Professor David Minnikin Memorial Lecture

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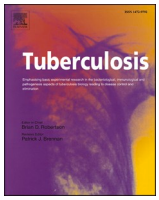

Review

Professor David Minnikin Memorial Lecture: An era of the mycobacterial cell wall lipid biomarkers

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ABSTRACT

This paper is dedicated to the memory of Professor David Ernest Minnikin (1939–2021). David was one of the key scientists who pioneered the field of *Mycobacterium tuberculosis* cell envelope research for over half a century. From the classification, identification, and extraction of the unusual lipids of the mycobacterial cell wall, to exploiting them as characteristic lipid biomarkers for sensitive detection, his ideas enlightened a whole world of possibilities within the tuberculosis (TB) field. In addition, his definition of the intricate models now forms a key milestone in our understanding of the *M. tuberculosis* cell envelope and has resolved many unanswered questions on the evolution of *M. tuberculosis*.

1. Background

Professor David Ernest Minnikin (1939–2021), the 'TB Lipid Man' sadly passed away aged 81 in 2021 following a short illness (Fig. 1). He was one of the principal scientists in the field of *M. tuberculosis* cell envelope research. He developed a broad interest across many different fields and published more than 400 original articles. Of which, some of his publications [1] have been cited more than 4800 times reflecting his significant contribution and impact on science.

1.1. Early life

David was raised by a farmer's daughter (Jane (nee Telfer)) and a gardener, Thomas Minnikin. He grew up in Newton, a village west of Newcastle upon Tyne. His determination and talent in academic studies began early in his childhood. He gained his first scholarship after sitting the entrance examination to the Lord Wandsworth College in Hampshire, UK. Later, he won his second scholarship to study chemistry at Trinity College, University of Oxford, UK and graduated in 1963. He developed an interest in *M. tuberculosis* research during his Ph.D. studies on 'The chemistry of the lipids of tubercle bacilli' under the supervision of Dr Nicholas Polgar at the Dyson Perrins Laboratory, University of Oxford in 1967. He continued as a Senior Research Officer in the Chemistry department at the University of Newcastle. During this time, David devised various extraction methods and used two-dimensional thin-layer chromatography to analyse the composition of polar lipids of the mycolic acid related taxa, including *Nocardia*, *Mycobacterium*, *Gordona*, *Corynebacterium*, *Bacterionema* and the "*Rhodochrous*" complexes [2]. He successfully exploited the difference in their lipid compositions to correlate to individual species. For example, polar lipids, such as diphosphatidylglycerol and phosphatidylinositol are found in all species; however, diacylated phosphatidylethanolamine was absent in *Corynebacterium* and *Bacterionema*. This suggests that both polar and apolar lipids could be used as fingerprints for the identification and classification of acid-fast bacteria after further extensive systematic studies in his later findings [3]. He successfully demonstrated growth conditions could be a factor interfering with the composition of the polar lipids [4]. In addition, David's novel lipid extraction methodology via a biphasic mixture to separate polar from apolar lipids in different phases within organisms, remained widely used in modern research, leading to more than 4800 citations [1]. Following his success and remarkable contribution, he was promoted to Senior Lecturer in 1985, and Professor at the University of Newcastle in 1995, before ending his career as a Professor at the University of Birmingham (Fig. 2).

1.2. The 'Minnikin' model

David deployed his novel biological chemistry approach to identify

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Fig. 1. A portrait of Professor David Ernest Minnikin painted by Dr Oona Y–C. Lee.

Fig. 2. Professor David Ernest Minnikin pictured with his son, Peter Minnikin at his 80-year-old's birthday.

and understand the lipids amongst several different mycobacterial species [5]. He successfully rewired the puzzle of the mycobacterial cell wall structure by proposing the first *M. tuberculosis* cell wall model (*aka* the 'Minnikin Model in 1982) [6]. Briefly, he proposed the presence of a tightly packed array of long-chain mycolic acids that esterified the arabinogalactan layer between the plasma membrane and outer membrane. David reported the presence of 'free' polar mycolate trehalose lipids in the outer leaflet as key virulence factors. This became a fundamental basis in *M. tuberculosis* research. This unusual hydrophobic barrier protects the pathogen, enabling it to survive within macrophages in the host's defence system. His insight into the mycobacterial cell wall and its lipid components led to the new developments underpinning drug treatment. For instance, the biosynthesis of mycolic acids is targeted by the first-line antibiotic isoniazid. As a chemist, David was enthused with studying the structure of the lipids in the mycobacterial cell wall, but his research passion extended into its biosynthesis and the role of other key lipids in the mycobacterial cell wall including the phthiocerols, mycocerosic acids, mycolipenic acids and phenolic glycolipids. These were further utilised to aid species identification. The structure of mycobacterial cell wall is still under ongoing extensive investigation [7,8].

David continued to devote his passion to the chemistry of mycobacterial lipids at the University of Newcastle from 1967 and further at the University of Birmingham in collaboration with Professor Gurdyal S. Besra FMedSci FRS since 2002 (Fig. 3). In 2005, he officially retired and stepped down to become an Emeritus Professor at the University of Birmingham to continue his research.

1.3. Retired with a new interest – the evolution of TB

Although, David officially retired in 2005, his passion for TB research never really ended. He embroiled himself in a new challenge and started his journey into the detection of ancient TB and charting its evolution across the field of archaeology/palaeontology/mycobacterial research. Owing to his strong chemistry background, David pioneered the introduction of the concept of deploying lipid biomarkers in archaeological research [9–12]. It is believed that both *M. tuberculosis* and *Mycobacterium leprae* are obligate common pathogens in ancient populations [13,14].

1.3.1. The challenges in the field of paleopathology

The common challenge in paleopathology is the insufficient osteolytic evidence to demonstrate infection caused by obligate *Mycobacterium* species. This is because.

1 Osteolytic changes (commonly found in ribs or weight-bearing joints) causing lesions in the anterior aspect of multiple vertebrates, classical collapsed spine or spinal angulation occurred only at the advanced stages of mycobacterial infection.
2 Only a low number (3–5%) of individuals infected with active TB possessed these osteolytic skeletal deformities in weight-bearing axial skeletons (e.g., vertebral bodies). In detail, the anterior aspect is compared to the posterior region, which involves up to four vertebrae, classic vertebral fusion only can be found in later stages, e.g., with the spine has collapsed and spinal angulation has occurred [15,16].
3 Ancient DNA (aDNA) studies remain very challenging due to their poor quality, low quantity and risk of contamination with modern DNA [17]. aDNA are susceptible to different forms of DNA damage, including fragmentation, blocking lesions and miscoding lesions [18]. In cases where aDNA was extracted from well-preserved archaeological remains, DNA amplification and genotyping methods were deployed initially to target small specific *M. tuberculosis* and *M. leprae* fragmented aDNA regions in detecting origins, spread and phylogeny. In spite of the introduction of Next Generation Sequencing (NGS) technology alongside sophisticated bioinformatic software, it is not always possible to obtain an analysis of the entire DNA within a sample without target enrichment approaches unless in exceptionally well-preserved material [13].

1.4. Mycolic acids – the first TB lipid biomarker

The cell envelope of *M. tuberculosis* consists of a thick waxy coat of unusual lipids arranged with a tightly packed polysaccharide and peptide layer, which makes up 40% of the total lipids of the bacterium. A high proportion of these unique lipids are present in the mycobacterial cell wall are the so-called mycolic acids. They are the most abundant lipids in the mycobacterial cell wall (up to 30% of the dry weight of the

Fig. 3. A recent photograph at the Institute of Microbiology and Infection, University of Birmingham of the TB group.

Mycobacterium) [8]. Mycolic acids vary in size and chemical type in different mycobacterial species [6], making it an ideal tool in mycobacterial species identification to support the identification of the causative organism in ancient infections for multiple reasons. Since the 1930s, several groups of scientists have been involved in resolving the structure of mycolic acids, including the research groups of Anderson, Lederer, Asselineau, Polgar, Goren, Brennan and Minnikin [6,19,20]. The pattern and the composition of mycolic acids in different mycobacterial species were analysed by two-dimensional thin-layer chromatography after degradation by both acid and alkaline methanolysis [21]. Later, David exploited them as unique lipid biomarkers as a tool to aid paleopathology and archaeological research. Through chemical derivatisation using a florescent probe, such as pyrenebutyric acid-pentafluorobenzy derivatives, the mycolic acid can be sensitively detected (up to femto-mole levels) in ancient samples using high-performance liquid chromatography (HPLC) without any amplification [12]. In addition, the unusually long carbon chains of mycolic acids are strongly resistant to extreme conditions and temperatures, protecting them from natural degradation. Through the use of such properties, lipid biomarker recognition has been decisive in pinpointing the oldest known cases of human and animal TB [9,12]; the former [9] through a woman and child from a pre-pottery settlement at Atlit-Yam, Israel (~9 kilo annum [ka]) and the latter [12] is an extinct *Bison antiquus* from Natural Trap Cave, Wyoming (~17 ka).

1.5. Other lipid biomarker - phthiocerol dimycocerosates

David was never short of innovative ideas, shortly following the establishment of mycolic acids as a biomarker for *M. tuberculosis* and *M. leprae*. He returned to the drawing board with his colleagues at the University of Birmingham, re-visited the mycobacterial cell wall model and searched for other unique lipid biomarkers [7,8,22,23]. Mycocerosates are long-chain multi methyl-branched fatty acids that are found in the strains of *M. tuberculosis*, *M. bovis*, *M. gastri*, *M. haemophilum*, *M kansasii*, *M. leprae*, *M. marinum* and *M. ulcerans* [24].

They are esterified mainly with phthiocerol and phenolphthiocerol long-chain diols, which form the free lipids termed phthiocerol dimycocerosates (PDIMs) and glycosyl phenolphthiocerol dimycocerosates, also known as phenolic glycolipids (PGLs) [6,8,10,25]. The main mycocerosates in *M. tuberculosis* vary between C_{27} to C_{32} with C_{32} being the major component. In contrast, mycocerosates in *M. leprae* vary between C_{27} to C_{34} with C_{30} being the major component. Their characteristic length and composition are used for species identification. Mycocerosic acid profiling was proven to be efficient and reliable in modern and ancient mycobacterial research via NICI-GCMS [10,12,22,26–29] and HPLC-MS [30].

Smaller phthiodiolone diesters are found in *M. kansasii*. Such biomarkers enlightened the opportunity to reveal the earliest mycobacterial infected case in animals e.g., detection of phtherceriols supported the observation of TB in case of bison [12]. The application of HPLC-HESI-MS has been also demonstrated in the Vác mummy collection (Váradi et al., 2021).

1.6. Significant ancient cases proven by multi-disciplinary approaches

The combination of various lipid biomarkers helped to differentiate and pinpoint the species in question. The analysis of a combination of mycolic, mycocerosic and mycolipenic acid and phthiocerol biomarkers provided incontrovertible evidence for TB in these landmark specimens. Even in those incidences with poorly preserved specimens, mycolipenic

acids potentially would be an extremely valubale biomarkers as they are very resistant to natural decay (Molnár et al., 2015 [31]). Some key studies are briefly discussed below. Ancient DNA together with lipid biomarkers helped confirmation of the paleopathology of TB and disease diagnosis in several cases. It was successfully proved that *M. tuberculosis* spread in ancient Egypt dating to about 600 BCE by using nested PCR of the IS6110 and mycolates, it was used as a disease diagnosis in an Egyptian mummy, a 50-year-old female, who proved more likely to die of TB rather than her original diagnosis of benign cystadenoma, which matched with the paleopathology of pulmonary exudate findings [32].

1.7. Co-existence relationship of host and pathogen

***M. tuberculosis*:** The studies of TB in archaeological specimens would help us to learn about the co-existence relationship between a host and a pathogen, with a view to tracing the evolution of pathogenic mycobacteria. By morphological and molecular methods, e.g., conventional PCR and lipid biomarkers, TB was proven in one of the oldest cases in a woman and infant, dating from 9250–8160 years ago. They were buried together in Atlit-Yam, Israel. It was believed that it was a pre-pottery settlement with early evidence of agriculture and animal domestication located in the Eastern Mediterranean. The lipid biomarkers were well preserved in an anaerobic environment due to the samples submerged under water with layers of soil. It supports the theory that the transmission of the tubercle bacillus is more likely to occur in a denser human population [9,33]. Similarly, *M. tuberculosis* was found in ancient Syria during early domestication [34]. The studies of TB in archaeology would help us to understand the evolution of this pathogen. Owing to a lack of solid TB infection in human cases before 9 ka BP (Before Present, BP), however an extinct bison skeleton aged 17 ka years old, it is proposed that mycobacterial infection could have evolved as a zoonosis [12,35].

***M. leprae*:** David always supported the idea of a multiple-disciplinary approach to analyse individual cases whenever possible. This includes adopting multiple methodologies ranging from pathological/morphological lesions, ancient DNA, whole genome sequencing and different lipid biomarkers. Scientists successfully proved the earliest leprosy case and learnt about how the disease spread and the evolution of *M. leprae* [36]. The diversity of genotypes and sub-genotypes of *M. leprae* provides information on how such a pathogen evolves but also the impact on the migration of the ancient human populations after infection, known as the migration-driven model [36]. It is believed that *M. leprae* has undergone an evolutionary bottleneck followed by clonal expansion. In which, Type 2F had also spread westwards to Britain by the early medieval period, the ancestral of the 3I strain were found in Great Chesterford in Britain and its isolates later found in southern Britain during the late medieval period and continental Europe and other parts of the world like Southern America [37]. The subsequent decline of *M. leprae* in Europe could be a result from a combination of factors e.g., increased host resistance. It is believed that individuals who are co-infected with both organisms would have a greater mortality death rate.

1.8. Significance

Although NGS technology would be an ideal tool to obtain genetic information in charting microbe evolution in ancient specimens [38,39], it is not readily available in poorly conserved imperative specimens. Alternative methodologies including the use of morphological/osteological analysis and lipid biomarkers should always be adopted and considered to provide evidence to the scientific community [25]. We have successfully proven TB and leprosy infections in numerous difficult cases, including identifying the oldest human case in Saujil, Argentina, (905–1030 CE), showing that the close social interaction within and between villages among the pre-Hispanic societies likely to be the contributing factor for TB transmission from northern Chile towards the northeast into the Yocavil valley [40]. Evidence of TB infection was also found in ancient Hungary in the Neolithic [41,42] and Medieval periods [43].

2. Conclusion

David successfully applied his knowledge of the mycobacterial cell envelope to develop novel sensitive detection methods based on the use of lipid biomarkers. Through the use of lipid biomarker-based diagnostic methods, he and his colleagues were able to demonstrate mycobacterial infection even in very challenging conditions (limited amount of poorly preserved ancient specimens). His contribution is not only to scientific research but his love and passion for teaching has indeed attracted a lot of young University students to eventually go into academic research, including Professor Gurdyal S. Besra (FMedSci FRS), Dr Houdini H.T. Wu (FIBMS PA-R), Dr Oona Lee (Fig. 4) and many more, who carried on with his spirit in scientific research. His positive thinking and attitude towards science have always been a great asset, he encouraged all scientists keep their minds open in research. The best quotation that summarises David's positivity in research would have to be 'the characterisation of ancient TB is not totally dependent on the recovery of intact genomes. Judicious combinations of ancient DNA fragments and specific lipid biomarkers provide unambiguous diagnosis, and these protocols are capable of refinement and extension' [44]. Without an intact whole genome, it might be not possible to find out the ancestor strain in the evolution directly, but this does not disprove or exclude the existence of the mycobacterial infection. He proposed that maybe pathogenic *M. tuberculosis* strains like those we recognize today emerged through association with the Pleistocene megafauna. Despite there being no direct proof of the co-evolution of humans and TB in the past to date [44], the diagnosis of TB infection in a Subalyuk Neanderthal child provides a signpost to a possible alternative explanation. In the late Pleistocene, there is mounting evidence that TB was widespread in a range of megafauna, with clear cases being observed in ancient bison metacarpals ([45]; [12,31,46]). 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 due to increased proportions of outer membrane apolar lipids providing enhanced whole cell hydrophobicity that correlates with facile aerosol transmission [7,23]. 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*" [23,25,31]. The emergence of clades of "rough" morphology TB bacilli, between ~50 and ~20 thousand years ago, may have contributed to the extinction of many classes of megafauna in that period [7,

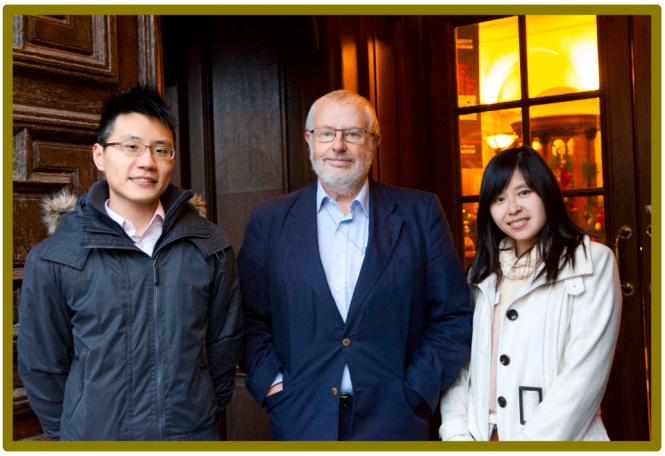

Fig. 4. A recent photograph of left to right: Dr Houdini H.T. Wu, Professor David Ernest Minnikin and Dr Oona Y–C. Lee at the Institute of Microbiology and Infection, University of Birmingham.

31]. Many of these megafaunas coexisted with Neanderthals in Western Eurasia so it is quite feasible that TB may have influenced the demise of the genus *Homo neanderthalis*.

David has resolved countless mysteries in the TB cell wall during his scientific career and demonstrated the possibility of making the impossible possible through his positivity and wisdom in science. His contribution and effort enabled us to advance so many steps closer to understanding the chemical structure and biosynthesis of these cell wall lipids as well as revealing the truth behind TB evolution. This surely will continue to help us in our future disease control and management.

Ethics statement

No ethical issues to report.

CRediT authorship contribution statement

Oona Y-C Lee: Conceptualization, Writing – original draft, Writing – review & editing, Visualization. **Houdini H.T. Wu:** Conceptualization, Writing – original draft, Writing – review & editing, Visualization. **Gurdyal S. Besra:** Conceptualization, Writing – original draft, Writing – review & editing.

Conflicts of interest

None.

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Transparency declaration

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