Molecular Biomarkers for Ancient Tuberculosis

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1. Introduction

Tuberculosis is an ancient disease. It was recognised and described by Hippocratus (460–390 BCE) and Galen (2nd–3rd century CE) in the western Classical World (Xarchus & Bourandas, 2003), ancient Egypt, India and the Far East (Morse, 1961). The obvious symptoms that attracted attention were the late outcomes of skeletal tuberculosis, where collapsed vertebrae led to scoliosis and Pott's disease, plus the symptoms associated with pulmonary tuberculosis, such as fever, weight loss and haemoptysis (coughing up blood). In the UK, tubercular lesions of the lymph glands (cervical lymphadenitis) were formerly termed scrofula, or the King's evil, and tubercular skin lesions were described as Lupus vulgaris or tuberculous chancre. The palaeopathology of ancient skeletal remains, together with classical and historical reports, demonstrate that tuberculosis occurred in prehistory. However, tuberculosis is still the greatest cause of death from any single infectious disease in the world today, with over one third of the global population infected and an estimated 1.7 million deaths from the disease in 2009 (WHO, 2010). Therefore it is essential to understand the nature of tuberculosis in the past: its distribution, spread and relationship to human society.

The disease is caused by members of a group of very closely related bacteria, termed the *Mycobacterium tuberculosis* complex (MTBC). These are obligate parasites and have the ability to subvert the cell-mediated immune system of the host and to survive and multiply within macrophages. Most human infections are caused by *Mycobacterium tuberculosis* and are usually acquired via the aerosol route from an active case of pulmonary tuberculosis. Infectious aerosols lodge in the alveoli but, in the majority of cases, the bacilli are controlled by the host immune system to form a granuloma and the disease remains latent. Infection can also occur by ingestion – milk or meat from an infected animal can give rise to human zoonotic cases of tuberculosis caused by *Mycobacterium bovis* or other members of the MTBC. In endemic areas, infection takes place in early life and may remain latent throughout a lifetime or become re-activated due to lowered host resistance caused by physical or mental stress, immunosuppression or extreme age (Rustad et al., 2009). Active primary tuberculosis, estimated to occur in 2–5% of cases, normally causes lymphadenitis and subsequent spread via the blood stream can cause meningitis or miliary tuberculosis (Grange & Zumla, 2009). Post-primary tuberculosis is estimated to occur in a similar proportion of people and these

individuals in ancient and historical times would be the recognisable cases of skeletal tuberculosis. Therefore, it is highly significant that these historical cases, diagnosed by skeletal pathology, represent only around 5% of the total number of individuals with the disease.

Because of their very slow growth-rate and clinical significance, the MTBC was one of the first groups of microorganisms to benefit from the introduction of the polymerase chain reaction (PCR) and molecular diagnostics. This led to a an understanding of the epidemiology of tuberculosis (Reed et al., 2009; Smith et al., 2006), the evolution of the MTBC (Brosch et al., 2002; Ernst et al., 2007; Gordon et al., 2009; Gutierrez et al., 2005) and to the realisation that particular lineages of *M. tuberculosis* are associated with the country of origin of their human hosts (Hershberg et al., 2008; Hirsh et al. 2004; Wirth et al., 2008). Total sequencing of the genomes of *M. tuberculosis* (Cole et al., 1998), *M. bovis* (Garnier et al., 2003) and attenuated *M. bovis* Bacille Calmette-Guérin (BCG) vaccine strains (Pan et al., 2011; Seki et al., 2009) has elucidated the relationship between MTBC strain, lineage and pathogenicity. We now understand that the MTBC represents a clonal expansion of pathogenic strains or ecotypes (Smith et al., 2006) each of which is associated with a parallel clonal expansion of their mammalian hosts (Maiden, 2009).

The MTBC is distinct from the large number of environmental mycobacteria, which are generally non-virulent or cause opportunist infections in hosts, especially those with increased susceptibility. A characteristic feature of the mycobacteria is their cell envelope, which contains a high proportion of lipid-rich molecules, such as mycolic acids and phthiocerol dimycocerosate waxes (Minnikin, 1982; Minnikin et al., 2002). These result in a hydrophobic bacterial cell wall with decreased permeability and susceptibility to degradation, that may partially explain the very slow growth rate of the MTBC and persistence of viable organisms after the death of the host (Sterling et al., 2000; Weed & Baggenstoss, 1951). The mycobacteria are members of a taxonomic clade typified by organisms with a high percentage of guanidine and cytosine residues in their DNA. It is believed that the DNA of GC-rich bacteria is structurally more stable than that of other microbes because of the additional hydrogen bond cross-links between the DNA strands.

The wealth of information on the genomics of *M. tuberculosis* strains present in the world today, coupled with our understanding of the co-evolution of *M. tuberculosis* with its human host, has attracted interest in determining the origins and timescale of this relationship. Relevant information can be obtained from archaeology, anthropology and palaeopathology, which provide details on past human populations, societies and the occurrence of infectious diseases. The relative robustness of *M. tuberculosis* biomarkers enables the well-established molecular methods used in diagnostic clinical microbiology to be applied, with appropriate modification, to the study of historical and archaeological remains. Originally the emphasis was on the detection and characterisation of *M. tuberculosis* ancient DNA (aDNA), as this enables the evolution of this group of pathogenic bacteria to be directly investigated by the detection and characterisation of their DNA (Fletcher et al., 2003a, 2000b; Matheson *et al.* 2009; Zink et al., 2001). However, it was soon appreciated that the unique lipid biomarkers found in the MTBC, in addition to enabling the independent verification of aDNA studies (Donoghue et al., 1998, 2010a; Gernaey et al., 2001; Hershkovitz *et al.,* 2008), have the potential to illuminate deep into human prehistory due to their particular stability (Gernaey & Minnikin, 2000; Redman *et al.,* 2009).

2. Ancient DNA (aDNA) from the *M. tuberculosis* complex (MTBC): Background and basics

2.1 DNA degradation and persistence

Within living cells, DNA is subjected to enzymatic repair processes, but this ceases after death. Thereafter, host DNA is rapidly degraded by enzymes derived both from the host and the macro and microbial flora that form part of the natural decay process (Pääbo et al., 2004). As a result of the cumulative changes over time (diagenesis) ancient DNA may develop hydrolytic and oxidative lesions. The breakdown of the N-glycosyl bond between the sugar and the base, in the presence of water, leads to hydrolytic cleavage and DNA fragmentation. Hydrolytic depurination causes a preferential loss of guanine and adenine, whereas the pyrimidines cytosine and thymine are 40-fold more susceptible to hydrolytic deaminisation (O'Rourke et al., 2000). Oxidative damage, especially to pyrimidines, can result in the formation of substances such as hydantoins, that block extension during PCR (Höss et al., 1996). DNA strands may also become chemically cross-linked due to Maillard products (Poinar et al., 1998), formed by condensation reactions between sugars and primary amino-groups in proteins and nucleic acids (Pääbo et al., 2004). Local environmental conditions have a strong impact on the persistence of aDNA, such as the temperature, the pH at the site, the availability of water and oxygen and fluctuations of all these factors over time (Poinar, 2003). Indeed, these factors outweigh the impact of the chronological age of samples. For example, a 20^0C decrease in temperature reduces base degradation 10- to 25-fold (Höss et al., 1996). Mycobacterial DNA is more robust than that of mammals, but its persistence depends not only upon the local environmental conditions but also the nature of the infection at the time of death of its host. Therefore, *M. tuberculosis* aDNA is often highly localized and DNA extraction protocols may have to be optimized for specimens from different sites.

2.2 Selection of specimens and sampling

In the early days of palaeomicrobiology, the criteria drawn up by researchers working on ancient mammalian DNA were recommended. These included preliminary screening tests, such as using the degree of amino acid racemization (Poinar & Stankiewicz, 1999) or collagen yield (Götherström et al., 2002), as an indication of the extent of DNA preservation. However, it appears that these are not reliable indicators, even of mammalian aDNA (Fernañdez et al, 2009; Kaestle & Horsburgh, 2002). More recently, Ottoni et al. (2009) discovered that there was better recovery of aDNA from animal bones that showed evidence of cooking, concluding that parameters based on protein diagenesis are not always useful for predicting ancient DNA survival. Work on other microbial pathogens, such as *Yersinia pestis* – the cause of bubonic plague – demonstrate that the dental pulp cavity in sound adult teeth is an excellent source of aDNA (Drancourt et al., 1998). It is believed that adsorption to hydroxyapatite increases the stability of aDNA (Götherström et al., 2002; Tuross, 1994) and any microorganisms present in the blood will potentially be present (Donoghue, 2008a).

For the examination of material for tuberculosis, the most common specimens available are bones. Most active cases of tuberculosis present as a lung disease, so ribs are a good source of *M. tuberculosis* DNA. Tuberculous lesions in the ribs arise by extension from spinal

lesions, from haematogenous spread from some remote soft tissue focus, or by direct spread from disease in the lungs, pleura, or chest wall lymphatic system (Mays et al., 2002). Initially, only bones with lesions were examined (Spigelman & Lemma, 1993), but it is now clear that in the majority of ancient cases of tuberculosis there are no lesions, but *M. tuberculosis* aDNA is present due to haematologous spread or by direct contact with infected tissue (Donoghue, 2011; Donoghue et al., 2011). Mummified tissue (Salo et al., 1994), skin (Faerman et al., 1997; Konomi et al., 2002), dental pulp (Faerman et al, 1997; Matheson et al, 2009) and calcified pleura (Donoghue et al., 1998) have also yielded MTBC aDNA.

Unfortunately, many published protocols based on human and animal aDNA research recommend that bones are pre-treated with bleach, ultraviolet light, or the outer bone surface is removed. The aim is thereby to remove surface contamination, but such procedures may inadvertently remove the very *M. tuberculosis* aDNA that is being sought (Donoghue, 2008a).

2.3 MTBC aDNA extraction and detection by conventional PCR

The amount of material examined by different investigators varies greatly (Donoghue et al., 2009), but the subsequent extraction procedures follow a similar pattern. Mineralized tissue is powdered and demineralized, enzymes are used to remove proteins, the samples are disaggregated with agents such as phenol-chloroform or guanidium thiocyanate, and DNA is captured by silica, on to filters or membranes, or simply precipitated with isopropanol. Due to the persistent mycobacterial cell wall, robust techniques such as bead beating, freeze-thaw cycles in liquid nitrogen, and incubation for longer time periods and at temperatures such as 56^0C are often used. The reagent N-phenacylthiazolium bromide may be used to overcome the problem of Maillard products and enable strand separation (Pääbo et al., 2004). Reagents used for DNA capture may need modification to allow for DNA fragments that are <200 base pairs (bp) in length. In poorly preserved samples the fragment length may be less than 100 bp. Experience has shown that aDNA is unstable in aqueous solution so extraction preparations are best stored as dried silica or precipitates and only re-constituted immediately prior to examination. Thereafter, the aDNA extracts should be aliquoted and stored at -80^0C, if possible, so the number of freeze-thaw cycles can be minimized.

DNA amplification by PCR enables targeted and specific recovery of informative genetic loci. Amplification of MTBC aDNA is usually based on MTBC-specific regions of repetitive sequences in the genome of all members of the complex, such as IS*6110* (Eisenach et al, 1990). The sensitivity of conventional PCR can be increased by further amplification of the amplified PCR product via a nested reaction (Taylor et al., 1996). The primers devised by these two primer sets give rise to amplicons of 123 bp and 92 bp, respectively. IS*6110* may have up to 24 copies/cell in *M. tuberculosis* (Tanaka et al., 2000), although a small percentage of strains have no copies at all and *M. bovis* strains have only a single copy per cell. The alternative specific PCR, based on IS*1081* (Taylor et al., 2005), is preferable in most cases, as there are six copies/cell in each member of the MTBC so quantification is possible. Optimization of the PCR is necessary and modification of the PCR reaction mix is recommended for work with aDNA. Inclusion of stabilizers such as bovine serum albumin is often beneficial, probably due to a variety of effects such as masking non-specific binding sites, stabilizing DNA fragments and binding or otherwise inactivating co-purified PCR

inhibitors (Donoghue, 2008a). Hot-start PCR and excess enzyme can also drive the reaction and overcome residual inhibitors. Detection of amplicon has traditionally been based on agarose gel electrophoresis. Detection is also possible by hybridization of labelled amplicons to a membrane, using a dot block technique, for example. However, real-time PCR enables amplified product with an incorporated fluorescent marker or probe to be monitored directly via a computer screen and the methodology facilitates quantification. For the future, *M. tuberculosis* diagnostics is moving towards isothermal and array technology. Microarrays are not ideal for the direct detection of ancient *M. tuberculosis* in crude extracts, due to the extensive fragmentation of target sequences. However, the introduction of new platforms based on surface interactions and nanotechnology offer exciting possibilities for the future.

2.4 Further developments in molecular diagnosis

In recent years, the field of molecular diagnostics of tuberculosis has expanded rapidly and a wide variety of new techniques have been introduced, or are currently being validated for clinical use. This is beginning to have an impact on the specialized field of palaeomicrobiology (Section 3.2 below) and, no doubt, there will be many more studies in the future that will be based on such technology.

2.4.1 Real-time PCR methods of MTBC aDNA detection and quantification

Real-time PCR (RT-PCR) or, more correctly, quantitative PCR (qPCR) enables amplified product with an incorporated fluorescent marker or probe to be monitored directly via a computer screen. The underlying principle of non-specific double-stranded DNA binding dye chemistry is that fluorescent dyes such as SYBR Green intercalate with any double stranded DNA. This enables the progress of the amplification to be followed as it progresses, whereas conventional PCR relies upon the detection of amplicon once the reaction has completed. By use of standards and specific primers, the number of copies of amplicon or the absolute amount of DNA can be quantified. Normally a series of peaks are visible on the computer screen, so on completion a melt analysis is performed and the temperature at which strand separation occurs (T_m) is used to determine the targeted sequence. Greater clarity and specificity is conferred by the use of specific DNA probes, which incorporate a fluorescent reporter that is normally quenched (Nazarenko et al., 1997). The fluorescence is only released once the probe has bound to the specific target sequence.

2.4.2 Other methods of MTBC aDNA detection in liquid systems

Several isothermal target amplification methods, which avoid the use of a thermocycler machine, have been developed in the two past decades (Karami et al., 2011). Loop-mediated isothermal amplification (LAMP) is an isothermal molecular method of DNA amplification that has been successfully implemented in the detection of *M. tuberculosis* in clinical specimens (Notomi et al., 2000; Neonakis et al., 2011). The reaction is driven by outer primers leading to strand displacement DNA synthesis, production of a single-stranded template, further DNA synthesis initiated by additional primers, and hybridization to the other end of the target sequence to produce a loop. In subsequent cycles further strand displacement leads to multiple copies of the target sequence. LAMP has several advantages, such as rapidity, high sensitivity, ease of application and cost-effectiveness.

The change of scale by the use of nanoparticles reduces the need for multiple rounds of DNA amplification. For example, direct examination of clinical samples has successfully demonstrated *M. tuberculosis* DNA after an initial round of PCR, using a colorimetric method based on an *M. tuberculosis* probe linked to gold nanoparticles (Baptista et al., 2006).

2.4.3 Detection of non-amplified DNA

An alternative approach to conventional PCR is to directly detect non-amplified DNA by amplification of the detection system such as labelled probes (Bhatt et al., 1999). However, the technology has now been developed to enable direct detection of sequences in non-amplified genomic DNA by means of various sensors. In one example, a piezoelectric biosensor enables real-time and label-free detection of the hybridization reaction between an immobilized probe and the complementary sequence in solution. The DNA probe is immobilized on the sensing surface (10 MHz quartz crystals), while the complementary sequence is present in the genomic DNA, previously fragmented with restriction enzymes (Minunni et al., 2005). This approach has been developed for the detection of *M. tuberculosis* (Kaewphinit et al., 2010). Another specific DNA detection method uses fluorescent semiconductor quantum dots and magnetic beads for fast detection of mycobacteria without any DNA amplification. Two biotinylated oligonucleotide probes are used to recognize and detect specific complementary mycobacterial target DNA through a sandwich hybridization reaction. Quantum dots conjugated with streptavidin and specific probes are used to produce a fluorescent signal. Magnetic beads, conjugated with streptavidin and a genus-specific probe are used to isolate and concentrate the DNA targets (Gazouli et al., 2010). Surface primer extension reactions may also be used to quantitatively detect unamplified, double-stranded genomic DNA (Martins et al., 2010). This methodology, by eliminating the need for pre-target labeling or amplification procedures, constitutes an alternative for the direct detection of genomic DNA from solution.

2.5 Authentication and precautions

Lists of precautions to take when working with mammalian aDNA have dominated palaeomicrobiology even though the recommendations may not be appropriate. For example, this is a summary of the "top ten list" drawn up by Poinar (2003):

1. a physically isolated work area, preferably a separate building where no genetic work is carried out;
2. PCR control amplifications, including non-template PCRs, multiple DNA and extraction controls;
3. molecular behaviour i.e. an inverse relationship between amount of PCR amplicon (bp) and length of target sequence;
4. quantification – the copy number of DNA should be assessed;
5. reproducibility – results should be repeatable from both the same and different DNA extracts of a specimen;
6. clone – direct sequencing should be confirmed by cloning amplicons and sequencing at least 10 clones to check for damage-induced errors and the ratio of endogenous to exogenous sequences;
7. independent replication – preferably by the independent examination of separate samples of the same specimen in independent laboratories;

8. biochemical preservation – use indirect assessment of the extent of DNA preservation by assessing the amount of diagenic change in other biomolecules, such as amino acids or lipids;
9. associated remains such as those of animals can be used to check for comparable aDNA survival to human DNA;
10. phylogenetic sense – sequences should be compared with others in appropriate databases to ensure authenticity.

Several of these recommendations have been accepted by palaeomicrobiologists, but a few are problematic:

1. The suggestion of a separate building and clean rooms with filtered air supplies may be advisable for work on human aDNA, where every investigator is a potential source of contamination with modern DNA. However, the MTBC has no environmental reservoir, so provided that researchers do not suffer from tuberculosis it is quite possible to perform palaeomicrobiological research by following good microbiological practice, including plentiful negative controls and independent verification (Donoghue et al., 2009).
2. Findings may not be reproducible because aDNA from tubercle bacilli will be localized. Even repeat samples from the same specimen may not yield a positive result in every case. Therefore, an additional criterion for work on a DNA of pathogenic microorganisms is proposed – that samples should be taken from sites appropriate to what is known of the natural history of the infection (Donoghue & Spigelman, 2006).
3. There is no evidence that cloning is necessary for verification of mycobacterial aDNA. Indeed, the opposite is true as work on *Mycobacterium leprae* (Taylor et al., 2006) showed that cloning gave no added value to data obtained by direct sequencing, but did introduce some errors, which were ascribed to *Taq* polymerase error and slipped strand mispairing. Similar conclusions have been reached in a recent study of mammalian aDNA (Winters et al., 2010).
4. Independent replication of aDNA may give discordant results due to localization of pathogen biomarkers within samples (see point (5) above). MTBC-specific lipid biomarkers may be more sensitive and can verify aDNA data without the need for amplification (Section 4 below) even though determined sceptics (Wilbur et al., 2009) may ignore this (Donoghue et al., 2009).
5. Comparison with different host biomolecular markers is discussed above (Section 2.2) and the conclusion is that aDNA can be found even in samples where other biomolecules are damaged.
6. Comparison with the recovery of aDNA from associated faunal remains is inappropriate for MTBC aDNA for at least two reasons. First, mycobacterial DNA appears to be more robust than mammalian DNA (Section 2.1). In addition, faunal remains are often a poorer source of aDNA than associated human remains (Mays et al, 2001), possibly due to treatment of carcasses after death and the absence of burial (Taylor et al., 2010).

3. Palaeomicrobiology of tuberculosis

3.1 Early studies 1993–2002 and initial conclusions

The earliest molecular studies on aDNA of the MTBC demonstrated proof of principle, but also answered historical questions about the occurrence of tuberculosis in the pre-colonial

Far East (Spigelman & Lemma, 1993) and whether tuberculosis occurred in the Americas before Columbus (Salo et al., 1994). During the first decade of such research it was demonstrated that MTBC aDNA could be found in bone and mummified tissue, from body sites in specimens without lesions and of a broad age range, from locations around the world (Donoghue, 2011). Additional methods of examination included pathology, microscopy and radiology. Authentication was provided by the direct detection of MTBC-specific cell wall lipid markers (Donoghue et al., 1998, Gernaey et al., 2001). Use of additional PCR target sites, including *rpoB*, *mtp40*, *oxyR* and spoligotyping – which uses a dot-blot method based on the MTBC Direct Repeat (DR) region (Kamerbeek et al., 1997), enabled confirmation of the principal human pathogen *M. tuberculosis sensu stricto* (Taylor et al., 1999). The oldest confirmed case of tuberculosis was reported in a Pleistocene bison (17,870 BP) from the Natural Trap Cave, Wyoming, USA. A metacarpal showed suggestive pathology and spoligotyping indicated that the infecting organism was a member of the MTBC, but the species was not confirmed at the time (Rothschild et al., 2001).

3.2 Recent findings and increased understanding

The increased understanding arising from total genome sequencing of *M. tuberculosis* (Cole et al., 1998) led to an appreciation that this group of organisms exhibits sequential deletions that can be used to distinguish between strains and lineages. Therefore, molecular typing protocols were developed based on a combination of synonymous single nucleotide polymorphisms (SNPs) in the *katG* codon 463 (*katG463*), *gyrA* codon 95 (*gyrA95*) and deletions (Brosch et al., 2002). The TbD1 deletion was identified as specific to the human pathogen *M. tuberculosis* and a significant marker of "ancestral" and "modern" strains. Therefore, both SNP typing and deletion analysis have been incorporated into MTBC aDNA studies, provided that the DNA preservation was sufficiently good for such single-copy markers to be amplified and detected.

The next decade included population studies and early epidemiological findings. A well-documented group of over 200 naturally mummified individuals from the 18th century was discovered in a church crypt in Vác, Hungary (Fletcher et al., 2003a). DNA preservation was particularly good and there was a high level of both active and presumed latent infections (Donoghue et al., 2011). It was possible to perform molecular fingerprinting and genotyping based on SNPs and to identify the *M. tuberculosis* aDNA as of "modern" strains. These techniques were used to demonstrate that in a small family group each person was infected with a different *M. tuberculosis* strain (Fletcher et al., 2003b). Interim epidemiological data have also been obtained from an on-going study of early Christian Nubians (550–750 and 750–1500 CE) and it is clear that tuberculosis was widespread, although there are no contemporaneous records and the DNA preservation is much less good (Donoghue, 2008b; Spigelman et al., 2005). Meanwhile, Zink, Nerlich and colleagues have produced a series of papers from a long-term study of burials in Thebes-West in ancient Egypt (Zink & Nerlich, 2004; Zink et al., 2003a, 2003b, 2004), spanning the pre-Dynastic period (5500–3100 BCE) to the New Kingdom (1550–1070 BCE). Molecular typing and spoligotyping indicated human *M. tuberculosis* and there was also evidence of another member of the MTBC, *Mycobacterium africanum*. However, no *M. bovis* was found. Indeed, there has only been one reported case of human tuberculosis associated with *M. bovis* aDNA (Taylor et al., 2007). This was found in a small group of pastoralists in south Siberia, dating from approximately 1761 to 2199 years

BP, placing the remains within the Iron Age period. Further work on the same specimens used qPCR to detect, quantify and characterize the *M. bovis* DNA (Murphy et al., 2009).

The use of qPCR with specific fluorescent reporters should enable the detection of highly fragmented aDNA. This was demonstrated by the detection of a 63 bp *IS6110* target sequence specific for the MTBC in a pre-Hispanic (900–1100 CE) adult from the north coast of Peru (Klaus et al., 2010). Both conventional and qPCR were used to examine skeletal material from western Hungary with palaeopathology suggestive of tuberculosis (Évinger et al., 2011). Samples were dated from 800–1200 CE and the qPCR with a specific 75 bp IS*6110* target sequence was positive in six cases including two from the 9th century, whereas conventional PCR was negative. However, conventional PCR with a 113 bp target sequence for IS*1081* was positive in two of these cases plus one other, but a qPCR probe with a 72 bp target sequence was negative, thus demonstrating the lack of consistency when seeking aDNA from microbial pathogens in human tissue.

3.3 Association of tuberculosis with other diseases

3.3.1 Co-infections

There has been no systemic examination of archaeological or historical material for co-infections, but our current understanding is that a pre-existing infection can increase susceptibility to another. A recent historical example is the influenza pandemic of 1918 where a major cause of death is believed to have been secondary bacterial pneumonia (Morens et al., 2008). It is very likely that additional examples will be found.

For example, parallel developments in the molecular detection of *M. tuberculosis* and *M. leprae* aDNA enabled co-infected individuals to be identified. These were cases of lepromatous leprosy with very typical palaeopathology, who were subsequently discovered to have systemic *M. tuberculosis* aDNA in their skeletal remains (Donoghue et al., 2005). An extensive literature search revealed that such co-infections had been reported in historical times prior to the introduction of chemotherapy; the findings led to a hypothesis that tuberculosis might have been a major factor in the elimination of leprosy from Western Europe.

An example of an association of tuberculosis with a parasite infection comes from pre-colonial northern Peru, where Chaga's disease, caused by the protozoan parasite *Trypanosoma cruzii*, was widespread (Aufderheide et al., 2004). Palaeopathology and aDNA analysis demonstrated both Chaga's disease and tuberculosis in the population and one 12 year-old girl from 910-935 BP was shown to have a co-infection (Arriaza et al., 2008). Another such association between tuberculosis and *Leishmania* spp. infection, possibly also linked to nutritional stress, was reported in preliminary data from early Christian Nubia (Spigelman et al., 2005).

3.3.2 Co-morbidity

There are many examples of increased susceptibility to infection associated with poor nutrition, a compromised immune system e.g. in neonates or the elderly, physical or mental stress due to wars and relocation, and underlying other medical conditions. An example of an association of tuberculosis with reduced lung function due to a massive vertebral

deformity, probably developmental, was described by Kustár et al. (2011) in an 18th century mummified lung from Vác, Hungary. Another individual from the same population, a child aged 1.5 – 2.5 years, showed numerous bony lesions throughout the body (Spigelman et al., 2006). A differential diagnosis, based upon the palaeopathology led to the conclusion that this infant suffered from Langerhans' cell histiocytosis (LCH), also referred to as histiocytosis-X. The aetiology and pathogenesis of LCH are still unknown but it is now thought to be a neoplasm, so the finding of tuberculosis in this child is not surprising. The child would have a repressed immune system, due to marrow replacement by the malignant cells, and thus be vulnerable to tuberculosis, which was widespread in this community.

3.4 MTBC lineages, evolution and timescale

The association of *M. tuberculosis* lineage with that of their human host has been convincingly illustrated in modern populations (Section 1). There is low DNA sequence variation in the MTBC and little, if any, horizontal gene exchange, which prevents reacquisition of genomic regions that have been lost. Therefore, deletions and functionally neutral SNPs are ideal markers for inferring deep phylogenies (Donoghue, 2009). The SNPs in the *katG* codon 463 (*katG463*), *gyrA* codon 95 (*gyrA95*) and the TbD1 deletion (Brosch et al., 2002) enable differentiation of three principal genetic groups within the MTBC. It is believed that these organisms have undergone an evolutionary bottleneck, associated with the adoption of a parasitic lifestyle. Thereafter, both host and pathogen have undergone clonal expansion. The timescale during which this has occurred is of interest, not least because of the realization that the evolution of the MTBC appears to be increasing exponentially today and the underlying factors need to be understood.

Although tuberculosis is still the greatest single cause of death caused by a single microbial pathogen, the high proportion of infected persons with latent infection indicates that host and pathogen have co-existed for a considerable length of time (Donoghue, 2009; Rustad et al., 2009). It is believed that the emergence of human pathogens is related to population density and tuberculosis has long been recognized as associated with the development of agriculture and animal domestication during the Neolithic transition. Palaeopathological data alone cannot distinguish between *M. tuberculosis* and *M. bovis* infection, and the earlier belief that human tuberculosis was derived from the animal disease has proved difficult to shift. However, palaeomicrobiology provides convincing evidence that *M. bovis* is rare in past human populations. Direct evidence of infection with human lineages was provided from ancient Egypt by genotyping and deletion analysis (Zink & Nerlich, 2004, Zink et al., 2003a) and spoligotyping (Zink et al., 2003b). Demonstration of the oldest infection with human lineages of the MTBC (Hershkovitz et al., 2008) was based on five different target sequences, and was confirmed by direct detection of *M. tuberculosis*-specific mycolic acid markers (see Section 4.2 below). The population was from a Pre-Pottery Neolithic site in the Eastern Mediterranean, dated around 9000 years ago, with plentiful evidence of animal domestication. It is therefore of special interest that the *M. tuberculosis* lineage is of a TbD1-deleted strain.

Ancestral sequence inference is a process used in bioinformatics to estimate the rate of evolutionary change under different scenarios. Combination with the direct evidence obtained from palaeomicrobiology, enables confirmation of the presence of particular *M. tuberculosis* lineages in the past, which can strengthen and inform existing models. This has

led to a significant extension of the timescale for the evolution of the MTBC. For example, when Brosch et al. (2002) first published their evolutionary model, it was noted that the 18th century Vác mummies were of *M. tuberculosis* of principal genetic groups 2 and 3, thus proving that these had not evolved during recent times. Six years later it was appreciated that the "modern" TbD1-deleted lineages existed 9000 years ago (Hershkovitz et al., 2008). It appears likely that further extension of the timescale will require the introduction of the more sensitive immobilized DNA technologies to enable detection of the highly fragmented material in such ancient samples.

4. Lipid biomarkers for the *M. tuberculosis* complex (MTBC)

4.1 Established lipid biomarkers for MTBC

The cell envelope of *M. tuberculosis* is based on complex macromolecules linked to produce a mycoloyl arabinogalactan-peptidoglycan organelle (Minnikin 1982; Barry et al., 2007). This organelle is the foundation for a characteristic mycobacterial outer membrane based on covalently bound mycolic acids, interacting with a range of unusual free lipids (Minnikin, 1982; Minnikin et al., 2002). This outer membrane has now been visualized directly (Hoffmann et al., 2008; Zuber et al., 2008) and given the label "mycomembrane". Mycolic acids are, therefore, an integral part of mycobacterial cell envelopes, with proven biomarker value, both in classification and identification, due to variations in the individual mycolate types expressed (Butler & Guthertz, 2001; Dobson et al., 1985). The mycolic acids produced by *M. tuberculosis* are composed of five principal types, as illustrated in Fig. 1A, each type having a range of homologues with different chain lengths (Minnikin, 1982; Minnikin & Polgar, 1967a, 1967b; Watanabe et al., 2001, 2002). This general mycolate fingerprint is shared by *M. tuberculosis* and other members of the MTBC, whose best-studied members include *M. bovis, M. africanum* and *Mycobacterium microti*. MTBC mycolic acids are relatively stable biomarkers, which have been detected in archaeological material up to 9,000 years old (Hershkovitz et al., 2008). The use of mycolate biomarkers in the identification of tuberculosis depends on the clear recognition of profiles characteristic of the MTBC; this will be discussed in detail below (Sections 4.2 & 4.3).

The so-called "free" lipids, which associate with the "polysaccharide-bound" mycolic acids to form the outer myco-membrane (Minnikin, 1982; Minnikin et al., 2002), are also a source of diagnostic lipid biomarkers. The mycocerosic and mycolipenic acids (Fig. 1B) are the best-studied examples, the former being components of phthiocerol dimycocerosate waxes and the latter being part of pentaacyl trehalose glycolipids (Minnikin et al., 1983, 1985a, 1985b, 2002). Mycocerosic acids are found in a limited number of mycobacterial species, including *Mycobacterium kansasii, M. leprae* and *Mycobacterium haemophilum* in addition to members of the MTBC; *Mycobacterium marinum* and *Mycobacterium ulcerans* have closely related acids (Minnikin et al., 2002). The distribution of the different mycocerosate types has been defined (Daffé & Lanéelle, 1988; Minnikin et al., 1985a; Minnikin et al., 1993a), with MTBC having a characteristic pattern composed of mainly C_{29}, C_{30} and C_{32} components (Fig. 1B). In contrast, only a single C_{27} mycolipenate (Fig. 1B) is usually encountered and principally in only *M. tuberculosis*. The value of mycocerosic and mycolipenic acid lipid biomarkers in the diagnosis of ancient tuberculosis has been investigated by Redman et al. (2009). An important aspect of identifying lipid biomarkers in archaeological samples is that such lipids are being increasingly implicated as virulence factors in the pathogenesis of *M.*

tuberculosis (Gordon et al. 2009; Neyrolles & Guilhot, 2011); this is a further avenue for research into the evolution of the host/pathogen relationship.
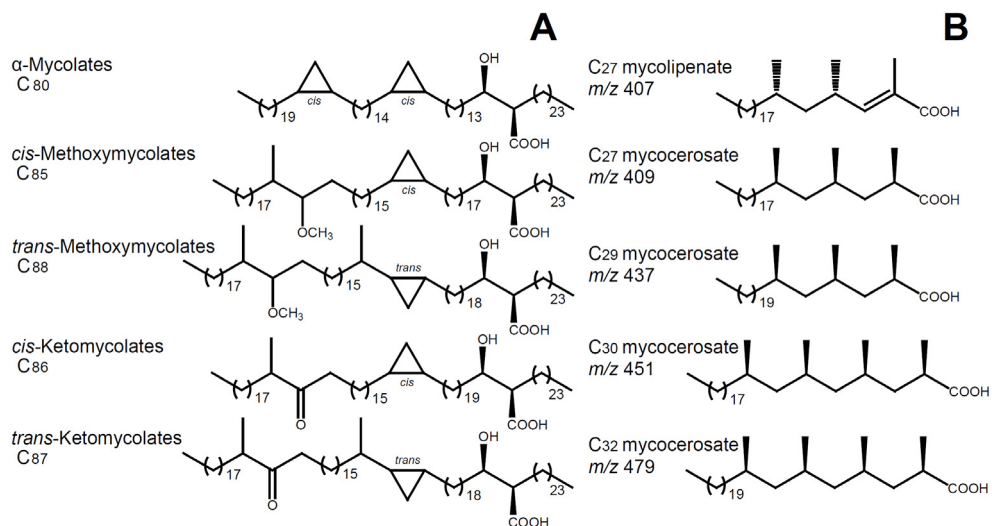

Fig. 1. Structures of selected lipid biomarkers for *M. tuberculosis*. **A.** The main components of each mycolic acid class are shown; each class comprises a limited range of homologous components with different chain lengths. **B.** Mycolipenic and mycocerosic acids; for each component, the ions (*m/z*) monitored on negative ion-chemical ionization gas chromatography-mass spectrometry (NICI-GCMS) of pentafluorobenzyl esters of these acids are given.

4.2 HPLC recognition of MTBC mycolic acid patterns in archaeological samples

The mycolic acids from *M. tuberculosis* (Fig. 1A) comprise three principal classes, α-, methoxy- and keto-, which can be separated from each other by simple so-called "normal phase" chromatography. Such separations are readily achieved by thin-layer chromatography (Dobson et al., 1985; Minnikin, 1993) or by high performance liquid chromatography (HPLC) on silica gel media (Minnikin, 1993; Qureshi et al., 1978; Steck et al., 1978). Normal phase profiles of total mycolates, simply showing α-, methoxy- and ketomycolate classes, are not diagnostic for the MTBC, as they are shared with a range of other mycobacterial species (Dobson et al., 1985; Minnikin, 1982; Watanabe et al., 2001, 2002). "Reverse phase" HPLC separates mycolates both according to chain length and polarity (Minnikin, 1993; Qureshi et al., 1978; Steck et al., 1978), and a characteristic "tight envelope" of peaks is produced by members of the MTBC (Donoghue et al., 2010a; Gernaey et al., 1998, 2001; Hershkovitz et al., 2008). This diagnostic profile has been found to be sufficient for the routine diagnosis of modern clinical tuberculosis, using computerized comparison to an internal standard (Butler & Guthertz, 2001). It is conceivable, however, that a combination of various factors could produce an envelope of peaks mimicking that

characteristic of tuberculosis. Extra dimensions of information are readily available if reverse phase HPLC is carried out on the individual α-, methoxy- and keto-mycolate classes (Donoghue et al., 2010a; Gernaey et al., 1998, 2001; Hershkovitz et al., 2008; Minnikin et al., 1993b). As shown in Fig. 1A, methoxy- and keto-mycolates have sub-classes depending on the presence of the alternative *cis*-cyclopropane or methyl branched *trans*-cyclopropane moieties; these sub-classes are not readily separable by normal phase chromatography (Donoghue et al., 2010a; Gernaey et al., 1998, 2001; Hershkovitz et al., 2008; Watanabe et al., 2001).

The first detection of mycolic acid biomarkers in archaeological skeletal material was performed in studies by Gernaey et al., (1998, 1999, 2001, 2002) and extended to calcified pleura (Donoghue et al., 1998). The sensitive HPLC analysis used mycolate methylanthryl esters, previously developed to diagnose modern tuberculosis in human sputum (Minnikin et al., 1993b). The derivatized mycolic acids were isolated by reverse phase HPLC and the total mycolate fraction was collected and analyzed by normal phase HPLC to separate the α-, methoxy- and ketomycolate classes. These individual mycolate classes were then resolved into envelopes of peaks diagnostic for MTBC by further reverse phase HPLC. The value of this effective sequential protocol will be demonstrated below (Fig. 2) for a more recent example (Hershkovitz et al., 2008). The archaeological material investigated by Gernaey et al. (1998, 1999, 2002) was a collection of 19th century skeletons excavated from the site of the old Newcastle upon Tyne Infirmary, UK; there was a good correlation with burial records. The power of combining aDNA and mycolate analyses was first demonstrated for 1,400 year old calcified pleura from Karkur in the Negev desert (Donoghue et al., 1998, 2004). In another combined study, Gernaey et al. (2001) showed that mediaeval skeletons from 1,000 years ago in Addingham, Yorkshire, UK had evidence of tuberculosis. These particular landmark samples were in fact the first in which mycolic acid, or any other, lipid biomarkers had been seen. Some of these early investigations have been reviewed by Gernaey & Minnikin (2000).

The methylanthryl derivatives used in the above pioneering studies were not ideal as their relative instability required that the sequential HPLC analyses must be done quickly, with minimum storage. A systematic exploration of derivatization protocols resulted in the selection of pentafluorobenzyl (PFB) mycolic acid esters, further esterified with pyrenebutyric acid (PBA). Ample justification of this selection was provided by confirmation of the oldest proven case of tuberculosis in ribs from a woman and child from a neolithic pre-pottery settlement at Atlit-Yam in the Eastern Mediterranean, dated at around 9,000 BP (Hershkovitz et al., 2008). The mycolic acid profiles for these extracts are shown in Fig. 2. The immaculate preservation of the mycolic acid biomarkers is illustrated by the remarkable similarity of the total mycolate profiles from the three skeletal extracts with that from authentic *M. tuberculosis* (Fig 2A). It must be remembered that the profiles in Fig. 2A represent a conglomerate of all five classes shown in Fig. 1, but it is a characteristic of MTBC mycolates that they cluster together to give a "tight envelope" of distinct peaks. Subjecting the collected total mycolates to normal phase HPLC (Fig. 2B) shows the proportions of the α-, methoxy- and ketomycolate classes but the methoxy- and keto- components having either *cis*- or *trans*- cyclopropane rings (Fig. 1A) are not separated.

Reverse phase HPLC of the collected α-mycolates (Fig. 3A) gives a profile of simple regularly spaced peaks corresponding to the single class of α-mycolates with two *cis*-cyclopropane rings; the main C_{80} component corresponds to the structure shown in Fig. 1A. The reverse phase HPLC profiles for the methoxymycolates from *M. tuberculosis* and the Atlit-Yam skeletons (Fig. 3B) are particularly informative. The main C_{85} component is the *cis*-methoxymycolate shown in Fig. 1A and the minor C_{88} component (Fig. 3B) is the principal *trans*-methoxymycolate given in Fig. 1A. Again the excellent correlation between the methoxymycolate profiles from standard *M. tuberculosis* and the archaeological samples illustrates the remarkable preservation of these samples. The main C_{87} *trans*-ketomycolate (Fig. 1A) dominates the reverse phase HPLC of the ketomycolates (Fig. 3C); the C_{86} *cis*-ketomycolate (Fig. 1A) is a very minor component (Fig. 3C). The particular mosaic of patterns, shown in Figs. 2 and 3, appears to be very characteristic for *M. tuberculosis sensu stricto*. In continuing unpublished studies, it is becoming apparent that the patterns recorded for *M. bovis* may possibly be distinguished by different proportions of the *cis*- and *trans*-methoxy- and ketomycolates; this is in accord with detailed structural studies (Watanabe et al., 2001, 2002).
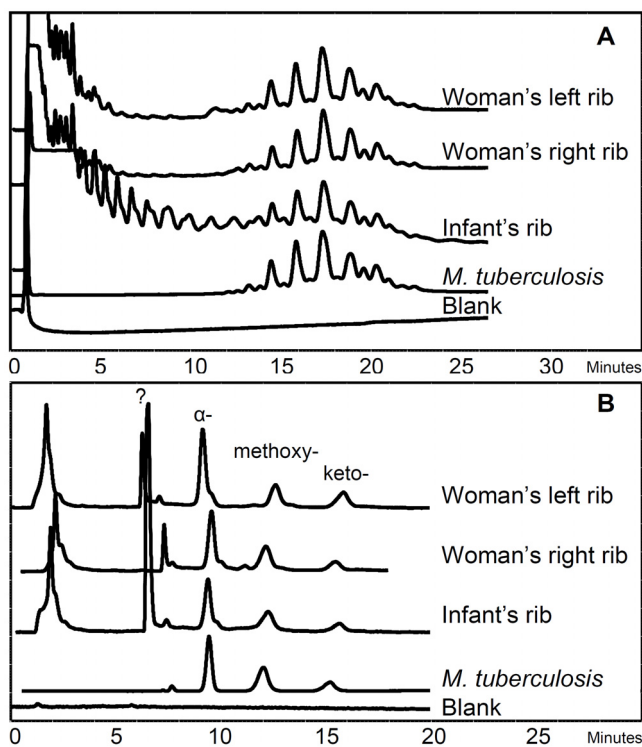

Fig. 2. HPLC of pyrenebutyric acid (PBA) esters of pentafluorobenzyl (PFB) esters of mycolic acids extracted from skeletons from Atlit-Yam and standard *M. tuberculosis*. Reproduced from Hershkovitz et al. (2008). **A.** Reverse phase HPLC of total mycolates. **B.** Normal phase HPLC of total mycolates, collected from 2A.

In addition to providing diagnostic profiles, HPLC allows quantitative data to be recorded. In particular, the relative proportions of the α-, methoxy- and ketomycolate types (Fig. 1A) are readily determined, as exemplified (Table 1) for the mycolates from Atlit-Yam (Hershkovitz et al., 2008). The α-mycolates contribute about half the mixture, with one quarter to one third being methoxymycolates and one tenth to one fifth being ketomycolates (Table 1). This good correlation of mycolate class proportions in the skeletal extracts with the *M. tuberculosis* standard is a clear indicator of good sample preservation. As will be discussed below for another study (Minnikin et al., 2011), degraded samples appear to lose their methoxy- and ketomycolates, particularly the latter. It is also possible to estimate the
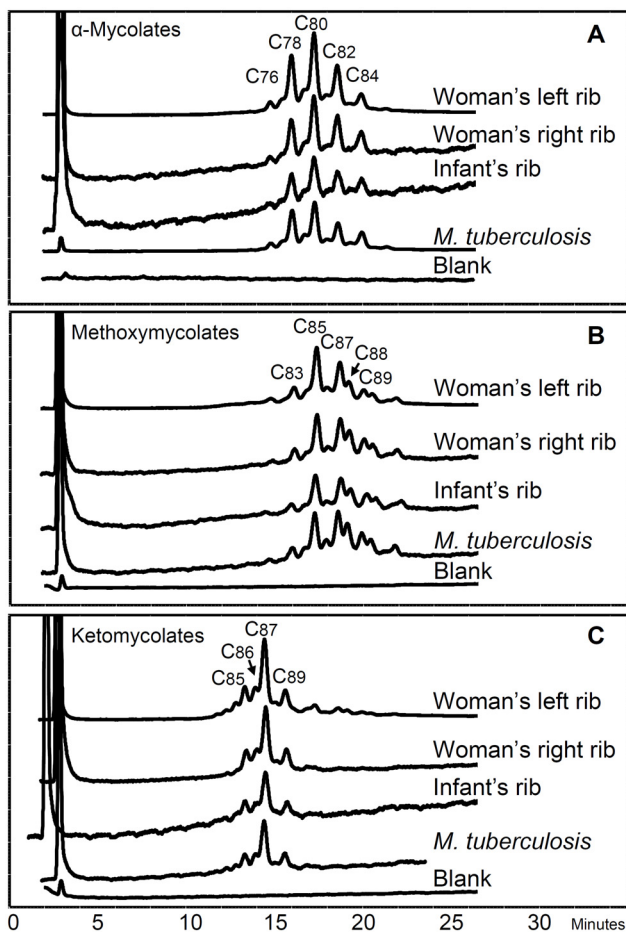

Fig. 3. HPLC of pyrenebutyric acid (PBA) esters of pentafluorobenzyl (PFB) esters of mycolic acids extracted from skeletons from Atlit-Yam and standard *M. tuberculosis*. Reproduced from Hershkovitz et al. (2008). **A, B**, & **C.** Reverse phase HPLC of α-mycolate, methoxymycolate and ketomycolate classes, respectively, collected from the normal phase separation illustrated in Fig. 2B.

absolute amounts of mycolic acids present, as shown (Table 2) for the Atlit-Yam extracts (Hershkovitz et al., 2008). The amount of mycolate in the 635 mg Woman left rib (12.80 µg) is most exceptional, equating to an almost weighable and visible one hundredth of a milligram! This suggests a very heavy tuberculosis infection in the bone from the Woman, as compared to that from the Infant, which is 168 times less; such snapshot comparisons are not statistically valid, however, as it would be necessary to examine a comparable range of bones from each individual several times over.

Mycolate	Woman's left rib	Woman's right rib	Infant's rib	*M. tb.* standard
Alpha-	56.3	52.65	53.2	48.2
Methoxy-	25.2	34.50	32.2	37.4
Keto-	18.5	12.85	14.6	14.4

Table 1. Percentage ratios of alpha-, methoxy- and ketomycolates in skeletons from Atlit-Yam. Reproduced from Hershkovitz et al. (2008).

Bone sample	Bone mass	Mycolate in bone	Mycolate/bone load
Woman's left rib	635 mg	12.80 µg	20.14 µg/g
Woman's right rib	483 mg	1.697 µg	3.51 µg/g
Infant's rib	589 mg	0.073 µg	0.12 µg/g

Table 2. Absolute amounts of mycolic acids in skeletons from Atlit-Yam. Reproduced from Hershkovitz et al. (2008).

Another example of good mycolic acid biomarker preservation is provided by the interesting case of Dr. Granville's mummy (Donoghue et al., 2010a). The subject is the lady Irtyersenu of the 26th Dynasty (*ca.* 2600 BP) from the necropolis of Thebes. Although aDNA from the MTBC was detected, the material was very difficult to work with, possibly due to the embalming method, which appears to have been unusual. In contrast, the mycolic acid HPLC traces were almost as pristine as those from the Atlit-Yam samples (Figs. 2 and 3) (Hershkovitz et al., 2008). These results, in conjunction with aDNA detection, clearly confirmed tuberculosis infection, which may have made a major contribution to the death of Irtyersenu (Donoghue et al., 2010a). However, pristine mycolic acid HPLC traces are by no means the norm in archaeological samples from subjects suspected to have suffered from tuberculosis. In a study aimed to test the possibility of detecting lipid biomarkers in archaeological material, after aDNA has been extracted and analyzed, a range of samples from subjects suspected to have been infected with tuberculosis, leprosy or both were investigated (Minnikin et al., 2011). Evidence of mycolic acids was found in all samples, thereby proving that it was possible to isolate lipid biomarkers from aDNA analysis residues; the hydrophobic lipids were not extracted by the aqueous media used to release aDNA. The total mycolate reverse phase HPLC profiles were complex and the normal phase HPLC traces all had α-mycolates but the methoxy- and ketomycolates were variable to non-existent, excepting two cases where good clear peaks for ketomycolates were recorded. The results in this paper are too complex to summarize concisely. For the 12 cases, suffice it to say that five diagnoses agreed with the aDNA (three tuberculosis, two leprosy), five cases did not correlate clearly and for two extracts the mycolic acids were so degraded that it was impossible to support positive aDNA diagnoses. This instructive study suggests that it is

important to obtain as much biomarker information as possible, particularly where mixed tuberculosis/leprosy cases are a probability. For one particular 7th century skeleton from the Avar period in Hungary, it was possible to recognise mycolic and mycocerosic acids from both *M. tuberculosis* and *M. leprae* (Lee et al., 2012). The quantitative data suggested a predominance of tuberculosis over leprosy in contrast to the bone pathology which indicated only leprosy. The presence of mycolic acids supported an aDNA diagnosis of leprosy in a 1st to 4th century CE skeleton from Uzbekistan (Taylor et al., 2009).

4.3 Mass spectrometry in detection of mycolic acid biomarkers for ancient tuberculosis

Mass spectrometry was a key technique in establishing the essential structures (Fig. 1A) of the mycolic acids of *M. tuberculosis* for the first time (Minnikin & Polgar, 1967a, 1967b; Minnikin, 1982), providing accurate molecular weights. Initially, the individual α-, methoxy- and ketomycolates were analysed separately by the pioneering but rather cumbersome high energy Electron Ionisation (EI) mode of mass spectrometry, which produces complex spectra with characteristic fragmentation patterns. Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-TOF) mass spectrometry is a convenient lower energy technique that often gives peaks corresponding to molecular weights augmented by ubiquitous sodium ions (M + Na$^+$). MALDI-TOF mass spectrometry was used to characterize a range of individual mycolate types in a comprehensive study (Watanabe et al., 2001) aimed at determining the precise location of functional groups in mycolic acids (Watanabe et al., 2002). In a parallel study, Laval et al. (2001) demonstrated that MALDI-TOF mass spectrometry of mycolic acid methyl esters can be used to provide a characteristic profile of total mycolic acid composition.

Based on the previous study by Laval et al. (2001), Mark et al. (2010) used MALDI-TOF mass spectrometry to search for tuberculosis mycolic acids in ancient bones. However, the profiles recorded by Mark et al. (2010) did not resemble those expected for mycolic acids, so a response to this paper was published (Minnikin et al., 2010). This response suggested guidelines for the satisfactory recognition of mycolic acid profiles diagnostic for *M. tuberculosis.* Remarkably, the original clearly inadequate conclusions were vigorously defended by Mark et al. (2011), without providing any new convincing data. It is most important, therefore, to demonstrate beyond doubt that the data provided by Mark et al. (2010, 2011) are not evidence for tuberculosis infection in any of the analyzed archaeological samples, whatsoever; the problems raised by these papers are considered in detail below.

The paper of Minnikin et al. (2010) provided a considered in-depth constructive analysis of the inadequacies of the data shown in Mark et al. (2010), so these criticisms will not be repeated in detail. The essence of the problems in both papers published by Mark et al. (2010, 2011) is exemplified by the data shown in Figs. 4 and 5. The profile shown in Fig. 4A (Mark et al., 2010) is suggested to be that of standard *M. tuberculosis* mycolic acids, but it only shows a series of regularly spaced peaks, more suggestive of polymeric material than distinct components of the family of mycolic acids shown in Fig. 1A. Similarly, the profile for an extract of a skeleton from Sükösd-Ságod (grave 19) (Fig. 4B) again showed a regular series of peaks, with limited correspondence between the data in Figs. 4A and 4B. The different classes of mycolic acids (Fig. 1A) occur naturally as groups of peaks with one or

two major components accompanied by several minor homologues (Minnikin & Polgar, 1967a, 1967b; Minnikin, 1982; Watanabe et al., 2001, 2002). Mycolic acids (Fig. 1A) are long-chain fatty acids, with series of homologues whose biosynthetic pathways dictate a general spacing of two methylene groups ($-CH_2.CH_2-$) amounting to 28 atomic mass units (amu). It is, therefore, totally impossible that the patterns presented in Figs. 4A and 4B, with spacings of 44 amu, can be assigned to homologous series of mycolic acids. Mark et al. (2010) hypothesize that the ion spacings are a result of carbon dioxide (44 amu) loss but this does not correlate with any known properties or published behaviour of any mycolic acids or, indeed, any other fatty acids. As suggested previously (Minnikin et al., 2010), an alternative explanation for the regular 44 amu spacing of the components is that the peaks are derived from a material incorporating polyethylene glycol repeating units. These polymeric polyethylene glycol-based preparations are in widespread industrial use and the risk of their appearance in mass spectra is well-known, as emphasized by, for example, by Keller et al. (2008) and Schiller et al. (2004), the latter reproducing a representative MALDI-TOF mass spectrum. Another general point, which comprehensively disqualifies the profiles in Figs. 4A and 4B, is the undisputed fact that mycolic acids do not have recorded molecular weights greater than 1350 amu (Laval et al., 2001; Watanabe et al., 2001, 2002) so the presence of alien substances is suspected. As noted above, these and other criticisms have been thoroughly aired by Minnikin et al. (2010) in a constructive attempt to establish reliable guidelines for the recognition of mycolic biomarkers in the diagnosis of ancient tuberculosis.
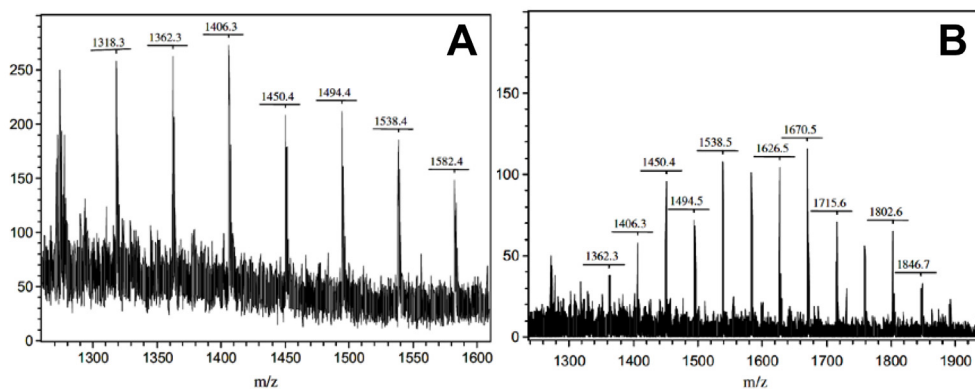

Fig. 4. MALDI-TOF mass spectra, reproduced with permission from Mark et al. (2010). **A**. Mycolic acid standard; Fig. 2 of Mark et al. (2010). **B**. Extract of bone sample from Sükösd-Ságod grave 19; Fig. 3A of Mark et al. (2010).

However, Mark et al. (2011) declined to acknowledge the obvious and indisputable errors and deficiencies, raised by Minnikin et al. (2010), in the original paper of Mark et al. (2010). In a most unsatisfactory manner, Mark et al. (2011) were able to submit their response on 17th December 2009, precisely one calendar month after the receipt of Minnikin et al. (2010) on 19th November 2009. This indisputably proves that unauthorized privileged information had been provided to Mark et al. (2011), well in advance of the due confidential reviewing process for the manuscript of Minnikin et al. (2010). Disregarding the deficiencies and practises in the publication process, attention must be focussed on the data and arguments advanced in the publication of Mark et al. (2011). As shown in Fig. 5A, the mass spectrum of

an authentic sample of *M. tuberculosis* mycolic acids was recorded (Mark et al. 2011) and this is very different from that shown in Fig. 4A, for supposedly the same material (Mark et al., 2010). The mass spectrum of an extract from the skeleton from Sükösd-Ságod (grave 19) (Fig. 5B) is again clearly distinct from the spectrum recorded for the same bones (Fig 4B) by Mark et al. (2010). More disturbingly, the supposedly positive spectrum recorded in Fig. 5B has little resemblance to that of the authentic standard (Fig. 5A). The most significant, but unknown, peak at *m/z* 1361.1 (Fig 5B) is clearly too large in mass to be a mycolic acid, as the
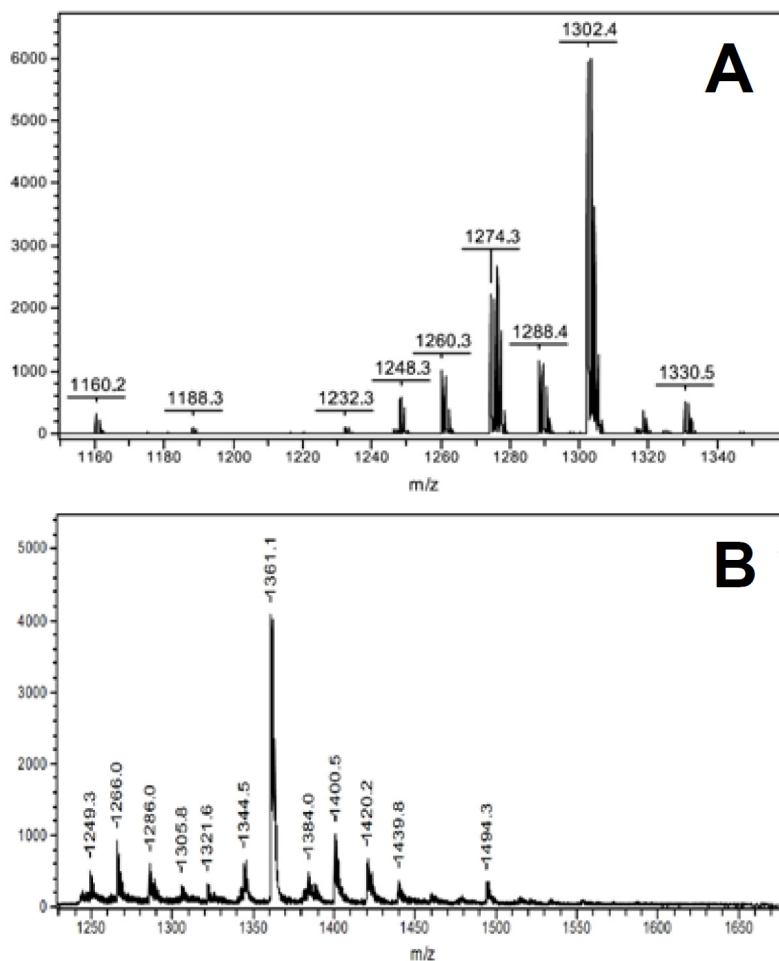

Fig. 5. MALDI-TOF mass spectra, reproduced with permission from Mark et al. (2011). **A**. Mycolic acid standard, provided by David E. Minnikin (University of Birmingham, UK); Fig. 3B of Mark et al. (2011). Peaks at *m/z* 1160 and 1188 are C_{78} and C_{80} α-mycolates, *m/z* 1274 is C_{85} *cis*-methoxymycolate and *m/z* 1302 is C_{87} *trans*-ketomycolates (see Minnikin et al., 2010 for a spectrum of the same sample). **B**. Extract of bone sample from Sükösd-Ságod grave 19; Fig. 4A of Mark et al. (2011).

highest component in the authentic standard is centred around *m/z* 1330 (Fig. 5A). In three other supposedly positive mass spectra, reported by Mark et al. (2011), the unknown, non-mycolic acid, peak at *m/z* 1361 is the main component. Incontrovertibly, the spectrum of the extract of the skeleton from Sükösd-Ságod (grave 19), shown in Fig. 5B, does not provide any evidence for the presence of tuberculosis, providing a double negative diagnosis for the same skeleton as the profile in Fig. 4B was also woefully inadequate.

The science displayed by Mark et al. (2010) has been thoroughly discussed by Minnikin et al. (2010) but it is also necessary, for the record, to draw critical attention to claims made in Mark et al. (2011). In this paper, there is an extensive discussion (page 1112, section 3) about whether it is likely to be possible to detect unmodified mycolic acids in archaeological samples. The key statement is "it would be very surprising if the mass spectra of ancient mycolic acid biomarkers were exactly the same as those of recent standards and clinical samples"; presumably this is aimed at providing some justification for claiming that the spectra exemplified in Figs. 4B and 5B (this Chapter) represent naturally modified mycolic acids. This argument is taken further in the statement "the identification of ancient mycolic acids and their metabolites could only be carried out with accurate and systematic chemical modelling of the mycolic acid post-mortem diagenesis". The implication of this statement is that diagenetic studies are a prerequisite but there are no suggestions about how this might be done. One way to approach this is to make extracts of suspected infected archaeological material and examine them by established objective protocols, such as HPLC, to determine if mycolic acids or their degradation products are recognisable. This is precisely the approach taken by Gernaey et al. (2001), Hershkovitz et al. (2008), Donoghue et al. (2010) and Minnikin et al. (2011); in the latter publication substantial degradation of mycolic acids is clearly recorded but in the others clearly recognisable mycolic acid patterns are documented. In the same paragraph (page 1112, section 3) of Mark et al. (2010), the paper of Donoghue et al. (2010a) is criticised by writing that "several significant differences can be observed on the standard chromatograms and the results of the bone samples". Such small differences are, however, valuable in showing that the extracts of bone samples are not contaminated by material from the standards. To suggest that such small differences might invalidate a diagnosis is contradictory to the arguments rehearsed elsewhere by Mark et al. (2011), which favoured a degree of diagenesis as a positive indication. Another criticism, levelled by Mark et al. (2011), concerns the presence of unknown peaks (labelled '?'), such as that in Figure 4 of Donoghue et al. (2010a); this particular unknown peak is in the same category as those shown in Fig. 2B (this Chapter) for the normal phase HPLC of mycolate derivatives from the Atlit-Yam skeletons (Hershkovitz et al., 2008). This unknown material, labelled '?' in Fig. 2B, represents residual material from the initial reverse phase isolation of the total mycolates (Fig. 2A) and there is no necessity to know its identity. Indeed one purpose of the normal phase HPLC analysis (Fig. 2B) of the total mycolates is simply to remove this contaminating material and obtain purified α-, methoxy- and ketomycolate classes for diagnostic reverse phase HPLC (Figs. 3A-C). Quite incredibly, the whole concept of normal phase chromatography is dismissed by Mark et al. (2011) as "not a 'simple' technique for the accurate separation of the components"; the reference (Neue, U.D., 1997. HPLC Columns. Wiley-VCN, New York.), quoted in support of this opinion, is only a document describing different types of chromatographic columns. However, normal phase chromatography is the bedrock of chemical research, being performed literally thousands of times each day in laboratories worldwide!

Mark et al. (2011) attempt to rationalize the disparate results presented in Mark et al. (2010, 2011) by claiming that choice of MALDI-TOF mass spectral matrices dramatically influences the resulting spectrum. In Mark et al. (2011) (page 1113, section 3) it is stated that "The results with using fullerene as the matrix are incomparable with the mass spectra made by using CHCA or 2,5-DHB". The essential conclusion is that the use of 2,5-DHB (2,5-dihydroxybenzoic acid) results in spectra such as those in Fig. 5, but using fullerene produces the spectra shown in Fig. 4, for supposedly the same samples. The explanation given by Mark et al. (2011) (page 1117, section 3.2) for the spectra in Fig. 5 is "On these spectra the *m/z* 44.01 fragmentation pattern could not be observed, because the fullerene needed much higher laser energy for ionization than the 2,5-DHB matrix, thus the fragmentation was stronger in the first case"; this "first case" refers to the spectra shown in Fig. 4. This hypothesis is linked to the problem of explaining the 44 amu spacing of the peaks in the spectra (Fig. 4) recorded by Mark et al. (2010). According to Mark et al. (2010, 2011) in profiles, such as that shown in Fig. 4A, a peak such as *m/z* 1406.3 should decarboxylate to give *m/z* 1362.3, losing 44 amu (CO_2). How then does *m/z* 1362.3 lose a further 44 amu to produce *m/z* 1318, as it would have already lost its carboxyl group? This is a scientific *non sequitur* unworthy of further consideration. The suggestion by Minnikin et al. (2010) that the sequences of peaks in Fig. 4 are characteristic of contaminating polymers based on polyethylene glycol (PEG) (Keller et al., 2008; Schiller et al., 2004) is a much more plausible explanation. The proposal by Mark et al. (2011) is that an authentic MALDI-TOF spectrum (Fig. 5A) of standard mycolic acids, using 2,5-DHB as matrix, can be changed to that shown in Fig. 4A simply by using C_{60} fullerene as matrix. This is a revolutionary but unlikely proposal that, if it is to be believed, must be substantiated by systematic research.

It is instructive to review the information required, using existing methods, to positively identify a mycolic acid pattern diagnostic for tuberculosis. The use of sequential reverse/normal/reverse phase HPLC analysis (Figs. 2 & 3) produces diagnostic patterns that, if the mycolates are undegraded, correlate well with standard material. Importantly, however, this protocol enables the key C_{80} (C_{78}) α-mycolates (Fig. 3A), C_{85} *cis*-methoxymycolates (Fig. 3B) and C_{87} *trans*-ketomycolates (Fig. 3C) to be recognised. It must be understood that related mycolic acids are present in many mycobacteria, but extensive studies have been carried out (Minnikin et al. 2010; Watanabe et al. 2001, 2002) to establish that the above combination of principal mycolic acid components (Fig. 1A, Fig. 3) is characteristic for *M. tuberculosis*. Any diagnoses, using mass spectrometry or any other technique, must recognise the presence of these characteristic components. In the case of MALDI-TOF mass spectrometry, the key C_{80} (C_{78}) α-mycolates, C_{85} methoxymycolates and C_{87} ketomycolates can be recognised in standard extracts, as shown in Fig. 5A, but this pattern must be recognisable in extracts of archaeological material. The studies of Mark et al. (2010, 2011) claim that recognisable MALDI-TOF mass spectra have been obtained for mycolic acids extracted from archaeological material. Simple visual comparison of the authentic mycolic acid spectrum, shown in Fig. 5A, with the spectra exemplified in Figs. 4B & 5B, or any other spectra published in Mark et al. (2010, 2011), reveals nothing remotely comparable. The bottom line conclusion, therefore, is that Mark et al. (2010, 2011) have not identified *M. tuberculosis* mycolic acids in bone samples and literature reference to these papers must never support the claim that mycolic acids were detected.
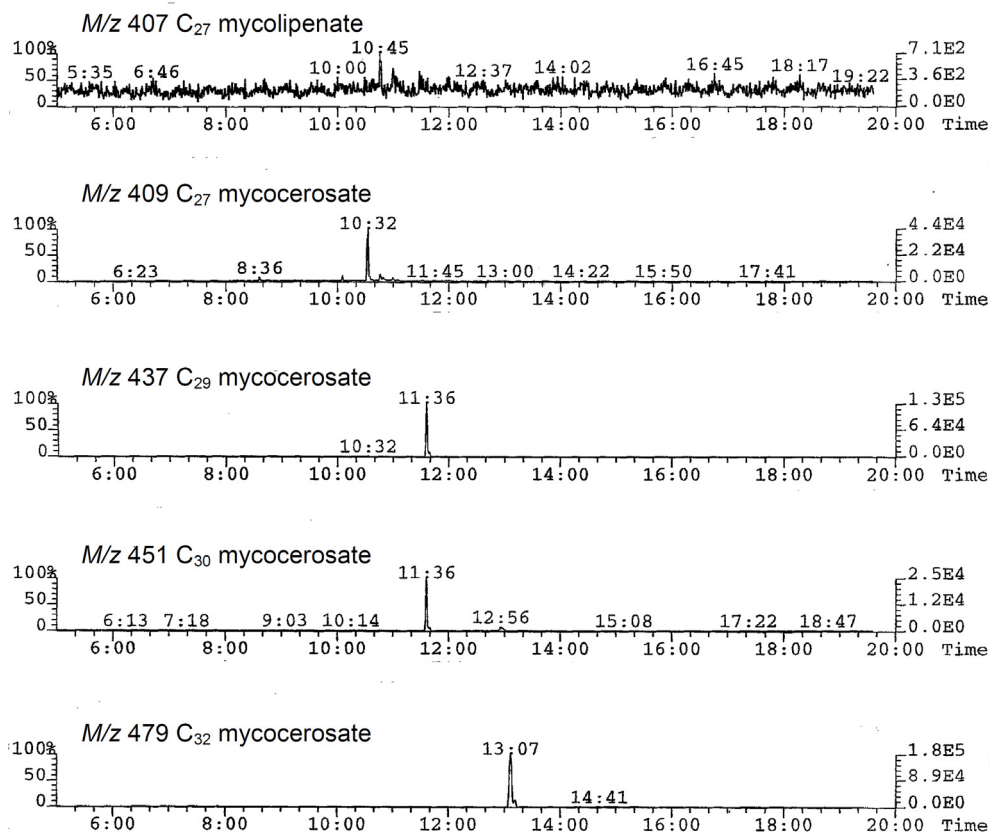
Fig. 6. Selected ion monitoring negative ion-chemical ionization gas chromatography-mass spectrometry (NICI-GCMS) of pentafluorobenzyl esters from Coimbra skeleton C8, reproduced with permission from Redman et al. (2009). The C_{27} M/z 407 ion is for mycolipenic acid; the remainder are for the indicated C_{27} to C_{32} mycocerosic acids (Fig. 1B).

The detailed analysis given above highlights the serious problem of perpetuating the conclusions in the papers of Mark et al. (2010, 2011). This demonstrates the potential risks and hazards for workers coming into a field of research and attempting to utilize a particular unproven technique to provide quick answers to a particular question. It is imperative that such researchers obtain basic knowledge and carry out the groundwork to enable them to perform effective objective science before producing premature publications. It is apparent, in the broad field of "archaeological science", that some "artistic license" is more than acceptable to some researchers and certain editors of learned journals. In the present scenario, however, where specific chemical biomarkers are being used to diagnose ancient tuberculosis, there is absolutely no latitude in the identification of these distinct well-characterized chemical compounds. They are either positively identified or they are not and well-proven methods must be used along the guidelines advocated by Minnikin et al. (2010). Another most unsatisfactory aspect of the unconstructive publication by Mark et al.

(2011) is that, while the senior authors, on this paper, publically condemned the constructive paper of Minnikin et al. (2010), they were keen to collaborate in the use of HPLC analysis of mycolic acids to support their osteological studies. These collaborative enterprises, performed in good faith, have produced a joint publication (Lee et al., 2012) and joint conference presentations (Donoghue et al., 2010b; Pálfi et al., 2010).

4.4 Mycocerosic and mycolipenic acid biomarkers for tuberculosis

The current integrated strategy for the use of lipid biomarkers in tuberculosis diagnosis involves alkaline hydrolysis followed by conversion of released fatty acids to pentafluorobenzyl esters, which are separated into non-hydroxylated and mycolate fractions (Redman et al., 2009; Hershkovitz et al., 2008). The latter are derivatized and examined by HPLC, as described above but the former are analyzed by negative ion-chemical ionization gas chromatography-mass spectrometry (NICI-GCMS), using selected ion monitoring to detect the diagnostic mycocerosic and mycolipenic acids (Minnikin et al., 1993a). The protocol was applied to extracts of 49 skeletons from the 1837–1936 Coimbra Identified Skeletal Collection and an example of a positive tuberculosis diagnosis is shown in Fig. 6 (Redman et al., 2009). The m/z 407 peak (10.45 min) corresponds to C_{27} mycolipenate (Fig. 1B) with the others representing C_{27}, C_{29}, C_{30} and C_{32} mycocerosates (Fig. 1B). This pattern is characteristic of the MTBC complex (Minnikin et al., 1993a; Redman et al., 2009), a particularly diagnostic feature being the co-chromatography (11.36 min) of the C_{29} and C_{30} acids (Fig. 6). This phenomenon results from the larger C_{30} acid being relatively more volatile due to the additional methyl branch (Fig. 1B). There was a 72% correlation of the mycocerosic acid profiles with the Coimbra burial records (Redman et al., 2009). Mycolipenic acids were detected only in skeletons, which were positive for mycocerosates but only in one third of these. Mycolipenic acids are components of pentaacyl trehalose glycolipids, which are likely to be degraded more rapidly than the phthiocerol dimycocerosate waxes (Minnikin et al., 2002).

5. Conclusion

After a slow beginning, almost two decades ago, characterization of *M. tuberculosis* aDNA has been systematically developed in a limited number of laboratories. The analysis of aDNA can provide a wealth of information about the particular strain of *M. tuberculosis* diagnosed, but the exact information is governed by the degree of preservation. The fact that aDNA analyses are now firmly established is due partly to technological advances but mainly due to established skills in key laboratories, built up over an extended period. Conclusive results can be obtained by aDNA analyses alone, but confirmatory biomarkers are valuable in completing the overall diagnosis. The tubercle bacillus and related mycobacteria are rich in unusual lipids, which are not produced in mammalian tissues. Building on sensitive methods, previously developed to detect lipid biomarkers for modern tuberculosis, and inspired by the initial aDNA results, mycolic acid biomarkers for tuberculosis were detected for the first time in archaeological bone just over a decade ago. After a flurry of initial studies, time was taken to explore more robust mycolic acid methods and expand the range of lipids to include characteristic mycocerosic and mycolipenic acids. Currently, aDNA and lipid analyses are established as a powerful combination to diagnose both tuberculosis and leprosy and, indeed, co-infections; these analyses can be performed on

the same sample. This is not a static situation, however, as new powerful methods are becoming available for analyses of aDNA and lipids; additionally, the range of lipids available for detection is by no means exhausted.

The availability of established aDNA and biomarker protocols and expertise provides avenues into a range of interlocking research areas. As noted above, evolutionary pathways can be verified for tuberculosis, also providing evidence about virulence as the key lipid biomarkers are implicated in this process. The very existence of apparently intact key lipids in ancient samples is also of basic chemical interest; for example, the mycolic acids from the 9,000 year old Atlit-Yam skeletons probably represent the oldest known cyclopropane rings! It is important also to study leprosy, as the evolutionary processes of these two mycobacterial diseases are fatally intertwined with many clear co-infections. The relative prevalence of tuberculosis and leprosy can give clear indications of the prevailing social environment. In tuberculosis/leprosy co-infections it is becoming possible to estimate the relative bacterial load in particular bones and thereby obtain indications regarding which disease was the immediate cause of death. For each disease and co-infections it will be instructive to examine bones throughout particular individual skeletons in order to gain information about dissemination. Evidence of tuberculosis and leprosy can be quite clear in the osteological record, with characteristic bone lesions and deformations and bone loss in the case of leprosy. Biomarker analysis can help illuminate and reinforce the diagnoses of skilled osteologists and compare bacterial loads in bones with and without disease indication. An appealing aspect of lipid biomarker analyses is the possibility of extracting the lipids with neutral solvents, avoiding any chemical or physical damage to particularly valuable bone samples. It must be borne in mind, however, that biomarker analyses for the diagnosis of ancient mycobacterial disease are currently sophisticated procedures, which cannot be easily attempted without time being spent in accumulating the necessary skills and experience.

The evolution of life on earth is a complex web of competitive and/or symbiotic interactions. Humans, related primates and all mammals are dependent on a symbiosis with many microorganisms, whose cells outnumber those of the host. From the perspective of the microorganism, the mammalian host provides an ecological niche in which it can multiply and evolve to improve its prospects for survival. In some cases, the interaction of microorganisms with mammals is a rapid, pathogenic process resulting in the demise of the host and the infecting agent passes on to a new subject. Early hominids and humans with a hunter/gatherer lifestyle had a low population density, so an alternative relationship with slow-growing organisms such as the pathogenic mycobacteria, emerged. Such pathogens have a long-term relationship with their host, thus enabling persistence of the organism until transmission is possible. This is likely to occur at the extremes of life, when the host immune response is immature or less effective, and at times of physical or mental stress, often associated with war, famine, poverty or social unrest. This latter scenario is typical of the ancient scourges of tuberculosis and leprosy, caused by *M. tuberculosis* and *M. leprae*, respectively.

Several decades ago, it appeared that BCG vaccination and combinations of effective drugs were conquering tuberculosis. However, declining vaccination efficacy, misuse of drug regimens and the rapid spread of HIV/AIDS-related immunodeficiency, together with increased urbanisation and population density, have provided the opportunity for

accelerated evolutionary changes to *M. tuberculosis* and the emergence of highly drug-resistant and readily transmissible strains. Modern genomic approaches are also highlighting the great diversity existing within the inhomogeneous species labelled as *M. tuberculosis*. The developing science of paleogenomics is enabling approximate timelines for the evolution of *M. tuberculosis* to be laid down. The analysis of biomarkers is the prime way to verify the various proposed evolutionary pathways and to provide a direct timescale, rather than one inferred from bioinformatic analysis. In this review the contributions of ancient DNA (aDNA) and lipid biomarker analyses have been elaborated and critically assessed.

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7. References

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