An isotopic case study of individuals with syphilis from the pathological-anatomical reference collection of the national museum in Prague (Czech Republic, 19th century A.D.)

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A B S T R A C T

Objective: This paper aims at investigating the possible existence of isotopic offsets in $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values in relation to tertiary syphilis.
Material: Based on materials from the 19th c. A.D. deriving from the pathological-anatomical reference collection (the Jedlička collection) of the National Museum in Prague (Czech Republic), a comparative approach of ten individuals with syphilis and nine without the disease was undertaken.
Methods: Bone powder samples were defatted according to the protocol of Liden et al. (1995). Bone collagen was extracted following the protocol of Bocherens et al. (1991).
Results: Our results show that individuals with syphilis have lower $\delta^{13}C_{col}$ values than individuals without the disease; the observed difference between the two groups is about 0.3-0.4‰, which is relatively small but still meaningful. However, no difference between $\delta^{15}N_{col}$ values of the two groups has been noticed.
Conclusions: Either diets prescribed by physicians to syphilitic patients or nutritional stress caused by cyclic appetite disturbance due to the disease itself or the administered medical treatment appeared to be possible explanations of the observed isotopic pattern. Overall, the response of the two isotopic proxies could argue for relatively limited nutritional restrictions.
Significance: This is the first study examining bone collagen isotopic response to syphilis based on clinically documented human skeletal materials.
Limitations: The sample sizes are relatively small and cautiousness must be taken regarding the interpretations of the data.
Suggestions for further research: Compound-specific stable isotope investigations and analysis of mercury content could be helpful to better understand the observed isotopic effects.

1. Introduction

Analysis of stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in soft and hard human tissues is a widely used and powerful tool to investigate modern and past diets (DeNiro and Epstein, 1976; Kaupová et al., 2014, 2018; Polet and Katzenberg, 2003; Richards et al., 1998; Salesse et al., 2013, 2018; Schwarcz et al., 1985). Isotopic values of human bodies derive from values of consumed food but are generally shifted due to predictable diet-tissue spacing specific to each element.

In collagen, stepwise enrichments in ^{13}C and ^{15}N occur between trophic levels. Correspondingly, $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values are expected to increase between diet and collagen consumer (Bocherens and Drucker, 2003; DeNiro and Epstein, 1978, 1981; Salesse, 2015; Schoeninger and DeNiro, 1984). Therefore, $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values, when used in conjunction with one another, provide semi-independent lines of evidence for understanding an individual's trophic position in a precise ecosystem. However, many environmental parameters (e.g., Hobbie et al., 2001; Stewart et al., 1995), cultural factors (e.g., Fraser et al.,

2011; Warinner and Tuross, 2009) and physiological processes (e.g., Ambrose and DeNiro, 1986; Fuller et al., 2004; Mekota et al., 2009; Waters-Rist and Katzenberg, 2010) may influence the isotopic signatures in different ways and make dietary reconstruction a challenge.

The health condition of a person can affect appetite and dietary habits, as well as metabolism, which controls the assimilation of macronutrients. Fair or poor health status may result in isotopic offsets and significantly modify the isotopic signatures recorded in proteinaceous tissues (e.g., Fuller et al., 2005; Mekota et al., 2006; Petzke et al., 2006). Surprisingly, pathology-influenced isotopic fractionation in bone collagen has been poorly explored in both archaeological and modern material (for detailed reviews on this topic, see Reitsema (2013), Richards and Montgomery (2012)). Thus far, only one study from clinically documented human skeletal remains has been published by Katzenberg and Lovell (1999). This pioneering work was explorative, which explains the restricted number of individuals sampled (n = 7). It aimed at examining the bone collagen responses to pathological conditions and changes in $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values for various bone disorders: i.e., atrophy, fracture, periostitis and osteomyelitis. The authors indicate that three pathological lesions engendered stable isotope shifts. Osteomyelitis led to an increase of the $\delta^{15}N_{col}$ values (~ 2‰), whereas post-paralytic atrophy and healed fracture were associated with limited isotopic changes compared to defined normal variation. Despite calls to replicate the study on additional bone disorders (e.g., Richards and Montgomery, 2012), it has never been repeated.

Hence, investigations to determine whether, and to what extent, treponemal diseases, and more specifically syphilis, influence stable isotope signatures in bone collagen is currently missing. The few studies include isotopic data of individuals with syphilis are based on archaeologically-derived skeletal material (Dent, 2017; Mays et al., 2003, 2012; Olsen, 2013; Olsen et al., 2011; Rissech et al., 2013; Roberts et al., 2013; Santos et al., 2013; Schwarz et al., 2013). Only two works paid attention to the potential alteration of the original stable isotope values due to the health condition of the sampled individuals (Dent, 2017; Olsen, 2013; Olsen et al., 2011). Dent (2017) showed that the $\delta^{13}C_{col}$ values of the putative individuals with syphilis (n = 2) did not differ from the rest of the sample, whereas the $\delta^{15}N_{col}$ values were lower. The study initiated by Olsen et al. (2011) and extended by Olsen (2013) revealed that dentin $\delta^{13}C_{col}$ values of presumptive individuals with syphilis (n = 4) were slightly higher than those of rib $\delta^{13}C_{col}$ values (~ 0.4‰), while no significant difference in intra-skeletal $\delta^{15}N_{col}$ values were observed. It appears from the literature that no clear isotopic pattern for syphilis emerges and that there is a need for an in-depth examination.

This paper aims to better understand the effects of tertiary syphilis on $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values. To achieve this objective, individuals with and without syphilis (n = 19) dating from 19th c. A.D. from the pathological-anatomical reference collection (the Jedlička collection) of the National Museum in Prague (Czech Republic) have been used.

2. Stable isotope analysis principles

Collagen is composed of amino acids (AA) deriving essentially from dietary materials (Chisholm, 1989). As nitrogen from dietary intake comes from proteins exclusively, the collagen $\delta^{15}N$ values accordingly reflect the protein fraction of diet (Schwarcz and Schoeninger, 1991). The nitrogen balance of an organism was proved to impact nitrogen isotopic variation in the contexts of eating disorders and pregnancy among humans, as well as in a number of controlled-feeding experiments on different animal species (Richards and Montgomery, 2012; Reitsema, 2013). A negative nitrogen balance results from efforts by the organism to compensate for protein insufficiency by catabolising its own tissues. Recycled body tissues repeat the fractionating processes of transamination and deamination, leaving the body tissues of a stressed individual more enriched in ^{15}N (Reitsema, 2013). However, some studies observed exceptions to this pattern, suggesting the existence of a

threshold level of stress below which $\delta^{15}N$ values are unaffected (Hatch, 2012). Interestingly, increased bioavailability of protein – caused either by an excess of protein in the diet or by a physiological issue – has a similar impact on $\delta^{15}N$ values. This is due to deamination resulting from the efforts of the organism to discharge the protein surplus (Sponheimer et al., 2003; Reitsema, 2013). In contrast to nitrogen, the origin of carbon atoms from collagen has been the subject of much debate since the latter might be obtained from dietary proteins or other ingested macronutrients under certain conditions (Sillen et al., 1989). The essential AAs (eAAs) contribute $\sim 22\%$ of the carbon atoms in the collagen structure. As eAAs cannot be indigenously generated, they must be assimilated whole – or as skeletons to be transaminated – from the protein fraction of diet, and their isotopic composition is directly inherited from the ingested foods. The remaining $\sim 78\%$ of carbon atoms in collagen is contributed by non-essential AAs (neAAs). There are many pathways for the synthesis of neAAs in the body, with or without dietary input. The composition of the internal metabolic pools of carbon used to build up the neAAs has been long discussed (Burleigh and Brothwell, 1978; Van Der Merwe, 1982; Krueger and Sullivan, 1984; Ambrose and Norr, 1993). Based on results from controlled animal feeding experiments, recent modelling studies proposed relatively fixed contributions of carbon from protein and energy (lipid and carbohydrate) fractions of diet to collagen (Craig et al., 2013; Fernandes et al., 2012; Froehle et al., 2010). For instance, an energetic contribution of 26 ± 4% was assumed by Fernandes et al. (2012). Furthermore, controlled feeding studies highlighted that under condition of low protein intake, neAAs concentrations in extracellular fluids could decrease below the threshold that is required to activate the enzymes to form the needed neAAs for collagen synthesis (Ambrose and Norr, 1993; Hare et al., 1991; Howland et al., 2003; Jim et al., 2004; Tieszen and Fagre, 1993; Warinner and Tuross, 2009). Consequently, substantial amounts of carbon deriving from dietary carbohydrates, and to a lesser extent from dietary lipids, may influence the isotopic composition of collagen (Schwarcz, 2000). Experimental studies involving nutritionally stressed animals have also shown that carbon in proteinaceous tissues could derive from non-protein dietary sources (Podlesak and McWilliams, 2006; Polischuk et al., 2001; Robb et al., 2015; Williams et al., 2007). Thus, under conditions of nutritional restrictions, the collagen $\delta^{13}C$ values of humans could be better correlated with whole diet.

Thus, the typical reaction of an organism to nutritional stress or disruption of protein metabolism appears to be: 1) an increase of $\delta^{15}N_{col}$ values, mainly as a result of protein catabolism; and 2) a decrease of $\delta^{13}C_{col}$ values, as a result of the endogenous synthetization of AAs and the mobilization of fat stores (Schwarcz, 2000; Mekota et al., 2006; Neuberger et al., 2013). Here, we propose to explore, taking into account the symptoms of tertiary syphilis, if this disease influences the protein metabolism of the afflicted individuals significantly enough to provoke systemic isotopic response in the slow remodeling tissue of bone collagen.

3. Skeletal manifestation of syphilis

Syphilis is a chronic and multistage disease caused by *Treponema pallidum* subsp. *pallidum*, a bacterium classified under the *Spirochaetaceae* family (De Melo et al., 2010; Hackett, 1963). Controversy surrounds the origin and antiquity of this disease. A plethora of papers proposing numerous hypotheses on these topics has been generated but failed to end the debate (Aufderheide and Rodríguez-Martín, 1998; Baker and Armelagos, 1988; Blondiaux, 2008; Crane-Kramer, 2002; Crosby, 2003; Dutour et al., 1994; Gaul et al., 2015; Guerra, 1978; Harper et al., 2011; Harrison, 1959; Quétel, 1990; Rothschild, 2005; Wood, 1978; Zuckerman et al., 2016). For a brief history of the disease in the Czech lands, the reader is encouraged to consult the works of Mikalová (2013) and Vargová et al. (2017). Of interest is the geographical and climatic area of the Czech lands. Located in the mild

climatic zone of central Europe, it is assumed that only venereal syphilis occurs, either in its acquired (i.e. sexually transmitted) or congenital form (Vargová et al., 2017). The causative agent of syphilis was discovered in 1905 (Schaudinn and Hoffmann, 1905). Up until 1909, the year of the discovery of the antisyphilitic effects of arsenicals, mercury was the mainstay of treatment for syphilis (Forrai, 2011; O'Shea, 1990). Arsenic compounds were widely used in Europe until they were superseded by penicillin in the 1940s (Douglas, 2009; Mahoney et al., 1943; Stokes et al., 1944).

Based on clinical findings, syphilis has been divided into three progressive stages: i.e. primary, secondary, and tertiary (LaFond and Lukehart, 2006; Radolf et al., 2006). Clinical data has established that approximately 20–50% of untreated cases of venereal syphilis progress to the tertiary stage (Aufderheide and Rodríguez-Martín, 1998). Although transient cranial periostitis may appear during the secondary stage of syphilis, the majority of bone changes appear during the tertiary stage (Harper et al., 2011; Ortner, 2003). Syphilis, as other treponemal diseases, leaves an osseous signature, most prominently marked by periosteal reaction, tibial remodeling and, occasionally, by bone destruction, referred to as gumma (Rothschild and Rothschild, 1995). The tibia followed by the nasal-palatal area and the cranial vault appear to be the bones affected most often (Buckley and Dias, 2002; Fournier, 1906; Ortner, 2003; Marden and Ortner, 2009; Cook and Powell, 2012).

4. Materials

For this study, we had access to the Jedlička collection curated at the depositories of the Department of Anthropology of the National Museum in Prague. The collection consists of about 6000 anatomical preparations of soft and hard tissue disorders gathered between approximately 1840 and 1950 during clinical human dissections. It contains unique manifestations of inflammatory and infectious bone diseases from the pre-antibiotic era, as well as numerous examples of skeletal abnormalities, bone damage, and other osteological changes. In most instances, the medical diagnoses have been made on living patients. Clinical reports and medical publications describing the anatomized cases have been preserved to this day. Recently, an exhaustive review of all the pathological dry bones has been performed and confirmed the previous diagnoses of the diseases. The remarkable pathological cases on upper and lower extremities and their differential diagnoses have been published in a peer-reviewed atlas by Smrčka et al. (2009).

We selected bones belonging to ten individuals with syphilis and nine without the disease. The relatively limited number of samples can be explained by the status of the Jedlička materials. As a museum collection, long-term safety and sustainability regulations protect these pathological-anatomical preparations. Since sampling is destructive, the research agreement restricted sampling of the bone in order to protect their museum value. Hence, multisampling of individuals with syphilis to understand intra-skeletal or intra-bone isotopic variations and perhaps short-term dietary changes was not achievable. First, bones with pathological lesions are generally the only skeletal segments preserved from the anatomized individuals. Second, the preserved bones display extensive lesions (gumma, periosteal reactive bone, *etc.*) intended to be used for teaching. Sampling areas of bone macroscopically unaffected by pathological change was virtually impossible.

The ten sampled pathological bones are all tibiae (Table 1) and present identifiable macroscopic lesions of tertiary syphilis (cf. Fig. 1). Only periosteal lesions have been targeted for sampling. Six of these cases (AJ2913, AJ2914, AJ2918, AJ2943, AJ3155 and AJ3162) are described in Ortner and Putschar (1981) and Smrčka et al. (2009). The group displaying syphilis comprises one subadult and nine adults (Table 1). The biological sex is reported only for three individuals, i.e. one male and two females (Table 1). All these sampled cases date from the second half of the 19th c. A.D., corresponding to the era preceding the application of penicillin therapy.

The nine bones without syphilitic lesions constitute a negative control group. No conditions except healed fractures are recorded in clinical documents and are visible on the selected bones. The control groups consists of femora only (Table 1). The bone sections sampled are distant to any recorded lesions. Seven of the control cases (AJ2863, AJ2877, AJ3297, AJ3298, AJ3300, AJ3306 and AJ3312) are published in Smrčka et al. (2009). This group contains seven adult males and two adult females (Table 1). All these individuals have been anatomized between 1847 and 1896 (Table 1).

Remodeling rates of cortical bone may substantially vary between the different skeletal segments (Cox and Sealy, 1997; Hedges et al., 2007; Manolagas, 2000; Shagina et al., 2012; Valentin, 2002). However, according to Ruff and Hayes (1988), it can be demonstrated that the tibia and femur have similar average bone remodeling rates, regardless the sex of the individuals and the bone areas under study ($\sim$ 4.1–4.5% per decade for cortical bone). The $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values acquired from tibiae of individuals with syphilis and femora of individuals without syphilis are therefore worthy of comparison.

Table 1
Presentation of isotopic and biological data of syphilitic and non-syphilitic individuals from the Jedlička collection (Prague, 19th c. A.D.).

#ID	Bone disorder	Sex	Age at death	Age category[*]	Sampled bone segment	Date of anatomization	$\delta^{13}C_{col}$	$\delta^{15}N_{col}$	%C	%N	C:N	%Col
AJ2913	Syphilis	Female	31	YA	Tibia	1891	−20.0	11.2	45.5	16.5	3.2	23.9
AJ2914	Syphilis	Female	27	YA	Tibia	1872	−19.9	11.0	42.4	14.5	3.4	17.6
AJ2918	Syphilis	–	18+	A	Tibia	[1850-1900]	−20.4	9.1	45.3	16.1	3.3	24.6
AJ2943	Syphilis	–	18+	A	Tibia	[1850-1900]	−19.8	12.1	44.8	15.9	3.3	23.4
AJ2951	Syphilis	Male	54	OA	Tibia	1879	−20.3	11.0	45.5	15.3	3.5	24.6
AJ3149	Syphilis	–	15-18	J	Tibia	[1850-1900]	−20.1	11.6	43.7	15.8	3.2	26.3
AJ3155	Syphilis	–	18+	A	Tibia	[1850-1900]	−20.5	10.8	45.9	16.1	3.3	24.3
AJ3162	Syphilis	–	18+	A	Tibia	[1850-1900]	−19.1	13.1	44.7	16.3	3.2	24.1
AJ3173	Syphilis	–	18+	A	Tibia	[1850-1900]	−19.9	12.2	45.9	16.7	3.2	24.9
AJ3176	Syphilis	–	18+	A	Tibia	[1850-1900]	−20.1	12.0	44.5	16.0	3.2	25.4
AJ2863	Control	Male	41	MA	Femur	1881	−19.5	11.4	44.6	16.2	3.2	22.8
AJ2876	Control	Male	49	MA	Femur	1862	−19.5	11.8	41.7	15.2	3.2	21.6
AJ2877	Control	Female	18+	A	Femur	1871	−19.6	12.1	41.2	15.0	3.2	23.1
AJ3297	Control	Male	23	YA	Femur	1883	−19.7	11.2	44.7	16.4	3.2	22.7
AJ3298	Control	Male	22	YA	Femur	1847	−20.3	12.3	42.3	15.4	3.2	23.4
AJ3299	Control	Male	52	OA	Femur	1888	−19.8	10.4	45.0	16.5	3.2	22.0
AJ3300	Control	Male	45	MA	Femur	1865	−19.9	10.5	44.8	16.5	3.2	21.5
AJ3306	Control	Female	73	OA	Femur	1870	−19.5	11.5	43.6	15.8	3.2	24.3
AJ3312	Control	Male	58	OA	Femur	1896	−19.5	11.0	45.3	16.5	3.2	23.0

* Age categories – J = Juvenile; A = Adult; YA = Young adult; MA = Middle adult; OA = Old adult.
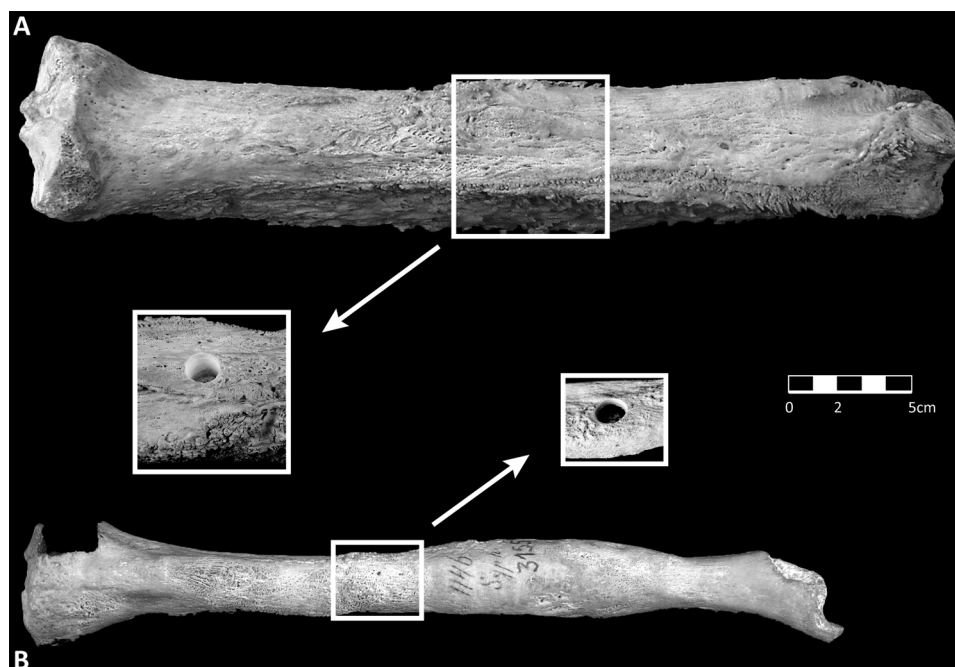
Fig. 1. Sampled tibias of two adult individuals infected by *Treponema pallidum pallidum*. A: case n°2918. B: case n°3155. Note – Bones are at the same scale.

However, bone remodeling process can be altered by certain bone disorders (Feng and McDonald, 2011; Ortner, 2003). In the case of tertiary syphilis, remodeling rates of affected bone areas can greatly increase and engender thickening of the cortical bone. Bone changes attributed to syphilis may develop and be remodeled throughout the tertiary stage (Ortner, 2003).

5. Methods

5.1. Bone collagen preparation and isotope measurements

Sampled bone was cleaned using a tungsten carbide drill bit to retain compact parts. Bone fragments were crushed into a powder using a ball mill, and particles ranging between 0.3 and 0.7 mm were collected. Prior to collagen extraction, bone powder samples were defatted according to the protocol developed by Kates (1986) and tested by Liden et al. (1995). Powdered bone was soaked in 10 ml of a methanol-chloroform mixture (2:1, v/v) and placed in an ultrasonic bath. After 10 min, the supernatant containing lipids was discarded and the solution was renewed. This step was repeated several times until there was complete elimination of fatty acids and their derivatives. Then, the samples were thoroughly rinsed and oven-dried at 65 °C for 7 h. Bone collagen was extracted following the protocol of Longin (1971), which was adapted by DeNiro and Epstein (1981) and Bocherens et al. (1988, 1991). Bone powder samples ($\approx$ 200 mg) were decalcified in 40 ml of 1 M hydrochloric acid at room temperature for 20 min. Gelatins were retrieved by filtration via Fisherbrand membranes (0.5 µm pores), rinsed, and then placed into 0.125 M sodium hydroxide at room temperature for 20 h to remove fulvic and humic acids. The samples were filtered as previously described and rinsed. Gelatins were subsequently solubilized in 0.01 M hydrochloric acid at 100 °C for 17 h and filtered using a 5–8 mm Ezee-filter to trap possible impurities. Finally, collagen samples were freeze-dried at −110 °C for a minimum of 48 h and extraction yields (%Col) were calculated (expressed as a weight percentage, wt.%). Bone preparations and chemical treatments were conducted at the Department of Anthropology of the National Museum in Prague.

Bone collagen samples were analyzed for $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ using a Europa Scientific Roboprep elemental analyzer coupled with a Europa Scientific 20-20 isotope ratio monitoring mass spectrometer at Iso-

Analytical Limited (Crewe, UK). Duplicate measurements were performed on 20% of samples to test reproducibility. International and in-house standards (IA-R042: NBS-1577B, IA-R038:L-alanine, IA-R006/IA-R046: mixture of cane sugar and ammonium sulfate) were also analyzed for quality control. Carbon and nitrogen contents of samples are expressed as percentages. Measurement errors calculated from 14 duplicates of the IA-R042 standard were ± 2.49 (1σ) for %C and ± 0.55 (1σ) for %N. Weight percent carbon and nitrogen were used to calculate atomic C:N ratios. The $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values were reported as per mil (‰) deviation relative to VPDB and AIR, respectively. Analytical errors calculated from 28 duplicates of the three standards were better than ± 0.10‰ (1σ) for $\delta^{13}C_{col}$ and ± 0.14‰ (1σ) for $\delta^{15}N_{col}$.

5.2. Collagen quality indicators

In modern bones, extraction yields are around 20.4 ± 3.9 wt% (1σ) (Bocherens et al., 1991), and samples containing less than 1 wt.% of collagen are considered unreliable (Dobberstein et al., 2009; Van Klinken, 1999). Moreover, carbon and nitrogen contents of modern bone range from 15.3 to 47% and from 5.5 to 17.3%, respectively (Ambrose, 1990). Bone collagen samples with carbon and nitrogen contents below 13% and 4.8%, respectively, are generally recognized as severely altered (Ambrose, 1990; Garvie-Lok, 2001; Iacumin et al., 1998; Reitsema, 2012; Van Klinken, 1999). Finally, atomic C:N ratios of modern bones are generally around 3.2–3.3 (Ambrose, 1990; Van Klinken, 1999), but can vary between 2.9 and 3.6 (DeNiro, 1985), and samples presenting values below or above these thresholds indicate alteration or contamination (Ambrose, 1990; DeNiro and Weiner, 1988; Grupe, 2001). Samples that fail to pass any of these criteria must be withdrawn from the study (Salesse et al., 2014; Salesse, 2015).

6. Results

The collagen quality indicators, as well as the isotopic results for the individuals with syphilis and those without are presented in Table 1. Collagen was successfully extracted from all of the bone samples. Collagen extraction yields vary between 17.6 and 26.3 wt.% (mean = 23.3 ± 1.9 wt.%, 1σ), and all exceed the minimum threshold of 1 wt.% indicating a satisfactory sample preservation. With values between 41.2
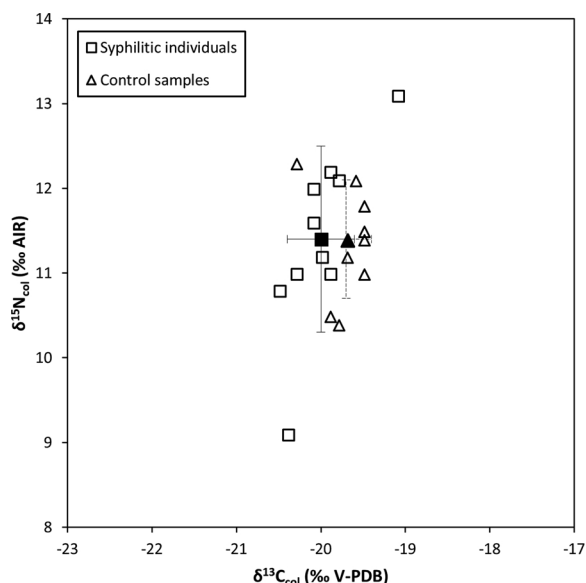
Fig. 2. Biplot showing the $\delta^{13}C_{col}$ against $\delta^{15}N_{col}$ values of syphilitic individuals and control samples from the Jedlička collection (Prague, 19th c. A.D.). Mean and standard deviation (1σ) for each group are presented as well.

and 45.9% (mean = 44.3 ± 1.4%, 1σ) and 14.5 and 16.7% (mean = 15.9 ± 0.6%, 1σ), carbon and nitrogen values fall in the upper part of the reference range for well-preserved collagen samples. The atomic C:N ratios fluctuate between 3.2 and 3.5 (mean = 3.2 ± 0.1%, 1σ), which is well within the acceptable range of variation for unaltered and uncontaminated collagen samples. The collagen extracts of individuals with syphilis and those without met all the criteria for good-quality collagen described above. Isotopic results of both groups of individuals can be considered reliable and used for data analysis and interpretation.

The $\delta^{13}C_{col}$ values of the individuals with syphilis range from -20.5 to -19.1‰ (mean = –20.0 ± 0.4‰, 1σ), while the $\delta^{15}N_{col}$ values range from 9.1 to 13.1‰ (mean = 11.4 ± 1.1‰, 1σ) (Fig. 2). The $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values of the control group range from -20.3 to -19.5‰ (mean = –19.7 ± 0.3‰, 1σ) and from 10.4 to 12.3‰ (mean = 11.4 ± 0.7‰, 1σ), respectively (Fig. 2). The diseased and control groups have significantly different mean $\delta^{13}C_{col}$ values (Mann–Whitney U test; W = 18, Z = –2.18, p-value = 0.03). On average, the individuals with syphilis exhibit lower $\delta^{13}C_{col}$ values than those without the disease (Fig. 2). The mean difference between these two groups is 0.3‰. In contrast, there is no significant difference in $\delta^{15}N_{col}$ values between these two groups (Mann–Whitney U test; W = 42, Z = –0.21, p-value = 0.83). The mean $\delta^{15}N_{col}$ values of both groups are identical (Fig. 2).

The individuals with syphilis exhibit a slightly greater dispersion of their $\delta^{13}C_{col}$ values (s² = 0.2; σ = 0.4‰) compared to the control group (s² = 0.1; σ = 0.3‰). Similarly, the variability of the $\delta^{15}N_{col}$ values in the group with the disease are much larger (s² = 1.2; σ = 1.1‰) than the values in the control group (s² = 0.4; σ = 0.7‰). This is mainly due to the presence of individuals with syphilis (AJ2918 and AJ3162) presenting the most atypical combinations of isotopic values. Specifically, AJ2918 presents the lowest $\delta^{15}N_{col}$ value (9.1‰) while AJ3162 has the highest $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values (−19.1‰ and 13.1‰, respectively) of all the sampled individuals. When AJ2918 and AJ3162 are removed from the sample, the dispersion of the $\delta^{13}C_{col}$ values in the diseased group becomes extremely low (s² = 0.1; σ = 0.2‰). In this case, the diseased and control groups still have significantly different mean $\delta^{13}C_{col}$ values (Mann–Whitney U test; W = 9, Z = −2.58, p-value = 0.01). The discrepancy between mean $\delta^{13}C_{col}$ values increased to 0.4‰. In contrast, the difference in $\delta^{15}N_{col}$ values between these two groups is still not significant (Mann–Whitney U test; W = 33, Z = −0.24, p-value = 0.80).

7. Discussion

To understand the observed isotopic pattern of the individuals with syphilis, sources of isotopic variation must be explored: 1) postmortem contamination of collagen extracts by embalming or biocidal products; 2) different diets based on biological and socioeconomic parameters; and 3) changes in protein metabolism related to the pathological condition itself or its therapy.

Diagenetic alteration can be easily ruled out since the sampled bones were never buried. If postmortem contamination was present, it would have occurred only through embalming solutions used for preserving dissected specimens or biocides used for removing soft tissues on processed bone segments. Unfortunately, documentation does not provide information on the products employed for tissue processing or preservation. However, embalming mixtures and cleaning agents present complex chemical compositions with carbon-rich but nitrogen-poor compounds (Brenner, 2014; McDonnell and Russell, 1999; Van Sint Jan and Rooze, 1992). Serious contamination affecting collagen integrity and causing shifts in isotopic values can be dismissed since all the sampled bones show collagen quality indicators akin to those of unaltered modern or fresh bones. Nevertheless, minor contamination particularly affecting collagen carbon contents or atomic C:N ratios, while still remaining inside the acceptable ranges of variation, might be the source of slight modification of the biogenic isotopic signals. However, an in-depth investigation of these indicators refutes the hypothesis that contamination occurred due to embalming or biocidal products. All bone collagen samples show a very limited dispersion of carbon contents (mean = 44.3 ± 1.4%, 1σ). Samples from patients with syphilis present more restricted distributions of carbon concentrations (mean = 44.8 ± 1.1%, 1σ) than samples from unaffected individuals (mean = 43.7% ± 1.6%, 1σ). Additionally, the atomic C:N ratios of the samples are consistent (mean = 3.2 ± 0.1%, 1σ) and no aberrant enrichment of carbon atoms appears compared to nitrogen contents. Furthermore, no significant relationship between collagen carbon contents or atomic C:N ratios and $\delta^{13}C_{col}$ values is observed at group levels (R² ≤ 0.2). Finally, there is no relationship between the $\delta^{13}C_{col}$ values and dates of dissection (R² = 0.08). Such a relationship would have been expected if contaminating products had changed over time (Fig. 3). Therefore, the discrepancy regarding the $\delta^{13}C_{col}$ values between the individuals with syphilis and those without cannot be explained by minor postmortem contamination and/or different
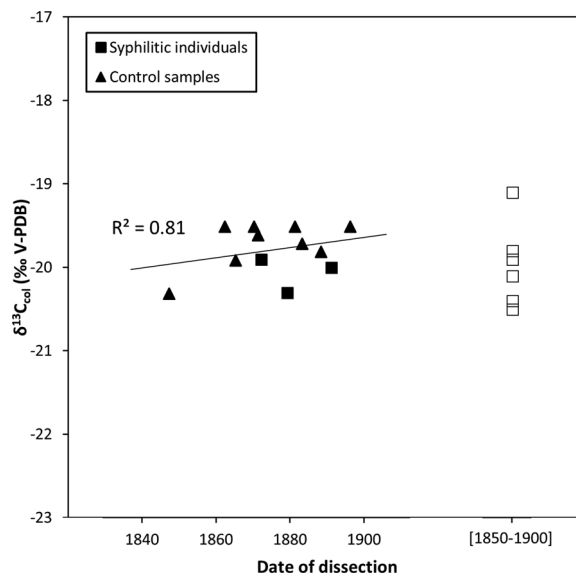

Fig. 3. Biplot showing the $\delta^{13}C_{col}$ values against the dates of anatomization of sampled syphilitic and non-syphilitic individuals. Note – Open symbols: Dissections occurred between 1850 and 1900.

chemical treatments of processed cadavers and bones. These results are similar to the conclusions of Urzel (2014), who established through solid-state ^{13}C nuclear magnetic resonance spectroscopy that embalming solutions and biocidal agents did not change the physicochemical properties of the bone samples, and that no accumulation of exogenous carbon atoms was perceptible in the collagen matrix.

Differential access to food by the sampled individuals should be also explored. The shift in $\delta^{13}C_{col}$ without a corresponding deviation in $\delta^{15}N_{col}$ observed between these two human groups from the same geographic area might be explained by different compositions of C_3/C_4 plant portions in diet, or by consumption of various plants within a C_3 plant spectrum (DeNiro and Epstein, 1978; Cernusak et al., 2009; Sjögren, 2017). Differential access to food due to socioeconomic status has been frequently highlighted in isotopic studies (e.g., Herrscher et al., 2017; Kaupová et al., 2018; Linderholm and Kjellström, 2011; Reitsema and Vercellotti, 2012). Available medical documentation does not provide much information about occupation or social class of the sampled individuals. However, based on reports from the Faculty of Medicine, as well as from the general hospital (1875, 1881, 1885, 1890, 1895 and 1899) in Prague, brief insight into patients suffering from syphilis during the second half of the 19th c. A.D. can be made. It appears that the incidence of syphilis from 1875 to 1899 varied between 150 to 950 new cases per year, with a mean of 550 annual cases (Kružicová, 2013). The afflicted patients were primarily from the working class (Brázdová, 2015; Kružicová, 2013). Anatomized subjects who were included in the Jedlička collection were likely individuals from the lowest socioeconomic classes. Cadaver procurement among the poor and marginalized segments of society was a common practice across Europe (Ghosh, 2015; Mitchell et al., 2011; Richardson, 2001). Based on these elements we can assert that the socioeconomic background is not a parameter explaining the discrepancy between the two groups with regard to the $\delta^{13}C_{col}$ values. Furthermore, it has been established that no physiological basis for the incorporation of the carbon and nitrogen atoms into bone collagen exists associated with age or sex differences among adults (DeNiro and Schoeniger, 1983; Lovell et al., 1986). However, age- and sex-related variation of $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values are often observed within human populations due to other factors influencing food choices, such as preferential access of males or females to certain food groups (Prowse, 2011; Reitsema and Vercellotti, 2012; Reitsema, 2013). The sex and age clusters within the individuals with syphilis and those without are too small to allow statistical testing. However, visual inspection of these groups does not reveal an obvious relationship between age or sex categories and isotopic values (Figs. 4 and 5). This could cautiously suggest that neither biological profile – at least sex and age at death – nor socioeconomic background of the sampled individuals, influenced the access to food resources and subsequently the distribution of the $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values.

Nevertheless, it is possible that the food choices of the individuals could have been modified as a part of a medical treatment. Treatises on syphilis from the 19th c. A.D. indicate that some patients were put on strict diets during the course of medical treatment (e.g., Colles, 1881; Cooper, 1895; Egan, 1853; Fournier, 1906; Hutchinson, 1877; Keyes, 1877; Lagneau, 1853). These diets could be a factor influencing the observed difference in mean $\delta^{13}C_{col}$ values between individuals with syphilis and those without, but details of the effects are unknown. Moreover, it is documented that medical practitioners often modified the diet of patients on a case-by-case basis according to the type of syphilitic lesions present, the degree of pain, and the body's response to infection.

Finally, physiological responses to the pathological condition or aftereffects of the administered medical treatments must be considered. As a systemic disease, tertiary syphilis often involves several bones and infects several organs. Oral and gastrointestinal manifestations are common (McConnell, 1911). During stages of late infection, ulcers, leukoplakia and gummas in the oral cavity can occur, which in extreme cases can lead to the partial destruction of the soft and hard palate
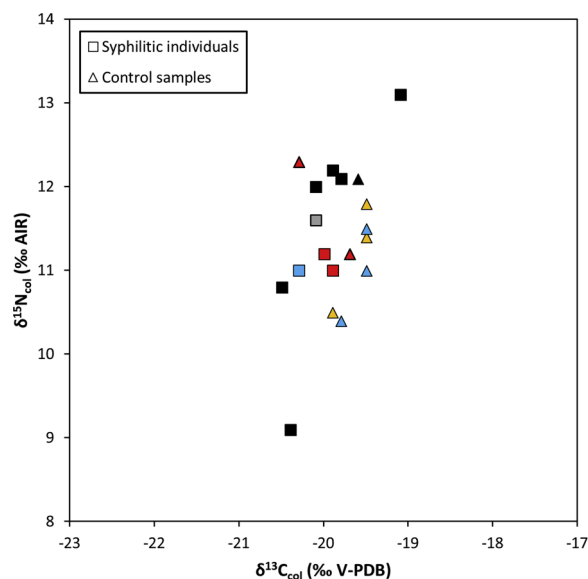

Fig. 4. Biplot showing the $\delta^{13}C_{col}$ against $\delta^{15}N_{col}$ values of sampled syphilitic and non-syphilitic individuals according to age categories. Note – Grey: juvenile; Red: young adult; Green: middle adult; Blue: old adult; Black: adult (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
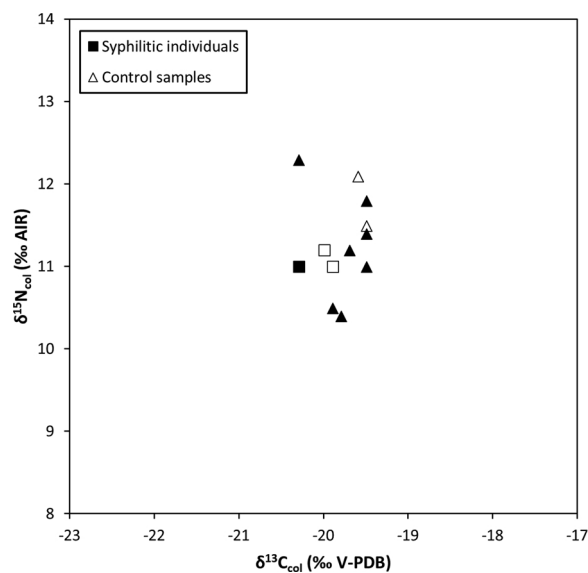

Fig. 5. Biplot showing the $\delta^{13}C_{col}$ against $\delta^{15}N_{col}$ values of sampled syphilitic and non-syphilitic individuals according to sex. Note – Filled symbols: male; Open symbols: female.

(Bains and Hosseini-Ardehali, 2005; Fournier, 1906; Huebsch, 1955; Keogh, 1913; Leão et al., 2006; Leuci et al., 2013). Ulceration of walls of the stomach and the lower gastrointestinal tract was also commonly recorded (Fournier, 1906; McNee, 1936; Modena et al., 1979). These lesions can lead to food intake disturbances (Bains and Hosseini-Ardehali, 2005; Di Cosola et al., 2007; McClain et al., 1993). Appetite disturbance is also a frequent symptom recorded in individuals with late-stage syphilis as a consequence of the disease itself (Barton, 1870; Lane, 1873; Sinkulová, 1971; Trow, 1920, 1922) or its therapy (Gerstner and Huff, 1977; Goldwater, 1957; Kedziora and Duflou, 1995; Reade, 1860). Recurrent or chronic loss of appetite over a very long period of time was regularly observed in patients during the 19th c. A.D. (e.g., Fournier, 1906; Hunter, 1845). Moreover, remedies during the pre-antibiotic era consisted primarily of mercurial care (e.g., injections,

inunctions, fumigations, oral administrations) (Cooper, 1895; Fournier, 1906; Hutchinson, 1877; Keyes, 1877). These treatments were provided to patients continuously with intervals lasting several years (up to six years after Keogh (1913) and Fournier (1906)). Such therapies could cause buccal lesions of the mouth, which may damage – temporarily or permanently – the sense of taste (Fournier, 1906; Hunter, 1845; Liagre, 1897). They could also disturb the digestive system – mainly the stomach and intestines – and cause intoxication, nausea and diarrhea (*e.g.,* Hunter, 1845; O'Shea, 1990). These aftereffects of medical treatment can intensify appetite disturbance in individuals with syphilis (Fournier, 1906; Hunter, 1845; Liagre, 1897; O'Shea, 1990).

Long-term appetite loss may lead to undernutrition. In this scenario, the ratio between routed dietary neAAs and endogenously formed neAAs in extracellular fluids of individuals with syphilis may change. Low concentrations of exogenous neAAs in fluids could activate the enzymes to build up the needed neAAs for collagen synthesis (Ambrose and Norr, 1993; Hare et al., 1991; Howland et al., 2003; Jim et al., 2004; Tieszen and Fagre, 1993; Warinner and Tuross, 2009). Consequently, substantial amounts of carbon derived from non-protein dietary sources may influence the isotopic composition of collagen (Schwarcz, 2000). Physiologically, dietary carbohydrates are hydrolyzed in glucose, which is broken down (through glycolysis) as 3-phosphoglycerate, phosphoenolpyruvate, and then pyruvate. Both 3-phosphoglycerate and pyruvate can be used to generate glycolytic neAAs (Fernandes et al., 2012; Schwarcz, 2000). Pyruvate can also be oxidized into acetyl-CoA, a key intermediate in the citric acid cycle for numerous other neAAs (e.g., aspartate, tyrosine, glutamate). During this process, significant isotopic fractionations occur, inducing a depletion in the ^{13}C of byproducts (DeNiro and Epstein, 1977). The endogenously built neAAs from pyruvate/acetyl-CoA could have lower δ^{13}C values than exogenous ones (DeNiro and Epstein, 1977). Furthermore, excess glucose is converted into glycerol and fatty acids (via lipogenesis) before being stored as triglycerides (Fernandes et al., 2012). These lipids are generally depleted in ^{13}C due to isotopic fractionations. Thus, reserve lipids have generally lower δ^{13}C values than proteins (DeNiro and Epstein, 1977, 1978; Roth and Hobson, 2000). Under certain conditions, these lipids can reintegrate the glycolysis pathway as 3-Phosphoglycerate (via gluconeogenesis) or acetyl-CoA (via beta oxidation) to participate in neAAs biosynthesis (DeNiro and Epstein, 1977; Fernandes et al., 2012; Salway, 2012). Newly formed neAAs could present more negative δ^{13}C values than routed dietary ones. During a period of nutritional restrictions, as experienced by individuals with syphilis, carbon from energy sources may be incorporated into carbon skeletons of endogenously synthesized neAAs, and all or part of the latter can be depleted in ^{13}C. This could explain the observed difference in mean $\delta^{13}C_{col}$ values between individuals with syphilis and the control group.

Such an isotopic effect on the $\delta^{13}C_{col}$ values is supported by several experimental studies involving nutritionally stressed animals (e.g., Podlesak and McWilliams, 2006; Polischuk et al., 2001; Robb et al., 2015; Williams et al., 2007). However, one might wonder why similar effects are not observed in $\delta^{15}N_{col}$ values, even though $\delta^{15}N_{col}$ is often considered as a promising isotopic indicator of disease or nutritional stress (e.g., Fuller et al., 2005; Katzenberg, 2012). Some authors argued that instead of a continuum where the level of change in ^{15}N contents corresponds to the level of nutritional restriction, there may be a threshold level of nutritional stress below which isotopic changes are likely to be negligible (Ben David et al., 1999; Hatch, 2012; Kempster et al., 2007; Polischuk et al., 2001). This threshold effect would be unique to each isotopic proxy; $\delta^{15}N_{col}$ would respond differently and/or could be less sensitive than $\delta^{13}C_{col}$ to certain nutritional stresses (see review of Hatch (2012)). Furthermore, the internal metabolic pools of carbon and nitrogen used to build up neAAs may be affected in different ways during episodes of prolonged nutritional restriction. Carbon balance may become negative faster than nitrogen. A neutral or positive carbon balance may be acquired by using carbon atoms that derive, first, from glucose and reserve triglycerides, and second, from body proteins that are rich in nitrogen (e.g., Podlesak and McWilliams, 2006; Williams et al., 2007). These two phenomena may explain why the $\delta^{15}N_{col}$ values of the individuals with syphilis are not affected by nutritional stress while the $\delta^{13}C_{col}$ values are. The observed isotopic pattern may also suggest that the nutritional stress was relatively restricted. This could explain why the results of our study differ from those of previous works focusing on humans, where individuals were generally subjected to acute starvation episodes (e.g., Fuller et al., 2005; Mekota et al., 2006, 2009).

8. Conclusion

Individuals with syphilis (n = 10) appear to have lower $\delta^{13}C_{col}$ values than the individuals in our control group (n = 9). The discrepancy between the two groups is about 0.3-0.4‰. However, there is no difference in $\delta^{15}N_{col}$ values between these two groups. Changes in diet might explain these findings. Strict diets were commonly prescribed to patients with syphilis by physicians during the course of their medical treatment. This practice could lead to the observed isotopic pattern. Alternatively, nutritional stress caused by persistent and cyclic appetite disturbance – as a consequence of the disease itself or of the administered medical treatment – could be also responsible for the observed isotopic pattern. Long-term nutritional stress could affect metabolism controlling the assimilation of macronutrients. In this scenario, the normal routing of neAAs may have been disrupted due to an insufficient intake of carbon atoms coming from dietary protein. A greater amount of carbon derived from ^{13}C depleted triglycerides or even glucose may have been incorporated into endogenously formed neAAs. However, a nitrogen threshold effect, or a preserved positive nitrogen balance despite a decrease of protein intake, may explain the unmodified $\delta^{15}N_{col}$ values at the group level. Overall, the response of the two isotopic proxies could indicate relatively limited nutritional restrictions. We are aware that we must remain cautious regarding our interpretation of the data since our sample sizes are small.

Although prescribed diets, as well as modes of administration and dosage of mercury varied over time and place (see Grünpeck, 1947 and O'Shea, 1990), isotopic patterns similar to the ones observed in this study could be expected for earlier populations. However, we recommend excluding individuals with syphilis from sampling strategies designed to reconstruct population diets in the past, even though the observed shift in $\delta^{13}C_{col}$ values is minor.

Finally, this research has posed new hypotheses and opened new research topics: analysis of mercury content in our skeletal material of syphilis sufferers could be helpful, especially to verify whether they received treatment with medicine containing the agent and how long they were exposed to mercury during treatment (cf. Kepa et al., 2012). Additionally, stable carbon and nitrogen isotope analysis of compound specific bone collagen amino acids could be useful to further understand the degree of routing of non-essential amino acids, as well as the provenance of the carbon and nitrogen atoms used for collagen synthesis in individuals with syphilis.

Acknowledgements

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