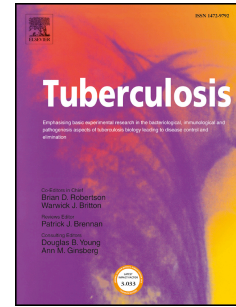


# Accepted Manuscript

7000 year-old Tuberculosis Cases from Hungary – Osteological and Biomolecular Evidence

Muriel Masson, Zsolt Berezcki, Erika Molnár, Helen D. Donoghue, David E. Minnikin, Oona Y.-C. Lee, Houdini H.T. Wu, Gurdyal S. Besra, Ian D. Bull, György Pálfi



PII: S1472-9792(15)00008-6

DOI: [10.1016/j.tube.2015.02.007](https://doi.org/10.1016/j.tube.2015.02.007)

Reference: YTUBE 1268

To appear in: *Tuberculosis*

Please cite this article as: Masson M, Berezcki Z, Molnár E, Donoghue HD, Minnikin DE, Lee OY-C, Wu HHT, Besra GS, Bull ID, Pálfi G, 7000 year-old Tuberculosis Cases from Hungary – Osteological and Biomolecular Evidence, *Tuberculosis* (2015), doi: 10.1016/j.tube.2015.02.007.

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## TITLE

7000 year-old Tuberculosis Cases from Hungary – Osteological and Biomolecular Evidence

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## SUMMARY

This study derives from the macroscopic analysis of a Late Neolithic population from Hungary. Remains were recovered from a tell settlement at Hódmezővásárhely-Gorzsa from graves within the settlement as well as pits, ditches, houses and as stray finds. One of the most important discoveries from these remains was evidence of tuberculosis. Pathological analysis of the seventy-one individuals revealed numerous cases of infections and non-specific stress indicators on juveniles and adults, metabolic diseases on juveniles, and evidence of trauma and mechanical changes on adults. Several cases showed potential signs of tuberculosis and further analyses were undertaken, including biomolecular studies. The five individuals were all very young adults and included a striking case of Hypertrophic Pulmonary Osteopathy (HPO) with rib changes, one case with resorptive lesions on the vertebrae, two cases with hypervascularisation on the vertebrae and periosteal remodelling on the ribs, and one case with abnormal blood vessel impressions and a possible lesion on the endocranial surface of the skull. The initial macroscopic diagnosis of these five cases was confirmed by lipid biomarker analyses, and three of them were corroborated by DNA analysis. At present, these 7000-year-old individuals are among the oldest palaeopathological and palaeomicrobiological cases of tuberculosis worldwide.

## KEYWORDS

Tuberculosis

Neolithic

Hungary

aDNA

Lipid biomarkers

ACCEPTED MANUSCRIPT

## INTRODUCTION

This research is based on the analysis of human skeletal remains recovered from the Late Neolithic tell settlement of Hódmezővásárhely-Gorzsa in the South of Hungary. This site, located about 15 miles North East of Szeged and 9 miles South West of Hódmezővásárhely, was occupied through six settlement phases starting from the Early Tisza culture. The naturally elevated settlement was surrounded by streams and marshes.

The Tisza Culture occupation of the settlement occurred during the first half of the fifth millennium BC, with a time span of at least 300 years. Twenty samples from the site provided a calibrated range of 4970 - 4594 BC,<sup>1</sup> recalibrated most recently by Masson to 4932 to 4602 BC with a 95.4% confidence interval using the calibration curve IntCal04 for Northern Hemisphere in the dating programme OxCal 4.1.<sup>2</sup> Bone fragments of HGO-53, one of the individuals presented in this study, were also analysed most recently at the Hertelendi AMS C-14 Lab in Debrecen, Hungary (AMS Lab code DeA-2485.1.1), and confirmed that this individual dated back to the start of the fifth millennium BC, with a calibrated age range of 4780-4715 BC with 1 sigma, based on HGO-53 radiocarbon age of 5872±32 BP and the intcal09.14c calibration data set.<sup>3</sup>

Only two percent of the site has been investigated to date, first by Gazdapusztai in the 1950s,<sup>4</sup> and then by Horváth in the 1970s, 1980s and 1990s.<sup>5</sup> Unfortunately, there are no published maps of the site, and there is no information currently available on the location of the graves and other remains in relation to the settlement and to each other. Seventy-one individuals dating from the Tisza Culture were recovered from Hódmezővásárhely-Gorzsa, including 56 who had been buried in graves within the settlement and the partial remains of a further possible fifteen recovered from pits, ditches, houses and as stray finds. Their remains are housed in the collection of the Biological Anthropology Department of the University of Szeged, on loan from the Móra Ferenc Múzeum in Szeged.

Macroscopic analysis revealed that juveniles accounted for a third of the Late Neolithic remains, while two-thirds of the adult remains where sex could be determined were female. This population appeared to have been mostly non-violent, leading a physically stressful life, prone to infections and with a high rate of dental disease.<sup>6</sup> The pathological analysis revealed a case of Hypertrophic Pulmonary Osteopathy (HPO). Although its most common causes nowadays are intrathoracic cancer and chronic intrathoracic infection,<sup>7</sup> tuberculosis would have been a more likely cause in the past. Tuberculosis has already been successfully identified as a possible primary cause of HPO in the archaeological record,<sup>8</sup> and was found to strongly correlate to HPO in a historic population from a pre-antibiotic era.<sup>9</sup> In modern cases, HPO has also been associated with severe and untreated pulmonary tuberculosis.<sup>10</sup> Based on the strong link between HPO and tuberculosis, aDNA and lipid biomarker analyses were carried out on this individual to confirm the presence of tuberculosis among this ancient population. The biomolecular results confirmed that tuberculosis had been present at this Late Neolithic site, and this case was published in full earlier this year.<sup>11</sup> Four further probable cases of tuberculosis were discovered through macroscopic analysis and were similarly tested for *Mycobacterium tuberculosis* aDNA, and mycolic and mycocerosic acid cell wall lipid biomarkers.

## MATERIALS AND METHODS

### Morphological analysis

All examinations were carried out macroscopically at the Biological Anthropology Department of Szeged University. All remains were damaged and fragmentary. Sex was estimated based on several morphological methods principally concentrating on the morphological traits of the skull and the pelvis, while also giving some consideration to bone dimensions.<sup>12,13</sup> Age was estimated from skeletal and dental development, particularly concentrating on state of epiphyseal fusion in addition to tooth wear, and giving some consideration to the morphology of the pubis and the auricular surface.<sup>14,15</sup> The palaeopathological analysis, based on macromorphological observations (Figure 1), was undertaken at the same laboratory.<sup>16</sup>

### aDNA analysis

The DNA analysis was undertaken in the former Department of Medical Microbiology at University College London, which has considerable experience of working with *Mycobacterium tuberculosis* aDNA. The recommended protocols for aDNA were followed and approximately 55mg of bone powder taken from each sample of ribs and vertebrae. Following DNA extraction,<sup>17,18</sup> PCR was used to amplify any DNA from specific regions of the multicopy IS6110 and IS1081 regions of the *M. tuberculosis* complex. Amplified DNA was examined initially by agarose gel electrophoresis and these primers were subsequently used on a Real-Time platform, to enable the detection of DNA using SYBR Green and melt analysis.<sup>19</sup> Sequencing was attempted after extraction of DNA from gel slices. Details of the aDNA methodologies utilised in this study have already been described further elsewhere.<sup>11</sup>

### Lipid biomarker analysis

Robust lipid biomarkers complement aDNA detection.<sup>17,18,20</sup> As they do not require amplification, they also suffer much less from contamination.<sup>18,21</sup> Although mycolic acids can suffer from degradation in ancient remains, mycocerosate biomarker fatty acids appear to be stronger and more resistant.<sup>21</sup> Lipid biomarkers from the samples were extracted, derivatised and fractionated, as described in detail previously.<sup>11,18,21</sup>

## RESULTS

HGO-53 has already been described previously.<sup>11</sup> He was a young adult male, probably between 19 and 20 years old, with pathology on his skull, thorax, shoulder, upper limbs, spine, lower limbs and feet. Hypertrophic Pulmonary Osteopathy (HPO) was diagnosed based on the strikingly symmetrical diffuse periostitis with active woven bone formation on the bones of this young adult male, a characteristic sign of HPO. In addition, the analysis also brought to light revealing changes on the ribs of the left chest, cavitations in the vertebral bodies and signs of porotic hyperostosis. Although DNA was very hard to recover from this damaged and fragmentary sample, it was nevertheless successfully extracted from the bones. Analysis provided a

partial sequence for *Mycobacterium tuberculosis*, albeit fragmented and of poor quality. A PCR using primers specific for *Mycobacterium tuberculosis* gave an amplicon of 113bp. This sample was also analysed for lipid biomarkers and revealed a mycocerosate profile typical of *M. tuberculosis* in addition to a weak mycolic acid trace with severe degradation.<sup>11</sup>

HGO-08 was a young female aged 17-22 years. Light *cribra orbitalia* was visible in both her orbits. Resorptive lesions were observed on the anterior aspect of all thoracic vertebrae from T5 as well as on two lumbar vertebrae. Schmorl's nods were found on each thoracic vertebra from T7 to T12. An extensive lesion on both the inferior side of the first lumbar vertebra and the superior side of the second lumbar vertebra was found with adjacent osteophytes. The case was confirmed by lipid biomarkers analyses with strong mycocerosate (Figure 2) and clear mycolate (Figure 3) profiles typical of *M. tuberculosis*.<sup>21</sup>

HGO-10, a male in his early twenties, was also diagnosed as a possible case of tuberculosis based on the evidence of hypervascularisation (pits and grooves) on the anterior aspect of five consecutive thoracic vertebrae and two lumbar (pits only), in addition to some level of periosteal remodeling and a slight hypervascularisation on the visceral surface of his ribs (Figure 1A). Linear enamel hypoplasia was visible on his dentition. The case was confirmed by lipid biomarker analyses with strong mycocerosate (Figure 2) and clear mycolate (Figure 3) profiles corresponding well to those from *M. tuberculosis*.<sup>21</sup>

HGO-21 was a female in her early twenties. Small endocranial pits, a resorptive lesion on the ninth thoracic vertebra (Figure 1B), increased vascularisation on the ventral side of one rib, *cribra orbitalia*, and light periostitis on the external surface of two fragments of ribs towards their sternal end, all pointed to a possible case of tuberculosis. This was confirmed by a strong mycocerosate profile (Figure 2), supported by a weak mycolate profile (Figure 3), and the results of DNA analysis.



The last confirmed case of tuberculosis is HGO-48, a young adult female showing abnormal blood vessel impressions (abvi) over most of her frontal endocranial surface with SES-like pattern (*Serpens endocrania symmetrica*) (Figure 1C), indicator of meningitis caused by infection, metabolic disease and particularly tuberculosis. A large round depression around 1 cm in diameter on the endocranial surface of the right parietal could be a sign of tuberculous lesion. Very slight *cribra orbitalia* was also found in her left orbit. The strong mycocerosate profile (Figure 2) and DNA analyses were all positive for *M. tuberculosis*, but the mycolate trace was very weak (Figure 3) and possibly degraded.

## DISCUSSION

The 25-year old female and a 12-month old infant from Atlit-Yam provide the earliest biomolecular evidence of tuberculosis in humans confirmed by both DNA and lipid biomarkers analyses.<sup>18</sup> The osteological pathological evidence was very scarce on the adult female and consisted of endocranial changes (SES) and periostitis on the tubular bones of the infant. Prior to this study, the oldest recognised osteological cases of tuberculosis came from Neolithic Europe and dated back to the 4<sup>th</sup> millennium BC,<sup>22-24</sup> while the first cases of tuberculosis confirmed by DNA dated back to pre-dynastic Egypt (3500-2650 BC).<sup>25</sup> In Hungary, a case of Pott's disease was discovered recently at the site of Alsónyék-Bátaszék in an adult male, dating from the Late Neolithic / Early Copper Age (5<sup>th</sup> millennium BC).<sup>26</sup> The morphological observations unequivocally indicate an advanced stage of vertebral tuberculosis and results of the DNA analysis are published in the same volume as this study. Several other possible tuberculosis cases have also been discovered recently from the 5000 year-old site of Vésztő-Mágor,<sup>27</sup> with presence of *M. tuberculosis* aDNA confirmed in one case by palaeomicrobial analysis of the dental pulp region in the teeth.<sup>28</sup>

With the successful combination of different scientific methods, including morphological observations and palaeomicrobiological analyses, we were able to conclusively verify the presence of the *Mycobacterium tuberculosis* complex in Neolithic Europe as early as 7000 years ago. Mycocerosic acids, in particular, have

proven to be robust biomarkers, offering a much more definitive diagnosis for tuberculosis of great antiquity.<sup>21</sup> Hódmezővásárhely-Gorzsa therefore presents the oldest confirmed cases of TB in Hungary and Europe to date, second only to Atlit-Yam worldwide. It is also unprecedented in the archaeological record by providing so many ancient cases of tuberculosis, with five confirmed already and several more potential cases still to be biochemically analysed.

This study also shows the importance of not restricting the diagnosis of tuberculosis in palaeopathological cases to the modern clinical diagnostic criteria for tuberculosis, as skeletal changes may have differed in the past. Classical tuberculosis pathology includes vertebral fusion and collapse leading to Pott's disease, knee joint ankylosis, hip joint destruction, cold abscess on the sacrum or vertebrae and endocranial TB. Other osseous change probably related to tuberculosis include rib periostitis mostly on the ventral side and particularly in the left chest, hypervascularisation, diffuse symmetrical periostitis (HPO), endocranial changes such as *serpens endocrania symmetrica* (SES) and abnormal blood vessel impressions.<sup>29,30</sup> All of these atypical changes were present in this sample of confirmed TB cases, while none of the classical signs of tuberculosis could be observed. These five Neolithic cases are therefore of considerable importance, not only for the palaeopathological record but also as an encouragement to osteoarchaeologists to look for less typical or obvious osteological signs of tuberculosis than for example the classic Pott's disease. Porotic hyperostoses, such as *cribra orbitalia* and *cribra cranii*, may also be associated with tuberculosis as these are generally attributed to iron-deficiency anaemia, which can develop from the interaction of several factors, such as weaning practices, diet, hygiene, parasites and infectious diseases. By discovering more palaeopathological cases of tuberculosis, it will help us gain a unique insight into the evolution of this still ever present disease, second only to HIV/AIDS as the greatest killer worldwide due to a single infectious agent.

## ACKNOWLEDGEMENTS

No ethical approval was required.

This research was supported by the Hungarian Scientific Research Fund (OTKA) Grant no. 78555 and by the Leverhulme Trust Project Grant F/00 094/BL. The UK National Environmental Research Council (NERC) provided funding for the mass spectrometry facilities at Bristol (Contract no. R8/H12/15; [www.lsmsf.co.uk](http://www.lsmsf.co.uk)). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

There are no competing interest.

Thanks to Dr. Ferenc Horváth from the Móra Ferenc Múzeum in Szeged and Dr. Antónia Marcsik from the Department of Biological Anthropology, University of Szeged, for providing access to the skeletal material. A Leverhulme Trust Emeritus Fellowship to DEM is acknowledged.

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## CAPTIONS TO ILLUSTRATIONS

Figure 1: Atypical osteological evidence of tuberculosis

A. Hypervascularisation of the vertebrae of HGO-10; B. Resorptive lesion on vertebra of HGO-21; C. Abnormal blood vessel impressions (abvi) with SES-like pattern (*Serpens endocrania symmetrica*) on the endocranial surface of HGO-48.

Figure 2: Mycocerosate profiles

Negative ion chemical ionisation gas chromatography (NI-CI GC-MS) profiles of pentafluorobenzyl esters of mycocerosic acids. The diagnostic signals at  $m/z$  437, 451 and 479 correspond to the carboxylate ions, identified by selected ion monitoring, derived from  $C_{29}$ ,  $C_{30}$  and  $C_{32}$  mycocerosates. The mycocerosates are recognisable by their appearance as double peaks following racemisation.

Figure 3: Mycolate profiles

Reverse phase fluorescence high performance liquid chromatography (HPLC) of total mycolic acid pyrenebutyryl pentafluorobenzyl (PBA-PFB) derivatives for extracts of all samples and standard *M. tuberculosis*. HGO-18 is a non Neolithic specimen from the same burial site, which was included for comparison.

Figure 4: Initial results of aDNA analysis

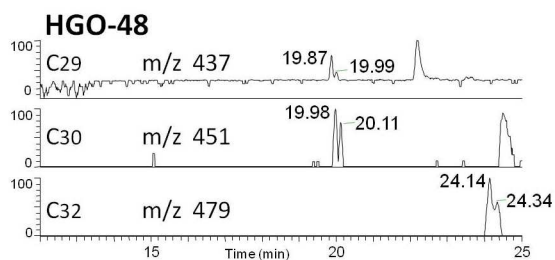
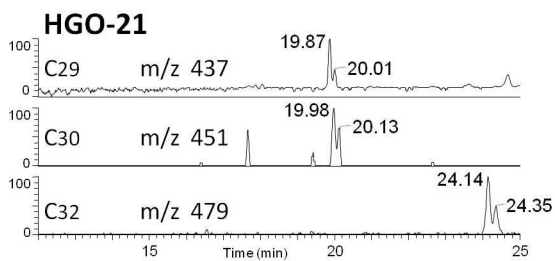
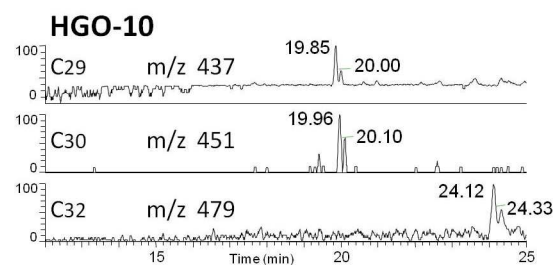
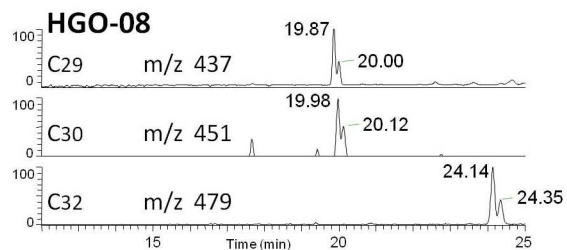


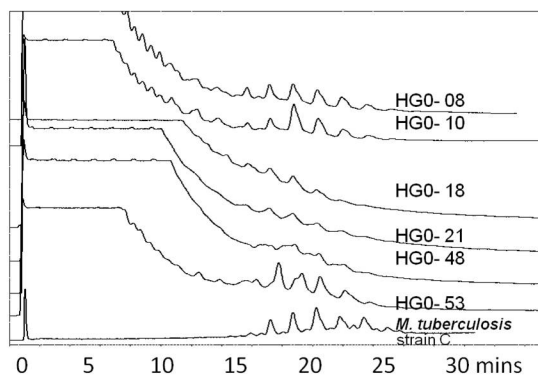
HGO-21: These PCRs used primers and a specific fluorescent probe for the *Mycobacterium tuberculosis* complex region IS1081 (6 copies/cell), as confirmatory as the more traditional sequencing used for HGO-53 but better suited to fragmentary DNA as this probe target a shorter 72bp sequence. The + indicates that the DNA extraction included treatment with PTB to enhance strand separation of the DNA.

HGO-48: These PCRs used primers and a fluorescent specific probe for the *MTb* complex region IS1081 (6 copies/cell). The + indicates that the DNA extraction included treatment with PTB to enhance strand separation of the DNA.



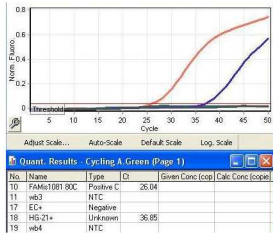
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HGO-21

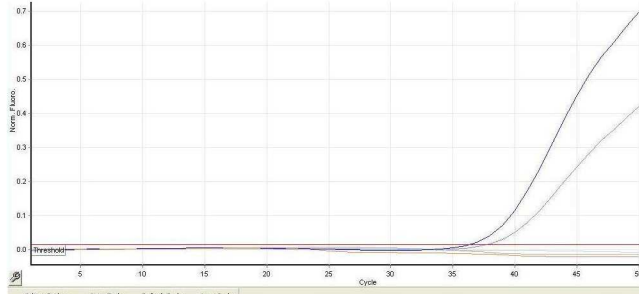


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Quant. Results - Cycling A, Green (Page 1)

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11	rs3	NTC			
17	EC+	Negative			
18	HG-21+	Unknown		36.85	
19	rs4	NTC			

HGO-48



Adjust Scale... Auto-Scale Default Scale Log Scale

Quant. Results - Cycling A, Green (Page 1)

No.	Name	Type	Cl	Given Conc (copy)	Calc Conc (copies)	3 Var	Rep. Cl	Rep. Cl Sk	Rep. Cl (95% Cl)	Rep. Calc. Conc	Rep. Calc. Conc (95%)
2	rs1	NTC									
3	HG48	Unknown		37.77					37.77		
4	HG48+	Unknown		36.39					36.39		
8	rs2	NTC									
10	EC+	Negative									

ACCEPTED MANUSCRIPT