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1 **Diet and disease in Tomar, Portugal:**  
2 **comparing stable carbon and nitrogen isotope ratios between skeletons with and**  
3 **without signs of infectious disease**  
4

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1 **Abstract**

2 **Objectives:** This study explored the correspondence between stable isotope ratios and  
3 indicators of non-specific (periostitis and/or osteomyelitis) and specific (venereal syphilis)  
4 disease in a sample of human skeletons from a Portuguese archaeological collection.  
5 Additionally, this study examined stable carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) isotope ratios  
6 between individuals at different disease stages.

7 **Materials and Methods:**  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  data from previously analysed skeletons without signs  
8 of infectious disease or physiological stress (n=32) were compared to new data from skeletons  
9 with active (n=6), healed (n=7) or a combination of both lesions (n=10). Skeletons with lesions  
10 (n=23) were also grouped as having only healed tibial periostitis (n=7), generalised non-  
11 specific (n=5) and generalised specific infections (n=2). The skeletons with lesions that did not  
12 fit into these groups (n=9) were not used in this analysis.

13 **Results:** The  $\delta^{15}\text{N}$  from skeletons with non-specific generalised infections in several bones  
14 differed significantly when compared to skeletons that had either only healed tibial periostitis  
15 or were without lesions. Skeletons with venereal syphilis had similar mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  to  
16 either skeletons without signs of disease or those with only healed tibial periostitis.

17 **Discussion:** These results suggest different diets may be linked into an individual's  
18 susceptibility to these pathogens. Diet influences resistance to infectious disease, while  
19 infections decrease nutrient availability, increase malabsorption and resting energy  
20 expenditure. Potentially therefore, combining isotopic evidence of diet with pathology may  
21 contribute to a new understanding of health and lifestyle in the past.

22

## 1 HIGHLIGHTS

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

9  
10 **KEYWORDS:** paleodiet; paleopathology; periostitis; infectious disease

11

## 12 1| INTRODUCTION

### 13 1.1. Effect of diet on health

14 Nutritional stress may result in either greater susceptibility to physiological stress or greater  
15 resilience to stress later in life (Bogin et al., 2007). Malnutrition impairs the immune system  
16 (e.g. Calder, 1991; Calder & Jackson, 2000; Scrimshaw & SanGiovanni, 1997). Individuals  
17 with poorer nutrition are less resistant to infectious diseases, and infectious disease decreases  
18 nutrient availability (e.g. Martorell, 1980; Mata et al., 1971). The effect of protein-energy  
19 malnutrition on aspects of immune function and susceptibility to infection (e.g. Calder &  
20 Jackson, 2000; Kuvibidila et al., 1993; Scrimshaw & SanGiovanni, 1997; Woodward, 1998;  
21 Woodward, 2001) affects practically all forms of immunity, in particular cell mediated  
22 immunity (Kuvibidila et al., 1993; Woodward, 1998; 2001), immune barrier function (Deitch  
23 et al., 1990; Sherman et al., 1985) and the functioning of lymphoid organs (Lee & Woodward,  
24 1996; Woodward & Miller, 1991). On the other hand, infections can decrease nutrient  
25 availability due to malabsorption (e.g. Mitra et al., 1997) and increase resting energy  
26 expenditure, altering the metabolism and redistribution of nutrients (Calder, 2013). However,

1 if nutrition is adequate, diseases like tuberculosis may have a less severe infection, instead of  
2 an exacerbated infection, resulting in prolonged chronic infections with a higher probability to  
3 affect the skeleton (Ulijaszek et al., 2012).

#### 4 **1.2. Skeletal lesions as health indicators**

5 Health is a complex state that can be reflected through skeletal indicators of physiological stress  
6 (Temple et al., 2014). Physiological stress can be related to a wide variety of factors such as  
7 disease and nutritional deficiencies (Armelagos, 2003; Goodman & Martin, 2002; Huss-  
8 Ashmore et al., 1992; Zuckerman & Armelagos, 2011). Even though systemic physiological  
9 stress is not directly observable in the skeleton their consequences, in some cases, are (Klaus,  
10 2014).

11 Infectious diseases were a significant cause of death in past populations, particularly  
12 prior to the antibiotic era (Ortner & Putschard, 1985). Pathogens can reach the skeleton by  
13 direct infection through wounds, extensions from adjacent soft tissue infections or spread by  
14 the blood from the site of a remote infection (Ortner & Putschard, 1985; Ortner, 2003). The  
15 body reacts to infection through an inflammatory response which aims to neutralize the  
16 pathogen and repair the resultant damage (Weston, 2012). Infection damages the normal cells  
17 and accelerates the cell turnover (inflammatory process) (Ragsdale & Lehmer, 2012).  
18 Inflammation affects the bone tissue at some level through the production of pathological  
19 skeletal phenotypes (e.g. Ragsdale & Lehmer, 2012; Redlich & Smolen, 2012). However,  
20 inflammation can be caused by other factors (e.g. Larsen, 1987; Ortner, 2003; Ortner &  
21 Putschard, 1985). Bone reacts in a limited number of ways (production or destruction of bone,  
22 or a combination of production and destruction of bone) for either infection or other causes  
23 such as trauma (e.g. Ragsdale & Lehmer, 2012; Weston, 2008; 2009). However, by analysing  
24 the skeleton as a whole and taking into account other bone-forming disorders, systemic non-  
25 specific infection remains a contextually plausible diagnostic option (Klaus, 2014).

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

10           In case of infection leading to pathological new bone formation, inflammation-derived  
11 pathological periosteal new bone formation is rooted in biological stress (Klaus, 2014).  
12 Osteomyelitis is the result of the introduction of infectious agents into bone, affecting the  
13 medullar cavity (Ortner & Putschard, 1985; Ortner, 2003). Bones with osteomyelitis can  
14 present a combination of cloacae, sequestered bone and involucrum or only reactive bone  
15 formation in the marrow and outer cortex that can result in smooth or lumpy compact bone  
16 (Ortner & Putschard, 1985; Ortner, 2003; Pinhasi, 2008). The expression of osteomyelitis can  
17 vary depending on age, nature of the initial infection and immunity of the individual (Pinhasi,  
18 2008).

19           Acute infections are usually associated with rapid death rarely affecting the skeleton  
20 but it may also stimulate new bone formation (Ortner & Putschard, 1985; Ortner, 2003). Rapid  
21 bone formation produces woven bone (active lesions) that typically is the initial stage in many  
22 abnormal bone forming lesions caused by infection (Ortner & Putschard, 1985; Ortner, 2003).  
23 In chronic or healing stages (healed lesions) the woven bone is remodelled into compact bone  
24 (Ortner & Putschard, 1985; Ortner, 2003). However, chronic infectious diseases often have  
25 various acute phases. Chronic infections are very informative about the nutritional adequacy

1 of the diet, the state of waste disposal and hygiene in a specific community (Goodman &  
2 Martin, 2002). Infectious pathologies, especially when linked with malnutrition, are the largest  
3 contributor to morbidity and mortality worldwide (Keusch & Farthing 1986). The study of  
4 nutrition-infection interactions is important to understand the complexity of the relationships  
5 of these factors with immunological status, co-morbidity and mortality (Ulijaszek et al. 2012),  
6 especially in pre-antibiotic societies.

7         New bone formation can also be considered an indicator of physiological stress and has  
8 been associated with lower socioeconomic status (e.g. Goodman & Martin, 2002; Peck, 2013;  
9 Robb et al., 2001), systematic infections (e.g. Goodman & Martin, 2002; Larsen, 2002; Ortner,  
10 2003), malnutrition (e.g. Weston, 2012) and niacin deficiency (Paine & Brenton, 2006), which  
11 can leave the individuals more susceptible to pathogens. Deposits of new bone may also be  
12 associated with elevated risks of mortality and are therefore informative about ill health (e.g.  
13 DeWitte & Wood, 2008).

### 14 **1.3. Stable isotope analysis**

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

19         There is enrichment in  $\delta^{13}\text{C}$  in an animal’s body tissues relative to its diet due to the  
20 fractionation that occurs during the tissue’s formation (van der Merwe & Vogel, 1978).  
21 Consumers have a carbon fractionation factor (enrichment in  $\delta^{13}\text{C}$ ) of approximately 5‰ in  
22 their bone collagen relative to their diet (Ambrose & Norr, 1993; van der Merwe & Vogel,  
23 1978) and an enrichment of 1‰ between trophic levels (DeNiro & Epstein, 1978; Tieszen et  
24 al., 1983). There is an increment in  $\delta^{15}\text{N}$  of 3‰ to 5‰ between trophic levels when compared  
25 with consumer’s diet (Bocherens & Drucker, 2003; Minagawa & Wada, 1984; Schoeninger &

1 DeNiro, 1984; Schoeninger et al., 1983). This fractionation enables the use of stable nitrogen  
2 isotopes ( $\delta^{15}\text{N}$ ) to infer trophic level and high  $\delta^{15}\text{N}$  recorded in bone collagen usually indicates  
3 high-protein diets (Sponheimer et al., 2003). There are other factors that can raise bone  $\delta^{15}\text{N}$ ,  
4 such as aridity (Ambrose & DeNiro, 1986; Heaton, 1987; Heaton et al., 1986; Sealy et al.,  
5 1987), physiological (Deschner et al., 2012; D’Ortenzio et al., 2015; Gaye-Siesseger et al.,  
6 2004; Katzenberg & Lovell, 1999; Oelbermann & Scheu, 2001) or protein stress (Hobson et  
7 al., 1993; Steele & Daniel, 1978).

8         Previous research on archaeological samples with and without lesions indicative of  
9 leprosy showed no significant differences in  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$ , suggesting that there were not dietary  
10 differences between the two groups (Bayliss et al., 2004; Linderholm & Kjellström, 2011).  
11 However, other studies showed marked differences between individuals who survived  
12 childhood and those who did not (Beaumont et al., 2015; Reitsema et al., 2016), with the ones  
13 who survived having higher animal protein in their post-weaning diets (Reitsema et al., 2016)  
14 suggesting that investigation of dietary protein, using stable isotopic analysis, might be used to  
15 better understand disease and physiological stress in past populations. Skeletal indicators of  
16 physiological stress, such as low stature and cribra orbitalia, have also been related to long-  
17 term effects on health throughout reduced lifespan (Watts, 2013) and increased risk of death  
18 during epidemics (DeWitte & Hughes-Morey, 2012; DeWitte & Wood, 2008).

#### 19 **1.4. Diet at Tomar**

20 People living in Tomar had a complex diet, low in terrestrial animal protein and high in aquatic  
21 protein intake, despite its inland location (Curto et al., 2018). Being controlled by religious  
22 military orders (Conde, 1996; Valente, 1998), it is possible that their presence in the town  
23 would have an impact on the general population particularly on their diet (Curto et al., 2018),  
24 due to religious fasting (Barber & Bate, 2002; Müldner et al, 2009; Müldner & Richards, 2007;  
25 Salamon et al., 2008). Fish was an expensive food source, particularly further away from the



1 coast (Gonçalves, 2004; Vicente, 2013), therefore higher amounts of fish consumption may  
2 reflect higher socio-economic status (Curto et al., 2018).

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

#### 10 **1.4. Research questions and predictions**

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

17 Protein malnutrition over a long period of time impairs the immune system and  
18 increases the likelihood of an individual contracting an infectious disease (e.g. Calder, 1991;  
19 Scrimshaw & SanGiovanni, 1997; Woodward, 1998; Calder & Jackson, 2000; Woodward,  
20 2001). Therefore, individuals with skeletal signs of infectious diseases might have had different  
21 diets than those without skeletal lesions. Skeletons with signs of infection might have had a  
22 diet poorer in animal protein, than the individuals without lesions, which might have lowered  
23 their resistance to disease (e.g. Calder, 1991; Kuvibidila et al., 1993; Scrimshaw &  
24 SanGiovanni, 1997; Woodward, 1998; Calder & Jackson, 2000; Woodward, 2001; Ulijaszek  
25 et al., 2012; Weston, 2012).

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

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

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

1 is considered a lesion which was active perimortem, while compact bone is considered a lesion  
2 which was healed perimortem (Ortner & Putschard, 1985; Ortner, 2003). Chronic infectious  
3 diseases can also have various acute phases and be very informative about the nutritional  
4 adequacy of the diet in a specific community (Goodman & Martin, 2002). Therefore, bone  
5 collagen  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  from skeletons without lesions (and other skeletal markers of  
6 physiological stress) will be compared with bone collagen  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  from 1) skeletons  
7 with only active lesions, 2) skeletons with only healed lesions and 3) skeletons with both healed  
8 and active lesions. Since Protein malnutrition impairs the immune system (e.g. Calder, 1991;  
9 Scrimshaw & SanGiovanni, 1997; Woodward, 1998; Calder & Jackson, 2000; Woodward,  
10 2001), we predict that skeletons without lesions have higher  $\delta^{15}\text{N}$  than those with lesions, with  
11 the ones with only active lesions having the lowest  $\delta^{15}\text{N}$ . The skeletons with only healed lesions  
12 are expected to have  $\delta^{15}\text{N}$  similar to the skeletons without lesions as they survived the disease  
13 long enough for the bone to remodel into compact bone (Ortner & Putschard, 1985; Ortner,  
14 2003; Wood et al., 1992).

## 15 **2 | MATERIALS AND METHODS**

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

22 Bone collagen stable isotope data (carbon, nitrogen and sulphur) from 32 human adult  
23 tibiae (15 females; 18 males) and 13 faunal remains (2 wild Sus; 2 domestic Sus; 1 juvenile  
24 Sus; 1 Canidae; 3 Bos; 1 Equus; 3 Ovicapridae) from Tomar (11<sup>th</sup> – 17<sup>th</sup> century) were  
25 previously analysed to reconstruct the general diet of the population (Curto et al., 2018). These

1 are reused here and compared to new isotope data from skeletons with signs of disease (Table  
2 1). These data are compared to new isotope ratios from 23 adult individuals (8 females; 14  
3 males; 1 undetermined) with skeletal lesions compatible with non-specific (n=21) and specific  
4 (venereal syphilis, n=2) infectious diseases.

5 All samples are from Santa Maria do Olival graveyard (areas 13 to 20; 11<sup>th</sup> to 17<sup>th</sup>  
6 centuries) in Tomar. The individuals without lesions (n=32), previously analysed (Curto et al.,  
7 2018), were used to estimate the baseline diet at Tomar and were selected based on the absence  
8 of skeletal lesions or skeletal stress markers (see Curto et al., 2018 for more detail; the outlier  
9 was not considered for this study).

## 10 **2.1. Estimating age and sex**

11 Sex was estimated based on pelvic (Phenice, 1969; Buikstra & Ubelaker, 1994) and cranial  
12 features (Buikstra & Ubelaker, 1994). Adult age at death estimates employed a combination of  
13 skeleton maturation (Scheuer & Black, 2000), pubic symphysis degeneration (Brooks &  
14 Suchey, 1990; Buikstra & Ubelaker, 1994) and auricular surface degeneration (Lovejoy et al.,  
15 1985). The skeletons analysed were grouped as young (18 to 30 years; n=5), mature (31 to 60  
16 years; n=8) and old (60+ years; n=4) adults; for six skeletons it was not possible to estimate  
17 age.

## 18 **2.2. Signs of infection**

19 From the 23 skeletons with lesions (Table 1), 21 have signs of non-specific infectious diseases  
20 and 2 have lesions compatible with specific infections (venereal syphilis). The 23 individuals  
21 were grouped in two different ways: a) active (n=6), healed (n=7) and a combination of both  
22 active and healed lesions (n=10); b) Skeletons with only healed tibial periostitis (n=7), those  
23 with non-specific (n=5) and specific (n=2) infectious diseases, while individuals who did not  
24 fit into these groups (n=9) were not considered for this analysis. Figures 2, 3 and 4 show  
25 examples of the different lesion stages analysed.

1           Skeletal lesions were considered to be from possible infectious causes if abnormal bone  
2 formation or bone formation and destruction, compatible with periostitis or osteomyelitis  
3 (Ortner & Putschard, 1985; Buikstra & Ubelaker, 1994; Aufderheide & Rodríguez-Martín,  
4 1998; Ortner, 2003), were present and not associated with trauma. Periostitis usually represents  
5 pathologic changes resulting in new bone growth, which is remodelled into lamellar bone  
6 during the healing process, but it can also result from inflammation of the surrounding tissues  
7 following a trauma (Ortner & Putschard, 1985; Ortner, 2003).

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

14           Lesions with unremodelled woven bone were considered active at the time of death  
15 (Ortner & Putschard, 1985; Ortner, 2003). Rapidly formed woven bone is poorly organized  
16 and has a porous appearance due to the loose organization of the mineralized osteoid fibres  
17 (Ortner & Putschard, 1985; Ortner, 2003). Markedly accentuated longitudinal striations and  
18 compact bony growth, without the presence of woven bone, were considered healed lesions  
19 (Ortner & Putschard, 1985; Ortner, 2003). The presence of both compact bony growth and  
20 woven bone was considered a combination of both healed and active lesions. The skeletons  
21 with only active lesions represent infectious diseases active perimortem and the ones with only  
22 healed lesions represent healed individuals. Skeletons with a combination of both types of  
23 lesions represent chronic infections, to which the individuals survived long enough to the  
24 disease for the bone to heal but with the disease still present. The skeletons with the different  
25 lesions (healed, active and both) were combined and compared with the individuals without

1 lesions, by age group: young without lesions (n=8); young with lesions (n=5); mature without  
2 lesions (n=13); mature with lesions (n=8); old without lesions (n=4) and old with lesions (n=4).

3         Since tibial periostitis is frequently used as an indicator of physiological stress (e.g.  
4 DeWitte, 2010; Robb et al., 2001) and can be caused by a variety of factors, including trauma,  
5 only individuals with bilateral healed periostitis on the tibiae were selected (markedly  
6 accentuated longitudinal striations; score 2; Steckel et al., 2006). The cases of venereal syphilis  
7 were diagnosed due to the presence of caries sicca, a sign specifically characteristic of venereal  
8 syphilis (Ortner & Putschard, 1985; Aufderheide & Rodriguez-Martin, 1998; Ortner, 2003).  
9 These groups with signs of infections were then compared with the skeletons without lesions  
10 (n=32; Curto et al., 2018).

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

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

### 21 **2.3. Collagen extraction and analysis**

22 Collagen extraction was done following Login (1971), Brown et al. (1988) and Richards and  
23 Hedges (1999). The collagen samples were weighed into tin capsules and combusted into CO<sub>2</sub>  
24 and N<sub>2</sub> in an isotope-ratio mass spectrometer at NERC Isotope Geosciences Facility and  
25 HERCULES laboratory. At NERC,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were calibrated using an in-house reference

1 material M1360p (powdered gelatine from British Drug Houses) with expected  $\delta$  values of –  
2 20.32‰ (calibrated against CH<sub>7</sub>, IAEA) and +8.12‰ (calibrated against N-1 and N-2, IAEA)  
3 for carbon and nitrogen respectively. Samples were run in duplicate and the 1 $\sigma$  reproducibility  
4 for mass spectrometry controls for these analyses were  $\delta^{15}\text{N} = \pm 0.08\text{‰}$  and  $\delta^{13}\text{C} = \pm 0.07\text{‰}$ . At  
5 HERCULES Laboratory,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were calibrated using IAEA-CH-6 (sucrose,  
6 –10.449‰), IAEA-CH-7 (polyethylene, –32.151‰), IAEA-N-1 (ammonium sulphate,  
7 +0.4‰) and IAEA-N-2 (ammonium sulphate, +20.3‰). Measurement errors were less than  
8  $\pm 0.1\text{‰}$  for  $\delta^{13}\text{C}$  and  $\pm 0.2\text{‰}$  for  $\delta^{15}\text{N}$ .

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

### 13 **3 | RESULTS**

#### 14 **3.1. Bone collagen $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of skeletons with generalised infections or healed tibial 15 periostitis compared to skeletons without lesions**

16 Osteomyelitis was only observed in the skeletons with venereal syphilis (skeletons 16.225 and  
17 18.158; Appendices: Figure A.1) and skeleton 16.255 ( $\delta^{13}\text{C} = -18.7\text{‰}$ ;  $\delta^{15}\text{N} = 10.0\text{‰}$ ), a mature  
18 male with osteomyelitis on the right tibia. Therefore, the results from this study are focused  
19 mainly on lesions within the scope of periostitis.

20 Figure 5 illustrates the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  for skeletons without lesions (n=32; Curto et al.,  
21 2018), with only healed tibial periostitis (n=7) and those with generalised specific (n=2) and  
22 non-specific (n=5) infections. There is one outlier with healed tibial periostitis ( $\delta^{13}\text{C} = -15.6\text{‰}$ ;  
23  $\delta^{15}\text{N} = 11.5\text{‰}$ ) that seems to have very different diet from the general population and therefore  
24 was not considered for the statistical analysis. Among the individuals with skeletal lesions, the  
25 ones with healed tibial periostitis (n=6; one is an outlier) have the highest mean values for both

1  $\delta^{13}\text{C}$  ( $-18.0\pm 1.1\%$ ; Table 2) and  $\delta^{15}\text{N}$  ( $10.9\pm 0.7\%$ ; Table 2), while those with non-specific  
2 generalised infections ( $n=5$ ) have the lowest mean for  $\delta^{13}\text{C}$  ( $-18.7\pm 0.8\%$ ; Table 1) and  $\delta^{15}\text{N}$   
3 ( $9.9\pm 0.4\%$ ; Table 1). The skeletons with venereal syphilis ( $n=2$ ) have similar mean values  
4 ( $\delta^{13}\text{C}=-18.5\pm 0.2\%$ ;  $\delta^{15}\text{N}=11.2\pm 0.3\%$ ) to the skeletons without lesions ( $n=32$ ;  $\delta^{13}\text{C}=-$   
5  $18.6\pm 0.5\%$ ;  $\delta^{15}\text{N}=10.8\pm 0.8\%$ ) and those with only healed tibial periostitis ( $n=6$ ), however the  
6 sample size is too small for an appropriate statistical analysis. The difference in  $\delta^{15}\text{N}$  between  
7 skeletons with non-specific generalised infection ( $\delta^{13}\text{C}=-18.7\pm 0.8\%$ ;  $\delta^{15}\text{N}=9.9\pm 0.4\%$ ) and  
8 healed periostitis ( $\delta^{13}\text{C}=-18.1\pm 1.2\%$ ;  $\delta^{15}\text{N}=11.2\pm 0.4\%$ ) is highly significant ( $p<0.003$ ; Table  
9 2) as is the difference between skeletons with non-specific generalised infection and those  
10 without lesions ( $\delta^{13}\text{C}=-18.5\pm 0.7\%$ ;  $\delta^{15}\text{N}=10.9\pm 0.9\%$ ) ( $p<0.004$ ; Table 1). There are no  
11 statistically significant differences for  $\delta^{13}\text{C}$  ( $p>0.53$ ; Table 2) or between skeletons without  
12 lesions and skeletons with only healed tibial periostitis for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  ( $p>0.20$ ; Table  
13 2).

### 14 **3.2. Bone collagen $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of skeletons with lesions compared to skeletons without** 15 **lesions, by age groups**

16 Figure 6 illustrates  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  for individuals with (including healed, active or a  
17 combination of both lesions) and without lesions by age group (Table 3). Young adults without  
18 lesions ( $n=8$ ) have higher  $\delta^{13}\text{C}$  ( $-18.5\pm 0.4\%$ ) and  $\delta^{15}\text{N}$  ( $11.4\pm 0.7\%$ ) than the ones with lesions  
19 ( $n=5$ ;  $\delta^{13}\text{C}=-18.8\pm 0.4\%$ ;  $\delta^{15}\text{N}=10.5\pm 0.8\%$ ) but still falling within the two standard deviations  
20 of each other and the general sample without lesions. There is no statistically significant  
21 differences in  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  for the mature (without lesions:  $n=13$ ;  $\delta^{13}\text{C}=-18.6\pm 0.6\%$ ;  
22  $\delta^{15}\text{N}=10.5\pm 0.7\%$ ; with lesions:  $n=8$ ;  $\delta^{13}\text{C}=-18.5\pm 0.5\%$ ;  $\delta^{15}\text{N}=10.7\pm 0.7\%$ ) and old adults  
23 (without lesions:  $n=4$ ;  $\delta^{13}\text{C}=-18.6\pm 0.3\%$ ;  $\delta^{15}\text{N}=10.7\pm 1.2\%$ ; with lesions:  $n=4$ ;  $\delta^{13}\text{C}=-$   
24  $18.4\pm 0.3\%$ ;  $\delta^{15}\text{N}=10.3\pm 0.4\%$ ) ( $p>0.38$ ; Table 3).



1 **3.3. Bone collagen  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of skeletons with active, healed or a combination of both**  
2 **lesions compared to skeletons without lesions**

3 The only healed lesions were found within the mature adults group (Figure 6). Results show  
4 there is no statistically significant difference in  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  when the skeletons without visible  
5 lesions (n=32;  $\delta^{13}\text{C}=-18.6\pm 0.5\text{‰}$ ;  $\delta^{15}\text{N}=10.8\pm 0.8\text{‰}$ ; Table 4) were compared with the  
6 skeletons with healed (n=6;  $\delta^{13}\text{C}=-18.4\pm 0.4\text{‰}$ ;  $\delta^{15}\text{N}=10.8\pm 0.7\text{‰}$ ; p=0.53; Table 4), active  
7 (n=6;  $\delta^{13}\text{C}=-18.5\pm 0.7\text{‰}$ ;  $\delta^{15}\text{N}=10.5\pm 0.7\text{‰}$ ; p=0.72; Table 4) or a combination of both lesions  
8 (n=10;  $\delta^{13}\text{C}=-18.4\pm 0.2\text{‰}$ ;  $\delta^{15}\text{N}=10.7\pm 0.8\text{‰}$ ; p=0.24; Table 4).

9 **4 |Discussion**

10 **4.1. Bone collagen  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of skeletons with generalised infections or healed tibial**  
11 **periostitis compared to skeletons without lesions**

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

21 The only skeleton with osteomyelitis (16.255), besides the ones with venereal syphilis,  
22 has similar  $\delta^{13}\text{C}$  ( $-18.7\text{‰}$ ) and  $\delta^{15}\text{N}$  ( $10.0\text{‰}$ ) to the individuals with non-specific generalised  
23 infections ( $\delta^{13}\text{C}=-18.7\pm 0.8\text{‰}$ ;  $\delta^{15}\text{N}=9.9\pm 0.4\text{‰}$ ; Table 2), suggesting that a diet lower in animal  
24 protein might have made him more susceptible to infectious disease (e.g. Kuvibidila et al.,  
25 1993; Scrimshaw & SanGiovanni, 1997; Woodward, 1998; Calder & Jackson, 2000;

1 Woodward, 2001). Venereal syphilis is a sexually transmitted disease and human hosts have  
2 no natural immunity to pathogenic treponemes (Kiple, 1993). Therefore, the immune system  
3 of the individuals before the disease is not as relevant to the individuals' susceptibility to these  
4 infections. However, good health prior to venereal syphilis infection may prolong the  
5 individual's survival (not only to the treponeme but also to other infections through skin ulcers  
6 which increase exposure to other pathogens) and increase the amount and severity of the lesions  
7 (Wood et al., 1992).

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

14         The osteological paradox (Wood et al., 1999) may explain the higher  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  for  
15 the skeletons with only healed tibial periostitis when compared to the ones with non-specific  
16 generalised infections (Figure 5 & Table 2). It is possible that the skeletons with only healed  
17 tibial periostitis had a diet richer in animal protein and therefore were more resistant to diseases  
18 (e.g. Calder, 1991; Kuvibidila et al., 1993; Scrimshaw & SanGiovanni, 1997; Woodward,  
19 1998; Calder & Jackson, 2000; Woodward, 2001; Ulijaszek et al., 2012; Weston, 2012) than  
20 those who had non-specific generalised infections. It has been argued that individuals with  
21 healed periostitis are of lower frailty, having a lower risk of death (e.g. DeWitte, 2010; Ortner,  
22 2003; Wood et al., 1992).

23         The diet of the population under study was complex and likely included food sources  
24 from outside Tomar (Curto et al., 2018). The diet of these individuals was poor in terrestrial  
25 protein and rich in aquatic protein ( $\delta^{13}\text{C}=-18.6\text{‰}$ ;  $\delta^{15}\text{N}=10.8\text{‰}$ ;  $\delta^{34}\text{S}=13.1\text{‰}$ ; Curto et al.,

1 2018). Stable isotope values are similar for males and females but the young adults have higher  
2  $\delta^{15}\text{N}$  ( $11.4\pm 0.6\text{‰}$ ) than the old adults ( $10.6\pm 0.8\text{‰}$ ), suggesting a higher animal protein intake  
3 for the young individuals (Curto et al., 2018). The high  $\delta^{15}\text{N}$  from skeletons without lesions  
4 seem to be related with higher aquatic protein intake (Curto et al., 2018), which may be related  
5 with these individuals having better health than those with signs of infection. Since fish was  
6 expensive (Gonçalves, 2004) and the military orders had angling rights (Vicente, 2013) it is  
7 also possible that the individuals without skeletal stress markers, or only healed tibial  
8 periostitis, had a higher socioeconomic status. Socioeconomic status may also have an impact  
9 on an individual's diet, not only directly on their diet but also the type of pathogens they would  
10 be exposed to.

11         The effect of protein malnutrition on the immune system is well known (Calder, 1991;  
12 Kuvibidila et al., 1993; Scrimshaw & SanGiovanni, 1997; Woodward, 1998; Calder & Jackson,  
13 2000; Woodward, 2001) and the possibility of dietary differences being present before the  
14 disease cannot be excluded.  $\delta^{15}\text{N}$  were significantly different between skeletons with non-  
15 specific generalised infections and those without lesions ( $p<0.004$ ) or with only healed tibial  
16 periostitis ( $p<0.003$ ). The higher  $\delta^{15}\text{N}$  observed in the two individuals with venereal syphilis,  
17 may not be related to physiological stress but may be due to the nature of the disease instead  
18 (sexually transmitted infection) and the  $\delta^{15}\text{N}$  might suggest a richer diet that could have allowed  
19 survival despite the disease and susceptibility to other pathogens. The possibility of these  $\delta^{15}\text{N}$   
20 differences being related with social status cannot be excluded. Various studies suggest dietary  
21 differences between sex and social status in Medieval times (e.g. Adamson 2004, Kjellström  
22 et al. 2009, Linderholm et al. 2008, Polet and Katzenberg 2003, Schutkowski et al. 1999,  
23 Reitsema et al. 2010, Reitsema and Vercellotti 2012). However, a previous study showed no  
24 significant stable isotope data between individuals of different sex or social status in Tomar  
25 (Curto et al., 2018).

1           There are two outliers among the skeletons sampled for isotopic analysis (Figure 5),  
2 one without lesions and another one with healed tibial periostitis. The skeleton without lesions,  
3 a young adult male, might be an outsider as his sulphur isotopes ratios (9.3‰) differ from the  
4 other individuals without lesions (mean  $\delta^{34}\text{S}$ =13.1‰; Curto et al., 2018). This skeleton was not  
5 considered for the statistical analysis. There are no sulphur isotopes values for the outlier with  
6 healed tibial periostitis but  $\delta^{13}\text{C}$  (-15.6‰) and  $\delta^{15}\text{N}$  (11.5‰) are similar to those of the outlier  
7 without lesions ( $\delta^{13}\text{C}$ =-15.4‰;  $\delta^{15}\text{N}$ =12.3‰).

#### 8 **4.2. Bone collagen $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of skeletons with lesions compared to skeletons without** 9 **lesions**

10 The values for the young adults show a statistical trend towards a significance ( $p<0.09$ ; Table  
11 3) difference in both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  between skeletons with ( $n=5$ ) and without ( $n=8$ ) lesions.  
12 Young individuals without lesions have higher  $\delta^{13}\text{C}$  ( $-18.5\pm 0.4\text{‰}$ ) and  $\delta^{15}\text{N}$  ( $11.4\pm 0.7\text{‰}$ ) than  
13 those with lesions ( $\delta^{13}\text{C}=-18.8\pm 0.4\text{‰}$ ;  $\delta^{15}\text{N}=10.5\pm 0.8\text{‰}$ ), which may suggest that the  
14 individuals with lesions may have had a diet with lower animal protein (Figure 6). There is no  
15 difference for mature ( $p>0.49$ ; Table 3) and old ( $p>0.39$ ; Table 3) individuals with or without  
16 lesions. Previous research on archaeological samples showed marked differences between  
17 individuals who survived childhood and those who did not (Beaumont et al., 2015; Reitsema  
18 et al., 2016), with the ones who survived having higher animal protein in their post-weaning  
19 diets (Reitsema et al., 2016) suggesting that diet at younger ages can have a high impact on the  
20 health status of an individual. The impact of diet on an individual's health might be prolonged  
21 throughout adult life as well. The young adult skeletons analysed do not have healed lesions,  
22 only active or a combination of both active and healed lesions, meaning that they died during  
23 acute phases of the disease (Ortner & Putschard, 1985; Ortner, 2003; Turner-Walker, 2008).

#### 24 **4.3. Bone collagen $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of skeletons with active, healed or a combination of both** 25 **lesions compared to skeletons without lesions**

1 The absence of significant differences in  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  between individuals without lesions  
2 (n=32;  $\delta^{13}\text{C}=-18.6\pm 0.5\text{‰}$ ;  $\delta^{15}\text{N}=10.8\pm 0.8\text{‰}$ ; Table 4) and those with healed (n=6;  $\delta^{13}\text{C}=-$   
3  $18.4\pm 0.4\text{‰}$ ;  $\delta^{15}\text{N}=10.8\pm 0.7\text{‰}$ ; Table 4), active (n=6;  $\delta^{13}\text{C}=-18.5\pm 0.7\text{‰}$ ;  $\delta^{15}\text{N}=10.5\pm 0.7\text{‰}$ ;  
4 Table 4) or a combination of both lesions (n=10;  $\delta^{13}\text{C}=-18.4\pm 0.2\text{‰}$ ;  $\delta^{15}\text{N}=10.7\pm 0.8\text{‰}$ ; p=0.24;  
5 Table 4) suggests that diet may have a higher impact on the susceptibility to chronic generalised  
6 infections than to infectious disease in general. It is therefore important to take into account the  
7 severity and stage of the disease. The  $\delta^{15}\text{N}$  average is slightly higher for the individuals without  
8 lesions (10.8‰; n=32) than for the one ones with active lesions (10.5‰; n=6; Table 4). This  
9 slight difference may indicate that the individuals without lesions had a diet richer in animal  
10 protein than those with active lesions, however the sample size is too small to make  
11 conclusions.

## 12 **5 | STUDY LIMITATIONS**

13 One of the limitations of this study is the impossibility of knowing the cause of death for the  
14 individuals analysed, alongside it not being possible to know which diseases caused most of  
15 the lesions and how long the individuals survived with the infections. The presence of skeletal  
16 lesions can represent an adaptation to a pathological condition (Ortner, 2003) indicating that  
17 the individual survived long enough for evidence to manifest in the skeletal tissues (Wood et  
18 al., 1992). The absence of skeletal lesions is ambiguous; it can indicate either good health, or  
19 a fast death as result of an acute disease (DeWitte & Stojanowski, 2015; Siek, 2013; Ortner,  
20 2003; Wood et al., 1992). Another limitation is that, while individuals with poorer nutrition are  
21 less resistant to infectious diseases, infectious disease further lowers nutritional status (e.g.  
22 Mata et al., 1971; Martorell, 1980; Calder, 1991; Scrimshaw & SanGiovanni, 1997; Calder &  
23 Jackson, 2000; Keusch, 2001).

## 24 **6 | CONCLUSION**

1 This study is part of a larger project that will compare intra-bone stable isotopic data from sites  
2 with and without skeletal lesions compatible with diseases and/or physiological stress. This  
3 study explored the dietary differences between individuals with and without skeletal lesions  
4 compatible with infectious diseases to better understand the impact of diet on individuals'  
5 health status and their susceptibility to infectious disease. There is a highly significant  
6 difference in  $\delta^{15}\text{N}$  between skeletons with healed tibial periostitis and non-specific generalised  
7 infection, as well as a difference at the margin of statistical significance between skeletons  
8 without lesions and those with generalised infections. These results demonstrate that the  
9 individuals with non-specific generalised infections had diets lower in animal protein than  
10 those without lesions or with only healed tibial periostitis. Poorer diets may increase  
11 susceptibility to pathogens leading more frequently to generalised infections while richer diets  
12 might increase the survivorship and ability to heal from infectious diseases. However, the  
13 possibility of these isotope ratios being a result of the disease cannot be excluded and more  
14 data from different periods of time within the individual's' life is necessary to understand when  
15 these differences started to manifest. These results indicate that diet has a higher impact on the  
16 health status of young people than mature or old individuals, being linked to selective mortality.  
17 Our results demonstrate that while non-specific generalised infections are a sign of ill health  
18 and poor diet, only healed tibial periostitis indicate a state of comparatively good overall health  
19 and diet.

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1           **Figure legends**

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

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

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

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

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

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

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