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# THE TALKING DEAD

NEW RESULTS FROM  
CENTRAL AND  
EASTERN EUROPEAN  
OSTEOARCHAEOLOGY

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# THE TALKING DEAD

New results from Central- and Eastern  
European Osteoarchaeology

Proceedings of the First International Conference  
of the Török Aurél Anthropological Association  
from Târgu Mureş

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**Szilárd Sándor GÁL**

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# CASES OF TUBERCULOSIS INFECTION VERIFIED BY LIPID BIOMARKER ANALYSIS IN HUNGARIAN ARCHAEOLOGICAL SAMPLES

Orsolya Anna Váradi<sup>1,2</sup>, Anita Kecskeméti<sup>2</sup>, Olga  
Spekker<sup>1</sup>, Erika Molnár<sup>1</sup>, Zsolt Bereczki<sup>1</sup>, András  
Szekeres<sup>2</sup>, Csaba Vágvolgyi<sup>2</sup>, György Pálfi<sup>1</sup>

<sup>1</sup>University of Szeged, Faculty of Science and Informatics,  
Department of Biological Anthropology  
<sup>2</sup>University of Szeged, Faculty of Science and  
Informatics, Department of Microbiology

## Abstract

*Since tuberculosis still causes more deaths than any other infectious disease, better understanding of the pathomechanism and the evolution of its infectious agent is surpassingly important. The disease is caused by members of Mycobacterium tuberculosis complex including several species, but the major infectious agent is M. tuberculosis.*

*Several methods exist in the clinical practice to diagnose this causative agent. One of them is an integrated protocol based on the unique lipid rich cell wall structure of Mycobacteria. The applied lipid biomarkers can be detected by HPLC and GC-MS. This protocol can also be used with a great efficiency in palaeopathological investigations.*

*The Department of Biological Anthropology at the University of Szeged has collaborated with David E. Minnikin's research group in the School of Biosciences at the University of Birmingham since 2012 in the investigation of archaeological samples from the viewpoint of tuberculosis infection. In this review, we give a short summary of the published results of the collaborative research work, which are good examples for the excellent application possibilities of lipid biomarker analysis. The reviewed samples were taken from three cemeteries in Hungary within a wide time range, from the Late Neolithic to the 7<sup>th</sup>-8<sup>th</sup> centuries AD.*

## Introduction

Nowadays, tuberculosis (TB) caused by bacteria belonging to the *Mycobacterium tuberculosis* complex (MTBC) is responsible for more deaths than any other infectious disease.<sup>1</sup> This complex includes several species, but the main infectious agents among them are *Mycobacterium tuberculosis* strains. The *Mycobacterium* genus is the member of the *Mycobacteriaceae* family belonging to the *Actinomycetales* order.<sup>2</sup> One of the most common features among *Mycobacteria* is their unique staining that requires special dyes (e.g. carbolfuchsin) because of the complex structure of their cell envelope containing lipid-rich molecules in high concentration.<sup>3</sup> The identification of these

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WHO, 2016.

<sup>1</sup> Somoskovi, 2007.

<sup>2</sup> Minnikin, 1982; Minnikin et al., 2002.

infectious agents in archeological samples has a great importance, and besides DNA analyses, the detection of characteristic cell wall components of the causative microbes can also provide reliable diagnostic possibilities.

### 1.1. Mycobacterial cell wall structure

*Mycobacteria* have a remarkably complex cell wall structure, described by the dual membrane model,<sup>4</sup> where a mycobacterial inner and outer membrane (MIM and MOM) can be distinguished, and according to David E. Minnikin and his colleagues,<sup>5</sup> there might be an extensive “periplasmic space” between them. Furthermore, there is a suspected peptidoglycan-galactan layer in the cell wall, which can provide anchorage points for the arabinan moieties. Mycolic acids (MAs) are esterified with these arabinan moieties in the MOM, which also contains other unusual free lipids interacting with mycolic acids such as mycocerosic and mycolipenic acids.<sup>6</sup>

We can use several components of this cell wall structure as lipid biomarkers in the identification of *Mycobacterium* species both in anthropology and in clinical practice. In this review the possibilities of analytical detection of lipid biomarkers are summarized through a few bioarchaeological samples investigated during the course of collaboration between the Department of Biological Anthropology at the University of Szeged and the laboratory of David E. Minnikin in the School of Biosciences at the University of Birmingham.

### 1.2. Lipid biomarkers

#### 1.2.1. Mycolic acids

MAs are integral part of mycobacterial cell envelopes, with a remarkable biomarker value both in identification and classification.<sup>7</sup> The MAs in *M. tuberculosis* can be classified into three principal groups, and all of them have a range of homologues with different chain lengths.<sup>8</sup> The three main types of MAs are  $\alpha$ -, methoxy- and keto-mycolates, which can be distinguished from each other in a simple normal phase chromatographical separation.<sup>9</sup> The amount of  $\alpha$ -mycolates is the highest, approximately 50 percent of all MAs.<sup>10</sup> Furthermore, chemical sub-classes can be differentiated within both methoxy- and keto-mycolates based on presence of alternative methyl branched trans-cyclopropane or cis-cyclopropane moieties, but the separation of them is not possible by normal phase chromatography.<sup>11</sup> MA-groups can be separated chromatographically from each other on normal stationary phase,<sup>12</sup> but it is still not diagnostic for the MTBC because a lot of other mycobacterial species also produce these three groups of MAs. Fortunately, separation within the three major groups of MA components is possible based on their chain length and polarity using reverse phase HPLC after a normal phase separation.<sup>13</sup> After this, computerized comparison with an appropriate standard seems to be adequate for diagnostic purposes.<sup>14</sup> The applicability of this method has been proved also in archaeological materials, for example in the case of samples

<sup>4</sup> Christensen et al., 1999; Minnikin, 1982.

<sup>5</sup> Minnikin et al. 2015.

<sup>6</sup> Minnikin, 1982; Minnikin et al., 2002.

<sup>7</sup> Butler & Guthertz, 2001; Dobson et al., 1985.

<sup>8</sup> Minnikin, 1982; Minnikin & Polgar, 1967a, 1967b; Watanabe et al., 2001, 2002.

<sup>9</sup> Dobson et al., 1985; Minnikin, 1993.

<sup>10</sup> Watanabe et al., 2001; 2002.

<sup>11</sup> Donoghue et al., 2010a; Gernaey et al., 1998, 2001; Hershkovitz et al., 2008; Watanabe et al., 2001.

<sup>12</sup> Dobson et al., 1985; Minnikin, 1993.

<sup>13</sup> Minnikin, 1993; Qureshi et al., 1978; Steck et al., 1978.

<sup>14</sup> Butler & Guthertz, 2001.

collected from Atlit Yam, a circa 9000 years-old archaeological site.<sup>15</sup> The unique lipid biomarker pattern of the members of the MTBC is usually complemented with DNA analysis<sup>16</sup> providing excellent possibility to conduct complex paleoepidemiological investigations with a broad time window in human prehistory due to the MAs' particular stability.<sup>17</sup> Furthermore, it is important to consider that some *Mycobacteria* can also be found in the soil and can contaminate the bone samples, but these are also distinguishable by their lipid biomarker pattern.<sup>18</sup>

One of the most popular lipid biomarker based method used in anthropological practice was developed by David E. Minnikin and his colleagues.<sup>19</sup> They published a multistep procedure for the detection of characteristic lipids.<sup>20</sup> This protocol was built from different previous methods and rigorously optimised to diagnose TB infection originally in clinical samples (sputum). The procedure starts with a saponification and a methylation step, followed by different liquid-liquid extractions, and a 9-anthrylmethyl derivatisation. After this, a solid phase extraction is carried out on C-18 cartridges and the derivatised MAs are separated at first with normal phase HPLC (Plate no. I, Fig. 1A). The anthrylmethyl-esters are detected via fluorescence detector, and the three MA groups are fractionated. The collected fractions are separated according to their chain length and polarity with reverse phase HPLC<sup>21</sup> (Plate no. I, Fig. 1B). To examine archaeological samples, this method was supplemented further with an initial reverse phase HPLC separation before the normal phase HPLC in case of the samples from a Byzantine basilica in the Negev desert at Karkur<sup>22</sup> (Plate no. I, Fig. 1C). In this site a calcified pleura fragment was found in the grave of a 35–45 years old male dated approximately to 1400 BP. The sample was taken from this pleura fragment.

The exact method was published by Gernaey and colleagues in 1998.<sup>23</sup> For the examination of biomarker patterns the samples were collected from the burial ground of the Newcastle Infirmary. This institute was used actively between 1753 and 1845 and in most cases the burials were well documented.

The lipid biomarker method was renewed in 2008,<sup>24</sup> when samples for both DNA analysis and lipid biomarker detection were taken from the skeletal remains of a woman and an infant, buried together at the approx. 9000 years old archaeological site of Atlit Yam. The earlier method<sup>25</sup> was changed at the derivatisation phase. Pentafluorobenzyl (PFB) was introduced instead of methylan-thryl derivatisation,<sup>26</sup> because the derivatives were relatively instable.<sup>27</sup> Mycolate-PFB esters were purified by solid phase extraction (the purification was done on normal phase silica) and esterified further with pyrenebutyric acid (PBA).<sup>28</sup>

### 1.2.2. Mycocerosic and mycolipenic acids

Mycocerosic and mycolipenic acids are further groups of lipids in the MOM, which can be applied as lipid biomarkers to diagnose TB infection. These are so-called "free" lipids, which are

<sup>15</sup> Hershkovitz et al., 2008.

<sup>16</sup> Donoghue et al., 1998, 2010a; Gernaey et al., 2001; Hershkovitz et al., 2008.

<sup>17</sup> Gernaey and Minnikin, 2000; Redman et al., 2009.

<sup>18</sup> Gernaey et al., 1999.

<sup>19</sup> Donoghue et al., 1998.

<sup>20</sup> Minnikin et al., 1993b.

<sup>21</sup> Minnikin, 1993b.

<sup>22</sup> Donoghue et al., 1998.

<sup>23</sup> Gernaey et al., 1998.

<sup>24</sup> Hershkovitz et al., 2008.

<sup>25</sup> Gernaey et al., 1998.

<sup>26</sup> Hershkovitz et al., 2008.

<sup>27</sup> Minnikin et al., 2012.

<sup>28</sup> Hershkovitz et al., 2008.

associated with anchored mycolic acids to form the outer myco-membrane.<sup>29</sup> Mycocerosic acids are long-chain multimethyl-branched-chain fatty acids belonging to phthiocerol dimycocerosate waxes.<sup>30</sup> Different mycocerosate types and their distribution among a range of *Mycobacterium* species has already been defined.<sup>31</sup> The pattern of C29, C30 and C32 mycocerosates is the most characteristic for the MTBC (Redman et al., 2015). Mycolipenates are members of pentaacyltrehalose glycolipids, and among them the C27 mycolipenate is specific to *M. tuberculosis*.

According to Larsson and colleagues, C32 mycocerosates can be detected by GC-MS<sup>32</sup> from 5-day cultures of sputum samples collected from tuberculosis patients. The analysis carried out using selected ion monitoring (SIM) negative ion chemical ionisation gas chromatography mass spectrometry (NI-CI GC-MS). When SIM is used, only the target analytes are monitored, thus the analytical sensitivity improves. The method, which can also be used in paleopathological research, was described by Redman and colleagues in 2009<sup>33</sup> during the analysis of samples from the Coimbra Collection dated to the 19<sup>th</sup>–20<sup>th</sup> centuries AD. This collection consists of the remains of more than 500 individuals who died in the Coimbra University Hospital. For this special investigation 49 individuals' bone samples were used. The cause of death was probably tuberculosis in the case of about 50% of the examined individuals.

The GC-MS method starts with similar steps like the HPLC protocol from the saponification to the derivatisation procedures.<sup>34</sup> After derivatisation, the mycocerosic acid PFB esters are purified by solid phase extraction using normal phase SPE cartridges before HPLC separation. To define the retention time of the sought mycocerosic acid esters on the normal phase, decafluorobenzhydrol and decafluorobenzophenoneco-markers are used. A range of decafluorobenzhydrol esters of long-chain fatty-acids are eluted before mycocerosate, while decafluorobenzophenone is eluted after them. The mycocerosic peak is collected and examined by selected ion monitoring (SIM) negative ion chemical ionisation gas chromatography mass spectrometry (NI-CI GC-MS).

## II. Detection of *M. tuberculosis* infection from Hungarian samples on the basis of lipid biomarker patterns

The Department of Biological Anthropology at the University of Szeged had already investigated specific infectious diseases in archaeological series since the 1970's.<sup>35</sup> The most outstanding cases are discussed in this review grouped by excavation sites.

### II.1. Szeged-Kiskundorozsma, Daruhalom-dűlő

During the course of former investigations at the Department of Biological Anthropology mainly macromorphological and aDNA techniques were used,<sup>36</sup> but from 2010 collaboration was built with the laboratory of David E. Minnikin, opening new possibilities in the identification of TB infections with the application of lipid biomarker pattern analysis. One of the first cooperations was the examination of bone material from grave no. 517(KD 517) in the Szeged-Kiskundorozsma, Daruhalom-dűlő archaeological site.<sup>37</sup> The cemetery is dated to the Avar Age (7<sup>th</sup> century AD),

<sup>29</sup> Minnikin, 1982; Minnikin et al., 2002.

<sup>30</sup> Minnikin et al., 1983, 1985a, 1985b, 2002.

<sup>31</sup> Daffé & Lanéelle, 1988; Minnikin et al., 1985a; Minnikin et al., 1993a.

<sup>32</sup> Larsson et al., 1981.

<sup>33</sup> Redman et al., 2009.

<sup>34</sup> Redman et al., 2009.

<sup>35</sup> Marcsik, 1972; Pálfi-Molnár, 2009.

<sup>36</sup> Haas et al., 2000; Pósa et al., 2015; Zink et al., 2007.

<sup>37</sup> Lee et al., 2012.



where 94 skeletons had been excavated.<sup>38</sup> This material is special for the high number of individuals who probably suffered in mycobacterial infection. Eight of them showed macromorphological features of *M. leprae* infection in different phases. The person who rested in grave KD 517 was a 35–40 years old male.<sup>39</sup> In the rhinomaxillary region considerable remodelling can be noticed. There is slight alveolar resorption, the os palatinus shows abnormal porosity and the nasal spine is completely obliterated. Furthermore, there are slight changes on the tibia and the fibula. Bilateral porotic *cribra orbitalia* was also observed. To support the diagnosis, aDNA and lipid biomarker analysis were performed. A scraped sample was taken earlier from the nasal region and aDNA investigation was done by Donoghue and colleagues.<sup>40</sup> The residuals of this samples were used for the lipid biomarker examination.<sup>41</sup> Symptoms in other skeletons from this site implied leprosy and/or tuberculosis, so KD 517 was tested for both *M. tuberculosis* and *M. leprae*. The HPLC profiles of total mycolates from KD 517 showed a closer similarity to *M. tuberculosis* than to *M. leprae* on reverse phase, which could mean the predominance of tuberculosis infection. Moreover, methoxymycolate fraction was recognized on the normal phase HPLC chromatogram. It suggested the presence of *M. tuberculosis*, as this kind of MAs are absent in the of *M. leprae*.<sup>42</sup> The second reverse phase profiles of the three main mycolate types suggested the presence of both *M. tuberculosis* and *M. leprae*.<sup>43</sup>

Mycocerosic acid content of this sample was also investigated with NI-CI-GCMS<sup>44</sup> and their four major characteristic components, C29, C30, C32 and C34 were detected. The high amount of C34 components suggested *M. leprae* infection, while the enhanced amount of C29 mycocerosates confirmed the presence of *M. tuberculosis*, implying a case of leprosy-TB coinfection.

## II.2. Hódmezővásárhely-Gorzsa

Hódmezővásárhely-Gorzsa was a Late Neolithic tell settlement belonging to the early Tisza culture, which was located in southern Hungary.<sup>45</sup> The most interesting symptoms were found in the fragmentary bones of a probably 19–20 years old male (grave no. 64, code HGO-53). According to the results of radiocarbon analysis of bone fragments, this individual can be dated back to the start of the fifth millennium BC. During the macroscopical observation the following pathological changes were noticed: light *cribra orbitalia* and *cribra cranii*, small patch of periostitis on the mandible. Cavitations were also found on the fragments of vertebral bodies. On the ventral surface of the heads of left ribs, active diffuse periostitis was noticed, heads of right ribs were not recovered. Other rib fragments from indeterminable sides also showed active diffuse periostitis, and one of them showed focal lytic lesion with reactive new bone surface. Signs of widespread active periosteal new bone formation were found along the shafts of the long bones, which was strikingly symmetrical both on the upper and the lower limbs. These periosteal changes can refer to HPO (Hypertrophic Pulmonary Osteopathy). On the foot bones, the signs of bilateral periostitis were also recognised.

After macromorphological observations, aDNA and lipid biomarker analysis were conducted.<sup>46</sup> First the IS1081 DNA region had been tested showing positive results. However, the application

<sup>38</sup> Molnár et al., 2006; Pálfi and Molnár, 2009.

<sup>39</sup> Lee et al., 2012; Molnár et al. 2006; Pálfi and Molnár 2009.

<sup>40</sup> Donoghue et al., 2005; Molnár et al., 2006.

<sup>41</sup> Lee et al., 2012.

<sup>42</sup> Minnikin et al., 1985.

<sup>43</sup> Lee et al., 2012.

<sup>44</sup> Lee et al., 2012.

<sup>45</sup> Masson et al., 2013.

<sup>46</sup> Masson et al., 2013.

of IS6110 complex-specific insertion sequence did not refer to the presence of *Mycobacterium* species. The detection of lipid biomarkers was more successful, since the mycolate fractions on the first reverse phase HPLC separation indicated the presence of long-chain mycolic acids. Although the profile was weak, it correlated with the standard profile for *M. tuberculosis*. The normal phase HPLC of the total mycolate fraction gave only a small peak for  $\alpha$ -mycolates, but the preservation of methoxy- or ketomycolates was not sufficient enough for the diagnosis. In contrast to this, the mycocerosic and the mycolipenic profiles confirmed the presence of the ancient tuberculosis infection measured by NI-CI-GC-MS.

Four additional cases were also examined later from this site with complex aDNA/HPLC/GC-MS strategy.<sup>47</sup> At first, these individuals were diagnosed as probable cases of tuberculous infection on the basis of macro-morphological examination, later also confirmed by molecular and chemical methodologies. HGO-08 involved in this work was a young, 17–22 years old female. On her skull light bilateral *cribra orbitalia* was recognised and other lesions were found in the thoracic region such as resorptive lesions on the anterior side of all thoracic vertebrae from the T5 to the T12 and also on two lumbar vertebrae. Furthermore, Schmorl's nodes were also visible on each thoracic vertebra from the T7 to the T12 (Plate no. I, Fig. 2). On the inferior side of the L1 and the superior side of the L2, an extensive lesion was observed with adjacent osteophytes. Following the macro-morphological identification of the disease, strong and clear mycocerosate- and mycolate profiles were also found indicating MTBC infection.

The next examined case was HGO-10, a male individual in the beginning of his twenties.<sup>48</sup> Hypervascularisation was observed on the anterior side of five successive thoracic vertebrae and two lumbar ones. Slight hypervascularisation was found on the visceral surface of the ribs. Furthermore, linear enamel hypoplasia was also found on the remains of this individual. The result of the lipid biomarker analysis also gave confirmation of a MTBC infection similarly to the previous case.

The third examined case was HGO-21, a female in her early twenties.<sup>49</sup> On her skulls small endocranial pits and *cribra orbitalia* were found. Resorptive lesions were observed on the T9 vertebra. Symptoms were also observed on her ribs such as increased vascularisation on the ventral side of one rib and light periostitis on the external surface of two fragments of other ribs towards their sternal end. The mycolate profile was weak, however, the positive aDNA result and the strong mycocerosate profile gave good confirmation of the infection.

The last examined individual from this site, HGO-48 was a young adult female. Abnormal blood vessel impressions were visible mainly on the frontal endocranial surface with a SES-like pattern (*serpens endocraniasym metrica*) (Plate no. I, Fig. 3). These signs refer to meningitis, possibly caused by infectious diseases including tuberculosis. A large round depression (circa 1 cm in diameter) on the endocranial surface of the right parietal might also be a tuberculous lesion. Slight *cribra orbitalia* was also recognised in the left orbit by Masson and colleagues. The mycolate profile was weak, however the strong mycocerosate profile and the result of the aDNA analysis confirmed the infection.

### 11.3. BÉLMÉGYER–CSÖMÖKIDOMB

The BÉLMÉGYER–CSÖMÖKIDOMB archaeological site contained the remains of 240 individuals. The cemetery was used between 670 and 800 AD during the Late Avar Age.<sup>50</sup> Nineteen tuberculosis

<sup>47</sup> Masson et al., 2015.

<sup>48</sup> Masson et al., 2015.

<sup>49</sup> Masson et al., 2015.

<sup>50</sup> Molnár et al., 2015.

infected individuals had been found based on earlier macroscopic and radiological examinations, including both classical and atypical/early stage TB cases.

The remains of a 40–60 years old female excavated from the grave no. 65 showed osteolytic lesions on the anterior aspect of the thoracic and lumbar vertebral bodies.<sup>51</sup> The lesions led to collapse of vertebrae causing angular kyphosis of the spine.<sup>52</sup> The aDNA analysis gave positive result for TB, which was also confirmed by the lipid biomarker analysis targeted to both mycolipenic- and mycocerosic acids, but the mycolic acid profile was weak.

The bones of a 57–62 years old male from grave no. 90 showed pathological remodeling and fusion of the lumbosacral region.<sup>53</sup> Furthermore, irregular *ante mortem* erosion was visible on the ventral surface of the sacrum suggesting earlier presence of cold abscess (Plate no. II, Fig. 4). Probable signs of *coxituberculosis* or tuberculous arthritis of the left hip was observed involving the left innominate and the femur (Plate no. II, Fig. 5). The aDNA analysis,<sup>54</sup> the mycolic acid and the mycolipenic acid profile all confirmed the MTBC infection, but the mycocerosic acid profile gave a less convincing evidence.<sup>55</sup>

In the case of the elderly male resting in grave no. 215, complete ankylosis of the right knee was reported.<sup>56</sup> This lesion strongly indicated the *gonitis tuberculosa*. The aDNA results were negative, the mycocerosic acid investigation was not effective enough, and the mycolic acid profile was also weak, but the mycolipenic detection diagnosed the *M. tuberculosis* infection.

Probable *coxituberculosis* of the right hip joint was found in the case of a 16–18 years old female individual from grave no. 38.<sup>57</sup> The aDNA analysis did not give conclusive results, nor did the mycolic acid separation, and the mycolipenate and the mycocerosate profiles recorded by NI-CI-GCMS were also only giving a weak signal.

The last case with classical TB changes is an adult male from the grave no. 189.<sup>58</sup> In this case, the main pathological changes affected the vertebrae suggesting *spondylitis tuberculosa* and ankylosis of the T9–T10 and the L1–L4 vertebrae. Furthermore, osteophytes and new bone formation were detected on the ventral surface of all lumbar vertebral bodies and long bone periostitis was detected on both femurs and tibias. The aDNA analysis along with the mycolipenic and mycocerosic analyses gave positive results, but the mycolic acid profile was not clear enough.

Further 14 examined individuals exhibited mainly atypical or early-stage macromorphological TB alterations and one of them did not show any lesion.<sup>59</sup> Most of the affected individuals belonged to younger age cohorts. 8 cases out of 14 showed rib periostitis (Plate no. II, Fig. 6) and 10 cases showed superficial vertebral changes. Long bone periostitis was found in the skeleton of 6 individuals, from which two showed diffuse periostitis. Endocranial lesions were observed in 5 cases. The presence of *cribra orbitalia* was observed on skulls of four individuals. The aDNA analysis gave positive result in 8 cases of out 14.<sup>60</sup>

Molnár and colleagues classified the individuals from Bélmegyer–Csömökidomb site into 6 groups on the base of lipid biomarker results. The first group includes the cases where

<sup>51</sup> Pálfi, 1991.

<sup>52</sup> Haas et al., 2000; Molnár et al., 2015.

<sup>53</sup> Molnár et al., 2015; Pálfi et al., 1992.

<sup>54</sup> Molnár et al., 2015; Haas et al., 2000.

<sup>55</sup> Molnár et al., 2015.

<sup>56</sup> Molnár et al., 2015; Pálfi–Csernus, 1990.

<sup>57</sup> Marcsik et al., 2007; Molnár et al., 2015.

<sup>58</sup> Marcsik et al., 2007; Molnár et al., 2015.

<sup>59</sup> Molnár et al., 2015.

<sup>60</sup> Haas et al., 2000; Molnár et al., 2015.

evidence of TB was found based on the mycolic, mycolipenic and mycocerosic acid profiles as well.<sup>61</sup> This group consists of 4 cases, among which the remains of a 16–18 years old individual excavated from grave no. 22 exhibited signs of leprosy-tuberculosis co-infection. The second group consists of 7 cases, where the presence of mycolipenate was clear, but the mycolic and mycocerosic acid signals gave less convincing evidence. This group included some individuals with classical TB changes. The third group contained 2 individuals with complete mycolipenate profiles, but the mycocerosic patterns were weak, while the mycolic acid examination gave no result. The fourth group included only one case, where even the mycolipenate signal was poor. The fifth group with 4 members showed weak and inconclusive evidence for both mycolipenic and mycocerosic acids. 2 samples were placed in the last group, where no mycobacterial lipid biomarker was detected. It is also important to mention that the individual, who did not show any classical or atypical TB associated alteration, was classified as a member of the first group.

### III. Discussion

It is important to understand the evolutionary history of *Mycobacterium tuberculosis* because tuberculous infections are becoming very frequent nowadays. In this question, examinations of archaeological samples may have a crucial role and could provide significant new information. The application of mycobacterial lipid biomarkers in the identification of *Mycobacterium tuberculosis* infections has a long history. However, this field is still developing nowadays considerably. The method developed by David E. Minnikin and his colleagues, can be used successfully in clinical practice and biological anthropology as well. Based on the formerly introduced examples, the method works well in a long time scale and in the case of leprosy co-infections as well. The application of lipid biomarker examination and a DNA analysis together with macromorphological methods provides a very effective and successful combination of diagnostic tools that will hopefully aid further important new discoveries in the future.

### References

- Alugupalli et al. 1998 Alugupalli, S., – Sikka, M. K., – Larsson, L., – White, D. C. (1998): Gaschromatography massspectrometry methods for the analysis of mycocerosic acids present in *Mycobacterium tuberculosis*. *J Microbiol Methods* 31, 143–50.
- Butler – Guthertz 2001 Butler, W. R., – Guthertz, L. S. (2001): Mycolic acid analysis by high-performance liquid chromatography for identification of *Mycobacterium* species. *ClinMicrobiolRev* 14, 704–726.
- Christensen et al. 1999 Christensen, H., – Garton, N. J., – Horobin, R. W., – Minnikin, D. E., – Barer M.R. (1999): Lipid domains of mycobacteria studied with fluorescent molecular probes. *Molecular Microbiology* 31, 1561–1572.
- Daffé – Lanéelle 1988 Daffé, M., – Lanéelle, M. A. (1988): Distribution of phthioceroldiester, phenolic mycosides and related compounds in mycobacteria. *J Gen Microbiol* 134, 2049–2055.
- Dobson et al. 1985 Dobson, G., – Minnikin, D. E., – Minnikin, S. M., – Parlett, J. H., – Goodfellow, M. – Ridell, M., – Magnusson, M. (1985): Systematic analyses of complex mycobacterial lipids. In: *Chemical Methods in Bacterial Systematics*. Eds. Goodfellow, M., – Minnikin, D. E. Academic Press. London, UK, 237–265.

<sup>61</sup> Molnár et al., 2015.

- Donoghue et al. 1998 Donoghue, H. D., - Spigelman, M., - Zias, J., - Gernaey-Child, A. M., - Minnikin, D. E. (1998): *Mycobacterium tuberculosis* complex DNA incalcified pleura from remains 1400 years old. *Lett Appl Microbiol* 27, 265-269.
- Donoghue et al. 2005 Donoghue, H. D., - Marcsik, A., - Matheson, C., - Vernon, K., - Nuorala, E., - Molto, J. E., - Greenblatt, C. L., - Spigelman, M. (2005): Co-infection of *Mycobacterium tuberculosis* and *Mycobacterium leprae* in human archaeological samples: a possible explanation for the historical decline of leprosy. *Proceedings of the Royal Society B* 272, 389-394.
- Donoghue et al. 2010 Donoghue, H. D., - Lee, O. Y.-C., - Minnikin, D. E., - Besra, G.S., - Taylor, J. H., - Spigelman, M. (2010): Tuberculosis in Dr Granville's Mummy: a molecular-examination of the first Egyptian mummy to be scientifically examined and given a medical diagnosis. *Proc Roy Soc B* 277, 51-56.
- Gernaey et al. 1998 Gernaey, A. M., - Minnikin, D. E., - Copley, M.S., - Power, J. J., - Ahmed, A.M.S., - Dixon, R. A., - Roberts, C. A., - Robertson, J.D., - Nolan, J., - Chamberlain, A. (1998): *Detecting ancient tuberculosis*. *Internet Archaeol*, [http://intarch.ac.uk/journal/issue5/gernaey\\_index.html](http://intarch.ac.uk/journal/issue5/gernaey_index.html).
- Gernaey et al. 1999 Gernaey, A. M., - Minnikin, D. E., - Copley, M.S. - Ahmed, A.M.S., - Robertson, D.J., - Nolan, J. - Chamberlain, A. T. (1999): Correlation of the occurrence of mycolic acids with tuberculosis in an archaeological population. In: *Tuberculosis Past and Present*. Eds. Pálfi, Gy., - Dutour, O., - Deák, J., - Hutás, I. Golden Book Publisher Ltd., TB Foundation. Szeged, Hungary, 275-282.
- Gernaey - Minnikin 2000 Gernaey, A. M., - Minnikin, D. E. (2000): Chemical methods in paleopathology. In: *Human Osteology in Archaeology and Forensic Science*. Eds. Cox, M., - Mays, S. Greenwich Medical Media Ltd. London, UK, 239-253
- Gernaey et al. 2001 Gernaey, A. M., - Minnikin, D. E., - Copley, M. S., - Dixon, R. A., - Middleton, J. C., - Roberts, C. A. (2001): *Mycolic acids and ancient DNA confirm an osteological diagnosis of tuberculosis*. *Tuberculosis*, 81, 259-265.
- Hershkovitz et al. 2008 Hershkovitz, I., - Donoghue, H. D., - Minnikin, D. E., - Besra, G. S., - Lee, O. Y.-C., - Gernaey, A. M., - Galili, E., - Eshed, V., - Greenblatt, C. L., - Lemma, E., - KahilaBar-Gal, G., - Spigelman, M. (2008): Detection and molecular characterization of 9000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in the Eastern Mediterranean. *PLoS ONE* 3, 3426-3426.
- Haas et al. 2000 Haas, J. C., - Zink, A., - Molnár, E., - Szeimies, U., - Reischl, U., - Marcsik, A., - Ardagna, Y., - Dutour, O., - Pálfi, Gy., - Nerlich, A.G. (2000): Molecular evidence for different stages of tuberculosis in ancient bone samples from Hungary. *Am J Phys Anthropol* 113, 293-304.
- Larsson et al. Larsson, L., - Mardh, P., - Odham, G., - Westerdahl, G. (1981): Use of selected ion monitoring for detection of tuberculostearic and C32 mycocerosic acid in mycobacteria and in five-day-old cultures of sputum specimens from patients with pulmonary tuberculosis. *Acta Pathol Microbiol Scand [B]* 89, 245-51.
- Lee et al. Lee, O. Y.-C., - Bull, I. D., - Molnár, E., - Marcsik, A., - Pálfi, Gy., - Donoghue, H. D., - Besra, G. S., - Minnikin, D. E. (2012): Integrated strategies for the use of lipid biomarkers in the diagnosis of ancient mycobacterial disease. In: *Proceedings of the 12th Annual Conference of the British Association for Biological Anthropology and Osteoarchaeology*. Eds. Mitchell, P. D., - Buckberry, J. Archaeopress. Oxford, UK, 63-69.
- Marcsik 1972 Marcsik, A. (1972): Generalizált TBC megbetegedés diagnózisa egy avar kori csontvázon. *Anthropológiai Közlemények* 16, 99-103.
- Marcsik et al. 2007 Marcsik, A., - Molnár, E., - Ósz, B. (2007): Specifikus fertőző megbetegedések csontelváltozásai történeti népesség körében. Szeged.

- Masson et al. 2013 Masson, M., – Molnár, E., – Donoghue, H. D., – Besra, G.S., – Minnikin, D. E., – Wu, H.H.T., – Lee, O. Y-C., – Bull, I.D., – Pálfi, Gy. (2013): Osteological and Biomolecular Evidence of a 7000-Year Old Case of Hypertrophic Pulmonary Osteopathy Secondary to Tuberculosis from Neolithic Hungary. *PLoS ONE* 8 (10) doi:10.1371/journal.pone.0078252.
- Masson et a. 2015 Masson, M., – Bereczki, Zs., – Molnár, E., – Donoghue, H. D., – Minnikin, D. E., – Lee, O. Y-C., – Wu, H. H. T., – Besra, G. S., – Bull, I. D., – Pálfi, Gy. (2015): 7000 year-old tuberculosis cases from Hungary Osteological and bio-molecular evidence, *Tuberculosis* 95(1) 13–17 doi: <http://dx.doi.org/10.1016/j.tube.2015.02.007>.
- Minnikin – Polgar 1967a Minnikin, D. E., – Polgar, N. (1967a): Mycolic acids from human and a vian tubercle bacilli. *Chemical Communications* 18, 916–918 doi: 0.1039/C19670000916.
- Minnikin – Polgar 1967b Minnikin, D. E., – Polgar, N. (1967b): The methoxymycolic and ketomycolic acids from human tubercle bacilli. *Chemical Communications* 22, 1172–1174 doi: 10.1039/C19670001172.
- Minnikin 1982 Minnikin, D. E. (1982): Lipids: Complex lipids, their chemistry, biosynthesis and role. In: *The Biology of Mycobacteria*. Eds. Ratledge, C., – Stanford, J. Academic Press. London, UK, 95–184
- Minnikin et a. 1983 Minnikin, D. E., – Dobson, G., – Hutchinson, I. G. (1983): Characterization of phthiocerol dimycocerosates from *Mycobacterium tuberculosis*. *Biochim Biophys Acta* 753, 445–449.
- Minnikin et a. 1985 Minnikin, D. E., – Dobson, G., – Goodfellow, M., – Draper, P., – Magnusson, M. (1985): Quantitative comparison of the mycolic and fattyacid composition of *Mycobacterium leprae* and *Mycobacterium gordonae*. *Journal of General Microbiology* 131. 2013–2021.
- Minnikin et a. 1985a Minnikin, D. E., – Dobson, G., – Goodfellow, M., – Magnusson, M., – Ridell, M. (1985a): Distribution of some mycobacterial waxes based on the phthiocerol family. *J Gen Microbiol* 131, 1375–1381.
- Minnikin 1993 Minnikin, D. E. (1993): Mycolicacids. In: *CRC Handbook of Chromatography: Analysis of Lipids*. Eds. K. D. Mukherjee–N. Weber. CRC Press, BocaRaton. Florida, USA, 339–348
- Minnikin et al. 1993a Minnikin, D. E., – Besra, G. S., – Bolton, R.C., – Datta, A. K., – Mallet, A. I., – Sharif, A., – Stanford, J. L., – Ridell, M., – Magnusson, M. (1993a): Identification of the leprosy bacillus and related mycobacteria by analysis of mycocerosate profiles. *AnnSocBelgMédTrop* 73 (Suppl. 1) 25–34.
- Minnikin et al. 1993b Minnikin, D. E., – Bolton, R. C., – Hartmann, S., – Besra, G. S., – Jenkins, P. A., – Mallet, A. I., – Wilkins, E., – Lawson, A.M., – Ridell, M. (1993b): An integrated procedure for the direct detection of characteristic lipids in tuberculosis patients. *AnnSocBelgMédTrop* 73 (Suppl. 1) 13–24.
- Minnikin et al. 2002 Minnikin, D. E., – Kremer, L., – Dover, L. G., – Besra, G. S. (2002): The methyl-branched fortifications of *Mycobacterium tuberculosis*. *ChemBiol* 9 545–553.
- Minnikin et al. 2012 Minnikin, D. E., – Lee, O. Y-C., – Wu, H. H. T., – Besra, G. S., – Donoghue, H. D. (2012): Molecular biomarkers for ancient tuberculosis. In: *Understanding Tuberculosis – Deciphering the Secret Life of the Bacilli*. Eds. P.-J. Cardona. InTech-Open Access Publisher. Rijeka, Croatia, 1–36 <http://www.intechopen.com/books/understanding-tuberculosis-deciphering-the-secret-life-of-the-bacilli>.
- Molnár et al. 2006 Molnár, E., – Marcsik, A. – Bereczki, Zs. – Donoghue, H. D. (2006): Pathological cases from the 7th century in Hungary. In: 16th European Meeting of the Paleopathology Association, 28 August – 1 September 2006. *Paleopathology Newsletter Supplement*. Eds. Sotiris K Manolis. Fira, Santorini, Greece, 33 (ISSN 0148–4737).

- Molnár et al. 2015 Molnár, E., - Donoghue, H. D., - Lee, O. Y-C., - Wu, H.H.T., - Besra, G. S., - Minnikin, D. E., - Bull, I. D., - Llewellyn, G., - Williams, C. M., - Spekker, O., - Pálfi, Gy. (2015): Morphological and biomolecular evidence for tuberculosis in 8th century AD skeletons from Bélmegyer-Csömöki domb, Hungary. *Tuberculosis* 95(1) 35–41 doi:http://dx.doi.org/10.1016/j.tube.2015.02.032.
- Pálfi et al. 1991 Pálfi, Gy. (1991): The osteoarchaeological evidence of vertebral tuberculosis in the 8th century. *Acta Biol Szeged* 37. 101–5.
- Pálfi – Csernus 1990 Pálfi, Gy., - Csernus, Z. (1990): Arthrite infectieuse an kylosante dans une série du VIII esiecle en Hongrie. *Paléobios* 6, 37–41.
- Pálfi et al. 1992 Pálfi, Gy., - Marcsik, A., - Kovács, J. (1992): Lumbosacral and hip tuberculosis in a migration peroid skeleton. *J Paleopathol* 4, 179–84.
- Pálfi – Molnár 2009 Pálfi, Gy., - Molnár, E. (2009): The Paleopathology of specific infectious diseases from Southeastern Hungary: a brief overview. *Acta Biol Szeged* 53 (2), 111–116.
- Pósa et al. 2015 Pósa, A., - Maixner, F., - Sola, C., - Berezki, Zs., - Molnár, E., - Masson, M., - Lovász, G., - Spekker, O., - Wicker, E., - Perrin, P., - Dutour, O., - Zink, A., - Pálfi, Gy. (2015): Tuberculosis infection in a late-medieval Hungarian population. *Tuberculosis* 95 (1), 1–5 http://dx.doi.org/10.1016/j.tube.2015.02.010.
- Masson et al. 2015 Masson, M., - Berezki, Zs., - Molnár, E. - Donoghue, H. D., - Minnikin, D. E., - Lee, O. Y-C., - Wu, H. H. T., - Besra, G. S., - Bull, I. D., - Pálfi, Gy. (2015): 7000 year-old tuberculosis cases from Hungary Osteological and biomolecular evidence. *Tuberculosis* 95 (1), 13–17 doi:http://dx.doi.org/10.1016/j.tube.2015.02.007.
- Qureshi et al. 1978 Qureshi, N., - Takayama, K., - Jordi, H. C., - Schnoes, H. K. (1978): Characterization of the purified components of a new homologous series of α-mycolic acids from *Mycobacterium tuberculosis* H37Ra. *J BiolChem* 253, 5411–5417.
- Redman et al. 2009 Redman, J. E., - Shaw, M. J., - Mallet, A. I., - Santos, A. L., - Roberts, C. A., - Gernaey, A.M., - Minnikin, D. E. (2009): Mycocerosic acid biomarkers for the diagnosis of tuberculosis in the Coimbra skeletal collection. *Tuberculosis* 89, 267–277.
- Steck et al. 1978 Steck, P. A., - Schwartz, B. A., - Rosendahl, M. S., - Gray, G. R. (1978): Mycolic acids. A reinvestigation. *J BiolChem* 253, 5625–5629.
- Somoskövi 2007 Á. Somoskövi: A *Mycobacterium*ok. In: *Pulmonáris és extrapulmonáris tuberkulózis*. Eds. P. Magyar – Á. Somoskövi. *Medicina*. Budapest 2007, 27–32.
- Watanabe et al. 2002 Watanabe, M., - Aoyagi, Y., - Ridell, M., - Minnikin, D. E. (2002): Separation and characterization of individual mycolic acids in representative mycobacteria. *Microbiology* 147 (2001) 1825–1837. Watanabe, M., - Aoyagi, Y., - Mitome, H., - Fujita, T., - Naoki, H., - Ridell, M., - Minnikin, DE. : Location of functional groups in mycobacterial meromycolate chains; the recognition of new structural principles in mycolic acids. *Microbiology* 148, 1881–1902.
- WHO (2016) http://www.who.int/mediacentre/factsheets/fs104/en/Downloaded: 2016.06.28. 16:49.
- Zink et al. 2007 Zink, A.R., - Molnár, E., - Motamedi, N., - Pálfi, Gy., - Marcsik, A., - Nerlich, A.G. (2007): Molecular History of Tuberculosis From Ancient Mummies and Skeletons. *International Journal of Osteoarchaeology* 17(4) 380–391.

## Figures

Fig. 1: HPLC PBA-PFB esters of mycolic acids extracted from skeletons from Atlit Yam and standard *M. tuberculosis*. A. Reverse phase HPLC of total mycolates; B. Normal phase HPLC of total

mycolates, collected from the former reverse phase HPLC measurement; C. Reverse phase HPLC of  $\alpha$ -mycolate, methoxymycolate and ketomycolate classes. Taken from Hershkovitz et al. (2008), modified by the authors.

- Fig. 2: Schmorl's node on the 12<sup>th</sup> thoracic vertebra. Hódmezővásárhely-Gorzsa, inv. no.: HGO-08, 17–22 years old female.
- Fig. 3: SES-like pattern. Hódmezővásárhely-Gorzsa, inv. no.: HGO-48, young adult female.
- Fig. 4: Pathological remodeling and fusion of the lumbosacral region. Further irregular *ante-mortem* erosion on the ventral surface of the sacrum. Békéscsaba-Csömökidomb, gr. no.: 90, 57–62 years old male.
- Fig. 5: Destruction on the left hip bone and on the left proximal femur. Békéscsaba-Csömökidomb, gr. no.: 90, 57–62 years old male.
- Fig. 6: Periostitis on a right rib fragment. Békéscsaba-Csömökidomb, gr. no.: 12, 33–39 years old male.



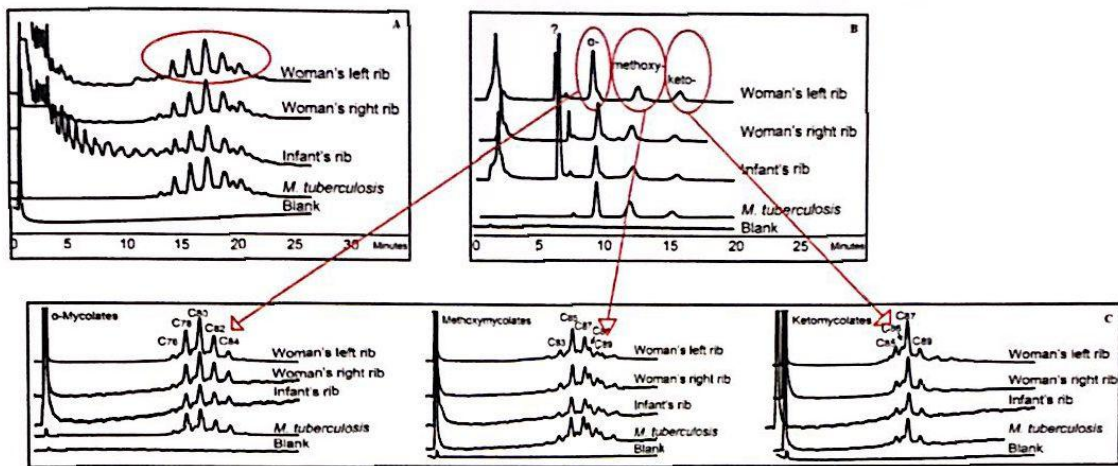


Figure 1

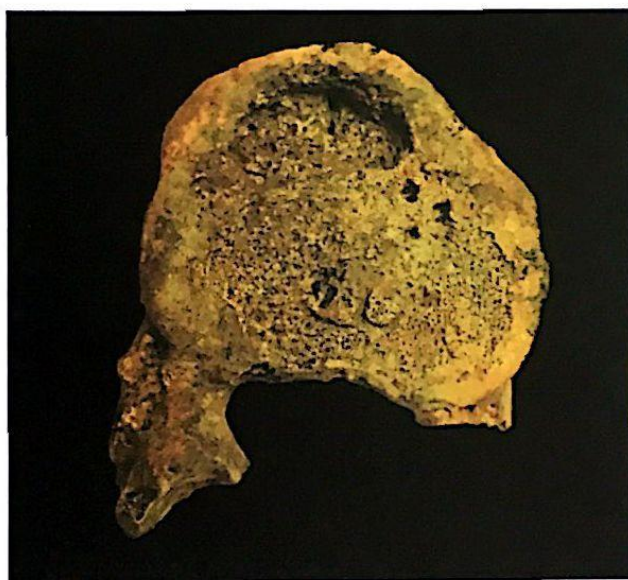


Figure 2

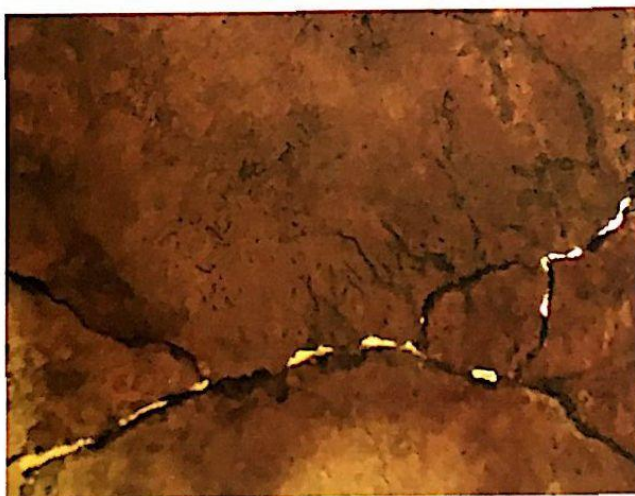


Figure 3

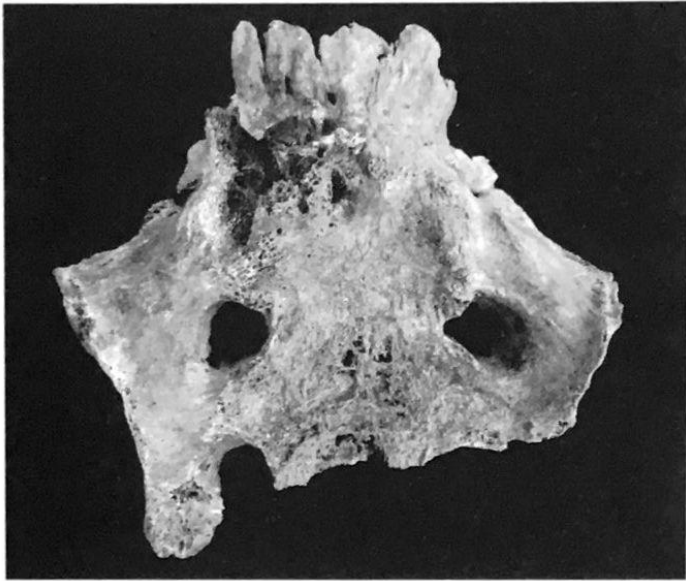


Figure 4



Figure 5



Figure 6