

## Ancient DNA analysis – An established technique in charting the evolution of tuberculosis and leprosy

Donoghue, Helen D.; Spigelman, Mark; O'grady, Justin; Szikossy, Ildikó; Pap, Ildikó; Lee, Oona Y.-c.; Wu, Houdini H.t.; Besra, Gurdyal S.; Minnikin, David E.

DOI:

[10.1016/j.tube.2015.02.020](https://doi.org/10.1016/j.tube.2015.02.020)

License:

Other (please specify with Rights Statement)

*Document Version*

Peer reviewed version

*Citation for published version (Harvard):*

Donoghue, HD, Spigelman, M, O'grady, J, Szikossy, I, Pap, I, Lee, OY, Wu, HHT, Besra, GS & Minnikin, DE 2015, 'Ancient DNA analysis – An established technique in charting the evolution of tuberculosis and leprosy', *Tuberculosis*, vol. 95, no. Supplement 1, pp. S140-144. <https://doi.org/10.1016/j.tube.2015.02.020>

[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.2015.02.020.

Eligibility for repository checked March 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**

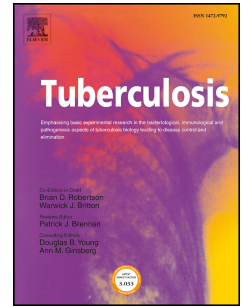
While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

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

# Accepted Manuscript

Ancient DNA analysis – an established technique in charting the evolution of tuberculosis and leprosy

Dr Helen D. Donoghue, Mark Spigelman, Justin O’Grady, Ildikó Szikossy, Ildikó Pap, Oona Y-C. Lee, Houdini H.T. Wu, Gurdyal S. Besra, David E. Minnikin



PII: S1472-9792(15)00021-9

DOI: [10.1016/j.tube.2015.02.020](https://doi.org/10.1016/j.tube.2015.02.020)

Reference: YTUBE 1281

To appear in: *Tuberculosis*

Please cite this article as: Donoghue HD, Spigelman M, O’Grady J, Szikossy I, Pap I, Lee OY-C, Wu HHT, Besra GS, Minnikin DE, Ancient DNA analysis – an established technique in charting the evolution of tuberculosis and leprosy, *Tuberculosis* (2015), doi: 10.1016/j.tube.2015.02.020.

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.

1 **Ancient DNA analysis – an established technique in charting the evolution of tuberculosis and**  
2 **leprosy**

3  
4  
5 Helen D. Donoghue<sup>a,b\*</sup>, Mark Spigelman<sup>a,c</sup>, Justin O’Grady<sup>a,1</sup>, Ildikó Szikossy<sup>d</sup>, Ildikó Pap<sup>d</sup>, Oona Y-  
6 C. Lee<sup>e</sup> Houdini H.T. Wu<sup>e</sup>, Gurdyal S. Besra<sup>e</sup> and David E. Minnikin<sup>e</sup>  
7  
8

9 <sup>a</sup>Centre for Clinical Microbiology, Division of Infection & Immunity, University College London,  
10 London, UK

11 <sup>b</sup>Centre for The History of Medicine, Division of Biosciences, University College London, UK

12 <sup>c</sup>Department of Anatomy and Anthropology, Sackler Faculty of Medicine, Tel Aviv University, Tel  
13 Aviv, Israel

14 <sup>d</sup>Department of Anthropology, Hungarian Natural Science Museum, Budapest, Hungary

15 <sup>e</sup>Institute of Microbiology and Infection, School of Biosciences, University of Birmingham,  
16 Edgbaston, Birmingham, UK  
17  
18

19 Email addresses:

20 [h.donoghue@ucl.ac.uk](mailto:h.donoghue@ucl.ac.uk); [spigelman@btinternet.com](mailto:spigelman@btinternet.com); [Justin.OGrady@uea.ac.uk](mailto:Justin.OGrady@uea.ac.uk);

21 [mailto:szikossy@nhmus.hu](mailto:mailto:szikossy@nhmus.hu); [mailto:papildi@hotmail.com](mailto:mailto:papildi@hotmail.com); [mailto:o.y.lee@bham.ac.uk](mailto:mailto:o.y.lee@bham.ac.uk);

22 [h.wu.2@bham.ac.uk](mailto:h.wu.2@bham.ac.uk); [g.besra@bham.ac.uk](mailto:g.besra@bham.ac.uk); [d.e.minnikin@bham.ac.uk](mailto:d.e.minnikin@bham.ac.uk)  
23

24 \*Corresponding author: Dr Helen D. Donoghue, Centre for Clinical Microbiology, Division of  
25 Infection & Immunity, Royal Free Campus, University College London, London NW3 2PF, UK

26 Email: [h.donoghue@ucl.ac.uk](mailto:h.donoghue@ucl.ac.uk) Telephone: +44(0)2077940500 ext. 31146  
27

28 <sup>1</sup>Current address: Norwich Medical School, University of East Anglia, Norwich, UK  
29  
30

31 Word count summary: 200

32 Word count (excluding summary and references): 3031  
33

34 **Summary**

35 Many tuberculosis and leprosy infections are latent or paucibacillary, suggesting a long time-scale for  
36 host and pathogen co-existence. Palaeopathology enables recognition of archaeological cases and PCR  
37 detects pathogen ancient DNA (aDNA). *Mycobacterium tuberculosis* and *Mycobacterium leprae* cell  
38 wall lipids are more stable than aDNA and restrict permeability, thereby possibly aiding long-term  
39 persistence of pathogen aDNA. Amplification of aDNA, using specific PCR primers designed for short  
40 fragments and linked to fluorescent probes, gives good results, especially when designed to target  
41 multi-copy loci. Such studies have confirmed tuberculosis and leprosy, including co-infections. Many  
42 tuberculosis cases have non-specific or no visible skeletal pathology, consistent with the natural history  
43 of this disease. *M. tuberculosis* and *M. leprae* are obligate parasites, closely associated with their  
44 human host following recent clonal distribution. Therefore genotyping based on single nucleotide  
45 polymorphisms (SNPs) can indicate their origins, spread and phylogeny. Knowledge of extant genetic  
46 lineages at particular times in past human populations can be obtained from well-preserved specimens  
47 where molecular typing is possible, using deletion analysis, microsatellite analysis and whole genome  
48 sequencing. Such studies have identified non-bovine tuberculosis from a Pleistocene bison from 17,500  
49 years BP, human tuberculosis from 9000 years ago and leprosy from over 2000 years ago.

50

51 **Key words:** Ancient DNA; evolution; *Mycobacterium leprae*; *Mycobacterium tuberculosis*; molecular  
52 typing

53

54

## 55 1. Introduction

56 According to the World Health Organisation,<sup>1</sup> one third of the global human population is infected  
57 with tuberculosis (TB) but most are latent infections. In people with no underlying risk factors,  
58 approximately 10% will develop an active infection during their lifetime.<sup>1</sup> However, underlying  
59 deficiencies in immunity caused by co-infections such as HIV, or co-morbidities such as cancer, greatly  
60 increase the chance of active infection. This high level of latent infection suggests a period of close co-  
61 evolution of *Mycobacterium tuberculosis* and its human host.<sup>2</sup> Phylogenetics indicate that the  
62 *Mycobacterium tuberculosis* complex (MTBC) emerged via an evolutionary bottleneck and that  
63 existing lineages have emerged after a succession of unidirectional deletion events.<sup>3</sup> *M. tuberculosis* is  
64 an obligate pathogen and has no environmental reservoir. There appears to be an association between  
65 *M. tuberculosis* lineages with different human populations around the globe and this persists within  
66 modern cities with a population of diverse origins.<sup>4</sup> An association has been found between population  
67 density and the emergence of human infectious diseases.<sup>5</sup> This association is apparent in the early  
68 Neolithic period of human development and in the case of TB appears to be continuing with the  
69 emergence of highly transmissible and virulent strains of *M. tuberculosis* in major cities that have a  
70 long record of continuous habitation.<sup>6</sup>

71 Leprosy is a chronic human infection caused by *Mycobacterium leprae*. This has declined in recent  
72 years but caused approximately 219,000 new cases in 2011,<sup>7</sup> mainly in South East Asia, Africa and  
73 South America. It is a major cause of preventable disability and of social exclusion due to stigma. *M.*  
74 *leprae* is extremely slow growing and requires to be in an intracellular environment within a host,  
75 primarily human. *M. leprae* also appears to have experienced an evolutionary bottleneck and  
76 subsequent clonal expansion between pathogen and host.<sup>8</sup> Different strains of *M. leprae* can be  
77 distinguished by variable number tandem repeat (VNTR) and short tandem repeat typing. These can  
78 indicate short-term transmission via microsatellite analysis but are unstable due to poor DNA repair by  
79 *M. leprae*.<sup>9</sup> Stable long-term changes can be monitored by synonymous single nucleotide  
80 polymorphisms (SNPs) and these have identified lineages that are also associated with different human  
81 populations.<sup>10</sup>

82 Much can be inferred by the study of modern isolates of both *M. tuberculosis* and *M. leprae*.  
83 However, the direct study of ancient specific biomarkers for these pathogens, such as ancient DNA  
84 (aDNA) and cell wall lipid biomarkers, has distinct advantages.<sup>11</sup> These biomarkers enable  
85 confirmation of infection in skeletal or soft tissue remains with non-specific or no palaeopathology, as  
86 only about 5% of TB cases are believed to result in bony changes. Ancient biomarkers may also answer

87 historical questions, such as the nature of pre-Columbian TB or the role played by the slave trade  
88 across the Atlantic in the dispersal of TB and leprosy to the Americas. Mixed infections can also be  
89 detected. Analysis of aDNA may permit the determination of genetic lineages, genotypes or sub-  
90 genotypes in specimens of known age, thus providing real time calibration of the date of their  
91 emergence. The association of ancient pathogen genotypes with different host populations may also  
92 pinpoint and date human migrations.<sup>10,12</sup>

93 A useful approach in palaeomicrobiology is to obtain independent verification of findings by seeking  
94 different specific biomarkers in individual specimens. Our group has concentrated on initial  
95 examination for aDNA and subsequent independent analysis of mycobacterial specific cell wall  
96 lipids.<sup>13</sup> Until recently, our aDNA data have been obtained by DNA amplification using PCR, first  
97 conventional single-stage or nested PCR, and more recently using real-time PCR with specific  
98 fluorescent probes and primers for selected target regions. As aDNA is often highly fragmented, the  
99 use of specific probes has been very productive as they enable specific detection of selected target  
100 regions of as little as 60–80 base pairs (bp). The development of Next Generation Sequencing (NGS)  
101 and sophisticated bioinformatic analysis has enabled sequencing and analysis of non-amplified DNA  
102 using targeted enrichment approaches.<sup>14,15</sup> Rarely, in exceptionally well-preserved material, it is  
103 possible to perform shot-gun sequencing without target enrichment and to obtain an analysis of the  
104 entire DNA within a sample. This has been achieved for *M. leprae* found in mediaeval dental pulp<sup>15</sup>  
105 and in extremely well preserved lung tissue from a naturally mummified individual from 18<sup>th</sup> century  
106 Vác, Hungary.<sup>16</sup>

107

## 108 **2. Ancient DNA methodology**

### 109 *2.1 Extraction of M. tuberculosis and M. leprae aDNA*

110 The following protocol gives sufficient time for samples to be disaggregated, but includes vigorous  
111 bead beading and snap freezing in dry ice to release aDNA from association with any residual lipids  
112 from the lipid-rich mycobacterial cell wall. Small samples (bone scrapings 20–80 mg; mummified  
113 tissue 10–40 mg) are taken from human remains, according to recommended protocols for aDNA with  
114 separate rooms and equipment for different stages of the process.<sup>17</sup> Skeletal material is crushed in a  
115 sterile pestle and mortar and samples are added to 400  $\mu$ l of Proteinase K/EDTA. The slurry is  
116 incubated at 56°C and mixed on a bead beater daily until solubilised. An aliquot is treated with 40  $\mu$ l of  
117 0.1 mol<sup>-1</sup> of *N*-phenacylthiozolium bromide (PTB), to cleave any covalent cross-links thus enabling

118 DNA strand separation and amplification.<sup>17</sup> As PTB is inhibitory in the PCR reaction, an aliquot  
119 without PTB is processed in parallel, so that short DNA fragments can be precipitated from PTB-free  
120 silica supernatants (see below). Sample tube contents are transferred into lysis buffer containing 5 mol<sup>-1</sup>  
121 guanidium thiocyanate and incubated for 1–3 days at 56°C. To complete the disruption of bone and any  
122 mycobacterial remnants, samples are boiled, then snap-frozen in liquid nitrogen and thawed in a 65°C  
123 water bath. This procedure is repeated twice. Sample tubes are centrifuged at 5000g for 15 mins at 5°C  
124 and the supernates carefully removed into clean, sterile tubes. DNA is captured with silica suspension  
125 (NucliSens<sup>®</sup>) and mixed on a rotator wheel for 1 hour. Tube contents are centrifuged and silica pellets  
126 washed once with wash buffer (NucliSens<sup>®</sup>), twice with 70% (v/v) ethanol (-20 °C) and once with  
127 acetone (-20 °C). After drying in a heating block, DNA is eluted using 60µl elution buffer  
128 (NucliSens<sup>®</sup>), aliquoted and used immediately or stored at -20°C. Silica supernates (500 µl) from PTB-  
129 negative samples are also taken from the lysis buffer, and 2.0 ml screw-capped Eppendorf tubes used to  
130 wash the silica. After chilling at 5°C, supernates are mixed vigorously for 20 sec with 200 µl of Protein  
131 Precipitation Solution (SLS Ltd, UK) and centrifuged for 3 min at 10,000 g. Any pellet is discarded and  
132 600 µl isopropanol (-20°C) added to 550 µl of each supernate. Tubes are mixed by inversion 50 times  
133 and spun 3 min. Supernates are discarded and tubes washed once with 500 µl 70% ethanol (-20°C).  
134 After draining, tubes are dried in a heating block. Any precipitated DNA is re-hydrated with 60µl  
135 elution buffer (NucliSens<sup>®</sup>), aliquoted and used immediately or stored at -20°C. Negative extraction  
136 controls are processed in parallel with the test samples.

## 137 2.2 DNA amplification and detection

138 In the current protocol, two specific regions of each organism are targeted, using repetitive elements  
139 to increase the likelihood of detection of pathogen aDNA. For the *M. tuberculosis* complex, IS6110 (1–  
140 25 copies/cell) and IS1081 (6 copies/cell) are used.<sup>18</sup> For *M. leprae*, RLEP (37 copies/cell) and  
141 REPLEP (15 copies/cell) are used.<sup>19</sup> Initially, conventional PCR was used, with primers targeting DNA  
142 regions of around 90-bp to 123-bp. PCR was performed in two stages, with 45 rounds of amplification  
143 followed, if necessary, by a nested reaction using internal primers, with a further 25 cycles of  
144 amplification. PCR products were detected by agarose gel amplification, gel slices were removed, the  
145 PCR products purified and sequenced. As aDNA is highly fragmented specific primers and fluorescent  
146 probes have since been designed to enable shorter DNA fragments to be specifically detected (Table 1).  
147 The Qiagen QuantiTect<sup>®</sup> Probe reaction mix is used with additional 2 mM BSA to reduce PCR  
148 inhibition and additional 2.0 mM MgCl<sub>2</sub> to facilitate primer binding. A hot-start *Taq* polymerase is  
149 used to minimise non-specific primer and template binding. Negative DNA extraction and PCR

150 controls are processed alongside the test sample. Amplification is performed in a final volume of 25  $\mu$ l  
151 using the Qiagen RotorGene<sup>®</sup> real-time platform. After enzyme activation for 15 min at 95°C,  
152 amplification consists of 50-55 cycles of strand separation at 94°C for 10 sec, primer binding at 60°C  
153 for 20 sec and strand extension at 72°C for 10 sec. The probes enable direct observation of specific  
154 amplicons and the determination of cycle threshold (Ct) indicates relative concentration of template.  
155 Findings may be confirmed by sequencing. Analysis of cell wall lipid biomarkers, based on the direct  
156 detection of cell wall components without any amplification of the signal, enables independent  
157 verification of the presence of the target pathogen in the sample.<sup>13,20</sup>

158

### 159 3. Significant questions answered by aDNA studies

#### 160 3.1 The *M. tuberculosis* lineages prevalent in early human history

161 TB is spread by infectious aerosols from an infected person, which results in lung infections,  
162 although bacteria may spread via the bloodstream to all other parts of the body. Infection of the  
163 vertebrae results in pathology typical of TB, such as Pott's disease, that was recognized in ancient  
164 Egypt and early Neolithic communities. However, in the majority of cases there is non-specific  
165 palaeopathology or none at all, so the extent of past TB infections was greatly underestimated.<sup>11</sup>  
166 However, it was noted that there was an increase in TB in the Neolithic compared with hunter-  
167 gatherers and this appeared to be associated with animal domestication. This led initially to an  
168 assumption that human TB was derived from animals and that *Mycobacterium bovis*, the principal  
169 cause of TB in domesticated animals, was the ancestor of *M. tuberculosis*. This was disproved once the  
170 phylogenomics of the MTBC was determined,<sup>2</sup> that demonstrated *M. tuberculosis* was more ancestral  
171 than *M. bovis*. The earliest demonstration of the MTBC, in Pleistocene bison, suggested that the  
172 pathogen resembled *M. tuberculosis* or *M. africanum*,<sup>21</sup> but at that time the significance of the smooth  
173 colony "*Mycobacterium canettii*" group was not realized. This very diverse group is believed to be  
174 most similar to the original ancestor of the MTBC and demonstrates greater variability than any other  
175 member of the MTBC.<sup>2,22</sup>

176 *M. tuberculosis* aDNA from ancient Egypt that had not experienced the TbD1 deletion has been  
177 reported from Ancient Egypt.<sup>23</sup> Such strains are still extant in the Far East around the Pacific Rim and  
178 are believed to be the oldest *M. tuberculosis* lineage. However, the majority of modern *M. tuberculosis*  
179 strains are TbD1-deleted and this lineage was demonstrated 9000 years ago in the pre-pottery Neolithic  
180 site of Atlit-Yam, in the Eastern Mediterranean.<sup>24</sup> *M. africanum* was found in Middle Kingdom ancient



181 Egypt<sup>23</sup> but *M. bovis* is very rare in the archaeological record. It was found in a group of Iron Age  
182 Siberian pastoralists (4<sup>th</sup> century BC – 4<sup>th</sup> century AD) who over-wintered in huts with their animals.<sup>25</sup>

183 The interest in *M. tuberculosis* lineages has been driven partly because of the realization that the  
184 clinical presentation of TB results from a combination of factors related to host susceptibility and *M.*  
185 *tuberculosis* virulence.<sup>2</sup> This can be studied directly in past populations, allowing the effects of  
186 industrialization, population density and large population movements to be examined.<sup>3,11</sup>

### 187 3.2 The nature of past *M. leprae* infections

188 Leprosy was recognized in antiquity by the characteristic clinical symptoms. *M. leprae* targets  
189 nerves and the bacteria invade the Schwann cells that are essential for the transmission of nerve  
190 impulses. The clinical presentation depends upon the host immune reactions. A predominant humeral  
191 response leads to multibacillary, or lepromatous leprosy, where there is a strong antibody response that  
192 is useless. This is the form of leprosy that results in gross changes to the nasopharyngeal region, the  
193 hands and feet, with destruction of tissue and gross deformity. However, in the presence of an effective  
194 cell-mediate host response, symptoms are minor although late autoimmune reactions can lead to  
195 destruction of nerve function and disability. The disease has been described in ancient China, Egypt  
196 and India<sup>7</sup> although it is sometimes difficult to distinguish between leprosy and other diseases with  
197 similar external symptoms. In archaeological cases, it is lepromatous leprosy that is recognized.  
198 Leprosy occurred during the Roman empire<sup>10</sup> and was spread by traders and invading armies.

199 A phylogenetic study of global *M. leprae*, that included both modern and aDNA, demonstrated a  
200 clear link between global populations and the *M. leprae* genotype and subgenotypes, as determined by  
201 SNPs.<sup>10,19</sup> It appears that SNP type 2 strains are associated with early strains that migrated from the  
202 Middle East to South East Asia but recent work shows they also spread westwards to northern and  
203 western Europe.<sup>12</sup> SNP 3 strains are found in North Africa and the Eastern Mediterranean and were  
204 very common in Mediaeval Europe. Different migratory routes were suggested for the spread of *M.*  
205 *leprae* from the proposed source near the Horn of Africa – a land route from the Mediterranean east to  
206 central China, and sea routes via India and South East Asia. The genotypes found today in these  
207 regions support this theory of dispersal.<sup>26</sup>

### 208 3.3 Past co-infections

209 The ability of aDNA studies to detect and characterize mixed infections had not been an original aim  
210 and it was by chance that co-infections of *M. tuberculosis* and *M. leprae* were detected.<sup>27</sup> Using  
211 conventional PCR, a decision has to be made on which organisms to target in a sample and choice of

212 primers is made accordingly. Mixed infections were discovered when mediaeval leprosy samples were  
213 examined for evidence of *M. tuberculosis* after contemporary co-infections were reported. This has led  
214 to an on-going debate on the possible sequence of events in the decline of leprosy in late Mediaeval  
215 Europe and whether TB had any role in bringing this about.

216 Thereafter, other examples of co-infections were sought. Another disease known in antiquity was  
217 malaria, in particular that caused by *Plasmodium falciparum*. Co-infection of *M. tuberculosis* and  
218 parasites is an important public health problem in co-endemic areas of the world today, and is therefore  
219 likely to have been so in the past. This has been demonstrated in ancient Lower Egypt dating to c. 800  
220 BC, where four mummies were found with aDNA from both *M. tuberculosis* and *P. falciparum*.<sup>28</sup>  
221 Intestinal and systemic parasites were widespread in the past, also was the carriage of ectoparasites. In  
222 addition, it is highly likely that future whole genome studies will identify multiple bacterial and viral  
223 infections within individual human archaeological remains, in addition to associations of infection with  
224 co-morbidities such as host immune or genetic disorders and cancer. Such complex scenarios form the  
225 backdrop to the emergence of modern pathogens and we should endeavour to increase our  
226 understanding of the factors involved.

227 Whole genome sequencing makes it feasible to detect different strains of the same pathogen within a  
228 host. This scenario was demonstrated in 18<sup>th</sup> century naturally mummified lung, where two *M.*  
229 *tuberculosis* strains were detected, apparently one more ancestral than the other and both resembling a  
230 modern outbreak strain, which is closely related to modern Haarlem and Erdman strains.<sup>16</sup> This may be  
231 relevant as a recent study of current mixed strain infections<sup>29</sup> found that the Haarlem and Beijing  
232 genotypes are more likely to occur in a mixed infection than any of the other genotypes tested  
233 suggesting pathogen-pathogen compatibility. There is evidence for intra-strain gene flux in *M.*  
234 *tuberculosis*<sup>22</sup> and this is likely to be significant in the emergence of modern *M. tuberculosis* strains  
235 that are rapidly diversifying, due to mutation, recombination and natural selection.<sup>30</sup>

236

#### 237 **4. Conclusions**

238 During the past twenty years, since study of the palaeomicrobiology of human infectious diseases  
239 became feasible, the nature of the research questions addressed has broadened in scope and become far  
240 more sophisticated. Early palaeopathologists wished for validation of their diagnoses that were based  
241 on skeletal morphological changes. However, it soon became clear that the scale of past TB infections  
242 was far greater than previously envisaged, as many infections do not have skeletal involvement.

243 Palaeomicrobiology has been used to answer historical questions, such as whether the European  
244 colonialists brought TB to South America. Indeed they did, but pre-Columbian TB existed and its  
245 nature is still the subject of study. Population studies of past TB and leprosy enable epidemiological  
246 studies from the pre-antibiotic era. Information on living and burial conditions can highlight whether  
247 there was social stigma or whether infected individuals were integrated into their society.

248 As *M. tuberculosis* and *M. leprae* are obligate pathogens, their geographical distribution illustrates  
249 past human migrations or dispersal around the globe. Recent developments in genomics have increased  
250 our understanding of modern strains of *M. tuberculosis* and *M. leprae*, thereby providing comparators  
251 for pathogen aDNA. It is now appreciated that palaeomicrobiology enables direct comparison of  
252 ancient and modern lineages. One of the greatest benefits is that palaeomicrobiology enables direct  
253 calibration of the timescale over which changes have occurred, in the absence of modern evolutionary  
254 pressures caused by antimicrobial therapy and mass transport around the globe.

255

#### 256 **Acknowledgements**

257 The authors appreciate the contribution made by the many collaborating laboratories, researchers and  
258 students on this project – also the authorities that made this work possible.

259

#### 260 **Ethical approval**

261 Not required

262

#### 263 **Funding**

264 Recent research has been partly supported by Hungarian National Scientific Research Foundation  
265 (OTKA) Grant No. 61166 and The LeverhulmeTrust Project Grant F/00 094/BL (GSB, DEM, OL)

#### 266 **Author contributions**

267 HD and MS conceived the original aDNA studies and DM and GB the lipid work. MS, IP and IS  
268 collected or supplied specimens. HD performed experiments and analyzed aDNA data. JO'G designed  
269 the PCR probes and primers. OL and HW performed lipid experiments. DM, GB, OL and HW  
270 analyzed data. HD wrote the manuscript and all authors approved the final version.

#### 271 **Competing interests**

272 None declared.

273 **References**

- 274 1. WHO (World Health Organization). Tuberculosis. Fact sheet No. 104. Reviewed February  
275 2013. <http://www.who.int/mediacentre/factsheets/fs104/en/index.html#>
- 276 2. Gagneux S. Host–pathogen coevolution in human tuberculosis. *Phil Trans R Soc B* 2012;**367**  
277 (1590):850–9.
- 278 3. Smith NH, Hewinson RG, Kremer K, Brosch R, Gordon SV. Myths and misconceptions: the  
279 origin and evolution of *Mycobacterium tuberculosis*. *Nature Rev Microbiol* 2009;**7**:537–44.
- 280 4. Reed MB, Pichler VK, McIntosh F, Mattia A, Fallow A, Masala S, Domenech P, Zwerling A,  
281 Thibert L, Menzies D, Schwartzman K, Behr MA. Major *Mycobacterium tuberculosis* lineages  
282 associate with patient country of origin. *J Clin Microbiol* 2009;**47**(4):1119–28.
- 283 5. Blaser MJ, Kirschner D. The equilibria that allow bacterial persistence in human hosts. *Nature*  
284 2007;**449**:843–9.
- 285 6. Barnes I, Duda A, Pybus OG, Thomas MG. Ancient urbanization predicts genetic resistance to  
286 tuberculosis. *Evolution* 2010;**65**(3):842–8.
- 287 7. WHO (World Health Organization). Leprosy. Factsheet No. 101. Reviewed September 2012.  
288 <http://www.who.int/mediacentre/factsheets/fs101/en/>
- 289 8. Maiden MCJ. Putting leprosy on the map. *Nat Genet* 2009;**41**(12):1264–6.
- 290 9. Taylor GM, Donoghue HD. Multiple loci variable number tandem repeat (VNTR) analysis  
291 (MLVA) of *Mycobacterium leprae* isolates from European archaeological human remains with  
292 lepromatous leprosy. *Microb Infect* 2011;**13**:923–9.
- 293 10. Monot, M Honoré N, Garnier T, Zidane N, Sherafi D, Paniz-Mondolfi A, Matsuoka M, Taylor  
294 GM, Donoghue HD, Bouwman A, Mays S, Watson C, Lockwood DN, Khamispour A, Dowlati  
295 Y, Jianping S, Rea TH, Vera-Cabrera L, Stefani MM, Banu S, Macdonald M, Sapkota BR,

- 296 Spencer JS, Thomas J, Harshman K, Singh P, Busso P, Gattiker A, Rougemont J, Brennan PJ,  
297 Cole ST. Phylogeography of leprosy. *Nat Genet* 2009;**41**:1282–1289.
- 298 11. Donoghue HD. Insights gained from palaeomicrobiology into ancient and modern tuberculosis.  
299 *Clin Microbiol Infect* 2011;**17**:821–9.
- 300 12. Taylor GM, Tucker K, Butler R, Pike AWG, Lewis J, Roffey S, Marter P, Lee OY-C, Wu HHT,  
301 Minnikin DE, Besra GS, Singh P, Cole ST, Stewart GR. Detection and strain typing of ancient  
302 *Mycobacterium leprae* from a Medieval leprosy hospital. *PLoS ONE* 2013;**8**(4): e62406.
- 303 13. Lee OY-C, Bull ID, Molnár E, Marcsik A, Pálfi G, Donoghue HD, Besra GS, Minnikin DE.  
304 Integrated strategies for the use of lipid biomarkers in the diagnosis of ancient mycobacterial  
305 disease. In: Mitchell PD, Buckberry J, editors. *Proceedings of the Twelfth Annual Conference of*  
306 *the British Association for Biological Anthropology and Osteoarchaeology, Department of*  
307 *Archaeology and Anthropology University of Cambridge 2010*. BAR International Series 2380,  
308 Archaeopress, Oxford, UK: 2012. p. 63–9.
- 309 14. Bouwman AS, Kennedy SL, Müller R, Stephens RH, Holst M, Caffell AC, Roberts CA, Brown  
310 TA. Genotype of a historic strain of *Mycobacterium tuberculosis*. *PNAS* 2012;**109**:18511–6.
- 311 15. Schuenemann VJ, Singh P, Mendum TA, Krause-Kyora B, Jäger G, Bos KI, Herbig A,  
312 Economou C, Benjak A, Busso P, Nebel A, Boldsen J, Kjellström A, Wu H, Stewart GR,  
313 Taylor GM, Bauer P, Lee OY-C, Wu HHT, Minnikin DE, Besra GS, Tucker K, Roffey S, Sow  
314 SO, Cole ST, Nieselt K, Krause J. Genome-wide comparison of Medieval and modern  
315 *Mycobacterium leprae*. *Science* 2013;**341**(6142):179–183.
- 316 16. Chan JZ-M, Sergeant MJ, Lee OY-C, Minnikin DE, Besra GS, Pap I, Spigelman M, Donoghue  
317 HD, Pallen MJ. Metagenomic analysis of tuberculosis in a mummy. *N Engl J Med* 2013;**369**(3):  
318 289–90.
- 319 17. Taylor GM, Mays SA, Huggett JF. Ancient DNA (aDNA) studies of man and microbes: general  
320 similarities, specific differences. *Int J Osteoarchaeol* 2010;**20**:747–51.

- 321 18. Évinger S, Bernert Zs, Fóthi E, Wolff K, Kóvári I, Marcsik A, Donoghue HD, O'Grady J, Kiss  
322 KK, Hajdu T. New skeletal tuberculosis cases in past populations from Western Hungary  
323 (Transdanubia). *HOMO – J Comp Hum Biol* 2011;**62**:165–183.
- 324 19. Singh P, Cole ST. *Mycobacterium leprae*: genes, pseudogenes and genetic diversity. *Future*  
325 *Microbiol* 2011;**6**(1):57–71.
- 326 20. Lee OY-C, Wu HHT, Donoghue HD, Spigelman M, Greenblatt CL, Bull ID, Rothschild BM,  
327 Martin LD, Minnikin DE, Besra GS. *Mycobacterium tuberculosis* complex lipid virulence  
328 factors preserved in the 17,000-year-old skeleton of an extinct bison, *Bison antiquus*. *PLoS*  
329 *ONE* 2012;**7**(7): e41923
- 330 21. Rothschild BM, Martin LD, Lev G, Bercovier H, Kahila Bar-Gal G, Greenblatt C, Donoghue H,  
331 Spigelman M, Brittain D. *Mycobacterium tuberculosis* complex DNA from an extinct bison  
332 dated 17,000 years before the present. *Clin Infect Dis* 2001;**33**:305–11.
- 333 22. Supply P, Marceau M, Mangenot S, Roche D, Rouanet C, Khanna V, Majlessi L, Criscuolo A,  
334 Tap J, Pawlik A, Fiette L, Orgeur M, Fabre M, Parmentier C, Frigui W, Simeone R, Boritsch  
335 EC, Debrie A-S, Willery E, Walker D, Quail MA, Ma L, Bouchier C, Salvignol G, Sayes F,  
336 Cascioferro A, Seemann T, Barbe V, Loch C, Gutierrez M-C, Leclerc C, Bentley SD, Stinear  
337 TP, Brisse S, Médigue C, Parkhill J, Cruveiller S, Brosch R. Genomic analysis of smooth  
338 tubercle bacilli provides insights into ancestry and pathoadaptation of *Mycobacterium*  
339 *tuberculosis*. *Nat Genet* 2013;**45**(2):172–9.
- 340 23. Zink AR, Molnár E, Motamedi N, Pálffy G, Marcsik A, Nerlich AG. Molecular history of  
341 tuberculosis from ancient mummies and skeletons. *Int J Osteoarchaeol* 2007;**17**:380–91.
- 342 24. Hershkovitz I, Donoghue HD, Minnikin DE, Besra GS, Lee OY-C, Gernaey AM, Galili E,  
343 Eshed V, Greenblatt CL, Lemma E, Kahila Bar-Gal G, Spigelman M. Detection and molecular

- 344 characterization of 9000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in  
345 the Eastern Mediterranean. *PLoS ONE* 2008;**3**(10), e3426.
- 346 25. Murphy EM, Chistov YK, Hopkins R, Rutland P, Taylor GM. Tuberculosis among Iron Age  
347 individuals from Tyva, South Siberia: palaeopathological and biomolecular findings. *J Archaeol*  
348 *Sci* 2009;**36**:2029–38.
- 349 26. Weng X, Xing Y, Liu J, Wang Y, Ning Y, Li M, Wu W, Zhang L, Li W, Vander Heiden J,  
350 Vissa V. Molecular, ethno-spatial epidemiology of leprosy in China: novel insights for tracing  
351 leprosy in endemic and non endemic provinces. *Infect Genet Evol* 2013;**14**:361–8.
- 352 27. Donoghue HD, Marcsik A, Matheson C, Vernon K, Nuorala E, Molto JE, Greenblatt CL,  
353 Spigelman M. Co-infection of *Mycobacterium tuberculosis* and *Mycobacterium leprae* in  
354 human archaeological samples: a possible explanation for the historical decline of leprosy. *Proc*  
355 *R Soc Lond B* 2005;**272**:389-94.
- 356 28. Lalremruata A, Ball M, Bianucci R, Welte B, Nerlich AG, Kun JFJ, Pusch CM. Molecular  
357 identification of falciparum malaria and human tuberculosis co-infections in mummies from the  
358 Fayum Depression (Lower Egypt). *PLoS ONE* 2013;**8**(4):e60307.
- 359 29. Hanekon M, Streicher EM, Van de Berg D, Cox H, McDermid C, Bosman M, Gey van Pittius  
360 NC, Victor TC, Kidd M, van Soolingen D, van Heiden PD, Warren RM. Population structure of  
361 mixed *Mycobacterium tuberculosis* infection is strain genotype and culture medium dependent.  
362 *PLoS One* 2013;**8**(7):e70178.
- 363 30. Namouchi A, Didelot X, Schöck U, Gicquel B, Rocha EPC. After the bottleneck: Genome-wide  
364 diversification of the *Mycobacterium tuberculosis* complex by mutation, recombination, and  
365 natural selection. *Genome Res* 2012;**22**:721-34.

366

367 **Table 1**368 PCR primers and probes to detect *M. tuberculosis* (IS6110, IS1081) and *M. leprae* (RLEP, RepLep)

PCR locus	Primer name	Primer sequence (5'-3')	Amplicon size (bp)
IS6110	6110 Probe	5'-FAM-ACCTCACCTATGTGTCGACCTG-BHQ1-3'	
IS6110	6110F	CACCTAACCGGCTGTGG	
IS6110	6110R	TGACAAAGGCCACGTAGG	75
IS1081	1081 Probe	5'-FAM-GGGCTACCGCGAACGCA-BHQ1-3'	
IS1081	NF	TGATTGGACCGCTCATCG	
IS1081	NR	CTTGATGGGGGCTGAAGC	72
RLEP	RLEP Probe	FAM-5' - CTCAGCCAGCAAGCAGGCAT-3'-BHQ2	
RLEP	RLEPF	CGCTGGTATCGGTGTCG	
RLEP	RLEPR	ACACGATACTGCTGCACC	80
REPLEP	REPLEP Probe	5'-FAM-CATGTCTATCTCCGTACGCAGCTG-BHQ1-3'	
REPLEP	REPLEPF	GACTGTACTTCTTGGCCAGC	
REPLEP	REPLEPR	GCAAGGTGAGCGTTGTGG	66

369