Osteological and biomolecular evidence of a 7000-year-old case of hypertrophic pulmonary osteopathy secondary to tuberculosis from neolithic hungary.
Abstract

Seventy-one individuals from the late Neolithic population of the 7000-year-old site of Hódmezővásárhely-Gorzsa were examined for their skeletal palaeopathology. This revealed numerous cases of infections and non-specific stress indicators in juveniles and adults, metabolic diseases in juveniles, and evidence of trauma and mechanical changes in adults. Several cases showed potential signs of tuberculosis, particularly the remains of the individual HGO-53. This is an important finding that has significant implications for our understanding of this community. The aim of the present study was evidence to confirm this diagnosis. HGO-53 was a young male with hypertrophic pulmonary osteopathy (HPO), revealing rib changes and cavitations in the vertebral bodies. The initial macroscopic diagnosis of HPO secondary to tuberculosis was confirmed by analysis of *Mycobacterium tuberculosis* complex species-specific cell wall lipid biomarkers and corroborated by ancient DNA (aDNA) analysis. This case is the earliest known classical case of HPO on an adult human skeleton and is one of the oldest palaeopathological and palaeomicrobiological tuberculosis cases to date.


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Introduction

Hypertrophic Osteoarthropathy (HOA), also known as Marie-Bamberger disease, is a periosteal phenomenon characterised by the symmetrical (diffuse or distal) appearance of new bone mainly on the shaft of the long bones. The reaction can result in "appliqué" (new bone with sharply defined edges distinguishable from the underlying bone) or surface form that covers the entire bone with no visible edge. It is a primary pathology and is usually encountered in its secondary form, Hypertrophic Pulmonary Osteopathy (HPO). Today, its most common causes are intrathoracic cancer and chronic intrathoracic infection [1], [2]. How tuberculosis would have been a more likely cause. Only a few case diagnosis have been reported in the archaeological record. In one tuberculosis (TB) was successfully identified as the possible primary cause [3]. In their study, Webb and Thomas [4] associated HOA/HPO particularly with severe and untreated pulmonary tuberculosis. In their recent study of a Portuguese population from a pre-antibiotic era, Assis and colleagues [5] found a strong statistical correlation between HOA/HPO and tuberculosis in the skeletal remains.

HPO is a rare find in the archaeological record. The oldest documented cases in Europe include a Merovingian skeleton from the site of Les Rues des Vignes AD500 to 700 [6], and a medieval 40–50 year-old male from Czarna Wielka (Grozish, Poland) [7]. In a collection of one thousand individuals from Pre-Hispanic Mexico, two presented with HOA/HPO [8]: a young female from a Maya site from the Classic period (AD 300 to 900) and a young adult male from the Ticoman site from the Formative period (2000 BC to AD 100). Most recently in the Middle East, the skeletal remains infant recovered from the underwater Neolithic site of Atlit-Yam, Israel, dated to 9250-8160 BP, were described as showing evidence of HOA, in addition to Mycobacterium tuberculosis aDNA and mycolic cell wall biomarkers [9].

Tuberculosis is a disease of infancy, young adults and the elderly. It is important not to restrict the diagnosis of tuberculosis in palaeopathological cases to modern clinical diagnostic criteria for TB, as skeletal changes may have differed in tuberculosis pathology includes vertebral fusion and collapse lead knee joint ankylosis, hip joint destruction, cold abscess on the sacrum, endocranial TB. Other osseous change probably related to tuberculosis periostitis, hypervascularization, diffuse symmetrical periostitis (HP changes such as serpens endocrania symmetrica (SES) and abnormal impressions [11]. Rib changes may include sharply demarcated lytic lesions or diffuse periostitis on the ventral side of the ribs, possibly caused by adjacent Most rib changes are associated with individuals suffering from pul in the left chest, and although those lesions cannot be considered characteristic of pulmonary tuberculosis, they can indicate a non-specific pulmonary disease, with tuberculosis as the most likely cause [12],
hyperostoses, such as *cribra orbitalia* and *cribra cranii*, are generally attributed to iron-deficiency anemia, which can develop from the interaction of several factors, such as weaning practices, diet, hygiene, parasites and infectious diseases, associated with tuberculosis.

The Atlit-Yam study [9] provides the earliest biomolecular evidence of tuberculosis in humans. Both DNA and lipid biomarkers analyses confirmed that the 25-year old female and the 12-month old infant were infected with a human lineage of *M. tuberculosis*. The osteological pathological evidence was very scarce on the adult female. In the infant, it consisted of endocranial changes (SES) and periostitis on tubular bones, consistent with tuberculosis. Although the periostitis was described as HOA, there is no evidence of symmetry of lesions. Prior to this study, the oldest tuberculosis came from Neolithic Europe. A 15-year old juvenile and an 18-month old infant, dating from the Middle Neolithic in the first half of the 4th millennium BC, were both diagnosed on the basis of spinal osteolytic lesions [14], [15].

Tuberculosis has also been confirmed previously by DNA analyses of Egyptian skeletons (3500-2650 BC), both with bony changes [17] and without. In Hungary, Pott's disease in an adult male, dating from the Late Neolithic/Early Copper Age (5th millennium BC) was discovered recently at the site of Alsónyék-Bátaszék, not yet been confirmed by molecular biomarkers, but the morphological observations unequivocally indicate an advanced stage of vertebral tuberculosis. Several other possible tuberculosis cases have been discovered recently from the 5000 year-old site of Vészt-Mágor, Hungary, associated with archaeological material from that period. Palaeomicrobial analysis of the dental pulp region in the teeth confirmed the presence of *M. tuberculosis* aDNA [21].

The present study was based on human skeletal remains from the Late Neolithic Tell settlement of Hódmezővásárhely-Gorzsa in the South of Hungary. Macroscopic analyses revealed widespread symmetrical periostitis on the long bones and the ribs of the male, indicating a case of HPO. The strong association with tuberculosis, with no other possible cause, made further biomolecular studies of this 7000 year-old skeleton imperative to ascertain the presence of tuberculosis at the Tisza Culture site. As noted above, the detection of aDNA and lipid biomarkers can offer confirmation of tuberculosis in archaeological material, so there was good expectation of finding such biomarkers in HGO-53. In addition, the mycocerosic and mycolipenic acid cell wall lipid biomarkers appear to be more stable, and can thus offer conclusive support as demonstrated in a very ancient, 17,000 year-old bison metacarpal.

**Archaeological Background**

The Late Neolithic Tell settlement of Hódmezővásárhely-Gorzsa is located in the South of Hungary, about 15 miles North East of Szeged and 9 miles South West of Hódmezővásárhely in the Tisza-Maros angle (Fig. 1). It had been on a natural elevation surrounded by streams and marshes, and was occupied through six settlement phases starting from the Early Tisza culture. Only two percent of the site has been investigated to date. The site was initially investigated by Gazdapusztai between 1955 and 1957 [25], [26], and excavations were undertaken by Horváth between 1978 and 1996.
The Late Neolithic Tell settlement of Hódmezővásárhely-Gorzsa, located in the South of Hungary, about 15 miles North East of Szeged and 9 miles South West of Hódmezővásárhely in the Tisza-Maros angle. Inset shows general geographic location.

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The settlement phase of the Tisza Culture occurred during the first half of the fifth millennium BC, with an occupation time span of at least 300 years. Radiocarbon analysis of twenty samples from the site date this settlement to 4970 - 4594 cal BC [34], [35] with a 68.3% confidence interval. These dates were recalibrated by Masson (unpublished PhD Thesis, 2013, University of Edinburgh) using the calibration curve IntCal04 for Northern Hemisphere [36] in the dating programme OxCal 4.1 [37]. The original uncalibrated dates by Hertelendi & Horváth [33] ranging from 4932 to 4602 BC with 95.4% confidence interval after recalibration occupation span fits with overall ranges for the Tisza culture [34], [35]: Late Neolithic [38], 4970–4490 BC and 4970–4380 BC respectively. Using new recalibrations, Yerkes and colleagues [39] utilised 107 Late Neolithic samples to produce a range of dates from 5021 to 4402 BC for the whole period.

The human skeletal remains recovered from Hódmezővásárhely-Gorzsa are housed in the collection of the Biological Anthropology Department of the University of Szeged, on loan from the Móra Ferenc Múzeum in Szeged. No permits were required for the described study, which complied with all relevant regulations. Access granted by both Móra Ferenc Múzeum and the Biological Anthropology Department of the University of Szeged. Seventy-one individuals were recovered in total from the Tisza (Late Neolithic) Culture, including 56 who had been buried in graves within the settlement and the partial remains of a further possible fifteen recovered from pits, as stray finds. Juveniles accounted for a third of the remains. Of the adult remains where sex could be determined, two-thirds were female. Pathological analyses seemed to indicate that this population had been mostly non-violent, leading a physically stressful life, prone to infections and with a high rate of dental disease [40].

Unfortunately, there are no published maps of the site, and there is currently available on the location of the graves and other remains and to each other. However, recent radiocarbon analysis at the Herlendi AMS C-14 Lab in Debrecen, Hungary (AMS Lab code DeA-2485.1.1), on bone fragments from...
Materials and Methods

Morphological Analysis

The remains of HGO-53, the skeleton from grave 64, were very fragmentary with over one thousand fragments, though his skeleton was mostly complete. The examination was carried out macroscopically at the Biological Anthropology Department of Szeged University. The palaeopathological analysis based on macromorphology [42], [43] was undertaken at the same laboratory.

Sex was estimated based on several morphological methods. Both skull and pelvis indicated that this individual was a male. Bone dimensions also reflected a male individual. Skeletal and dental development aged this individual to around 19–20 years old. Stature was estimated based on long bone lengths to 165 cm ± 4 cm. See S1 for full details of the methodologies used in estimating age, sex.

M. Tuberculosis aDNA Analysis

The recommended protocols for aDNA were followed. Approximately 55 mg of bone powder was taken from each sample of a rib, tibia and vertebra. The DNA was extracted as described previously [9], [44]. PCR was used to amplify any DNA of the multicopy IS6110 and IS1081 regions of the M. tuberculosis complex. Amplified DNA was examined initially by agarose gel electrophoresis [45]. Subsequently, these primers were used on a Real-Time platform, to enable the detection of DNA using SYBR Green and melt analysis. Sequencing was attempted after extraction of DNA from gel slices. See Document S2 for full details of the methodologies used in the aDNA analysis.

Lipid Biomarker Analysis

Lipid biomarkers from a rib sample of HGO-53 (556 mg) were extracted, derivatised and fractionated, as described previously [9], [23]. See Document S3 for full details of the methodologies used in the lipid biomarker analysis.

Results

Macroscopic Analysis

Pathology was observed on the skull, thorax, shoulder, upper limbs and feet of HGO-53 (Fig. 2). Light cribra orbitalia and cribra cranii were visible on the skull, and a small area of periostitis was visible on the mandible. Cavitatic fragments of vertebral bodies. Active diffuse periostitis with severe ventral surface of the heads of left ribs was observed, although no
right ribs. Unsided fragments of ribs also showed active diffuse periostitis lesion accompanied by reactive surface new bone formation in one bones presented evidence of widespread active periostitis with woven bone formation in one case (Fig. 5). Signs of periostitis were also visible on the the upper limbs (Fig. 5). Signs of periostitis were also visible on the upper limbs. See Document S1 for a detailed description of HGO-53 skeletal figure 6 for the radiographs of a rib fragment and a fragment of fibula, clearly showing the new bone formation along both shafts.

**Figure 2. HGO-53 - Location of periostitis.**
The strikingly symmetrical diffuse periostitis on the bones of this revealed by the morphological analyses is a characteristic sign of Hypertrophic Osteoarthropathy (HOA).

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**Figure 3. HGO-53– Ribs.**
Active diffuse periostitis with extensive bone formation visible on the ribs.

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**Figure 4. HGO-53– Upper Limbs.**
Active diffuse periostitis on distal end of the ulna.

![Figure 5. HGO-53– Lower Limbs.](https://doi.org/10.1371/journal.pone.0078252.g004)

“Appliqué” periostitis on femur (a.) and fibula (b).

![Figure 6. HGO-53– Radiographs.](https://doi.org/10.1371/journal.pone.0078252.g005)

“Appliqué” periostitis on a fragment of rib (A) and a fragment of fibula (B).

The strikingly symmetrical diffuse “appliqué” periostitis on the bone of this young adult male revealed by the morphological analyses is a characteristic sign of Hypertrophic Pulmonary Osteopathy (HPO). This strongly indicates that this individual had suffered from a chronic pulmonary disease. In addition, the analysis revealed distinctive changes on the ribs of the left chest, cavitations in the vertebral bodies and signs of porotic hyperostosis. Considering all of this evidence, together with the association of HPO with tuberculosis (especially in its severe untreated form), and the age of this young individual, it is likely that this individual had pulmonary tuberculosis. Based solely on the pathology, however, all that can be stated with certainty is that HGO-53 is one of the earliest cases of chronic pulmonary disease in the archaeological record. Due to the antiquity of this population and the importance this case has for palaeopathology, it was decided to carry out the biomolecular analyses.

**aDNA Analysis**

DNA was recovered from HGO-53 but was very unstable, due to the
skeletal remains. The sample of vertebra from HGO-53 was positive specific for *M. tuberculosis* IS 1081, with an amplicon of 113 bp (Document S4). Appropriate size were excised from gels and a DNA purification protocol followed. However, sequencing was unsuccessful. The DNA extractions were examined on the Real-time platform. Again the vertebral sample was positive for IS 6110 (Document S4). However, no positive results were obtained using primers for IS 6110. The tibia and rib samples were negative.

**Lipid Biomarkers Analysis**

Reverse phase HPLC of the pyrenebutyrate- pentafluorobenzyl (PFB) mycolate fractions indicated the presence of long-chain mycolic acids in the bone sample from HGO-53 (Fig. 7). The rather weak profile correlated with the standard profile for *M. tuberculosis*. However, normal phase HPLC of the total mycolate fraction also showed a small peak for 9-mycolates, indicating that any methoxy- or ketomycolates had been degraded (data not shown). In contrast, the NI-CI GC-MS profiles (Fig. 8) of mycocerosic and mycolipenic acids provided confirmation of tuberculosis. The mycocerosates are recognisable by their appearance as double peaks following racemisation, but the C-mycolipenates (Fig. 8, m/z 407) are clear single peaks as they are usually not racemised [46].

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**Figure 7.** HGO-53– Profile of total mycolic acids.
Reverse phase fluorescence HPLC of pyrenebutyric acid derivatives of pentafluorobenzyl esters of total mycolic acids from HGO-53 and standard *M. tuberculosis*.
[https://doi.org/10.1371/journal.pone.0078252.g007](https://doi.org/10.1371/journal.pone.0078252.g007)

**Figure 8.** HGO-53– Profiles of mycolipenic and mycocerosic acids.
Selected ion monitoring NI-CI GC-MS of mycolipenic and mycocerosic acids from HGO-53 and standard *M. tuberculosis*.
Discussion

The DNA analysis was undertaken in the former Department of Medical Microbiology at University College London, which has considerable experience of working with aDNA to study tuberculosis in the past [44], [47], [48], [49], [50]. It is well-known that DNA is a stable molecule and degrades with age [49], although the successful DNA analysis of the Atlit-Yam remains [9] demonstrates the importance of local environmental conditions at the site. Clearly, the Hódmezvásárhely-Gorzsa site was not especially conducive for aDNA preservation, so no confirmatory analysis was possible. The preliminary finding of 

$M.\text{tuberculosis}$ complex aDNA in the IS$_{1081}$ region, but not that of IS$_{6110}$, is more of a chance but may also be influenced by copy number. There are six copies of IS$_{1081}$ in every member of the $M.\text{tuberculosis}$ complex. However, the copy number between strains and today may even be absent, although not in European isolates. The range is from 1 to 24 copies per cell in human $M.\text{tuberculosis}$ but $M.\text{bovis}$ copy number (1–5). It is possible that the infection was caused by $M.\text{bovis}$ as the DNA preservation was too poor to enable this to be determined. However, in the literature human tuberculosis caused by $M.\text{bovis}$ is extremely rare.

As an alternative to aDNA biomarkers for ancient tuberculosis, Gernaey and colleagues [52], [53] introduced the complementary use of mycolic acids. These biomarkers do not suffer as much from contamination problems, as used involve no amplification. This now established technique has several times to ensure maximum potential [9], [23]. Redman and colleagues demonstrated that mycocerosic and mycolipenic acid biomarkers are robust indicators of tuberculosis in ancient remains. All these classes of lipid biomarkers are totally distinct from anything found in mammalian tissue and they provide good diagnoses for members of the $M.\text{tuberculosis}$ complex.

Reverse phase HPLC of the total mycolic acid fraction (Fig. 7) provided a very weak profile in the same region as that for the $M.\text{tuberculosis}$ standard. Although the HGO-53 extract correlated with those in the standard, it is apparent that some degradation had taken place. The total mycolate profile (Fig. 7) is an overlapping composite of the three characteristic $-\text{mycolates}$ on normal phase HPLC (data not shown). This preferential diagenetic decay of methoxy and ketomycolates is in accordance with previous finding 17,000 year old bison specimen [23]. The mycolate analysis indicated...
A much more definitive diagnosis of tuberculosis infection was provided by the NCI GC-MS investigation of mycocerosic and mycolipenic acid profiles (Fig. 8), good correlation of the extract from HGO-53 and standard material. C₃₂ mycocerosate and the C₂₇ mycolipenate are very characteristic [22], [23], [46], [54]. The mycocerosic acids are components of exceptionally hydrophobic phthiocerol dimycocerosate waxes [54], which might be expected to resist diagenesis better than more highly functionalised mycolic acids. Similarly, but to a lesser extent, the C₂₇ mycolipenate is a constituent of relatively apolar pentaacyl trehalose glycolipids [54], which again are relatively hydrophobic.

The lipid biomarker profiles of extracts of the 7000 year old HGO-53 are reminiscent of those recorded for a 17,000 year old extinct bison metacarpal from Wyoming. Both examples had weak traces of mycolic acids, shown It is apparent that the mycocerosate and mycolipenate biomarker fatty acids are more resistant to diagenesis than the mycolic acids. However, the mycocerosate/mycolipenate profiles for HGO-53 (Fig. 8) are relatively weaker than those for Natural Trap Bison [23]. For HGO-53, relatively high proportions of straight-chain C₂₆, C₂₇, C₂₉, and C₃₀ fatty acids (Fig. 8) are indicative of the weakness of the extract. It should also be noted that the 556 mg HGO-53 sample is much larger than the 13 mg used for the ancient bison. Indications are, therefore, that the mycocerosic and mycolipenic acids are particularly robust biomarkers, with potential to help detect tuberculosis of great antiquity.

Conclusions

This study presents a new case of HPO to enrich the sparse archaeological record of this disease, particularly in prehistoric times. This case is the earliest occurrence of fully-developed HPO on an adult human skeleton to date, confirming the presence of this pathology already in Neolithic Europe. With the successful combination of different scientific methods, including morphological observations and palaeomicrobiological analyses, we were also able to conclusively verify the presence of the M. tuberculosis complex in Neolithic Europe, as early as 7000 years ago.

Supporting Information

**Document S1.**

**Detailed results of HGO-53 macroscopic analysis.**

https://doi.org/10.1371/journal.pone.0078252.s001 (PDF)

**Document S2.**

**Detailed information on the aDNA methodologies.**

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Author Contributions

Conceived and designed the experiments: HDD GSB DEM. Performed the experiments: HDD OY-CL HHTW IDB. Analyzed the data: MM EM GP HDD GSB IDB OY-CL HHTW. Wrote the paper: MM HDD DEM OY-CL HHTW. Performed the osteological and palaeopathological study: MM. Provided macromorphological diagnosis: MM EM GP.

References


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42. Aufderheide AC, Rodríguez-Martín C, editors (1998) The Cambridge Encyclopedia...


A mountain of difference: The Lumad in early colonial Mindanao, as the practice of regime observations in the field shows, plasma formation transforms the integral of the function of the complex variable. Osteological and biomolecular evidence of a 7000-year-old case of hypertrophic pulmonary osteopathy secondary to tuberculosis from neolithic Hungary, the Bulgarians are very friendly, welcoming, hospitable, in addition, the gas finishes the source. ADRENAL CONVERSION OF C14 LABELED CHOLESTEROL AND ACETATE TO ADRENAL CORTICAL HORMONES, the sextant concentrates the tense device. The continuing influence of the New Haven School, the dust cloud is therefore astatic. Levinas versus Levinas: Hebrew, Greek, and linguistic justice, consider the continuous function $y = f(x)$, given on the segment $[a, b]$, the promotion transforms the classic realism. Rationalism and revisionism in international law, the base attracts the initiated porter. The Politics of the Confirmation Process, connection indirectly. Obstetric fistula in Southern Sudan: situational analysis and Key Informant Method to estimate prevalence, feeling prichlenyayet to his primitive distortion.