# UNIVERSITY<sup>OF</sup> BIRMINGHAM University of Birmingham Research at Birmingham

# Lipid biomarkers provide evolutionary signposts for the oldest known cases of tuberculosis

Lee, Oona Y-c.; Wu, Houdini H.t.; Besra, Gurdyal S.; Rothschild, Bruce M.; Spigelman, Mark; Hershkovitz, Israel; Bar-gal, Gila Kahila; Donoghue, Helen D.; Minnikin, David E.

DOI: 10.1016/j.tube.2015.02.013

License: Other (please specify with Rights Statement)

Document Version Peer reviewed version

#### Citation for published version (Harvard):

Lee, OY, Wu, HHT, Besra, GS, Rothschild, BM, Spigelman, M, Hershkovitz, I, Bar-gal, GK, Donoghue, HD & Minnikin, DE 2015, 'Lipid biomarkers provide evolutionary signposts for the oldest known cases of tuberculosis', *Tuberculosis*, vol. 95, no. Supplement 1, pp. S127-132. https://doi.org/10.1016/j.tube.2015.02.013

Link to publication on Research at Birmingham portal

#### **Publisher Rights Statement:**

NOTICE: this is the author's version of a work that was accepted for publication in Tuberculosis. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in Tuberculosis, DOI: 10.1016/j.tube.2015.02.013.

Eligibility for repository checked April 2015

#### **General rights**

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

•Users may freely distribute the URL that is used to identify this publication.

•Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.

•User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?) •Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

#### Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

# Accepted Manuscript

Lipid biomarkers provide evolutionary signposts for the oldest known cases of tuberculosis

Oona Y-C. Lee, Houdini H.T. Wu, Gurdyal S. Besra, Bruce M. Rothschild, Mark Spigelman, Israel Hershkovitz, Gila Kahila Bar-Gal, Helen D. Donoghue, David E. Minnikin, Professor

PII: S1472-9792(15)00014-1

DOI: 10.1016/j.tube.2015.02.013

Reference: YTUBE 1274

To appear in: Tuberculosis

Please cite this article as: Lee OY-C, Wu HHT, Besra GS, Rothschild BM, Spigelman M, Hershkovitz I, Bar-Gal GK, Donoghue HD, Minnikin DE, Lipid biomarkers provide evolutionary signposts for the oldest known cases of tuberculosis, *Tuberculosis* (2015), doi: 10.1016/j.tube.2015.02.013.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Short communication (2500 words 15 refs 3 figures)

# Lipid biomarkers provide evolutionary signposts for the oldest known cases of tuberculosis

Oona Y-C. Lee<sup>a</sup>, Houdini H.T. Wu<sup>a</sup>, Gurdyal S. Besra<sup>a</sup>, Bruce M. Rothschild<sup>b</sup>, Mark Spigelman<sup>c</sup>, Israel Hershkovitz<sup>d</sup>, Gila Kahila Bar-Gal<sup>e</sup>, Helen D. Donoghue<sup>f</sup> and David E. Minnikin<sup>a,\*</sup>

<sup>a</sup> Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK

<sup>b</sup>Biodiversity Institute and Departments of Anthropology and Geology, University of Kansas, Lawrence KS 66045, USA

<sup>c</sup>Kuvin Center for the Study of Infectious and Tropical Diseases and Ancient DNA, Hadassah Medical School, Hebrew University, Jerusalem, Israel

<sup>d</sup>Department of Anatomy and Anthropology, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

<sup>e</sup>Koret School of Veterinary Medicine, Hebrew University of Jerusalem, Rehovot, Israel <sup>f</sup>Centres for Clinical Microbiology and the History of Medicine, University College London, UK

Email addresses

leeoy@bham.ac.uk; <u>h.wu.2@bham.ac.uk; d.e.minnikin@bham.ac.uk; g.besra@bham.ac.uk;</u> <u>bmr@ku.edu; spigelman@btinternet.com; anatom2@post.tau.ac.il;</u> <u>gila.kahila@mail.huji.ac.il; h.donoghue@ucl.ac.uk</u>

#### \*Corresponding author

Professor David E. Minnikin, School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Email: d.e.minnikin@bham.ac.uk Telephone: +44(0)1214158126

#### SUMMARY

Studies on the evolution of tuberculosis, and the influence of this disease on human and animal development and interaction, require the accumulation of indisputable biomarker evidence. Ideally, the determination of full genomes would provide all the necessary information, but for very old specimens DNA preservation may be compromised and only limited DNA amplification may be a possibility. *Mycobacterium tuberculosis* is characterised by the presence of unusual cell envelope lipids, with specific biomarker potential. Lipid biomarker recognition has been decisive in pinpointing the oldest known cases of human and animal tuberculosis; the former are a woman and child from a pre-pottery settlement at Atlit-Yam, Israel (~9,000 ka) and the latter is an extinct *Bison antiquus* from Natural Trap Cave, Wyoming (~17,000 ka). Including some new data, it is demonstrated how analysis of a combination of mycolic, mycocerosic and mycolipenic acid and phthiocerol biomarkers provide incontrovertible evidence for tuberculosis in these landmark specimens.

Keywords: Ancient tuberculosis; Lipids; Biomarkers

#### 1. Introduction

Exploration of the origins and evolution of tuberculosis necessarily relies on the clear unambiguous identification of ancient well-characterised archaeological specimens. It would be advantageous to identify an extended population of infected individuals but, given the likely scarcity of the oldest examples, investigation of single landmark cases may well be a productive option. If studies of isolated cases are well-conducted and published in established peer-reviewed journals, the results achieved must be properly respected. In this report, the incontrovertible evidence for tuberculosis in the oldest human<sup>1</sup> and animal<sup>2</sup> cases<del>, described to date,</del> will be reviewed and some new data included.

The widest possible combination of complementary methods should be used to diagnose ancient mycobacterial disease. For skeletal material, considerable expertise has been developed in recognising characteristic bone changes linked to tuberculosis infection.<sup>3,4</sup> The precise diagnosis of tuberculosis disease requires recognition of decisive biomarkers<sup>5</sup> for the causative agent *Mycobacterium tuberculosis*. The amplification of key DNA sequences has been systematically developed into widely applied protocols, during the past two decades During the past twenty years, DNA fragment analysis has been extensively utilised.<sup>5</sup> Major advances in determining full genomic data have been recently provided by the application of so-called "Next Generation Sequencing"<sup>6</sup> and the more direct "Metagenomic" approach.<sup>7</sup>

The predominant feature of the tubercle bacillus is the presence of high proportions of long-chain lipids, easily distinguishable from any mammalian lipids. In a pioneering study, the 70 to 90 carbon mycolic acids (MAs) (Figure 1A) were clearly identified in a mediaeval bone from Addingham, UK, complementing DNA amplification and skeletal indications.<sup>8</sup> The biomarker range has been extended to include multi-methyl-branched mycocerosic and mycolipenic acids (Figure 1B).<sup>2,9</sup> MAs were originally analysed by fluorescence High Performance Liquid Chromatography (HPLC) of slightly unstable methylanthryl esters,<sup>8</sup> so a special robust derivatisation protocol, involving pyrenebutyrates of pentafluorobenzyl (PFB) esters was systematically developed.<sup>1,2,9</sup> The mycocerosate and mycolipenate PFB esters can be exquisitely detected by Selected Ion Monitoring (SIM) Negative Ion Chemical Ionisation (NI-CI) Gas Chromatography Mass Spectrometry.<sup>2,9</sup> The value of lipid biomarkers in providing decisive confirmation of tuberculosis in key archaeological specimens will be reviewed and emphasised. The cases under consideration are Lipid biomarker detection has been particularly decisive in diagnosing tuberculosis in ribs from a woman and child from a

pre-pottery settlement at Atlit-Yam, Israel (~9,000 ka)<sup>1</sup> and an extinct *Bison antiquus* metacarpal from Natural Trap Cave, Wyoming (~17,000 ka).<sup>2</sup> However, these clear diagnostic data are occasionally overlooked<sup>4</sup>, so in this communication the data for these two landmark cases are presented together and reinforced by some new lipid results.

#### 2. Landmark studies

#### 2.1 Nine thousand year old woman and child, Atlit-Yam, Israel

Archaeological investigations off the coast at Atlit-Yam revealed a submerged coastal pre-pottery, post-domestication Neolithic settlement, which included skeletal material from a woman and infant with lesions suggestive of tuberculosis.<sup>1,10</sup> In particular, the inner aspect of the infant cranial bones had serpentine engravings (*serpens endocrania symmetrica*, SES), considered to be diagnostic for intra-thoracic inflammation associated with tuberculosis.<sup>1,11</sup> The tubular bones from the infant, and to a lesser extent from the adult, also demonstrated lesions identified as hypertrophic osteoarthropathy (HOA), highly suggestive of tuberculosis.<sup>1,12</sup> Encouraging results were also achieved for the PCR amplification and sequencing of the *M. tuberculosis* DNA insertion elements IS*6110* and IS*1081*,<sup>1</sup> but additional confirmation was desirable.

Carefully crafted robust lipid biomarker studies were found to be ideal complements to the above compelling evidence for TB in the Atlit-Yam skeletons.<sup>1</sup> The chosen bone samples were degraded by a proven alkaline hydrolysis,<sup>1,2,8</sup> designed to release the maximum amount of mycobacterial lipid biomarkers. Quantitative conversion of the acidic fatty components to pentafluorobenzyl (PFB) esters gave lipid biomarkers the ability to be separated reproducibly on silica gel cartridges into fractions containing PFB mycolipenate/mycocerosates, PFB mycolates and free phthiocerols (Figure 1). The latter two lipid classes were converted to stable pyrenebutyric acid (PBA) esters, ideal for sensitive fluorescence HPLC, with the PFB mycocerosate/mycolipenates being amenable to NI-CI GC-MS. The assembled lipid biomarker profiles for the Atlit-Yam specimens are shown in Figure 2.

A simple logical sequence is followed for the HPLC characterisation of mycolic acid derivatives. The initial "reverse phase" HPLC (Figure 2A) serves to isolate and observe any  $C_{70} - C_{90}$  mycolates free from any smaller mammalian lipids. A "tight envelope" of total mycolate peaks, as recorded in Figure 2A, is very characteristic for members of the *M*. *tuberculosis* complex and, indeed, such clear profiles are immediately very positive indications of tuberculosis infections.<sup>5,8</sup> The next stage is to subject the collected total

4

mycolates (Figure 2A) to "normal phase" HPLC to separate the α-, methoxy- and ketomycolate classes (Figure 1A) according to their polarity (Figure 2B); if clear peaks are seen for the individual mycolates, this strengthens the diagnosis of TB. Reverse phase HPLC of each of the collected mycolate types (Figure 2B) can provide very diagnostic profiles (Figure 2C-E). The α-mycolates are relatively homogeneous, a regular series of C<sub>76</sub> to C<sub>84</sub> homologues (Figure 2C) having two *cis*-cyclopropane rings (Figure 1A). The oxygenated mycolates, in contrast, comprise two overlapping homologous series with either *cis*- or *trans*cyclopropane rings (Figure 1A). A very diagnostic feature of the methoxymycolates from *M. tuberculosis* (Figure 2D) is the presence of a double peak comprising the C<sub>87</sub> *cis*-cyclopropyl and C<sub>88</sub> *trans*-cyclopropyl methoxymycolates. Similarly, the ketomycolates from *M. tuberculosis* are characteristically dominated by the presence of the C<sub>87</sub> *trans*-cyclopropyl ketomycolate (Figure 2E). This sequential analysis enables a close correlation to be observed between the mycolate patterns for the Atlit-Yam specimens and standard *M. tuberculosis*.

To support the positive mycolate results, the mycocerosate/mycolipenate (Figure 1B) and phthiocerol family (Figure 1C) profiles have been prepared, in unpublished studies, using NI-CI GC-MS of PFB esters for the former (Figure 2F-I) and fluorescence HPLC of PBA esters for the latter (Figure 2J). Again, the mycocerosate/mycolipenate traces (Figure 2F-I) correspond well with those for standard *M. tuberculosis*. Interestingly, the infant showed a stronger presence of mycolipenate (Figure 2H,  $C_{27}$  *m/z* 407). The presence of members of the phthiocerol family (Figure 1C) was revealed by reverse phase HPLC (Figure 2J) for ribs from the Atlit-Yam Infant and Woman. The main components corresponded to the characteristic *M. tuberculosis*  $C_{34}$  and  $C_{36}$  phthiocerol As (PA) and  $C_{33}$  and  $C_{35}$  phthiodiolones (PO); there was insufficient material to carry out confirmatory normal phase HPLC.

#### 2.2. Seventeen thousand year old bison Natural Trap Cave Wyoming USA

Excavations directed by the late Larry D. Martin (1943-2013) of the Biodiversity Institute, University of Kansas, in Natural Trap Cave, Wyoming, revealed the presence of a variety of well-preserved Pleistocene animal bones<sup>2,3</sup>. In particular, a metacarpal from a 17 ka extinct *Bison antiquus* showed evidence of lesions "undermining the articular surface", associated with tuberculosis both in humans and animals.<sup>2,3</sup> In a landmark study, aDNA characteristic of *M. tuberculosis* rather than *M. bovis* was clearly identified.<sup>12</sup>

A search for lipid biomarkers provided weak but encouraging profiles of mycolic acids (Figure 3A-E). Remarkably, absolutely pristine profiles of mycolipenic ( $C_{27}$  m/z 407) and

mycocerosic acids (Figure 1B) were recorded for specimens taken both at the lesion (Bison 1) and a remote site (Bison 2) (Figure 3F-H). The initial reverse phase HPLC profiles of the PBA derivatives of the phthiocerol family fraction (Figure 3I) were not as clean as those for the Atlit-Yam skeletons (Figure 2J). Normal phase analysis (Figure 3J) of the collected material from the reverse phase separation (Figure 3I) confirmed the presence of members of the phthiocerol family, as published previously.<sup>2</sup> In addition, to the phthiocerols, however, the full normal phase profiles of the PBA derivatives showed the presence of ill-defined more-polar components, labelled X, Y and Z in Figure 3J. The presence of these unknown components was not reported in the initial study,<sup>2</sup> as their identity could not be readily understood. One possible explanation is that components X - Z are derived from glycosyl phenolphthiocerols, the deacylated so-called "phenolic" glycolipids (PGLs). To investigate this hypothesis, the PGL from M. bovis BCG was processed through the hydrolysis and extraction protocols and it was found that the resulting PBA derivatives chromatographed in a similar fashion to the components X, Y and Z (data not shown). Unfortunately, none of the X - Z material remained from the bison samples, so it was not possible to make a precise direct comparison. The key conclusion, however, is that the material from Atlit-Yam does not show the presence of these potential biomarkers, possibly derived from PGLs (Figure 2J). Many modern isolates of *M. tuberculosis* do not produce PGLs, but they are found in *M*. bovis, M. africanum, certain M. tuberculosis Beijing strains and smooth morphology isolates labelled "Mycobacterium canettii". A recent detailed study<sup>13</sup> has confirmed that "M. canettii" strains are likely ancient progenitors of modern tuberculosis. The aDNA studies<sup>12</sup> ruled out M. bovis, but indicated a similarity with M. africanum. Further studies will be needed to determine the precise strain of tubercle bacillus infecting the Natural Trap Cave bison.

#### 3. Conclusions

Sound evidence has been assembled, therefore, for the clear diagnosis of tuberculosis in ~9ka human skeletons and a ~17ka bison metacarpal; these examples provide solid reference points for tracing the evolution of tuberculosis back into antiquity. Surprisingly, the actual validity of the aDNA results in the properly peer-reviewed Atlit-Yam study<sup>2</sup> has been directly challenged,<sup>14</sup> with no mention being made of the very conclusive MA lipid biomarker evidence. Equally, in an otherwise generally comprehensive review on ancient tuberculosis and leprosy,<sup>4</sup> the critique was repeated but no reference whatsoever was made to a range of published lipid biomarker results,<sup>5</sup> even though the Atlit-Yam<sup>1</sup> and Addingham<sup>8</sup> studies were cited.<sup>4</sup> The inclusion of new mycocerosate/mycolipenate and phthiocerol data for the Atlit-

6

Yam specimens (Figure 2F-J) and additional components in the phthiocerol area for the Natural Trap Cave bison (Figure 3J) adds further weight to the power of lipid biomarkers in the diagnosis of ancient tuberculosis.

Genomic studies suggest a rapid expansion of modern biotypes of the tubercle bacillus following an apparent evolutionary bottleneck at the end of the Pleistocene.<sup>13</sup>. In a detailed examination of the parallel evolution of genomes from 186 members of the *M. tuberculosis* complex and 4,995 human mitochondria, extrapolations were interpreted to suggest that TB and humans co-evolved "out of Africa", commencing ~70 ka ago.<sup>15</sup> However, parallel evolution is not necessarily linked co-evolution and absolutely no evidence was advanced to substantiate the presence of any human tuberculosis going back to 70 ka. Indeed, not a single citation was made to any previous publication on the diagnosis of ancient tuberculosis in archaeological material! The current lack of any identified cases of Pleistocene TB in *Homo sapiens* and the apparent abundance of the disease in Pleistocene megafauna has led to the suggestion that animals may well have been prime vectors to facilitate the emergence of a viable tuberculosis pathogen from an environmental ancestor.<sup>2</sup>

**Dedication:** This paper is dedicated to the memory of Larry Martin (1943 – 2013), formerly of the Biodiversity Institute, University of Kansas, whose informed selection of specimens from Natural Trap Cave, Wyoming, led directly to the first diagnosis of tuberculosis in Pleistocene megafauna.

Funding: The Leverhulme Trust Project Grant F/00 094/BL (OY-CL, DEM, GSB)

#### References

- Hershkovitz I, Donoghue HD, Minnikin DE, Besra GS, Lee OY-C, Gernaey AM, Galili E, Eshed V, Greenblatt CL, Lemma E, Kahila Bar-Gal G, Spigelman M. Detection and molecular characterization of 9000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in the Eastern Mediterranean. *PLoS ONE* 2008;**3**:e3426.
- Lee OY-C, Wu HHT, Donoghue HD, Spigelman, M, Greenblatt CL, Bull ID, Rothschild, BM, Martin, LD, Minnikin, DE, Besra, GS. *Mycobacterium tuberculosis* complex lipid virulence factors preserved in the 17,000-year-old skeleton of an extinct bison, *Bison antiquus*. *PLoS ONE* 2012;7:e41923.
- Rothschild BM, Rothschild C. Evolution of osseous/radiologic signs of tuberculosis.
  In: Palfi G, Dutour O, Deak J, Hutas I, editors. *Tuberculosis Past and Present*. Golden Book Publisher Ltd., Tuberculosis Foundation, Budapest, Hungary; 1999 p. 293-298.
- 4. Stone AC, Wilbur AK, Buikstra JE, Roberts CA. Tuberculosis and leprosy in perspective. *Yearbook of Physical Anthropology* 2009;**52**:66–94.
- Minnikin DE, Lee OY-C, Wu HHT, Besra GS, Donoghue HD. Molecular biomarkers for ancient tuberculosis. In: Cardona P-J, editor. *Understanding Tuberculosis – Deciphering the Secret Life of the Bacilli*. Rijeka, Croatia.: InTech -Open Access Publisher. 2012. p. 1-36. http://www.intechopen.com/books/understanding tuberculosis-deciphering-the-secret-life-of-the-bacilli
- Bouwman AS, Kennedy SL, Muller R, Stephens RH, Holst M, Caffell AC, Roberts CA, Brown TA. Genotype of a historic strain of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* 2012;**109**:18511-18516.

- Chan JZ-M, Sergeant MJ, Lee OY-C, Minnikin DE, Besra GS, Pap I, Spigelman M, Donoghue HD, Pallen MJ. Metagenomic analysis of tuberculosis in a mummy. *N Engl J Med* 2013;**369:**289-290.
- Gernaey AM, Minnikin DE, Copley MS, Dixon RA, Middleton JC, Roberts CA. Mycolic acids and ancient DNA confirm an osteological diagnosis of tuberculosis. *Tuberculosis* 2001;81:259–265.
- Galili E, Weinstein-Evron M, Hershkovitz I, Gopher A, Kislev M, Omri Lernau O, Kolska-Horwitz L, Lernau H. Atlit-Yam: A prehistoric site on the sea floor off the Israeli coast. *J Field Archaeol* 1993;20:133–157.
- Hershkovitz I, Greenwald CM, Latimer B, Jellema LM, Wish-Baratz S, Eshed V, Dutour O, Rothschild BM. *Serpens endocrania symmetrica* (SES): A new term and possible sign of cranial tuberculosis. *Amer J Phys Anthropol* 2002;**118**:201-216.
- Rothschild BM, Rothschild C. Recognition of hypertrophic osteoarthropathy in skeletal remains. *J Rheumatol* 1998;25:2221-2227.
- Rothschild BM, Martin LD, Lev G, Bercovier H, Bar-Gal GK, Greenblatt C, Donoghue H, Spigelman M, Brittain D. *Mycobacterium tuberculosis* complex DNA from an extinct bison dated 17,000 years BP. *Clin Infect Dis* 2001;**33**:305–311.
- 13. Supply P, Marceau M, Mangenot S, Roche D, Rouanet C, Khanna V, Majlessi L, Criscuolo A, Tap J, Pawlik A, Fiette L, Orgeur M, Fabre M, Parmentier C, Frigui W, Simeone R, Boritsch EC, Debrie AS, Willery E, Walker D, Quail MA, Ma L, Bouchier C, Salvignol G, Sayes F, Cascioferro A, Seemann T, Barbe V, Locht C, Gutierrez MC, Leclerc C, Bentley SD, Stinear TP, Brisse S, Médigue C, Parkhill J, Cruveiller S, Brosch R. Genomic analysis of smooth tubercle bacilli provides insights into ancestry and pathoadaptation of *Mycobacterium tuberculosis. Nat Genet* 2013;45:172-179.

- 14. Wilbur AK, Bouwman AS, Stone AC, Roberts CA, Pfister L-A, Buikstra JE, Brown TA. Deficiencies and challenges in the study of ancient tuberculosis DNA. *J Archaeol Sci* 2009;36:1990–1997.
- 15. Comas I, Coscolla M, Luo T, Borrell S, Holt KE, Kato-Maeda M, Parkhill J, Malla B, Berg S, Thwaites G, Yeboah-Manu D, Bothamley G, Mei J, Wei L, Bentley S, Harris SR, Niemann S, Diel R, Aseffa A, Gao Q, Young D, Gagneux S. Out-of-Africa migration and Neolithic coexpansion of *Mycobacterium tuberculosis* with modern humans. *Nat Genet* 2013;45:1176-1182.

10

#### **Figure legends**

**Figure 1.** Lipid biomarkers for *M. tuberculosis*. (A) Generalized structures of α-, methoxyand ketomycolates; the main components are in brackets. (B) Structures of mycolipenate and mycocerosates, showing negative carboxylate ions used for selected ion monitoring on NI-CI GC-MS analysis of pentafluorobenzyl (PFB) esters. (C) Structures of members of the phthiocerol family.

**Figure 2.** Mycobacterial lipid profiles from standard *M. tuberculosis* and Atlit-Yam Woman and Infant. (A – E) Reverse and normal phase fluorescence HPLC of mycolate pyrenebutyric acid (PBA) derivatives of pentafluorobenzyl (PFB) esters. (F –I) NI-CI GC-MS of pentafluorobenzyl (PFB) esters of mycolipenate and mycocerosates; relative intensities are shown normalised to the major component [100]. (J) Reverse phase fluorescence HPLC of pyrenebutyric acid (PBA) derivatives of members of the phthiocerol family: PA phthiocerol A, PB phthiocerol B, PO phthiodiolone.

**Figure 3.** Mycobacterial lipid profiles from standard *M. tuberculosis* and Natural Trap Cave Bison metacarpal specimens. (A – E) Reverse and normal phase fluorescence HPLC of mycolate pyrenebutyric acid (PBA) derivatives of pentafluorobenzyl (PFB) esters. (F –H) NI-CI GC-MS of pentafluorobenzyl (PFB) esters of mycolipenate and mycocerosates; relative intensities are shown normalised to the major component [100]. (I and H) Reverse (I) and normal (J) phase fluorescence HPLC of pyrenebutyric acid (PBA) derivatives of components recovered with members of the phthiocerol family: PA phthiocerol A, PB phthiocerol B, PO phthiodiolone, X, Y & Z unknown components.







15

20

25

Blank

30 Minutes

Figure 2





Figure 3