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Lipid biomarker-based verification of TB infection in mother's and daughter's mummified human remains (Vác Mummy Collection, 18th century, CE, Hungary)

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ABSTRACT The perpetual burden of tuberculosis (TB) keeps drawing the focus of research on this disease. Among other risk factors (e.g., poor living conditions, malnutrition, smoking, HIV infection, etc.), being in close contact with a TB infected person requires special attention. For a better understanding of the disease, paleopathological investigations concerning TB have been carried out with various techniques for a long a time; nevertheless, analysis of incidence among family members is hardly possible in past populations. An exceptional group of naturally mummified individuals, the collection of the Vác mummies (Hungary, 18th century CE), is known about the large TB incidence rate, which has been revealed by aDNA analysis. Besides the high rate of TB infection, another interesting aspect of the collection is that in some cases, the family connections could be reconstructed. In this paper, we present the mycocerosic acid profiles gained by HPLC-HESI-MS measurements of two Vác mummies, who were mother and daughter according to the personal records. Earlier metagenomic analysis already revealed mixed *M. tuberculosis* infection with the same bacterial strains in both individuals; moreover, the same bacterial strains were recorded in both cases.
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Introduction

Tuberculosis (TB) is a well-known infectious disease, which can be caused by members of *Mycobacterium tuberculosis* complex (MTBC), in humans, and also several animal species (Brites et al. 2018; WHO 2020). The 12 species/varieties, included in the complex, are genetically closely related, presenting 99.9% similarity in the nucleotide level (Brosch et al. 2002; Brites and Gagneux 2017; Brites et al. 2018; Riojas et al. 2018). Up to now, the following species are considered as members of the complex: *M. tuberculosis, M. africanum, "M. canettii", M. bovis, M. microti, M. pinnipedii, M. orygis, M. mungi, M. suricattae, M. caprae,* "chimpanzee bacillus", and "dassie

bacillus" (Brites et al. 2018; Riojas et al. 2018), but only the first three are specifically human pathogens (Castets et al. 1968; Brosch et al. 2002; Niemann et al. 2004; Bañuls et al. 2015). In human infections, the most common scenario is when transmission occurs via inhalation of bacilli-filled droplets, which are projected in the air while coughing, sneezing or even talking (Flynn and Chan 2001; Bañuls et al. 2015; Getahun et al. 2015).

Several risk factors contribute to the spread of TB: some of them affect the transmission itself (e.g. poor living and working condition, household overcrowding, etc.), and some increase the host's susceptibility (e.g., HIV infection, malnutrition, smoking, diabetes) (Lönnroth et al. 2009; WHO 2020). To reduce TB burden, the prevention of new infections is crucial, and a key factor is

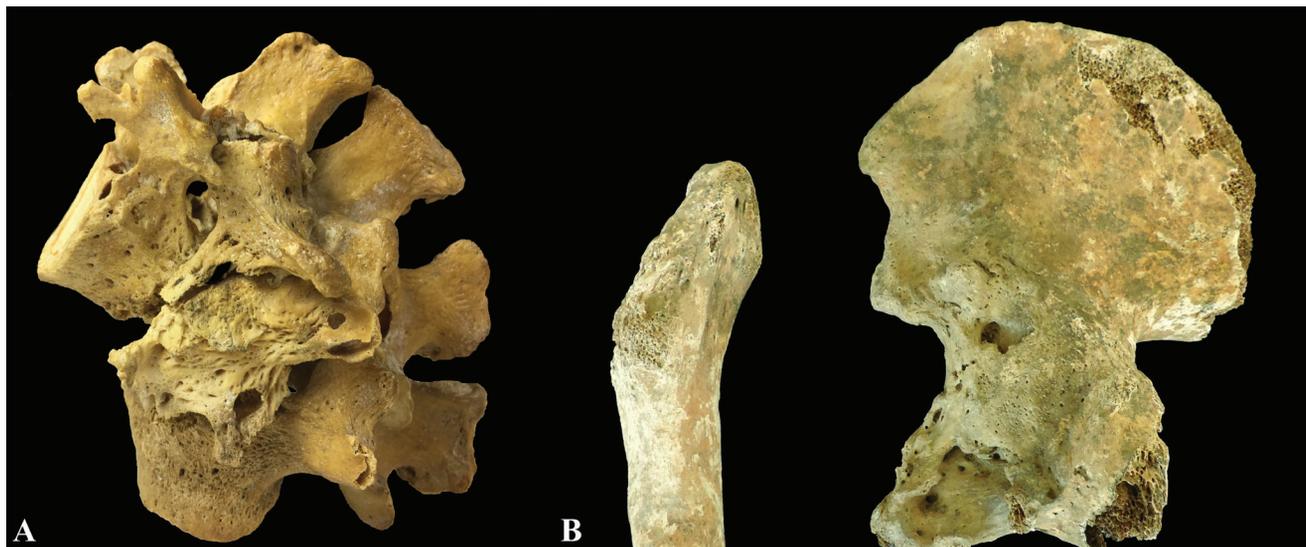[#]The two last authors contributed equally.

Figure 1. Examples of osteoarticular TB associated lesions. **A:** Pott's gibbus (Robert J. Terry Anatomical Skeletal Collection, Terry No. 1124R). **B:** *Coxitis tuberculosa* (Bélmegyer–Csömöki domb, Grave No. 90).

providing TB preventive treatment for high-risk groups (WHO 2020). Being in close contact with a person who has active TB – especially in newly diagnosed TB patients – means a high risk for transmission; thus, close family members, principally those from the same household, need particular attention (Vidal et al. 1997; Wang and Lin 2000; Horsburgh and Rubin 2011; Augustynowicz-Kopeć et al. 2012; Acuña-Villaorduña et al. 2018; WHO 2020).

According to WHO estimates, approximately 1.2 million HIV-negative and an additional 208000 HIV-positive people died of TB and about 10 million people fell ill with

the disease globally in 2019 (WHO 2020). The relatively high incidence, the appearing rifampicin- and multidrug-resistant TB (MDR-TB) strains and the co-infection cases (especially with HIV) highlight the necessity of extensive TB research. This disease is mainly remembered due to its devastating effects during the 18th - 19th century, when it was highly spread in Europe, implying an extraordinary burden (Bello et al. 1999; Vuorinen 1999, Glaziou et al. 2018; Loddenkemper et al. 2018; Roberts 2020), but has been a recognised threat for thousands of years (Gutierrez et al. 2005; Daniel 2006; Baker et al. 2015; Barberies et
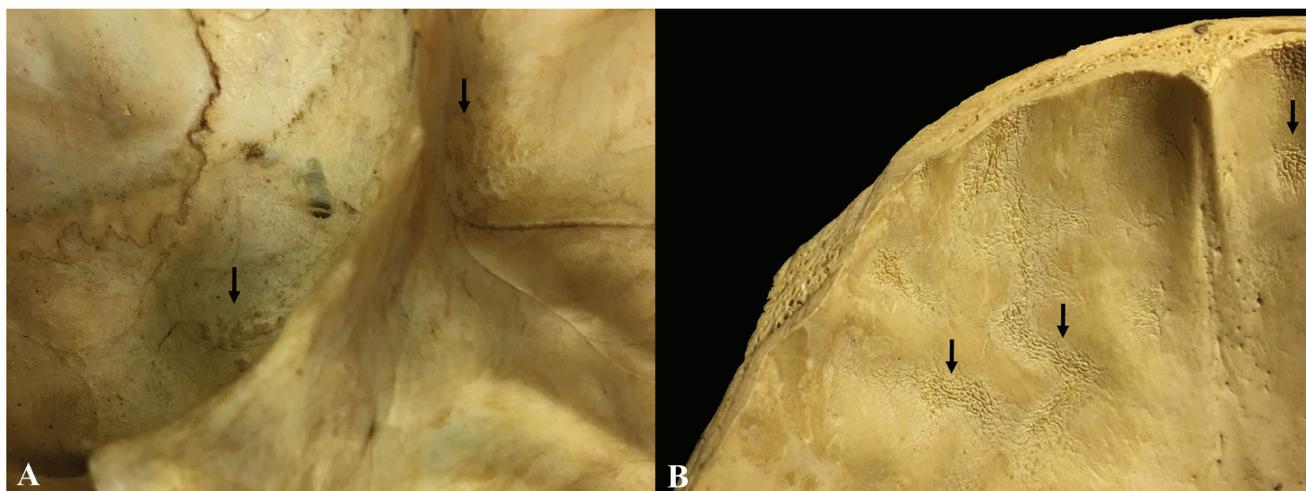

Figure 2. Examples of TBM associated lesions. **A:** Granular impressions on the greater wing of the sphenoid bone (Robert J. Terry Anatomical Skeletal Collection, Terry No. 566). **B:** Abnormal blood vessel impressions on the endocranial surface of the frontal bone (Robert J. Terry Anatomical Skeletal Collection, Terry No. 254).

al. 2017). To achieve a better understanding of the evolution of the infectious agent, paleopathological research investigating the epidemiology of TB in past populations is highly important.

The paleopathological signs of TB include Pott's gibbus (Fig. 1A) and *coxitis tuberculosa* (Fig. 1B), traces of cold abscess, and endocranial lesions caused by tuberculous meningitis (TBM) e.g. granular impressions (Fig. 2A), and abnormal blood vessel impressions (Fig. 2B) (Schultz 1993, 1999, 2001, 2003; Aufderheide and Rodríguez-Martín 1998; Marcsik et al., 1999; Pálfi and Marcsik 1999; Hershkovitz et al. 2002; Maczel 2003; Ortner 2003; Pálfi and Molnár 2009; Pálfi et al. 2012, 2015; Spekker et al. 2012; Kajdocsi Lovász 2015; Masson et al. 2015; Molnár et al. 2015; Paja et al. 2015; Schultz and Schmidt-Schultz 2015; Spekker 2018; Spekker et al. 2020a, 2020b). Moreover, new bone formation on the long bones and on the visceral surface of ribs are used as TB-related markers (Roberts et al. 1994; Marcsik et al. 2009; Santos and Roberts 2001, 2006; Hershkovitz et al. 2002; Maczel 2003; Matos and Santos 2006; Pálfi and Molnár 2009; Pálfi et al. 2012, 2015; Kajdocsi Lovász 2015; Masson et al. 2015; Molnár et al. 2015). Since skeletal TB and CNS TB develop in only a few cases (Golden and Vikram 2005; Rock et al. 2008; Spekker et al. 2018; Rodriguez-Takeuchi et al. 2019; Spekker et al. 2020a; Spekker et al. 2020b), the simultaneous application of molecular biological and analytical techniques are useful tools to draw a clearer picture about the paleoepidemiology of TB (Molnár et al. 2015; Pálfi et al. 2015; Donoghue et al. 2017). Since the 1990s, two approaches are commonly applied to supplement the morphological TB-related paleopathological investigations, namely aDNA based and lipid biomarker-based methods (Spigelman and Lemma 1993; Donoghue et al. 1998; Gernaey et al. 1998; Hershkovitz et al. 2008; Redman et al. 2009; Chan et al. 2013; Kay et al. 2015; Donoghue et al. 2017).

The lipid biomarker-based methods benefit from the lipid-rich cell wall, characteristic of mycobacteria (Minnikin and Goodfellow 1980; Minnikin 1982; Daffé and Lanéelle 1988; Minnikin et al. 1993; Hershkovitz et al. 2008; Redman et al. 2009; Lee et al. 2012; Minnikin et al. 2015a; Donoghue et al. 2017). Most commonly, the mycolic acid (MA) and mycocerosic acid (MC) components, and the C27 mycolipenic acid are used. MAs, MCs, and mycolipenic acids can be found in the so-called Mycobacterial Outer Membrane (MOM) (Minnikin et al. 2015b). MAs are long chain α-alkyl-β-hydroxy fatty acids, which are covalently bound to the mycoloylarabinogalactan-peptidoglycan macromolecules (Watanabe et al. 2001; Minnikin et al. 2015b; Abrahams and Besra 2016; Batt et al. 2020; Dulberger et al. 2020). MCs are long-chain multimethyl-branched-chain fatty acids esterified mainly

Figure 3. Dominican Church of Vác. Photo taken by András Thumbasz.

with phthiocerol and phenolphthiocerol long-chain diols (Minnikin 1982; Daffé and Lanéelle 1988; Redman et al. 2009; Minnikin et al. 2015b; Batt et al. 2020). In contrast with MAs, MCs can be found only in a smaller group of mycobacteria, namely in *M. tuberculosis*, *M. bovis*, *M. gastri*, *M. haemophilum*, *M. kansasii*, *M. leprae*, *M. marinum,* and *M. ulcerans* (Draper et al. 1983; Minnikin et al. 1985; Daffé and Lanéelle 1988; Hartmann and Minnikin, 1992; Minnikin et al. 1993; Redman et al. 2009). MCs have been detected for paleopathological investigations traditionally via NICI-GCMS (Redman et al. 2009) and a HPLC-MS method has been newly introduced (Váradi et al. 2021).

In 1994, a group of naturally mummified individuals were found in a long-forgotten crypt during the renovation of the Dominical Church of Vác (1994–1995) (Fig. 3) (Pap et al. 1999). The discovered Vác mummy collection is well-documented, with many available individual data (Szikossy et al. 1997). The mummies are curated in the Department of Anthropology, Hungarian Natural History Museum, Budapest, Hungary. The collection is known for the high presence of TB infected cases, that drew the focus of several studies on this group (Szikossy et al. 1997; Pap et al. 1999; Fletcher et al. 2003; Donoghue et al. 2011; Chan et al. 2013; Kay et al. 2015; Pap et al. 2017). Most of these individuals lived in the 18th century CE, which is from the pre-antibiotic era; therefore, they represent a well-characterised link between recent and archaeological samples. Besides the broad pathological investigations, demographical analysis and exploration of family connections were carried out, as well as attempts to reconstruct individual life stories (Kustár et al. 2011a, 2011b; Szikossy et al. 2015; Szikossy 2020).

The aim of this study is to present and compare the results of mycocerosic acid profiling of two TB infected Vác mummies, a mother (Fig. 4A) and her daughter (Fig.

4B). Earlier both individuals were proved to have mixed infection with the same *M. tuberculosis* strains via aDNA analysis (Kay et al. 2015), and samples taken from the daughter presented positive MC profiles in previous HPLC-MS measurements (Váradi et al. 2021).

Materials and Methods

MTBC strains and mummy samples used in this study

For reference, in an earlier study, we used five MTBC strains (laboratory IDs of the isolated strains MTBC-1/2015; MTBC-254/2000; MTBC-3910/2014; MTBC-242/2000; and MTBC-1/8508/2014), isolated from patients, who had been diagnosed with pulmonary tuberculosis. The average distribution of the reference strains has been evaluated and published in our earlier report (Váradi et al. 2021). The isolation of the reference strains was carried out in the Institute of Clinical Microbiology, University of Szeged, Szeged, Hungary and the National Korányi Institute of TB and Pulmonology, Budapest, Hungary, according to the national recommendations (EMMI, State Secretariat for Healthcare, 2018). The identification and growing conditions followed a previously described protocol (Váradi et al. 2021). The harvested bacterial samples were stored in freeze-dried form at -20 °C.

The examined human sample was taken from the chest region of the late Anna Schőner (body number: #28, inventory number: 2009.19.28., age at death: 55 years). The rib sample was removed by sanitized tweezers and stored in a tightly closed bag at room temperature. The bone was powdered in the clean laboratory of the Institute

for Mummy Studies, EURAC Research, Bolzano, Italy.

Sample preparation and instrumental analysis

In the case of the sample pre-treatment and measurement of the MTBC strains, 20 mg of bacterial material was utilized and the previously described method was applied (Váradi et al. 2021). For the lipid analysis of the mummy sample, 434 mg of bone powder was used. The sample pre-treatment was carried out briefly as follows: the samples were heated at 100 °C overnight with the addition of 20% KOH in MeOH (2 mL; m/V) and toluene (1 mL) in PTFE capped glass tubes. Samples were acidified to pH 1 with the addition of 10% HCl and 37% HCl solutions. Thereafter, the samples were extracted with the addition of toluene (1 mL) three times, and one more time with the addition of hexane isomer mixture (1 mL). The removed and combined organic layers were evaporated to dryness in vacuum with a Savant SC250EXP SpeedVac concentrator (Thermo Scientific, Waltham, Massachusetts, USA). The mummy sample was dissolved in 1000 µl and the bacterial samples were dissolved in 200 µL of the following mixture: isopropanol (IPA):heptane:acetonitrile (MeCN) (4:1:5). The sample solutions were filtered by PTFE syringe filters (pore size: 2.0 µm; diameter: 13 mm).

The measurements were carried out on a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Waltham, Massachusetts, USA), which was coupled with a Q-Exactive Plus (Thermo Scientific, Waltham, Massachusetts, USA) mass spectrometer (MS). For the separation, a Gemini – NX C18 (3 µm, 110A, 50 mm x 2 mm) column (Phenomenex, Torrance, California, USA) was used at 30 °C.

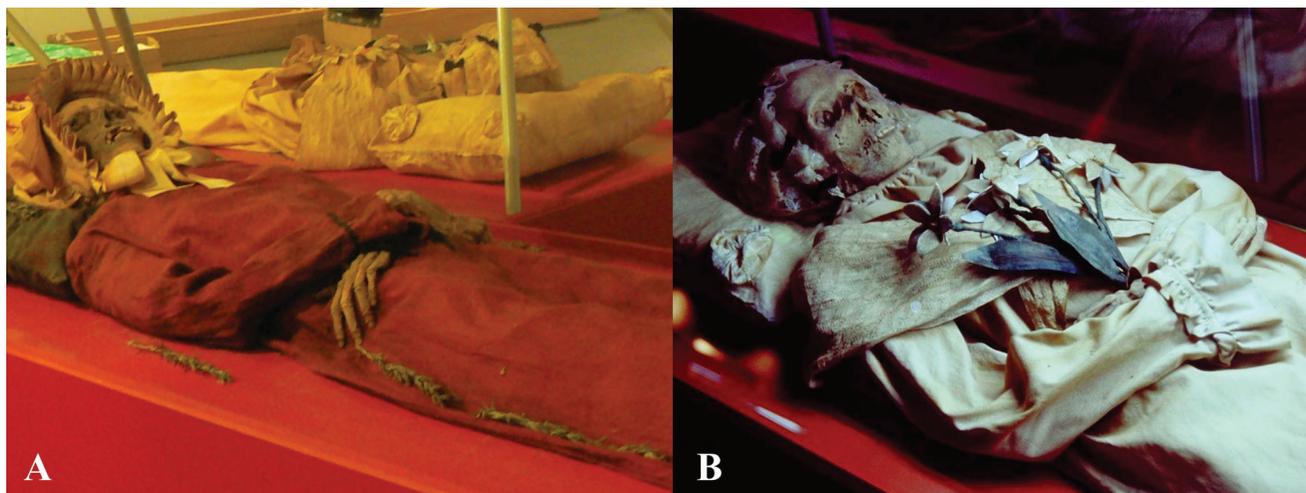The separation was carried out with gradient elution

Figure 4. Sources of the mummy samples. **A:** Anna Schőner (Body No: 28, Inv. No: 2009.19.28.). **B:** The late Terézia Hausmann (Body No: 68, Inv. No: 2009.19.68.). Photos were taken on the exhibition of the Hungarian Natural History Museum.

Table 1. Gradient elution program applied for the HPLC separation of mycocerosic acids.

Time (min)	Eluent A (%): MeCN + 0.1% acetic acid	Eluent B (%): IPA:heptane (8:2) + 0.1% acetic acid
0	90	10
5	85	15
15	30	70
16	10	90
18	10	90
18.5	80	20
19.5	80	20
20	10	90
23	10	90
24	90	10
28	90	10

(see details in Table 1), and 200 µL/min flow rate was applied. The injection needle was thoroughly washed before and after injection, with 150 µL IPA:heptane (8:2). Solvent blank injections were made between each sample investigation.

Regarding the MS parameters, the following settings were applied: the sheath gas flow and the auxiliary gas rates were set to 35 L/min and 10 L/min, respectively, the capillary and the auxiliary gas was heated to 350 °C, the spray voltage was set to 4 kV, while the S-lens voltage was 70 V. The targeted MCs (Fig. 5) were monitored in negative Selected Ion Monitoring (SIM) mode: 409.40510 (C27, $C_{27}H_{53}O_2$), 437.43640 (C29, $C_{29}H_{57}O_2$), 451.45205 (C30, $C_{30}H_{59}O_2$), 479.48335 (C32, $C_{32}H_{63}O_2$), and 493.49900 (C33, $C_{33}H_{65}O_2$), with 0.4 m/z isolation window. The area values of the MC peaks were calculated with TraceFinder 4.0 General Quan Software (Thermo Scientific, Waltham, Massachusetts, USA). The maximum mass deviation from the calculated [M-H]⁻ ion mass was 5 ppm for peak identification. The detected MC peaks were normalised to the peak with the highest area value.

Results and Discussion

Three members of the Hausmann family were identified in the Vác Mummy Collection of the Hungarian Natural History Museum. The mother, Anna Schőner (1738-1793) and her two daughters, Terézia (1769-1797) and Barbara Hausmann (1780-1795). Their father, János Hausmann is not in the collection. According to the aDNA examinations (Fletcher et al. 2003; Chan et al. 2013; Kay et al. 2015), all three female members of the Hausmann family were infected by tuberculosis. First, Anna Schőner died at the age of 55 in 1793, December 16. Two years later, she was

followed by her younger daughter, Barbara Hausmann, who died at the age of 15, on March 2, 1975. In another two years, the elder daughter, Terézia Hausmann passed away at the age of 28, on December 25, 1797 (Exploration documentation of the Dominican Church of Vác, 1994-1995, Tragor Ignác Múzeum.). Both girls were emaciated, suggesting a long-lasting illness. It is possible that the thirteen-year-older Terézia took care of her sister before the illness made her too weak (Cseplák et al. 2015, Donoghue et al. 2021). In this study, we present the mycocerosate profiles observed by the analysis of samples taken from Anna Schőner (#28) and Terézia Hausmann (#68).

The lipid profile observed for the extract of the rib sample taken from the #28 individual is shown in Fig. 6A and Fig. 7. The *M. tuberculosis* C27 MC minor component was not detected in the extract. In the MC profile, the main peak was the C32 (100), and it was accompanied by a relatively high peak of C30 (49). The C29 MC was presented in a relatively high ratio (25), as well. The C33 MC was also detectable but was a minor component in the extract (3). The first eluting component was the C29 MC with the retention time of 8.20 min, followed by the C30 and C32 MCs at 8.75 and 10.64 min, respectively. The retention time of the C33 MC peak was 10.97. The general distribution of the detected MCs was in accord with others from the same group of mummies, and with the average profile gained by the analysis of clinical samples (e.g., Fig. 5B, Fig. 6B), and with the MTB profiles published using different approaches (Minnikin et al. 1993; Redman et al. 2009; Váradi et al. 2021). Based on our previous results, the observed lipid profile fulfills the requirement to be identified as a positive case, as the three most presented MCs were the C32, C30, and C29, with a principal amount of the C32 component (Fig. 6C, Fig. 7). The samples belonging to the daughter (#68) of this individual were found earlier to be positive with HPLC-MS analysis (Váradi et al. 2021). In that case, both soft tissue and rib samples were included in the investigation. The MC profile of the soft tissue presented provided clean peaks for C32, C29, C30 and C33 MCs. The main peak was the C32 (100), which was accompanied by major C29 (38) and C30 (97) peaks and with C33 MC (5) as minor
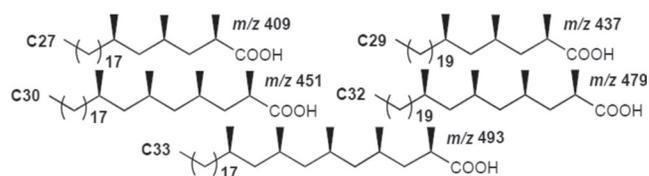
Figure 5. Structures of mycocerosic acids. The mass spectral *m/z* values correspond to carboxylate anions (M – H⁺).
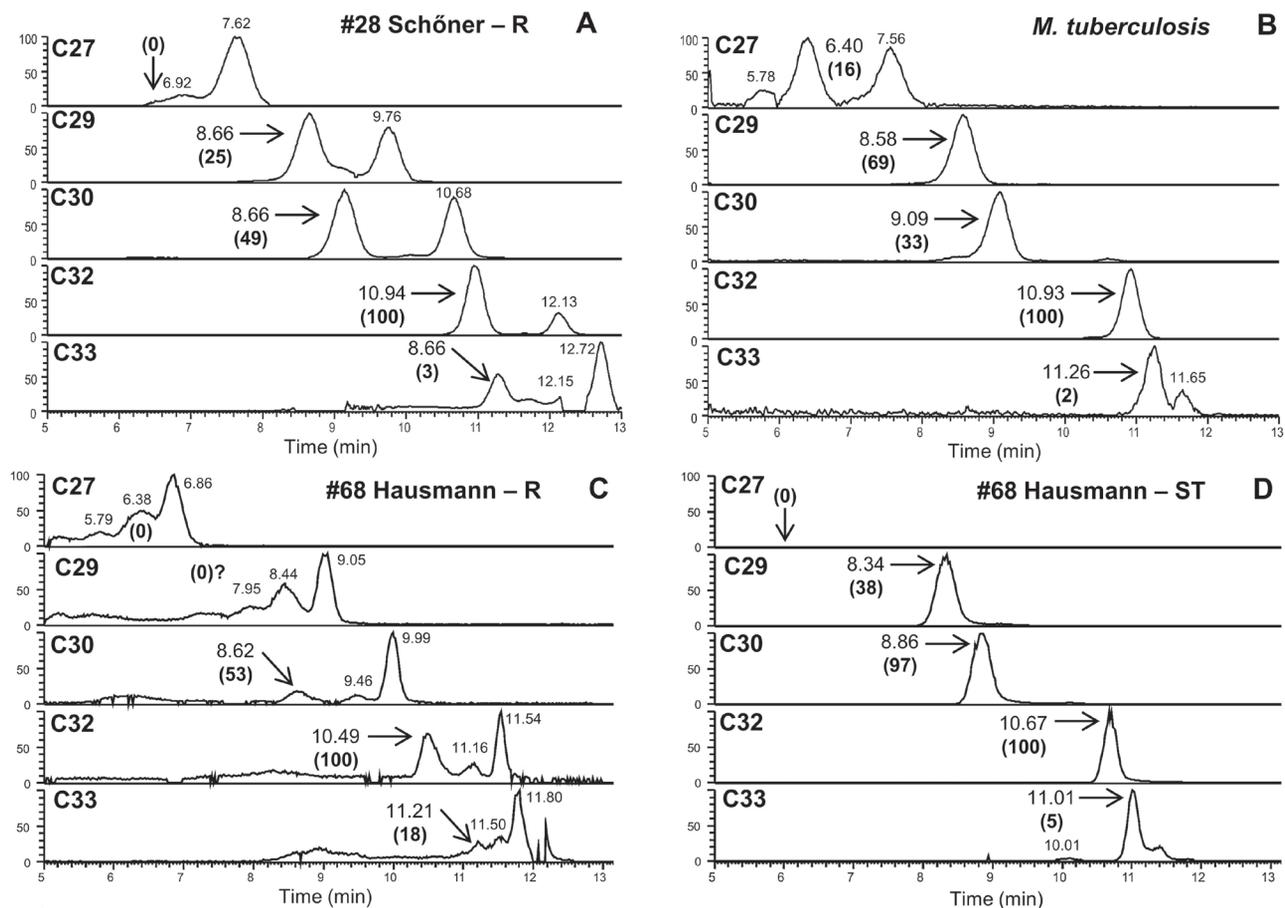
Figure 6. Mycocerosic acid profile of M. tuberculosis and positive mycocerosate (MC) profiles from mummy samples #28 and #68. **A:** Rib (R) sample from Anna Schőner (#28). **B:** Mycocerosate profile of M. tuberculosis 254-2000. **C** and **D:** Rib (R) and Soft Tissue (ST) samples from Terézia Hausmann (#68). MC distributions are shown normalised to the main component (100).

component (Fig. 6D, Fig. 7). The extract of the rib sample of #68 individual had clear main C32 (100) and major C30 components (53), but the minor C33 was indistinct (~18) and the area for the expected C29 was obscured.

Samples taken from the same individuals were earlier analysed with two different aDNA techniques. Firstly, as part of an extensive study, the examination of over 350 samples covering 168 individuals were screened for the 123-bp region of the IS*6110* insertion sequence (Fletcher et al. 2003). The positive samples were investigated for silent point mutations of the *gyrA* 95 and *katG* 463 genes; for the differentiation of the infectious agents into three genotypes, following the work of Sreevatsan et al. (1997). Our positive MC profile matched the result of the DNA-based analysis; traits of *gyrA* 95 and *katG* 463 genes were found in the abdomen, with the silent point mutations characteristic to the group 2 genotype of *M. tuberculosis* (Fletcher et al. 2003). In the case of the same study, during the analysis of the samples taken from the

#68 individual's left chest, the silent point mutations of the *gyrA* 95 and *katG* 463 genes were characteristic of the group 3 genotype of *M. tuberculosis*. Metagenomic analysis carried out on the samples taken from the abdomen region of the #28 individual and on the samples taken from the left chest region of the #68 individual presented similar results (Kay et al. 2015). In both cases, a mixed MTB infection was revealed, but although the detected genotypes were the same, they were detected in different proportions. One of the *M. tuberculosis* strains belonged to sublineage 4.1.2.1, also known as the Haarlem lineage (Kay et al. 2015; Stucki et al. 2016), and the other was assigned by Kay et al. (2015) to sublineage 4.7, which is currently part of sublineage 4.10 according to Stucki et al. (2016). The metagenomic results suggest a transmission between mother and child or infection from the same source (Kay et al. 2015).

Although in this case the applied MC profiling method cannot provide the same resolution regarding the *M.*
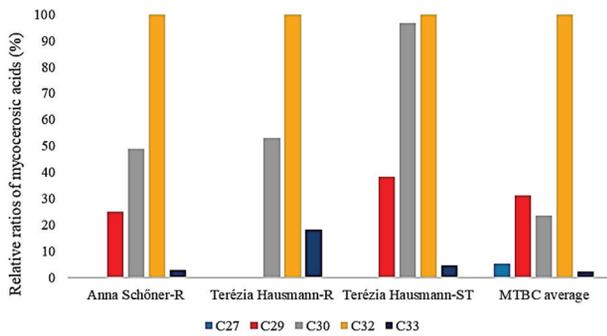
Figure 7. The normalised distribution of positive mycocerosate profiles from mummy rib (R) and soft tissue (ST) samples. Also shown is an "MTB average", calculated in previous examinations of clinical samples.

tuberculosis strains, it demonstrates the widespread occurrence of TB infection among the Vác mummies. The combined application of morphology, aDNA and lipid biomarker analysis is important to gain a clearer picture in paleopathological practice as the results can support each other, and help to reveal positive cases, which would possibly remain hidden if only one method was applied (Hershkovitz et al. 2008; Lee et al. 2012; Baker et al. 2015; Masson et al. 2015; Molnár et al. 2015; Donoghue et al. 2017; Luna et al. 2020). The examination of mummified human remains with a variety of different approaches is especially useful and provides a unique insight to TB research (Salo et al. 1994; Zink et al. 2003; Donoghue et al. 2004; Donoghue et al. 2009; Minnikin et al. 2011; Chan et al. 2013; Lalremruata et al. 2013; Kay et al. 2015; Piombino-Mascali et al. 2015; Szikossy et al. 2015).

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