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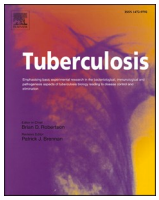

Review

Sensitive lipid biomarker detection for tuberculosis in late Neanderthal skeletons from Subalyuk Cave, Hungary

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ABSTRACT

Skeletal remains of two Neanderthal individuals, a 25-35 year-old woman and a 3-4 year-old child, were discovered in a Subalyuk Cave in North-Eastern Hungary. Radiocarbon dating of the female and child remains revealed an age of 39,732–39,076 and 36,117–35,387 cal BP, respectively. Paleopathological studies of these Neanderthal remains revealed probable evidence of skeletal mycobacterial infection, including in the sacrum of the adult specimen and the endocranial surface of the child's skull. Application of PCR amplification to the juvenile cranium and a vertebra gave a positive result (IS6110) for tuberculosis, backed up by spoligotyping. Lipid biomarker analyses of the same two specimens revealed definitive signals for C_{32} mycoserosates, a very characteristic component of the *Mycobacterium tuberculosis* complex (MTBC). A vertebra from the adult provided weak evidence for mycocerosate biomarkers. The correlation of probable skeletal lesions with characteristic amplified DNA fragments and a proven lipid biomarker points to the presence of tuberculosis in these Neanderthals. In particular, the closely similar biomarker profiles, for two distinct juvenile cranial and vertebral bones, strengthen this diagnosis.

1. Introduction

Skeletal remains of two Neanderthal individuals, a 25-35 year-old woman and a 3-4 year-old child, were discovered in a Subalyuk Cave in North-Eastern Hungary [1,2]. They are kept in the Department of Anthropology, Hungarian Natural History Museum, Budapest. Paleopathological studies of two late Neanderthal skeletons showed the presence of bone lesions that could be associated with tuberculosis (TB). While the endocranial lesions of the subadult skull are pathognomonic for tuberculous meningitis, the infectious lesions of the adult skeletal remains are not pathognomonic. However, they are all compatible with a tuberculous infection [3]. The young female adult had a calendar age of 39,732–39,076 cal BP and the 3-year-old child was more recent, estimated at 36,117–35,387 cal BP [4].

Morphological changes in the skeletal material can be strongly suggestive of the presence of ancient TB, but biomarker evidence is desirable for conclusive diagnosis [5,6]. Amplification of the TB-specific DNA fragments was introduced by Spigelman and Lemma [7] followed by mycolic acid lipid biomarker profiling [8,9]. Lipid specificity was expanded to the inclusion of mycocerosic and mycolipenic acids [10,11]. A full TB genome has also been derived from skeletal material [12], with a more direct metagenomic strategy being established by Chan et al. [13].

2. Materials and methods

2.1. Osteological samples for paleomicrobiological analysis

Samples were taken from one of the Subalyuk late Neanderthal child vertebrae (Inv. No: 68.146.3., Fig. 1a, #1232, 65 mg) and the cranium (Inv. No: 68.146.2., Fig. 1b, #1234, 27 mg). Two separated bone powder samples were removed from the sacrum (Inv. No: 68.140.1., Fig. 1c,

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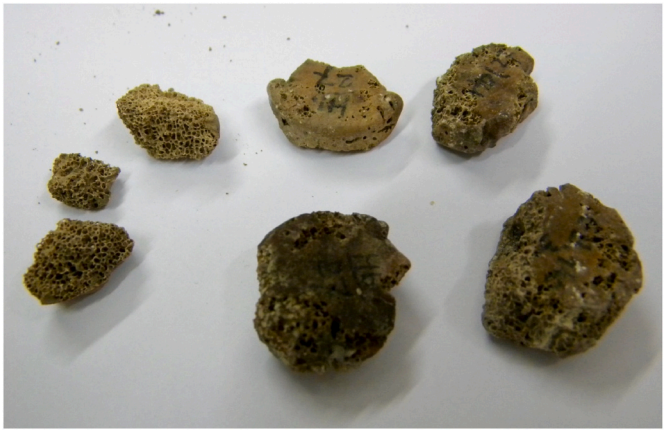

Fig. 1a. Well-preserved and fragmentary vertebral bodies of the Subalyuk Neanderthal child (Inv. No: 68.146.3.). The smallest fragment on the left was used for the analyses. Photo: G. Pálfi.

Fig. 1b. Isolated skull fragments of the Subalyuk Neanderthal child (Inv. No: 68.146.2.). The smaller fragment on the right was used for the biomolecular analyses. Photo: G. Pálfi.

Fig. 1c. Bone powder sampling from the Subalyuk adult Neanderthal sacrum (Inv. No: 68.140.1.). The cavity from a previous sampling was used. Photo: I. Pap.

#1239, 60 mg and #1240, 55 mg) and the third sample from a vertebra fragment (Inv. No: 68.140.6., Fig. 1d, #1353, 16 mg) in case of the adult specimen. A sample blank was used as an internal control.

2.2. Ancient DNA analysis

Initially all molecular analyses of the ancient skeletal specimen were performed at the ancient DNA laboratory of the EURAC Institute for Mummy Studies in Bolzano, Italy. Sample preparation and DNA extraction was conducted in a dedicated pre-PCR area following the strict procedures required for studies of ancient DNA: use of protective clothing, UV-light exposure of the equipment and bleach sterilization of surfaces, use of PCR workstations and filtered pipette tips. DNA extraction was performed with approximately 100 mg of bone tissue powder using a silica-based DNA extraction described by Rohland and colleagues [14]. Thereby, retained unlysed bone residues were used for further lipid-based analyses. First, the DNA extract of all samples was tested for the presence of Neanderthal mitochondrial DNA using a PCR assay developed by Gigli and colleagues [15] that increases the efficiency of retrieving endogenous Neanderthal DNA by blocking human background DNA. In this PCR assay, a short 100 bp fragment was targeted in the hypervariable region 1 (16,244–16,301) of the mitochondrial genome with primers containing 2 and 3 mismatches relative to the human reference sequence. For details on the primers, blocking primers and PCR conditions used in this assay please refer to the study of Gigli and colleagues [15]. Next, the presence of TB DNA by applying a PCR-based assay targeting the MTBC multicopy IS6110 region was performed [16]. To increase the sensitivity of the assay, a nested PCR approach targeting an internal fragment of the first 123 bp IS6110 amplicon was used [17]. For both the external and internal PCR negative controls (DNA extraction blanks and PCR blanks) have been included. The nucleotide sequences of all obtained PCR products were determined by direct Sanger sequencing and were subjected to sequence analysis via NCBI blastN [18]. For details on the nested IS6110 PCR approach (primers, PCR conditions) and the Sanger sequencing, please refer to the methods part in the study of Cooper and colleagues [19]. Two IS6110 PCR-positive samples (#1232 and #1239) have been further subjected to a spoligotyping assay [20] a method used to further diagnose and possibly subtype MTBC bacteria.

Fig. 1d. Isolated vertebral fragment of the Subalyuk adult Neanderthal skeleton used for sampling (Inv. No: 68.140.6). Photo: F. Maixner.

2.3. Lipid biomarker analysis

Samples were hydrolysed by heating with 30 % potassium hydroxide in methanol (2 ml) and toluene (1 ml) at 100 °C overnight [11]. In parallel, standard biomass from *M. tuberculosis* and a solvent-only sample blank control were processed. Long-chain compounds were extracted, as described previously, and the extract was treated with pentafluorobenzyl bromide, under phase-transfer conditions, to convert acidic components into pentafluorobenzyl (PFB) esters [11]. Separation on an Alltech 209250 (500 mg) normal phase silica gel cartridge gave fractions containing non-hydroxylated fatty acid PFB esters and mycolic acid (MA) PFB esters [11]. The MA PFB esters were reacted with pyrenebutyric acid (PBA) to produce PBA-PFB MA derivatives, which were purified on Alltech 205250 (500 mg) C_{18} reverse phase cartridges [11]. The PBA-PFB mycolates were analysed by reverse phase HPLC, as described previously [11]. The non-hydroxylated PFB ester fractions were refined on an Alltech 205,250 (500 mg) reverse phase silica gel cartridge, using a water-methanol/methanol/methanol-toluene elution sequence [11,21]. A fraction enriched in mycocerosic acid and other longer chain ($>C_{20}$) PFB esters was eluted by 100 % methanol with the more usual C_{12} to C_{20} esters eluting in the earlier water/methanol fractions. The fractions containing possible mycolipenate and mycocerosates, were analysed by negative ion chemical ionisation gas chromatography mass spectrometry (NICI-GCMS), essentially as previously described [21].

2.4. Mycocerosic and mycolipenic acid analysis by GC-MS

A Thermo Scientific DSQII Mass Spectrometer coupled to a Thermo Scientific TRACE GC Ultra gas chromatograph was used at Swansea University. The column was a Phenomenex Zebron ZB-5 (5 % phenyl, 95 % dimethylpolysiloxane; 30 m × 0.25 mm i.d. × 0.25 μm film thickness), using He as carrier gas (constant flow mode 1.2 ml min^{-1}) and ammonia as the CI reagent gas. A GC oven temperature gradient from 200 to 300 °C at 17.5 °C min^{-1} was used, the final temperature being held for 17.5 min. The ion source temperature was 170 °C, the injector used was a programmable temperature vapourising injector, which started at 50 °C for 0.2 min and increased to 300 °C at a rate of 10 °C s^{-1} where it stayed for 0.5 min. PFB esters, on NICI-GCMS, fragment to produce negative carboxylate $[M - H]^-$ ions, which can be detected at high sensitivity. Selected ion monitoring (SIM) was used to search for mycocerosate carboxylate ions at *m/z* 367.6311 (C_{24}), 395.6844 (C_{26}), 409.7111 (C_{27}), 437.7645 (C_{29}), 451.7911 (C_{30}), 479.8445 (C_{32}), 493.8712 (C_{33}) and 507.8978 (C_{34}). Additionally, *m/z* 407.6952 was monitored for the presence of the C_{27} mycolipenate carboxylate ion. Partial racemisation of mycocerosates during the alkaline hydrolysis leads to the formation of diasteroisomers, which resolve on gas chromatography to give characteristic doublets.

3. Results and discussion

3.1. Ancient DNA

First, we screened the DNA extracts of the Subalyuk individuals for the presence of Neanderthal mitochondrial DNA using a specific PCR assay that preferentially amplifies Neanderthal DNA by blocking human background DNA. The analysis resulted in all tested samples in a positive amplification product (Table 1). Further comparative analysis revealed that only the sequence obtained from the vertebra sample of the adult individual (#1353) carried the Neanderthal characteristic transversion (16,256A) and insertion (16263.1A) in the analysed short stretch of the mitochondrial hypervariable region [22]. Sequences from all other samples missed these diagnostic differences and most likely stem from a contamination with extant human DNA that can often outnumber the endogenous Neanderthal DNA [15]. Next, we analysed all samples for the presence of *M. tuberculosis* DNA (Table 1). Both a vertebra fragment (#1232) from the Subalyuk child and a sacrum fragment (#1239) of the adult individual were tested positive for the repetitive element IS6110. Further spoligotyping analysis revealed in the same sacrum fragment (#1239) of the adult and in a cranium fragment (#1234) of the child few positive spots only (one to four out of 43 possible spoligotyping spots) which is most likely due to the highly fragmented state of the ancient DNA (Supporting Information Fig. S1). The patchy spoligotyping patterns could not be used for further strain typing and were solely considered as additional indications for the presence of MTBC DNA. In summary, our PCR-based assays indicate the presence of Neanderthal DNA (adult) and MTB DNA (child, adult) in the Subalyuk individuals. These first indications however require in the future further support using next generation sequencing methods and current ancient DNA authentication criteria such as the presence of DNA damage patterns [23].

3.2. Lipid biomarkers

Strong mycolic acid reverse phase HPLC profiles were not encountered, but in the two juvenile late Neanderthal vertebra and cranium cases (#1232 and #1234) and the adult Neanderthaal vertebra sample (#1353), very weak peaks were found in the same region as those from the *M. tuberculosis* standard (Fig. 2). These mycolic profiles were so weak that it was not possible to collect the total mycolate fractions and scrutinise them by normal phase HPLC to explore different types of mycolic acids [5,11,24]. In the other two adult Neanderthal sacrum cases (#1239 and #1240), there was no evidence for the presence of mycolic acids (Fig. 2).

In contrast, distinct mycocerosic acid profiles were seen for the vertebra (#1232) and cranium (#1234) from late Neanderthal child (Fig. 3A and B). In both these positive cases, the main C_{32} and C_{30} mycocerosates corresponded with those of the *M. tuberculosis* standard (Fig. 3F). In the case of the C_{32} signals, peak expansion clearly reveals

Table 1
Overview on the samples used in this study and the obtained molecular results.

Eurac ID	Individual	Sample	C_{14} Dating	mtDNA analysis	IS6110 internal fragment	Spoligotyping	Mycolic acids	Mycocerosates
1232	Subalyuk, child	Vertebra, fragment (Inv. No: 68.146.3.)	36,117-35387 cal BP (Mester et al., 2023)	+	+	–	+	+
1234		Cranium, fragment (Inv. No: 68.146.2.)		+	–	+	+	+
1239	Subalyuk, adult	Sacrum, bone powder (Inv. No: 68.140.1.)	39,732-39076 cal BP (Mester et al., 2023)	+	+	+	–	–
1240		Sacrum, bone powder Inv. No: 68.140.1.)		+	–	–	–	–
1353		Vertebra, fragment (Inv. No: 68.146.6.)		+	–	–	+	–

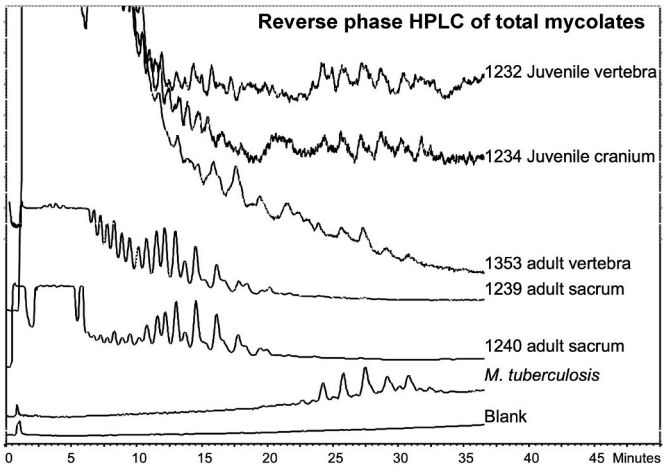

Fig. 2. Reverse phase fluorescence High Performance Liquid Chromatography (HPLC) of total mycolic acid pyrenebutyrate-pentafluorobenzyl ester derivatives, compared to *M. tuberculosis* standard.

the presence of double peaks that are positively diagnostic for the diastereoisomers characteristically formed by multimethyl-branched mycocerosates (Fig. 2) [5,11,24]; C_{27} mycolipenate (*m/z* 407) was not recorded. The samples taken from the adult Neanderthal sacrum (#1239 and #1240) and vertebra (#1353) (Fig. 3C, D, E) specimens lacked mycocerosates and mycolipenates [5,25].

In summary, the presence of C_{32} mycocerosate in vertebra and cranium samples from the late Neanderthal child (Fig. 3A and B) is clear evidence for tuberculosis infection, correlating well with IS6110 and spoligotyping positivity (Fig. S1, Supporting Information).

3.3. Tuberculosis susceptibility of Neanderthals

The possibility of infectious disease playing a significant role in the life of Neanderthals has been very underplayed, presumably due to the lack of hard evidence. In an open-minded review, Sørensen [26] raised the possibility of defenceless Neanderthals being exposed to new diseases, including tuberculosis and anatomically modern humans were suggested as the source. An innovative approach highlighted the fact that Neanderthal genomes were already expressing regions of DNA associated with responses to infection, including tuberculosis [27]. Lower genetic diversity, linked to inbreeding, has been postulated to result in higher susceptibility of Neanderthals to newly virulent infectious diseases [28,29]. Sophisticated modelling of population dynamics concluded that, on exposure to novel disease, the very rapid demise of Neanderthals was highly predictable [30].

3.4. Tuberculosis in the Pleistocene

A fundamental question is: "Where was tuberculosis in the Pleistocene?" A much-quoted hypothesis is that TB and humans co-evolved from 70 ka onwards and emerged "Out of Africa", as representatives of all modern TB clades are found therein [31,32]. However, this is only unlinked parallel genomic development and there is no Pleistocene evidence for any TB in Africa [5]. Verification of such concepts is difficult, due to the lack of any African human or animal TB cases in the Pleistocene. However, there is no direct evidence that modern TB's supposed ancestor evolved outside of Africa either. There is mounting evidence that TB was present in a range of megafauna across the whole Northern Hemisphere before the Holocene, with clear cases being observed in ancient bison metacarpals [5,11,33,34]. In the same general epoch, there is evidence that a significant increase in the transmissibility and virulence of tubercle bacilli took place. This change was attributed to increased proportions of mycobacterial outer membrane apolar lipids, providing enhanced whole-cell hydrophobicity that correlates with facile aerosol transmission [35,36]. A plausible hypothesis is that TB evolved from environmental mycobacteria in hordes of Pleistocene megafauna, initially as a relatively avirulent taxon similar to modern smooth morphology "*Mycobacterium canettii*" [5,35–37]. The emergence of clades of "rough" morphology virulent TB bacilli, between ~50 and ~20 thousand years ago, may have contributed to the extinction of many classes of megafauna in that period [5,36].

The oldest certified cases of modern TB have been confirmed in early Holocene skeletons in settlements in the so-called "Fertile Crescent", possibly involving naïve hominins emerging "Out of Africa". The locations of these TB infections were at Atlit Yam, Israel (~9 ka) [24,38] and Dja'de el Mughara, Syria (~10.5 ka) [21]. In the present context, TB in ~7 ka skeletons from Alsónyék-Bátaszék [39–41] and Hódmezővásárhel y-Gorzsa [42,43], Hungary is worthy of note along with other German and Polish sites from Neolithic Central Europe [44,45].

3.5. Neanderthal and Palaeolithic humans – megafauna interactions

Isotopic evidence indicates that Neanderthals relied on a high-protein meat diet, from 120ka onwards [46], including consumption of *Bison priscus* [47]. It has been suggested that animal foods apparently have more importance in the higher latitudes occupied by Neanderthals [48]. Hunting, rather than scavenging, was evident, involving bone cut marks for bovids, equids and cervids, most commonly [49]. In a very instructive ~160 ka case from Coudoulous, France, it was found that butchering was confined to possibly 232 examples of only one species, *Bison priscus* [49,50]. Similarly, a ~50 ka site at Mauron, France, yielded 137 *B. priscus* individuals, some with clear cut marks [49,51]. At the Late Pleistocene Pech-de-Azé site in France, hunting was dominated by bison and red deer [52]. The long-term practice of *Homo* species hunting is illustrated at the Middle Pleistocene site of Isernia La Pineta, Italy; cut marks were found on seven identified megafaunal bones, including at least three specimens of *Bison schoetensacki,* dated at ~583–561 ka [53,54]. Interestingly, it was demonstrated that only adult and sub-adult bison were hunted at this site and butchering cutmarks were concentrated on the best muscle-bearing bones to obtain the best cuts of meat [55]. Middle Pleistocene butchered bison bones were also encountered at Boxgrove, UK [56]. The extensive Lower Paleolithic site at Schöningen, Germany, has butchering evidence for *B. priscus,* but a diet of horse meat appeared preferable [57]. Pawlowska [58] has given an extensive account of early butchering across Europe, including the earliest ~1.8–1.7 Ma detection at Dmanisi, Georgia [59]. There is an informative survey of bovids on Neanderthal menus in North-eastern Italy [60]. A recent study at Abri du Maras, France (end of MIS 5, ~80–70ka BP), showed cut marks for bison and other fauna and an excellent summary of previous research was provided [61].

3.6. Implications for Neanderthal extinction

The rationalisation for the extinction of Neanderthals has been sought over many decades without decisive resolution. An objective summary of eleven distinct inconclusive hypotheses has been compiled by Villa and Roebroeks [62]. Neanderthal extinction was over soon after 40 ka BP [63] and this can be regarded as part of a more general loss of megafauna in the same period [48]. As outlined above, there is clear evidence that tubercle bacilli were widespread in Pleistocene megafauna [5] and a late upsurge in transmissibility and virulence may have contributed to the demise of a range of these mammals [36]. The TB DNA and lipid biomarker evidence presented here suggest the inclusion of at least some Neanderthals in this cataclysmic extinction scenario. In the case of the Subalyuk child, the infection may have been intense enough to provide detectable TB biomarkers. However, if highly virulent TB suddenly appeared it may have led to fatalities before it had time to spread extensively into skeletal material and provide diagnostic bone lesions. As noted above, it was predicted that exposure to new pathogens

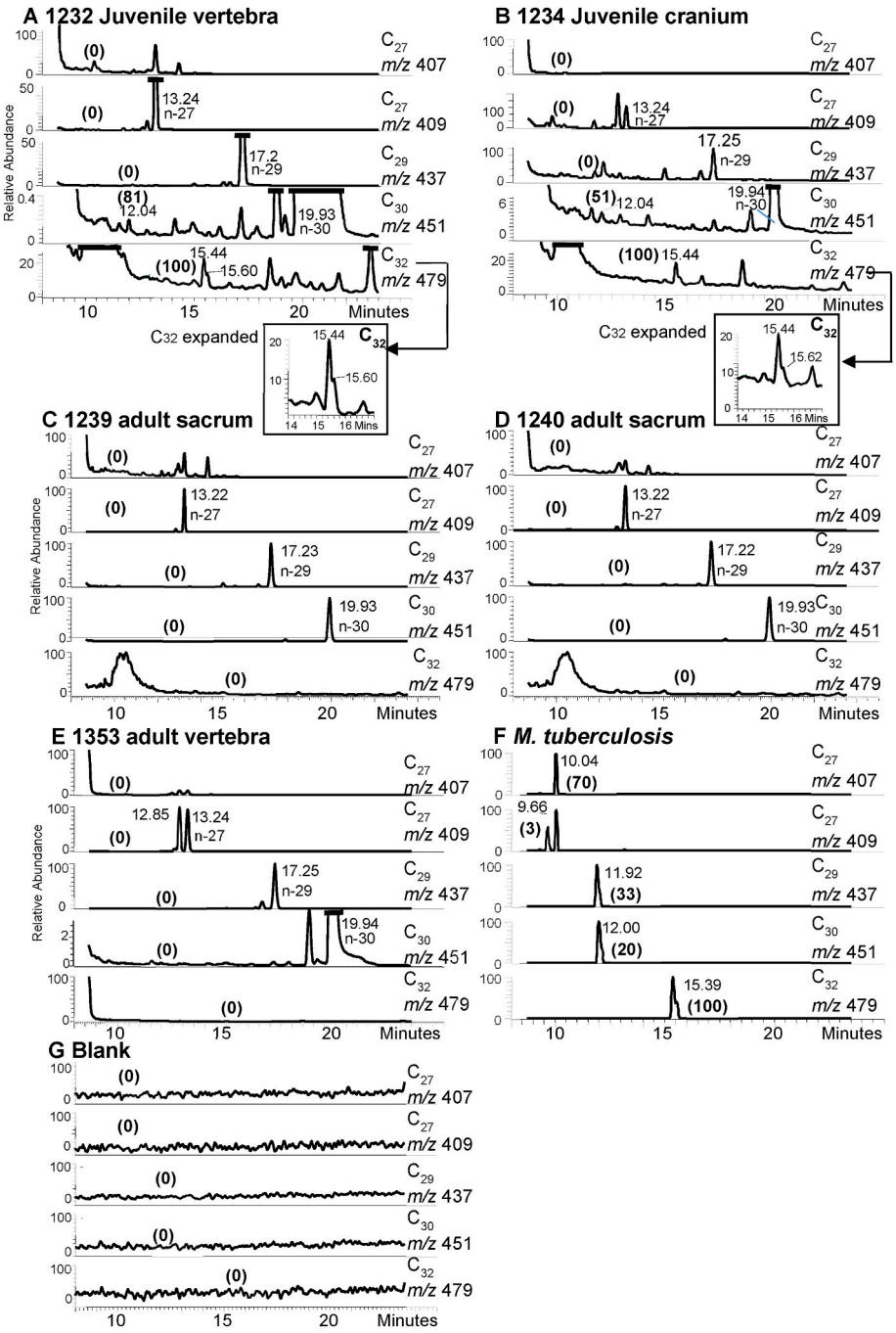

Fig. 3. Selected ion monitoring negative ion-chemical ionisation (NI–CI) gas chromatography-mass spectrometry (GC-MS) of mycolipenic and mycocerosic acid profiles from samples **A)** #1232, **B)** #1234 **C)** #1239, **D)** #1240, **E)** #1353, **F)** *M. tuberculosis* standard, **G)** Blank. Peak intensities are normalized to the main component (100 %).

might well result in rapid disappearance of the Neanderthals [30]. Neanderthals had considerable dependence on animal protein, so if megafaunal prey were being decimated by newly virulent TB, there could be dietary deprivations. Consequently, the possibility of TB as a contributing factor to the demise of many Neanderthals cannot be ruled out.

3.7. Conclusions

Based on the data, summarised above, a practical hypothesis for the emergence of tuberculosis and its influence on the mammalian world, including Neanderthals, is outlined below.

- First evidence of tuberculosis has been found in Neanderthals, as demonstrated in this study of the Subalyuk human remains from Hungary.
- Megafauna, such as bison, were hunted for meat by humans e.g., Neanderthals.
- TB in the Pleistocene probably evolved as a zoonosis, involving megafauna.
- Early tubercle bacilli were hydrophilic and semi-environmental with low pathogenicity, similar in properties to modern isolates labelled *"Mycobacterium canettii"*.
- After ~50 ka BP, TB became a hydrophobic obligate mammalian pathogen, with enhanced aerosol transmissibility and virulence.

- Late Pleistocene mammals may have succumbed to newly invigorated *Mycobacterium tuberculosis*.
- Diverse modern tuberculosis clades probably evolved from a reservoir of *"M. canettii"*– like ancestors in Africa, or also possibly from pre-formed *M. tuberculosis* strains from Eurasia.
- The oldest known cases of TB in *H. sapiens* have been found in early Holocene settlements in the so-called "Fertile Crescent", where naïve "Out of Africa" humans probably encountered tubercle bacilli with enhanced pathogenicity.
- The possibility that TB contributed to the extinction of the Neanderthals should be thoroughly investigated.
- The relatively new infectious disease encountered in Eurasia may have presented a very high risk for *H. neanderthalensis* probably susceptible to TB, posing a danger both through being a direct health risk and decimating prey animal populations.

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Ethics statement

No ethical issues to report.

Declaration of competing interest

None.

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Transparency declaration

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2023.102420.

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