table A4. Individual isotopic data and collagen integrity for long bones of individuals with skeletal lesions.

Skeleton number	Sex	Age	Distant to lesion					Active lesions					Healed lesions					Fractures				
			$\delta^{13}C$	%C	$\delta^{15}N$	%N	C/N	$\delta^{13}C$	%C	$\delta^{15}N$	%N	C/N	$\delta^{13}C$	%C	$\delta^{15}N$	%N	C/N	$\delta^{13}C$	%C	$\delta^{15}N$	%N	C/N
3.73	Male	Mature	-18.3	15.1	9.6	4.8	3.4	-	-	-	-	-	-18.2	32.4	9.4	11.5	3.3	-	-	-	-	-
14.121	Female	Mature	-19.2	28.6	10.5	9.4	3.5	-	-	-	-	-	-	-	-	-	-	-18.3	36.1	10.4	12.8	3.3
14.130	Male	Old	-18.7	21.4	11.0	7.0	3.4	-18.5	7.6	11.0	21.6	3.3	-	-	-	-	-	-	-	-	-	-
14.21	Male	Young	-19.4	42.9	10.1	16.7	3.3	-19.0	34.2	10.7	11.7	3.4	-	-	-	-	-	-	-	-	-	-
14.301	-	-	-18.7	14.4	10.7	5.4	3.4	-	-	-	-	-	-	-	-	-	-	-18.1	41.6	11.6	14.8	3.3
14.392	Female	Mature	-19.0	17.5	10.7	5.8	3.5	-	-	-	-	-	-18.3	17.6	10.2	5.9	3.5	-	-	-	-	-
14.407	Male	Mature	-17.6	17.5	11.6	5.7	3.5	-	-	-	-	-	-	-	-	-	-	-18.4	18.1	11.9	6.5	3.3
14.72	Female	Old	-18.4	28.9	10.1	9.8	3.4	-18.9	29.5	9.7	10.0	3.4	-18.1	15.6	11.5	5.2	3.5	-	-	-	-	-
15.191	Male	-	-15.6	34.0	11.5	11.9	3.3	-	-	-	-	-	-16.8	32.8	10.9	13.0	3.4	-18.0	33.7	9.8	11.9	3.3
15.96	Female	Old	-18.4	15.0	10.0	5.0	3.3	-	-	-	-	-	-18.3	28.0	10.3	9.4	3.5	-	-	-	-	-
16.169	Female	Young	-18.6	41.9	9.7	15.8	3.3	-19.8	39.4	11.0	14	3.3	-	-	-	-	-	-	-	-	-	-
16.225	Male	Young	-18.6	27.0	11.0	9.2	3.4	-19.5	14.0	10.3	5.4	3.3	-	-	-	-	-	-	-	-	-	-
16.255	Male	Mature	-18.7	34.4	10.0	11.8	3.4	-19.2	23.6	10.5	4.0	3.5	-	-	-	-	-	-	-	-	-	-
17.170	Female	-	-18.5	28.0	10.2	9.6	3.3	-	-	-	-	-	-	-	-	-	-	-18.9	38.8	10.6	14.3	3.3
17.350	Male	Mature	-18.9	20.7	10.7	6.4	3.5	-	-	-	-	-	-	-	-	-	-	-20.1	22.3	10.8	7.7	3.4
17.366	Male	-	-21.9	14.5	12.4	4.0	3.5	-18.7	16.9	11.9	5.6	3.5	-	-	-	-	-	-	-	-	-	-
17.556	Male	Young	-18.5	22.1	11.7	7.5	3.4	-18.4	13.6	12.4	4.6	3.5	-	-	-	-	-	-	-	-	-	-
18.158	Male	Mature	-18.3	27.5	11.4	9.5	3.4	-17.9	21.0	12.9	6.9	3.5	-18.4	31.4	11.2	10.9	3.4	-	-	-	-	-
18.160	Male	Mature	-17.7	17.5	10.9	5.8	3.4	-18.5	23.0	11.0	7.7	3.5	-	-	-	-	-	-	-	-	-	-
18.250	Male	Mature	-18.6	17.8	11.6	6.0	3.5	-	-	-	-	-	-18.4	9.0	9.8	2.9	3.5	-	-	-	-	-
18.464	-	-	-19.1	38.0	11.9	12.3	3.5	-18.1	34.4	12.3	12.0	3.3	-	-	-	-	-	-	-	-	-	-
19.42	Male	-	-18.4	21.4	10.7	7.0	3.3	-18.7	36.0	10.7	12.5	3.4	-18.1	32.4	10.2	11.4	3.3	-	-	-	-	-
19.45	Male	Old	-18.0	28.9	10.0	9.8	3.3	-18.3	23.8	10.7	7.7	3.5	-	-	-	-	-	-	-	-	-	-
20.596	-	-	-17.9	28.3	11.0	9.9	3.3	-	-	-	-	-	-17.9	34.0	10.6	12.1	3.3	-	-	-	-	-
16.196	Male	-	-18.7	31.4	12.0	11.1	3.3	-	-	-	-	-	-	-	-	-	-	-18.0	3.2	11.7	11.3	3.3
17.553	Male	-	-19.3	27.4	10.9	9.5	3.3	-	-	-	-	-	-	-	-	-	-	-18.6	28.1	11.1	9.6	3.4
21.092285	-	-	-18.8	20.6	10.6	6.2	3.5	-	-	-	-	-	-	-	-	-	-	-19.0	21.1	9.6	6.7	3.3
V5.22	Male	Mature	-18.5	33.8	9.8	11.6	3.4	-18.4	34.2	10.2	11.8	3.4	-19.4	38.8	10.5	11.2	3.5	-	-	-	-	-

Table A5. Individual isotopic data and collagen integrity for ribs of individuals with skeletal lesions.

Skeleton number	Sex	Age	Distant to lesion					Active lesions					Healed lesions					Fractures				
			$\delta^{13}C$	%C	$\delta^{15}N$	%N	C/N	$\delta^{13}C$	%C	$\delta^{15}N$	%N	C/N	$\delta^{13}C$	%C	$\delta^{15}N$	%N	C/N	$\delta^{13}C$	%C	$\delta^{15}N$	%N	C/N
14.132	Male	Mature	-19.4	13.6	9.7	4.9	3.3	-19.4	34.4	10.4	12.0	3.3	-	-	-	-	-	-	-	-	-	-
14.31	Male	Mature	-18.9	30.0	10.7	10.5	3.3	-	-	-	-	-	-19.1	33.0	11.1	11.8	3.3	-19.1	29.9	10.6	10.4	3.3
14.392	Female	Mature	-19.1	39.4	10.7	14.0	3.3	-	-	-	-	-	-18.3	34.4	10.3	12.0	3.3	-	-	-	-	-
14.50	Male	Mature	-19.6	37.4	11.1	10.2	3.2	-	-	-	-	-	-	-	-	-	-	-18.9	28.5	10.4	9.4	3.5
14.72	Female	Old	-19.1	38.0	10.2	9.9	3.3	-	-	-	-	-	-18.4	21.8	12.2	7.6	3.4	-	-	-	-	-
15.96	Female	Old	-18.7	38.6	10.5	13.8	3.3	-18.4	32.1	10.1	11.1	3.4	-18.3	38.7	10.1	13.6	3.3	-18.7	21.8	10.6	7.5	3.4
16.225	Male	Young	-20.7	17.0	11.0	5.1	3.5	-	-	-	-	-	-20.8	20.7	10.9	6.4	3.5	-	-	-	-	-
17.556	Male	Young	-19.5	39.5	10.9	13.3	3.5	-	-	-	-	-	-19.4	18.0	10.7	5.2	3.5	-	-	-	-	-
18.158	Male	Mature	-18.5	28.5	12.0	10.2	3.3	-18.2	33.1	11.7	12.2	3.4	-18.5	39.9	10.5	14.5	3.2	-	-	-	-	-
18.4	Male	Old	-18.4	40.6	10.9	15.0	3.2	-	-	-	-	-	-18.4	32.2	11.1	11.5	3.3	-	-	-	-	-
V5.22	Male	Mature	-18.6	37.6	10.3	13.7	3.2	-18.7	12.2	11.8	3.7	3.5	-18.2	38.7	9.9	13.6	3.3	-	-	-	-	-

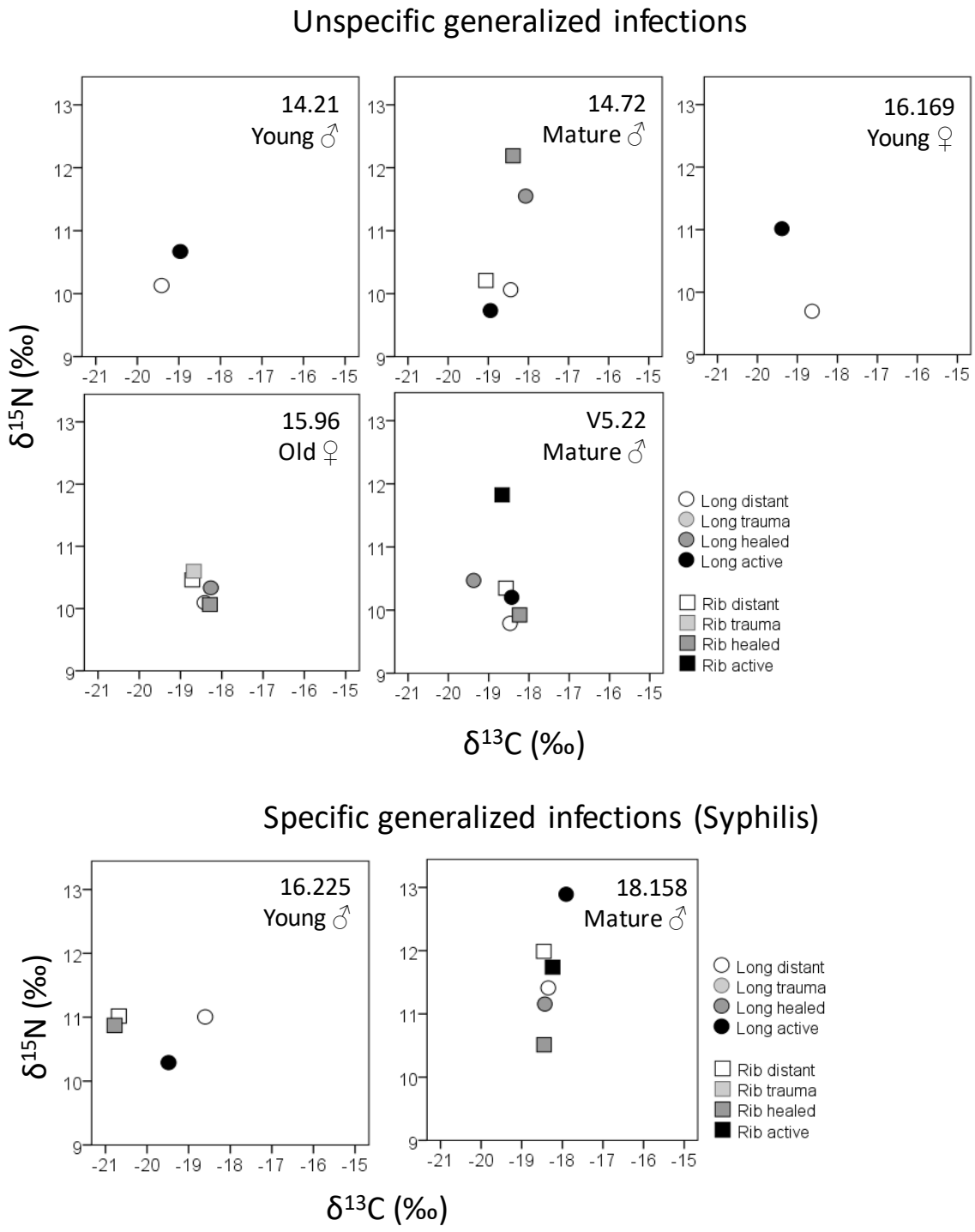


Figure A3. Intra-skeleton carbon and nitrogen stable isotope ratios.

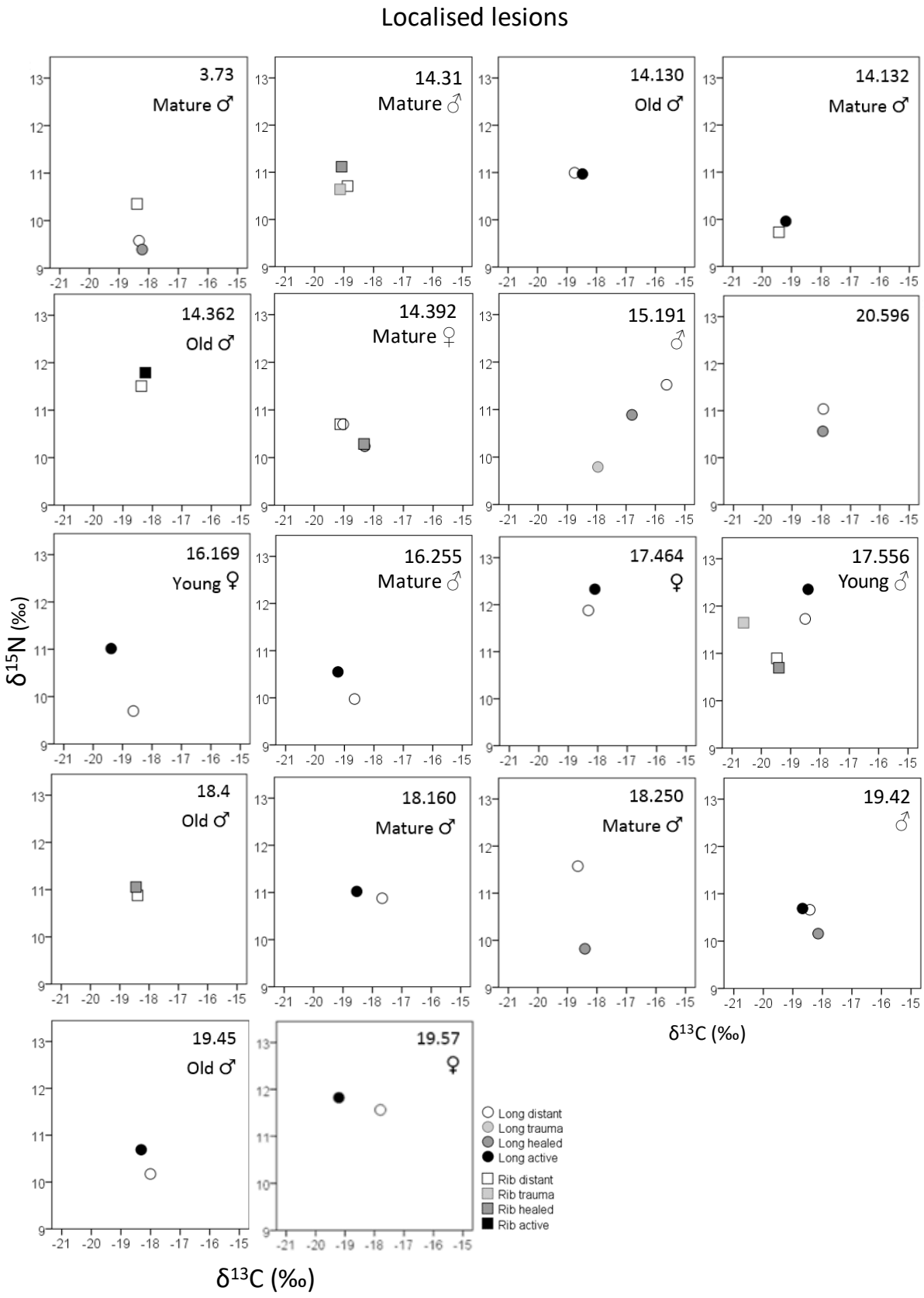


Figure A3 (continuation). Intra-skeleton carbon and nitrogen stable isotope ratios.