Mycolic acids as markers of osseous tuberculosis in the Neolithic skeleton from Kujawy region (central Poland)

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ABSTRACT: The subject of analysis is the male skeleton from a double burial of the Globular Amphora Culture, derived from the Neolithic site at Brześć Kujawski in Kujawy region (central Poland). Within the spine of the individual advanced lesions are observed (destruction of the vertebral bodies, symptoms of the periostitis in the thoracic region) which are characteristic of skeletal tuberculosis. To check whether the observed morphological changes resulted from infection with *Mycobacterium tuberculosis* (*M.tb*), the bone material was tested positively for the presence of mycolic acids, the specific components of the cell wall of pathogenic *M.tb* bacilli, by mass spectrometry.

KEY WORDS: mycolic acids, paleopathology, Neolithic

Introduction

It is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis* (*M.tb*), the causative agent of tuberculosis (TB). Despite the introduction of TB control strategies, the disease still remains one of the most common causes of death due to the infectious agent. In 2012, there were an estimated 8.6 million new cases of TB and 1.3 million people died from the dis-

ease (WHO, Global Tuberculosis Report; 2013). The emergence of multi-drug-resistant (MDR) *M.tb* strains and HIV co-infection make the TB epidemiological situation much worse. In 2012, an estimated 1.1 million (13%) of people who developed TB were HIV-positive globally. Data from drug resistance surveys of the World Health Organization (WHO) have shown that 3.6% of newly diagnosed cases and 20% of those previously treated for TB had MDR-TB. All these facts indicate the need for new diagnostics, medicines, and vaccines that could help control the global TB epidemic.

There is evidence for TB in animals prior to the Mesolithic (Lee et al. 2012), but no evidence of human TB. Tuberculosis (TB) is thought to have emerged in the period of transition from the Mesolithic to the Neolithic, when human populations became to grow thanks to the formation of food-producing communities based on animal farming, agriculture, and a sedentary way of life. These factors also resulted in the spread of many parasitic diseases (Brothwell 1981). TB has been documented to have occurred throughout subsequent centuries and re-emerged during the 17th to 19th centuries because of intensive urbanization, population growth, and progressing overcrowding (Roberts and Buikstra 2003; Müller et al. 2013). Diseases with TB-like symptoms were mentioned, amongst others, in Egyptian tomb inscriptions, in the Old Testament, Rig Veda books, and ancient Greek writings (Aufderheide and Rodriguez-Martin 1998; Gładykowska-Rzeczycka 2008).

Similarly as in the case of other diseases that may reach epidemic proportions, the spread of TB may be facilitated by a poor diet, impairment of an immune system by other diseases or simply by age, inadequate living conditions, densely crowded living conditions, unhygienic behavior, and even occupational risks, which may directly affect one's immunity (Fijałek and Supady 2002). The development of antibiotic therapy in the 1940s reduced the TB incidence rate, but did not completely eradicate the disease. According to Sajduda et al. (2004), in 2000 there were 10,049 new cases and 1,428 previously treated cases of TB (notification rate, 29.7 of 100,000). This increasing incidence of TB is accompanied by the spread of new bacterial strains resistant to one or more medicines.

As a disease known for ages TB affected millions of human beings over many millennia. Consequently, lesions caused by osseous tuberculosis are some of the first detected diseases by paleopathological research, and they have been richly documented. The oldest human skeletons with lesions attributable to tuberculosis of bones date back to the Neolithic (Bartels 1907; Sager et al. 1972; Formicola et al. 1987; Canci et al. 1996; El-Najjar et al. 1997; Roberts and Buikstra 2003; Hershkovitz et al. 2008), which seems to corroborate the key role of the agricultural revolution in the life of human populations and in the development of this disease. Cases of skeletons exhibiting bone destruction characteristic of TB (especially spine deformity in Pott's disease – collapse involving the anterior part of the vertebral body in the thoracic or lumbar regions of the spine, leading to pathologic angular kyphosis and hunchback) are known from later periods for all continents inhabited by humans except for Australia (Holloway et al. 2011). The main limitation on tuberculosis research in ancient populations is the non-specific nature of many bone lesions caused by this disease. Many pathologi-

cal conditions (e.g. chronic pyogenic osteomyelitis, *Brucella* osteomyelitis, fungal infections, typhoid spine, vertebral fractures, septic, traumatic, and rheumatoid arthritis, malignant bone tumors, ankylosing spondylitis) could be erroneously diagnosed as tuberculosis (Aufderheide and Rodríguez-Martin 1998). Therefore, previously, TB could be diagnosed with a high degree of certainty in archaeological specimens only in its most characteristic form affecting the spine as lesions in other locations may be indistinguishable from those of other etiologies.

The next milestone in TB research was the use of biochemical methods that made it possible to directly confirm the presence of the pathogen in the tissues of the studied individuals. An *M.tb* aDNA sequence was first obtained in 1993 from the archaeological remains of four individuals with tuberculosis diagnosed morphologically ranging in age from 300 to 1400 years (Donoghue et al. 2004). Since that time, many cases of TB confirmed using this method have been reported for ancient populations (an up-to-date list is given by Holloway et al. 2011), with the oldest cases dating back to the Neolithic (Hershkovitz et al. 2008; Masson et al. 2013). Isolation of *M.tb* aDNA makes it possible to verify traditional paleopathological diagnoses and also give a more complete picture of the spread of *M.tb* in archeological populations, providing new opportunities for studying its origin, evolution, virulence, and host-pathogen interaction. Another step in the advancement of PCR methods for studying mycobacterial aDNA was the development of the spoligotyping technique enabling differentiation between the various strains of the *M. tuberculosis* complex. One of the most important results attained using this method is

undoubtedly the fact that it challenged the view that *M.tb* evolved from *M. bovis* as a result of transmission of bacteria to humans from newly domesticated cattle about 10,000–15,000 years BC (Brosch et al. 2002; Donoghue et al. 2004; Zink et al. 2007). Detailed genomic data, confirming ancient tuberculosis, have been recently obtained by use of so-called Next Generation Sequencing (Bouwman et al. 2012) and direct Metagenomics (Chan et al. 2013).

Despite its advantages, aDNA analysis, using spoligotyping and sequencing techniques, faces a number of research limitations – most importantly, it is prone to the contamination of ancient material with environmental DNA, leading to false-positive results (Tran et al. 2011). Moreover, aDNA-based identification of microorganisms in ancient specimens might be limited by chemical modifications and fragmentation of nucleic acids during postmortem degradation. Other possible biomarkers of *M.tb* infection are β-hydroxy long-chain fatty acids known as mycolic acids. These molecules are major cell wall components and constitute from 40% to 60% of cell dry weight (Laval et al. 2001; Takayama et al. 2005). They are covalently attached to mycobacterial cell wall arabinogalactan and participate in the formation of a low-permeability barrier to many substances, including antibiotics (Takayama et al. 2005). Mycolic acids are involved in the first-line recognition of mycobacteria during host–pathogen interactions, contribute to bacterial survival within phagocytes and play a crucial role in the structure and function of the mycobacterial cell envelope (Takayama et al. 2005). Most mycobacterial species produce a combination of different types of mycolic acids, and their characteristic pro-

files have been used for taxonomic and diagnostic purposes. The acids differ in the length of the terminal alkyl group as well as the number of methylene groups between the cyclopropane rings and the carboxyl group. Due to the occurrence of various chemical groups within the main chain of these fatty acids, three mycolate subclasses, called alpha-, keto- and methoxy-mycolic acids, have been distinguished. In *M.tb*, the most abundant forms are alpha-mycolates, whereas methoxy- and keto-mycolates are less common (Watanabe et al. 2002; Takayama 2005).

Mycobacteria are characterized by the presence of mycolic acids consisting of 70–90 carbon atoms. They are composed of a longer β-hydroxy chain with a shorter α-alkyl side chain with two functional positions - a distal and a proximal containing various functional groups (Watanabe et al. 2002; Takayama et al. 2005). In *M. tuberculosis* the dominant mycolic

acid type are α-mycolates, whereas methoxy- and keto-mycolic acids are the minor components (Watanabe et al. 2002; Takayama et al. 2005). The α-mycolates are cis, cis-dicyclopropyl fatty acids that differ in the length of the terminal alkyl group and contain different number of methylene groups between the carboxyl group and the cyclopropane rings. The methoxy- and keto-mycolates may contain either cis- or trans-cyclopropane rings (Fig. 1).

Since the first use of mycolic acids for diagnosing TB in archaeological human remains (Gernaey et al. 1999; 2001), they have been more and more often applied as robust biomarkers of this disease in past populations (e.g., Hershkovitz et al. 2008; Lee et al. 2012; Minnikin et al. 2012). This is largely due to the fact that mycolic acid analyses are less susceptible to contamination than aDNA-based analysis thanks to their greater specificity.
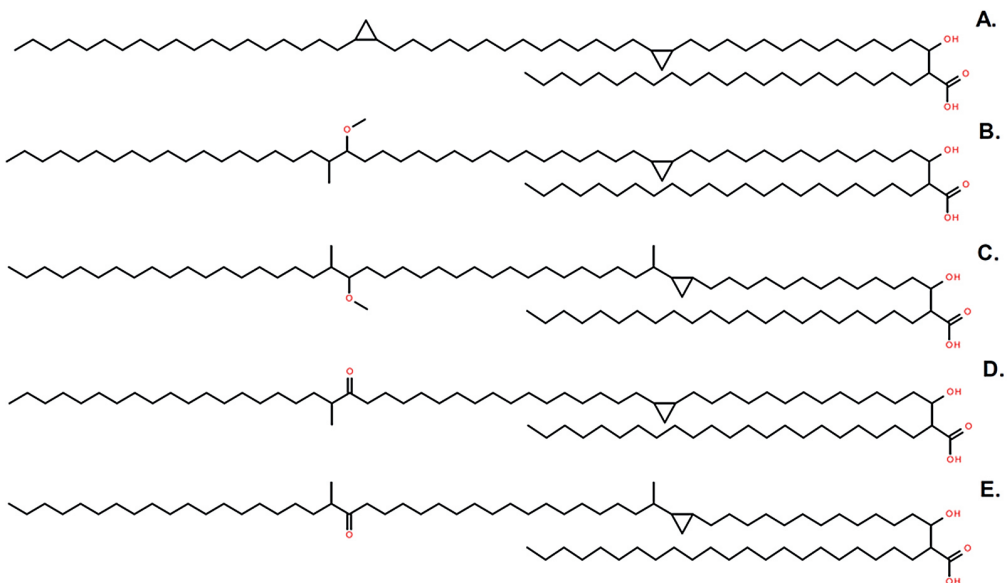

Fig. 1. Different forms of mycolic acids in *Mycobacterium tuberculosis* (Minnikin et al. 2012)

There are two objectives of this paper: first to confirm, by means of mycolic acid analysis, the macroscopically diagnosed tuberculosis on the skeleton from a Neolithic site in Brześć Kujawski (site 4), and second to determine the strain of the identified *Mycobacterium*.

Materials and Methods

The studied skeleton comes from a double burial discovered during the archeological works conducted by Jażdżewski in the area of a settlement and burial ground of the Brześć Kujawski group of the Lengyel Culture in the 1930s. The grave was initially classed with other materials belonging to that culture, but due to some distinct features of the burial other researchers later proposed that it represents the middle and late-Neolithic Globular Amphora Culture (oral communication from prof. Ryszard Grygiel). Shallow burial location below the surface was the cause of his severe destruction by ploughing, including the displace-

Fig. 2. Sketch showing the condition of the Neolithic burial. Objects hatched mean residues of bodyguards stone tomb, arrows indicate the position of the skulls of both individuals (after: Jażdżewski 1938, tab. IX)

ment and mixing of bone skeletons of both individuals (Fig. 2).

The macroscopic analysis was carried out in the osteological laboratory of the Department of Anthropology, University of Łódź. Sex and age were estimated based of the macroscopic methods commonly used in bioarchaeology (White and Folkens 2005). Differences in the morphology of the bones indicates that examined remains come from a male (the age of the individual was estimated about 30–50 years) and a young female (incomplete ossification process of the ilium suggest the age of 20–25 years). The paleopathological analysis of the bones belonging to male skeleton revealed distinct destructive changes of the vertebrae, which could be diagnosed as tuberculosis. On the other parts of this skeleton no pathological changes were observed, but the skeleton was incomplete, which limited possibilities of the research. The skeleton of woman was better preserved and without any visible lesions.

Samples were collected from the thoracic vertebra of the middle part of the spine of the male skeleton, adjacent two vertebrae reveal characteristic lesions of tuberculosis (the exact location of the lesions was not possible because the spine was incomplete).

Mycolic acid isolation from bone samples

Biochemical analysis was performed in the Department of Immunology and Infectious Biology, Institute of Microbiology, University of Łódź. The bone samples were ground by hand using an agate mortar. 100 mg of bone powder was homogenized three times with 300 μL of hexane (1 min, 4 m/s) and glass matrix

($\varnothing$ 1 mm) using a FastPrep 24 homogenizer. After centrifugation (2000 rpm, 10 min), the supernatants were evaporated to dryness under a vacuum evaporator. The dry residue was dissolved in 1 ml of the HPLC mobile phase and transferred to HPLC vials for LC-MS/MS analysis. The isolation of mycolic acids from *M. tuberculosis* $H_{37}R_v$ strain (ATCC 27294) was done in accordance with the optimized method described by Szewczyk et al. (2013) based on the alkaline hydrolysis of the cell wall lipids and the chloroform extraction of mycolic acids.

Mass spectrometry analyses were carried out as previously described by Szewczyk et al. (2013) with the flow injection analysis (FIA) negative electrospray ionization (ESI) Multiple Reaction Monitoring (MRM) MS/MS detection mode. LC-MS/MS analyses were performed on an Agilent 1200 LC system coupled with an AB Sciex 3200 QTRAP mass detector equipped with Turbo V ESI ion source. The LC settings were: injection volume 10 μl, draw speed 200 μl/min, eject speed and eluent flow 400 μl/min. The mobile phase consisted of a mixture of methanol-chloroform-acetonitrile (20/40/40 v/v/v) with an addition of 5 mM ammonium formate. To perform system conditioning and avoid carry-over effect the run cycle consisted of the following injections and method runs: 100 μl of chloroform, 10 μl of the blank and 10 μl of the tested sample with the needle wash for 3 s in chloroform in the flush port before and after every injection.

Results

Macroscopic analysis

As it was mentioned above the pathological alterations were observed within the spine of the male skeleton. Two vertebrae reveal characteristic lesions of tuberculosis: collapsed vertebral body (particularly its ventral part) of the vertebra from the middle of thoracic region of the spine which is characteristic of the Pott's disease and the compression fracture of the fifth lumbar vertebra (Fig. 3).

Mycolic acid analysis

Analysis of the presence and profile of long-chain mycolic acids extracted from the bone sample of the male skeleton was performed with the use of LC-MS/MS methodology according to Szewczyk et al. (2013), in the Institute of Microbiology, Biotechnology and Immunology,
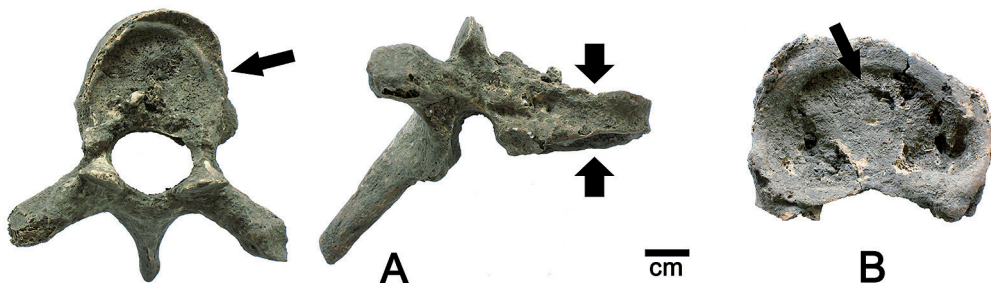

Fig. 3. The affected vertebrae of the analysed skeleton: A – collapsed vertebral body one of the thoracic vertebra (Pott's disease), B – compression fracture of the fifth lumbar vertebra

Table 1 Mycolic acids MRM pairs characteristic for *M. tuberculosis* $H_{37}R_v$ strain and found in the extract of bone (the thoracic vertebra) sample of the male skeleton

Mycolic acid			*M. tuberculosis* $H_{37}R_v$		Bone sample of the male skeleton
MRM transition	Chemical formula	Abbreviation	Mean	SD	
1136/395	$\alpha(C24)C_{78}H_{152}O_3$	α-C78 (C24)	13.28%	0.31%	11.11%
1136/367	$\alpha(C22)C_{78}H_{152}O_3$	α-C78 (C22)	2.67%	0.13%	3.36%
1164/395	$\alpha(C24)C_{80}H_{156}O_3$	α-C80 (C24)	15.92%	1.21%	19.04%
1192/395	$\alpha(C24)C_{82}H_{160}O_3$	α-C82 (C24)	9.23%	0.76%	13.76%
1220/395	$\alpha(C24)C_{84}H_{164}O_3$	α-C84 (C24)	3.98%	0.64%	7.90%
1236/395	$k(C24)C_{84}H_{164}O_4$	k-C84 (C24)	3.71%	0.60%	2.93%
1252/395	$m(C24)C_{85}H_{168}O_4$	m-C85 (C24)	11.43%	1.69%	8.18%
1252/367	$m(C22)C_{85}H_{168}O_4$	m-C85 (C22)	2.20%	0.12%	1.94%
1264/395	$k(C24)C_{86}H_{168}O_4$	k-C86 (C24)	4.81%	0.22%	4.45%
1278/395	$k(C24)C_{87}H_{170}O_4$	k-C87 (C24)	7.43%	0.37%	7.31%
1280/395	$m(C24)C_{87}H_{172}O_4$	m-C87 (C24)	13.83%	0.82%	11.03%
1292/395	$k(C24)C_{88}H_{172}O_4$	k-C88 (C24)	1.59%	0.28%	1.94%
1294/395	$m(C24)C_{88}H_{174}O_4$	m-C88 (C24)	4.90%	0.36%	2.74%
1308/395	$m(C24)C_{89}H_{176}O_4$	m-C89 (C24)	5.01%	0.43%	4.31%

α, alpha; k, keto; m, methoxy; C24, 24 carbon atoms; C22, 22 carbon atoms; SD, standard deviation.
The percentages shown represent the intensity of each peak in relation to the total intensity of all tested mycolic acids.

University of Łódź. Mycolic acid profile was established on the basis of the precursor (Prec) spectrometer scan mode on the basis of fragment ions (395.4, 367.3) from the 24- or 22- carbon α-alkyl chains. The analysis of the extract revealed the presence of 14 types of mycolic acids (MRM pairs: 1136/395, 1136/367, 1164/395, 1192/395, 1220/395, 1236/395, 1252/395, 1252/367, 1264/395, 1278/395, 1280/395, 1292/395, 1295/395, 1308/395) (Fig. 3). The profile and the quantitative relationship of individual fatty acids were found to be compliant to the profile of the standard *M. tuberculosis* $H_{37}R_v$ strain (Table 1).

Thus, both macroscopic and biochemical analysis indicate that the individual suffered from TB. This finding was also confirmed by aDNA analysis which was performed in the Laboratory of Mycobacteria: Physiology and Genetics, Institute for Medical Biology, Polish Academy of Sciences, Łódź.

The sample of vertebra from the analysed skeleton was positive for PCR
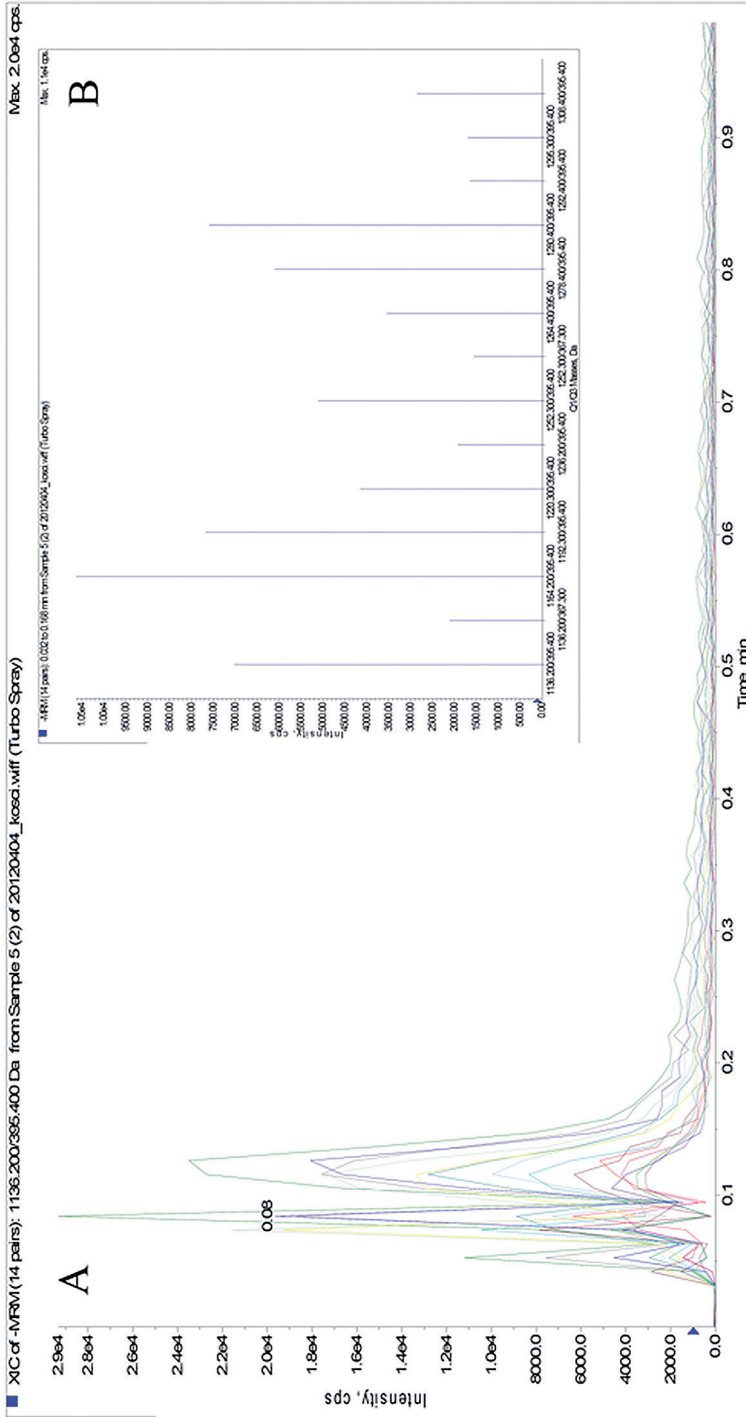
Fig. 4. MS/MS analysis of mycolic acids extracted from bone sample of the male skeleton. Chromatogram (A) and MRM profiles (B)

using primers specific for *M. tuberculosis* IS6110, with an amplicon of 123pz (Is P1 and IsP2), 93pz (is3 and is4).

Discussion

Tuberculosis may affect any organ of the body. The most frequently recorded cases of extrapulmonary TB involve the pleura, lymph nodes, bones and joints, and the genitourinary system. Nowadays, tuberculosis of bones and joints accounts for a small percentage of all TB cases (by different authors 1%–5%). According to Holloway et al. (2011) tuberculosis of bones in the past could be more frequent but due to the condition of the prehistoric material it is difficult to assess. Bone and joint lesions often concur with TB in another location. This type of TB affects the spine in 40%–60% of the cases, as well as hip joints (13%–15%), and knee joints (10%–15%). Therefore analysis of morphological lesions in bone material usually consists of description vertebral fusion and collapse leading to Pott's disease, knee joint ankylosis and hip joint destruction (Ortner 2003; Holloway et al. 2011).

However, some bone lesions, such as those in the ribs and long bones, may be non-specific, as other conditions (e.g., brucellosis and tumors) may cause similar changes (Roberts et al. 1994; Roberts and Buikstra 2003; Aufderheide and Rodriguez-Martin 1998), which makes them difficult to interpret. Moreover, it should be remembered that TB is primarily a disease of the respiratory tract, and as such may cause bone lesions only in a small number of individuals (Resnick 1995; Golden and Vikram 2005). Thus, additional methods of examination are necessary to give a conclusive diagnosis. Over the past several years, many bone remains have been analyzed to detect morphological lesions that may have arisen as a result of TB (Roberts and Buikstra 2003; Holloway et al. 2011; Masson et al. 2013). It has been found that some bone lesions, such as periostitis and hypertrophic osteoarthropathy, are significantly correlated with the presence of the *Mycobacterium tuberculosis complex* (Hershkovitz et al. 2008; Masson et al. 2013).

Paleopathological research makes use of both macro- and microscopic examinations, as well as genetic and biochemical testing. As it was already mentioned in the introduction, aDNA studies often pose many technical problems related to the degradation of bone remains and their contamination. Gernaey et al. (1999, 2001) proposed the use of mycolic acids as an alternative to aDNA biomarkers in studies concerning the occurrence of TB in prehistoric populations. Redman et al. (2009) and Lee et al. (2012) showed that mycocerosic and mycolipenic acid biomarkers are also valuable indicators of tuberculosis in prehistoric bone material. The authors suggested that these lipid biomarkers may be more stable and less susceptible to contamination, and provide a good diagnostic tool for detecting *M.tb* due to the fact that they differ considerably from mammalian tissues. Mycolic, mycocerosic and mycolipenic acids not only allow the identification of the presence of *Mycobacterium* but also to determine its species. Widely used gas chromatography technique, one or two-dimensional thin layer chromatography (1D/2D-TLC) or high performance liquid chromatography (HPLC) are slowly being replaced by tandem mass spectrometers MS/MS type with electrospray type ion source (ESI) allowing more detailed analysis of the components of the

bacterial cell wall. While a few literature reports suggest a potential use of LC-MS/MS method for the differentiation of mycolic acids isolated from living cells of mycobacteria (Song et al. 2009; Shui et al. 2007, 2012), this technique has not yet been extensively applied in the analysis of these structures in the material of archaeological origin. The presented results are one of the first that suggest the possibility of the use of LC-MS/MS method in the identification of mycobacterial infections in the ancient prehistorical material. We showed that the profile of mycolic acids isolated from the bone sample showed full compliance with the profile of these compounds present in the cell wall of the virulent standard *M. tuberculosis* $H_{37}R_v$ strain. Extracted mycolic acids mixture contained 14 types of mycolic acids characterized by MRM pairs: 1136/395, 1136/367, 1164/395, 1192/395, 1220/395, 1236/395, 1252/395, 1252/367, 1264/395, 1278/395, 1280/395, 1292/395, 1295/395, 1308/395 occurring in the ratio characteristic for *M. tuberculosis*. This findings allow to suggest that the cause of the observed changes in bone debris was probably *M. tuberculosis*.

Conclusions

Obtained results confirm the potential of using the method of MS/MS in the study of archaeological material. Methods based on the isolation of mycolic acids from the skeletal remains together with the analysis of characteristic profiles of these compounds appear to be promising tools not only in detecting *Mycobacterium* infections, but also in the differentiation of species within the genus.

Sequential reverse and normal phase fluorescence HPLC has been developed into an effective protocol for the recognition of mycolic acid biomarkers for tuberculosis in archaeological samples (Hershkovitz et al. 2008; Lee et al. 2012; Minnikin et al. 2012). However, it is important to develop the use of mass spectrometry to provide a higher level of confirmatory information. An attempt was made by Mark et al. (2010) to detect ancient mycolic acids by MALDI-TOF MS, but it is clear (Minnikin et al. 2012) that the recorded profiles do not correspond to mycolic acids from *M. tuberculosis*. The data presented here represent the first positive detection of mycobacterial mycolic acids by mass spectrometry in an archaeological specimen. Further studies will be needed to determine whether the procedure can be used routinely for samples in which mycolic acids are less abundant and possibly degraded.

It should also be noted the considerable antiquity of the skeleton: the individual comes from a Neolithic population of the Globular Amphora Culture dated to 3500–3000 BC. Therefore, the fact that a positive result was obtained for such old material is of great importance. So far, the only case of TB in Neolithic material from Złota in Poland (in the Sandomierz region) was described by Gładykowska-Rzeczycka, but aDNA analysis did not confirm this diagnosis (Gładykowska-Rzeczycka 2008). It should be emphasized that using mycolic acids, in our study, it is possible to determine not only the *Mycobacterium*, but also the species (which was *Mycobacterium tuberculosis* in the case presented herein).

Authors' contributions

BBS conceived and designed the paper, performed the research project; WL interpreted the data, edited the manuscript; MD, RSZ designed and carried out microbiologic laboratory analysis and interpreted the results; EŻ served as principal investigator for the research, edited the manuscript. All authors were involved in drafting the manuscript and approved the final manuscript.

Conflict of interest

The Authors declare that there is no conflict of interests regarding the publication of this article.

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