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

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Abstract

Objectives: 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.

Materials and Methods: $\delta^{13}C$ 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.

Results: The $\delta^{15}N$ 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 $\delta^{15}N$ to either skeletons without signs of disease or those with only healed tibial periostitis.

Discussion: 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.

HIGHLIGHTS

- Individuals with healed periostitis had similar diets to those without lesions;
- Significant difference ($p<0.003$) between skeletons with healed local periostitis and unspecific generalised infection (various bones affected);
- Dietary differences between healthy and diseased skeletons more noticeable in young adults;
- Individuals with unspecific generalised infections potentially had less access to animal protein than those without lesions or only healed periostitis.

KEYWORDS: paleodiet; paleopathology; periostitis; infectious disease

1| INTRODUCTION

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; Calder & Jackson, 2000; Scrimshaw & 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. Calder & Jackson, 2000; Kuvibidila et al., 1993; Scrimshaw & 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 & Woodward, 1996; Woodward & Miller, 1991). 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).

1.2. Skeletal lesions as health indicators

Health is a complex state that can be reflected through skeletal indicators of physiological stress (Temple et al., 2014). Physiological stress can be related to a wide variety of factors such as disease and nutritional deficiencies (Armelagos, 2003; Goodman & Martin, 2002; Huss-Ashmore et al., 1992; Zuckerman & 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 & 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 & 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 & Lehmer, 2012). Inflammation affects the bone tissue at some level through the production of pathological skeletal phenotypes (e.g. Ragsdale & Lehmer, 2012; Redlich & Smolen, 2012). However, inflammation can be caused by other factors (e.g. Larsen, 1987; Ortner, 2003; Ortner & 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 & 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 & 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 & 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 & 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, 2008).

Acute infections are usually associated with rapid death rarely affecting the skeleton but it may also stimulate new bone formation (Ortner & 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 & Putschard, 1985; Ortner, 2003). In chronic or healing stages (healed lesions) the woven bone is remodelled into compact bone (Ortner & 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 & Martin, 2002). Infectious pathologies, especially when linked with malnutrition, are the largest contributor to morbidity and mortality worldwide (Keusch & 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 socioeconomic status (e.g. Goodman & Martin, 2002; Peck, 2013; Robb et al., 2001), systematic infections (e.g. Goodman & Martin, 2002; Larsen, 2002; Ortner, 2003), malnutrition (e.g. Weston, 2012) and niacin deficiency (Paine & 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 & Wood, 2008).

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 & Epstein, 1976), as a consumer's tissues reflect the isotopic array of the ingested foods.

There is enrichment in $\delta^{13}C$ in an animal's body tissues relative to its diet due to the fractionation that occurs during the tissue's formation (van der Merwe & 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 & Norr, 1993; van der Merwe & Vogel, 1978) and an enrichment of 1‰ between trophic levels (DeNiro & Epstein, 1978; Tieszen et al., 1983). There is an increment in $\delta^{15}N$ of 3‰ to 5‰ between trophic levels when compared with consumer's diet (Bocherens & Drucker, 2003; Minagawa & Wada, 1984; Schoeninger &

DeNiro, 1984; Schoeninger et al., 1983). This fractionation enables the use of stable nitrogen isotopes ($\delta^{15}N$) to infer trophic level and high $\delta^{15}N$ recorded in bone collagen usually indicates high-protein diets (Sponheimer et al., 2003). There are other factors that can raise bone $\delta^{15}N$, such as aridity (Ambrose & 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 & Lovell, 1999; Oelbermann & Scheu, 2001) or protein stress (Hobson et al., 1993; Steele & Daniel, 1978).

Previous research on archaeological samples with and without lesions indicative of leprosy showed no significant differences in $\delta^{13}C$ or $\delta^{15}N$, suggesting that there were not dietary differences between the two groups (Bayliss et al., 2004; Linderholm & 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 & Hughes-Morey, 2012; DeWitte & Wood, 2008).

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 (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 (Curto et al., 2018), due to religious fasting (Barber & Bate, 2002; Müldner et al, 2009; Müldner & 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 (Curto et al., 2018).

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

1.4. Research questions and predictions

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, we 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, 1991; Scrimshaw & SanGiovanni, 1997; Woodward, 1998; Calder & Jackson, 2000; 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, 1991; Kuvibidila et al., 1993; Scrimshaw & SanGiovanni, 1997; Woodward, 1998; Calder & Jackson, 2000; Woodward, 2001; Ulijaszek et al., 2012; Weston, 2012).

$\delta^{15}N$ in particular are very informative of trophic level (Schoeninger et al. 1983; Minagawa & Wada 1984; Schoeninger & DeNiro 1984; Bocherens & Drucker, 2003) and high $\delta^{15}N$ usually indicate high-protein diets (Sponheimeret al., 2013). Therefore we predict that skeletons without signs of infectious disease have higher $\delta^{15}N$ than the ones with skeletal lesions. However, there are other factors that can raise the $\delta^{15}N$ including physiological (Katzenberg & Lovell, 1999; Oelbermann & Scheu, 2001; Gaye-Siessegeret al., 2004; Vogel et al., 2012; Deschner et al., 2012; D'Ortenzio et al., 2015) and/or nutritional stress (Steele & Daniel, 1978; Hobson et al., 1993; Hatch et al., 2006; Warriner & Turross, 2010), which have been associated with $\delta^{15}N$ increase due to protein catabolism.

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 & 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 & 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 $\delta^{13}C$ from skeletons without lesions (and other skeletal markers of physiological stress; Curto et al., 2018) will be compared with bone collagen $\delta^{15}N$ and $\delta^{13}C$ 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 & 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 & 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 & 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 $\delta^{13}C$ 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, 1991; Scrimshaw & SanGiovanni, 1997; Woodward, 1998; Calder & Jackson, 2000; Woodward, 2001), we predict that skeletons without lesions have higher $\delta^{15}N$ than those with lesions, with the ones with only active lesions having the lowest $\delta^{15}N$. The skeletons with only healed lesions are expected to have $\delta^{15}N$ similar to the skeletons without lesions as they survived the disease long enough for the bone to remodel into compact bone (Ortner & Putschard, 1985; Ortner, 2003; Wood et al., 1992).

2 | MATERIALS AND METHODS

Santa Maria do Olival necropolis, at Tomar (Figure 1), 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 (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 (Curto et al., 2018). These

are reused here and compared to new isotope data from skeletons with signs of disease (Table 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; 11th to 17th centuries) in Tomar. The individuals without lesions (n=32), previously analysed (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 Curto et al., 2018 for more detail; the outlier was not considered for this study).

2.1. Estimating age and sex

Sex was estimated based on pelvic (Phenice, 1969; Buikstra & Ubelaker, 1994) and cranial features (Buikstra & Ubelaker, 1994). Adult age at death estimates employed a combination of skeleton maturation (Scheuer & Black, 2000), pubic symphysis degeneration (Brooks & Suchey, 1990; Buikstra & 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 old (60+ years; n=4) adults; for six skeletons it was not possible to estimate age.

2.2. Signs of infection

From the 23 skeletons with lesions (Table 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. Figures 2, 3 and 4 show examples of the different lesion stages analysed.

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 & Putschard, 1985; Buikstra & Ubelaker, 1994; Aufderheide & 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 & 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 were considered active at the time of death (Ortner & 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 & Putschard, 1985; Ortner, 2003). Markedly accentuated longitudinal striations and compact bony growth, without the presence of woven bone, were considered healed lesions (Ortner & 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); old without lesions (n=4) and old 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 & Putschard, 1985; Aufderheide & Rodriguez-Martin, 1998; Ortner, 2003). These groups with signs of infections where then compared with the skeletons without lesions (n=32; 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.

2.3. Collagen extraction and analysis

Collagen extraction was done following Login (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 $\delta^{15}N$ 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 $\delta^{15}N$ 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.

3 | RESULTS

3.1. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ 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; Appendices: Figure A.1) 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 5 illustrates the $\delta^{13}C$ and $\delta^{15}N$ for skeletons without lesions (n=32; 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 2) and $\delta^{15}N$ (10.9±0.7‰; Table 2), while those with non-specific generalised infections (n=5) have the lowest mean for $\delta^{13}C$ (-18.7±0.8‰; Table 1) and $\delta^{15}N$ (9.9±0.4‰; Table 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 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 1). There are no statistically significant differences for $\delta^{13}C$ ($p>0.53$; Table 2) 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 2).

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

Figure 6 illustrates $\delta^{13}C$ and $\delta^{15}N$ for individuals with (including healed, active or a combination of both lesions) and without lesions by age group (Table 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 $\delta^{15}N$ 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 old 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 3).

3.3. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ 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). Results show there is no statistically significant difference in $\delta^{13}C$ or $\delta^{15}N$ when the skeletons without visible lesions (n=32; $\delta^{13}C$=-18.6±0.5‰; $\delta^{15}N$=10.8±0.8‰; Table 4) 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 4), active (n=6; $\delta^{13}C$=-18.5±0.7‰; $\delta^{15}N$=10.5±0.7‰; p=0.72; 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).

4 |Discussion

4.1. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ 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 & 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 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 & SanGiovanni, 1997; Woodward, 1998; Calder & Jackson, 2000;

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 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 (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 5).

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 5 & Table 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, 1991; Kuvibidila et al., 1993; Scrimshaw & SanGiovanni, 1997; Woodward, 1998; Calder & Jackson, 2000; 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 (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‰; 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 old adults (10.6±0.8‰), suggesting a higher animal protein intake for the young individuals (Curto et al., 2018). The high $\delta^{15}N$ from skeletons without lesions seem to be related with higher aquatic protein intake (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 socioeconomic status. Socioeconomic 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, 1991; Kuvibidila et al., 1993; Scrimshaw & SanGiovanni, 1997; Woodward, 1998; Calder & Jackson, 2000; Woodward, 2001) and the possibility of dietary differences being present before the disease cannot be excluded. $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 (Curto et al., 2018).

There are two outliers among the skeletons sampled for isotopic analysis (Figure 5), 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‰; 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‰) are similar to those of the outlier without lesions ($\delta^{13}C$=-15.4‰; $\delta^{15}N$=12.3‰).

4.2. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ 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 3) difference in both $\delta^{13}C$ and $\delta^{15}N$ 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).There is no difference for mature ($p>0.49$; Table 3) and old ($p>0.39$; Table 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 & Putschard, 1985; Ortner, 2003; Turner-Walker, 2008).

4.3. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ 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 had a diet richer in animal protein than those with active lesions, however the sample size is too small to make conclusions.

5 | STUDY LIMITATIONS

One of the limitations 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 infections. 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). The absence of skeletal lesions is ambiguous; it can indicate either good health, or a fast death as result of an acute disease (DeWitte & Stojanowski, 2015; Siek, 2013; Ortner, 2003; 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. Mata et al., 1971; Martorell, 1980; Calder, 1991; Scrimshaw & SanGiovanni, 1997; Calder & Jackson, 2000; Keusch, 2001).

6 | CONCLUSION

This study is part of a larger project that will compare intra-bone stable isotopic data from sites with and without skeletal lesions compatible with diseases and/or physiological stress. 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 $\delta^{15}N$ 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 demonstrate 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 old individuals, being linked to selective mortality. Our results demonstrate that while non-specific generalised infections are a sign of ill health and poor diet, only healed tibial periostitis indicate a state of comparatively good overall health and diet.

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Figure legends

Figure 1. Map of Portugal showing the location of Tomar. Adapted from d-maps.com.

Figure 2. Example of healed tibial periostitis (skeleton 15.96).

Figure 3. Example of a lesion combining active and healed periosteal reactions (skeleton v5.22).

Figure 4. Example of healed osteomyelitis from an individual with syphilis (skeleton 20.240). It is possible to observe a detachable new layer of bone growing on top of the *periosteum*.

Figure 5. $\delta^{13}C$ and $\delta^{15}N$ (‰) for individuals without lesions, with only healed periostosis, with non-specific generalised infections and with treponematosis. Data from skeletons without lesions previously analysed in Curto et al. (2018).

Figure 6. $\delta^{13}C$ and $\delta^{15}N$ (‰) for individuals with and without lesions, by age group (means calculated without outliers). Data from skeletons without lesions previously analysed in Curto et al. (2018).