# Development of lipid biomarker based diagnostic method for TB research in archaeological samples via HPLC-HRMS

**Ph.D. thesis**

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## INTRODUCTION

The diagnosis of ancient mycobacterial disease in archaeological material requires a multidisciplinary approach. Most of the TB-related bony lesions are the result of extra-pulmonary TB (Aufderheide & Rodríguez-Martín, 1998; Marcsik et al., 1999, 2009; Pálfi & Marcsik, 1999; Hershkovitz et al., 2002; Maczel, 2003; Ortner et al., 2003; Paja et al., 2015; Pálfi & Molnár, 2009; Pálfi et al., 2012; 2015; Mariotti et al., 2015; Spekker, 2018; Spekker et al., 2018; Spekker et al., 2020a; Spekker et al., 2020b), which was registered only in 16% of all incident cases in 2019 (WHO, 2020). Moreover, only a low proportion of extrapulmonary TB develops specific and detectable bony lesions. Macromorphological paleopathological studies lead necessarily to the underestimation of ancient TB prevalence. For the better estimation of TB incidence in past populations an array of specific biomarkers can be brought into play to confirm initial skeletal diagnoses and search in the soft tissue of suspected cases (Donoghue et al., 2017). The pioneering application of whole-genome sequencing (Chan et al., 2013) has opened the prospect of obtaining comprehensive information regarding the nature and number of infecting *M. tuberculosis* strains (Kay et al., 2015; Bos et al., 2014). Confidence in aDNA diagnoses has been greatly reinforced by recording profiles of specific lipid biomarkers, which are particularly robust and accessible (Donoghue et al., 2017).

An integrated procedure for the detection of characteristic lipid biomarkers was published in 1993 by David E. Minnikin and colleagues (Minnikin et al., 1993). The method included the detection of MAs, tuberculostearic acid (TSA) and MCs. After the lipid extraction lipids were purified by solid phase extraction (SPE) on normal phase (NP) cartridge. MA detection was carried out via HPLC-FLD (high performance liquid chromatograph connected to fluorescence detector) by the application of 9-anthrylmethyl ester derivatisation after reverse phase (RP) SPE purification. MAs were first separated according to type on NP-HLPC, then individual MA types have been further separated on RP-HPLC. TSA and MCs were derivatised with pentafluorobenzyl bromide (PFB) for GC-FID-ECD (gas chromatograph coupled with flame ionisation and electron capture detector) and GC-ECD, respectively.

However, the previously described method was developed for clinical diagnosis shortly after, it was used for the detection of ancient TB infection with minor changes (e.g. an initial RP-HPLC separation was added before preceding the NP-HPLC separation) by detecting MAs from a 1400 years old calcified pleura sample and two approximately 1000 years old rib samples of morphologically TB positive cases along with aDNA analysis (Gernaey et al., 1998; Donoghue et al., 1998; Gernaey et al., 2001). A new and more sensitive version of MA detection was

published in 2008 by Hershovitz and colleagues (Hershovitz et al., 2008). The samples used for the demonstration were taken from a woman and an infant buried together having TB-related bone lesions. The individuals were unearthed from a 9000 years old, Neolithic excavation site, called Atlit-Yam. The extracted lipids were first derivatised with PFB, then purified on NP-SPE. Then PFB-MAs were further derivatised with PBA and purified on C18 RP-SPE. PBA-PFB mycolates were further extracted and analysed first by RP-HPLC-FLD. Components corresponding to PBA-PFB mycolates were collected for further analysation on NP-HPLC-FLD, where α-, methoxy- and ketomycolates were separated. The individual mycolate types were collected separately and examined on RP-HPLC-FLD, where individual mycolates were separated. In this study both individuals have been proven to be positive to TB by aDNA as well as mycolic acid examination (Donoghue et al., 1998; Gernaey et al., 1998; Gernaey et al., 2001; Hershkovitz et al., 2008).

A different approach was also introduced in first half of the last decade. This method was also principally developed for clinical diagnosis of TB (Szewczyk et al., 2013). The method based on HPLC-MS/MS examination, with electrospray ionisation (ESI). The analyses targeted 10 of the most abundant and characteristic MAs of *M. tuberculosis*, the multiple reaction (MRM) pairs included α-, methoxy- and ketomycolates. The preparation did not include any derivatisation, furthermore, the samples were analysed by flow injection analyses, both features contributed in the establishment of a rapid procedure. The method was first used on archaeological samples in the next year (Borowska-Strugińska et al., 2014). The samples derived from a 30-50 years old male, buried in the Neolithic period, whose macromorphological examination described him as a TB positive individual. The previously described clinical method was applied, but the MS detection was supplemented with further MA targets, monitoring 14 MRM transition pairs. The MA profile recorded from the examined samples presented a similar distribution to *M. tuberculosis*, moreover the lipid biomarker based results were confirmed by PCR analysis of the extracted aDNA.

A further optimised method was introduced for establishing mycocerosate profile and detecting C27 mycolipenic acid from historical bone samples, which was published in 2009 (Redman et al., 2009). The optimisation results were tested and demonstrated on samples taken from individuals of the Coimbra Identified Skeletal Collection. The examined biomarkers were C26, C27, C29, C30, C32, C33, C34 mycocerosic acids and C27 ML. The extracted MC and ML containing fraction was first derivatised with PFB and further extracted, and then purified on NP-SPE. The collected fractions were analysed on NP-HPLC due to the separation of

multimethyl-branched fatty acid (FA)-PFB esters (e.g. the mycocerosates) from other PFB FA esters, and the multimethyl-branched FA-PFB esters were collected. For the proper timing of the fraction collection from the HPLC, two co-markers, decafluorobenzophenone (DFBP) and long-chain (C6, C8, C12, C16 and C18) esters of decafluorobenzhydrol were applied, which are eluting after and before the targeted fraction, as follows. The collected PFB-esters were measured by negative ion chemical ionisation gas chromatograph coupled with mass spectrometer (NI-CI GC-MS) in selected ion monitoring (SIM) mode. The C26, C33, C34 mycocerosic acids were excluded from their study as no definitive ion signals were observed. From the standard *M. tuberculosis* PN cells the C29, C30 and C32 MCs were extracted in the highest ratio, along with the C27 ML. Among the 49 examined bone samples 33 mycocerosate positive samples were found. 22 out of the 33 correlated with a positive diagnosis for tuberculosis, and only 2 individuals who were diagnosed with pulmonary tuberculosis presented negative results for mycocerosates.

When both mycobacterial aDNA and lipid biomarker analysis is carried out, it is possible to recover the latter one from the samples that have already undergone DNA extraction (Lee et al., 2012; Donoghue et al., 2017). The combined application of macroscopic, aDNA and lipid biomarker based methods resulted outstanding results (e.g. Donoghue et al., 1998; Gernaey et al., 1998; Hershkovitz et al., 2008; Donoghue et al., 2010; Lee et al., 2012; Baker et al., 2015; Lee et al., 2015; Masson et al., 2015; Minnikin et al., 2015; Molnár et al., 2015; Luna et al., 2020; Donoghue et al., 2017).

For the verification of new methods, and the estimation of reliability of new markers in macroscopic analysis paleopathologists usually use well-documented collections of skeletons and mummies from the pre-antibiotic era (Roberts et al., 1994; Santos & Roberts, 2001; 2006; Pálfi et al., 2012; Spekker, 2018; Spekker et al., 2020a; 2020b). The Vác mummy collection is one of the well-documented and broadly studied collections. Among other advantages of this collection, several results of TB-related investigations and individual data are available regarding this group (Szikossy et al., 1997; Pap et al., 1999; Fletcher et al., 2003; Chan et al., 2013; Kay et al., 2015; Pap et al., 2017). As these mummies are dated to the modern age, but they are from the pre-antibiotic era, they also represent an excellent link between recent and archaeological samples. As TB was already a growing burden all around Europe, including Hungary and the suspicion of TB-infected cases arose based on macroscopic observations, an extended screening was carried out searching for DNA traits of the infectious agent (Fletcher et al., 2003). 350 samples belonging to 168 individuals were examined by Fletcher and her

colleagues. The samples were taken from the lungs, pleura, abdomen, ribs, hair, teeth, and clothing. In their study 55% of the individuals proved to be infected with *Mycobacterium tuberculosis* complex (MTBC).

## THE AIMS AND OBJECTIVES OF THE DISSERTATION

Two of the above mentioned methods for the detection of MA, MC and ML C27 are used for a long time (Hershkovitz et al., 2008; Redman et al., 2009; Lee et al., 2012; Donoghue et al., 2017) and proved their outstanding relevance in many cases, however they both include a very complex sample pre-treatment procedure.

Our aim was to establish a lipid biomarker based HPLC-MS method, for TB diagnosis in historical human samples, as this instrumentation is available in many laboratories, and has the potential of a quick and sensitive and at the same time affordable measurement protocol. The main objectives of this PhD dissertation are the following:

1. Development and optimisation of a HPLC-MS method for the detection of the two most commonly used lipid biomarkers, namely the mycolic acids and mycocerosic acids.

2. Development of a lipid profile library, regarding both mycolic and mycocerosic acids, which can be used as reference for diagnosis.

3. Testing the efficiency of the established lipid biomarker based method on a well-studied collection of mummified human remains.

## MATERIALS AND METHODS

Two reference strains from the American Type Culture Collection (ATCC), provided by BEI Resources (Manassas, Virginia, USA), namely the *M. tuberculosis* H37Rv (NR-49098) and *M. bovis* (NR-31210) and MA standard provided by David E. Minnikin were used for primary verification. For the method testing, five *M. tuberculosis* complex strains were used (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 diagnosed with pulmonary tuberculosis. Also, eight different NTM species, namely *M. kansasii* 1959/2018, *M. chelonae* 16/2018, *M. gordonae* 389/2018, *M. intracellulare* 7802/2017, *M. avium* 16229/2018, *M. chimaera* 619/2018, *M. abscessus ssp. abscessus* 180/2018, and *M. fortuitum* complex 3/2018, from various clinical specimens were included. For the test of the developed mycocerosic acid detection method bone and soft tissue samples were taken from six individuals of the Vác mummy collection, selected on the base of previous positive results gained by aDNA

examination. The sampled adult individuals were four females and two males. The analysed mummy samples (both bone and soft tissue samples) derived from the chest region of the examined individuals. Measurements were carried out on a Shimadzu LC-10AD VP HPLC coupled with a Shimadzu LCMS-2010A Single Quadrupole mass spectrometer (MS) and on a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Waltham, Massachusetts, USA), which was coupled with a Q-Exactive Plus MS (Thermo Scientific, Waltham, Massachusetts, USA). For the separation, a Gemini – NX C18 (3 μm, 110A, 50 mm x 2 mm) column (Phenomenex, Torrance, California, USA) was used.

In the case of the MA detection method development 5 different eluent compositions were tested on the single quadrupole LCMS system, and 14 eluent compositions on the HPLC-Orbitrap MS system in FIA. Ionisation was carried out with ESI ion source. For the verification of the observed MA peaks ions were fragmented in parallel reaction monitoring (PRM) with the application of collision energy of 70 kV. As for the MC detection method, 1 eluent composition (acetonitrile:isopropanol:heptane (5:4:1)+ 0.1% AcOH) was applied both in FIA and gradient elution. During the method optimisation ESI and APCI ion sources were applied. All the lipids were detected in negative ion mode.

## RESULTS AND DISCUSSION

The verification of the targeted MA peaks was carried out with two approaches. On the mass spectrum both the targeted and not targeted MAs could be included in series of molecules with 28 Dalton differences (two-carbon unit), characteristic to the homologous series of MAs, due to their biosynthesis pathway (Takayama et al., 2005). With the application of PRM measurements the two most characteristic fragments were the 365.35767 and the 395.38901, identified as the C24 and C26 α-alkyl chain, respectively. During the optimisation procedures with the increased capillary and auxillary gas temperature, as well as with the increased capillary and S-lens voltage, the intensity of the targeted peaks become higher regardless of the applied solvent composition in the case of MAs and MCs.

In contrast with aDNA, obviously there is no option for amplifying the extracted lipid biomarkers, thus the efficiency of the extraction is surpassingly important, hence we optimised the sample pre-treatment protocol. The application of 20% or 30% KOH in the saponification mixture did not presented any difference, however further decrease to 10% resulted lower MA outcome. The application of hexane isomer mixture resulted higher efficiency regarding the extraction of α- and methoxy-MA specimens, while affected negatively the efficiency of the

extraction of keto-MAs, hence extraction 3 times with toluene was followed by one additional extraction step with hexane.

As a reference for later investigations the development of a mycobacterial MA profile library has been started, including the profile of MTBC-1/2015, MTBC-254/2000, MTBC-3910/2014, MTBC-242/2000, MTBC-1/8508/2014, *M. gordonae* 389/2018, *M. intracellulare* 7802/2017, *M. abscessus ssp. abscessus* 180/2018, *M. kansasii* 1959/2018 and *M. chelonae* 16/2018. The α-C78 was found to be the most abundant MA in all the included MTBC strains. The second highest representation varied between the α-C80 and m-C85, moreover m-C87 was also highly represented. The α-C82, m-C88, k-C87 and m-C89 MAs were also represented in higher, than 5% in most of the analysed MTBC strains. Regarding the summarised values of individual MA-s based in their type the α-MAs were represented in the highest ratio in the involved MTBC strains, ranging between 40%-50%, however in one exception, the highest summarised area values belonged to methoxy-MA specimens, which were slightly less represented in the other 4 strains, adding up to approximately 40% of the overall area values. The least represented MA type was the keto-MA in every cases. In the case of the NTM species, the most abundant MA specimen was the α-C78 MA, as well. MAs with higher alkyl-chain and oxygenated MAs were less represented in most of NTM strains. In our measurements the oxygenated MA-s of *M. intracellulare*, *M. abscessus* and *M. chelonae* showed different distribution to the published profiles (Minnikin & Brennan, 2020), which implies the need of further investigation. However, the published MA profiles of NTM species presents differences from our results (Song et al., 2009; Shui et al., 2011; Szewczyk et al., 2013; Minnikin & Brennan, 2020), with the low representation of oxygenated mycolates the differentiation of MTBC from NTM representatives seems possible with the application of lipid profiling via HPLC-MS.

In the development of MC based detection method, a MC profile library was established. Among the representatives of MTBC in 4 out of 5 strains the C32 MC was the most prominent, while C29 and C30 MCs were present with similar distribution, and C33 MC was observed in the lowest ratio, similarly to those detected in the *M. tuberculosis* H37Rv reference strain. The one strain excluded from the establishment of the average MC profile of MTB presented a MC distribution more characteristic to *M. bovis*. Among the analysed NTM species (*M. avium* 16229/2018, *M. chelonae* 16/2018, *M. intracellulare* 7802/2017, *M. fortuitum* 3/2018, *M. gordonae* 389/2018, *M. abscessus sp. abscessus* 180/2018, *M. chimaera* 619/2018, *M. kansasii* 1959/2018) only the profile of *M. kansasii* was established, as the remainder species are not expressing mycocerosates.

Based on the observed bacterial profiles the detection of prominent C32 MC content, accompanied with significant C29 and C30 MC peaks were used as criteria for MTB diagnosis in the following analysis. In the case of four mummified individuals, profiles of characteristic *M. tuberculosis* MCs were recorded, but two other mummies did not yield diagnostic MC profiles. In the case of 6 samples (4 soft tissue, 2 rib) 4 MCs have been detected, and only 3 MCs were recorded in 2 samples. The C27 MC did not reach the limit of detection in any of the mummy samples. In the two cases lacking significant mycocerosate presence, but with good indications of aDNA biomarkers, the discrepancy may be due to differences in the samples investigated. Mummified individuals provide a much greater choice of areas for sampling than in skeletons without soft tissue. Even though the cases of #25 and #79 were deemed negative, the very weak signals observed could be sufficient to encourage investigation of a wider range of samples from these mummies.

## CONCLUSIONS, RESEARCH SIGNIFICANCE, AND PERSPECTIVES

1. The first step towards establishing a lipid biomarker based HPLC-HRMS method for TB diagnosis in historical human samples was the tuning of the applied HPLC and the MS parameters, for the efficient detection of mycolic acids and mycocerosic acids and the optimisation of the sample pre-treatment process. In the case of the MA detection method development 5 different eluent compositions were tested on the single quadrupole LCMS system, and 14 eluent compositions on the HPLC-Orbitrap MS system. In our initial measurements on the single quadrupole LCMS system the application of chloroform seemed beneficial. However, the presence of increased chloroform concentration affected negatively the ionisation of MAs on the Orbitrap MS. With the application of heptane, as non-polar component we could increase the peak intensity, moreover we were able to establish a method for the separation and parallel detection of MAs and MCs. The MS parameters were optimised with one eluent composition on the single quadrupole LCMS system, and with the application of two eluent compositions on the HPLC-Orbitrap MS system for MA detection. During the optimisation of the MS parameters, the positive effect of the increased capillary and auxiliary gas temperature, as well as the increased capillary and S-lens voltage was observed. The intensity of the targeted peaks become higher regardless of the applied solvent composition. For the detection of MCs one eluent composition was used. During the optimisation of the MC detection protocol APCI and ESI ion sources were compared. For the detection of mycocerosic acids ESI has been observed as more efficient ion source, which is known to provide softer ionisation in

contrast with the APCI (Yunker et al., 2014). As for the optimisation of the sample pre-treatment protocol of MA extraction the KOH concentration of the initial saponification mixture was tested. The comparison of 20% or 30% KOH content did not presented any difference, however, further decrease to 10% resulted lower MA outcome. Beside the optimisation of KOH concentration, 5 different extraction solvents/solvent combinations were compared. The results suggested that nonetheless the application of non-polar solvents are needed for the extraction, the lowest polarity is not equal with the best efficiency.

2. With the application of the optimised methods, the development of lipid profile library, regarding both mycolic and mycocerosic acids has been carried out. The MA profiles of 5 MTBC clinical isolates (MTBC-1/2015; MTBC-254/2000; MTBC-3910/2014; MTBC-242/2000; and MTBC-1/8508/2014) and 5 NTM clinical isolates (*M. gordonae* 389/2018, *M. intracellulare* 7802/2017, *M. abscessus ssp. abscessus* 180/2018, M. *kansasii 1959/2018* and *M. chelonae* 16/2018) have been established. However, data regarding MA profiles of NTM species in the literature presents a higher variety (Song et al., 2009; Shui et al., 2011; Szewczyk et al., 2013), and our sample set is not adequate for statistical conclusions, with the low representation of oxygenated mycolates the differentiation of MTBC from NTM representatives seems possible with the application of lipid profiling via HPLC-MS, but the confirmation requires the extension of the library. The MC profile development included 5 MTBC clinical isolates (MTBC-1/2015; MTBC-254/2000; MTBC-3910/2014; MTBC-242/2000; and MTBC-1/8508/2014), *M. tuberculosis H37Rv* and *M. bovis* (ATCC) reference strains and 8 NTM clinical isolates (*M. avium* 16229/2018, *M. chelonae* 16/2018, *M. intracellulare* 7802/2017, *M. fortuitum* 3/2018, *M. gordonae* 389/2018, *M. abscessus sp abscessus* 180/2018, *M. chimaera* 619/2018, *M. kansasii* 1959/2018). Among the representatives of MTBC in 4 out of 5 strains and in the case of *M. tuberculosis* H37Rv reference strain, the same tendency of MC distribution has been observed as the literature is suggesting for *M. tuberculosis* (Redman et al., 2009; Lee et al., 2012). One of the 5 strains presented a MC distribution more characteristic to *M. bovis*. Among the analysed NTM species only the profile of *M. kansasii* was established, as the remainder species are not expressing mycocerosates. The analysed *M. avium* strain presented a peak with the same retention time of C27 MC, which will need further investigation. Based on the observed profiles the detection of prominent C32 MC content, accompanied with significant C29 and C30 MC peaks were used as criteria for MTB diagnosis in the following analysis.

3. The efficiency of the established lipid biomarker based method was tested on a well-studied collection of mummified human remains, namely on the Vác mummy collection. Regarding the MA detection, despite of the applied non-polar solvent composition the elution of MAs was not complete. We could not afford even a minimal carry-over during our measurements, hence only the MC method was tested on the mummy samples. In the case of four mummified individuals, profiles of characteristic *M. tuberculosis* MCs were recorded, but two other mummies did not yield diagnostic MC profiles. However, all the mummy samples had been found, previously, to be positive by aDNA examination, but the samples in this study were not identical with those used previously and the sampling site may affect the results. An extended study of confirmed cases, with multiple sampling sites of skeletal material and soft tissue, where available, is suggested to clarify the situation. The present results, however, provide further confirmation of widespread tuberculosis in the Vác mummies. The HPLC-ESI-MS method, developed for the detection of MCs, opens up a new avenue for the detection of ancient mycobacterial disease, encompassing both tuberculosis and leprosy and joint cases. Currently, the instrumentation required is available in many laboratories and technical developments are likely to reduce complexity and increase sensitivity and affordability. A particular advantage is that no chemical derivatisation of the particular lipid biomarkers is needed, the free acids being utilised. The method could be extended to include the related *M. tuberculosis* lipid biomarker, mycolipenic acid (Donoghue et al., 2017). However, in our method we could not overcome with the carry-over of MAs yet, involving new solvent compositions and testing new approaches for separation, a comprehensive protocol could be developed to add profiles of free mycolic acids (Borowska-Strugińska et al., 2014). In the well-established HPLC procedure coupled with fluorescence detection, the recognition of diagnostic mycolate profiles requires extensive derivatisation and sequential reverse and NP-HPLC analyses (Hershkovitz et al., 2008; Donoghue et al., 2017). In contrast, in a single selected ion monitoring HPLC run, all the diagnostic mycolic acid data are collected and are available for further processing.

## LIST OF PUBLICATIONS (MTMT ID: 10053024)

### THE 2 PAPERS PROVIDING BASIS FOR THE DISSERTATION

**Váradi OA**, Rakk D, Spekker O, Terhes G, Urbán E, Berthon W, Pap I, Szikossy I, Maixner F, Zink A, Vágvölgyi Cs, Donoghue HD, Minnikin DE, Szekeres A, Pálfi Gy. (2020) Verification of tuberculosis infection among Vác mummies (18th century CE, Hungary) based on lipid biomarker profiling with a new HPLC-HESI-MS approach. Tuberculosis. Accepted, in press. **IF$_{2019}$: 2.576**

Spekker O, Schultz M, Paja L, **Váradi OA**, Molnár E, Pálfi Gy, Hunt DR. (2020) Tracking down the White Plague. Chapter two: The role of endocranial abnormal blood vessel impressions and periosteal appositions in the paleopathological diagnosis of tuberculous meningitis. PLOS ONE. 15(9): e0238444. DOI: 10.1371/journal.pone.0238444. **IF$_{2019}$: 2,740**

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**TOTAL IF: 7.724**

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