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The molecular mechanisms of *Mycobacterium tuberculosis* curli pili (MTP) in regulation of fatty acid metabolism, carbon metabolism, and bioenergetics of the pathogen and host during infection

by

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Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

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December 2024

DECLARATION 1: PLAGIARISM

I, Shinese Ashokcoomar, declare that:

- i. The work presented in this thesis is my original research;
- ii. This thesis has not been submitted to any university for the obtainment of an academic qualification;
- iii. No text/images/tables have been included in this thesis without references or consent and permission from the respective authors;
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- vi. There was no conflict of interests amongst the authors involved in any of the manuscripts/publications.



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As the candidate's supervisor, I agree with all aspects of this Doctoral thesis.



Supervisor: Professor Manormoney Pillay

13 December 2024

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DECLARATION 2: MANUSCRIPTS AND PUBLICATIONS

This Doctoral thesis consists of five manuscripts, three of which have been published in peer reviewed journals, one which has been accepted for publication in a peer reviewed journal and is in press, and one which has been reviewed and requires some revision prior to consideration for publication. Author contributions are stipulated below.

Manuscript 1 (Published):

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Author contribution:

Prof M. Pillay conceptualised and funded the study. Prof M. Pillay, Prof B. Pillay and Prof Loots designed the study. Ms Ashokcoomar and Ms Reedoy contributed equally to the study, conducted experiments, analysed the data and drafted the manuscript. Ms Beukes processed the samples and performed GC×GC-TOFMS. Ms van Reenen conducted statistical bioinformatic analyses. Dr Senzani provided RT-qPCR guidance and analytical support. All authors contributed to and approved the manuscript.

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Author contribution:

Prof M. Pillay conceptualised and funded the study. Prof M. Pillay, Prof B. Pillay and Prof Loots designed the study. Ms Ashokcoomar conducted experiments, analysed and interpreted the data, and drafted the manuscript. Ms Beukes processed the samples and performed GC×GC-TOFMS. Ms van Reenen conducted statistical bioinformatic analyses. All authors contributed to and approved the manuscript.

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Oral Presentation

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Poster presentation

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Mycobacterium tuberculosis curli pili (MTP) reduces the beneficial respiratory parameters of an infected THP-1 macrophage host.

Oral presentation

DEDICATION

To my beloved parents, Pradeep and Maureen Ashokcoomar

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LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Full name</u>
AIM2	Absent in melanoma 2
AECs	Alveolar epithelial cells
AES	Allelic exchange substrate
AMs	Alveolar macrophages
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
BCA	Bicinchoninic acid
BCG	Bacillus Calmette-Guérin
BDQ	Bedaquiline
BH-adjustment	Benjamini & Hochberg adjustment to control the rate of false
bp	Base pairs
°C	Degrees Celsius
¹³ C	Carbon-13 labelled
CCM	Central carbon metabolism
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CFUs	Colony forming units
CFZ	Clofazimine
cGAS	Cyclic guanosine monophosphate–adenosine monophosphate synthase
CI	Confidence interval
CLRs	C-type lectin receptors
CMST	Cell mitochondrial stress test
CO ₂	Carbon dioxide
COVID-19	Coronavirus disease 2019
COX	Cyclooxygenase
CRs	Complement receptors
C _t	Cycle threshold
CXCL	C-X-C motif chemokine ligand
2D	Two-dimensional
3D	Three-dimensional
2-DG	2-deoxy-D-glucose
DIM/PDIM	Phthiocerol dimycocerosate
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ECAR	Extracellular acidification rate
ESX	Early secreted antigenic target 6 secretion system
ETC	Electron transport chain
FAO	Fatty acid oxidation
Fc	Fragment crystallisable

FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazine
<i>g</i>	Gravitational force
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GC×GC-TOFMS	Two-dimensional gas chromatography time-of-flight mass spectrometry
GlycoPER	Glycolytic proton efflux rate
GRA	Glycolytic rate assay
HIF	Hypoxia-inducible factor
HIV	Human immunodeficiency virus
HIV/AIDS	Human immunodeficiency virus and acquired immunodeficiency syndrome
HLA	Human leukocyte antigen
hMDM	Human monocyte-derived macrophage
H ⁺ V-ATPase	Vesicular proton-pump ATPase
IFN	Interferon
Ig	Immunoglobulin
IGRA	Interferon gamma release assay
IL	Interleukin
IMs	Interstitial macrophages
INH	Isoniazid
iNOS	Inducible nitric oxide synthase
kDa	Kilo Dalton
KEGG	Kyoto Encyclopaedia of Genes and Genome
KZN	KwaZulu-Natal
LAM	Lipoarabinomannan
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LM	Lipomannan
LV	Latent variable
LZD	Linezolid
ManLAM	Mannosylated lipoarabinomannan
MATLAB	Matrix laboratory
Mce	Mammalian cell entry
MDR	Multidrug-resistant
MHC	Major histocompatibility complex
miRNAs	micro ribonucleic acids
mL	Millilitre
mm	Millimetre
MOI	Multiplicity of infection
MOPS	3-(N-morpholino) propanesulfonic acid
MR	Mannose receptor
mRNA	Messenger ribonucleic acid

MS	Mass spectrometry
MTA	5'-S-Methyl-5'-thioadenosine
mTOR	Mammalian target of rapamycin
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
MTBC	<i>Mycobacterium tuberculosis</i> complex
MTP	<i>Mycobacterium tuberculosis</i> curli pili
Δmtp	<i>mtp</i> -knockout mutant
<i>mtp</i> -complement	Complemented <i>mtp</i> -knockout strain
m/z	Mass-to-charge ratio
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide dehydrogenase
NADPH	Nicotinamide adenine dinucleotide phosphate
NF	Nuclear transcription factor
NIST	National Institute of Standards and Technology
nm	Nanometre
NMR	Nuclear magnetic resonance
NRLP	Nuclear oligomerization domain-like receptor protein
OCR	Oxygen consumption rate
OD _{600nm}	Optical density at 600 nanometres
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PER	Proton efflux rate
PIMs	Phosphatidyl-myo-inositol mannosides
PLS-DA	Partial least squares discriminant analysis
PMA	Phorbol 12-myristate 13-acetate
POC	Point-of-care
PPP	Pentose phosphate pathway
PRRs	Pattern recognition receptors
QC	Quality control
RIF	Rifampicin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
RR	Rifampicin resistant
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
16S rRNA	16S ribosomal ribonucleic acid
SEM	Standard error mean
SRs	Scavenger receptors
TB	Tuberculosis

TCA	Tricarboxylic acid
TDM	Trehalose dimycolate
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
TOF	Time-of-flight
μL	Microlitre
μm	Micrometre
V	Volts
VIP	Variable importance in projection
v/v	Volume/volume
WHO	World Health Organization
WT	Wildtype
XF	Extracellular
XDR	Extensively drug-resistant

ABSTRACT

Background/Aim:

The initial contact between pathogen and host is a vital step in the establishment of infection. *Mycobacterium tuberculosis* (*M. tuberculosis*) mediates adhesion to target hosts cells such as macrophages via various proteinaceous molecules known as adhesins, found on the cell surface. A distinguished adhesin of *M. tuberculosis* is *M. tuberculosis* curli pili (MTP). Previous functional genomics studies have described the involvement of MTP in bacterial aggregation and biofilm formation in addition to its role as an adhesin and invasin of epithelial cells and macrophages. Transcriptomic studies elucidated the importance of MTP in modulating host immune response *in vitro* and *in vivo*. Moreover, MTP was found to be present exclusively in the *M. tuberculosis* complex, and to bind to antibodies in patient sera. The use of metabolomics, bioenergetics and molecular biology will further improve current understanding of MTP as a virulence factor, thereby corroborating its role as a biomarker for the development of better tuberculosis diagnostics and therapeutics. Therefore, this study aimed to elucidate the effect of MTP on *M. tuberculosis* metabolism and expression of selected genes involved in fatty acid transport, β -oxidation, lactate oxidation, and gluconeogenic carbon flow. Furthermore, this study aimed to investigate the metabolomic and bioenergetic role that MTP plays in the pathogen-host infection model.

Methods:

Confirmed wildtype (WT) *M. tuberculosis*, *mtp*-deletion mutant (Δmtp) and *mtp*-complemented strains were used throughout this study. Bacterial cultures were standardised prior to harvesting of wet cell mass for metabolite and ribonucleic acid (RNA) extraction. The bacterial metabolite extraction was performed using a whole metabolome extraction method and the metabolites were detected using untargeted two-dimensional gas chromatography time-of-flight mass spectrometry (GC $\times$ GC-TOFMS) (Chapter 2). The RNA was extracted via the TriZol method for reverse transcription quantitative polymerase chain reaction (RT-qPCR) on selected genes to support metabolomic data (Chapters 2 and 6).

Host THP-1 monocytes were differentiated into macrophages prior to infection. Each of the bacterial strains were used to infect macrophages at a multiplicity of infection (MOI) of five. After four hours of incubation, host cells were used for: a) metabolomics via whole metabolome extraction for either detection with untargeted GC $\times$ GC-TOFMS (Chapters 3 and 4) or targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) with stable isotope carbon-13 ($^{13}\text{C}_6$)-glucose tracing (Chapter 5); b) bioenergetics through cell mitochondrial stress tests (CMSTs) and glycolytic rate assays (GRAs) performed in an extracellular flux analyser (Chapter 5); and c) lysis to harvest intracellular bacteria for RNA extraction to perform RT-qPCR (Chapter 6). All experiments were repeated to ensure reproducibility of the data, which were analysed by the appropriate statistical tests to highlight significant differences or similarities amongst the models, groups and samples.

Results/Discussion:

Bacterial RT-qPCR and functional metabolomics through GC $\times$ GC-TOFMS revealed that the Δmtp was associated with an altered cell wall composition. Of the 28 metabolites shortlisted, 19 of them were detected in elevated concentrations in the Δmtp compared to the WT, thus indicating that the lack of MTP reduces the overall ability of the pathogen to utilise these metabolites for biological processes. This was deduced from the high concentrations of carbohydrates involved in cell wall biogenesis and functions, and fatty acid metabolism. Additionally, alterations to amino acid concentrations further explained the previously reported growth retardation compared to the WT. *Mycobacterium tuberculosis* infected THP-1 macrophage models revealed significant alterations to central carbon metabolism (CCM), fatty acid metabolism and amino acid metabolism in the absence

of MTP. In particular, the Δmtp infected THP-1 macrophages demonstrated that nine of the GC $\times$ GC-TOFMS detected metabolites were significantly higher relative to the WT infected THP-1 macrophages. These altered concentrations were observed for metabolites involved in glycolysis/gluconeogenesis, tricarboxylic acid (TCA) cycle, fatty acid and amino acid metabolic pathways that feed into or are fed by CCM.

Given that *M. tuberculosis* establishes contact with both macrophages and epithelial cells during infection, a comparison of two different host cells were performed. Metabolite concentrations compared between the THP-1 macrophage infection model and A549 epithelial infection model demonstrated that the macrophages are more likely to advocate for pathogen clearance through a more efficient pro-inflammatory response, particularly in the absence of MTP, relative to the epithelial cells. Data presented here demonstrated that the MTP adhesin plays a role in securing a protective environment for *M. tuberculosis* during initial stages of infection.

Therefore, the THP-1 macrophage infection model was further investigated by targeted LC-MS/MS and bioenergetics to ascertain a more comprehensive understanding of the carbon flux, energy profiles and macrophage polarity in the absence and presence of MTP. The Δmtp infection model mimicked the oxygen consumption rate (OCR) for basal respiration, adenosine triphosphate (ATP) production, maximal respiration, and spare respiratory capacity of the uninfected THP-1 macrophages and had the highest compensatory glycolytic rate. Furthermore, the tracing experiments revealed that MTP has no effect on the flux of glucose, but rather has an impact on the total concentrations of the macrophage infection model. These findings indicate that MTP plays a role in modulating the macrophage respiratory parameters and dampens the pro-inflammatory response of the host. Hence, the macrophage host will be more proficient at launching an immune response if the adhesin is absent. The RT-qPCR performed on selected genes from pure bacterial culture and intracellular bacteria demonstrated that removal of MTP from *M. tuberculosis* alters lipid import, β -oxidation, and lactate oxidation of *M. tuberculosis*. Moreover, in all models in which the *mtp*-complement was used, partial restoration of the phenotype was observed as a result of the overexpressing episomal plasmid. Cumulatively, MTP was evidenced to play a multifunctional role for *M. tuberculosis*.

Conclusion:

The surface-located adhesin, MTP, is a virulence factor of *M. tuberculosis* as it initiates and facilitates attachment to, and invasion of hosts in addition to providing structural integrity to the pathogen. This adhesin enhances bacterial survival as it reprograms the *M. tuberculosis* and THP-1 macrophage metabolism to manipulate host effector functions. Collectively, this study provided metabolomic, bioenergetic and molecular evidence that MTP significantly contributes to the success of *M. tuberculosis* as an intracellular pathogen. Hence, the focus of TB intervention strategies should include intercepting the MTP interaction with the host to offset *M. tuberculosis* pathogenicity and confer an advantage to the host.

INTRODUCTION

Despite being a preventable and curable disease, tuberculosis (TB) is the leading cause of death caused by a single infectious agent. Globally ranking below coronavirus disease (COVID-19) and above human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) in the previous three years, TB returned to its first ranking in 2023 by causing 1.25 million deaths [1]. Annually, more than 10 million people, including men, women and children, fall ill with TB. Customarily, TB affects the lungs and manifests as pulmonary disease particularly during the first two years following infection [1]. Moreover, the persistence of drug resistance continues to exacerbate current TB treatment challenges with a globally estimated 400 000 people developing either multidrug-resistant (MDR) or rifampicin resistant (RR) TB in 2023 [1]. In an attempt to meet the WHO End TB Strategy targets for 2030 and 2035, urgent action is required to improve currently developed and implemented TB detection, prevention and treatment strategies.

Although the causative agent of TB, *Mycobacterium tuberculosis* (*M. tuberculosis*), was first identified in the late 1880s, the development and implementation of the first effective TB vaccine was seen in early 1920s. This vaccine, *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), is efficacious during infancy and early childhood and is not protective against pulmonary TB during adolescence and adulthood [1]. Regardless of the numerous vaccine candidates developed [1], only a few promising candidates make it to higher phases of clinical trials and obtain approval for TB prevention strategies [2]. Current TB treatment regimens span over months and is further aggravated by pre-existing conditions, degree of drug susceptibility/resistance and potential drug interactions [3]. The upsurge in drug resistance is due to the improper use of antibiotics in treatment of drug-susceptible patients as well as poor adherence by patients due to the long duration and toxicity of the regimens [4]. Advancements in TB treatments are desperately needed to shorten the current four-to six-month standard TB treatment regimens, thus, increasing the likelihood of patients completing treatment and clearing off *M. tuberculosis* infection.

The pathogenesis of *M. tuberculosis* comprises a series of events [5]. To understand *M. tuberculosis* pathogenesis, each stage of the infection process needs to be scrutinised. Characterising each stage of *M. tuberculosis* infection involves investigating the complex interplay between the immune response of the host and the immensely adaptable virulence factors of the pathogen. Thorough understanding of each stage of host-pathogen interactions prompts the identification of novel biomarker [6], pathway flux and adaptations to metabolic profiles [7; 8], which will enable the development of robust tools that could potentially control and eradicate TB.

A crucial stage during *M. tuberculosis* infection is the initial adherence of the bacterium to host-target tissues. In the case of pulmonary TB, host epithelial cells of the alveolar space and resident phagocytic macrophages are involved in the initial stages of pathogenesis [9]. Mediating the adherence of a pathogen to its host cell are specific externally exposed proteinaceous molecules, called adhesins [10]. Adhesins are notable virulence factors in *M. tuberculosis* pathogenicity [11]. Bacterial species do not rely on just one adhesin, hence produce multiple adhesins [12]. A notable adhesin of *M. tuberculosis* is *M. tuberculosis* curli pili (MTP), which surprisingly is encoded by *Rv3312A*, which is located between intermediary metabolism associated genes [13; 14]. Previous studies have shown that mutant strains lacking MTP were unable to bind to laminin *in vitro* [14]. Functional genomics and crystal violet quantification showed that the MTP mutant had a 68.4% reduction in biofilm mass in comparison to the wildtype (WT) strain [15]. Over and above its role as an adhesin and invasin of THP-1 macrophages [16], MTP was demonstrated to adhere to and invade A549 pulmonary epithelial cells [12]. Studies have reported differences in adherence and invasion levels between MTP-deficient and MTP-proficient strains of *M. tuberculosis*, which were derived from a drug-susceptible clinical strain of the F15/LAM4/KZN family [12; 16]. The *mtp* gene was shown to be exclusive to members of the *M. tuberculosis* complex (MTBC), and highly conserved among clinical strains of the pathogen

[17]. Furthermore, two separate studies that used a slot blot assay or an enzyme-linked immunoassay and demonstrated that MTP synthetic peptide or immobilised MTP fibres were able to respectively detect anti-MTP antibodies in serum/plasma samples from TB patients [13; 17]. These findings confirmed expression of MTP during infection, including HIV-TB co-infection [18]. Hence, studies that focus on adhesins and their initial interaction with the host during the infection process may provide useful information for the development of new and effective diagnostics and drugs or novel vaccine approaches. If the contact between *M. tuberculosis* and host is detected and compromised, it may be possible to halt the infection cascade at the initial point of host-pathogen contact.

Insight into the infection process and discovery of biomarkers can be achieved with a combination of research methodologies. The use of high-throughput processes underpinning “omics” technologies, which include genomics, transcriptomics, proteomics and more recently metabolomics, assists in achieving a holistic view of systems biology during the infection process [19; 20]. Individually, each technique produces specific data that reflect perturbations associated with the disease. The advancement of “omics” technologies in recent years have allowed scientists to understand the evolutionary history of *M. tuberculosis*, diversity between MTBC lineages, drug resistance and profiling, as well as host responses to infection and discovery of potential biomarkers [21].

Metabolomics is the identification and quantification of all metabolites (amino acids, lipids, sugars and fatty acids) within a biological system at a given time [22]. Perturbation of a biological system may result in differences in the composition of metabolites produced; hence metabolomics is a pivotal tool in understanding disease progression. There are different types of analytical techniques used in metabolomics. “Hyphenated techniques” are used to combine chromatographic and spectral technologies to identify metabolites [23]. One such hyphenated technique is comprehensive two-dimensional gas chromatography (GC×GC) coupled with time-of-flight mass spectrometry (TOFMS). The use of GC×GC-TOFMS is known to be a robust and powerful technique when investigating group-type and within-group separations [24]. Another technique is liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), which assists in the separation of specific analytes from matrix components. Application of LC-MS/MS facilitates the improvement of sensitivity and specificity through the separation of interferences, which usually cannot be differentiated by the mass spectrometer [25]. Moreover, metabolomics can use either untargeted or targeted approaches for metabolome profiling or ¹³C-isotopologue analysis of substrate breakdown/incorporation [26; 27].

Linking metabolomics and bioenergetics allows for the assessment of disease progression and these non-invasive technologies can be employed in TB research. Bioenergetics involves investigating the molecular physiology and chemistry of energy metabolism of a system [28]. Dysfunction caused by disease states have an adverse effect on energy profiles, which can be detected in real-time by extracellular flux analysers [29]. Bioenergetics provides insight into the relative functioning of electron transport chain (ETC) components during cellular respiration and acidification [28]. This technique enables a deeper understanding of energetic phenotypes and the subsequent signalling molecules within a system especially if used with infection models.

By combining reverse transcription polymerase chain reaction (RT-PCR) with quantitative PCR (qPCR), it is possible to measure ribonucleic acid (RNA) levels through the synthesis of complementary deoxyribonucleic acid (cDNA). This technique is termed reverse transcription quantitative PCR (RT-qPCR) and is the current “gold standard” in molecular biology when there is a need for rapid detection of gene expression changes [30]. Therefore, RT-qPCR is a widely acceptable technology that allows for the investigation of changes to gene expression in different host model systems and between knockout mutants.

Merging of various techniques, such as those described above, can potentially improve current knowledge regarding *M. tuberculosis* pathogenicity and virulence. This will be advantageous to the fight against TB. By combining gene knockout mutants, *in vitro* infection models, quantitative transcriptomics, global metabolomics, and bioenergetic profiling, it is possible to gather insight into

the process of *M. tuberculosis* infection. Consequently, this study aimed to investigate the impact of MTP in the regulation of pathogen and host metabolic profiles, gene expression and whole cell bioenergetics by exploiting targeted and untargeted metabolomics, RT-qPCR and bioenergetics. Data presented in this study will motivate for the use of MTP and putative pathway intermediates that are modulated by this adhesin, as potentially suitable biomarkers for the development of novel diagnostics, effective drugs and reliable vaccines against TB.

CHAPTER 1: Literature review

1.1. Tuberculosis

Tuberculosis (TB) is a contagious infection, caused by the bacterium *Mycobacterium tuberculosis* (*M. tuberculosis*) [1]. This disease can affect the bone and central nervous system of animals and humans, but it most prominently manifests as a pulmonary disease [31]. The pathogenesis of TB is initiated when *M. tuberculosis*, present in aerosol droplets, are deposited onto the alveolar surfaces of the lung [31]. Thereafter, a complex host immune response is triggered, which involves various immune cells that play a crucial role in determining if an active disease develops or the infection is controlled [32]. Approximately 90% of individuals exposed to *M. tuberculosis* do not develop the disease but potentially develop a latent infection [33]. This latent infection has a 5% chance of developing into active disease within the first two years of exposure, and an additional 5% chance of it developing into active TB later on, particularly for individuals with high risk factors [33; 34]. High risk individuals are those that have pre-existing health conditions that lead to an immunocompromised state [33]. Regardless of TB being a curable disease, its persistence is not only attributed to latency, but also accelerated by microbiological complexities of *M. tuberculosis* strains, drug resistance and effectiveness of the human immune response [34; 35]. Moreover, despite the progress made in TB diagnostics and treatments, there is still a lack of affordable, efficient and rapid point-of-care (POC) diagnostic tests, fast and effective treatment and a lifelong efficacious vaccine [36]. Therefore, anti-TB treatment strategies are considerably hindered and has led to major global concerns for public health.

1.1.1. Epidemiology

In 2023, TB was globally ranked as the leading infectious killer with an estimated 1.25 million deaths [1]. Of the estimated 10.8 million individuals who fell ill with TB in 2023, 5.9 million (55%) were male, 3.6 million (33%) were female and 1.3 million (12%) were children below the age of 15 years [1]. The newly diagnosed cases for 2023 were reported to be 8.2 million, the highest number seen since TB reporting began in 1995 and exceeded the previous peak of 7.5 million in 2012 [1; 37]. Thirty of the world's high TB burden countries account for 87% of TB cases reported in 2023 [1]. Moreover, there was a globally estimated 400 000 individuals who developed multidrug-resistant or rifampicin resistant TB (MDR/RR-TB) in 2023 [1].

Africa reported close to a quarter of the global TB burden for 2023, with 400 000 deaths making up 32% of the global deaths [1]. As one of the 30 countries, South Africa reported a TB incidence rate of an estimated 427 000 cases per 100 000 population, with estimated totals of 13 000 cases of MDR/RR-TB and 56 000 deaths in 2023 [1]. The high prevalence rate of human immunodeficiency virus infection (HIV) in South Africa has mainly driven the high TB incidence and mortality rates prior to 2009 [38]. Since then, the upscaling of antiretroviral treatment along with the country's efforts to meet the WHO's End TB Strategy milestones (for 2020 and 2025) and targets (for 2030 and 2035) has resulted in a decrease in TB incidence by more than 50% between 2015 and 2023 (Figure 1.1)[1]. However, despite the improvement in statistics, TB remains a major concern as 56% of South Africans suffer catastrophic costs associated with current TB treatment [38]. This could potentially cause a reversal of the downward trend observed in reported TB cases since the disease governs poverty-stricken populations that have biosocial risk factors such as undernutrition and overcrowding [35].

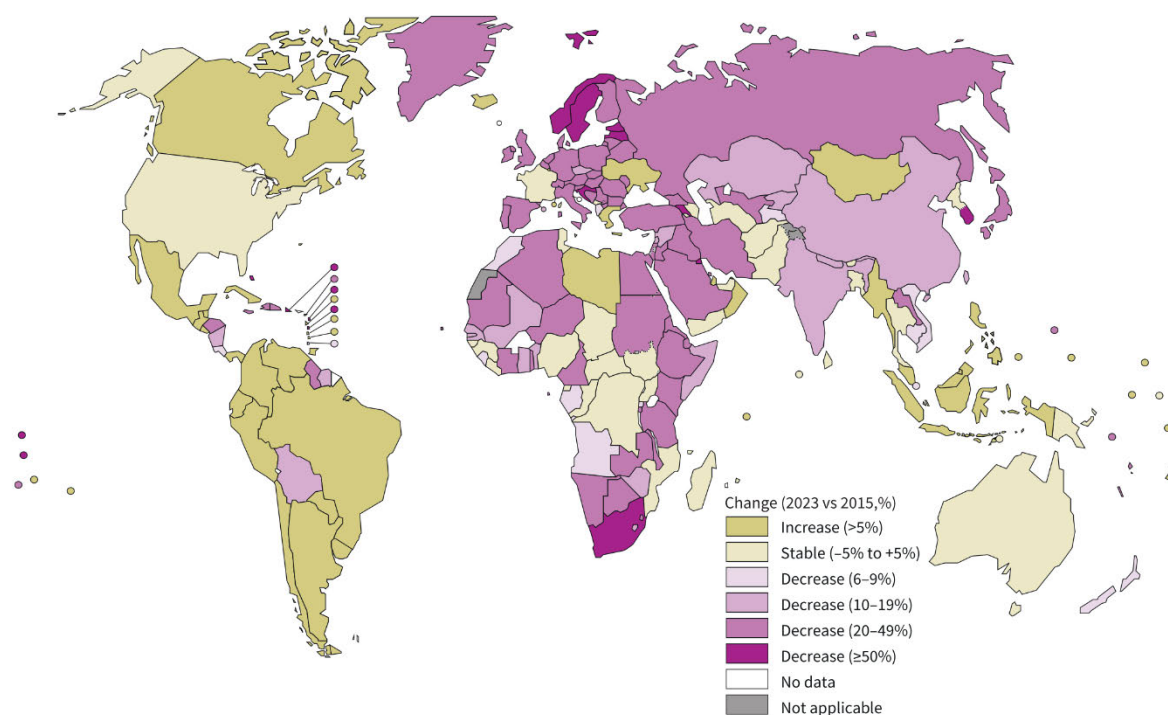


Figure 1.1. Percentage change in the global estimated TB incidence (new cases per 100 000 population) from 2015 to 2023. South Africa is one of the few countries that has reduced TB incidence rates by more than 50% in the last eight years [1].

1.1.2. Drug resistance

The person-to-person transmission, especially of drug-resistant strains of *M. tuberculosis*, is a huge challenge in the fight against TB [39]. *Mycobacterium tuberculosis* drug resistance can be classified as mono-resistant, poly-resistant, MDR, pre-extensively drug-resistant (pre-XDR TB), and extensively drug-resistant (XDR) TB. Mono-resistant and poly resistant TB disease is caused by *M. tuberculosis* strains that are resistant to one or at least two TB treatment drugs, respectively [39]. However, poly-resistant bacteria cannot be resistant to both isoniazid (INH) and rifampicin (RIF) [39]. When the pathogen is resistant to at least INH and RIF, the disease is considered to be MDR TB [39]. Pre-XDR is a result of *M. tuberculosis* being resistant to first-line INH, RIF, and a fluoroquinolone or second-line injectable such as amikacin, capreomycin, and kanamycin [39]. Lastly, most severe is XDR, and this is a result of the bacteria being resistant to INH, RIF, a fluoroquinolone, and a second-line injectable or bedaquiline (BDQ)/linezolid (LZD) [39]. In 2023, 3.2% of the world's new TB cases were MDR/RR-TB and, of them, 19% were pre-XDR [1].

Over decades, the development of drug resistance is a result of inappropriately prescribed and misused anti-TB medicines, poor quality drugs, and the incompleteness of the full treatment regimen by patients once TB symptoms subside [4]. Antimicrobial drug resistance in *M. tuberculosis* is mainly attributed to mutational events within the genome of the ever-evolving pathogen, as the presence of the cell wall makes acquisition of external genetic material rather difficult [40]. These evolutionary mutational events include deletion and duplication events, and single nucleotide polymorphisms [41]. Therefore, the *M. tuberculosis* population remains strongly clonal and if a resistant sub-population survives drug treatment, anti-microbial resistance becomes apparent [40]. Thus, varying degrees of drug resistance have been reported to be associated with specific *M. tuberculosis* lineages [42].

1.1.3. *Mycobacterium tuberculosis* complex and lineages

Mycobacterium tuberculosis makes up part of the *M. tuberculosis* complex (MTBC) that is responsible for animal and human TB. The other MTBC members include *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium canettii*, *Mycobacterium caprae*, *Mycobacterium microti*, and *Mycobacterium pinnipedii* [43]. The MTBC has been classified as a single complex as these species share a common ancestor, since they display genetic homogeneity with approximately 99% nucleotide identity [43]. However, within the MTBC there are distinct phylogenetic lineages that account for diversity in pathogenic virulence, transmissibility, host response and antibiotic resistance that is believed to have co-evolved with humans over millennia [44].

Lineages of MTBC are classified by the geographical location that they predominantly dominate but are not limited to these areas: Lineage 1 (Indo-Oceanic lineage), Lineage 2 (East Asian lineage, which includes the Beijing families), Lineage 3 (Central Asian strain/Delhi [CAS/Delhi]), Lineage 4 (Euro-American, which includes Latin American-Mediterranean [LAM]), Lineage 5 (West African 1), Lineage 6 (West African 2), Lineage 7 (Ethiopian), Lineage 8 (Central Africa), and Lineage 9 (East Africa) [45]. The most widespread lineages across the globe are Lineages 2 and 4, which are believed to be more virulent than other lineages of the MTBC as they are responsible for outbreaks and MDR/XDR TB infections [44; 46]. Local evidence of this includes phylogenetic studies in the Western Cape of South Africa, which identified Lineages 2 and 4 as the causes of XDR TB infections, thus demonstrating the distinct virulence characteristics that aid in transmission of these lineages [46]. Moreover, a *M. tuberculosis* strain from the F15/LAM4/KZN family originating at the Tugela Ferry hospital in KwaZulu-Natal, South Africa, was reported to evolve from MDR to XDR within a decade [40; 47]. Therefore, high TB burdened countries harbour a wide spectrum of strain diversity and drug resistance phenotypes. For example, the F15/LAM4/KZN family of Lineage 4 includes the drug-susceptible V9124, the MDR V1435 as well as the XDR KZN605 [48]. The successful spread of drug-resistant strains has been attributed to gene mutations that have a minimal effect on the fitness cost of *M. tuberculosis* [48-50].

1.1.4. Unique phenotypic characteristics of *Mycobacterium tuberculosis*

Differences in the constitution of the “mycomembrane” are associated with adaptation to species-specific variation as a result of environmental interactions and ecological niches [51]. Favourable for *M. tuberculosis* pathogenicity, the adapted cell envelope is important for virulence and intrinsic antibiotic resistance [52]. A distinguishing feature of *M. tuberculosis* is its characteristic multilayered complex cell envelope composed of the universal phospholipid bilayer plasma membrane, the outer membrane, the unique mycomembrane and the outermost layer [52; 53]. Of the diverse range of structural components such as polysaccharides, proteins and lipids, the long-chain fatty acids form a waxy layer and accounts for approximately 60% of the cell wall weight [52; 54]. Therefore, apart from the structural integrity conferred by the *M. tuberculosis* cell architecture, the unique cell wall structure provides the pathogen with low permeability and extreme hydrophobicity, thus making it resistant to many antibiotics and host immune responses [52].

The typical plasma membrane enclosing the cellular contents is surrounded by a highly cross-linked peptidoglycan layer and forms covalent complexes with arabinogalactan [53]. These carbohydrate polymers are dynamically organised and provide a thick physical mesh-like barrier for protection against microenvironmental damage [53]. Arabinogalactans are esterified to the distinctive mycolic acids, which are long, high molecular weight α -alkyl and β -hydroxy fatty acids. These account for a major fraction of the cell envelope and confer further protection against hydrophilic antibiotics, oxidative damage, and complement deposition [53]. Lastly, mycosides make up the outermost layer, which comprise agglutinin antigens, identified as of sugar moieties [54]. Some complex glycolipids include, sulfolipids, phthiocerol dimycocerosates (DIM/PDIM), trehalose dimycolate

(TDM), glycopeptidolipids, phosphatidyl-myo-inositol mannosides (PIM), and lipoglycans such as lipomannan (LM), lipoarabinomannan (LAM) and mannose caps of mannosylated lipoarabinomannan (ManLAM) [53]. Mycobacterial glycolipids interact with receptors in host cells and thus, facilitates bacterial internalisation and host immune response modulation [53]. Additionally, protein transport across this unusual cell envelope is enabled by secretion systems such as the early secreted antigenic target 6 (ESAT-6) type VII secretion system (ESX/Type VII) [55; 56].

Mycobacterium tuberculosis is chemoorganotrophic, non-motile, non-sporing, acid-fast and weakly gram-positive bacilli [57]. The bacilli microscopically appear as straight or slightly curved rods, approximately 1 to 4 μm in length and 0.3 to 0.6 μm in width [57; 58]. This slow-growing aerobic bacterium replicates approximately every 24 hours, taking between 7 to 30 days to be visualised on specialised agar [57-59]. Specialised *M. tuberculosis* culture agar includes Lowenstein-Jensen media or Middlebrook 7H11/7H10 on which bacterial colonies appear either buff and granular or creamy white, raised, irregular in shape, dry and wrinkled on the surface, respectively [54; 60]. *Mycobacterium tuberculosis* is cultured at 37°C, thus emulating the human body temperature at which it thrives [57].

1.2. Host defence systems

Protection against disease is attributed to the host immune response, which can be categorised into innate immunity and adaptive immunity (Table 1.1) [61; 62]. These two systems work together, and involve specific host cell types with specific functions that determine disease outcome and/or progression.

Table 1.1. Components of innate and adaptive immune systems [62].

Innate Immune System		Adaptive Immune System
Physical Barriers	Internal Defenses	
• Skin, hair, cilia • Mucus membranes • Mucus and chemical secretions • Digestive enzymes in mouth • Stomach acid	• Inflammatory response • Complement proteins • Phagocytic cells • Natural killer (NK) cells	
		• Antibodies and the humoral immune response • Cell-mediated immune response • Memory response

1.2.1. Innate immunity

Known as a nonspecific system, the innate immune response is triggered within the first few hours of infection. This system provides protection against pathogens by either forming physical barriers or being the first responder immune cells [61; 62]. Physical barriers comprise outer and inner surfaces like the skin and mucus membranes, respectively [62]. These closed surfaces prevent pathogens from gaining entry into the body or produce acid, enzymes and mucus that stop growth of invaders [61; 62]. Additionally, certain movements of hair-like structure such as cilia in the lungs, prevent pathogens from settling [61]. Within the lung, alveolar epithelial cells (AECs) form an epithelial barrier [63]. If this barrier/epithelial lining is compromised, immune system cells like phagocytes (macrophages and neutrophils), dendritic cells, eosinophils, and natural killer cells come into play

with their particular functions [62; 64]. These cells recognise and eliminate the invading microorganism through various cellular components and signalling pathways.

Monocytes differentiate into macrophages, which are essential for phagocytosis of foreign particles and cell debris, a process that is initiated by activation of surface receptors [63; 65]. Macrophages signal other immune effector cells to the site of infection through the secretion of cytokines. Additionally, macrophages can present antigens to T cells of adaptive immunity [66]. Neutrophils are an abundantly found type of white blood cell that phagocytoses cells and releases antimicrobial substances like reactive oxygen species (ROS) and enzymes that are known to kill invading pathogens [67]. Neutrophils rapidly respond to the site of infection and thus are critical for innate immune response [63; 67]. However, it is argued that eosinophils are the first granulocytes to be recruited to the airways following *M. tuberculosis* infection and within a week they can outnumber neutrophils [63; 68]. Eosinophils release cytotoxic granules and secrete cytokines and chemokines, thus they are involved in a wide range of immune mediated responses [63]. Moreover, natural killer cells are lymphocytes that have well established roles in combating viral infections such as HIV and they recognise and kill infected cells without any prior sensitisation [63]. This is done by the release of cytotoxic granules that contain perforin and granzymes [69]. Additionally, natural killer cells secrete cytokines and interferon gamma (IFN- γ) to enhance host immune responses [70]. Lastly, dendritic cells are professional antigen-presenting cells, therefore, they are responsible for capturing and processing of pathogenic antigens to T cells in the lymph nodes [63; 70]. They can also shape the adaptive immune response by producing cytokines that influence differentiation and activation of T cells, thus creating crosstalk between innate and adaptive immunity [63; 70].

1.2.2. Adaptive immunity

The adaptive immune system is signalled to specifically target the invading pathogen. Although this defence mechanism is slower than the innate system, it is more accurate when it responds to the invading pathogen [61]. This type of immune response is a long-standing one, in which the invading pathogen is quickly identified in the event of re-infection [61]. Adaptive immunity can be triggered by natural or artificial means such as a naturally occurring infection or an artificially induced response via vaccination [61]. The adaptive immunity comprises of lymphocytes and antibodies [61].

B cells are the lymphocytes that play a role in humoral immunity, hence they differentiate into plasma cells to produce antibodies [62; 64]. The antibodies produced have the potential to neutralise the invading pathogen, opsonise it for phagocytosis, or activate the complement system [71]. B cells can also facilitate antigen-specific T cell activation and proliferation via antigen presentation [72]. T cells activate through antigen presentation and can be divided into subtypes such as helper T cells (CD4⁺ T cells), cytotoxic T cells (CD8⁺ T cells) and regulatory T cells (Tregs) [72-74]. The helper T cells prompt different aspects of immune response based on its subsets and they assist B cells and cytotoxic T cells through cytokines [73]. Infected cells are directly killed by cytotoxic T cells, which recognise antigens presented by major histocompatibility complex (MHC) class I molecules, thereby inducing apoptosis through the release of cytotoxic enzymes [72]. Immune tolerance is maintained by Tregs to ensure that the immune response does not become overly aggressive [74]. Furthermore, both B and T cells can differentiate into memory cells after an initial immune response [75]. This ensures that upon re-exposure to the same antigen, the host can quickly produce antibodies [75]. Hence, memory cells are critical to the effectiveness of vaccines that establish long-lasting immunity against specific diseases [62].

1.2.3. Macrophages and regulation of immune response

As briefly mentioned above, macrophages are responsible for the activation of innate and acquired host immune responses; therefore, macrophages are fundamental in the containment or elimination of infection. However, under certain circumstances, macrophages provide an opportunity for growth and survival of *M. tuberculosis*. These protective host immune cells are derived from hematopoietic cells present in the bone marrow [76]. In peripheral blood, pro-monocytic cells differentiate into mature monocytes and once migrated into tissue monocytes further differentiate into macrophages to respond to inflammation/infection or maintain homeostasis [77]. Differentiation of the macrophage cell line is largely dependent on tissue type, growth factors and cytokines present [78]. In the laboratory, natural systems can be replicated using cell lines. The human cell line THP-1 is one of the most frequently used model system with monocytic properties [79]. Essentially, differentiation of THP-1 monocytes into macrophage-like cells, which resemble mature macrophages is induced by activation of protein kinase C with phorbol 12-myristate 13-acetate (PMA) [80]. This ultimately increases adherence and results in loss of proliferative activity of cells [80].

Macrophages can broadly be divided into alveolar macrophages (AMs), present in the airways, and interstitial macrophages (IM), present in lung parenchyma [63]. In the lung, IMs produce interleukin 10 (IL-10), which regulates T cells and dendritic cells during infection [63]. Whereas, AMs comprise more than 95% of cells found in the bronchoalveolar lavage and are essential for maintaining barrier integrity, lubrication, and removal of compromised cells [78]. Therefore, pathogen recognition by AMs triggers the adaptive immune system, subsequently facilitating successful elimination of the pathogen with minimal damage to the host [81; 82].

Furthermore, based on the mode of activation, macrophages are broadly classified as M1 (pro-inflammatory) or M2 (anti-inflammatory). Pro-inflammatory macrophages heavily rely on glycolysis, whilst anti-inflammatory macrophages rely on oxidative phosphorylation (OXPHOS) [83]. During *M. tuberculosis* infection, pro-inflammatory macrophages display high levels of tumour necrosis factor alpha (TNF- α), IFN- γ , IL-6, IL-12, IL-1 β , IL-15, IL-23, C-X-C motif chemokine ligand 10 (CXCL-10), cyclooxygenase-2 (COX-2), and ROS/reactive nitrogen intermediates in addition to low levels of immune-regulatory molecules such as IL-10, IL-4, transforming growth factor beta, and COX-1 [84-87].

Anti-inflammatory mediators such as signal transducer and activator of transcription (STAT) 3 and 6, proto-oncogene C-Maf, and hypoxia-inducible factor (HIF) 2 α stimulate the production of immune effectors that create a permissive environment for the pathogen [88; 89]. Some of these effectors include IL-10, IL-1 receptor antagonists IL-1Ra, IL-4Ra, arginase 1 and 2, and peroxisome proliferator-activated receptor gamma [90]. Anti-inflammatory macrophage phenotype may favour persistent infections and give rise to MDR or XDR TB as a result of treatment failure [91].

1.3. Tuberculosis pathogenesis

The general process of successful disease establishment for most pathogens involves a series of events, which include exposure to/contact with pathogen, adhesion/colonisation of target cells, invasion, and infection of the host [92]. In the case of pulmonary TB, invasion of the host can be followed by dissemination of *M. tuberculosis*, latent infection and reactivation of disease [5].

1.3.1. *Mycobacterium tuberculosis* transmission, initial contact and invasion

Active TB patients propel *M. tuberculosis* bacilli in aerosolised droplets when coughing, sneezing or talking, thus allowing transmission of the disease to healthy individuals [5]. Once inhaled, *M. tuberculosis* travels through the respiratory tract to the alveoli of the lungs. Thereafter, the pathogen

makes initial contact with either AECs or resident AMs of the host, which make up the first line of defence as a physical barrier or professional phagocyte, respectively [93]. Phagocytic cells use pattern recognition receptors (PRRs) to detect *M. tuberculosis* pathogen-associated molecular patterns (PAMPs) [94]. Host PRRs include toll-like receptors (TLRs), C-type lectin receptors (CLRs), fragment crystallisable (Fc) receptors, complement receptors (CRs), scavenger receptors (SRs), and cytosolic deoxyribonucleic acid (DNA) sensors [94]. Cellular receptors facilitate the interaction between the pathogen and host, subsequently leading to adhesion, bacterial internalisation and immune activation [93; 95].

Interaction between host TLR2 and *M. tuberculosis* lipoproteins, LM, LAM and PIM facilitates phagocyte activation without immediate ingestion of the pathogen [96]. Furthermore, unmethylated cytosine-phosphate-guanine (CpG) motifs of bacterial DNA are recognised by host TLR9 [97]. These events cause a signalling cascade via myeloid differentiation primary response protein 88 (MyD88), which facilitated the production of nuclear transcription factor NF- κ B and pro-inflammatory cytokines [94].

Host mannose receptor (MR) is a type of CLR, which recognises mycobacterial LAM, ManLAM and PIM, thereby inducing the production of anti-inflammatory cytokines and failure of activation of host oxidative responses [94]. Moreover, MR ligation to TDM induces production of pro-inflammatory cytokines, generation of Th1/Th17 immune responses and induction of granuloma formation [98]. Hence, this pathogen-host interaction connects both the innate and adaptive immune mechanisms.

The Fc receptors and CR expressed on the host surface, particularly CR3 on macrophages, recognise *M. tuberculosis* PIMs and subsequently facilitate phagocytosis of the pathogen [78]. Moreover, SR known as macrophage receptor with collagenous structure (MARCO), recognises TDM and cooperates with TLR2 to activate NF- κ B and pro-inflammatory responses [99]. Cytosolic DNA sensors recognise the mycobacterial DNA present in the cytosol and are mediated via the cytosolic sensors cyclic guanosine monophosphate–adenosine monophosphate synthase (cGAS) or absent in melanoma 2 (AIM2) [100].

1.3.2. Immune evasion and dissemination of *Mycobacterium tuberculosis*

Hosts innate immune systems often immediately eliminate the invading pathogen through different bactericidal mechanisms. The efficacy of these mechanisms depends on the intrinsic microbicidal capacity of the host phagocytes (dictated by genetics of the individual infected) along with the innate virulence factors of the *M. tuberculosis* strain inhaled. Both of these factors are accountable for the initial containment of the infection or lack thereof [101]. Remarkable strategies employed by *M. tuberculosis* to evade host killing include the stimulation of PRRs, which triggers bacteria internalisation, interference of phagosome maturation, cytosol access and control of host cell death [94].

Following internalisation, *M. tuberculosis* gets trapped in a phagosome which undergoes fusion with lysosomes in a process termed phagosome maturation. This process is regulated by host Ras-associated binding protein guanosine triphosphate enzymes (Rab GTPases) [102]. Concurrently, phagosomal acidification occurs by the sudden drop of pH from neutral to 5 via vesicular proton-pump ATPase (H^+ V-ATPase) to ensure optimal activity of lysosomal digestive enzymes and ROS production [103]. Additionally, infected cells undergo autophagy triggered by cytosolic lectin (which identifies damaged pathogen-containing vacuoles for anti-bacterial autophagy) or ubiquitin ligases, which clears the pathogen via ubiquitin-mediated autophagy [104; 105].

Mycobacterium tuberculosis interference with phagosome maturation occurs through prevention of phagolysosome biogenesis and phagosomal acidification. This is achieved when the mycobacterial vacuole fails to mature because Coronin1 (an actin binding protein) is recruited to the phagosomal surface where it is retained to activate calcium-calcieneurin signalling, which blocks phagosome-lysosome fusion [106]. The host membrane trafficking regulatory lipid, phosphatidylinositol 3-

phosphate (PI3P), is inhibited by ManLAM and secreted acid phosphatase, thereby preventing phagosomal acquisition of the lysosomal cargo and delivery of hydrolytic enzyme [107-109]. Phagosome acidification is blocked by inhibiting the assembly of the H⁺ V-ATPase complex and its fusion with the phagosomal membrane [110; 111]. *Mycobacterium tuberculosis* secreted tyrosine phosphatase, PtpA, directly interacts with subunit H of the H⁺V-ATPase complex and results in the stabilisation of the pH between 6.2 and 6.5 [110; 111]. Moreover, *M. tuberculosis* causes the depletion of H⁺ V-ATPase from its phagosome by subverting the function of host immune regulator, to selectively target H⁺ V-ATPase subunit A for ubiquitination and degradation by the proteasome [112]. In addition, TDM interacts with CLR Mincle, which causes delays to phagosomal maturation and acidification [113].

Cytosolic access is established by *M. tuberculosis* through the ESX-1/ Type VII system and intact functional DIM/PDIM production [114; 115]. It occurs when DIM/PDIM favour intracellular bacterial replication, phagosomal damage and rupture [115]. *Mycobacterium tuberculosis* entry into the cytosol is detected by cytosolic receptors, which affect the fate of host and pathogen by influencing the innate and adaptive immune responses [56]. *Mycobacterium tuberculosis* is given the advantage since its DNA is sensed by cGAS, subsequently leading to the expression of type I interferons (IFN- α/β), believed to be disadvantageous to the host [94]. Alternatively, cytosolic *M. tuberculosis* DNA is detected by AIM2, partially activating the nuclear oligomerisation domain (NOD)-like receptor protein (NLRP3) inflammasome to release inflammatory cytokines IL-1 β and IL-18 [116]. Lastly, ESX-1 mediated cytosolic translocation of bacterial DNA results in the activation of Tank-binding kinase 1 (TBK-1), which initiates autophagic activity [94].

Host cell death can occur via apoptosis (programmed cell death) or necrosis [117]. Apoptosis is an energy-dependent process mediated by the caspase cascade and occurs via TNF- α activation as well as caspase 3 and 8 [94; 118]. Apoptosis is caused by apoptotic vesicle formation and is associated with suppression of inflammation and T-cell immunity [94]. *Mycobacterium tuberculosis* genes that have been reported to influence the induction or inhibition of host cell death include those encoding superoxide dismutase A (*sodA*) and nicotinamide adenine dinucleotide dehydrogenase (NADH) 1 subunit G (*nuoG*) [119; 120]. Induction of macrophage apoptosis can result in a reduction in bacterial viability of *M. tuberculosis*. Necrosis on the other hand enhances bacterial replication and allows the release of viable mycobacteria for dissemination and subsequent re-infection [121]. Necrotic cell death is involved in granuloma formation and inflammatory tissue damage which ultimately results in transmission of *M. tuberculosis* [118].

1.3.3. Granuloma formation and latent *Mycobacterium tuberculosis* infection

Host recruitment of immune cells to the site of infection continues in attempts to sequester *M. tuberculosis* and limit further multiplication by the formation of granulomas [122]. During this stage, the infected individual may develop latent TB in which the bacteria remain viable, but infection is kept at bay [5]. Due to chronic cytokine stimulation, macrophages differentiate into epithelioid cells, which fuse to become giant cells that make up part of the granuloma structure [123]. Granulomas are characteristically well-organised collections of immune cells (neutrophils, macrophages and T lymphocytes) that restrict the spread of a *M. tuberculosis* infection to additional tissue [101]. Pro-inflammatory cytokines (IFN- γ , TNF- α , IL-6, IL-12, IL-17, and IL-23) play an important role in the formation and stability of granulomas [124]. Additionally, chemokines like CCL2, CCL3, CCL5, CXCL8, and CXCL10 play a key role in granuloma formation and are required for the recruitment of inflammatory cells [123]. However, granulomas are safe microenvironments for *M. tuberculosis* to establish latency as *M. tuberculosis* continues growing at a controlled rate within granulomas [101]. Newly formed granulomas are highly vascularised and the vessels have extensive lymphocytic cuffs [125]. *Mycobacterium tuberculosis* dysregulates host lipid metabolism by disruption of influx and efflux of lipid particles from the serum, thereby contributing to the formation of foam cells [126].

Mycolic acids contribute to the differentiation of foamy macrophages, which are characterised by accumulated lipid droplets [126]. As the granuloma matures, macrophages differentiate into foamy macrophages and various morphotypes [126]. Caseous granulomas form when the centre of the granuloma undergoes necrotic lysis, which facilitates the distribution of foamy macrophages around the necrotic debris [127]. Caseous lesions act as reservoirs by encasing and sheltering the *M. tuberculosis*, thus allowing the pathogen to maintain dormancy [128]. The series of the above-mentioned events are illustrated in the adapted figure below (Figure 1.2) [129].

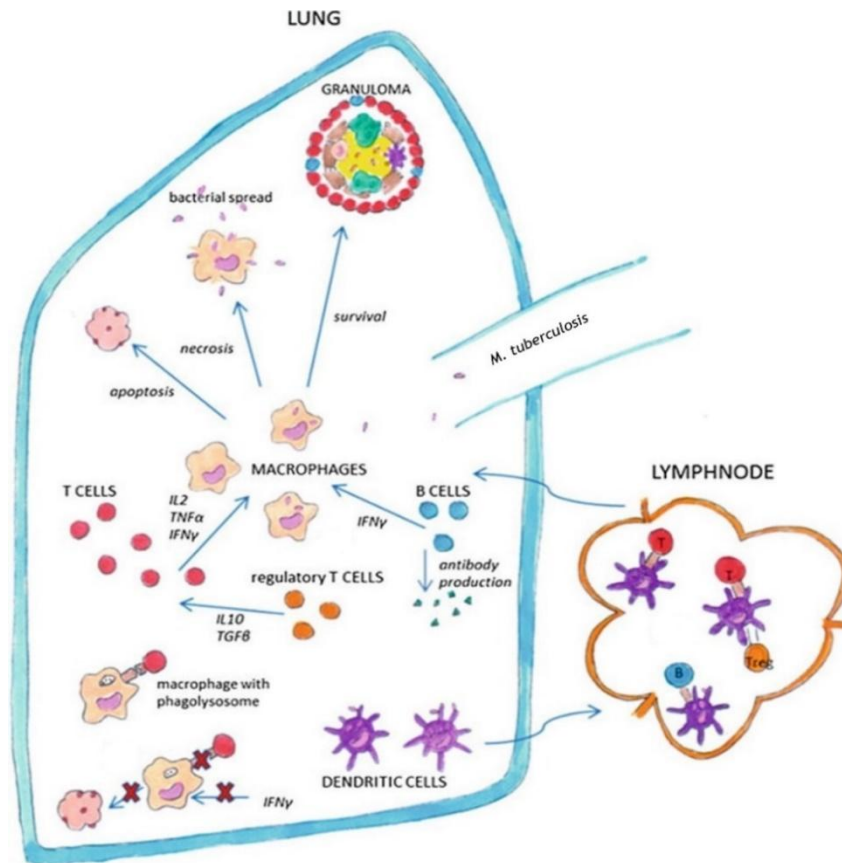


Figure 1.2. Schematic representation of basic immunological antimycobacterial mechanisms in the lung and lymph node. *Mycobacterium tuberculosis* inhalation into the lungs triggers activation of immune response through immune effector cells such as macrophages, dendritic cells, T cells and B cells via signalling molecules [129].

1.3.4. Reactivation and progression to active tuberculosis

Mycobacterium tuberculosis infection can be reactivated by mycobacterial persistence and inadequacy of the immunosurveillance system [101]. Reactivation can be caused by genetic or environmental factors, which weaken a host's immunity [101]. A mutation in genes coding for particular host C-type lectins, cytokines, chemokines and specific receptors, which influence important immune signalling pathways are examples of genetic causes [101]. An environmental or exogenous cause is the emergence of a co-infection with HIV as hosts become immune-compromised [130]. Alternatively, reactivation can be caused by failure of the host to develop and maintain immune signals [101]. Other triggering factors include chemotherapy, immune suppressive medications, malnutrition, drug or alcohol addiction and smoking [5]. Once immunocompromised, the dormant bacilli present in the granulomas, reactivate and replicate [131]. This is accompanied by granuloma liquefaction and cavitation, which results in the release of *M. tuberculosis*, formation of cavity lesions

and lung damage seen in TB patients [132]. Furthermore, resulting caseous material serve as nutrient sources, which promote the growth of the *M. tuberculosis* to an overwhelming burden for the host [133]. The bacilli subsequently spread throughout the lung and travel to blood capillaries, allowing transmission to new hosts, as well as dissemination to other organs [133]. Hence, the infected hosts develop symptomatic/active TB and are highly infectious.

1.4. Managing the spread of tuberculosis

Given the contagious nature of TB, prevention, detection and treatment are vital in the control and spread of TB. Active TB symptoms may be mild for several months, which lead to delays in seeking health care. Active TB symptoms include weakness/fatigue, weight loss, fever and night sweats, coughing with sputum and blood, and chest pain [39]. At this point, the infection is well established, and the patient is highly infectious. Therefore, the turnaround times of diagnosis and treatment should be rapid.

1.4.1. Diagnosis of tuberculosis

Classically, a pulmonary TB infection may be accurately diagnosed by sputum microscopy, culture in both liquid and solid media, chest X-rays, and nucleic acid amplification [134]. However, some of these methods detect TB at a later stage of infection, require long waiting periods or fail to identify drug-resistant TB. Nonetheless, some countries still rely on sputum smears by microscopy to diagnose TB [1]. Commonly used TB tests are the tuberculin skin test (TST), TB blood tests (IFN- γ release assays (IGRAs)) or the newer antigen-based skin test such as SIILTIBCY [135]. These tests take anywhere between 24 to 72 hours to deliver a result and are unable to differentiate between active and latent TB [135; 136]. Rapid molecular diagnostic tests for early detection of TB include the use of the Xpert MTB/RIF Ultra (Cepheid, Sandton, GP, ZA) and Truenat (Molbio Diagnostics, Goa, India) assays. These highly accurate tests simultaneously detect TB and resistance to the first-line RIF within two hours [1]. This near-patient cartridge-based diagnostic assay utilises real-time qPCR of *M. tuberculosis* DNA, and was recommended by the WHO for initial pulmonary and extrapulmonary TB diagnosis [137].

Development of alternative detection systems include investigation of urinary LAM in suspected TB cases of both HIV-infected [138] and uninfected individuals [139]. Additionally, biomarkers present in blood are being investigated to discriminate between active and latent TB. Human leukocyte antigen (HLA)-DR, expressed on *M. tuberculosis*-specific CD4 T cells, is proposed to be a robust marker capable of distinguishing active TB and latent TB in HIV-infected populations [140]. Therefore, better understanding of antigen-specific responses to *M. tuberculosis* during infection can be harnessed in the development of diagnostics to monitor infection and its treatment responses.

Despite the development and current use of these genotypic and serodiagnostic tools, there is no routinely implemented POC test at the patient's bedside. This type of test needs to be quick, highly sensitive and specific, affordable and have a long shelf life [36]. Additionally, this type of diagnostic tool should not require specialised equipment and trained personal as most areas requiring these tools are resource-constrained settings [36]. Hopes of development of such tools heavily relies on the discovery of novel, accurate, and reliable biomarkers.

1.4.2. Current tuberculosis treatment

The treatment of TB requires a rigorous antibiotic course that is significantly impacted by the drug susceptibility profile of the *M. tuberculosis* strains. The type of antibiotics used during treatment and its duration is dependent on the infecting strain of *M. tuberculosis* and the patient's predisposition.

Drug-susceptible, active TB can be treated and cured with four antimicrobial drugs (INH, RIF, ethambutol and pyrazinamide) over a standard four-to-six month course, given that the course of treatment is adhered to [1; 39; 137]. During the first two months, all four drugs are administered, followed by a continuation of INH and RIF for the remaining four months [5]. It is crucial that patients adhere to this regimen, as non-compliance can potentially lead to treatment failure and the development of drug resistance [5]. However, if therapy is being initiated after drug susceptibility testing, exclusion of some of these drugs during the intensive phase is preferred [141]. In addition, all patients at risk of neuropathy (pregnant woman; breast-feeding infants; HIV-positive patients; people with alcoholism, diabetes, chronic renal failure, malnutrition or old age) are given pyridoxine (vitamin B6) in conjunction with INH [141].

Drug-resistant TB is more complicated and requires treatment with a combination of antibiotics (fluoroquinolones) and injectable medications [1; 142]. Treatment of drug-resistant TB can range from nine to 24 months [5; 137]. A promising treatment advancement of MDR-TB utilises a combination of BDQ, pretomanid and LZD (BPaL) and had a treatment success rate of 90% [143]. However, this treatment regimen comes with significant side effects, thus necessitates careful monitoring and dose adjustments [143]. Moreover, treatment of XDR TB occurs over 24 or more months and requires tailoring to the specific *M. tuberculosis* strain. Therefore, it is a challenge to treat and manage and is associated with high rates of treatment failure and potential of increasing drug resistance [64].

Moreover, TB treatment may be accompanied by short-term and long-term side effects. Some of these include nausea, vomiting, diarrhoea, dizziness, rashes, changes in vision, abdominal pain, anxiety or confusion, depression, jaundice, hematologic toxicity, hepatitis and neurotoxicity [39; 142]. Therefore, regardless of the drug susceptibility profile of the *M. tuberculosis* strain being treated, continuous monitoring is required during TB treatment. This aids patients to adhere to treatment and prevent the emergence of further drug resistance by improving patient outcomes.

1.4.3. Vaccine development

Since its first introduction in the 1920s, the Bacillus Calmette-Guérin (BCG) vaccine is currently the only licensed TB vaccine available [1]. The BCG vaccine is primarily administered to newborns and high-risk children to prevent severe forms of TB, such as meningitis and disseminated disease. Unfortunately, BCG is not reliable against pulmonary TB in adults and adolescence, with an effectiveness around 80% and lower [144].

The pipeline for TB vaccine development includes a range of platforms such as adjuvanted proteins, whole cell vaccines and recombinant subunit vector vaccines. In late 2024, the WHO reported that there were 15 vaccine candidates in clinical trials [1]. Four of these candidates were undergoing phase I trials, five were undergoing phase II trials, and six were undergoing phase III trials [1].

Of these candidates, the M72/AS01_E vaccine comprising of a recombinant fusion protein derived from *M. tuberculosis* antigens (Mtb32A and Mtb39A), combined with the AS01_E adjuvant system is currently being investigated in phase III trials [145; 146]. Previously, this vaccine was significantly protective against TB, in individuals with evidence of latent TB in South Africa, Kenya and Zambia over a two year follow-up [145]. The current double-blind trial is scheduled to enrol up to 20 000 participants at around 60 trial sites in seven countries including South Africa, Zambia, Malawi, Mozambique, Kenya, Indonesia and Vietnam [146]. Although this candidate is promising, its 50% efficacy is borderline for the WHO's target of protection from a vaccine [147].

Other protein subunit vaccine investigations include H4:IC31, H56:IC31, and ID93/GLA-SE, which aim to boost the immune response generated by BCG [148-150]. Initial studies indicated that these candidates are safe and immunogenic, but their efficacy needs to be fully established [151]. Amongst viral-vectored vaccines, results from the MVA85A phase IIb clinical trial showed heightened antigen-specific CD4 T-cell responses, but no additional protection against TB in infants [152]. The promising whole-cell vaccine, VPM1002, aims to improve BCG immunogenicity [153]. The VPM1002 vaccine is a recombinant urease C-deficient, listeriolysin expressing BCG vaccine, designed to escape the phagosome through expression of listeriolysin and facilitate phagosomal acidification [153]. Therefore, this vaccine enhances MHC class I antigen presentation to CD8 T-cells [153]. Furthermore, MTBVAC (genetically attenuated *M. tuberculosis* lacking *phoP* and *fadD26*), is the first and only live attenuated vaccine that contains all the necessary antigens to stimulate an immune response [154]. Although all of these vaccine candidates hold promise, they could be ruled out as viable options during clinical trials. Therefore, the need for novel biomarker discovery remains urgent.

1.5. Biomarkers

A key component for discovery and advancement of TB prevention, diagnosis, treatment, and monitoring interventions, is the discovery of biomarkers. A biomarker is a substance that can be detected in the body that is indicative of disease and is measured accurately and reproducibly [155]. Biomarkers can be of host or pathogen origin and can play important roles in mechanisms of action as well as drug resistance [156]. Biomarkers are classified into four types that include predisposition, predictive, prognostic and diagnostic biomarkers [157]. The development of an effective biomarker involves four important steps that include discovery, analytical validation, evaluation of clinical efficacy, and clinical use [158].

Although various single biomarkers have been successfully implemented in diagnostics and disease prognosis, the dawn of “omics” technologies has shed light on the prospect of using biosignatures (combined biomarker profiles) for the same purpose [159]. The use of biosignatures may be valuable when individual biomarkers cannot be identified or simply, do not exist [159]. Given that the interplay of various molecules (including DNA, ribonucleic acid (RNA), proteins and metabolites) determine the pathological state, multi-level biomarkers are a desirable option [158].

1.5.1. Tuberculosis biomarkers

Various biomarkers have been identified and explored in TB research and they cover a vast range of biological molecules such as cytokines, microRNAs (miRNAs), and protein signatures. Cytokines, particularly IFN- γ and IL-2, have been extensively studied as biomarkers for TB diagnosis. T-cell populations that produce these cytokines have shown promise in differentiating latent TB from active TB infection [160]. Moreover, the antigen-specific chemokine, CXCL10, has been flagged to effectively differentiate between active and latent TB [161]. This IFN- γ inducible protein, is a biomarker that was detected in increased concentrations in plasma of active TB patients [162; 163]. Furthermore, the use of IGRAs has been motivated for as a method of monitoring anti-TB treatment [164].

Specific miRNA signatures in serum that are associated with active pulmonary TB have been motivated for use in early TB diagnosis [165]. Additionally, long noncoding RNAs (lncRNAs) show promise as novel diagnostic markers, particularly in macrophages infected with *M. tuberculosis* [166]. *Mycobacterium tuberculosis* DNA and Ag85 complex (family of proteins with enzymatic mycolyl transferase activity) are detected in blood and urine samples of pulmonary TB patients, whereas LAM is detected in urine [167; 168].

The expression of CD38, HLA-DR and proliferation marker Ki-67 on *M. tuberculosis*-specific CD4⁺ T-cells are associated with *M. tuberculosis* load [169]. The modulation of these markers distinguishes active TB from latent TB with high sensitivity and specificity [169; 170]. Patients who had successfully completed TB therapy were adequately classified by these markers [169]. Furthermore, a seven-marker serum protein biosignature demonstrated high diagnostic performance for active TB [171]. Therefore, instead of a single biomarker, a panel of biomarkers, may increase diagnostic sensitivity and specificity. This approach underscores the potential of proteomic analyses in developing non-invasive diagnostic tests that pave the way for enhancement of diagnostic performance. A similar argument holds true for vaccine development, in that multi-epitope candidates may be more effective than a single-epitope one [172].

Despite increasing evidence motivating the use of *M. tuberculosis* adhesins as suitable vaccine and diagnostic targets [18; 173-176], their role in modulating the pathogen and host metabolomic signatures as well as bioenergetic profiles during TB pathogenesis has not been fully elucidated. Such knowledge would be pertinent to support the validation of adhesins as notable biomarkers, as well as the identification of novel metabolomic signatures associated with them, to be used as diagnostic, drug target, and vaccine candidates.

1.5.2. Adhesins

The initial contact between the pathogen and its target cell is crucial for entry into the host during the infection process. Adhesins are extracellular surface molecules or appendages that facilitate adherence of the pathogen to the host. These structures promote interaction with receptors of host cells or soluble macromolecules [173]. Adhesins prevent sheer stress removal of the microorganism from the colonised surface [176]. Furthermore, adhesins facilitate host cell invasion, manipulate host cell signalling, promote the spread of the pathogen and evasion of the host immune response [176; 177]. Hence, adhesins are at the core of pathogens' ability to contaminate, transmit, establish residency within a host, and instigate an infection [177].

Adhesins may be assembled into hair-like appendages (pili/fimbriae), which extend out from the surface of bacteria or are directly associated with microbial cell surfaces (non-pilus adhesion) [178]. Pili are defined as hydrophobic, polymeric, proteinaceous structures commonly composed of a major repeating subunit (pilin) and occasionally a minor tip-associated adhesin subunit [14]. These structures are involved in many virulence-associated functions, which include bacterial aggregation and biofilm formation, agglutination of erythrocytes, adherence, and mucosal surface colonisation [179; 180]. Additionally, PAMPs, such as flagella or pili, stimulate pro-inflammatory signals [181]. Non-pilus adhesins can interact with host molecules, including transmembrane proteins and extracellular matrix (ECM) components [178]. Microbial surface components recognise adhesive matrix molecules and activate signal transduction cascades of the host, by binding to receptors of the ECM [182]. These receptors include integrins that adhere to molecules, including bone sialoprotein, collagens, elastin, fibronectin, glycosaminoglycans, laminin, thrombospondin, and vitronectin [182]. During infection, *M. tuberculosis* interacts with and acquires entry to host cells, such as epithelial cells, macrophages, and dendritic cells, and this process is essential to the bacterium's replication, survival and dissemination [31; 177]. Nonetheless, adhesins involved in these fundamental processes have mainly been unexplored and underappreciated [175]. To effectively infect a host, bacterial species cannot rely on just one adhesin. Subsequently bacteria are known to produce multiple adhesins [12]. Several *M. tuberculosis* proteins functioning in attachment to host have been documented previously and are displayed in the adapted Table 1.2 [174].

Table 1.2. Currently known *M. tuberculosis* adhesins and their associated binding structure [174].

Adhesin	Mediates adhesion to
19-kDa antigen	Monocytes and macrophages
Alanine- and proline-rich antigen (Apa)	Pulmonary surfactant protein A and macrophages
Antigen 85 complex	Fibronectin and macrophages
Cpn60.2 molecular chaperone	Macrophages
Curli pili (MTP)	Laminin, macrophages and epithelial cells
DnaK molecular chaperone	Macrophages
Early secreted antigen ESAT-6	Laminin
Glutamine synthetase A1	Fibronectin
Glyceraldehyde-3-phosphate dehydrogenase	Possibly fibronectin (as occurs in group A streptococci)
Heparin-binding hemagglutinin adhesin	Epithelial cells
Laminin-binding protein	Laminin
L,D-transpeptidase	Fibronectin and laminin
Malate synthase	Fibronectin, laminin, and epithelial cells
Membrane protein	Collagen, fibronectin, and laminin
<i>Mycobacterium</i> cell entry-1 protein	Epithelial cells
N-acetylmuramoyl-L-alanine amidase	Fibronectin and laminin
PE-PGRS proteins	Fibronectin and macrophages
Protein kinase D	Brain endothelia and laminin
PstS-1 (38-kDa antigen)	Macrophages
Type IV pili	Possibly macrophages and epithelial cells

1.5.3. *Mycobacterium tuberculosis* curli pili (MTP)

Novel strategies of TB prevention and treatment necessitate targeting bacterial determinants of infection, instead of those of survival [174]. By identifying and treating the pathogen at the point of contact, it may be possible to incapacitate and ensure natural clearance of the bacilli, without imposing selective pressures that allow numerous drug-resistant forms of the bacterium to multiply [174]. A notable adhesin of *M. tuberculosis* is MTP, which has shown potential as a suitable biomarker for diagnostic tests [18; 174; 183].

Curli pili are coiled, highly aggregative adhesive fibres that are approximately 5 nm wide [184]. Pili are assembled via a nucleation pathway, which requires the major and minor pilin subunits [184]. Pilin subunits produced by *M. tuberculosis* are encoded by the open reading frame designated as *Rv3312A* (*mtp*) [13; 14]. Interestingly, *mtp*, is located between intermediary metabolism associated genes and is not arranged as part of an operon or cluster with curli/pili associated genes [13; 14]. Amongst clinical isolates, *mtp* is highly conserved and has been shown to be unique to *M. tuberculosis* complex strains [17]. Although there is no similarity between the primary sequence homology of MTP protein subunits and curli pili of bacteria, morphologically, the MTP adhesin has much in common with other bacteria [14]. The MTP adhesin is sticky, aggregative and comprised of insoluble structures largely containing proline and glycine residues, which account for the common hydrophobic properties of pilins [14]. Additionally, MTP are capable of binding to Congo red dye (a property associated with bacterial aggregation and human amyloidogenic proteins) and laminin [14].

The interplay between MTP and ECM protein, laminin, *in vitro* is ascribed to the occurrence of a collagen-like repeat beginning at residue 30 of the MTP protein [14]. It has been shown that *mtp*-mutants of *M. tuberculosis* H37Rv were unable to bind to laminin [14]. Therefore, MTP acts as an adherence factor, and mediates interaction and colonisation of host cells [14].

Using functional genomics, the *mtp* gene was confirmed to be essential for piliation and facilitated biofilm formation [15]. The MTP adhesin was demonstrated to act as an adhesin and invasin to THP-1 macrophages [16], and A549 pulmonary epithelial cells [12]. The MTP-proficient and MTP-deficient strains of *M. tuberculosis* V9124, derived from a drug-susceptible clinical strain of the F15/LAM4/KZN family, are illustrated below (Figure 1.3) [15; 16].

In contrast to other bacterial pili [181; 185; 186], MTP was shown to minimally modulate host immune response in A549 pulmonary epithelial cells [12]. The mechanism of interaction of MTP with THP-1 macrophages is currently unknown. It has been suggested that MTP adheres indirectly to THP-1 macrophages by forming a molecular bridge with the aid of host proteins or directly via membrane-bound ligands [16]. Direct adherence may involve receptors, such as complement, mannose, surfactant protein, or SRs [187-189]. Hence, further research is essential to better understand the role of MTP in the *M. tuberculosis* infection process.

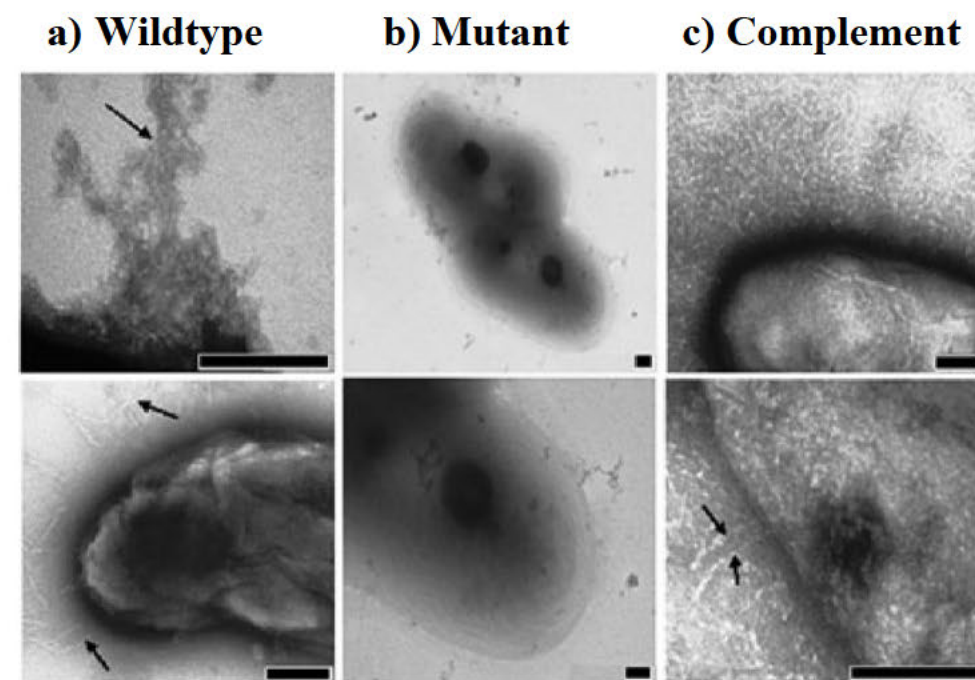


Figure 1.3. Electron micrographs of negatively stained *M. tuberculosis* strains with and without MTP pili. a) WT *M. tuberculosis* V9124 with MTP, b) Δmtp mutant without MTP, and c) *mtp*-complemented strain with MTP. Black arrows point to pili fibres. Scale bars 0.2 μm [15].

1.6. The use of “multi-omics” to improve anti-tuberculosis strategies

The basic understanding of central dogma, which is the flow of biological information from genetic material to transcripts and then to proteins, is in fact rather complex [190]. Instead of a linear flow of information, it is more accurately described as a network of interactions. This is supported by evidence that highlights epigenetic modifications, such as DNA methylation, that influences the regulation of genes and subsequent protein synthesis and metabolic interactions [191]. Hence, in

recent years, multi-omics approaches allow researchers to identify the more complicated relationships and regulatory networks that govern phenotypic outcomes [19].

Multi-omics approaches emerge as a powerful tool that provides insight into the intricate interplay between *M. tuberculosis* and host immune responses [20]. Multi-omics has been implemented in the discovery of biomarker targets for application in rational drug design, production of more effective vaccines, production of universally applicable rapid diagnostics and treatment monitoring [20]. Moreover, interest in multi-omics for the development of personalised treatment offers useful information by identifying *M. tuberculosis* clinical characteristics (strain and resistance profiles) and host genetic factors (immunological state and comorbidities) that determine the best treatment plan and help predict therapy outcomes for individuals [192]. This undoubtedly can enhance therapeutic results since the risk of drug resistance and disease progression can be lowered [192].

Multi-omics is the use of a combination of broad research methodologies such as genomics, transcriptomics, proteomics, and more recently, metabolomics [156; 158; 190; 193]. On its own, each type of “omics” produces specific data showing perturbations associated with the disease, but is restricted to correlations largely reflecting reactive rather than causative processes [190]. However, an integration of different data types of techniques achieves a holistic view of systems biology during the infection process that can expose potential causes of disease or find effective treatment targets, which may be further tested in molecular studies.

1.6.1. Genomics

Genomics plays an important role in the multi-omics framework as it provides foundational insights into the genetic makeup of *M. tuberculosis* and its host. Genomic technologies include exome sequencing, genotype arrays and next generation sequencing [194-196]. Genome-wide association studies have identified thousands of genetic variants associated with complex diseases in multiple human populations. Whole genome sequencing of clinical *M. tuberculosis* isolates have been utilised to retrospectively track pathogen transmission events [197; 198], identify drug resistance-conferring mutations [50; 198] as well as discriminate between re-infection and relapse cases [50; 199]. In addition to diagnostic confirmation of the presence of *M. tuberculosis*, whole genome sequencing provides information on antibiotic susceptibility by the identification of genetic variations that cause drug resistance [200]. Some studies have successfully detected and sequenced *M. tuberculosis* directly from uncultured sputum samples [201; 202]. However, cleared infections and active disease cannot be differentiated using DNA-based techniques, as DNA from non-viable mycobacteria remain detectable [202]. Moreover, studies have demonstrated that significant alterations in the microbiota of TB patients not only aids in understanding disease pathogenesis but opens avenues for novel therapeutic strategies that target both the pathogen and the host's microbiome [203].

1.6.2. Transcriptomics

Levels of RNA are qualitatively and quantitatively analysed by transcriptomics. Transcriptomic studies have shown that up to 80% of the genome is transcribed and approximately 3% of the genome encodes proteins [204]. Various techniques used in transcriptomics include RNA-sequencing, microarrays and qPCR [205]. Although relatively inexpensive, widely available and easily analysed, microarrays can only detect sequences homologous to those present on the array [205]. On the other hand, RNA-sequencing is unbiased but remains costly and complex. The technique best used for studying small amounts of transcripts in a sizable sample set is qPCR. More specifically, reverse transcription-quantitative PCR (RT-qPCR) is commonly used to determine the messenger RNA (mRNA) levels in various biological systems [206]. This technique is considered to have superior sensitivity, accuracy and rapid execution [207]. Nonetheless, amplification efficiency, quality of

mRNA, and choice of reliable reference genes critically affect interpretation and accuracy of RT-qPCR results [207; 208].

Blood transcriptomic profiling has been used to analyse perturbations in hosts upon infection and in active TB. In active TB patients, a whole blood transcriptional signature, governed by IFN-inducible genes, was identified [209]. This signature was not present in healthy controls and individuals with latent TB [209]. Genes included in the IFN-inducible gene signature were downstream of both IFN- γ and type I IFN and were downregulated upon successful treatment [209; 210]. This has been reiterated in numerous studies worldwide with independent clinical cohorts [211-213], and in meta-analyses [214]. Moreover, the integration of transcriptomic data with proteomic analyses can elucidate the relationship between mRNA levels and protein abundance, which can potentially provide insights into the regulatory mechanisms that control the host's response to TB infection [215]. This strategy can aid in the identification of specific genes and pathways that are upregulated or downregulated during infection, thereby enhancing discovery potential for biomarkers [215].

1.6.3. Proteomics

Peptide abundance, modifications and interactions can be investigated with proteomics [190]. Protein presence and interactions can be detected by the less biased phage display and two-hybrid assays or by affinity purification in which a single molecule is isolated using a tag or antibody [190]. Mass spectrometry (MS) can then be used to identify associated proteins, directly measure covalent and post translational modifications [190]. Post-translational modifications include phosphorylation, glycosylation, proteolysis, nitrosylation, and ubiquitination all of which are need in intracellular signalling, protein turnover and transport, control of enzyme activity, as well as maintenance of cell structure [216-218].

Proteomic fingerprinting has been used to identify active pulmonary TB from non-TB, different TB disease states and monitor the treatment response [193; 219; 220]. By employing proteomic profiling and protein structure prediction approaches, it was possible to identify the biomarkers such as C reactive protein, neopterin, serum amyloid A and transthyretin [219]. Differentially expressed proteins involved in apoptotic process, cellular process, immune system process, response to stimulus and select metabolic process were identified in patients with severe pulmonary TB [220]. The combination of specific plasma proteins achieved 90% sensitivity and 92% specificity to discriminate between patients with severe pulmonary TB and mild TB; and 90% sensitivity and 99% specificity to discriminate between healthy controls and patients with severe pulmonary TB [220]. Additionally, drug-sensitive and drug-resistant strains of *M. tuberculosis* were differentiated by specific proteins and altered pathways, thus providing an understanding of how the pathogen adapts to antibiotic pressure [221]. Moreover, proteomic studies highlighted the role of specific efflux pumps and metabolic enzymes in conferring drug resistance [190].

1.6.4. Metabolomics

Metabolomics is the identification and quantification of all metabolites within a biological system at a given time [22]. Metabolomic fingerprinting is the high-throughput global analysis of metabolites (amino acids, lipids and sugars with low molecular weights) that are mainly involved in biological process as a substrate or a product [22]. There are fewer classes of metabolites compared to genes or proteins, and as a result of this, it is possible to identify metabolites with a high degree of certainty [222]. In recent years, metabolomics has been employed in the search for biomarkers, and when combined with various cellular models, has been used to extensively study metabolomic flux [7; 159; 223]. Metabolomics can be separated into untargeted and targeted approaches depending on the goal that the researcher is aiming to achieve [27; 224]. Untargeted metabolomics is used when there is a

need to detect all metabolites from samples, including unknown compounds [27]. Hence, this technique can be employed to create metabolome fingerprints/signatures. Targeted metabolomics can be used to focus on specific categories of metabolites and allows the detection of substrates breakdown/flow through pathways [26; 27]. Therefore, the use of ^{13}C -isotopologue profiling analysis can directly measure the intracellular metabolism [26]. Untargeted and targeted metabolomics are often employed together in attempts to detect and accurately quantify metabolites [224].

1.6.4.1. Metabolomic platforms

Within the broad field of metabolomics, there are various types of analytical platforms that can be used in isolation or coupled with other techniques to improve data output and quality. A common technique is nuclear magnetic resonance (NMR), which is particularly useful for characterisation of unknown compounds when minimal destruction of the sample is required [225; 226]. However, NMR spectra are complex, especially when investigating a great diversity of metabolite classes and concentrations [227]. For this application, the use of MS is preferred as it simplifies detection and is a less time consuming approach [228]. In addition, the MS approach identifies and monitors metabolic pathways of a substrate and its intermediates involved in metabolism [228].

Capillary electrophoresis, liquid chromatography (LC) and gas chromatography (GC) are different techniques used in conjunction with MS. By combining different types of MS high chromatographic resolution of compounds, and a high sensitivity is achieved [22]. “Hyphenated techniques” are used to combine chromatographic and spectral methods; therefore, allowing researchers to exploit the advantages of both techniques being used [23]. Chromatography techniques are used to produce pure fractions of metabolites in a mixture and spectroscopy techniques are used to produce selective information for identification of metabolites [23].

Comprehensive GC×GC is a powerful and robust technique used for group-type and within-group separations [24]. This method allows for compounds to disperse in a two-dimensional (2D) space by making use of two connected columns (containing various stationary phases) with two independent modes of separations [24]. Normally, the first column is less polar and longer than the second one. A carrier gas is used in GC-MS, for example, helium, nitrogen or hydrogen, to propel a sample through a column causing various components to be separated [23] due to different retention times. A modulator focuses and releases packages of material for a second separation [24]. This event needs to occur quickly so that multiple samplings of each first-dimension peak can be obtained [24]. Hence, rapid detectors such as time-of-flight MS (TOFMS) are used.

The physical separation capabilities of LC are combined with successive MS capabilities in LC-MS/MS to create a powerful analytical technique. A mobile phase which uses a liquid solvent, flows through the first column and since compounds interact differently with the second stationary phase (non-mobile), separation of compounds occurs based on polarity and size [229]. Thereafter, compounds are converted into ions, typically by electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) and are directed into two stages of MS [230]. First compounds are sorted by mass-to-charge ratios (m/z). Following the initial analysis, selected ions are fragmented in a collision cell, which leads to the formation of smaller product ions. These product ions are analysed in the second MS. Therefore, this hyphenated technique is used for its sensitivity, specificity, and ability to analyse complex biological samples [231]. Hence, LC-MS/MS is extensively used in research for drug monitoring, biomarker discovery, and pathogen identification [7; 232; 233].

1.6.4.2. Metabolomics and *Mycobacterium tuberculosis*

Metabolomics has gained traction by enhancing researchers’ understanding of *M. tuberculosis* growth, survival, replication, metabolism, pathogenicity as well as drug resistance [228]. Investigation of *M. tuberculosis* tricarboxylic acid (TCA) cycle via LC-MS revealed that the pathogen

operates its TCA cycle in a half-cycle mode in which α -ketoglutarate and glutamate are synthesised by the oxidative branch, whilst succinate is synthesised by the reductive branch [234].

Metabolomic patterns of *M. tuberculosis* INH-resistant strains, which carry *katG* mutations, were differentiated from the WT by producing 29 different metabolites that included fatty acids, alkanes, surfactant protein, alcohols and other metabolites involved in the stress alternative energy pathway [235]. Fatty acid metabolomes of *rpoB* mutant *M. tuberculosis* strains were investigated by GC-MS to better characterise RIF resistance [223]. The *rpoB* mutant *M. tuberculosis* strains exhibited altered metabolite markers that demonstrated decreased synthesis of various 10-methyl branched-chain fatty acids and cell wall lipids, and increased use of the shorter-chain fatty acids as carbon sources [223]. Moreover, this study demonstrated that RIF resistance came with an overall fitness cost as *rpoB* mutants displayed reduced growth, replication and viability rates [223].

Metabolomics has also been used to characterise extracellular metabolomes of culture supernatant of non-TB mycobacteria and of hyper-virulent strains of *M. tuberculosis* [236]. Principal component analysis (PCA) was used to distinguish metabolome profiles of the various strains [236]. Data revealed that changes to the identified metabolites can be used to evaluate the biological mechanisms contributing to virulence of *M. tuberculosis*, whereas, unidentified metabolites can be considered as potentially novel compounds, since they do not match to current compound databases [236]. Additionally, the unidentified metabolites motivate the need for expansion of currently available databases to accurately and effectively identify metabolites.

1.6.4.3. Metabolomics of *Mycobacterium tuberculosis* during infection

Mycobacterium tuberculosis, acquires nutrients from its host environment, thus allowing it to generate biomass and energy for survival and replication during infection [237-239]. During the infection process, it is technically and conceptually challenging to disentangle host and pathogen metabolism since they share majority of metabolites and metabolic pathways [240]. Therefore, the majority of metabolomics approaches make use of absence/presence of metabolites and the changes in overall concentrations [236; 241], auxotrophic strains of intracellular *M. tuberculosis* [242] and carbon labelling [26].

Given that metabolomics is a non-invasive tool, it can be used in applications such as diagnostics and therapeutics. Several studies have identified tuberculostearic acid, 9-hexadecenoic acid, 11-eicosenoic acid, 13-docosenoic acid, eicosanoic acid, heptadecanoic acid, cadaverine and 15-tetracosenoic acid as metabolites that differentiate various infectious *Mycobacterium* species from one another [243-245]. These metabolites are associated with the unique cell wall structure of different *Mycobacterium* species. Moreover, 9-octadecenoic acid, 10-heptadecenoic acid, α -D-glucopyranoside, D-glucosamine and nonadecanoic acid are also associated with the cell wall, and were detected more than once as biomarkers [243; 245].

In addition, these biomarkers were confirmed when TB-positive and TB-negative patient sputum samples were compared using GC $\times$ GC-TOFMS [246]. The sera of TB patients could be differentiated from healthy controls by the significant difference in the concentrations of 17 metabolites [241]. Although docosanoic acid (behenic acid) was detected as an abundant fatty acid in *M. tuberculosis* [243], when using TB-positive patient samples, the diagnostic capacity was considered poor [159]. Nonetheless, when docosanoic acid was used in combination with lysophosphatidylcholine, presqualene diphosphate and threoninyl- γ -glutamate, TB patients could be differentiated from healthy controls in addition to patients with other lung diseases [247]. More recently, the versatility of LC-MS/MS has been motivated for the detection of mycolic acid in sputum and cell cultures, and for the exploration of circulating *M. tuberculosis* antigen peptides as diagnostic markers [248; 249]. Moreover, anti-TB first-line and second-line drugs were simultaneously quantified in plasma samples; therefore, supporting metabolomics for use in monitoring therapeutic drug levels to ensure

effective treatment regimens since low plasma drug concentrations can be attributed to malabsorption or co-infections like HIV [250; 251].

The surge of multi-omics technologies has allowed protein and transcript levels to supplement the metabolic changes during infection [252; 253]. Auxotrophic strains have been used to uncover intracellular nutrient usage through infection phenotypes. Studies utilising auxotrophic *M. tuberculosis* strains in cellular and animal infection models have identified active metabolic pathways and specific nutrient consumption [242]. Amino acid metabolism [254; 255], carbohydrate uptake and utilisation [256; 257], gluconeogenesis [258; 259], lipid-based fuelling of central carbon metabolism (CCM) [260] and mycobacterial lipid uptake and breakdown [261-263] were shown to play a crucial role in the infection process.

Interestingly, GC/MS analysis of metabolomic variation of human macrophages infected with different *M. tuberculosis* strains showed that most of the metabolites displayed similar regularity and variation tendencies after infection by different virulent strains [261]. This study suggested that *M. tuberculosis* invades and infects macrophages largely through three different pathways: glutathione anabolic metabolism, lipid biosynthesis and metabolism, and carbohydrate biosynthesis and metabolism [261]. The incorporation of ^{13}C into proteinogenic amino acids of bacteria infecting pre-labelled host cells has proved to be useful in providing insights into biomass precursors and host molecule consumption [26]. Additionally, *M. tuberculosis* utilises asparagine and aspartate as nitrogen sources for essential growth and adaptation within the host [254].

Although a vast number of potential biomarkers/biosignatures are identified, only a select few produce compelling data to suggest potential use for diagnostics and therapeutics. Selection of the correct biomarker is important as it will affect sensitivity, specificity, usability, cost and health outcomes in the long-run [264]. Therefore, the search for novel biomarkers, particularly involved in TB, should be continued.

1.7. Bioenergetics

Cellular energetics or “bioenergetics” refers to the study of energy flow and energy transformations in biological systems. This technique has the ability to provide great insight into cellular processes since all living cells harness energy conversions for catalytic and anabolic functions [265]. Bioenergetics assesses various cellular energetic parameters such as oxygen consumption rate (OCR), adenosine triphosphate (ATP)-production, and metabolic flexibility, all of which are vital in cellular health and disease states. In the context of TB research, bioenergetics can be employed to better understand the metabolic adaptations of both host and pathogen by investigating mitochondria and other cellular components involved in energy transformations.

1.7.1. Bioenergetic platforms and assays

A bioenergetic platform that has been developed to provide comprehensive assessments of energy fluxes are the Multiplexed Assays, such as Mitochondrial ToxGlo™ Assay (Promega Cooperation, WI, USA). This platform integrates various bioenergetic measurements, such as ATP synthesis rates and dehydrogenase activities, to offer a holistic view of mitochondrial function across different tissues [266]. Therefore, Multiplexed Assays are valuable in comparative bioenergetics studies of health and disease, as they allow for the identification of tissue-specific metabolic adaptations [266]. Another innovative approach involves microfluidic devices, which create a controlled environment to studying single-cell bioenergetics. These devices can be used to monitor mitochondrial transport and energy metabolism in response to specific stimuli allowing for detailed investigations into cellular responses [267]. Hence, microfluidic platforms have been used to analyse the energetic changes in

neurons following axonal injury, highlighting the importance of localised energy metabolism in regenerative processes [267].

Additionally, high-resolution respirometry enables detection of respiratory control ratios and other bioenergetic parameters in isolated mitochondria or permeabilised cells [268]. The Oroboros Oxygraph 2000 (O2k) (Oroboros Instruments, Innsbruck, Austria) is a commonly used instrument, which allows researchers to explore various metabolic states and their implications for health and disease [268].

The most widely used bioenergetic platform is probably the Seahorse Extracellular (XF) Analyzer (Agilent Technologies, CA, USA), which allows for measurements of cellular respiration and glycolysis in real-time [269]. This platform can assess mitochondrial function by measuring OCR and extracellular acidification rate (ECAR), which can provide insights into the balance between OXPHOS and glycolysis, the two major energy generating pathways, in different cell types [270]. The Seahorse technology has been applied in studies investigating drug treatment and disease models, thus making it a versatile tool for bioenergetic analysis [270].

1.7.2. Seahorse XF Analyzer assays

Detailed metabolic profiling can be achieved with Seahorse XF Analyzer, which is compatible with various assays. Two of the commonly used assays are the XF Cellular Mitochondrial Stress Test (CMST) and XF Glycolytic Rate Assay (GRA). These tests evaluate mitochondrial function through the simultaneous measurement of OCR and ECAR under different conditions, which are determined by the sequential addition of specific drug inhibitors from expertly designed ports on a cartridge that rests above a cell culture plate (Figure 1.4) [269]. These assays provide a comprehensive view of cellular energy metabolism, enabling researchers to discern the metabolic phenotype of various cell types under physiological and pathological conditions [271].

After initial baseline readings of OCR and ECAR, respiration modulating drugs such as oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), rotenone, and antimycin A present in drug ports are programmed to be injected in sequential order [269]. Oligomycin is an ATP synthase (complex V) inhibitor and is injected into the cell plate to decrease electron flow through the electron transport chain (ETC). The addition of FCCP is designed to cause a collapse in proton gradients, hence, disrupting mitochondrial membrane potential. Therefore, FCCP is considered as an uncoupling agent and its presence results in the maximal oxygen consumption by complex IV of the ETC. Lastly, the addition of rotenone and antimycin A in combination causes a complete drop of OCR given that rotenone is an inhibitor of complex I and antimycin A is an inhibitor of complex III [272]. From the real-time respiratory profiles generated, specific parameters such as basal respiration, maximal respiration, ATP production, spare respiratory capacity, proton leak, and non-mitochondrial respiration can be determined from the CMST [232].

Alternatively, the GRA utilises a different set of respiration modulating drugs and injection strategies [269]. Following basal readings of proton efflux rate (PER), rotenone and antimycin A (inhibitors of mitochondrial ETC) are injected into the system to determine compensatory PER through inhibition of mitochondrial oxygen consumption. This subsequently halts the CO₂ derived acidification. Hence, compensatory glycolysis provides insight on the ability to meet the energy demands via glycolysis when OXPHOS is inhibited. The addition of 2-deoxy-D-glucose (2-DG), a glucose analogue that competitively inhibits glycolysis, ensures that the PER preceding the injection is primarily caused by glycolysis and not mitochondrial-derived acidification [232]. Moreover, glycolytic proton efflux rate (glycoPER) is calculated from total PER [273]. Therefore, the GRA can provide information on basal glycolysis and compensatory glycolysis in different cell types.

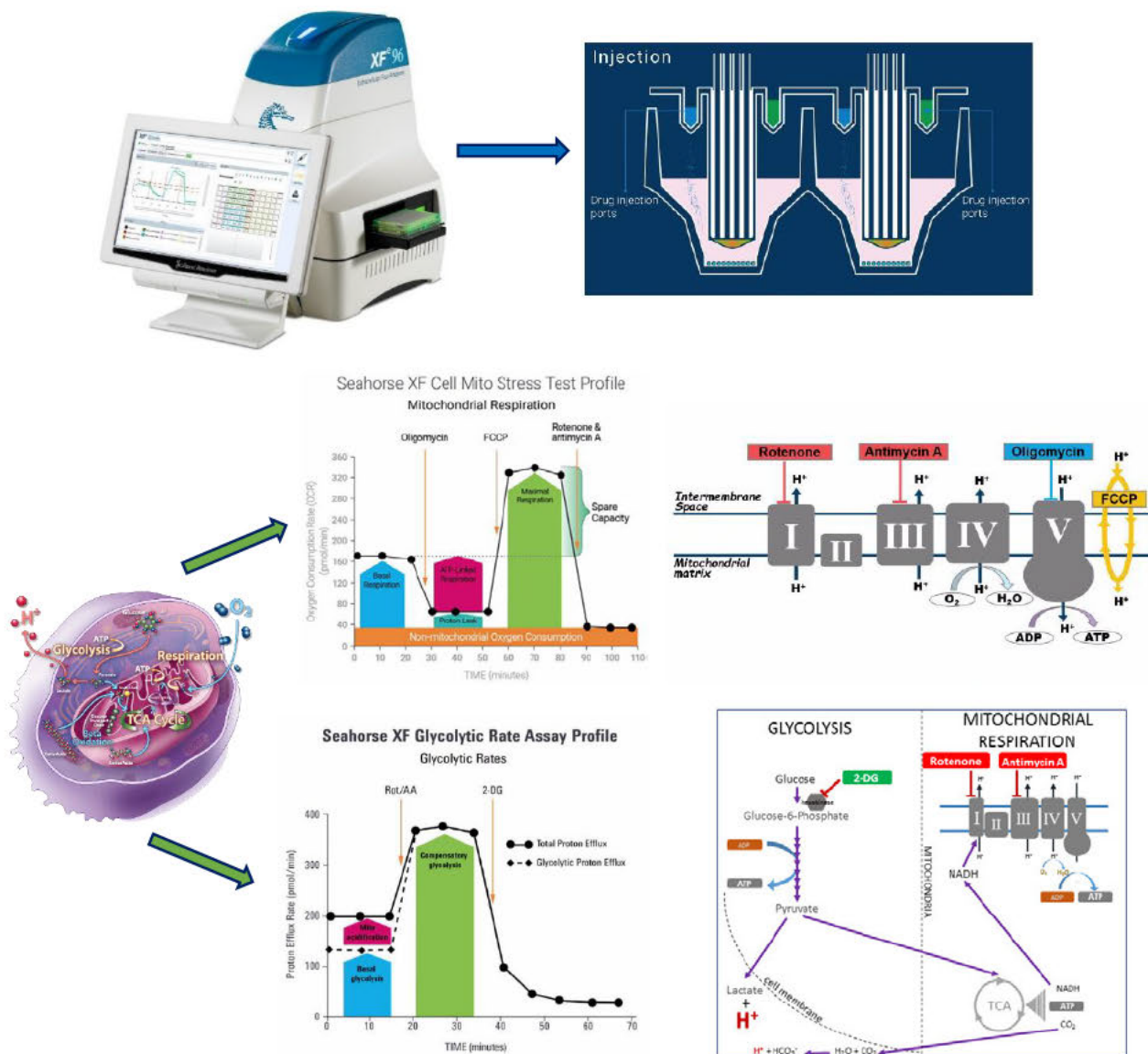


Figure 1.4. Seahorse XF Analyzer detects discrete changes in cellular bioenergetics. Drug ports inject respiration modulating drugs into cell culture plates to measure OCR and ECAR in real-time assays such as CMST and GRA. The CMST utilise ETC inhibiting drugs, whereas GRA takes advantage of ETC inhibitors and competitive binding analogues [269].

1.7.3. Understanding *Mycobacterium tuberculosis* pathogenesis through bioenergetics

Development of host-directed therapies aiming to restore normal metabolic functions and enhance the immune response against TB can be aided by a deeper understanding of bioenergetic pathways [90]. Currently, bioenergetics has proven to be a pivotal tool in understanding *M. tuberculosis* strategies to manipulate host metabolism. The Seahorse XF Analyzer has been used to demonstrate that *M. tuberculosis* infection leads to dysregulation of macrophage CCM through the action of hydrogen sulfide (H_2S), which suppresses glycolysis and impairs the immune response [274]. This manipulation of host metabolism aids bacterial survival but also presents potential therapeutic targets to be exploited [274].

Macrophages infected with *M. tuberculosis* are known to display a metabolic phenomenon, similar to the Warburg effect, in which they preferentially switch from OXPHOS to glycolysis for energy

production despite the presence of sufficient oxygen [275]. This switch has been noted in mouse lungs infected with *M. tuberculosis*, in which host immune cells exhibited enhanced glycolysis and pentose phosphate pathway (PPP) activity [232]. Enhanced glycolysis is indicative of a rapid generation of ATP and biosynthetic precursors needed for effective immune responses [90; 275]. Therefore, this metabolic reprogramming is vital for the survival and proliferation of immune cells in the hypoxic environment of granulomas formed during TB infection [232].

Additionally, the cytotoxic effects of anti-TB drugs on immune cells have been evaluated through generation of bioenergetic profiles. Recent studies have revealed that different antibiotics can have distinct effects on the respiration rates and overall energy metabolism of pathogen and host cells [276; 277]. Therefore, understanding cytotoxic effects can lead to the development of more effective treatment regimens, which consider the intertwined metabolic state of both pathogen and host.

1.7.4. Combining bioenergetics with multi-omics to advance tuberculosis research

The combination of targeted metabolomics with cellular energetics is an excellent approach to discern *M. tuberculosis* survival and pathogenic strategies. The cellular energetics tools have been adapted for use on *M. tuberculosis* culture and was used to critically evaluate the effect of BDQ, Q203 and clofazimine (CFZ) on the *M. tuberculosis* ETC, and the value of ETC as a drug target [7]. Data showed that *M. tuberculosis* increases total respiration to maintain ATP by rapidly rerouting around ETC inhibition by these drugs [7]. This is achieved through a switch between terminal oxidase and the increased ETC activity potentiates CFZ production of ROS that is associated with rapid killing *in vitro* and in macrophages [7]. Therefore, combination therapies targeting the ETC were motivated to be exploited to enhance bacterial killing [7]. Additionally, similar studies provided insight into the metabolic mechanism of BDQ-induced cell death and motivated for the development of combination anti-TB therapies that target OXPHOS and glycolysis [278].

A metabolomic and bioenergetic study demonstrated that *M. tuberculosis* infection induces a quiescent energy phenotype in human monocyte-derived macrophages (hMDMs) and decelerated flux through glycolysis and the TCA cycle [29]. Contrastingly, infection of macrophages with BCG or dead *M. tuberculosis* induced glycolytic phenotypes with greater flux. Furthermore, *M. tuberculosis* infection caused a reduction of the mitochondrial dependency on glucose and increased the mitochondrial dependency on fatty acids [29]. This study demonstrated how quantifiable bioenergetic parameters can be used to accurately measure and track disease [29].

Circulating lipids were identified as potential biomarkers for TB diagnosis by multi-omics-derived immunometabolism biosignatures [279]. The insights into the bioenergetic profiles associated with TB give rise to promising diagnostic enhancement. Moreover, the influence of gut microbiota on the bioenergetic landscape during TB pathogenesis is important for the identification of metabolic pathway targets for therapeutic interventions [203].

Integration of multi-omics data, including metabolomics, proteomics, and genomics, has been shown to redefine the virulence attributes of *M. tuberculosis*. These types of studies have uncovered metabolic pathways crucial for *M. tuberculosis* survival and pathogenicity, linking bioenergetics to the overall virulence mechanisms [280].

The metabolic state of macrophages is indicative of their functional polarisation, broadly classified as M1 and M2 phenotypes. The differential reliance on metabolic pathways of macrophage phenotypes can be quantitatively assessed using bioenergetic assays, allowing researchers to confirm macrophage polarisation [281]. Irradiated *M. tuberculosis* was used to infect human AMs and was reported to induce aerobic glycolysis, which is crucial for controlling intracellular bacterial replication via pro-inflammatory processes (M1 phenotype) [282]. Furthermore, HIF-1 α was demonstrated to be essential for the metabolic reprogramming of macrophages during *M. tuberculosis* infection as it promotes glycolysis and lactate production in M1 macrophages while inhibiting OXPHOS [283]. This was determined from the impaired ATP production and reduced macrophage

trafficking in absence of HIF-1 α , underscoring its role in determining macrophage phenotype and function [283].

All of the above-mentioned studies have taken advantage of the currently available tools to improve chances of advancing current TB diagnostics and therapeutics. Over time, multi-omics and bioenergetics have provided a robust framework for investigation and analysis of *M. tuberculosis* virulence and host defences. Exploring the correlation between genetic material, transcripts, metabolites, pathways and energetic processes holds promise in the discovery of new approaches to combat TB.

1.8. Study details

1.8.1. Problem statement

The gradually rising TB incidence rates remain a major public health concern. Currently implemented TB diagnostic and therapeutic regimens are not effective enough to eradicate TB. To increase the chances of stopping the spread of TB, research should prioritise identification of novel biomarkers to be used for prevention, early detection, and streamlined treatment strategies. Although the MTP adhesin has shown promise as a potential TB biomarker, its role in the pathogen's as well as its THP-1 macrophage host metabolism has not been investigated. This gap in knowledge, provides the rationale for this study. A better understanding of MTP's contribution to the metabolic profile of *M. tuberculosis* and its influence on THP-1 macrophages will provide insight into which molecular mechanisms are influenced by MTP during early infection.

1.8.2. Purpose of the study

This experimental research study was performed with the prospect of improving current knowledge of *M. tuberculosis* pathogenicity. This study will potentially validate MTP as a virulence factor and a suitable TB biomarker. Bacterial and *M. tuberculosis* infected THP-1 macrophage metabolic profiles will be determined, with regard to MTP. Moreover, metabolic perturbations observed in the absence of MTP will indicate which metabolic pathways are modulated by MTP, thereby allowing more focused investigation through gene expression levels, metabolic flux and cellular energetics. These efforts will aid in the development of innovative rapid diagnostic tools as well as effective therapies.

1.8.3. Significance of study

Gene knockout mutants, infection models, global and targeted metabolomics as well as RT-qPCR when utilised individually, can provide valuable information regarding *M. tuberculosis* pathogenesis. Thus, embracing a combination of these techniques will assist in expanding researchers' current understanding of the role of MTP in modulating *M. tuberculosis* survival and pathogenesis and the effect of this adhesin on host responses. Therefore, this novel study assessed the influence of MTP on the THP-1 macrophage metabolomic pathways, whole cell bioenergetics, and expression levels of selected genes. The findings are expected to substantiate the potential of MTP as a suitable biomarker for the development of intervention therapy against TB. Furthermore, this strategy may result in identifying specific MTP-modulated pathway metabolites during infection, thus facilitating further novel biomarker discovery.

1.8.4. Aims of the study

This study broadly aimed to investigate the molecular mechanisms of MTP in the regulation of cell surface associated functions, carbon metabolism, fatty acid metabolism, and cellular energetics of the pathogen and host.

The specific aims of this study are:

- 1) To compare the global metabolomic fingerprints of MTP-deficient and MTP-proficient strains of *M. tuberculosis*.
- 2) To determine if MTP influences the metabolic profile of THP-1 macrophages during the initial stages of infection.
- 3) To compare and evaluate the impact of MTP on the metabolic profiles of *M. tuberculosis* infected A549 pulmonary epithelial cells and THP-1 macrophages.
- 4) To investigate the impact of MTP on carbon flux through CCM and determine the bioenergetic profiles of MTP-deficient and MTP-proficient *M. tuberculosis* infected THP-1 macrophages.
- 5) To investigate the role of MTP on the regulation of genes that modulate lipid metabolism, carbon metabolism, and cellular import/transport functions in bacterial culture and intracellular bacteria from infected THP-1 macrophages.

1.8.5. Objectives

The objectives listed below are associated with the corresponding number from the specific aims listed in section 1.8.4. The objectives were:

- 1) To culture and extract metabolites from pelleted wet mass of WT (V9124), Δmtp -knockout mutant and *mtp*-complemented strains of *M. tuberculosis* for untargeted GC $\times$ GC-TOFMS and validate metabolomics findings with RT-qPCR of selected genes.
- 2) To extract metabolites from pelleted wet mass of WT (V9124), Δmtp -knockout mutant and *mtp*-complemented *M. tuberculosis* infected THP-1 macrophages for untargeted GC $\times$ GC-TOF-MS.
- 3) To use untargeted GC $\times$ GC-TOF-MS platform to compare the metabolomic profiles of WT (V9124), Δmtp -knockout mutant and *mtp*-complemented *M. tuberculosis* infected THP-1 macrophages and A459 epithelial cells.
- 4) To use targeted LC-MS/MS to determine the influence of MTP on carbon flux in WT (V9124), Δmtp -knockout mutant and *mtp*-complemented *M. tuberculosis* infected THP-1 macrophages and to use the Agilent Seahorse XF Analyzer to perform CMSTs and GRAs on these infection groups.
- 5) To perform RT-qPCR on genes involved in fatty acid import, β -oxidation, lactate oxidation, and gluconeogenic carbon flow, of pure bacterial culture and intracellular WT (V9124), Δmtp -knockout mutant and *mtp*-complement strains of *M. tuberculosis* and compare the gene expression levels of the two models.

1.8.6. Thesis overview

This thesis is divided into seven chapters. Chapter 1 comprises a review of the currently available and relevant literature. Chapter 2, 3, 4, 5 and 6 are research chapters written in manuscript format. Chapters 2, 3, and 4 have been published in peer reviewed journals. Chapters 5 and 6 have been

submitted to journals and are currently under review for publication. Each of the research chapters corresponds to a sequential aim and its respective objective. Therefore, Chapter 2 provides the first insight into the influence of MTP on the metabolomic fingerprint of *M. tuberculosis*. Chapter 3 maps out the effect of MTP on the intertwined metabolic signature of *M. tuberculosis* infected THP-1 macrophages. Chapter 4 highlights changes to A549 pulmonary epithelial cell and THP-1 macrophage metabolomic profiles, with regards to the absence/presence of MTP. Chapter 5 investigates the effect of MTP on carbon flux through CCM and cellular energetic profiles. Chapter 6 underscores MTP's role in the regulation of genes involved in lactate oxidation, fatty acid oxidation and transport. Lastly, Chapter 7 is a synthesis of research chapters and demonstrates the connection, conclusions, limitations of the current study and recommendations for future work.

References:

1. World Health Organization (2024). Global tuberculosis report. *World Health Organization* [Online]. Available: <https://www.who.int/publications/i/item/9789240101531> [Accessed October 2024].
2. Buis, J.S., Jerene, D., Gebhard, A., Bakker, R., Majidulla, A., Kerkhoff, A.D., Limaye, R.J., and Pelzer, P.T. (2024). Mapping the existing body of knowledge on new and repurposed TB vaccine implementation: A scoping review. *PLoS Global Public Health*, 4(8), e0002885. <http://dx.doi.org/10.1371/journal.pgph.0002885>.
3. Centers of Disease Control and Prevention (2023). Treatment for TB Disease. *Centers for Disease Control and Prevention* [Online]. Available: <https://www.cdc.gov/tb/topic/treatment/tbdisease.htm> [Accessed October 2024].
4. World Health Organization (2023). Antimicrobial resistance. *World Health Organization* [Online]. Available: <https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance> [Accessed November 2024].
5. Alsayed, S.S.R., and Gunosewoyo, H. (2023). Tuberculosis: Pathogenesis, current treatment regimens and new drug targets. *International Journal of Molecular Sciences*, 24(6), 5202. <http://dx.doi.org/10.3390/ijms24065202>.
6. Dix, A., Vlaic, S., Guthke, R., and Linde, J. (2016). Use of systems biology to decipher host-pathogen interaction networks and predict biomarkers. *Clinical Microbiology and Infection*, 22(7), 600-606. <https://doi.org/10.1016/j.cmi.2016.04.014>.
7. Lamprecht, D.A., Finin, P.M., Rahman, M.A., Cumming, B.M., Russell, S.L., Jonnala, S.R., Adamson, J.H., and Steyn, A.J. (2016). Turning the respiratory flexibility of *Mycobacterium tuberculosis* against itself. *Nature Communications*, 7, 12393. <https://doi.org/10.1038/ncomms12393>.
8. Loots, D.T. (2016). New insights into the survival mechanisms of rifampicin-resistant *Mycobacterium tuberculosis*. *Journal of Antimicrobial Chemotherapy*, 71(3), 655-660. <https://doi.org/10.1093/jac/dkv406>.
9. Mohammadnabi, N., Shamseddin, J., Emadi, M., Bodaghi, A.B., Varseh, M., Shariati, A., Rezaei, M., Dastranj, M., and Farahani, A. (2024). *Mycobacterium tuberculosis*: The mechanism of pathogenicity, immune responses, and diagnostic challenges. *Journal of Clinical Laboratory Analysis*, 38(23), e25122. <https://doi.org/10.1002/jcla.25122>.
10. Niemann, H.H., Schubert, W.D., and Heinz, D.W. (2004). Adhesins and invasins of pathogenic bacteria: A structural view. *Microbes and Infection*, 6(1), 101-112. <https://doi.org/10.1016/j.micinf.2003.11.001>.
11. Klemm, P., and Schembri, M.A. (2000). Bacterial adhesins: Function and structure. *International Journal of Medical Microbiology*, 290(1), 27-35. [https://doi.org/10.1016/s1438-4221\(00\)80102-2](https://doi.org/10.1016/s1438-4221(00)80102-2).
12. Ramsugit, S., Pillay, B., and Pillay, M. (2016). Evaluation of the role of *Mycobacterium tuberculosis* pili (MTP) as an adhesin, invasin, and cytokine inducer of epithelial cells. *The*

- Brazilian Journal of Infectious Diseases*, 20(2), 160-165. <http://dx.doi.org/10.1016/j.bjid.2015.11.002>.
13. Alteri, C. (2005). *Novel pili of Mycobacterium tuberculosis*. Doctoral thesis, The University of Arizona, United States of America. <https://repository.arizona.edu/handle/10150/195515?show=full>.
 14. Alteri, C.J., Xicohtencatl-Cortes, J., Hess, S., Caballero-Olín, G., Girón, J.A., and Friedman, R.L. (2007). *Mycobacterium tuberculosis* produces pili during human infection. *Proceedings of the National Academy of Sciences of the United States of America*, 104(12), 5145-5150. <https://doi.org/10.1073/pnas.0602304104>.
 15. Ramsugit, S., Guma, S., Pillay, B., Jain, P., Larsen, M.H., Danaviah, S., and Pillay, M. (2013). Pili contribute to biofilm formation *in vitro* in *Mycobacterium tuberculosis*. *Antonie Van Leeuwenhoek*, 104(5), 725-735. <https://doi.org/10.1007/s10482-013-9981-6>.
 16. Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* pili promote adhesion to and invasion of THP-1 macrophages. *Japanese Journal of Infectious Diseases*, 67(6), 476-478. <https://doi.org/10.7883/yoken.67.476>.
 17. Naidoo, N., Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* pili (MTP), a putative biomarker for a tuberculosis diagnostic test. *Tuberculosis*, 94(3), 338-345. <http://dx.doi.org/10.1016/j.tube.2014.03.004>.
 18. Naidoo, N., Pillay, B., Bubb, M., Pym, A., Chiliza, T., Naidoo, K., Ndung'u, T., Kasproicz, V.O., and Pillay, M. (2018). Evaluation of a synthetic peptide for the detection of anti-*Mycobacterium tuberculosis* curli pili IgG antibodies in patients with pulmonary tuberculosis. *Tuberculosis*, 109, 80-84. <https://doi.org/10.1016/j.tube.2018.01.007>.
 19. Vlachavas, E.I., Bohn, J., Ückert, F., and Nürnberg, S. (2021). A detailed catalogue of multi-omics methodologies for identification of putative biomarkers and causal molecular networks in translational cancer research. *International Journal of Molecular Sciences*, 22(6). <https://doi.org/10.3390/ijms22062822>.
 20. Otchere, I.D., Aboagye, S.Y., Arthur, P.K., and Asante-Poku, A. (2024). Viewpoint of multi-omics potential in tuberculosis research: Identifying biomarkers for biomanufacturing of efficient control tools. *Frontiers in Tropical Diseases*, 5, 1443248. <https://doi.org/10.3389/fitd.2024.1443248>.
 21. Warner, D.F., and Mizrahi, V. (2014). Translating genomics research into control of tuberculosis: Lessons learned and future prospects. *Genome Biology*, 15(11), 514. <https://doi.org/10.1186/s13059-014-0514-z>.
 22. Dunn, W.B., and Ellis, D.I. (2005). Metabolomics: Current analytical platforms and methodologies. *TrAC Trends in Analytical Chemistry*, 24(4), 285-294. <http://dx.doi.org/10.1016/j.trac.2004.11.021>.
 23. Patel, K.N., Patel, J.K., Patel, M.P., Rajput, G.C., and Patel, H.A. (2010). Introduction to hyphenated techniques and their applications in pharmacy. *Pharmaceutical Methods*, 1(1), 2-13. <https://doi.org/10.4103/2229-4708.72222>.
 24. Haglund, S., Peter, Löfstrand, K., Siek, K., and Asplund, L. (2013). Powerful GC-TOF-MS techniques for screening, identification and quantification of halogenated natural products. *Mass Spectrometry*, 2(Spec Iss), S0018. <https://doi.org/10.5702/massspectrometry.s0018>.
 25. Thomas, S.N., French, D., Jannetto, P.J., Rappold, B.A., and Clarke, W.A. (2022). Liquid chromatography–tandem mass spectrometry for clinical diagnostics. *Nature Reviews. Methods Primers*, 2(1), 96. <https://doi.org/10.1038/s43586-022-00175-x>.
 26. Beste, D.J., Noh, K., Niedenfuhr, S., Mendum, T.A., Hawkins, N.D., Ward, J.L., Beale, M.H., Wiechert, W., and McFadden, J. (2013). ¹³C-flux spectral analysis of host-pathogen metabolism reveals a mixed diet for intracellular *Mycobacterium tuberculosis*. *Chemistry and Biology*, 20(8), 1012-1021. <https://doi.org/10.1016/j.chembiol.2013.06.012>.

27. Roberts, L.D., Souza, A.L., Gerszten, R.E., and Clish, C.B. (2012). Targeted metabolomics. *Current Protocols in Molecular Biology*, Chapter 30, Unit 30.32.31-24. <http://dx.doi.org/10.1002/0471142727.mb3002s98>.
28. Bhatti, G.K., Gupta, A., Pahwa, P., Khullar, N., Singh, S., Navik, U., Kumar, S., Mastana, S.S., Reddy, A.P., Reddy, P.H., and Bhatti, J.S. (2022). Targeting mitochondrial bioenergetics as a promising therapeutic strategy in metabolic and neurodegenerative diseases. *Biomedical Journal*, 45(5), 733-748. <https://doi.org/10.1016/j.bj.2022.05.002>.
29. Cumming, B.M., Addicott, K.W., Adamson, J.H., and Steyn, A.J. (2018). *Mycobacterium tuberculosis* induces decelerated bioenergetic metabolism in human macrophages. *Elife*, 7, e39169. <https://doi.org/10.7554/elife.39169>.
30. Adams, G. (2020). A beginner's guide to RT-PCR, qPCR and RT-qPCR. *The Biochemist*, 42(3), 48-53. <http://dx.doi.org/10.1042/BIO20200034>.
31. Smith, I. (2003). *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clinical Microbiology Reviews*, 16(3), 463-496. <https://doi.org/10.1128/cmr.16.3.463-496.2003>.
32. Kinsella, R.L., Zhu, D.X., Harrison, G.A., Mayer Bridwell, A.E., Prusa, J., Chavez, S.M., and Stallings, C.L. (2021). Perspectives and advances in the understanding of tuberculosis. *Annual Review of Pathology Mechanisms of Disease*, 16, 377-408. <https://doi.org/10.1146/annurev-pathol-042120-032916>.
33. Tobin, E.H. , and Tristram, D. (2024). Tuberculosis Overview, in *StatPearls [Internet]*. StatPearls Publishing: <https://www.ncbi.nlm.nih.gov/books/NBK441916/#:~:text=About%2010.6%20million%20p,World%20TB%20Report%202024.%5D>.
34. Brophy, K.M., Scarlett-Ferguson, H., Webber, K.S., Abrams, A.C., and Lammon, C.B. (2010). *Clinical Drug Therapy for Canadian Practice*. Lippincott Williams & Wilkins
35. Mizrahi, V. (2024). A spotlight on the tuberculosis epidemic in South Africa. *Nature Communications*, 15(1), 1290. <https://doi.org/10.1038/s41467-024-45491-w>.
36. García, J.I., Allué-Guardia, A., Tampi, R.P., Restrepo, B.I., and Torrelles, J.B. (2021). New developments and insights in the improvement of *Mycobacterium tuberculosis* vaccines and diagnostics within the End TB Strategy. *Current Epidemiology Reports*, 8(2), 33-45. <https://doi.org/10.1007/s40471-021-00269-2>.
37. World Health Organization (2023). Global tuberculosis report. *World Health Organization [Online]*. Available: <https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2023> [Accessed May 2024].
38. TBThinkTank (2024). National TB recovery plan 3.0 April 2024 – March 2025. *The National Department of Health and Other TB Related Literature [Online]*. Available: <https://tbthinktank.org/ndoh-and-other-tb-related-literature/> [Accessed October 2024].
39. Centers of Disease Control and Prevention (2024). Tuberculosis (TB). [Online]. Available: https://www.cdc.gov/tb/hcp/clinical-overview/drug-resistant-tuberculosis-disease.html#cdc_clinical_overview_treat_pat-treatment-and-recovery [Accessed October 2024].
40. Pillay, M. , and Sturm, A.W. (2007). Evolution of the extensively drug-resistant F15/LAM4/KZN strain of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa. *Clinical Infectious Diseases*, 45(11), 1409-1414. <https://doi.org/10.1086/522987>.
41. Domenech, P., Kolly, G.S., Leon-Solis, L., Fallow, A., and Reed, M.B. (2010). Massive gene duplication event among clinical isolates of the *Mycobacterium tuberculosis* W/Beijing family. *Journal of Bacteriology*, 192(18), 4562-4570. <https://doi.org/10.1128/jb.00536-10>.
42. Ford, C.B., Shah, R.R., Maeda, M.K., Gagneux, S., Murray, M.B., Cohen, T., Johnston, J.C., Gardy, J., Lipsitch, M., and Fortune, S.M. (2013). *Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nature Genetics*, 45(7), 784-790. <https://doi.org/10.1038/ng.2656>.

43. Bespiatykh, D., Bespiatykh, J., Mokrousov, I., and Shitikov, E. (2021). A comprehensive map of *Mycobacterium tuberculosis* complex regions of difference. *mSphere*, 6(4), e0053521. <https://doi.org/10.1128/msphere.00535-21>.
44. Coscolla, M., and Gagneux, S. (2014). Consequences of genomic diversity in *Mycobacterium tuberculosis*. *Seminars in Immunology*, 26(6), 431-444. <https://doi.org/10.1016/j.smim.2014.09.012>.
45. Xu, A.M., He, C.J., Cheng, X., Abuduaini, A., Tuerxun, Z., Sha, Y.Z., Kaisaier, A., Peng, H.M., Zhen, Y.H., Zhang, S.J., Xu, J.R., Li, L., and Zou, X.G. (2022). Distribution and identification of *Mycobacterium tuberculosis* lineage in Kashgar prefecture. *BMC Infectious Diseases*, 22(1), 312. <https://doi.org/10.1186/s12879-022-07307-4>.
46. Oostvogels, S., Ley, S.D., Heupink, T.H., Dippenaar, A., Streicher, E.M., De Vos, E., Meehan, C.J., Dheda, K., Warren, R., and Van Rie, A. (2022). Transmission, distribution and drug resistance-conferring mutations of extensively drug-resistant tuberculosis in the Western Cape Province, South Africa. *Microbial Genomics*, 8(4), 000815. <https://doi.org/10.1099/mgen.0.000815>.
47. Gandhi, N.R., Moll, A., Sturm, A.W., Pawinski, R., Govender, T., Lalloo, U., Zeller, K., Andrews, J., and Friedland, G. (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet*, 368(9547), 1575-1580. [https://doi.org/10.1016/s0140-6736\(06\)69573-1](https://doi.org/10.1016/s0140-6736(06)69573-1).
48. Naidoo, C.C., and Pillay, M. (2014). Increased *in vitro* fitness of multi- and extensively drug-resistant F15/LAM4/KZN strains of *Mycobacterium tuberculosis*. *Clinical Microbiology and Infection*, 20(6), O361-O369. <https://doi.org/10.1111/1469-0691.12415>.
49. Otchere, I.D., Asante-Poku, A., Osei-Wusu, S., Baddoo, A., Sarpong, E., Ganiyu, A.H., Aboagye, S.Y., Forson, A., Bonsu, F., Yahayah, A.I., Koram, K., Gagneux, S., and Yeboah-Manu, D. (2016). Detection and characterization of drug-resistant conferring genes in *Mycobacterium tuberculosis* complex strains: A prospective study in two distant regions of Ghana. *Tuberculosis*, 99, 147-154. <https://doi.org/10.1016/j.tube.2016.05.014>.
50. Köser, C.U., Feuerriegel, S., Summers, D.K., Archer, J.A., and Niemann, S. (2012). Importance of the genetic diversity within the *Mycobacterium tuberculosis* complex for the development of novel antibiotics and diagnostic tests of drug resistance. *Antimicrob Agents Chemother*, 56(12), 6080-6087. <https://doi.org/10.1128/aac.01641-12>.
51. Chiaradia, L., Lefebvre, C., Parra, J., Marcoux, J., Burlet-Schiltz, O., Etienne, G., Tropis, M., and Daffé, M. (2017). Dissecting the mycobacterial cell envelope and defining the composition of the native mycomembrane. *Scientific Reports*, 7(1), 12807. <https://doi.org/10.1038/s41598-017-12718-4>.
52. Bernut, A., Viljoen, A., Dupont, C., Sapriel, G., Blaise, M., Bouchier, C., Brosch, R., De Chastellier, C., Herrmann, J.-L., and Kremer, L. (2016). Insights into the smooth-to-rough transitioning in *Mycobacterium bolletii* unravels a functional Tyr residue conserved in all mycobacterial MmpL family members. *Molecular Microbiology*, 99(5), 866-883. <https://doi.org/10.1111/mmi.13283>.
53. Jacobo-Delgado, Y.M., Rodríguez-Carlos, A., Serrano, C.J., and Rivas-Santiago, B. (2023). *Mycobacterium tuberculosis* cell-wall and antimicrobial peptides: a mission impossible? *Frontiers in Immunology*, 14, 1194923. <https://doi.org/10.3389/fimmu.2023.1194923>.
54. Kumar, S. (2015). *Essentials of Microbiology*. Jaypee Brothers, Medical Publishers Pvt. Limited, New Delhi, India.
55. Beckham, K.S., Ciccarelli, L., Bunduc, C.M., Mertens, H.D., Ummels, R., Lugmayr, W., Mayr, J., Rettel, M., Savitski, M.M., Svergun, D.I., Bitter, W., Wilmanns, M., Marlovits, T.C., Parret, A.H., and Houben, E.N. (2017). Structure of the mycobacterial ESX-5 type VII secretion system membrane complex by single-particle analysis. *Nature Microbiology*, 2, 17047. <https://doi.org/10.1038/nmicrobiol.2017.47>.

56. Gröschel, M.I., Sayes, F., Simeone, R., Majlessi, L., and Brosch, R. (2016). ESX secretion systems: Mycobacterial evolution to counter host immunity. *Nature Reviews. Microbiology*, 14(11), 677-691. <https://doi.org/10.1038/nrmicro.2016.131>.
57. Gordon, S.V. , and Parish, T. (2018). Microbe profile: *Mycobacterium tuberculosis*: Humanity's deadly microbial foe. *Microbiology (Reading)*, 164(4), 437-439. <https://doi.org/10.1099/mic.0.000601>.
58. Sakamoto, K. (2012). The pathology of *Mycobacterium tuberculosis* infection. *Veterinary Pathology*, 49(3), 423-439. <https://doi.org/10.1177/0300985811429313>.
59. Syrym, N., Turgenbaev, K., Yespembetov, B., Basybekov, S., Bazarbayev, M., Borsynbaeva, A., and Bekembayeva, G. (2014). The medium for acceleration of *Mycobacterium tuberculosis* growth, in *Health*, 1866-1873. Scientific Reseach An Accademic Publisher: <http://dx.doi.org/10.4236/health.2014.614219>.
60. Cui, Y., Zhao, D., Barrow, P.A., and Zhou, X. (2016). The endoplasmic reticulum stress response: A link with tuberculosis? *Tuberculosis*, 97, 52-56. <http://dx.doi.org/10.1016/j.tube.2015.12.009>.
61. Delves, P.J. (2020). Chapter 4 - Innate and adaptive systems of immunity, in *The Autoimmune Diseases (Sixth Edition)*, 45-61. Academic Press: <https://doi.org/10.1016/B978-0-12-812102-3.00004-X>.
62. Molnar, C. , and Gair, J. (2015). Chapter 12: Introduction to the immune system and disease, in *Concepts of Biology*. BCcampus: <https://opentextbc.ca/biology/chapter/12-2-innate-immunity/>. Licence: <https://creativecommons.org/licenses/by/4.0/>.
63. Sankar, P. , and Mishra, B.B. (2023). Early innate cell interactions with *Mycobacterium tuberculosis* in protection and pathology of tuberculosis. *Frontiers in Immunology*, 14, 1260859. <https://doi.org/10.3389/fimmu.2023.1260859>.
64. Marshall, J.S., Warrington, R., Watson, W., and Kim, H.L. (2018). An introduction to immunology and immunopathology. *Allergy, Asthma & Clinical Immunology*, 14((Suppl2)), 49. <https://doi.org/10.1186/s13223-018-0278-1>.
65. Lee, J.H., Yin, Q., and Park, H.H. (2023). Editorial: Structural immunology of molecular innate immunity. *Frontiers in Immunology*, 14, 1284121. <https://doi.org/10.3389/fimmu.2023.1284121>.
66. Sun, L., Wang, X., Saredy, J., Yuan, Z., Yang, X., and Wang, H. (2020). Innate-adaptive immunity interplay and redox regulation in immune response. *Redox Biology*, 37, 101759. <https://doi.org/10.1016/j.redox.2020.101759>.
67. Srinivasan, P. (2013). Neutrophils-the sentinels of periodontal innate immunity. *Journal of Clinical and Cellular Immunology*, 13, 1-6. <http://dx.doi.org/10.4172/2155-9899.S13-002>.
68. Bohrer, A.C., Castro, E., Hu, Z., Queiroz, A.T.L., Tocheny, C.E., Assmann, M., Sakai, S., Nelson, C., Baker, P.J., Ma, H., Wang, L., Zilu, W., du Bruyn, E., Riou, C., Kauffman, K.D., Moore, I.N., Del Nonno, F., Petrone, L., Goletti, D., Martineau, A.R., Lowe, D.M., Cronan, M.R., Wilkinson, R.J., Barry, C.E., Via, L.E., Barber, D.L., Klion, A.D., Andrade, B.B., Song, Y., Wong, K.W., and Mayer-Barber, K.D. (2021). Eosinophils are part of the granulocyte response in tuberculosis and promote host resistance in mice. *Journal of Experimental Medicine*, 218(10), e20210469. <https://doi.org/10.1084/jem.20210469>.
69. O'Brien, K.L. , and Finlay, D.K. (2019). Immunometabolism and natural killer cell responses. *Nature Reviews. Immunology*, 19(5), 282-290. <https://doi.org/10.1038/s41577-019-0139-2>.
70. Schmidt, C., Schneble, N., and Wetzker, R. (2014). The fifth dimension of innate immunity. *Journal of Cell Communication and Signalling*, 8(4), 363-367. <https://doi.org/10.1007/s12079-014-0246-6>.
71. Dong, X., Tu, H., Qin, S., Bai, X., Yang, F., and Li, Z. (2023). Insights into the roles of B cells in patients with sepsis. *Journal of Immunology Research*, 2023, 7408967. <https://doi.org/10.1155/2023/7408967>.

72. Zhu, L., Lin, A., Shao, T., Nie, L., Dong, W., Xiang, L., and Shao, J. (2014). B cells in teleost fish act as pivotal initiating APCs in priming adaptive immunity: An evolutionary perspective on the origin of the B-1 cell subset and B7 molecules. *The Journal of Immunology*, 192(6), 2699-2714. <https://doi.org/10.4049/jimmunol.1301312>.
73. Ross, P.J., Sutton, C.E., Higgins, S., Allen, A.C., Walsh, K., Misiak, A., Lavelle, E.C., McLoughlin, R.M., and Mills, K.H. (2013). Relative contribution of Th1 and Th17 cells in adaptive immunity to Bordetella pertussis: towards the rational design of an improved acellular pertussis vaccine. *PLoS Pathogens*, 9(4), e1003264. <https://doi.org/10.1371/journal.ppat.1003264>.
74. Cerutti, A., Puga, I., and Cols, M. (2012). New helping friends for B cells. *European Journal of Immunology*, 42(8), 1956-1968. <https://doi.org/10.1002/eji.201242594>.
75. Crooke, S.N., Ovsyannikova, I.G., Poland, G.A., and Kennedy, R.B. (2019). Immunosenescence and human vaccine immune responses. *Immunity & Ageing*, 16, 25. <https://doi.org/10.1186/s12979-019-0164-9>.
76. Hume, D.A., Ross, I.L., Himes, S.R., Sasmono, R.T., Wells, C.A., and Ravasi, T. (2002). The mononuclear phagocyte system revisited. *Journal of Leukocyte Biology*, 72(4), 621-627. <https://doi.org/10.1189/jlb.72.4.621>.
77. Ebert, R.H. , and Florey, H.W. (1939). The extravascular development of the monocyte observed *in vivo*. *British Journal of Experimental Pathology*, 20(4), 342-356. <https://pmc.ncbi.nlm.nih.gov/articles/PMC2065374/>.
78. Guirado, E., Schlesinger, L.S., and Kaplan, G. (2013). Macrophages in tuberculosis: Friend or foe. *Seminars in Immunopathology*, 35(5), 563-583. <https://doi.org/10.1007/s00281-013-0388-2>.
79. Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., and Tada, K. (1980). Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *International Journal of Cancer*, 26(2), 171-176. <https://doi.org/10.1002/ijc.2910260208>.
80. Schwende, H., Fitzke, E., Ambts, P., and Dieter, P. (1996). Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *International Journal of Cancer* 59(4), 555-561. <https://doi.org/10.1002/ijlb.59.4.555>.
81. Lambrecht, B.N. (2006). Alveolar macrophage in the driver's seat. *Immunity*, 24(4), 366-368. 10.1016/j.immuni.2006.03.008.
82. Lambrecht, B.N. (2006). Alveolar macrophage in the driver's seat. *Immunity*, 24(4), 366-368. <https://doi.org/10.1016/j.immuni.2006.03.008>.
83. Fraternali, A., Brundu, S., and Magnani, M. (2015). Polarization and repolarization of macrophages. *Journal of Clinical & Cellular Immunology*, 6(2), 1-10. <https://doi.org/10.4172/2155-9899.1000319>.
84. Ahmad, F., Rani, A., Alam, A., Zarin, S., Pandey, S., Singh, H., Hasnain, S.E., and Ehtesham, N.Z. (2022). Macrophage: A cell with many faces and functions in tuberculosis. *Frontiers in Immunology*, 13, 747799. <https://doi.org/10.3389/fimmu.2022.747799>.
85. Murray, P.J., Allen, J.E., Biswas, S.K., Fisher, E.A., Gilroy, D.W., Goerdts, S., Gordon, S., Hamilton, J.A., Ivashkiv, L.B., Lawrence, T., Locati, M., Mantovani, A., Martinez, F.O., Mege, J.L., Mosser, D.M., Natoli, G., Saeij, J.P., Schultze, J.L., Shirey, K.A., Sica, A., Suttles, J., Udalova, I., van Ginderachter, J.A., Vogel, S.N., and Wynn, T.A. (2014). Macrophage activation and polarization: Nomenclature and experimental guidelines. *Immunity*, 41(1), 14-20. <https://doi.org/10.1016/j.immuni.2014.06.008>.
86. Liu, C., Liu, H., and Ge, B. (2017). Innate immunity in tuberculosis: Host defense vs pathogen evasion. *Cellular & Molecular Immunology*, 14(12), 963-975. <https://doi.org/10.1038/cmi.2017.88>.
87. Gordon, S. , and Martinez, F.O. (2010). Alternative activation of macrophages: Mechanism and functions. *Immunity*, 32(5), 593-604. <https://doi.org/10.1016/j.immuni.2010.05.007>.

88. Thiriot, J.D., Martinez-Martinez, Y.B., Endsley, J.J., and Torres, A.G. (2020). Hacking the host: Exploitation of macrophage polarization by intracellular bacterial pathogens. *Pathogens and Disease*, 78(1), ftaa009. <https://doi.org/10.1093/femspd/ftaa009>.
89. Anand, N., Peh, K.H., and Kolesar, J.M. (2023). Macrophage repolarization as a therapeutic strategy for osteosarcoma. *Intional Journal of Molecular Science*, 24(3), 2858. <https://doi.org/10.3390/ijms24032858>.
90. Shi, L., Jiang, Q., Bushkin, Y., Subbian, S., and Tyagi, S. (2019). Biphasic dynamics of macrophage immunometabolism during *Mycobacterium tuberculosis* infection. *mBio*, 10(2). <https://doi.org/10.1128/mbio.02550-18>.
91. Huang, Z., Luo, Q., Guo, Y., Chen, J., Xiong, G., Peng, Y., Ye, J., and Li, J. (2015). *Mycobacterium tuberculosis*-induced polarization of human macrophage orchestrates the formation and development of tuberculous granulomas *in vitro*. *PLoS One*, 10(6), e0129744. <https://doi.org/10.1371/journal.pone.0129744>.
92. Pizarro-Cerdá, J. , and Cossart, P. (2006). Bacterial adhesion and entry into host cells. *Cell*, 124(4), 715-727. <https://doi.org/10.1016/j.cell.2006.02.012>.
93. De Waal, A.M., Hiemstra, P.S., Ottenhoff, T.H., Joosten, S.A., and Van Der Does, A.M. (2022). Lung epithelial cells interact with immune cells and bacteria to shape the microenvironment in tuberculosis. *Thorax*, 77(4), 408-416. <https://doi.org/10.1136/thoraxjnl-2021-217997>.
94. Queval, C.J., Brosch, R., and Simeone, R. (2017). The macrophage: A disputed fortress in the battle against *Mycobacterium tuberculosis*. *Frontiers in Microbiology*, 8, 2284. <https://doi.org/10.3389/fmicb.2017.02284>.
95. Orth, K. , and Krachler, A.M. (2013). *Made to Stick: Anti-adhesion therapy for bacterial infections*. Vol. 8.
96. Quesniaux, V., Fremond, C., Jacobs, M., Parida, S., Nicolle, D., Yermeev, V., Bihl, F., Erard, F., Botha, T., Drennan, M., Soler, M.N., Le Bert, M., Schnyder, B., and Ryffel, B. (2004). Toll-like receptor pathways in the immune responses to mycobacteria. *Microbes and Infection*, 6(10), 946-959. <https://doi.org/10.1016/j.micinf.2004.04.016>.
97. Boritsch, E.C., Frigui, W., Cascioferro, A., Malaga, W., Etienne, G., Laval, F., Pawlik, A., Le Chevalier, F., Orgeur, M., Ma, L., Bouchier, C., Stinear, T.P., Supply, P., Majlessi, L., Daffé, M., Guilhot, C., and Brosch, R. (2016). pks5-recombination-mediated surface remodelling in *Mycobacterium tuberculosis* emergence. *Nature Microbiology*, 1, 15019. <https://doi.org/10.1038/nmicrobiol.2015.19>.
98. Ishikawa, E., Ishikawa, T., Morita, Y.S., Toyonaga, K., Yamada, H., Takeuchi, O., Kinoshita, T., Akira, S., Yoshikai, Y., and Yamasaki, S. (2009). Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *Journal of Experimental Medicine*, 206(13), 2879-2888. <https://doi.org/10.1084/jem.20091750>.
99. Bowdish, D.M.E., Sakamoto, K., Kim, M.-J., Kroos, M., Mukhopadhyay, S., Leifer, C.A., Tryggvason, K., Gordon, S., and Russell, D.G. (2009). MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and *Mycobacterium tuberculosis*. *PLoS Pathogens*, 5(6), e1000474. <https://doi.org/10.1371/journal.ppat.1000474>.
100. Manzanillo, Paolo S., Shiloh, Michael U., Portnoy, Daniel A., and Cox, Jeffery S. (2012). *Mycobacterium tuberculosis* activates the DNA-dependent cytosolic surveillance pathway within acrophages. *Cell Host & Microbe*, 11(5), 469-480. <https://doi.org/10.1016/j.chom.2012.03.007>.
101. Sasindran, S. , and Torrelles, J. (2011). *Mycobacterium tuberculosis* infection and inflammation: What is beneficial for the host and for the bacterium? *Frontiers in Microbiology*, 2(2). <https://doi.org/10.3389/fmicb.2011.00002>.
102. Schnettger, L., Rodgers, A., Repnik, U., Lai, R.P., Pei, G., Verdoes, M., Wilkinson, R.J., Young, D.B., and Gutierrez, M.G. (2017). A Rab20-dependent membrane trafficking pathway

- controls *M. tuberculosis* replication by regulating phagosome spaciousness and integrity. *Cell Host Microbe*, 21(5), 619-628.e615. <https://doi.org/10.1016/j.chom.2017.04.004>.
103. Sun-Wada, G.H., Tabata, H., Kawamura, N., Aoyama, M., and Wada, Y. (2009). Direct recruitment of H⁺-ATPase from lysosomes for phagosomal acidification. *Journal of Cell Science*, 122(Pt 14), 2504-2513. <https://doi.org/10.1242/jcs.050443>.
 104. Thurston, T.L., Wandel, M.P., von Muhlinen, N., Foeglein, A., and Randow, F. (2012). Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. *Nature*, 482(7385), 414-418. <https://doi.org/10.1038/nature10744>.
 105. Sakowski, E.T., Koster, S., Portal Celhay, C., Park, H.S., Shrestha, E., Hetzenecker, S.E., Maurer, K., Cadwell, K., and Philips, J.A. (2015). Ubiquilin 1 promotes IFN- γ -induced xenophagy of *Mycobacterium tuberculosis*. *PLoS Pathogens*, 11(7), e1005076. <https://doi.org/10.1371/journal.ppat.1005076>.
 106. Jayachandran, R., Sundaramurthy, V., Combaluzier, B., Mueller, P., Korf, H., Huygen, K., Miyazaki, T., Albrecht, I., Massner, J., and Pieters, J. (2007). Survival of mycobacteria in macrophages is mediated by coronin 1-dependent activation of calcineurin. *Cell*, 130(1), 37-50. <https://doi.org/10.1016/j.cell.2007.04.043>.
 107. Fratti, R.A., Backer, J.M., Gruenberg, J., Corvera, S., and Deretic, V. (2001). Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. *Journal of Cell Biology*, 154(3), 631-644. <https://doi.org/10.1083/jcb.200106049>.
 108. Fratti, R.A., Chua, J., Vergne, I., and Deretic, V. (2003). *Mycobacterium tuberculosis* glycosylated phosphatidylinositol causes phagosome maturation arrest. *Proceedings of the National Academy of Sciences of the United States of America*, 100(9), 5437-5442. <https://doi.org/10.1073/pnas.0737613100>.
 109. Vergne, I., Fratti, R.A., Hill, P.J., Chua, J., Belisle, J., and Deretic, V. (2004). *Mycobacterium tuberculosis* phagosome maturation arrest: Mycobacterial phosphatidylinositol analog phosphatidylinositol mannoside stimulates early endosomal fusion. *Molecular Biology of the Cell*, 15(2), 751-760. <https://doi.org/10.1091/mbc.e03-05-0307>.
 110. Wong, D., Bach, H., Sun, J., Hmama, Z., and Av-Gay, Y. (2011). *Mycobacterium tuberculosis* protein tyrosine phosphatase (PtpA) excludes host vacuolar-H⁺-ATPase to inhibit phagosome acidification. *Proceedings of the National Academy of Sciences of the United States of America* 108(48), 19371-19376. <https://doi.org/10.1073/pnas.1109201108>.
 111. Sturgill-Koszycki, S., Schlesinger, P.H., Chakraborty, P., Haddix, P.L., Collins, H.L., Fok, A.K., Allen, R.D., Gluck, S.L., Heuser, J., and Russell, D.G. (1994). Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. *Science*, 263(5147), 678-681. <https://doi.org/10.1126/science.8303277>.
 112. Queval, C.J., Song, O.R., Carralot, J.P., Saliou, J.M., Bongiovanni, A., Deloison, G., Deboosère, N., Jouny, S., Iantomasi, R., Delorme, V., Debie, A.S., Park, S.J., Gouveia, J.C., Tomavo, S., Brosch, R., Yoshimura, A., Yeramian, E., and Brodin, P. (2017). *Mycobacterium tuberculosis* controls phagosomal acidification by targeting CISH-mediated signalling. *Cell Reports*, 20(13), 3188-3198. <https://doi.org/10.1016/j.celrep.2017.08.101>.
 113. Patin, E.C., Geffken, A.C., Willcocks, S., Leschczyk, C., Haas, A., Nimmerjahn, F., Lang, R., Ward, T.H., and Schaible, U.E. (2017). Trehalose dimycolate interferes with Fc γ R-mediated phagosome maturation through Mincle, SHP-1 and Fc γ RIIB signalling. *PLoS One*, 12(4), e0174973. <https://doi.org/10.1371/journal.pone.0174973>.
 114. Simeone, R., Sayes, F., Song, O., Groschel, M.I., Brodin, P., Brosch, R., and Majlessi, L. (2015). Cytosolic access of *Mycobacterium tuberculosis*: Critical impact of phagosomal acidification control and demonstration of occurrence *in vivo*. *PLoS Pathogens*, 11(2), e1004650. <https://doi.org/10.1371/journal.ppat.1004650>.
 115. Augenstreich, J., Arbues, A., Simeone, R., Haanappel, E., Wegener, A., Sayes, F., Le Chevalier, F., Chalut, C., Malaga, W., Guilhot, C., Brosch, R., and Astarie-Dequeker, C.

- (2017). ESX-1 and phthiocerol dimycocerosates of *Mycobacterium tuberculosis* act in concert to cause phagosomal rupture and host cell apoptosis. *Cellular Microbiology*, 19(7), e1276. <https://doi.org/10.1111/cmi.12726>.
116. Chen, Y., Ye, X., Escames, G., Lei, W., Zhang, X., Li, M., Jing, T., Yao, Y., Qiu, Z., Wang, Z., Acuña-Castroviejo, D., and Yang, Y. (2023). The NLRP3 inflammasome: Contributions to inflammation-related diseases. *Cellular & Molecular Biology Letters*, 28(1), 51. <https://doi.org/10.1186/s11658-023-00462-9>.
 117. Labbé, K. , and Saleh, M. (2008). Cell death in the host response to infection. *Cell Death & Differentiation*, 15(9), 1339-1349. <https://doi.org/10.1038/cdd.2008.91>.
 118. Butler, R.E., Brodin, P., Jang, J., Jang, M.-S., Robertson, B.D., Gicquel, B., and Stewart, G.R. (2012). The balance of apoptotic and necrotic cell death in *Mycobacterium tuberculosis* infected macrophages is not dependent on bacterial virulence. *PLoS One*, 7(10), e47573. <https://doi.org/10.1371/journal.pone.0047573>.
 119. Velmurugan, K., Chen, B., Miller, J.L., Azogue, S., Gurses, S., Hsu, T., Glickman, M., Jacobs, W.R., Jr., Porcelli, S.A., and Briken, V. (2007). *Mycobacterium tuberculosis* nuoG is a virulence gene that inhibits apoptosis of infected host cells. *PLoS Pathogens*, 3(7), e110. <https://doi.org/10.1371/journal.ppat.0030110>.
 120. Gengenbacher, M., Nieuwenhuizen, N., Vogelzang, A., Liu, H., Kaiser, P., Schuerer, S., Lazar, D., Wagner, I., Mollenkopf, H.J., and Kaufmann, S.H. (2016). Deletion of nuoG from the vaccine candidate *Mycobacterium bovis* BCG deltaureC::hly improves protection against tuberculosis. *mBio*, 7(3), e00679-00616. <https://doi.org/10.1128/mbio.00679-16>.
 121. Oddo, M., Renno, T., Attinger, A., Bakker, T., MacDonald, H.R., and Meylan, P.R. (1998). Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*. *Journal of Immunology*, 160(11), 5448-5454. <https://doi.org/10.4049/jimmunol.160.11.5448>.
 122. Sholeye, A.R., Williams, A.A., Loots, D.T., Tutu Van Furth, A.M., Van Der Kuip, M., and Mason, S. (2022). Tuberculous granuloma: Emerging insights from proteomics and metabolomics. *Frontiers in Neurology*, 13, 804838. <https://doi.org/10.3389/fneur.2022.804838>.
 123. Saunders, B.M. , and Britton, W.J. (2007). Life and death in the granuloma: Immunopathology of tuberculosis. *Immunology and Cell Biology*, 85(2), 1103-1111. <https://doi.org/10.1038/sj.icb.7100027>.
 124. Domingo-Gonzalez, R., Prince, O., Cooper, A., and Khader, S.A. (2016). Cytokines and chemokines in *Mycobacterium tuberculosis* infection. *Microbiology Spectrum*, 4(5), 10.1128/microbiolspec.TBTB1122-0018-2016. <https://doi.org/10.1128/microbiolspec.TBTB2-0018-2016>.
 125. Yang, J., Zhang, L., Qiao, W., and Luo, Y. (2023). *Mycobacterium tuberculosis*: Pathogenesis and therapeutic targets. *MedComm*, 4(5), e353. <https://doi.org/10.1002/mco2.353>.
 126. Russell, D.G., Cardona, P.J., Kim, M.J., Allain, S., and Altare, F. (2009). Foamy macrophages and the progression of the human tuberculosis granuloma. *Nature Immunology*, 10(9), 943-948. <http://dx.doi.org/10.1038/ni.1781>.
 127. Huszár, S., Chibale, K., and Singh, V. (2020). The quest for the holy grail: New antitubercular chemical entities, targets and strategies. *Drug Discovery Today*, 25(4), 772-780. <https://doi.org/10.1016/j.drudis.2020.02.003>.
 128. Grosset, J. (2003). *Mycobacterium tuberculosis* in the extracellular compartment: an underestimated adversary. *Antimicrob Agents and Chemotherapy*, 47(3), 833-836. 10.1128/aac.47.3.833-836.2003.
 129. Heemskerk, D., Caws, M., Marais, B., and Farrar, J. (2015). Pathogenesis, in *Tuberculosis in Adults and Children*. Springer International Publishing: <https://link.springer.com/book/10.1007/978-3-319-19132-4>. Licence: <https://s100.copyright.com/AppDispatchServlet?publisherName=SpringerNature&orderBea>

[nReset=true&orderSource=SpringerLink&title=Pathogenesis&author=Dorothee%20Heemskerker%2C%20Maxine%20Caws%2C%20Ben%20Marais%20et%20al&contentID=10.1007%2F978-3-319-19132-4_2©right=The%20Author%28s%29&publication=eBook&publicationDate=2015&startPage=9&endPage=16&imprint=The%20Author%28s%29&oa=CC%20BY-NC.](#)

130. Geldmacher, C., Ngwenyama, N., Schuetz, A., Petrovas, C., Reither, K., Heeregrave, E.J., Casazza, J.P., Ambrozak, D.R., Louder, M., Ampofo, W., Pollakis, G., Hill, B., Sanga, E., Saathoff, E., Maboko, L., Roederer, M., Paxton, W.A., Hoelscher, M., and Koup, R.A. (2010). Preferential infection and depletion of *Mycobacterium tuberculosis*-specific CD4 T cells after HIV-1 infection. *Journal of Experimental Medicine* 207(13), 2869-2881. <https://doi.org/10.1084/jem.20100090>.
131. Chai, Q., Zhang, Y., and Liu, C.H. (2018). *Mycobacterium tuberculosis*: An adaptable pathogen associated with multiple human diseases. *Frontiers in Cellular and Infection Microbiology*, 8. 10.3389/fcimb.2018.00158.
132. Luies, L. , and Du Preez, I. (2020). The echo of pulmonary tuberculosis: Mechanisms of clinical symptoms and other disease-induced systemic complications. *Clinical Microbiology Reviews*, 33(4), 10.1128/cmr. 00036-000200893-8512.
133. Gengenbacher, M. , and Kaufmann, S.H. (2012). *Mycobacterium tuberculosis*: Success through dormancy. *FEMS Microbiology Reviews*, 36(3), 514-532. <https://doi.org/10.1111/j.1574-6976.2012.00331.x>.
134. Ryu, Y.J. (2015). Diagnosis of pulmonary tuberculosis: Recent advances and diagnostic algorithms. *Tuberculosis and Respiratory Diseases*, 78(2), 64-71. <http://dx.doi.org/10.4046/trd.2015.78.2.64>.
135. Goscé, L., Allel, K., Hamada, Y., Surkova, E., Kontsevaya, I., Wang, T.T., Liu, W.-H., Matveev, A., Ziganshina, L.E., and Korobitsyn, A. (2024). Systematic review of the economic impact of novel *Mycobacterium tuberculosis* specific antigen-based skin tests for detection of TB infection compared with tuberculin skin test and interferon-gamma release assays. *PLoS Global Public Health*, 4(10), e0003655. <https://doi.org/10.1371/journal.pgph.0003655>.
136. Pahal, P. , and Sharma, S. (2023). PPD Skin Test, in *StatPearls [Internet]*. StatPearls Publishing: <https://www.ncbi.nlm.nih.gov/books/NBK556037/>.
137. World Health Organization (2024). WHO consolidated guidelines on tuberculosis. Module 3: diagnosis—rapid diagnostics for tuberculosis detection. *World Health Organization* [Online]. Available: <https://www.who.int/publications/i/item/9789240029415> [Accessed October 2024].
138. Lawn, S.D., Kerkhoff, A.D., Vogt, M., and Wood, R. (2012). Diagnostic accuracy of a low-cost, urine antigen, point-of-care screening assay for HIV-associated pulmonary tuberculosis before antiretroviral therapy: A descriptive study. *The Lancet. Infectious Diseases*, 12(3), 201-209. [https://doi.org/10.1016/s1473-3099\(11\)70251-1](https://doi.org/10.1016/s1473-3099(11)70251-1).
139. Paris, L., Magni, R., Zaidi, F., Araujo, R., Saini, N., Harpole, M., Coronel, J., Kirwan, D.E., Steinberg, H., and Gilman, R.H. (2017). Urine lipoarabinomannan glycan in HIV-negative patients with pulmonary tuberculosis correlates with disease severity. *Science Translational Medicine*, 9(420), eaal2807. <https://doi.org/10.1126/scitranslmed.aal2807>.
140. Lee, Y.H., Hyun, Y.S., Jo, H.A., Baek, I.C., Kim, S.M., Sohn, H.J., and Kim, T.G. (2022). Comprehensive analysis of *Mycobacterium tuberculosis* antigen-specific CD4(+) T cell responses restricted by single HLA class II allotype in an individual. *Frontiers in Immunology*, 13, 897781. <https://doi.org/10.3389/fimmu.2022.897781>.
141. Nahid, P., Dorman, S.E., Alipanah, N., Barry, P.M., Brozek, J.L., Cattamanchi, A., Chaisson, L.H., Chaisson, R.E., Daley, C.L., Grzemska, M., Higashi, J.M., Ho, C.S., Hopewell, P.C., Keshavjee, S.A., Lienhardt, C., Menzies, R., Merrifield, C., Narita, M., O'Brien, R., Peloquin, C.A., Raftery, A., Saukkonen, J., Schaaf, H.S., Sotgiu, G., Starke, J.R., Migliori, G.B., and Vernon, A. (2016). Official American Thoracic Society/Centers for Disease Control and

- Prevention/Infectious Diseases Society of America Clinical Practice guidelines: Treatment of drug-susceptible tuberculosis. *Clinical Infectious Diseases*, 63(7), e147-e195. <https://doi.org/10.1093/cid/ciw376>.
142. Garcia-Prats, A.J., Schaaf, H.S., and Hesselning, A.C. (2016). The safety and tolerability of the second-line injectable antituberculosis drugs in children. *Expert Opinion on Drug Safety*, 15(11), 1491-1500. <https://doi.org/10.1080/14740338.2016.1223623>.
 143. Bigelow, K.M., Tasneen, R., Chang, Y.S., Dooley, K.E., and Nuermberger, E.L. (2020). Preserved efficacy and reduced toxicity with intermittent linezolid dosing in combination with bedaquiline and pretomanid in a murine tuberculosis model. *Antimicrobial Agents and Chemotherapy*, 64(10), e01178-01120. <https://doi.org/10.1128/aac.01178-20>.
 144. Zhuang, L., Ye, Z., Li, L., Yang, L., and Gong, W. (2023). Next-generation TB vaccines: Progress, challenges, and prospects. *Vaccines (Basel)*, 11(8), 1304. <https://doi.org/10.3390/vaccines11081304>.
 145. Tait, D.R., Hatherill, M., Meeren, O.V.D., Ginsberg, A.M., Brakel, E.V., Salaun, B., Scriba, T.J., Akite, E.J., Ayles, H.M., Bollaerts, A., Demoitié, M.-A., Diacon, A., Evans, T.G., Gillard, P., Hellström, E., Innes, J.C., Lempicki, M., Malahleha, M., Martinson, N., Vela, D.M., Muyoyeta, M., Nduba, V., Pascal, T.G., Tameris, M., Thienemann, F., Wilkinson, R.J., and Roman, F. (2019). Final analysis of a trial of M72/AS01_E vaccine to prevent tuberculosis. *New England Journal of Medicine*, 381(25), 2429-2439. <https://doi.org/10.1056/nejmoa1909953>.
 146. Burki, T. (2024). Pivotal tuberculosis vaccine trial begins. *Lancet*, 403(10432), 1125. [https://doi.org/10.1016/s0140-6736\(24\)00579-8](https://doi.org/10.1016/s0140-6736(24)00579-8).
 147. Jiang, F., Sun, T., Cheng, P., Wang, J., and Gong, W. (2023). A summary on tuberculosis vaccine development-Where to go? *Journal of Personalized Medicine*, 13(3), 408. <https://doi.org/10.3390/jpm13030408>.
 148. Geldenhuys, H., Mearns, H., Miles, D.J.C., Tameris, M., Hokey, D., Shi, Z., Bennett, S., Andersen, P., Kromann, I., Hoff, S.T., Hanekom, W.A., Mahomed, H., Hatherill, M., Scriba, T.J., Van Rooyen, M., Bruce McClain, J., Ryall, R., and De Bruyn, G. (2015). The tuberculosis vaccine H4:IC31 is safe and induces a persistent polyfunctional CD4 T cell response in South African adults: A randomized controlled trial. *Vaccine*, 33(30), 3592-3599. <https://doi.org/10.1016/j.vaccine.2015.05.036>.
 149. Luabeya, A.K., Kagina, B.M., Tameris, M.D., Geldenhuys, H., Hoff, S.T., Shi, Z., Kromann, I., Hatherill, M., Mahomed, H., Hanekom, W.A., Andersen, P., Scriba, T.J., Schoeman, E., Krohn, C., Day, C.L., Africa, H., Makhethhe, L., Smit, E., Brown, Y., Suliman, S., Hughes, E.J., Bang, P., Snowden, M.A., McClain, B., and Hussey, G.D. (2015). First-in-human trial of the post-exposure tuberculosis vaccine H56:IC31 in *Mycobacterium tuberculosis* infected and non-infected healthy adults. *Vaccine*, 33(33), 4130-4140. <https://doi.org/10.1016/j.vaccine.2015.06.051>.
 150. Sagawa, Z.K., Goman, C., Frevol, A., Blazeovic, A., Tennant, J., Fisher, B., Day, T., Jackson, S., Lemiale, F., Toussaint, L., Kalisz, I., Jiang, J., Ondrejcek, L., Mohamath, R., Vergara, J., Lew, A., Beckmann, A.M., Casper, C., Hoft, D.F., and Fox, C.B. (2023). Safety and immunogenicity of a thermostable ID93 + GLA-SE tuberculosis vaccine candidate in healthy adults. *Nature Communications*, 14(1), 1138. <https://doi.org/10.1038/s41467-023-36789-2>.
 151. Bekker, L.-G., Dintwe, O., Fiore-Gartland, A., Middelkoop, K., Hutter, J., Williams, A., Randhawa, A.K., Ruhwald, M., Kromann, I., Andersen, P.L., DiazGranados, C.A., Rutkowski, K.T., Tait, D., Miner, M.D., Andersen-Nissen, E., De Rosa, S.C., Seaton, K.E., Tomaras, G.D., McElrath, M.J., Ginsberg, A., and Kublin, J.G. (2020). A phase 1b randomized study of the safety and immunological responses to vaccination with H4:IC31, H56:IC31, and BCG revaccination in *Mycobacterium tuberculosis* uninfected adolescents in Cape Town, South Africa. *eClinicalMedicine*, 21, 100313. <https://doi.org/10.1016/j.eclinm.2020.100313>.

152. Tameris, M.D., Hatherill, M., Landry, B.S., Scriba, T.J., Snowden, M.A., Lockhart, S., Shea, J.E., McClain, J.B., Hussey, G.D., Hanekom, W.A., Mahomed, H., and McShane, H. (2013). Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: A randomised, placebo-controlled phase 2b trial. *Lancet*, 381(9871), 1021-1028. [https://doi.org/10.1016/s0140-6736\(13\)60177-4](https://doi.org/10.1016/s0140-6736(13)60177-4).
153. Cotton, M.F., Madhi, S.A., Luabeya, A.K., Tameris, M., Hesselning, A.C., Shenje, J., Schoeman, E., Hatherill, M., Desai, S., Kapse, D., Brückner, S., Koen, A., Jose, L., Moultrie, A., Bhikha, S., Walzl, G., Gutschmidt, A., Kotze, L.A., Allies, D.L., Loxton, A.G., Shaligram, U., Abraham, M., Johnstone, H., Grode, L., Kaufmann, S.H.E., and Kulkarni, P.S. (2022). Safety and immunogenicity of VPM1002 versus BCG in South African newborn babies: A randomised, phase 2 non-inferiority double-blind controlled trial. *The Lancet. Infectious Diseases*, 22(10), 1472-1483. [https://doi.org/10.1016/s1473-3099\(22\)00222-5](https://doi.org/10.1016/s1473-3099(22)00222-5).
154. Aguilo, N., Uranga, S., Marinova, D., Monzon, M., Badiola, J., and Martin, C. (2016). MTBVAC vaccine is safe, immunogenic and confers protective efficacy against *Mycobacterium tuberculosis* in newborn mice. *Tuberculosis*, 96, 71-74. <https://doi.org/10.1016/j.tube.2015.10.010>.
155. Strimbu, K., and Tavel, J.A. (2010). What are biomarkers? *Current Opinion in HIV and AIDS*, 5(6), 463-466. <https://doi.org/10.1097/coh.0b013e32833ed177>.
156. Yu, Y., Jiang, X.X., and Li, J.C. (2023). Biomarker discovery for tuberculosis using metabolomics. *Frontiers in Molecular Biosciences*, 10, 1099654. <https://doi.org/10.3389/fmolb.2023.1099654>.
157. Simon, R. (2014). Biomarker based clinical trial design. *Chinese Clinical Oncology*, 3(3), 39. <https://doi.org/10.3978/j.issn.2304-3865.2014.02.03>.
158. Quezada, H., Guzmán-Ortiz, A.L., Díaz-Sánchez, H., Valle-Rios, R., and Aguirre-Hernández, J. (2017). Omics-based biomarkers: Current status and potential use in the clinic. *Boletín Médico Del Hospital Infantil de México (English Edition)*, 74(3), 219-226. <https://doi.org/10.1016/j.bmhime.2017.11.030>.
159. Du Preez, I., Luies, L., and Loots, D.T. (2017). Metabolomics biomarkers for tuberculosis diagnostics: Current status and future objectives. *Biomarkers in Medicine*, 11, 179-194. <https://doi.org/10.2217/bmm-2016-0287>.
160. Essone, P.N., Kalsdorf, B., Chegou, N.N., Loxton, A.G., Kriel, M., Preyer, R., Ernst, M., Walzl, G., and Lange, C. (2014). Bifunctional T-cell-derived cytokines for the diagnosis of tuberculosis and treatment monitoring. *Respiration*, 88(3), 251-261. <https://doi.org/10.1159/000365816>.
161. Jeong, Y.H., Hur, Y.-G., Lee, H., Kim, S., Cho, J.-E., Chang, J., Shin, J.S., Lee, H., Kang, A.Y., Cho, S.-N., and Ha, S.-J. (2015). Discrimination between active and latent tuberculosis based on ratio of antigen-specific to mitogen-induced IP-10 production. *Journal of Clinical Microbiology*, 53(2), 504-510. <https://doi.org/10.1128/jcm.02758-14>.
162. Petrone, L., Cannas, A., Aloï, F., Nsubuga, M., Sserumkuma, J., Nazziwa, R.A., Jugheli, L., Lukindo, T., Girardi, E., Reither, K., and Goletti, D. (2015). Blood or urine IP-10 cannot discriminate between active tuberculosis and respiratory diseases different from tuberculosis in children. *Biomed Research International*, 2015, 589471. <https://doi.org/10.1155/2015/589471>.
163. Azzurri, A., Sow, O.Y., Amedei, A., Bah, B., Diallo, S., Peri, G., Benagiano, M., D'Elis, M.M., Mantovani, A., and Del Prete, G. (2005). IFN-gamma-inducible protein 10 and pentraxin 3 plasma levels are tools for monitoring inflammation and disease activity in *Mycobacterium tuberculosis* infection. *Microbes and Infection*, 7(1), 1-8. <https://doi.org/10.1016/j.micinf.2004.09.004>.
164. Shaik, J., Pillay, M., and Jeena, P. (2014). The role of interferon gamma release assays in the monitoring of response to anti-tuberculosis treatment in children. *Paediatric Respiratory Reviews*, 15(3), 264-267. <https://doi.org/10.1016/j.prrv.2013.11.007>.

165. Miotto, P., Mwangoka, G., Valente, I.C., Norbis, L., Sotgiu, G., Bosu, R., Ambrosi, A., Codecasa, L.R., Goletti, D., Matteelli, A., Ntinginya, E.N., Aloï, F., Heinrich, N., Reither, K., and Cirillo, D.M. (2013). miRNA signatures in sera of patients with active pulmonary tuberculosis. *PLoS One*, 8(11), e80149. <https://doi.org/10.1371/journal.pone.0080149>.
166. Li, G., Feng, Z., Song, H., Wang, Y., Zhu, L., and Li, Y. (2023). Long non-coding RNA expression in PBMCs of patients with active pulmonary tuberculosis. *Frontiers in Microbiology*, 14, 1257267. <https://doi.org/10.3389/fmicb.2023.1257267>.
167. Seid, G., Alemu, A., Tsedalu, T., and Dagne, B. (2022). Value of urine-based lipoarabinomannan (LAM) antigen tests for diagnosing tuberculosis in children: Systematic review and meta-analysis. *IJID Regions*, 4, 97-104. <https://doi.org/10.1016/j.ijregi.2022.06.004>.
168. Cannas, A., Goletti, D., Girardi, E., Chiacchio, T., Calvo, L., Cuzzi, G., Piacentini, M., Melkonyan, H., Umansky, S.R., Lauria, F.N., Ippolito, G., and Tomei, L.D. (2008). *Mycobacterium tuberculosis* DNA detection in soluble fraction of urine from pulmonary tuberculosis patients. *International Journal of Tuberculosis and Lung Disease*, 12(2), 146-151. PMID: 18230246; <https://pubmed.ncbi.nlm.nih.gov/18230246/>.
169. Adekambi, T., Ibegbu, C.C., Kalokhe, A.S., Yu, T., Ray, S.M., and Rengarajan, J. (2012). Distinct effector memory CD4⁺ T cell signatures in latent *Mycobacterium tuberculosis* infection, BCG vaccination and clinically resolved tuberculosis. *PLoS One*, 7(4), e36046. <https://doi.org/10.1371/journal.pone.0036046>.
170. Goletti, D., Petruccioli, E., Joosten, S.A., and Ottenhoff, T.H.M. (2016). Tuberculosis biomarkers: From diagnosis to protection. *Infectious Disease Reports*, 8(2), 6568. <https://doi.org/10.4081/idr.2016.6568>.
171. Chegou, N.N., Sutherland, J.S., Malherbe, S., Crampin, A.C., Corstjens, P.L., Geluk, A., Mayanja-Kizza, H., Loxton, A.G., Van der Spuy, G., Stanley, K., Kotzé, L.A., Van Der Vyver, M., Rosenkrands, I., Kidd, M., Van Helden, P.D., Dockrell, H.M., Ottenhoff, T.H., Kaufmann, S.H., and Walzl, G. (2016). Diagnostic performance of a seven-marker serum protein biosignature for the diagnosis of active TB disease in African primary healthcare clinic attendees with signs and symptoms suggestive of TB. *Thorax*, 71(9), 785-794. <https://doi.org/10.1136/thoraxjnl-2015-207999>.
172. Zhang, L. (2018). Multi-epitope vaccines: A promising strategy against tumors and viral infections. *Cellular & Molecular Immunology*, 15(2), 182-184. <https://doi.org/10.1038/cmi.2017.92>.
173. Kline, K.A., Fälker, S., Dahlberg, S., Normark, S., and Henriques-Normark, B. (2009). Bacterial adhesins in host-microbe interactions. *Cell Host & Microbe*, 5(6), 580-592. <https://doi.org/10.1016/j.chom.2009.05.011>.
174. Ramsugit, S., and Pillay, M. (2016). Identification of *Mycobacterium tuberculosis* adherence-mediating components: A review of key methods to confirm adhesin function. *Iranian Journal of Basic Medical Sciences*, 19(6), 579-584. PMID: <https://pmc.ncbi.nlm.nih.gov/articles/PMC4951595/>.
175. Govender, V.S., Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* adhesins: Potential biomarkers as anti-tuberculosis therapeutic and diagnostic targets. *Microbiology*, 160(Pt 9), 1821-1831. <https://doi.org/10.1099/mic.0.082206-0>.
176. Stones, D.H., and Krachler, A.-M. (2015). Fatal attraction: How bacterial adhesins affect host signaling and what we can learn from them. *International Journal of Molecular Sciences*, 16(2), 2626-2640. <https://doi.org/10.3390/ijms16022626>.
177. Viljoen, A., Dufrêne, Y.F., and Nigou, J. (2022). Mycobacterial adhesion: From hydrophobic to receptor-ligand interactions. *Microorganisms*, 10(2), 454. <https://doi.org/10.3390/microorganisms10020454>.

178. Soto, G.E. , and Hultgren, S.J. (1999). Bacterial adhesins: Common themes and variations in architecture and assembly. *Journal of Bacteriology*, 181(4), 1059-1071. <https://doi.org/10.1128/jb.181.4.1059-1071.1999>.
179. Finlay, B.B. , and Falkow, S. (1997). Common themes in microbial pathogenicity revisited. *Microbiology and Molecular Biology Reviews*, 61(2), 136-169. <https://doi.org/10.1128/mmbr.61.2.136-169.1997>.
180. Strom, M.S. , and Lory, S. (1993). Structure-function and biogenesis of the type IV pili. *Annual Review of Microbiology*, 47, 565-596. <https://doi.org/10.1146/annurev.mi.47.100193.003025>.
181. Ledesma, M.A., Ochoa, S.A., Cruz, A., Rocha-Ramírez, L.M., Mas-Oliva, J., Eslava, C.A., Girón, J.A., and Xicohtencatl-Cortes, J. (2010). The hemorrhagic coli pilus (HCP) of *Escherichia coli* O157:H7 is an inducer of proinflammatory cytokine secretion in intestinal epithelial cells. *PLoS One*, 5(8), e12127. <https://doi.org/10.1371/journal.pone.0012127>.
182. Boland, T., Latour, R., A., and Stutzenberger, F., J. (2000). *Handbook of bacterial adhesion: Principles, methods, and applications*. Humana Press Inc, Totowa.
183. Mann, K.M., Pride, A.C., Flentie, K., Kimmey, J.M., Weiss, L.A., and Stallings, C.L. (2016). Analysis of the contribution of MTP and the predicted Flp pilus genes to *Mycobacterium tuberculosis* pathogenesis. *Microbiology*, 162(10), 1784-1796. <https://doi.org/10.1099/mic.0.000368>.
184. Hammar, M., Bian, Z., and Normark, S. (1996). Nucleator-dependent intercellular assembly of adhesive curli organelles in *Escherichia coli*. *Proceedings of the National Academy of Sciences*, 93(13), 6562-6566. <https://doi.org/10.1073/pnas.93.13.6562>.
185. Berin, M.C., Darfeuille-Michaud, A., Egan, L.J., Miyamoto, Y., and Kagnoff, M.F. (2002). Role of EHEC O157:H7 virulence factors in the activation of intestinal epithelial cell NF-kappaB and MAP kinase pathways and the upregulated expression of interleukin 8. *Cellular Microbiology*, 4(10), 635-648. <https://doi.org/10.1046/j.1462-5822.2002.00218.x>.
186. Xicohtencatl-Cortés, J., Lyons, S., Chaparro, A.P., Hernández, D.R., Saldaña, Z., Ledesma, M.A., Rendón, M.A., Gewirtz, A.T., Klose, K.E., and Girón, J.A. (2006). Identification of proinflammatory flagellin proteins in supernatants of *Vibrio cholerae* O1 by proteomics analysis. *Molecular and Cellular Proteomics*, 5(12), 2374-2383. <https://doi.org/10.1074/mcp.M600228-MCP200>.
187. Gaynor, C.D., McCormack, F.X., Voelker, D.R., McGowan, S.E., and Schlesinger, L.S. (1995). Pulmonary surfactant protein A mediates enhanced phagocytosis of *Mycobacterium tuberculosis* by a direct interaction with human macrophages. *Journal of Immunology*, 155(11), 5343-5351. <https://doi.org/10.4049/jimmunol.169.7.3565>.
188. Schlesinger, L.S. (1993). Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. *Journal of Immunology*, 150(7), 2920-29300022-1767 (Print) 0022-1767.
189. Schlesinger, L.S. (1998). *Mycobacterium tuberculosis* and the complement system. *Trends in Microbiology*, 6(2), 47-49; discussion 49-50. [https://doi.org/10.1016/s0966-842x\(97\)01203-1](https://doi.org/10.1016/s0966-842x(97)01203-1).
190. Hasin, Y., Seldin, M., and Lusis, A. (2017). Multi-omics approaches to disease. *Genome Biology*, 18(1), 83. <https://doi.org/10.1186/s13059-017-1215-1>.
191. Qin, H., Niu, T., and Zhao, J. (2019). Identifying multi-omics causers and causal pathways for complex traits. *Frontiers in Genetics*, 10, 110. <https://doi.org/10.3389/fgene.2019.00110>.
192. Guha, P., Dutta, S., Murti, K., Charan, J.K., Pandey, K., Ravichandiran, V., and Dhingra, S. (2024). The integration of omics: A promising approach to personalized tuberculosis treatment. *Medicine in Omics*, 12, 100033. <https://doi.org/10.1016/j.meomic.2024.100033>.

193. Haas, C.T., Roe, J.K., Pollara, G., Mehta, M., and Noursadeghi, M. (2016). Diagnostic ‘omics’ for active tuberculosis. *BMC Medicine*, 14, 37. <https://doi.org/10.1186/s12916-016-0583-9>.
194. Hirschhorn, J.N. , and Daly, M.J. (2005). Genome-wide association studies for common diseases and complex traits. *Nature Reviews. Genetics*, 6(2), 95-108. <https://doi.org/10.1038/nrg1521>.
195. Ragoussis, J. (2009). Genotyping technologies for genetic research. *Annual Review of Genomics and Human Genetics*, 10, 117-133. <http://dx.doi.org/10.1146/annurev-genom-082908-150116>.
196. Koboldt, D.C., Steinberg, K.M., Larson, D.E., Wilson, R.K., and Mardis, E.R. (2013). The next-generation sequencing revolution and its impact on genomics. *Cell*, 155(1), 27-38. <https://doi.org/10.1016/j.cell.2013.09.006>.
197. Walker, T.M., Ip, C.L., Harrell, R.H., Evans, J.T., Kapatai, G., Dedicoat, M.J., Eyre, D.W., Wilson, D.J., Hawkey, P.M., Crook, D.W., Parkhill, J., Harris, D., Walker, A.S., Bowden, R., Monk, P., Smith, E.G., and Peto, T.E. (2013). Whole-genome sequencing to delineate *Mycobacterium tuberculosis* outbreaks: a retrospective observational study. *The Lancet. Infectious Diseases*, 13(2), 137-146. [https://doi.org/10.1016/s1473-3099\(12\)70277-3](https://doi.org/10.1016/s1473-3099(12)70277-3).
198. Koleske, B.N., Jacobs, W.R., Jr., and Bishai, W.R. (2023). The *Mycobacterium tuberculosis* genome at 25 years: lessons and lingering questions. *Journal of Clinical Investigation*, 133(19), e173156. <https://doi.org/10.1172/jci173156>.
199. Bryant, J.M., Harris, S.R., Parkhill, J., Dawson, R., Diacon, A.H., van Helden, P., Pym, A., Mahayiddin, A.A., Chuchottaworn, C., Sanne, I.M., Louw, C., Boeree, M.J., Hoelscher, M., McHugh, T.D., Bateson, A.L., Hunt, R.D., Mwaigwisya, S., Wright, L., Gillespie, S.H., and Bentley, S.D. (2013). Whole-genome sequencing to establish relapse or re-infection with *Mycobacterium tuberculosis*: A retrospective observational study. *The Lancet. Respiratory Medicine*, 1(10), 786-792. [https://doi.org/10.1016/s2213-2600\(13\)70231-5](https://doi.org/10.1016/s2213-2600(13)70231-5).
200. Coll, F., McNerney, R., Guerra-Assuncao, J.A., Glynn, J.R., Perdigao, J., Viveiros, M., Portugal, I., Pain, A., Martin, N., and Clark, T.G. (2014). A robust SNP barcode for typing *Mycobacterium tuberculosis* complex strains. *Nature Communications*, 5, 4812. <https://doi.org/10.1038/ncomms5812>.
201. Doughty, E.L., Sergeant, M.J., Adetifa, I., Antonio, M., and Pallen, M.J. (2014). Culture-independent detection and characterisation of *Mycobacterium tuberculosis* and *M. africanum* in sputum samples using shotgun metagenomics on a benchtop sequencer. *PeerJ*, 2, e585. <https://doi.org/10.7717/peerj.585>.
202. Brown, A.C., Bryant, J.M., Einer-Jensen, K., Holdstock, J., Houniet, D.T., Chan, J.Z., Depledge, D.P., Nikolayevskyy, V., Broda, A., Stone, M.J., Christiansen, M.T., Williams, R., McAndrew, M.B., Tutill, H., Brown, J., Melzer, M., Rosmarin, C., McHugh, T.D., Shorten, R.J., Drobniowski, F., Speight, G., and Breuer, J. (2015). Rapid whole-genome sequencing of *Mycobacterium tuberculosis* isolates directly from clinical samples. *Journal of Clinical Microbiology*, 53(7), 2230-2237. <https://doi.org/10.1128/jcm.00486-15>.
203. Zhang, C., Li, M., Lyu, M., Zhou, Y., Wang, Y., You, L., Liu, T., Peng, W., Bai, H., Zhao, Z., and Ying, B.-w. (2024). *Exploring the relationship between tuberculosis and gut microbiota: A multi-omics approach.* SSRN: https://papers.ssrn.com/sol3/papers.cfm?abstract_id=4903609.
204. Encyclopedia of DNA Elements (ENCODE) Project Consortium (2012). An integrated encyclopedia of DNA elements in the human genome. *Nature*, 489(7414), 57-74. <https://doi.org/10.1038/nature11247>.
205. Ward, S.K., Abomoelak, B., Marcus, S.A., and Talaat, A.M. (2010). Transcriptional profiling of *Mycobacterium tuberculosis* during infection: Lessons learned. *Frontiers in Microbiology*, 1, 121. <https://doi.org/10.3389/fmicb.2010.00121>.

206. Bustin, S.A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): Trends and problems. *Journal of Molecular Endocrinology*, 29(1), 23-39. <https://doi.org/10.1677/jme.0.0290023>.
207. Derveaux, S., Vandesompele, J., and Hellemans, J. (2010). How to do successful gene expression analysis using real-time PCR. *Methods*, 50(4), 227-230. <https://doi.org/10.1016/j.ymeth.2009.11.001>.
208. Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., and Wittwer, C.T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, 55(4), 611-622. <https://doi.org/10.1373/clinchem.2008.112797>.
209. Berry, M.P., Graham, C.M., McNab, F.W., Xu, Z., Bloch, S.A., Oni, T., Wilkinson, K.A., Banchereau, R., Skinner, J., Wilkinson, R.J., Quinn, C., Blankenship, D., Dhawan, R., Cush, J.J., Mejias, A., Ramilo, O., Kon, O.M., Pascual, V., Banchereau, J., Chaussabel, D., and O'Garra, A. (2010). An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature*, 466(7309), 973-977. <https://doi.org/10.1038/nature09247>.
210. Bloom, C.I., Graham, C.M., Berry, M.P., Wilkinson, K.A., Oni, T., Rozakeas, F., Xu, Z., Rossello-Urgell, J., Chaussabel, D., Banchereau, J., Pascual, V., Lipman, M., Wilkinson, R.J., and O'Garra, A. (2012). Detectable changes in the blood transcriptome are present after two weeks of antituberculosis therapy. *PLoS One*, 7(10), e46191. <https://doi.org/10.1371/journal.pone.0046191>.
211. Blankley, S., Berry, M.P., Graham, C.M., Bloom, C.I., Lipman, M., and O'Garra, A. (2014). The application of transcriptional blood signatures to enhance our understanding of the host response to infection: the example of tuberculosis. *Philosophical Transactions of the Royal Society of London*, 369(1645), 20130427. <https://doi.org/10.1098/rstb.2013.0427>.
212. Cliff, J.M., Lee, J.S., Constantinou, N., Cho, J.E., Clark, T.G., Ronacher, K., King, E.C., Lukey, P.T., Duncan, K., Van Helden, P.D., Walzl, G., and Dockrell, H.M. (2013). Distinct phases of blood gene expression pattern through tuberculosis treatment reflect modulation of the humoral immune response. *J Infect Dis*, 207(1), 18-19. <https://doi.org/10.1093/infdis/jis499>.
213. Ottenhoff, T.H., Dass, R.H., Yang, N., Zhang, M.M., Wong, H.E., Sahiratmadja, E., Khor, C.C., Alisjahbana, B., Van Crevel, R., Marzuki, S., Seielstad, M., Van De Vosse, E., and Hibberd, M.L. (2012). Genome-wide expression profiling identifies type 1 interferon response pathways in active tuberculosis. *PLoS One*, 7(9), e45879. <https://doi.org/10.1371/journal.pone.0045839>.
214. Sambarey, A., Devaprasad, A., Baloni, P., Mishra, M., Mohan, A., Tyagi, P., Singh, A., Akshata, J.S., Sultana, R., Buggi, S., and Chandra, N. (2017). Meta-analysis of host response networks identifies a common core in tuberculosis. *NPJ Systems Biology and Applications*, 3, 4. <https://doi.org/10.1038/s41540-017-0005-4>.
215. Chen, C., Wang, J., Pan, D., Wang, X., Xu, Y., Yan, J., Wang, L., Yang, X., Yang, M., and Liu, G.P. (2023). Applications of multi-omics analysis in human diseases. *MedComm*, 4(4), e315. <https://doi.org/10.1002/mco2.315>.
216. Beck, H.C., Nielsen, E.C., Matthiesen, R., Jensen, L.H., Sehested, M., Finn, P., Grauslund, M., Hansen, A.M., and Jensen, O.N. (2006). Quantitative proteomic analysis of post-translational modifications of human histones. *Molecular & Cellular Proteomics*, 5(7), 1314-1325. <https://doi.org/10.1074/mcp.m600007-mcp200>.
217. Mann, M., and Jensen, O.N. (2003). Proteomic analysis of post-translational modifications. *Nature Biotechnology*, 21(3), 255-261. <https://doi.org/10.1038/nbt0303-255>.

218. Wu, R., Haas, W., Dephoure, N., Huttlin, E.L., Zhai, B., Sowa, M.E., and Gygi, S.P. (2011). A large-scale method to measure absolute protein phosphorylation stoichiometries. *Nature Methods*, 8(8), 677-683. [10.1038/nmeth.1636](https://doi.org/10.1038/nmeth.1636).
219. Agranoff, D., Fernandez-Reyes, D., Papadopoulos, M.C., Rojas, S.A., Herbster, M., Loosemore, A., Tarelli, E., Sheldon, J., Schwenk, A., Pollok, R., Rayner, C.F., and Krishna, S. (2006). Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. *Lancet*, 368(9540), 1012-1021. [https://doi.org/10.1016/s0140-6736\(06\)69342-2](https://doi.org/10.1016/s0140-6736(06)69342-2).
220. Liu, Q., Pan, L., Han, F., Luo, B., Jia, H., Xing, A., Li, Q., and Zhang, Z. (2018). Proteomic profiling for plasma biomarkers of tuberculosis progression. *Molecular Medicine Reports*, 18(2), 1551-1559. <https://doi.org/10.3892/mmr.2018.9134>.
221. Ivanisevic, T. , and Sewduth, R.N. (2023). Multi-omics integration for the design of novel therapies and the identification of novel biomarkers. *Proteomes*, 11(4), 34. <https://doi.org/10.3390/proteomes11040034>.
222. Wang, Q.Z., Wu, C.Y., Chen, T., Chen, X., and Zhao, X.M. (2006). Integrating metabolomics into a systems biology framework to exploit metabolic complexity: Strategies and applications in microorganisms. *Applied Microbiology and Biotechnology*, 70(2), 151-161. [10.1007/s00253-005-0277-2](https://doi.org/10.1007/s00253-005-0277-2).
223. Du Preez, I. , and Loots, D.T. (2012). Altered fatty acid metabolism due to rifampicin-resistance conferring mutations in the rpoB gene of *Mycobacterium tuberculosis*: Mapping the potential of pharmaco-metabolomics for global health and personalized medicine. *Omics*, 16(11), 596-603. <https://doi.org/10.1089/omi.2012.0028>.
224. Han, W., Ward, J.L., Kong, Y., and Li, X. (2023). Editorial: Targeted and untargeted metabolomics for the evaluation of plant metabolites in response to the environment. *Frontiers in Plant Science*, 14, 1167513. <https://doi.org/10.3389/fpls.2023.1167513>.
225. Halouska, S., Chacon, O., Fenton, R.J., Zinniel, D.K., Barletta, R.G., and Powers, R. (2007). Use of NMR metabolomics to analyze the targets of D-cycloserine in mycobacteria: Role of D-alanine racemase. *Journal of Proteome Research*, 6(12), 4608-4614. <https://doi.org/10.1021/pr0704332>.
226. Villas-Boas, S.G., Mas, S., Akesson, M., Smedsgaard, J., and Nielsen, J. (2005). Mass spectrometry in metabolome analysis. *Mass Spectrometry Reviews* 24(5), 613-646. <https://doi.org/10.1002/mas.20032>.
227. Kim, H.K., Choi, Y.H., and Verpoorte, R. (2011). NMR-based plant metabolomics: Where do we stand, where do we go? *Trends in Biotechnology*, 29(6), 267-275. <https://doi.org/10.1016/j.tibtech.2011.02.001>.
228. Swanepoel, C.C. , and Loots, D.T. (2014). The use of functional genomics in conjunction with metabolomics for *Mycobacterium tuberculosis* research. *Disease Markers*, 2014, 124218. <https://doi.org/10.1155/2014/124218>.
229. Chen, J., Lu, A., Tan, D., Zhang, Q., Lu, Y., Qin, L., and He, Y. (2022). Determination of scopolamine distribution in plasma and brain by LC-MS/MS in rats. *International Journal of Analytical Chemistry*, 2022, 8536235. <https://doi.org/10.1155/2022/8536235>.
230. Carter, M.D., Calcutt, M.W., Malow, B.A., Rose, K.L., and Hachey, D.L. (2012). Quantitation of melatonin and N-acetylserotonin in human plasma by nanoflow LC-MS/MS and electrospray LC-MS/MS. *Journal of Mass Spectrometry*, 47(3), 277-285. <https://doi.org/10.1002/jms.2051>.
231. Walvekar, A., Rashida, Z., Maddali, H., and Laxman, S. (2018). A versatile LC-MS/MS approach for comprehensive, quantitative analysis of central metabolic pathways. *Wellcome Open Research*, 3, 122. <https://doi.org/10.12688/wellcomeopenres.14832.1>.
232. Cumming, B.M., Reddy, V.P., and Steyn, A.J.C. (2020). The analysis of *Mycobacterium tuberculosis*-induced bioenergetic changes in infected macrophages using an extracellular flux analyzer. *Methods in Molecular Biology*, 2184(Methods in Molecular Biology), 161-184. https://doi.org/10.1007/978-1-0716-0802-9_12.

233. Mirsaedi, M., Banoei, M.M., Winston, B.W., and Schraufnagel, D.E. (2015). Metabolomics: Applications and promise in mycobacterial disease. *Annals of the American Thoracic Society*, 12(9), 1278-1287. <https://doi.org/10.1513/annalsats.201505-279ps>.
234. Tian, J., Bryk, R., Itoh, M., Suematsu, M., and Nathan, C. (2005). Variant tricarboxylic acid cycle in *Mycobacterium tuberculosis*: Identification of alpha-ketoglutarate decarboxylase. *Proceedings of the National Academy of Sciences of the United States of America*, 102(30), 10670-10675. <https://doi.org/10.1073/pnas.0501605102>.
235. Loots, D.T. (2014). An altered *Mycobacterium tuberculosis* metabolome induced by *katG* mutations resulting in isoniazid resistance. *Antimicrob Agents Chemother*, 58(4), 2144-2149. <https://doi.org/10.1128/aac.02344-13>.
236. Lau, S.K.P., Lam, C.-W., Curreem, S.O.T., Lee, K.-C., Lau, C.C.Y., Chow, W.-N., Ngan, A.H.Y., To, K.K.W., Chan, J.F.W., Hung, I.F.N., Yam, W.-C., Yuen, K.-Y., and Woo, P.C.Y. (2015). Identification of specific metabolites in culture supernatant of *Mycobacterium tuberculosis* using metabolomics: Exploration of potential biomarkers. *Emerging Microbes & Infections*, 4(1), e6. <https://doi.org/10.1038/emi.2015.6>.
237. Kwaik, A.Y. , and Bumann, D. (2013). Microbial quest for food *in vivo*: 'Nutritional virulence' as an emerging paradigm. *Cellular Microbiology*, 15(6), 882-890. <https://doi.org/10.1111/cmi.12138>.
238. Brown, S.A., Palmer, K.L., and Whiteley, M. (2008). Revisiting the host as a growth medium. *Nature Reviews. Microbiology*, 6(9), 657-666. 10.1038/nrmicro1955.
239. Eisenreich, W., Dandekar, T., Heesemann, J., and Goebel, W. (2010). Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. *Nature Reviews. Microbiology*, 8(6), 401-412. <https://doi.org/10.1038/nrmicro2351>.
240. Zimmermann, M., Kogadeeva, M., Gengenbacher, M., McEwen, G., Mollenkopf, H.J., Zamboni, N., Kaufmann, S.H.E., and Sauer, U. (2017). Integration of metabolomics and transcriptomics reveals a complex diet of *Mycobacterium tuberculosis* during early macrophage infection. *mSystems*, 2(4), e00057-00017. <https://doi.org/10.1128/mSystems.00057-17>.
241. Zhou, A., Ni, J., Xu, Z., Wang, Y., Lu, S., Sha, W., Karakousis, P.C., and Yao, Y.-F. (2013). Application of (1)H-NMR spectroscopy-based metabolomics to sera of tuberculosis patients. *Journal of Proteome Research*, 12(10), 4642-4649. <https://doi.org/10.1021/pr4007359>.
242. Ehrt, S., Rhee, K., and Schnappinger, D. (2015). Mycobacterial genes essential for the pathogen's survival in the host. *Immunological Reviews*, 264(1), 319-326. <https://doi.org/10.1111/imr.12256>.
243. Olivier, I. , and Loots, d.T. (2012). A metabolomics approach to characterise and identify various *Mycobacterium* species. *Journal of Microbiological Methods*, 88(3), 419-426. 10.1016/j.mimet.2012.01.012.
244. Du Preez, I. (2012). A comparison of two extraction methods for differentiating and characterising various *Mycobacterium* species and *Pseudomonas aeruginosa* using GC-MS metabolomics. *African Journal of Microbiology Research*, 6(13), 3159-3172. <http://dx.doi.org/10.5897/AJMR11.1269>.
245. Schoeman, J.C., du Preez, I., and Loots du, T. (2012). A comparison of four sputum pre-extraction preparation methods for identifying and characterising *Mycobacterium tuberculosis* using GCxGC-TOFMS metabolomics. *Journal of Microbiological Methods*, 91(2), 301-311. <https://doi.org/10.1016/j.mimet.2012.09.002>.
246. Du Preez, I. , and Loots, D.T. (2013). New sputum metabolite markers implicating adaptations of the host to *Mycobacterium tuberculosis*, and vice versa. *Tuberculosis*, 93(3), 330-337. <https://doi.org/10.1016/j.tube.2013.02.008>.
247. Feng, S., Du, Y.Q., Zhang, L., Zhang, L., Feng, R.R., and Liu, S.Y. (2015). Analysis of serum metabolic profile by ultra-performance liquid chromatography-mass spectrometry for

- biomarkers discovery: Application in a pilot study to discriminate patients with tuberculosis. *Chinese Medical Journal*, 128(2), 159-168. <https://doi.org/10.4103/0366-6999.149188>.
248. Szewczyk, R., Kowalski, K., Janiszewska-Drobinska, B., and Druszczyńska, M. (2013). Rapid method for *Mycobacterium tuberculosis* identification using electrospray ionization tandem mass spectrometry analysis of mycolic acids. *Diagnostic Microbiology and Infectious Disease*, 76(3), 298-305. <https://doi.org/10.1016/j.diagmicrobio.2013.03.025>.
 249. Liu, C., Zhao, Z., Fan, J., Lyon, C.J., Wu, H.J., Nedelkov, D., Zelazny, A.M., Olivier, K.N., Cazares, L.H., Holland, S.M., Graviss, E.A., and Hu, Y. (2017). Quantification of circulating *Mycobacterium tuberculosis* antigen peptides allows rapid diagnosis of active disease and treatment monitoring. *Proceedings of the National Academy of Sciences of the United States of America*, 114(15), 3969-3974. <https://doi.org/10.1073/pnas.1621360114>.
 250. Prahl, J.B., Lundqvist, M., Bahl, J.M.C., Johansen, I.S., Andersen, Å.B., Frimodt-Møller, N., and Cohen, A.S. (2016). Simultaneous quantification of isoniazid, rifampicin, ethambutol and pyrazinamide by liquid chromatography/tandem mass spectrometry. *APMIS: Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, 124(11), 1004-1015. <https://doi.org/10.1111/apm.12590>.
 251. Dijkstra, J.A., Sturkenboom, M.G., Sturkenboom, M.G., Hateren, K.V., Koster, R.A., Greijdanus, B., and Alffenaar, J.-W.C. (2014). Quantification of amikacin and kanamycin in serum using a simple and validated LC-MS/MS method. *Bioanalysis*, 6(16), 2125-2133. <https://doi.org/10.4155/bio.14.191>.
 252. Schmidt, F. , and Volker, U. (2011). Proteome analysis of host-pathogen interactions: Investigation of pathogen responses to the host cell environment. *Proteomics*, 11(15), 3203-3211. <https://doi.org/10.1002/pmic.201100158>.
 253. Westermann, A.J., Gorski, S.A., and Vogel, J. (2012). Dual RNA-seq of pathogen and host. *Nature Reviews. Microbiology*, 10(9), 618-630. <https://doi.org/10.1038/nrmicro2852>.
 254. Gouzy, A., Larrouy-Maumus, G., Bottai, D., Levillain, F., Dumas, A., Wallach, J.B., Caire-Brandli, I., de Chastellier, C., Wu, T.-D., Poincloux, R., Brosch, R., Guerquin-Kern, J.-L., Schnappinger, D., Sório de Carvalho, L.P., Poquet, Y., and Neyrolles, O. (2014). *Mycobacterium tuberculosis* exploits asparagine to assimilate nitrogen and resist acid stress during infection. *PLoS Pathogens*, 10(2), e1003928. <https://doi.org/10.1371/journal.ppat.1003928>.
 255. Zhang, H., Li, D., Zhao, L., Fleming, J., Lin, N., Wang, T., Liu, Z., Li, C., Galwey, N., Deng, J., Zhou, Y., Zhu, Y., Gao, Y., Wang, T., Wang, S., Huang, Y., Wang, M., Zhong, Q., Zhou, L., Chen, T., Zhou, J., Yang, R., Zhu, G., Hang, H., Zhang, J., Li, F., Wan, K., Wang, J., Zhang, X.E., and Bi, L. (2013). Genome sequencing of 161 *Mycobacterium tuberculosis* isolates from China identifies genes and intergenic regions associated with drug resistance. *Nature Genetics*, 45(10), 1255-1260. <https://doi.org/10.1038/ng.2735>.
 256. Kalscheuer, R., Weinrick, B., Veeraraghavan, U., Besra, G.S., and Jacobs, W.R. (2010). Trehalose-recycling ABC transporter LpqY-SugA-SugB-SugC is essential for virulence of *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences*, 107(50), 21761-21766. <https://doi.org/10.1073/pnas.1014642108>.
 257. Marrero, J., Trujillo, C., Rhee, K.Y., and Ehrt, S. (2013). Glucose phosphorylation is required for *Mycobacterium tuberculosis* persistence in mice. *PLoS Pathogens*, 9(1), e1003116. <https://doi.org/10.1371/journal.ppat.1003116>.
 258. Marrero, J., Rhee, K.Y., Schnappinger, D., Pethe, K., and Ehrt, S. (2010). Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for *Mycobacterium tuberculosis* to establish and maintain infection. *Proceedings of the National Academy of Sciences*, 107(21), 9819-9824. 10.1073/pnas.1000715107.
 259. Trujillo, C., Blumenthal, A., Marrero, J., Rhee, K.Y., Schnappinger, D., and Ehrt, S. (2014). Triosephosphate isomerase is dispensable in vitro yet essential for *Mycobacterium*

- tuberculosis* to establish infection. *MBio*, 5(2), e00085. <https://doi.org/10.1128/mbio.00085-14>.
260. Munoz-Elias, E.J., Upton, A.M., Cherian, J., and McKinney, J.D. (2006). Role of the methylcitrate cycle in *Mycobacterium tuberculosis* metabolism, intracellular growth, and virulence. *Molecular Microbiology*, 60(5), 1109-1122. <https://doi.org/10.1111/j.1365-2958.2006.05155.x>.
 261. Cheng, J., Che, N., Li, H., Ma, K., Wu, S., Fang, J., Gao, R., Liu, J., Yan, X., Li, C., and Dong, F. (2013). Gas chromatography time-of-flight mass spectrometry-based metabolomic analysis of human macrophages infected by *M. tuberculosis*. *Analytical Letters*, 46(12), 1922-1936. <https://doi.org/10.1080/00032719.2013.777924>.
 262. Lee, W., VanderVen, B.C., Fahey, R.J., and Russell, D.G. (2013). Intracellular *Mycobacterium tuberculosis* exploits host-derived fatty acids to limit metabolic stress. *The Journal of Biological Chemistry*, 288(10), 6788-6800. <https://doi.org/10.1074/jbc.m112.445056>.
 263. Pandey, A.K. , and Sasseti, C.M. (2008). Mycobacterial persistence requires the utilization of host cholesterol. *Proceedings of the National Academy of Sciences*, 105(11), 4376-4380. <https://doi.org/10.1073/pnas.0711159105>.
 264. Byrnes, S.A. , and Weigl, B.H. (2018). Selecting analytical biomarkers for diagnostic applications: A first principles approach. *Expert Review of Molecular Diagnostics*, 18(1), 19-26. <https://doi.org/10.1080/14737159.2018.1412258>.
 265. Deamer, D. , and Weber, A.L. (2010). Bioenergetics and life's origins. *Cold Spring Harbor Perspectives in Biology*, 2(2), a004929. <https://doi.org/10.1101/cshperspect.a004929>.
 266. Fisher-Wellman, K.H., Davidson, M.T., Narowski, T.M., Lin, C.T., Koves, T.R., and Muoio, D.M. (2018). Mitochondrial diagnostics: A multiplexed assay platform for comprehensive assessment of mitochondrial energy fluxes. *Cell Reports*, 24(13), 3593-3606.e3510. <https://doi.org/10.1016/j.celrep.2018.08.091>.
 267. Huang, N. , and Sheng, Z.H. (2022). Microfluidic devices as model platforms of CNS injury-ischemia to study axonal regeneration by regulating mitochondrial transport and bioenergetic metabolism. *Cell Regeneration*, 11(1), 33. <https://doi.org/10.1186/s13619-022-00138-3>.
 268. Walsh, M.A., Musci, R.V., Jacobs, R.A., and Hamilton, K.L. (2023). A practical perspective on how to develop, implement, execute, and reproduce high-resolution respirometry experiments: The physiologist's guide to an Oroboros O2k. *FASEB Journal*, 37(12), e23280. <https://doi.org/10.1096/fj.202301644RR>.
 269. Agilent (2024). Seahorse real-time cell metabolic analysis. [Online]. Available: <https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-analyzers> [Accessed April 2024].
 270. Duraj, T., Carrión-Navarro, J., Seyfried, T.N., García-Romero, N., and Ayuso-Sacido, A. (2021). Metabolic therapy and bioenergetic analysis: The missing piece of the puzzle. *Molecular Metabolism*, 54, 101389. <https://doi.org/10.1016/j.molmet.2021.101389>.
 271. Little, A.C., Kovalenko, I., Goo, L.E., Hong, H.S., Kerk, S.A., Yates, J.A., Purohit, V., Lombard, D.B., Merajver, S.D., and Lyssiotis, C.A. (2020). High-content fluorescence imaging with the metabolic flux assay reveals insights into mitochondrial properties and functions. *Communications Biology*, 3(1), 271. <https://doi.org/10.1038/s42003-020-0988-z>.
 272. Marchetti, P., Fovez, Q., Germain, N., Khamari, R., and Kluza, J. (2020). Mitochondrial spare respiratory capacity: Mechanisms, regulation, and significance in non-transformed and cancer cells. *FASEB Journal*, 34(10), 13106-13124. <https://doi.org/10.1096/fj.202000767R>.
 273. Bailey, J.D., Diotallevi, M., Nicol, T., McNeill, E., Shaw, A., Chuaiphichai, S., Hale, A., Starr, A., Nandi, M., Stylianou, E., McShane, H., Davis, S., Fischer, R., Kessler, B.M., McCullagh, J., Channon, K.M., and Crabtree, M.J. (2019). Nitric oxide modulates metabolic remodeling in inflammatory macrophages through TCA cycle regulation and itaconate

- accumulation. *Cell Reports*, 28(1), 218-230.e217. <https://doi.org/10.1016/j.celrep.2019.06.018>.
274. Rahman, M.A., Cumming, B.M., Addicott, K.W., Pacl, H.T., Russell, S.L., Nargan, K., Naidoo, T., Ramdial, P.K., Adamson, J.H., Wang, R., and Steyn, A.J.C. (2020). Hydrogen sulfide dysregulates the immune response by suppressing central carbon metabolism to promote tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America*, 117(12), 6663-6674. <http://dx.doi.org/10.1073/pnas.1919211117>.
275. Cumming, B.M., Pacl, H.T., and Steyn, A.J.C. (2020). Relevance of the Warburg effect in tuberculosis for host-directed therapy. *Frontiers in Cellular and Infection Microbiology*, 10, 576596. <https://doi.org/10.3389/fcimb.2020.576596>.
276. Cumming, B.M., Baig, Z., Addicott, K.W., Chen, D., and Steyn, A.J.C. (2021). Host bioenergetic parameters reveal cytotoxicity of antituberculosis drugs undetected using conventional viability assays. *Antimicrobial Agents and Chemotherapy*, 65(10), e0093221. <https://doi.org/10.1128/aac.00932-21>.
277. Cahill, C., Phelan, J.J., and Keane, J. (2020). Understanding and exploiting the effect of tuberculosis antimicrobials on host mitochondrial function and bioenergetics. *Frontiers in Cellular and Infection Microbiology*, 10, 493. <https://doi.org/10.3389/fcimb.2020.00493>.
278. Mackenzie, J.S., Lamprecht, D.A., Asmal, R., Adamson, J.H., Borah, K., Beste, D.J.V., Lee, B.S., Pethe, K., Rousseau, S., Krieger, I., Sacchettini, J.C., Glasgow, J.N., and Steyn, A.J.C. (2020). Bedaquiline reprograms central metabolism to reveal glycolytic vulnerability in *Mycobacterium tuberculosis*. *Nature Communications*, 11(1), 6092. <https://doi.org/10.1038/s41467-020-19959-4>.
279. Tien, N.T.N., Yen, N.T.H., Phat, N.K., Anh, N.K., Thu, N.Q., Dinh Hoa, V., Eunsu, C., Kim, H.-S., Nguyen, D.N., Kim, D.H., Oh, J.Y., and Long, N.P. (2024). Circulating lipids as biomarkers for diagnosis of tuberculosis: A multi-cohort, multi-omics data integration analysis. *medRxiv*, 24311536. <https://doi.org/10.1101/2024.08.06.24311536>.
280. Pinto, S.M., Verma, R., Advani, J., Chatterjee, O., Patil, A.H., Kapoor, S., Subbannayya, Y., Raja, R., Gandotra, S., and Prasad, T.S.K. (2018). Integrated multi-omic analysis of *Mycobacterium tuberculosis* H37Ra redefines virulence attributes. *Frontiers in Microbiology*, 9, 1314. <https://doi.org/10.3389/fmicb.2018.01314>.
281. Van den Bossche, J., Baardman, J., and de Winther, M.P. (2015). Metabolic characterization of polarized M1 and M2 bone marrow-derived macrophages using real-time extracellular flux analysis. *Journal of Visualized Experiments* (105), 53424. <https://doi.org/10.3791/53424>.
282. Gleeson, L.E., Sheedy, F.J., Palsson-McDermott, E.M., Triglia, D., O'Leary, S.M., O'Sullivan, M.P., O'Neill, L.A., and Keane, J. (2016). Cutting edge: *Mycobacterium tuberculosis* induces aerobic glycolysis in human alveolar macrophages that is required for control of intracellular bacillary replication. *The Journal of Immunology*, 196(6), 2444-2449. <https://doi.org/10.4049/jimmunol.1501612>.
283. Braverman, J., Sogi, K.M., Benjamin, D., Nomura, D.K., and Stanley, S.A. (2016). HIF-1 α Is an essential mediator of IFN- γ -dependent Immunity to *Mycobacterium tuberculosis*. *Journal of Immunology*, 197(4), 1287-1297. <https://doi.org/10.4049/jimmunol.1600266>.

The first chapter delved into currently existing literature detailing tuberculosis, from its epidemiology, lineages and development of drug resistance to the pathogen's ability to overcome challenges faced with host defence mechanisms. The chapter explored *Mycobacterium tuberculosis* defining pathogenic characteristics and highlighted the use of multi-omics approaches in the discovery of biomarkers and motivation for adhesins, particularly *M. tuberculosis* curli pili, as a suitable biomarker for the development of tuberculosis therapeutics. Furthermore, exploring beyond the traditional "omics", by incorporating bioenergetics into research strategies was argued to be beneficial for advancement of available knowledge.

Chapter 2 investigated the role of MTP in modulating bacterial metabolic pathways in wildtype *M. tuberculosis* V9124, the *mtp*-deletion mutant and its *mtp*-complemented strain. This chapter has been published as a journal article in *Metabolomics* (Springer, Nature), 2020 (DOI: <https://doi.org/10.1007/s11306-020-01720-z>). The joint first authors' contributions are as follows:

- Ms S. Ashokcoomar: Conducted experiments, analysed the data, drafted the manuscript and supplementary material.
- Ms K. S. Reedoy: Conducted experiments, analysed the data, drafted the manuscript and supplementary material.

CHAPTER 2: *Mycobacterium tuberculosis* curli pili (MTP) deficiency is associated with alterations in cell wall biogenesis, fatty acid metabolism and amino acid synthesis

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Authors conflict of interest statement: Ms Ashokcoomar declares that she has no conflict of interest. Ms Reedoy declares that she has no conflict of interest. Dr Senzani declares he has no conflict of interest. Prof Loots declares he has no conflict of interest. Ms Beukes declares she has no conflict of interest. Ms van Reenen declares she has no conflict of interest. Prof B. Pillay declares he has no conflict of interest. Prof M. Pillay declares she has no conflict of interest.

Authors contribution statement: Prof M. Pillay conceptualised and funded the study. Prof M. Pillay, Prof B. Pillay and Prof Loots designed the study. Ms Ashokcoomar and Ms Reedoy contributed equally to the study, conducted experiments, analysed the data and drafted the manuscript and supplementary information. Ms Beukes processed the samples and performed GC×GC-TOFMS. Ms van Reenen conducted statistical bioinformatic analyses. Dr Senzani provided RT-qPCR guidance and analytical support. All authors contributed to and approved the manuscript.

Consent for publication: All authors approved of this manuscript for publication.

Availability of data and material: The datasets generated during and/or analysed during the current study are not publicly available due to collaborative purposes and future publications but are available from the corresponding author on reasonable request. KEGG database reported in this study is accessible via [<https://www.genome.jp/kegg/>].

2.1. Abstract

Introduction: In an effort to find alternative therapeutic interventions to combat tuberculosis, a better understanding of the pathophysiology of *Mycobacterium tuberculosis* (*M. tuberculosis*) is required. The *M. tuberculosis* curli pili (MTP) adhesin, present on the surface of this pathogen, has previously been shown using functional genomics and global transcriptomics, to play an important role in establishing infection, bacterial aggregation, and modulating host response *in vitro* and *in vivo*.

Objective: This investigation aimed to determine the role of MTP in modulating the metabolism of *M. tuberculosis*, using *mtp* gene-knockout mutant and complemented strains.

Methods: Untargeted two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC-TOFMS), and bioinformatic analyses, were used to identify significant differences in the metabolite profiles among the wildtype (WT), Δmtp mutant and *mtp*-complemented strains, and validated with results generated by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Results: A total of 28 metabolites were found to be significantly altered when comparing the Δmtp mutant and the WT strains indicating decreased utilisation of metabolites in cell wall biogenesis, a reduced efficiency in the breakdown of fatty acids, and decreased amino acid biosynthesis in the former strain. Comparison of the WT to *mtp*-complemented strain and Δmtp to *mtp*-complemented strain revealed 10 and 16 metabolite differences, respectively. The RT-qPCR results supported the metabolomics findings. Complementation of the Δmtp mutant resulted in a partial restoration of MTP function.

Conclusion: The lack of the MTP adhesin resulted in various bacterial cell wall alterations and related metabolic changes. This study highlights the importance of MTP as a virulence factor and further substantiates its potential use as a suitable biomarker for the development of diagnostic tools and intervention therapeutics against TB.

2.2. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), still poses a global threat, not only to health, but also to socio-economics, particularly in low and middle income countries [1]. An estimated 10 million incident cases of TB, 1.2 million deaths among human immunodeficiency virus (HIV)-negative people and 251 000 deaths in HIV-co-infected individuals were reported in 2018 [1]. A reduction of these statistics requires improved TB diagnostic, treatment and prevention strategies, directed by a better understanding of *M. tuberculosis* physiology and pathogenicity, as well as TB pathogenesis in order to identify bacterial and host determinants of infection, that may serve as diagnostic and vaccine targets [2; 3].

Direct contact between inhaled *M. tuberculosis* and its target cells, which include macrophages, epithelial cells and dendritic cells [4], is required for the successful invasion and infection. Initial contact and adhesion of the pathogen to the host cell is mediated by extracellular surface molecules termed adhesins [5], which in turn also lead to the activation of key virulence factors, and subsequently invasion of the host cell [6; 7]. These events collectively promote the spread of the pathogen and evasion of the host immune response [7].

Mycobacterium tuberculosis curli pili (MTP), encoded by Rv3312A, was demonstrated to bind with laminin, and react with immunoglobulin G (IgG) antibodies in patients' sera [8]. In addition to promoting biofilm formation [9], and functioning as an adherence factor, MTP was reported to act as an invasin in THP-1 macrophages [10] and A549 pulmonary epithelial cells [11]. Global transcriptomics data from our research group demonstrated that this adhesin significantly modulates host immune responses post-infection in epithelial cells and BALB/C mice [12; 13]. The *mtp* gene is highly conserved amongst clinical isolates, and unique to *M. tuberculosis* complex strains (Naidoo et al., 2014). Furthermore, a synthetic MTP peptide demonstrated 97% accuracy in detecting anti-MTP IgG antibodies in serum samples of HIV-uninfected, as well as co-infected patients [14]. Taken together, the data affirms the importance of MTP in TB pathogenesis, and its potential use as a suitable biomarker for the design of TB diagnostics and intervention therapy.

Metabolomics, a relatively new but now well established research approach, analyses the metabolites produced or influenced by an organism with altered gene expression [15]. These can be quantified by the various types of analytical techniques, including the hyphenated two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-TOFMS), that combines chromatographic and spectral technologies to identify metabolites [16]. Metabolomics has been used in a number of studies to investigate *M. tuberculosis* physiology and metabolic pathways, in order to better understand carbon, lipid and amino acid metabolism [17-22]. It has also been able to better characterise active TB infection [23-25] and to identify significant biomarkers as indicators of virulence [15; 26-29], as well as indicators of TB disease detectable in sera [30] and in urine [31], all in an effort to develop simple, non-invasive and rapid diagnostic or novel therapeutics.

While much of the research on MTP supports it being a crucial determinant to the onset of the TB infection cycle, the metabolite changes associated with this adhesin have not yet been previously reported. Hence, this study reports on the use of a GC×GC-TOFMS metabolomics approach to elucidate the role of MTP in modulating *M. tuberculosis* metabolism and associated functions, that may lead to the identification of potential novel biomarkers. Furthermore, an understanding of the metabolic pathways involved in the infection cascade, would serve to further validate the use of the adhesin as a suitable biomarker for the advancement of intervention strategies against TB.

2.3. Materials and methods

2.3.1. Bacterial strains and culture conditions

The wildtype (WT) *M. tuberculosis* V9124 from the F15/LAM4/KZN family was previously isolated in Medical Microbiology, University of KwaZulu-Natal, from Tugela Ferry, KwaZulu-Natal, South Africa [32]. This strain has been used in a previous study to construct the *mtp* clean-deletion mutant (Δmtp) by the insertion of an allelic exchange substrate containing the *hyg^R-sacB* cassette using the protocol described by Bardorov et al. in 2002 [33], and the *mtp*-complemented strain containing the non-integrating plasmid, pMV261 with a copy of *mtp* [9]. All strains were initially polymerase chain reaction (PCR)-confirmed after culturing at 37°C on Middlebrook 7H11 or 7H9 (Difco, Becton-Dickinson, Franklin Lakes, NJ, USA) media containing hygromycin B (75 µg/mL) for Δmtp and kanamycin (30 µg/mL) for *mtp*-complement supplemented with 10% (v/v) oleic albumin dextrose catalase (Becton–Dickinson, Franklin Lakes, NJ, USA) and 0.5% (v/v) glycerol (Sigma-Aldrich, St. Louis, MO, USA) (Appendix B: Table S1 and Figure S1). For the experiment, antibiotic-free broth cultures for all strains were grown with additional supplementation with tween-80 (Sigma-Aldrich, St. Louis, MO, USA) (0.05%) and shaking (I-26 Shaking Incubator, New Brunswick Scientific, Canada) at 100 rpm for 7 - 8 days.

2.3.2. Sample preparation

Figure 2.1 summarises the workflow followed in the research design and statistical analysis. Prior to sample preparation, the inoculum for each strain was standardised by 10-fold serial dilutions of OD_{600nm} 1 (equivalent to $\sim 1 \times 10^8$ colony forming units (CFU)/mL) cultures [34], and were plated onto 7H11 agar and incubated (Shel Lab CO₂ Incubator, Cornelius, OR, USA) at 37°C for three weeks to determine the CFU (Appendix B: Table S2). GraphPad Prism version 8 software (GraphPad Software, La Jolla, CA, USA) was used to determine any significant differences between the strains (Appendix B: Table S3). Thereafter, ten biological replicates of each of the three strains were individually cultured (n = 30) in 40 mL Middlebrook 7H9 supplemented broth (Figure 2.1). Each biological replicate was standardised to an OD_{600nm} of 0.015 and incubated at 37°C with shaking at 100 rpm. Once an OD_{600nm} of between 0.95 – 1.1 was reached, 22 mL and 17 mL of culture from each sample was used for pellet collection (Appendix B: Table S4) and ribonucleic acid (RNA) extraction, respectively (Figure 2.1). Cultures were centrifuged (Mikro 200R, Hettich Zentrifugen, Tuttlingen, Germany) at 4°C at 1 792×g for 30 min. The pellets for RNA extraction were resuspended in 1 mL TriZol reagent (Ambion, Life Technologies, USA) and stored at - 80°C until use. The pellets for metabolite extraction were resuspended in 500 µL of phosphate buffered saline (PBS) (Merck, Darmstadt, Germany) and centrifuged in Eppendorf Lobind microcentrifuge tubes (Sigma-Aldrich,

St. Louis, MO, USA) at 4°C at 21 952×g for 10 min. This wash step was repeated twice and at the end of the third wash, PBS was removed, and the resulting pellets were stored at -80°C for GC×GC-TOFMS.

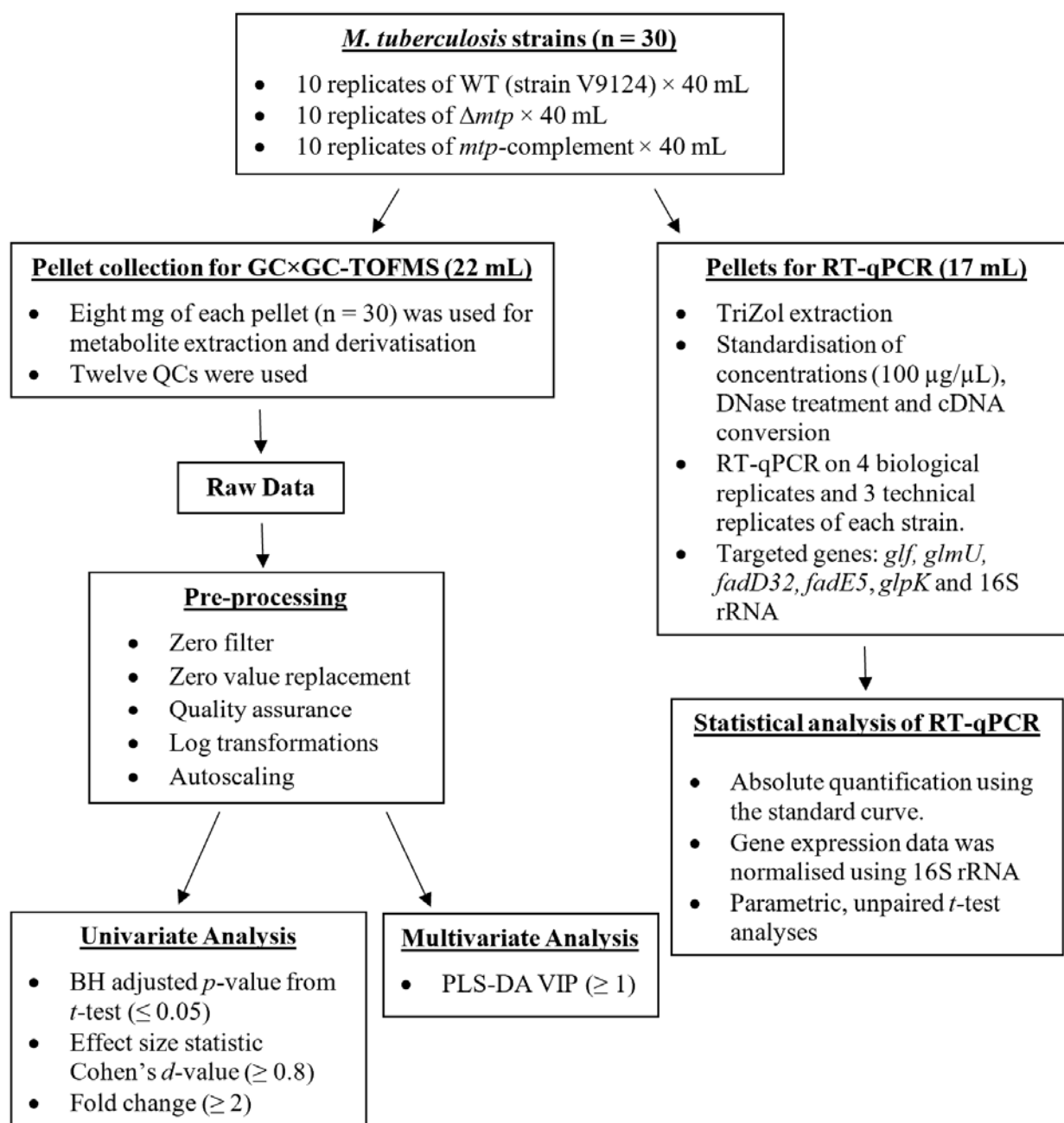


Figure 2.1. Flow diagram depicting study design and statistical workflow for data analysis including cut-off values used as selection criteria for shortlisting of metabolites. Each bacterial sample was used for two types of extraction: metabolite extraction and RNA extraction. Metabolite extracts were run through GC×GC-TOFMS and the RNA samples were used for cDNA conversion and RT-qPCR. Thereafter, the appropriate processing, statistics and analysis were conducted.

2.3.3. Sample extraction and derivatisation

Eight milligrams of pelleted wet cell mass of each sample were thawed and weighed into a 1.5 mL Eppendorf tube. A pooled quality control (QC) sample was made up by combining 8 mg of one randomly selected sample from each of the sample groups. Metabolites from various metabolite classes were extracted using whole metabolome extraction method [35] optimised to *M. tuberculosis* cells prior to the experiment and 0.13125 mg/mL 3-phenylbutyric acid (Sigma-Aldrich, St. Louis, MO, USA) was used as an internal standard. Ultra-pure chloroform:methanol:water (1:3:1) (Burdick and Jackson, Honeywell International Inc., Muskegon, MI, USA) was used as an extraction solvent [35]. Samples were derivatised using 50 μ L of methoxyamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in 15 mg/mL pyridine (Merck, Darmstadt, Germany) at 50°C for 90 minutes, followed by 40 μ L of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (Sigma-Aldrich, St. Louis, MO, USA) at 60°C for 60 minutes. The extracts were transferred to a 0.1 mL insert in a sample vial and capped prior to GC $\times$ GC-TOFMS analysis.

2.3.4. GC $\times$ GC-TOFMS analysis

The samples were analysed using a Pegasus 4D GC $\times$ GC-TOFMS (Leco Corporation, St. Joseph, MI, USA). One microliter of sample was injected in a two-split ratio on an Agilent 7890A GC (Agilent, Atlanta, GA) coupled to a time-of-flight mass spectrometer (Leco Corporation, St. Joseph, MI, USA) equipped with a Gerstel Multi-Purpose Sampler (Gerstel GmbH & co. KG, Eberhard-Gerstel- Platz 1, D-45473 Mülheim an der Ruhr). First dimensional separation was achieved with a Rxi-5Sil MS primary column (28.759 m, 0.25 mm internal diameter, 0.25 μ M film thickness) (Restch GmbH & co. KG, Haan, Germany) and a Rxi-17 capillary column (1.380 m, 0.25 mm internal diameter, 0.25 μ M film thickness) was fitted as the secondary column (Restch GmbH & co. KG, Haan, Germany). The front inlet temperature was held constant at 270°C for the entire run. For the primary oven, an initial GC oven temperature was set at 70°C for 2 minutes followed by an initial increase in oven temperature of 4°C/minute to a final temperature of 300°C, which was maintained for 2 minutes. The secondary column oven temperature was set at 85°C for 2 minutes, then increased by 4°C /min, until a final temperature of 300°C. The initial temperature of the modulator was 100°C for 2 minutes, followed by a 4°C/minute increase to a final temperature of 310°C held for 12 minutes. Cryomodulation and a hot pulse of nitrogen gas of 0.7 seconds, every 3 seconds was used to control the effluent from the primary to secondary column. The acquisition delay for each sample was 400 seconds and the transfer line temperature was held at 270°C, with the ion source temperature at 200°C. The detector voltage was adjusted to 1600 V with the filament bias - 70 eV. Spectra were collected from 50 to 800 m/z at an acquisition rate of 200 spectra per second. Peak finding and mass spectral deconvolution was achieved using ChromaTOF (Leco Corporation) software (version 4.51.6.0). Mass fragmentation patterns and their respective retention times screened against commercially available National Institute of Standards and Technology (NIST) spectral libraries (main library and replicate library) for peak identification. Level 3 identification was performed [36].

2.3.5. Statistical data analysis

A total of 30 experimental and 12 QC samples were included in the statistical analysis (Figure 2.1). Samples were randomised into three batches and quantified across 731 metabolites. If zero concentrations were detected in all samples for a specific metabolite, the metabolite was not included in the list. Outlier removal could not be justified. Matrix laboratory (MATLAB) software (MATLAB with Statistics Toolbox (2019), version 9.5.0 (R2018b), Natick, Massachusetts: The MathWorks Inc) along with the Eigenvector PLS_Toolbox 8.7 (Wenatchee, WA: Eigenvector Research Inc) was used to identify metabolites with the potential to differentiate among the three strains. For data pre-processing, a zero filter was applied to exclude metabolites with zero observations of 50% or more, thus removing 189 metabolites. The remaining zeros replaced by random numbers between the minimum and zero. The QC samples were injected intermittently throughout the GC $\times$ GC-TOFMS analysis to ensure analytical repeatability, and was considered successful as the variability between

experimental samples far exceeded the variability between QC sample analyses (Appendix B: Figure S2). Both parametric univariate and multivariate methods were employed to identify metabolites with discriminatory ability. Log transformation and auto-scaling was required to ensure all metabolites achieved normality and were regarded as equally important by multivariate models [37], respectively. Fold change values were calculated to assess the magnitude of differences on average. The independent samples *t*-test was used to determine the statistical significance of differences between groups for each metabolite. To supplement the findings of the *t*-test, Cohen's *d*-value was used as an effect size indicating practical significance of differences [38]. Corrections for multiple testing was affected by the Benjamini & Hochberg (BH) adjustment to control the rate of false discovery. Multivariate analyses incorporated principal component analysis (PCA) scores plots (Appendix B: Figure S3a, c, and e), partial least squares discriminant analysis (PLS-DA) scores plots (Appendix B: Figure S3b, d, and f) and associated variable importance in the projection (VIP) values, and correlation analysis using Spearman's Rho. The VIP and power values were used to rank metabolites according to their contribution to the PLS-DA and PCA models' respective ability to explain the observed variation. Univariate and multivariate criteria were used in combination to select important metabolites.

2.3.6. Reverse transcription quantitative PCR (RT-qPCR) validation of metabolomics data

In order to better understand the metabolomic changes at a transcriptomics level, RT-qPCR was performed on the three bacterial strains to assess gene expression levels as a result of the *mtp* gene deletion. Resources such as Kyoto Encyclopaedia of Genes and Genomes (KEGG), BioCyc and Universal Protein Resource (UniProt) were used for pathway interrogation and the selection of enzymes for RT-qPCR. Selection criteria for the enzymes were based on identifying patterns/anomalies within metabolite categories to further explain the metabolomics data. In addition, some metabolites are involved in bidirectional pathways, and gene expression data would clarify whether the metabolite had a role in degradation or biosynthesis. Hence, five genes (*glf* encoding UDP-galactopyranose mutase, *glmU* encoding a bifunctional N-acetyltransferase/uridylyltransferase, *fadD32* encoding acyl-AMP synthetase, *fadE5* encoding an acyl-CoA dehydrogenase and *glpK* encoding a glycerol kinase) were randomly selected for amplification. The RNA extraction was performed [34], with the modification of repeating the 70% ethanol wash step, and the use of a Precellys 24 lysis and homogenisation machine (Bertin Technologies, Montigny-le Bretonneux, France). The RNA concentrations (Appendix B: Table S5) were standardised to 100 µg/µL prior to checking RNA integrity (Appendix B: Table S5 and Figure S4) and DNase treatment (Thermo Fisher Scientific, Waltham, MA, USA). Conversion of complementary deoxyribonucleic acid (cDNA) using the High-Capacity cDNA Reverse Transcription kit (Roche Applied Sciences, Penzberg, Germany) was conducted as per the manufacturer's instructions. Primer efficiencies were tested for all primer sets (Appendix B: Table S6). The Ssoadvanced Universal SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA) was used to perform RT-qPCR using a total reaction volume of 10 µL, in a 7500 RT-qPCR Detection System (Applied Biosystems, Foster City, CA, USA). Cycling conditions were as follows for a total of 40 cycles; holding stage at 95 °C for 30 seconds, cycling stage at 95 °C for 5 seconds with an annealing temperature of 60 °C for 30 seconds. Melt curve analysis was measured as continuous fluorescence set at 95 °C for 15 seconds, 60 °C for 1 minute, 95 °C for 30 seconds, and 60 °C for 15 seconds. The resulting gene expression data was normalised using 16S ribosomal RNA (rRNA) and was analysed by absolute quantification using the standard curve method. Four biological replicates and three technical replicates were performed for each strain (Figure 2.1). Parametric, unpaired *t*-test analyses were performed using GraphPad Prism software to determine significance values.

2.4. Results

2.4.1. PLS-DA data produced group separation confirming model validation

The PLS-DA models showed complete group separation amongst all three stains (Figure 2.2), indicative of three different metabolome profiles. It was observed that the Δmtp showed a greater deviation from the WT, than the *mtp*-complement as depicted by the placement of the ellipsoids (Figure 2.2). The models produced R^2 and Q^2 values of 99% and 92% for WT versus Δmtp (Appendix B: Figure S3b), and 99% and 85% for WT versus *mtp*-complement (Appendix B: Figure S3d), and 99% and 85% for Δmtp versus *mtp*-complement (Appendix B: Figure S3f), respectively, indicating robust predictive ability not sensitive to small variations in case selection. The VIP values were therefore used to rank metabolites according to their joint ability to explain the group structure. Additionally, Spearman correlation plots were generated to ascertain the degree of similarity of the WT to the Δmtp and *mtp*-complement metabolite profiles (Appendix B: Figure S5). Interestingly, the dominant green colouring in the plot comparing the WT and Δmtp strains (Appendix B: Figure S5a) showed a low correlation of metabolite profiles, whereas the WT and *mtp*-complement comparison (Appendix B: Figure S5b) showed a dominant orange/red colouring, indicative of stronger correlation. These results were expected as the Δmtp metabolite profile should differ from the WT. Furthermore, the *mtp*-complement should, ideally, return to a similar phenotype to the parental WT strain, if not identical.

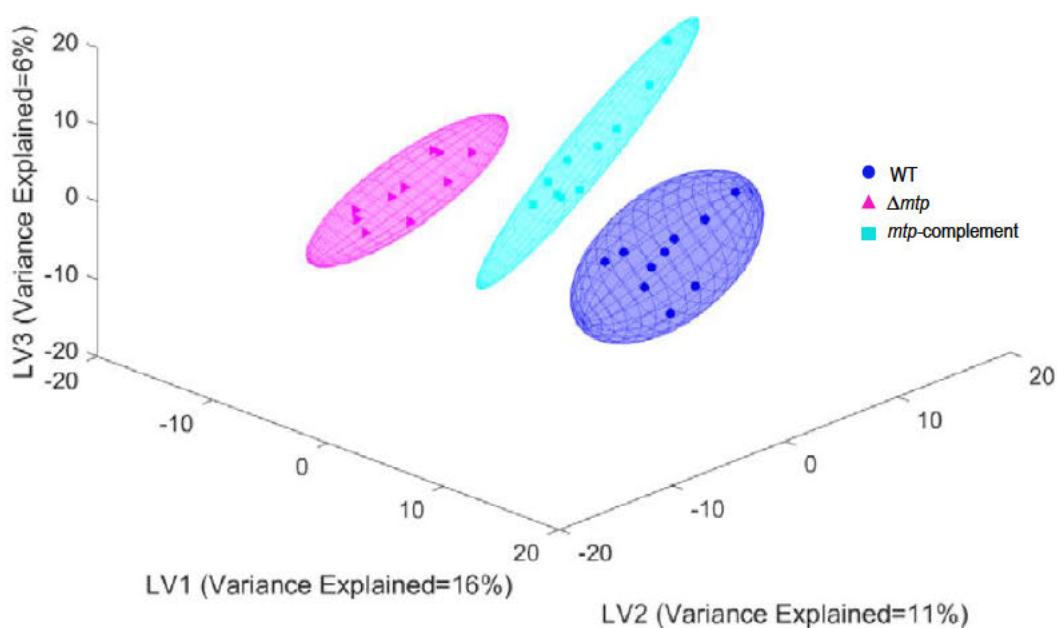


Figure 2.2. The 3D PLS-DA model comparing the three bacterial strains highlight the variation associated with genotype. The variations indicated on all axes were low, but were the maximum percentages associated with the group structure. Each point on a graph represents a sample as projected onto the new lower-dimensional space considering their group association (indicated in the figure key). Complete group separation was observed between the WT (blue), Δmtp (pink) and *mtp*-complement (cyan). Ellipsoids represent a 90% confidence interval (CI) of score centroids of each group. The percentage of the overall variation in the groups, as explained by each latent variable (LV), a linear combination of the metabolites which forms a new dimension in the modelled subspace, is indicated in brackets along each axis. The X variance relates to the observed data, while the Y variance relates to the grouping information supplied.

2.4.2. Selection of biologically significant metabolites

Analytes that were not assigned a name for identification were excluded from the analysis as the software was unable to match the spectra to existing metabolites in the library. Metabolites were shortlisted using the selection criteria; *t*-test *p*-value (after BH adjustment) ≤ 0.05 , Cohen's *d*-value effect size ≥ 0.8 , PLS-DA VIP ≥ 1 , and fold change ≥ 2 . A total of 51 metabolites met all criteria between WT and Δmtp ; however, only 28 of these were deemed biologically significant based on supporting literature and analysis of metabolites/pathways (KEGG and PubChem) and were categorised according to biological relevance (Table 2.1).

Metabolites not classified as biologically significant were listed in Appendix B: Table S7. There were 11 metabolites associated with cell wall biogenesis and function, 10 of which were increased in the Δmtp , except for D-galactofuranose. Eight metabolites were associated with fatty acid metabolism, of which seven had a higher relative concentration in Δmtp compared to WT, except for propanetriol. For the amino acid and protein synthesis category, seven of the nine metabolites had a higher relative concentration in the WT cells, except for dl-2-amino adipic acid and cadaverine. A total of 52 metabolites met all criteria between WT and *mtp*-complement. Only 10 of these metabolites were of biological relevance and exclusive to the WT and *mtp*-complement comparison (Table 2.2). With exception of 11-eicosenoic acid, all metabolites associated with cell wall biogenesis and function were found in lower concentrations in the *mtp*-complement relative to the WT. All metabolites, except D-gluconic acid, associated with amino acid and protein synthesis were found in lower concentrations in the *mtp*-complement relative to the WT. Metabolites that were not biologically significant nor exclusive to this model were listed in Appendix B: Table S8. A total of 32 metabolites met all statistical criteria between Δmtp and *mtp*-complement; however, only 16 of these were deemed biologically significant based on supporting literature and analysis of metabolites/pathways and were categorised according to biological relevance (Table 2.3). Of the 16 metabolites, seven (43.75%) were found to be similar to metabolites significantly altered in the comparison between WT and Δmtp (Table 2.1), and four metabolites (25%) were similar to those significantly altered in the comparison between WT and *mtp*-complement (Table 2.2). Given that the complementation is meant to restore phenotype to that of the WT, it is expected that the WT and *mtp*-complement should display similar alterations from the Δmtp metabolome. Therefore, the differences seen in Table 2.3 indicate that the metabolome of the *mtp*-complement is closer to the WT than Δmtp . The remaining five metabolites from the 16 (31.25%) were exclusive to the Δmtp and Δmtp -complement comparison. With the exceptions of D-glucose, butanoic acid and pyrrole-2-carboxylic-acid, all metabolites were found in relatively higher concentration in the Δmtp than *mtp*-complement. Metabolites not classified as biologically significant were listed in Appendix B: Table S9.

Table 2.1. Most significant metabolites when comparing the metabolomes of *M. tuberculosis* WT and Δmtp strains selected as per multivariate and univariate selection criteria together with their respective mean relative concentration and standard deviation, fold change with respect to the WT, PLS-DA VIP value, Cohen's *d*-value and BH-adjusted *p*-value.

Category	Metabolite	Mean relative concentration and standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
		WT	Δmtp				
Metabolites involved in cell wall functions and biogenesis	D-Galactose	3.52E-03 (1.22E-03)	1.63E-02 (4.08E-03)	4.635	7.867	3.164	<0.001
	D-Glucose	2.95E-04 (1.96E-04)	1.87E-03 (7.93E-04)	6.350	2.334	1.989	0.001
	D-Mannose	3.87E-03 (2.13E-03)	9.58E-03 (2.57E-03)	2.479	2.143	2.231	<0.001
	Glucopyranose	2.30E-03 (8.98E-04)	9.13E-03 (2.95E-03)	3.977	2.375	2.328	<0.001
	D-Mannopyranose	8.64E-05 (5.07E-05)	5.21E-04 (2.04E-04)	6.035	1.899	2.129	0.001
	D-Galactofuranose	2.82E-04 (1.24E-04)	1.36E-04 (3.03E-05)	-2.073	3.511	1.175	0.022
	D-Ribofuranose	2.70E-04 (1.87E-04)	6.74E-04 (2.43E-04)	2.495	1.348	1.665	0.005
	D-Erythro-pentofuranose	2.44E-03 (8.09E-04)	1.35E-02 (6.25E-03)	5.554	1.036	1.780	0.003
	N-Acetyl-D-glucosamine	1.38E-04 (4.23E-05)	6.10E-04 (1.19E-04)	4.414	6.958	3.963	<0.001
	D-Fructose	3.92E-03 (1.68E-03)	1.00E-02 (2.28E-03)	2.564	3.254	2.689	<0.001
	Phosphoric acid	5.22E-03 (1.31E-03)	1.30E-02 (1.64E-03)	2.485	6.720	4.753	<0.001
Metabolites associated with fatty acid metabolism	Docosanoic acid	3.37E-04 (1.16E-04)	1.01E-03 (1.13E-04)	2.988	5.057	5.796	<0.001
	13-Docosanoic acid	7.37E-05 (4.00E-05)	2.46E-04 (1.04E-04)	3.343	1.199	1.667	0.003
	Hexacosanoic acid	1.63E-03 (4.69E-04)	4.63E-03 (1.03E-03)	2.834	4.289	2.926	<0.001
	Tetracosanoic acid	5.12E-04 (1.23E-04)	1.33E-03 (2.74E-04)	2.591	3.593	2.970	<0.001
	Linolenic acid	2.65E-04 (1.00E-04)	6.07E-04 (1.91E-04)	2.288	1.100	1.789	0.002
	9-Octadecenoic acid	8.15E-04 (2.71E-04)	2.00E-03 (8.28E-04)	2.452	3.768	1.429	0.009
	Myo-inositol	2.19E-02 (6.55E-03)	5.93E-02 (7.66E-03)	2.701	4.593	4.968	<0.001
	Propanetriol	8.46E-04 (2.70E-04)	3.16E-04 (4.92E-05)	-2.678	3.157	1.960	0.002
Metabolites involved in amino acid and protein synthesis	L-Aspartic acid	9.23E-02 (2.84E-02)	2.74E-02 (1.09E-02)	-3.365	4.651	2.364	<0.001
	N-Acetylaspartic acid	5.14E-04 (4.10E-04)	7.18E-05 (1.99E-05)	-7.163	3.899	1.079	0.033
	Tryptophan	4.01E-03 (1.42E-03)	1.43E-03 (7.12E-04)	-2.800	4.110	1.815	0.002
	L-Proline	1.10E-03 (4.60E-04)	5.21E-04 (1.93E-04)	-2.110	3.079	1.257	0.017
	L-Alanine	0.15E-01 (7.81E-02)	6.25E-02 (2.76E-02)	-2.461	2.616	1.211	0.020
	dl-2-Aminoadipic acid	2.84E-04 (1.52E-04)	1.30E-03 (9.19E-04)	4.589	4.790	1.111	0.029
	Cadaverine	4.82E-05 (2.19E-05)	1.03E-04 (4.69E-05)	2.138	2.377	1.187	0.022
	3-Hydroxyisovaleric acid	4.43E-04 (2.48E-04)	1.48E-04 (6.1E-05)	-2.996	3.336	1.191	0.022
	Methylcitric acid	9.54E-04 (3.78E-04)	4.58E-04 (9.15E-05)	-2.082	2.807	1.312	0.014

WT: wildtype; Δmtp : *mtp*-gene knockout mutant; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg (BH) adjustment for false discovery rate; positive fold change = higher concentration in Δmtp ; negative fold change = higher concentration in WT.

Table 2.2. Most significant metabolites when comparing the metabolomes of *M. tuberculosis* WT and *mtp*-complemented strains selected as per multivariate and univariate selection criteria together with their respective mean relative concentration and standard deviation, fold change with respect to the WT, PLS-DA VIP value, Cohen's *d*-value and BH-adjusted *p*-value.

Category	Metabolite	Mean relative concentration and standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
		WT	<i>mtp</i> -complement				
Metabolites involved in cell wall functions and biogenesis	11-Eicosenoic acid	8.08E-05 (3.15E-05)	4.83E-04 (2.33E-04)	5.977	5.306	1.730	0.005
	α -D-Glucopyranose	2.02E-02 (6.62E-03)	6.83E-03 (1.18E-03)	-2.956	5.112	2.032	0.002
	Arabinofuranose	3.05E-04 (1.80E-04)	6.75E-05 (2.93E-05)	-4.561	2.103	1.324	0.015
	Glutamine	2.42E-04 (6.60E-05)	1.10E-04 (5.84E-05)	-2.197	3.925	1.999	0.003
Metabolites involved in amino acid and protein synthesis	Phenylalanine	1.98E-02 (6.69E-03)	8.56E-03 (4.56E-03)	-2.313	2.235	1.684	0.005
	Serine	2.45E-02 (5.39E-03)	1.21E-02 (3.50E-03)	-2.020	4.810	2.316	<0.001
	Alanine	2.39E-03 (1.18E-03)	6.87E-04 (6.48E-04)	-3.523	2.988	1.454	0.009
	5'-S-Methyl-5'-thioadenosine	9.04E-04 (2.19 E-04)	3.02E-04 (1.67E-04)	-2.995	3.482	2.744	<0.001
	N-Butylamine	4.85E-04 (2.25E-04)	8.35E-05 (4.94E-05)	-5.800	5.591	1.780	0.004
	D-Gluconic acid	5.75E-03 (2.73E-03)	1.44E-02 (4.71E-03)	2.501	1.922	1.841	0.003

WT: wildtype; *mtp*-complement: complemented *mtp*-knockout strain; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg (BH) adjustment for false discovery rate; positive fold change = higher concentration in *mtp*-complement; negative fold change = higher concentration in WT.

Table 2.3. Most significant metabolites when comparing the metabolomes of *M. tuberculosis* Δmtp and *mtp*-complement strains selected as per multivariate and univariate selection criteria together with their respective mean relative concentration and standard deviation, fold change with respect to the Δmtp , PLS-DA VIP value, Cohen's *d*-value and BH-adjusted *p*-value.

Category	Metabolite	Mean relative concentration and standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
		Δmtp	<i>mtp</i> -complement				
Metabolites involved in cell wall functions and biogenesis	α -D-Glucopyranose	1.50E-02 (2.83E-03)	6.83E-03 (1.18E-03)	-2.204	1.616	2.916	<0.001
	Arabinofuranose	2.91E-04 (2.12E-04)	7.01E-05 (2.75E-05)	-4.147	1.601	1.044	0.046
	D-Ribose	2.29E-04 (8.98E-05)	7.57E-05 (3.24E-05)	-3.029	1.008	1.711	0.005
	D-Ribofuranose	6.74E-04 (2.43E-04)	1.25E-04 (2.36E-05)	-5.379	1.868	2.263	0.001
	D-Erythro-pentofuranose	1.35E-02 (6.25E-03)	2.64E-03 (8.93E-04)	-5.137	1.170	1.748	0.005
	D-Galactose	1.63E-02 (4.08E-03)	6.39E-03 (3.16E-03)	-2.553	2.541	2.451	<0.001
	D-Glucose	8.03E-04 (2.82E-04)	9.84E-03 (6.55E-03)	12.265	1.283	1.385	0.016
	D-Mannopyranose	5.21E-04 (2.04E-04)	2.39E-04 (1.25E-04)	-2.179	1.045	1.380	0.017
Metabolites associated with fatty acid metabolism	Butanoic acid	2.25E-02 (1.27E-02)	1.02E-01 (4.35E-02)	4.509	2.019	1.868	<0.001
Metabolites involved in amino acid and protein synthesis	Alanine	1.85E-03 (7.95E-04)	6.79E-04 (6.48E-04)	-2.672	1.319	1.430	0.019
	Benzeneacetic acid	5.05E-05 (1.79E-05)	2.43E-05 (1.40E-05)	-2.080	1.086	1.465	0.017
	N-Butylamine	4.86E-04 (1.82E-04)	8.79E-05 (4.64E-05)	-5.530	2.704	2.187	0.001
	Cadaverine	1.28E-03 (6.52E-04)	2.76E-04 (1.65E-04)	-4.641	1.268	1.540	0.009
	dl-2-Aminoadipic acid	1.30E-03 (9.19E-04)	3.44E-04 (1.93E-04)	-3.785	2.488	1.043	0.046
	Pyrrole-2-carboxylic-acid	6.62E-05 (3.32E-05)	2.12E-04 (8.79E-05)	3.208	2.306	1.661	0.005
	Adenosine	1.20E-03 (5.11E-04)	4.67E-04 (5.65E-04)	-2.569	1.067	1.434	0.029

Δmtp : *mtp*-gene knockout mutant; *mtp*-complemented strain: complemented *mtp*-knockout strain; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg (BH) adjustment for false discovery rate; positive fold change = higher concentration in *mtp*-complement; negative fold change = higher concentration in Δmtp .

2.4.3. Gene expression data significantly correlates with metabolomics data

A higher concentration of D-galactose in the Δmtp relative to the WT (Table 2.1) and *mtp*-complement (Table 2.3) was consistent with the significantly higher gene expression of *glf* in the Δmtp when compared to the WT ($p = 0.003$) (Figure 2.3a) and *mtp*-complement ($p = 0.001$) (Figure 2.3a), respectively. Furthermore, the gene expression of *glf* ($p = 0.978$) in the *mtp*-complement did not differ from the WT (Figure 2.3a). The relatively higher gene expression of *glmU* in the Δmtp compared to the WT ($p = 0.007$) and *mtp*-complement ($p = 0.045$) (Figure 2.3a), respectively, in contrast to the similarity between the WT and *mtp*-complement ($p = 0.179$), explains the higher concentration of *N*-acetyl-d-glucosamine in the Δmtp compared to the WT (Table 2.1). The gene *fadD32*, encoding acyl-AMP synthetase, was significantly expressed at a higher level by the Δmtp compared to the WT ($p = 0.001$), in contrast ($p = 0.172$) to *fadE5*, encoding an acyl-CoA dehydrogenase. This data supports the accumulation of fatty acids in the Δmtp (Table 2.1). However, the inverse was observed when comparing Δmtp to the *mtp*-complement as *fadD32* ($p = 0.478$) showed no significant difference, in contrast to *fadE5* ($p = 0.015$). When comparing the *mtp*-complement to the WT, *fadD32* ($p = 0.120$) was not significant in contrast to *fadE5* ($p = 0.009$), which was expressed at a significantly higher level in the WT. Lastly, *glpK*, encoding a glycerol kinase, was significantly upregulated in the Δmtp compared to the WT ($p = 0.025$), corresponding to the higher relative concentration of propanetriol (or glycerol) (Table 2.1). Conversely, the gene expression levels of *glpK* in the *mtp*-complement when compared to the WT ($p = 0.418$) and Δmtp ($p = 0.114$) were both non-significant. This, together with the absence of fatty acid metabolism alterations (Table 2.2 and 2.3), indicate that complementation of the mutant partially restored the metabolism of the *mtp*-complement to that of its parental WT strain. The metabolite pathways in which the above genes are involved in are shown in Figure 2.3b, c and d.

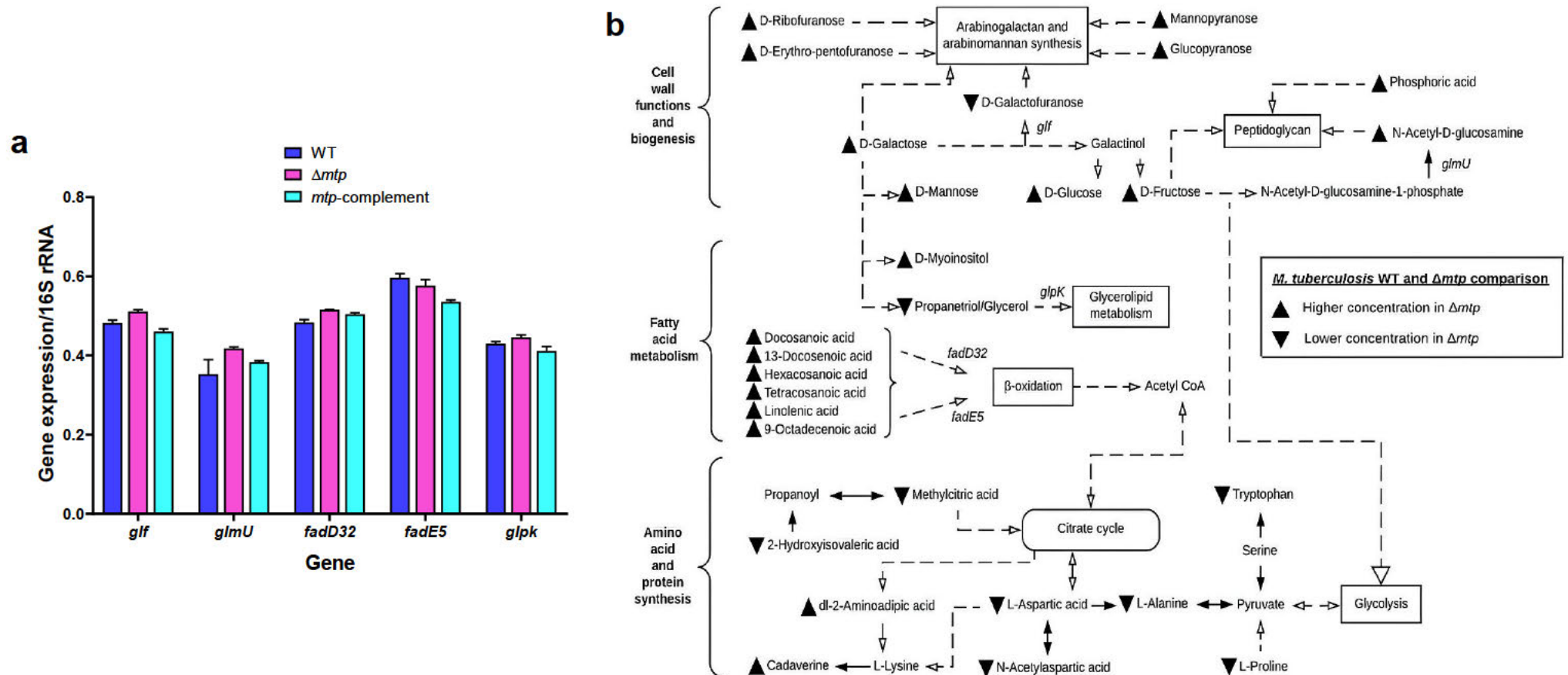


Figure 2.3. RT-qPCR to assess gene expression of five genes (*glf*, *glmU*, *fadD32*, *fadE5* and *glpK*) and their role in metabolic pathway alterations observed in MTP-proficient and MTP-deficient strains of *M. tuberculosis*. (a) Gene expression levels were normalised to 16S ribosomal RNA (16S rRNA) for each gene of interest. Significance levels were established using the unpaired sample *t*-test for parametric data between the WT and Δmtp strains, the WT and *mtp*-complement strains, and the Δmtp and *mtp*-complement strains. (a) Between the WT and Δmtp , four of the genes displayed statistical significance; *glf* ($p = 0.003$), *glmU* ($p = 0.007$), *fadD32* ($p = 0.001$) and *glpK* ($p = 0.025$) and the gene *fadE5* was non-significant ($p = 0.172$). Expression levels of four of the genes in the *mtp*-complement were restored to the WT levels; *glf* ($p = 0.978$), *glmU* ($p = 0.179$), *fadD32* ($p = 0.120$) and *glpK* ($p = 0.418$), while the gene *fadE5* was significantly expressed, compared to the WT ($p = 0.009$). Between the Δmtp and *mtp*-complement strains, three of the genes displayed statistical significance; *glf* ($p = 0.001$), *glmU* ($p = 0.045$) and *fadE5* ($p = 0.015$), and genes that were non-significant were *fadD32* ($p = 0.478$) and *glpK* ($p = 0.114$). Alterations in the metabolites involved in cell wall functions and biogenesis, fatty acid metabolism, or amino acid and protein synthesis are shown to be in a higher or lower concentration, indicated by the arrow, in the three comparisons of the study: (b) WT and Δmtp , (c) WT and *mtp*-complement, and (d) Δmtp and *mtp*-complement. The genes analysed during RT-qPCR are shown in italics in 2.3b.

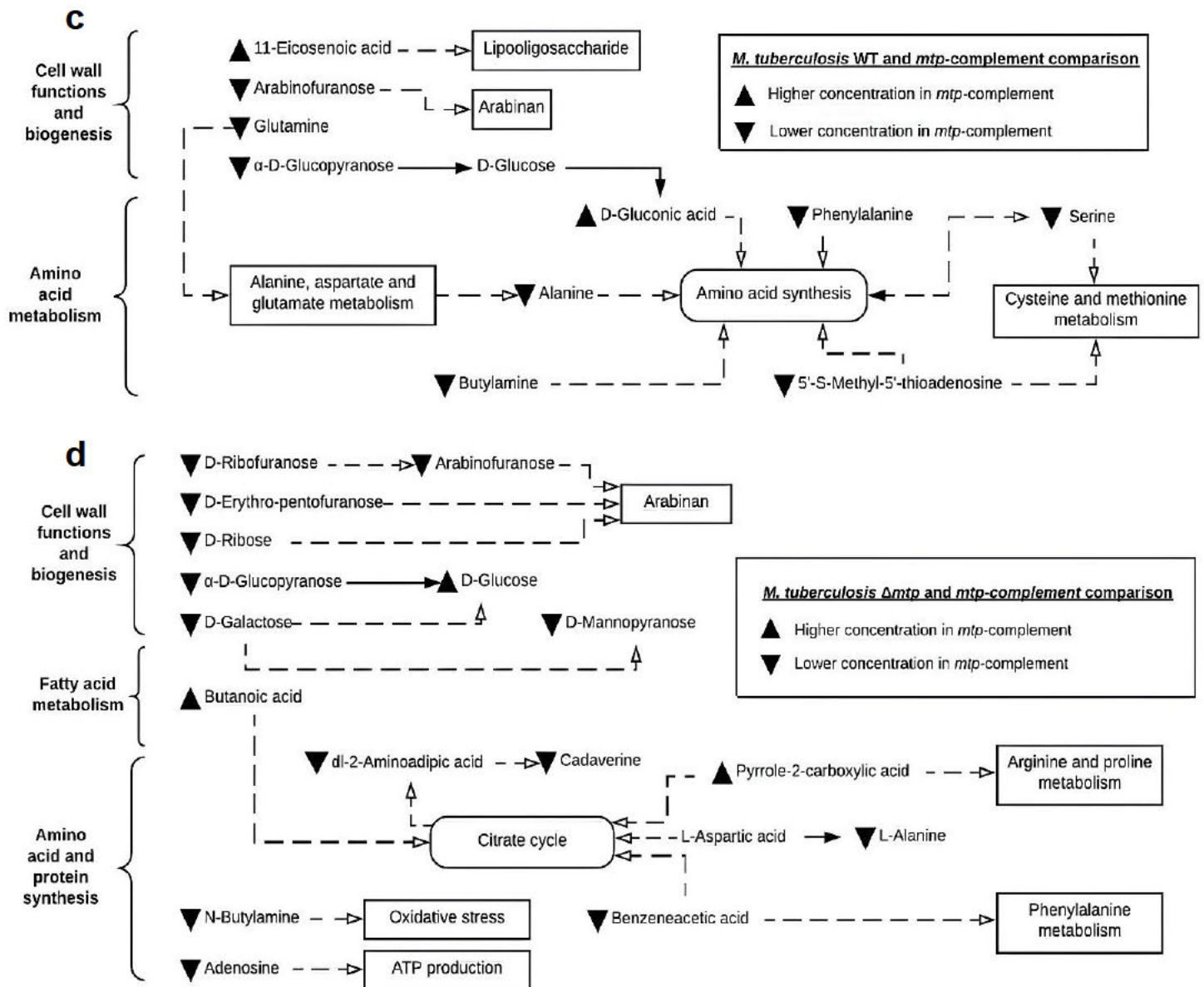


Figure 2.3. continued. RT-qPCR to assess gene expression of five genes (*glf*, *glmU*, *fadD32*, *fadE5* and *glpK*) and their role in metabolic pathway alterations observed in MTP-proficient and MTP-deficient strains of *M. tuberculosis*. (a) Gene expression levels were normalised to 16S ribosomal RNA (16S rRNA) for each gene of interest. Significance levels were established using the unpaired sample *t*-test for parametric data between the WT and Δmtp strains, the WT and *mtp*-complement strains, and the Δmtp and *mtp*-complement strains. (a) Between the WT and Δmtp , four of the genes displayed statistical significance; *glf* ($p = 0.003$), *glmU* ($p = 0.007$), *fadD32* ($p = 0.001$) and *glpK* ($p = 0.025$) and the gene *fadE5* was non-significant ($p = 0.172$). Expression levels of four of the genes in the *mtp*-complement were restored to the WT levels; *glf* ($p = 0.978$), *glmU* ($p = 0.179$), *fadD32* ($p = 0.120$) and *glpK* ($2.4p = 0.418$), while the gene *fadE5* was significantly expressed, compared to the WT ($p = 0.009$). Between the Δmtp and *mtp*-complement strains, three of the genes displayed statistical significance; *glf* ($p = 0.001$), *glmU* ($p = 0.045$) and *fadE5* ($p = 0.015$), and genes that were non-significant were *fadD32* ($p = 0.478$) and *glpK* ($p = 0.114$). Alterations in the metabolites involved in cell wall functions and biogenesis, fatty acid metabolism, or amino acid and protein synthesis are shown to be in a higher or lower concentration, indicated by the arrow, in the three comparisons of the study: (b) WT and Δmtp , (c) WT and *mtp*-complement, and (d) Δmtp and *mtp*-complement. The genes analysed during RT-qPCR are shown in *italics* in 2.3b.

2.5. Discussion

Previous studies have demonstrated that the surface-located adhesin, MTP, plays an important role as the first point of contact during the host-pathogen interaction [8-11], and elicits an antibody response in humans following infection [8; 14]. These findings collectively indicated that MTP is potentially suitable as a TB diagnostic target and vaccine candidate. This provided the impetus for the current study, which hypothesised that MTP contributes to the regulation of the metabolome of *M. tuberculosis*, and to identify potential metabolic pathways and/or specific metabolites that could be exploited as intervention targets.

A number of metabolites were significantly and differentially produced by the strains compared in this study. The majority of these metabolites were elevated in the Δmtp strain compared to the WT strain and *mtp*-complemented strains. These findings suggest that the deletion of *mtp* resulted in a significant perturbation of the *M. tuberculosis* metabolome, while gene complementation partially restored the metabolome to that of the WT. The 28 biologically significant metabolites identified in the Δmtp strain, compared to the WT strain, were categorised into three groups (cell wall functions and biogenesis, fatty acid metabolism, as well as amino acid and protein synthesis). The 10 biologically significant metabolites identified between the WT and *mtp*-complement were found to be associated with cell wall functions and amino acid metabolism, while the 16 biologically significant metabolites found between Δmtp and *mtp*-complement strains were involved in cell wall functions, amino acid and fatty acid metabolism. The functional relevance and potential roles of biologically relevant metabolites are discussed below and are summarised in a metabolite pathway diagram (Figure 2.3b, c and d).

2.5.1. The role of MTP and the utilisation of metabolites for cell wall biogenesis in *M. tuberculosis*.

The mycolic acid-arabinogalactan-peptidoglycan (mAGP) complex, composed of mycolic acid lipids, arabinofuranose and galactofuranose residues [39; 40], is a major structural element of the mycobacterial cell wall [41; 42]. Arabinogalactan, a mycobacterial cell wall polymer that facilitates covalent attachment of peptidoglycan and mycolic acids [43-45], comprises D-galactose and D-arabinose (Figure 2.3b). Moreover, D-ribofuranose and D-erythro-pentofuranose contribute to arabinan synthesis [39; 46; 47], while galactose, mannose and carbohydrate precursors, mannopyranose and glucopyranose [48; 49] play important roles in the synthesis of arabinogalactan and arabinomannan [43; 50] as seen in Figure 2.3b. Apart from necessitating the structure of bacterial peptidoglycan, N-acetyl-D-glucosamine, is involved in amino sugar and nucleotide sugar metabolism [51; 52]. Furthermore, cellular D-fructose [46] and phosphoric acid, involved in oxidative phosphorylation, function in peptidoglycan biosynthesis [52]. An overall accumulation of these metabolites in the MTP-deficient strain compared to the MTP-proficient strains, suggest that in the absence of *mtp* there is a reduction in energy requirement and utilisation of these substrates for peptidoglycan and cell wall biosynthesis. However, as a result of complementation, glutamine and 11-eicosenoic concentrations differed between the MTP-proficient strains, indicating minor perturbations in part of the cell wall [53] and lipooligosaccharide of the cell envelope [54; 55], respectively.

2.5.2. The role of MTP and the utilisation of fatty acids by *M. tuberculosis*

The lipid-rich nature of the mycobacterial cell wall requires the biosynthesis of unsaturated fatty acids. The chemical derivative of oleic acid, 9-octadecenoic acid, as well as docosanoic acid, 13-docosenoic acid and linolenic acid are involved in the biosynthesis of unsaturated fatty acids [52; 56]. These metabolites are necessary for mycobacterial survival where fatty acids are accumulated and stored in globules functioning as a carbon energy source reservoir [57]. Tetracosanoic acid and hexacosanoic acid have shown promise as diagnostic markers [58; 59], whereas myo-inositol, involved in forming phosphatidyl-myo-inositol mannosides (PIMs)

[60], are crucial for maintaining mycobacterial phospholipid membrane structure [61]. The elevated concentrations of these metabolites in the MTP-deficient strain (Figure 2.3b), indicates a dysfunctionality in the utilization of these fatty acids in the integration of their respective lipid by-products into the cell wall or cell membrane. These findings are in accordance with a similar study in which a hypo-virulent strain of *M. tuberculosis* compared to a hyper-virulent strain, revealed a reduced ability of the former to utilize metabolites in synthesising the characteristic lipid-rich cell wall [26]. *Mycobacterium tuberculosis* switches from carbohydrate metabolism (using glycerol or glucose) to lipid metabolism (using fatty acids and host lipids) [62]; therefore, the reduced concentrations of propanetriol (or glycerol) observed in the absence of MTP on *M. tuberculosis* indicates a greater utilisation of this metabolite as a carbon source for growth as a compensatory mechanism to the reduced fatty acid catabolism.

2.5.3. The role of MTP in amino acid and protein synthesis in *M. tuberculosis*

Aspartic acid, and its precursor *N*-acetylaspatic acid [49], are non-essential amino acids involved in the synthesis of nucleotides and other amino acids, including threonine, proline, alanine, glycine and isoleucine [49; 52]. Tryptophan, synthesised *de novo* by *M. tuberculosis*, is important for protein synthesis and confers an advantage to the pathogen during tryptophan starvation [63], thus aiding host immune evasion [64]. Apart from being a carbon and nitrogen source [65], L-proline facilitates the maintenance of cellular osmotic pressure necessary for bacterial growth [66] and characteristically forms part of pilin proteins, such as MTP [8]. Isoleucine degradation pathway gives rise to a hydroxyl fatty acid, 3-hydroxyisovaleric acid [67]. The above-mentioned metabolites showed a reduction in concentrations in the MTP-deficient strain (Figure 2.3b), indicative of compromised amino acid and protein synthesis in the absence of *mtp*. Additionally, dL-2-amino adipic acid, involved in synthesising lysine via the diaminopimelate pathway, was similarly observed in high concentrations in the hypo-virulent Beijing strain of *M. tuberculosis* [26] suggesting that upregulation of the lysine biosynthesis pathway functions as a survival/virulence mechanism in the less virulent strains [49; 52]. Cadaverine, synthesised from lysine, regulates acid tolerance, membrane permeability, RNA synthesis and glutathione metabolism [52; 68-70]. Furthermore, cadaverine also functions as a direct radical scavenger, and was previously reported to be upregulated in *M. tuberculosis* experiencing elevated oxidative stress [71].

2.5.4. Residual metabolic alteration induced by complementation

The *mtp*-complemented strain was investigated to confirm whether phenotypical alterations observed in the Δmtp were due to the genotypic differences caused by deletion of the *mtp* gene specifically. Given that complementation was achieved by the insertion of a non-integrating plasmid [9], some variability in the metabolome was expected. As indicated by the remaining significant metabolites in Tables 2.2 and 2.3, complementation appears to be successful in partially restoring fatty acid metabolism, as well as amino acid and protein synthesis to the parental strain. The metabolites, phenylalanine, serine, alanine, 5-S-methyl-5-thioadenosine and butylamine are involved in amino acid metabolism [49; 71-74], and were detected in reduced concentrations in the *mtp*-complement compared to the WT (Figure 2.3c), indicative of slightly altered metabolic pathways. A reduction in *N*-butylamine in some strains of *M. tuberculosis* was proposed to indicate increased energy, carbon, and nitrogen demands due to oxidative stress [71]. Furthermore, gluconic acid, an oxidative breakdown product of glucose [75], found in higher concentrations in the *mtp*-complement compared to WT, serves as a substrate in the pentose phosphate pathway.

The comparison between Δmtp and *mtp*-complemented strains additionally revealed higher relative concentrations of butanoic acid, a short-chain fatty acid utilised by *M. tuberculosis* as a carbon source [76], and pyrrole-2-carboxylic acid, an intermediate in arginine and proline

metabolism [49], in the latter strain. Moreover, benzeneacetic acid, adenosine and D-ribose were found in lower concentrations in the *mtp*-complement. Benzeneacetic acid (phenylacetic acid) is involved in phenylalanine metabolism [49]. Adenosine, comprised of adenine and D-ribose form part of adenosine triphosphate, required for energy production [77]. The alterations in metabolic profile of the *mtp*-complement (Figure 2.3d) point toward a higher demand for energy in the *mtp*-complement compared to the Δmtp .

2.5.5. Functional analysis using RT-qPCR support metabolomics results

The significantly higher gene expression (Figure 2.3a) of *glf* and *glmU* in the MTP-deficient *M. tuberculosis* strain, compared to the MTP-proficient stains, indicate that UDP-galactopyranose mutase and the bifunctional *n*-acetyltransferase/uridylyltransferase caused the increased concentrations of galactose and *N*-acetyl-d-glucosamine, respectively. This confirms an altered galactose metabolism [52] and UDP-*N*-acetyl-d-glucosamine biosynthesis I pathway to produce *N*-acetyl-d-glucosamine [50; 52] in the absence of MTP, potentially hindering utilisation in peptidoglycan synthesis. The accumulation of the fatty acids in the mutant paired with the upregulation of *fadD32* (involved in initial long chain fatty acid degradation) but similar expression levels of *fadE5* (involved in fatty acid β -oxidation, which splits into individual routes of breakdown [52] is suggestive of a faster fatty acid breakdown into lipid by-products in the absence of MTP. Furthermore, the gene, *glpK*, encodes a glycerol kinase that degrades glycerol (Figure 2.3b) during glycerolipid metabolism [52]. The results suggested a more rapid degradation of glycerol by the mutant to be used as a carbon source compared to the WT [62] or a reduced uptake as previously described.

A limitation of this study is that the use of only a single *M. tuberculosis* strain makes it difficult to extrapolate the findings to all clinical strains. However, since metabolomics analyses in general are expensive, labour intensive, and time consuming, the testing of multiple, virulent clinical *M. tuberculosis* strains is challenging. A difficulty experienced in the current study was the lack of annotated *M. tuberculosis* pathways and metabolites that were identifiable with this bacterium. Investigations into the molecular mechanisms driving some of these altered pathways to further substantiate MTP and some of the key metabolites as suitable biomarkers is recommended. These could include *in vitro* antibiotic killing curves coupled with curlicides to motivate as adjunctive treatment with the standard treatment regimen or gene regulation and confirmatory phenotypic analysis of altered amino acid and protein synthesis pathways observed.

2.6. Conclusion

Apart from being an adhesin and invasin, the current study demonstrates that MTP also facilitated changes to the *M. tuberculosis* metabolome. Using functional metabolomics, the metabolites detected in this study provided greater insight into the effect of the MTP deletion, on various metabolic pathways. The Δmtp was associated with an altered cell wall composition, as seen by the decreased utilisation of carbohydrates in cell wall biogenesis, a reduced efficiency in the breakdown of fatty acids, and decreased amino acid biosynthesis, which collectively explain the mechanisms driving the previously reported growth retardation compared to the WT. The elevation of the majority of the metabolites (19 out of 28) in the Δmtp strain compared to the WT, points towards an overall reduced ability for the utilisation of these metabolites for biological processes in the Δmtp strain. Taken together, these metabolomic findings further validate the MTP adhesin as an important contributor to TB pathogenesis, and therefore, an important biomarker for the design of a diagnostic tool and intervention therapy.

References:

1. World Health Organization (2019). Global tuberculosis report. *World Health Organization* [Online]. Available: <https://www.who.int/publications/i/item/9789241565714> [Accessed November 2019].
2. Forrellad, M.A., Klepp, L.I., Gioffré, A., Sabio y García, J., Morbidoni, H.R., de la Paz Santangelo, M., Cataldi, A.A., and Bigi, F. (2013). Virulence factors of the *Mycobacterium tuberculosis* complex. *Virulence*, 4(1), 3-66. <https://doi.org/10.4161/viru.22329>.
3. Smith, I. (2003). *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clinical Microbiology Reviews*, 16(3), 463-496. <https://doi.org/10.1128/cmr.16.3.463-496.2003>.
4. Bussi, C. , and Gutierrez, M.G. (2019). *Mycobacterium tuberculosis* infection of host cells in space and time. *FEMS Microbiology Reviews*, 43(4), 341-361. <https://doi.org/10.1093/femsre/fuz006>.
5. Bermudez, L.E. , and Goodman, J. (1996). *Mycobacterium tuberculosis* invades and replicates within type II alveolar cells. *Infection and Immunity*, 64(4), 1400-1406. <https://doi.org/10.1128/iai.64.4.1400-1406.1996>.
6. Kline, K.A., Fälker, S., Dahlberg, S., Normark, S., and Henriques-Normark, B. (2009). Bacterial adhesins in host-microbe interactions. *Cell Host & Microbe*, 5(6), 580-592. <https://doi.org/10.1016/j.chom.2009.05.011>.
7. Stones, D.H. , and Krachler, A.-M. (2015). Fatal attraction: How bacterial adhesins affect host signaling and what we can learn from them. *International Journal of Molecular Sciences*, 16(2), 2626-2640. <https://doi.org/10.3390/ijms16022626>.
8. Alteri, C.J., Xicohténcatl-Cortes, J., Hess, S., Caballero-Olín, G., Girón, J.A., and Friedman, R.L. (2007). *Mycobacterium tuberculosis* produces pili during human infection. *Proceedings of the National Academy of Sciences of the United States of America*, 104(12), 5145-5150. <https://doi.org/10.1073/pnas.0602304104>.
9. Ramsugit, S., Guma, S., Pillay, B., Jain, P., Larsen, M.H., Danaviah, S., and Pillay, M. (2013). Pili contribute to biofilm formation *in vitro* in *Mycobacterium tuberculosis*. *Antonie Van Leeuwenhoek*, 104(5), 725-735. <https://doi.org/10.1007/s10482-013-9981-6>.
10. Ramsugit, S. , and Pillay, M. (2014). *Mycobacterium tuberculosis* pili promote adhesion to and invasion of THP-1 macrophages. *Japanese Journal of Infectious Diseases*, 67(6), 476-478. <https://doi.org/10.7883/yoken.67.476>.
11. Ramsugit, S. , and Pillay, M. (2016). Identification of *Mycobacterium tuberculosis* adherence-mediating components: A review of key methods to confirm adhesin function. *Iranian Journal of Basic Medical Sciences*, 19(6), 579-584. PMID: <https://pubmed.ncbi.nlm.nih.gov/articles/PMC4951595/>.
12. Dlamini, M.T. (2016). *Whole transcriptome analysis to elucidate the role of mtp in gene regulation of pulmonary epithelial cells infected with Mycobacterium tuberculosis*. Masters dissertation University of KwaZulu-Natal, Durban, South Africa. <https://researchspace.ukzn.ac.za/items/71f0ed4e-aa63-4cca-8bf6-4d771326e616>.
13. Nyawo, G. (2016). *The role of Mycobacterium tuberculosis pili in pathogenesis: Growth and survival kinetics, gene regulation and host immune response, and in vitro growth kinetics*. Masters dissertation University of KwaZulu-Natal, Durban, South Africa. <https://researchspace.ukzn.ac.za/items/3628eab3-3e5f-47a6-9eb3-07c3f3c52ad7>.
14. Naidoo, N., Pillay, B., Bubb, M., Pym, A., Chiliza, T., Naidoo, K., Ndung'u, T., Kasprovicz, V.O., and Pillay, M. (2018). Evaluation of a synthetic peptide for the detection of anti-*Mycobacterium tuberculosis* curli pili IgG antibodies in patients with

- pulmonary tuberculosis. *Tuberculosis*, 109, 80-84. <https://doi.org/10.1016/j.tube.2018.01.007>.
15. Loots, D.T., Meissner-Roloff, R.J., Newton-Foot, M., and Gey Van Pittius, N.C. (2013). A metabolomics approach exploring the function of the ESX-3 type VII secretion system of *M. smegmatis*. *Metabolomics*, 9, 631-641. <https://doi.org/10.1007/s11306-012-0481-x>.
 16. Patel, K.N., Patel, J.K., Patel, M.P., Rajput, G.C., and Patel, H.A. (2010). Introduction to hyphenated techniques and their applications in pharmacy. *Pharmaceutical Methods*, 1(1), 2-13. <https://doi.org/10.4103/2229-4708.72222>.
 17. De Carvalho, L.P., Fischer, S.M., Marrero, J., Nathan, C., Ehrt, S., and Rhee, K.Y. (2010). Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalized co-catabolism of carbon substrates. *Chemical Biology*, 17(10), 1122-1131. <https://doi.org/10.1016/j.chembiol.2010.08.009>.
 18. Beste, D.J., Noh, K., Niedenfuhr, S., Mendum, T.A., Hawkins, N.D., Ward, J.L., Beale, M.H., Wiechert, W., and McFadden, J. (2013). ¹³C-flux spectral analysis of host-pathogen metabolism reveals a mixed diet for intracellular *Mycobacterium tuberculosis*. *Chemistry and Biology*, 20(8), 1012-1021. <https://doi.org/10.1016/j.chembiol.2013.06.012>.
 19. Chow, Eric D. , and Cox, Jeffery S. (2011). TB lipidomics - The final frontier. *Chemistry & Biology*, 18(12), 1517-1518. <https://doi.org/10.1016/j.chembiol.2011.12.003>.
 20. Eoh, H. (2014). Metabolomics: A window into the adaptive physiology of *Mycobacterium tuberculosis*. *Tuberculosis*, 94(6), 538-543. <https://doi.org/10.1016/j.tube.2014.08.002>.
 21. Halouska, S., Fenton, R.J., Zinniel, D.K., Marshall, D.D., Barletta, R.G., and Powers, R. (2014). Metabolomics analysis identifies d-Alanine-d-Alanine ligase as the primary lethal target of d-Cycloserine in mycobacteria. *Journal of Proteome Research*, 13(2), 1065-1076. <https://doi.org/10.1021/pr4010579>.
 22. Warner, D.F. , and Mizrahi, V. (2014). Translating genomics research into control of tuberculosis: Lessons learned and future prospects. *Genome Biology*, 15(11), 514. <https://doi.org/10.1186/s13059-014-0514-z>.
 23. Cha, D., Cheng, D., Liu, M., Zeng, Z., Hu, X., and Guan, W. (2009). Analysis of fatty acids in sputum from patients with pulmonary tuberculosis using gas chromatography-mass spectrometry preceded by solid-phase microextraction and post-derivatization on the fiber. *Journal of Chromatography A*, 1216(9), 1450-1457. <https://doi.org/10.1016/j.chroma.2008.12.039>.
 24. Mirsaeidi, M., Banoei, M.M., Winston, B.W., and Schraufnagel, D.E. (2015). Metabolomics: Applications and promise in mycobacterial disease. *Annals of the American Thoracic Society*, 12(9), 1278-1287. <https://doi.org/10.1513/annalsats.201505-279ps>.
 25. Collins, J.M., Walker, D.I., Jones, D.P., Tukvadze, N., Liu, K.H., Tran, V.T., Uppal, K., Frediani, J.K., Easley, K.A., Shenvi, N., Khadka, M., Ortlund, E.A., Kempker, R.R., Blumberg, H.M., and Ziegler, T.R. (2018). High-resolution plasma metabolomics analysis to detect *Mycobacterium tuberculosis*-associated metabolites that distinguish active pulmonary tuberculosis in humans. *PLoS One*, 13(10), e0205398. <https://doi.org/10.1371/journal.pone.0205398>.
 26. Meissner-Roloff, R.J., Koekemoer, G., Warren, R.M., and Loots, D.T. (2012). A metabolomics investigation of a hyper- and hypo-virulent phenotype of Beijing lineage *M. tuberculosis*. *Metabolomics*, 8, 1194-1203. <https://doi.org/10.1007/s11306-012-0424-6>.

27. Swanepoel, C.C. , and Loots, D.T. (2014). The use of functional genomics in conjunction with metabolomics for *Mycobacterium tuberculosis* research. *Disease Markers*, 2014, 124218. <https://doi.org/10.1155/2014/124218>.
28. Lau, S.K.P., Lam, C.-W., Curreem, S.O.T., Lee, K.-C., Lau, C.C.Y., Chow, W.-N., Ngan, A.H.Y., To, K.K.W., Chan, J.F.W., Hung, I.F.N., Yam, W.-C., Yuen, K.-Y., and Woo, P.C.Y. (2015). Identification of specific metabolites in culture supernatant of *Mycobacterium tuberculosis* using metabolomics: Exploration of potential biomarkers. *Emerging Microbes & Infections*, 4(1), e6. <https://doi.org/10.1038/emi.2015.6>.
29. Loots, D.T. (2016). New insights into the survival mechanisms of rifampicin-resistant *Mycobacterium tuberculosis*. *Journal of Antimicrobial Chemotherapy*, 71(3), 655-660. <https://doi.org/10.1093/jac/dkv406>.
30. Zhong, L., Zhou, J., Chen, X., and Yin, Y. (2016). Serum metabolomic study for the detection of candidate biomarkers of tuberculosis. *International Journal of Clinical and Experimental Pathology*, 9(3), 3256-3266ISSN:1936-2625/IJCEP0021658.
31. Mahapatra, S., Hess, A.M., Johnson, J.L., Eisenach, K.D., DeGroote, M.A., Gitta, P., Joloba, M.L., Kaplan, G., Walzl, G., Boom, W.H., and Belisle, J.T. (2014). A metabolic biosignature of early response to anti-tuberculosis treatment. *BMC Infectious Diseases*, 14, 53. <https://doi.org/10.1186/1471-2334-14-53>.
32. Gandhi, N.R., Moll, A., Sturm, A.W., Pawinski, R., Govender, T., Lalloo, U., Zeller, K., Andrews, J., and Friedland, G. (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet*, 368(9547), 1575-1580. [https://doi.org/10.1016/s0140-6736\(06\)69573-1](https://doi.org/10.1016/s0140-6736(06)69573-1).
33. Bardarov, S., Bardarov, S., Pavelka, M.S., Sambandamurthy, V., Larsen, M., Tufariello, J., Chan, J., Hatfull, G., and Jacobs, W.R. (2002). Specialized transduction: An efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. *Microbiology (Reading)*, 148(Pt 10), 3007-3017. <https://doi.org/10.1099/00221287-148-10-3007>.
34. Larsen, M.H., Biermann, K., Tandberg, S., Hsu, T., and Jacobs, W.R., Jr. (2007). Genetic manipulation of *Mycobacterium tuberculosis*. *Current Protocols in Microbiology*, Chapter 10, Unit 10A.12. <https://doi.org/10.1002/9780471729259.mc10a02s6>.
35. Beukes, D., Du Preez, I., and Loots, D.T. (2019). Total metabolome extraction from mycobacterial cells for GC-MS metabolomics analysis. *Methods in Molecular Biology*, 1859, 121-131. https://doi.org/10.1007/978-1-4939-8757-3_6.
36. Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., and Hollender, J. (2014). Identifying small molecules via high resolution mass spectrometry: Communicating confidence. *Environmental Science and Technology*, 48(4), 2097-2098. <https://doi.org/10.1111/j.1365-2958.2006.05279.x>.
37. Van Den Berg, R.A., Hoefsloot, H.C.J., Westerhuis, J.A., Smilde, A.K., and Van Der Werf, M.J. (2006). Centering, scaling, and transformations: Improving the biological information content of metabolomics data. *BMC Genomics*, 7, 142. <https://doi.org/10.1186/1471-2164-7-142>.
38. Sullivan, G.M. , and Feinn, R. (2012). Using effect size-or why the *p* value is not enough. *Journal of Graduate Medical Education*, 4(3), 279-282. <https://doi.org/10.4300/jgme-d-12-00156.1>.
39. Mikušová, K.n., Huang, H., Yagi, T., Holsters, M., Vereecke, D., D'Haese, W., Scherman, M.S., Brennan, P.J., McNeil, M.R., and Crick, D.C. (2005). Decaprenylphosphoryl arabinofuranose, the donor of the D-arabinofuranosyl residues

- of mycobacterial arabinan, is formed via a two-step epimerization of decaprenylphosphoryl ribose. *Journal of Bacteriology*, 187(23), 8020-8025. <https://doi.org/10.1128/jb.187.23.8020-8025.2005>.
40. Wesener, D.A., Levengood, M.R., and Kiessling, L.L. (2017). Comparing galactan biosynthesis in *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*. *The Journal of Biological Chemistry*, 292(7), 2944-2955. <https://doi.org/10.1074/jbc.m116.759340>.
 41. Joe, M. , and Lowary, T.L. (2016). Synthesis of a homologous series of galactofuranose-containing mycobacterial arabinogalactan fragments. *Canadian Journal of Chemistry*, 94(11), 976-988. <https://doi.org/10.1139/cjc-2016-0416>.
 42. Completo, G.C. , and Lowary, T.L. (2008). Synthesis of galactofuranose-containing acceptor substrates for mycobacterial galactofuranosyltransferases. *The Journal of Organic Chemistry*, 73(12), 4513-4525. <https://doi.org/10.1021/jo800457j>.
 43. Azuma, I., Ajisaka, M., and Yamamura, Y. (1970). Polysaccharides of *Mycobacterium bovis* Ushi 10, *Mycobacterium smegmatis*, *Mycobacterium phlei*, and Atypical *Mycobacterium* P1. *Infection and Immunity*, 2(3), 347-349. <https://doi.org/10.1128/iai.2.3.347-349.1970>.
 44. Ramakrishnan, T., Murthy, P.S., and Gopinathan, K.P. (1972). Intermediary metabolism of mycobacteria. *Bacteriological Reviews*, 36(1), 65-108. <https://doi.org/10.1128/br.36.1.65-108.1972>.
 45. Brennan, P.J. , and Nikaido, H. (1995). The envelope of mycobacteria. *Annual Review of Biochemistry*, 64, 29-63. <https://doi.org/10.1146/annurev.bi.64.070195.000333>.
 46. Alderwick, L.J., Harrison, J., Lloyd, G.S., and Birch, H.L. (2015). The mycobacterial cell wall-peptidoglycan and abinogalactan. *Cold Spring Harb Perspect Med*, 5(8), a021113. <https://doi.org/10.1101/cshperspect.a021113>.
 47. Rogacki, M.K., Pitta, E., Balabon, O., Huss, S., Lopez-Roman, E.M., Argyrou, A., Blanco-Ruano, D., Cacho, M., Vande Velde, C.M.L., Augustyns, K., Ballell, L., Barros, D., Bates, R.H., Cunningham, F., and Van Der Veken, P. (2018). Identification and profiling of hydantoins - A novel class of potent antimycobacterial DprE1 inhibitors. *Journal of Medicinal Chemistry*, 61(24), 11221-11249. <http://dx.doi.org/10.1021/acs.jmedchem.8b01356>.
 48. Pathak, A.K., Pathak, V., Riordan, J.M., Gurcha, S.S., Besra, G.S., and Reynolds, R.C. (2004). Synthesis of mannopyranose disaccharides as photoaffinity probes for mannosyltransferases in *Mycobacterium tuberculosis*. *Carbohydrate Research*, 339(3), 683-691. <https://doi.org/10.1016/j.carres.2003.10.031>.
 49. Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., and Morishima, K. (2017). KEGG: New perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Research*, 45(D1), D353-D361. <https://doi.org/10.1093/nar/gkw1092>.
 50. Titgemeyer, F., Amon, J., Parche, S., Mahfoud, M., Bail, J., Schlicht, M., Rehm, N., Hillmann, D., Stephan, J., Walter, B., Burkovski, A., and Niederweis, M. (2007). A genomic view of sugar transport in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. *Journal of Bacteriology*, 189(16), 5903-5915. <https://doi.org/10.1128/jb.00257-07>.
 51. Schleifer, K.H. , and Kandler, O. (1973). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriological Reviews*, 37(2), 258. <https://doi.org/10.1128/br.37.2.258-258.1973>.
 52. Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., Li, Q., Shoemaker, B.A., Thiessen, P.A., Yu, B., Zaslavsky, L., Zhang, J., and Bolton, E.E. (2019). PubChem 2019 update: Improved access to chemical data. *Nucleic Acids Research*, 47(D1), D1102-D1109. <https://doi.org/10.1093/nar/gky1033>.

53. Tripathi, D., Chandra, H., and Bhatnagar, R. (2013). Poly-L-glutamate/glutamine synthesis in the cell wall of *Mycobacterium bovis* regulated in response to nitrogen availability. *BMC Microbiology*, 13, 226. <https://doi.org/10.1186/1471-2180-13-226>.
54. Etienne, G., Malaga, W., Laval, F., Lemassu, A., Guilhot, C., and Daffé, M. (2009). Identification of the polyketide synthase involved in the biosynthesis of the surface-exposed lipooligosaccharides in mycobacteria. *Journal of Bacteriology*, 191(8), 2613-2621. <https://doi.org/10.1128/jb.01235-08>.
55. Angala, S.K., Belardinelli, J.M., Huc-Claustre, E., Wheat, W.H., and Jackson, M. (2014). The cell envelope glycoconjugates of *Mycobacterium tuberculosis*. *Critical Reviews in Biochemistry and Molecular Biology*, 49(5), 361-399. <https://doi.org/10.3109/10409238.2014.925420>.
56. Kinsella, R.J., Fitzpatrick, D.A., Creevey, C.J., and McInerney, J.O. (2003). Fatty acid biosynthesis in *Mycobacterium tuberculosis*: Lateral gene transfer, adaptive evolution, and gene duplication. *Proceedings of the National Academy of Sciences of the United States of America*, 100(18), 10320-10325. <https://doi.org/10.1073/pnas.1737230100>.
57. Schaefer, W.B. , and Lewis, C.W., Jr. (1965). Effect of oleic acid on growth and cell structure of mycobacteria. *Journal of Bacteriology*, 90(5), 1438-1447. <https://doi.org/10.1128/jb.90.5.1438-1447.1965>.
58. Jantzen, E., Tangen, T., and Eng, J. (1989). Gas chromatography of mycobacterial fatty acids and alcohols: Diagnostic applications. *APMIS*, 97(11), 1037-1045. <https://doi.org/10.1111/j.1699-0463.1989.tb00515.x>.
59. Chou, S., Chedore, P., and Kasatiya, S. (1998). Use of gas chromatographic fatty acid and mycolic acid cleavage product determination to differentiate among *Mycobacterium genavense*, *Mycobacterium fortuitum*, *Mycobacterium simiae*, and *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology*, 36(2), 577-579. <https://doi.org/10.1128/jcm.36.2.577-579.1998>.
60. Boldrin, F., Ventura, M., Degiacomi, G., Ravishankar, S., Sala, C., Svetlikova, Z., Ambady, A., Dhar, N., Kordulakova, J., Zhang, M., Serafini, A., Vishwas, K.G., Kolly, G.S., Kumar, N., Palù, G., Guerin, M.E., Mikusova, K., Cole, S.T., and Manganelli, R. (2014). The phosphatidyl-myo-inositol mannosyltransferase PimA is essential for *Mycobacterium tuberculosis* growth *in vitro* and *in vivo*. *Journal of Bacteriology*, 196(19), 3441-3451. <https://doi.org/10.1128/jb.01346-13>.
61. Sancho-Vaello, E., Albasa-Jové, D., Rodrigo-Unzueta, A., and Guerin, M.E. (2017). Structural basis of phosphatidyl-myo-inositol mannosides biosynthesis in mycobacteria. *Biochim Biophys Acta Molecular and Cell Biology of Lipids*, 1862(11), 1355-1367. <https://doi.org/10.1016/j.bbalip.2016.11.002>.
62. Cook, G.M., Berney, M., Gebhard, S., Heinemann, M., Cox, R.A., Danilchanka, O., and Niederweis, M. (2009). Physiology of mycobacteria. *Advances in Microbial Physiology*, 55, 81-182, 318-319. [https://doi.org/10.1016/s0065-2911\(09\)05502-7](https://doi.org/10.1016/s0065-2911(09)05502-7).
63. Zhang, Y.J., Reddy, M.C., Ioerger, T.R., Rothchild, A.C., Dartois, V., Schuster, B.M., Trauner, A., Wallis, D., Galaviz, S., Huttenhower, C., Sacchettini, J.C., Behar, S.M., and Rubin, E.J. (2013). Tryptophan biosynthesis protects mycobacteria from CD4 T-cell-mediated killing. *Cell*, 155(6), 1296-1308. [10.1016/j.cell.2013.10.045](https://doi.org/10.1016/j.cell.2013.10.045).
64. Zelante, T., Fallarino, F., Bistoni, F., Puccetti, P., and Romani, L. (2009). Indoleamine 2,3-dioxygenase in infection: The paradox of an evasive strategy that benefits the host. *Microbes and Infection*, 11(1), 133-141. <https://doi.org/10.1016/j.micinf.2008.10.007>.
65. Zhang, L., Alfano, J.R., and Becker, D.F. (2015). Proline metabolism increases katG expression and oxidative stress resistance in *Escherichia coli*. *Journal of Bacteriology*, 197(3), 431-440. <https://doi.org/10.1128/JB.02282-14>.

66. Csonka, L.N. (1989). Physiological and genetic responses of bacteria to osmotic stress. *Microbiological Reviews*, 53(1), 121-1470146-0749.
67. Landaas, S. (1975). Accumulation of 3-hydroxyisobutyric acid, 2-methyl-3-hydroxybutyric acid and 3-hydroxyisovaleric acid in ketoacidosis. *Clinica Chimica Acta: International Journal of Clinical Chemistry*, 64(2), 143-1540009-8981.
68. Kamio, Y., Itoh, Y., and Terawaki, Y. (1981). Chemical structure of peptidoglycan in *Selenomonas ruminantium*: Cadaverine links covalently to the D-glutamic acid residue of peptidoglycan. *Journal of Bacteriology*, 146(1), 49-53. <https://doi.org/10.1128/jb.146.1.49-53.1981>.
69. Neely, M.N. , and Olson, E.R. (1996). Kinetics of expression of the *Escherichia coli* cad operon as a function of pH and lysine. *Journal of Bacteriology*, 178(18), 5522-5528. <https://doi.org/10.1128/iai.00893-09>.
70. Samartzidou, H. , and Delcour, A.H. (1998). *E.coli* PhoE porin has an opposite voltage-dependence to the homologous *OmpF*. *The EMBO Journal*, 17(1), 93-100. <https://doi.org/10.1093/emboj/17.1.93>.
71. Loots, D.T. (2014). An altered *Mycobacterium tuberculosis* metabolome induced by *katG* mutations resulting in isoniazid resistance. *Antimicrob Agents Chemother*, 58(4), 2144-2149. <https://doi.org/10.1128/aac.02344-13>.
72. Bruning, J.B., Murillo, A.C., Chacon, O., Barletta, R.G., and Sacchettini, J.C. (2011). Structure of the *Mycobacterium tuberculosis* D-alanine:D-alanine ligase, a target of the antituberculosis drug D-cycloserine. *Antimicrob Agents Chemother*, 55(1), 291-301. <https://doi.org/10.1128/aac.00558-10>.
73. Buckoreelall, K., Sun, Y., Hobrath, J.V., Wilson, L., and Parker, W.B. (2012). Identification of Rv0535 as methylthioadenosine phosphorylase from *Mycobacterium tuberculosis*. *Tuberculosis*, 92(2), 139-147. <https://doi.org/10.1016/j.tube.2011.11.010>.
74. Namanja-Magliano, H.A., Evans, G.B., Harijan, R.K., Tyler, P.C., and Schramm, V.L. (2017). Transition state analogue inhibitors of 5'-deoxyadenosine/5'-methylthioadenosine nucleosidase from *Mycobacterium tuberculosis*. *Biochemistry*, 56(38), 5090-5098. <https://doi.org/10.1021/acs.biochem.7b00576>.
75. Ramachandran, S., Fontanille, P., Pandey, A., and Larroche, C. (2006). Gluconic acid: Properties, applications and microbial production. *Food Technology & Biotechnology*, 44(2). <https://hrcak.srce.hr/file/161891>.
76. Early, J.V., Casey, A., Martinez-Grau, M.A., Gonzalez Valcarcel, I.C., Vieth, M., Ollinger, J., Bailey, M.A., Alling, T., Files, M., Ovechkina, Y., and Parish, T. (2016). Oxadiazoles have butyrate-specific conditional activity against *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*, 60(6), 3608-3616. <https://doi.org/10.1128/aac.02896-15>.
77. Dunn, J. , and Grider, M.H. (2023). Physiology, adenosine triphosphate, in *StatPearls [Internet]*. StatPearls Publishing: <https://www.ncbi.nlm.nih.gov/books/NBK553175/>.

Chapter 2 investigated the role of *Mycobacterium tuberculosis* curli pili (MTP) in a bacterial model using wildtype *M. tuberculosis* V9124, the *mtp*-deletion mutant and *mtp*-complemented strain. Significant metabolic perturbations were observed in the MTP-deficient strain compared to MTP-proficient strains. Function of the *mtp* gene was mostly restored in the complemented strain. The differentially detected metabolites suggested that MTP influences cell wall biogenesis, fatty acid metabolism and amino acid synthesis of *M. tuberculosis*.

The influence of MTP in modulating the intertwined metabolic pathways of *M. tuberculosis* and its THP-1 macrophage host were investigated in Chapter 3. This chapter has been published as a journal article in *Microbial Pathogenesis*, (Elsevier) 2021 (DOI: <https://doi.org/10.1016/j.micpath.2021.104806>).

CHAPTER 3: *Mycobacterium tuberculosis* curli pili (MTP) is associated with alterations in carbon, fatty acid and amino acid metabolism in a THP-1 macrophage infection model

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3.1. Abstract

Introduction: The initial host-pathogen interaction is crucial for the establishment of infection. An improved understanding of the pathophysiology of *Mycobacterium tuberculosis* (*M. tuberculosis*) during macrophage infection can aid the development of intervention therapeutics against tuberculosis. *Mycobacterium tuberculosis* curli pili (MTP) is a surface-located adhesin, involved in the first point-of-contact between pathogen and host.

Objective: This study aimed to better understand the role of MTP in modulating the intertwined metabolic pathways of *M. tuberculosis* and its THP-1 macrophage host.

Methods: Metabolites were extracted from pelleted wet cell mass of THP-1 macrophages infected with *M. tuberculosis* wildtype V9124 (WT), Δmtp -deletion mutant and the *mtp*-complemented strains, respectively, via a whole metabolome extraction method using a 1:3:1 ratio of chloroform:methanol:water. Metabolites were detected by two-dimensional gas chromatography time-of-flight mass spectrometry. Significant metabolites were determined through univariate and multivariate statistical tests and online pathway databases.

Results: Relative to the WT, totals of nine and ten metabolites were significantly different in the Δmtp and *mtp*-complement strains, respectively. All nine significant metabolites were found in elevated levels in the Δmtp relative to the WT. Additionally, of the ten significant metabolites, eight were detected in lower levels and two were detected in higher levels in the *mtp*-complement relative to the WT.

Conclusion: The absence of the MTP adhesin resulted in reduced virulence of *M. tuberculosis* leading to alterations in metabolites involved in carbon, fatty acid and amino acid metabolism during macrophage infection, suggesting that MTP plays an important role in the modulation of host metabolic activity. These findings support the prominent role of the MTP adhesin as a virulence factor as well as a promising biomarker for possible diagnostic and therapeutic intervention.

3.2. Introduction

Tuberculosis (TB) is caused by the well-established pathogen *Mycobacterium tuberculosis* (*M. tuberculosis*). Although preventable and curable, this communicable disease remains a global threat and is the foremost cause of death by a single infectious agent [1]. In 2018, the recorded 10 million TB cases included 1.5 million deaths of which 251 000 people were positive for human immunodeficiency virus (HIV) [1]. Additionally, the threat of antimicrobial resistance has escalated globally with 18% of previously treated cases harbouring multidrug-resistant TB or rifampicin resistant TB (MDR/RR-TB) [1]. Currently implemented diagnostics and treatment regimens are, therefore, not effective enough to end TB.

Pathogenic bacilli present in aerosol droplets deposited onto the alveolar surfaces of the lung replicate when the immune system is compromised [2]. Resident alveolar macrophages (AMs) are recognised for their defensive roles against mycobacterial infections, as these cells typically engulf and destroy infected cells and pathogens in the lung, hence, they are involved in the initial stages of pathogenesis [3-5]. However, *M. tuberculosis* can overcome the host defences and survive the harsh environment within macrophages [6].

The adherence of a pathogen to its host cell is mediated by specific externally exposed proteinaceous molecules, termed adhesins [7]. Adhesins prevent the removal of microorganisms from colonised surfaces and set off events, which facilitate invasion of the host cell, conversely, leading to the induction of the host's defence mechanisms [8]. *Mycobacterium tuberculosis* produces multiple adhesins; one of which is *M. tuberculosis* curli

pili (MTP), composed of pilin subunits encoded by the open reading frame *Rv3312A (mtp)* [9]. Amongst clinical isolates, *mtp* was shown to be highly conserved and unique to *M. tuberculosis* complex strains [10]. Functional genomics, using a *mtp*-deletion mutant, has demonstrated that MTP acts as an adhesin and invasin to THP-1 macrophages [11] and A549 epithelial cells [12]. Global transcriptomics data demonstrated that MTP significantly modulates host immune responses post-infection in epithelial cells and BALB/C mice [13; 14]. Anti-MTP IgG antibodies in patients' serum samples were detectable by a synthetic MTP peptide with an accuracy of 97% [15]. These findings, together with its surface location suggest the potential of MTP as a biomarker for diagnostic tests, and for the design of an effective anti-TB agent that can interrupt the first point-of-contact between pathogen and host.

Insight into the infection process is achievable through metabolomics, which can detect and quantify metabolites within a biological system at a given time [16]. Hence, any perturbation of a biological system may result in variations in the composition/concentration of the metabolites produced [17]. Hyphenated two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-TOFMS) conjoins chromatographic and spectral technologies to identify metabolites [18]. Studies have used GC-MS to better characterise *M. tuberculosis* virulence and identify active TB infections [19-22].

Previously an *in vitro* functional metabolomics study elucidated pathogen metabolite changes associated with MTP using MTP-proficient and MTP-deficient strains of *M. tuberculosis* [23]. The current study used GC×GC-TOFMS to establish the influence of MTP on the intertwined host and pathogen metabolic pathways in an infection model of THP-1 macrophages to further validate the MTP adhesin and/or identified metabolites as potential biomarkers for enhancement of intervention strategies to combat TB.

3.3. Materials and methods

3.3.1. Ethical statement

The study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BE254/17) and the experimental work was performed in a Biosafety Level 2+ (BSL 2+) laboratory. Bacterial culture, cell culture and infection experiments were conducted at Medical Microbiology, University of KwaZulu-Natal, Durban South Africa. Metabolite extraction, derivatisation and analysis were conducted at the Centre for Human Metabolomics, North-West University, Potchefstroom, South Africa.

3.3.2. Bacterial strains and growth conditions

Wildtype (WT) *M. tuberculosis* V9124 from the F15/LAM4/KZN family [24], a marked *mtp*-deletion mutant (Δmtp) generated via specialised transduction [25] of WT in which a 188-bp region of the *mtp* gene was deleted and replaced with a $\gamma\delta res-sacB-hyg\gamma\delta res$ cassette [25; 26], and an electrotransformed *mtp*-complemented strain using the non-integrating plasmid pMV261 [25; 26] were used in the study. All bacterial strains were cultured at 37°C in Middlebrook 7H9 broth or 7H11 plates (Difco, Becton-Dickinson, Franklin Lakes, NJ, USA) supplemented with 10% (v/v) oleic albumin dextrose catalase supplement (Difco, Becton-Dickinson, Franklin Lakes, NJ, USA) and 0.5% (v/v) glycerol (Sigma-Aldrich, St. Louis, MO, USA). Cultures in 7H9 broth were additionally supplemented with 0.05% tween-80 (Sigma-Aldrich, St. Louis, MO, USA) and grown in a shaking incubator (I-26 Shaking Incubator, New

Brunswick Scientific, Canada) at 100 rpm for 7 – 8 days. Strains were cultured on antibiotic-containing broth prior to polymerase chain reaction (PCR) confirmation; hygromycin (75 µg/mL) for *Δmtp*; and kanamycin (30 µg/mL) for the complemented strain.

Prior to host cell infection, bacterial inocula for each of the three strains were prepared by centrifugation of log phase bacterial culture at 2 000×*g* for 10 minutes and resuspension of the pellet in 40 mL of complete cell culture media (comprised Roswell Park Memorial Institute 1640 medium, with L-Glutamine (Lonza, BioWhittaker, Basel, Switzerland) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France)) to achieve an optical density (OD_{600nm}) of approximately 1 (Lightwave II, Biochrom, Cambridge, UK), equivalent to 1×10⁸ cells/mL [27].

3.3.3. Infection of THP-1 macrophages

The THP-1 monocytic cell line (ATCC TIB-202) was cultured in complete cell culture media and incubated at 37°C in 5% CO₂ in a humidified incubator (Shel Lab CO₂ Incubator, Cornelius, OR, USA). THP-1 cell pellets were quantified by the trypan blue exclusion assay and seeded at a final concentration of 9×10⁵ cells/mL per 75 cm² tissue culture vented flasks (Nest Biotechnology, Jiangsu, China). Monocytes were differentiated into macrophages with the addition of phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) (Sigma-Aldrich, St. Louis, MO, USA). A total of 90 flasks were seeded; 30 flasks were assigned for each of the three strains, these included 10 flasks, which served as the uninfected controls, 10 flasks as experimental infection groups and 10 flasks to determine colony forming units (CFUs), respectively. All flasks were incubated (Shell Lab CO₂ Incubator, Cornelius, OR, USA) overnight at 37°C in 5% CO₂. Macrophage monolayers were washed once with phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) resuspended in cell culture media and infected with previously prepared bacterial inocula to achieve a multiplicity of infection (MOI) ratio of approximately 5:1 in the experimental and CFU flasks per bacterial strain. Only complete cell culture medium was added to the flasks containing uninfected macrophages. The flasks were incubated (Shell Lab CO₂ Incubator, Cornelius, OR, USA) at 37°C in 5% CO₂ and 95% humidity for four hours of infection (selected based on pilot study outlined in the Appendix C: Table S1 and Figure S1). Bacterial inocula were serially diluted and plated onto Middlebrook 7H11 agar plates to confirm the MOI (Appendix C: Table S2). Following four hours of infection, the monolayers were washed twice with PBS. For the uninfected macrophages and experimental flask, cell detachment was achieved with addition of a trypsin-versene mixture (Lonza, BioWhittaker, Basel, Switzerland) incubated (Shell Lab CO₂ Incubator, Cornelius, OR, USA) at 37°C for 3 minutes. Thereafter, the trypsin-versene mixture was inactivated with the addition of complete cell culture media. Host cells were washed three times with 500 µL of PBS and centrifugation at 200×*g* for 20 minutes (Mikro 200R, Hettich Zentrifugen, Tuttlingen, Germany). Pellets were immediately frozen to halt any additional metabolite turnover and stored at -80°C (Sigma-Aldrich, St. Louis, MO, USA) until metabolite extraction was performed. Infected host cells in the CFU flasks were lysed with 0.01% Triton X-100 (Millipore, Merck, Darmstadt, Germany) for 20 minutes. Ten-fold serial dilutions of lysates were plated onto Middlebrook 7H11 plates in triplicate to quantitate the invading bacteria and confirm reproducibility of the infection with CFUs, which were read after incubation at 37°C for three weeks (Appendix C: Figure S2 and Table S2).

3.3.4. Metabolite extraction and derivatisation and GC×GC-TOFMS analysis

A whole metabolome extraction method [28] was used to extract metabolites from an array of metabolite classes and was used on the THP-1 cell line prior to experimentation. Aliquots of 30 mg per sample were used for analyses and quantification was reported as ng/mg cell mass. For each sample group, a quality control (QC) sample was made up by combining 30 mg of one randomly selected sample from that group and 0.49218 mg/mL 3-phenylbutyric acid (Sigma-Aldrich, St. Louis, MO, USA) served as the internal standard for normalisation. As described in a previous study [23], metabolites were extracted using a 1:3:1 ratio of ultra-pure chloroform:methanol:water (Burdick & Jackson; Honeywell International Inc., Muskegon, MI, USA), in a MM400 mixer mill (Retsch GmbH & co. KG, Haan, Germany). Derivatisation was carried out in a solution of 50 μ L methoxyamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in 15 mg/mL pyridine (Merck, Darmstadt, Germany) at 50 °C for 90 minutes. This preceded 40 μ L of N,O-Bis (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (Sigma-Aldrich, St. Louis, MO, USA) at 60 °C for 60 minutes. The samples were analysed by untargeted GC×GC-TOFMS in a Pegasus 4D instrument (Leco Corporation, St. Joseph, MI, USA). A three-split ratio was used on the Agilent 7890A GC (Agilent, Atlanta, GA) coupled to a time-of-flight mass spectrometer (Leco Corporation, St. Joseph, MI, USA) equipped with a Gerstel Multi-Purpose Sampler (Gerstel GmbH & co. KG, Eberhard-Gerstel- Platz 1, D-45473 Mülheim an der Ruhr). First dimensional separation was achieved with a Restek RTX-1ms primary column (30 m, 250 μ m internal diameter, 0.25 μ m filament thickness) and a Restek RTX-17 secondary capillary column (1.4 m, 250 μ m internal diameter, 0.25 μ m filament thickness) (LECO Africa, JHB, SA). The transfer line temperature and ion source temperature were held at a constant 270 °C and 220 °C, respectively. The detector was adjusted to 1400 V and an acquisition rate of 200 spectra per second was used to collect spectra between the range 50 - 800 m/z. Leco Cooperation ChromaTOF software (version 4.51.6.0) was used to process the data, including baseline removal using the 'spanning' tracking method with offset of 1, and automatic smoothing was performed. An expected 2D peak width of 50 (5 seconds $\times$ 10 modulations; and 1 second on the 2nd dimension) and signal-to-noise ratio of 100 was used to detect the peaks with five apexing masses. Peak identities were determined by using both the MS-generated mass fragmentation patterns, and their GC retention times. Spectra were matched to in house data, compiled from previously injected standards, and the National Institute of Standards and Technology (NIST) commercial libraries (main library and replicate library) to identify the important metabolites. Statistical Compare was used to generate a data matrix through peak alignment.

3.3.5. Statistical data analysis

Experimental samples and 50 quality controls were randomized across three batches for the statistical analysis. MATLAB (MATLAB with Statistics Toolbox (2019), version 9.5.0 (R2018b) software (Natick, Massachusetts: The MathWorks Inc) along with the Eigenvector PLS_Toolbox 8.7 (Wenatchee, WA: Eigenvector Research Inc. Software available at <http://www.eigenvector.com>) was used to identify metabolites with the potential to differentiate amongst the sample groups. Prior to statistical analysis four analytical outlier samples were removed: two from the WT uninfected group, one from the Δmtp infected group and one from the *mtp*-complement infected group. This left a total of seven WT uninfected, nine WT infected, nine Δmtp uninfected, eight Δmtp infected, nine *mtp*-complement uninfected

and eight *mtp*-complement infected samples. The number of remaining samples in each group were still adequate for the comparisons, thus were used for statistical analysis. Statistical analysis was performed as previously reported [23]. Specifically, data pre-processing included zero filter and zero value replacement in which metabolites with zero observations of 50% or more were excluded and the remaining zeros were replaced by random numbers between the minimum and zero. Analytical repeatability was ensured through the intermittent injection of QC samples throughout the GC×GC-TOFMS analysis. The quality assurance was successful as the variability between experimental samples far exceeded the variability between QC samples (Appendix C: Figure S3). Log transformations were used for data normalisation and the metabolites were regarded as equally important by multivariate models [29] through autoscaling. Univariate analysis incorporated Benjamini & Hochberg adjustment for multiple testing to control the rate of false discovery (BH FDR) [30] resulting from the independent samples *t*-test (with $p \leq 0.05$ considered statistically significant) [31], Cohen's *d*-value effect size statistic (with $d \geq 0.8$ considered to be of practical relevance) [32] and fold changes (with fold change ≥ 2 considered noteworthy) [33; 34]. Multivariate analysis included principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) into the generation of PLS-DA variable importance in the projection (VIP) values (where metabolites with $VIP \geq 1$ were considered strong predictors) [35]. Metabolites which met all four selection criteria, were regarded as statistically significant, and were further investigated for biological relevance through PubChem [36] and/or KEGG [37], as well as literature. Thereafter, biological relevance was used to categorise metabolites.

3.4. Results

3.4.1. Volcano plots illustrate numerous univariate differences among sample groups

The metabolites produced by the WT, Δmtp and *mtp*-complement infected samples were compared to one another (Appendix C: Figure S4 a and b) as well as their respective uninfected controls (Appendix C: Figure S4 c, d, and e) to determine any practical or statistically significant differences in their profiles. Results were depicted through volcano plots which scatter the uncorrected log transformed *p*-values from the independent samples *t*-test against Cohen's *d*-values. Significant metabolites, depicted in red, were further investigated in the results that follow.

3.4.2. Partial least squares discriminant analysis (PLS-DA) data validation illustrates adequate group separation between models

The PLS-DA models were constructed to determine differences in the metabolome among the WT, Δmtp and *mtp*-complement infected and respective uninfected sample groups (Appendix C: Figure S5) and to describe the difference among the infection model group comparisons (Figure 1a and b). The R^2 (predictive accuracy) and Q^2 (leave-one-out predictive accuracy) values were scrutinised to understand the extent of PLS-DA overfit and assess the validity of models. Models were considered valid when R^2 and Q^2 values equalled or exceeded 80% and 60%, respectively. The models that validated were WT uninfected compared to WT infected (Appendix C: Figure S5a), Δmtp uninfected compared to Δmtp infected (Appendix C: Figure S5b), WT infected compared to Δmtp infected (Figure 3.1a) and WT infected compared to *mtp*-

complement infected (Figure 3.1b) indicating separation between groups within these models. The *mtp*-complement uninfected compared to *mtp*-complement infected model (Appendix C: Figure S5c) did not validate as it did not meet the Q^2 value criterion; therefore, it was not suitable for further interpretation. However, univariate data resulting from the comparison between the uninfected and infected THP-1 macrophages for each strain did reveal metabolomic changes in the latter in response to the infecting pathogen (Appendix C: Tables S3, S4 and S5). All three infection groups were compared to each other in a PCA and PLS-DA model (Appendix C: Figure S6) to show the unsupervised and supervised group separation profiles, respectively. The PLS-DA (Appendix C: Figure S6b) did not validate ($Q^2 < 60\%$); therefore, it was not suitable for further interpretation. Additionally, PLS-DAs for individual group comparisons validated (Figure 3.1). Compellingly, the differences in the metabolomic profiles between WT and Δmtp infected samples (Figure 1a) may be attributed to the absence of the MTP adhesin in the infecting strain. The unexpected variation observed between the WT infected and *mtp*-complement infected metabolite profiles (Figure 3.1b) is indicative of partial restoration of metabolic phenotype of *mtp*-complement to that of the WT in the infection group. However, the variation explained by each axis is low (Figure 3.1), and thus, represents a small proportion of the overall variation in the metabolite levels detected within the respective infection groups.

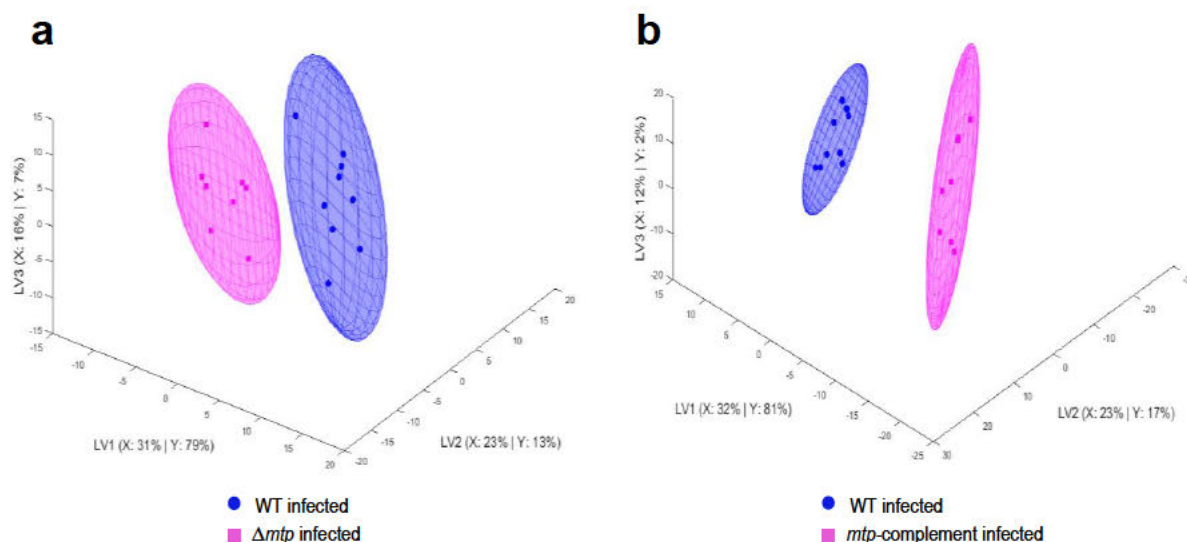


Figure 3.1. The 3D PLS-DA score plots of various group comparisons. Models comparing (a) WT infected macrophages to Δmtp infected macrophages ($R^2 = 98.51\%$; $Q^2 = 90.56\%$) and (b) WT infected macrophages to *mtp*-complement infected macrophages ($R^2 = 98.92\%$; $Q^2 = 93.90\%$). The WT infected macrophages are represented in royal blue and the Δmtp and *mtp*-complement infected macrophages are represented in pink, respectively. The R^2 = proportion of variance in the group labels explained by the model; Q^2 = proportion of variance in the group labels explained by the model given the leave-one-out cross validation. Ellipsoids represent 95% CI of score centroids of each group. Accuracy of group assignment (Y) and variation explained in measured metabolites (X), as achieved by each latent variable (LV), percentages are indicated along each axis.

3.4.3. Data processing and shortlisting of statistical and biologically relevant metabolites within each comparison group revealed metabolites of interest

Analytes that were not assigned a name for identification from spectral libraries were excluded from the analysis. Metabolites were stringently shortlisted by selecting only those that met all the statistical selection criteria (absolute fold change ≥ 2 ; BH FDR p -value ≤ 0.05 ; Cohen's d -value effect size ≥ 0.8 ; and PLS-DA VIP ≥ 1) [30-35] and were of biological relevance [36; 37]. Of the 229 metabolite differences between WT infected and Δmtp infected macrophages, nine metabolites were deemed both statistically and biologically relevant and were grouped according to their involvement in various metabolic pathways (Table 3.1). All the significant metabolites listed in Table 3.1 were elevated in the Δmtp infection group relative to the WT infection group, thereby indicating that the absence of the MTP adhesin in the mutant alters carbon, amino acid and fatty acid metabolism in this infection group. Of the 228 metabolites detected between the WT infected and *mtp*-complement infected macrophages, 10 metabolites were deemed both statistically and biologically relevant and exclusive to this comparison model (Table 3.2). In addition to the changes in carbon and amino acid metabolism, purine and pyrimidine metabolism was perturbed between the WT infected and *mtp*-complement infected model (Table 3.2). The metabolites in Table 3.2 were chiefly detected in lower levels in the *mtp*-complement infected group relative to the WT infected group, with exception of citric acid and fumaric acid involved in carbon metabolism. When comparing the WT infected with the Δmtp infected group and *mtp*-complement infected group, nine (approximately 4%) of the 229

metabolites and 10 (approximately 4%) of the 228 metabolites, respectively, detected in varying levels make up only a small percentage of the overall metabolome alterations (Appendix C: Figure S7).

3.4.4. Metabolic alterations to carbon, fatty acid, amino acid and purine and pyrimidine pathways in the absence/presence of MTP

The higher levels of glyceric acid (glycerate) in the Δmtp infected samples relative to WT infected samples (Table 3.1), indicate that the lack of the MTP adhesin causes inefficient utilisation of glyceric acid in pentose phosphate pathway (PPP) or glycolysis [37-39] (Figure 3.2). Alternatively, this could be a result of increased glycerate production. The glucogenic carbon flow from the tricarboxylic acid (TCA) cycle to phosphoenolpyruvate (PEP) is mediated by phosphoenolpyruvate carboxykinase (PEPCK) and is considered crucial for the survival of *M. tuberculosis* in macrophages and in mice [40] as the macrophage response to infection is to switch from pyruvate oxidation to pyruvate reduction, to form lactate (Figure 3.2) [41]. In the current study, the levels of lactic acid was approximately 13× higher in macrophages infected by the Δmtp relative to the WT strains (Table 3.1). The data suggests that with the lack of the MTP adhesin, lactic acid is not being utilised at the site of infection, thereby impeding intracellular growth of *M. tuberculosis*. The elevated levels of citrate (citric acid) and fumarate (fumaric acid) in the *mtp*-complement infected group relative to the WT infected group suggests that the TCA cycle may have not been affected by MTP itself (Figure 2), but by the over-piliation resulting from complementation with an over-expressing episomal plasmid containing *mtp*.

Fatty acids have been demonstrated to be essential carbon sources for *M. tuberculosis*, thereby permitting the co-metabolism of multiple carbon substrates [42-45]. In the current study, α -linolenic (ALA) acid metabolism (5-dodecenoic acid), fatty acid biosynthesis (decanoic acid, dodecanoic acid, palmitelaidic acid), biosynthesis of unsaturated fatty acids (eicosapentaenoic acid, 13-docosenoic acid) [38; 39] were seen to be affected (Figure 3.2). All of these metabolites were found in elevated levels in the Δmtp infection model relative to the WT infected, indicating an accumulation of these intermediates in the absence of the MTP adhesin (Table 3.1). The metabolism of fatty acids is essential for *M. tuberculosis* survival in the host, as the production of mycolic acids from elongated fatty acids, forms a protective lipid layer in the cell wall [46]. The data in the current study suggest that the absence of the MTP adhesin perturbs fatty acid metabolism, potentially resulting in impairment of the lipid layer of this pathogen's cell wall.

During infection, *M. tuberculosis* essentially synthesises carbohydrates through the gluconeogenic conversion of lipid-derived and/or amino acid-derived intermediates [47]. Metabolites detected in the current study are associated with an altered arginine and proline metabolism (spermine and 4-aminobutanoic acid or gamma-aminobutyric acid (GABA), produced via the decarboxylation of glutamic acid, and catalysed by glutamate decarboxylase) [48], nicotinate and nicotinamide metabolism (niacinamide), lysine biosynthesis and degradation (2-amino adipic acid, cadaverine) and tyrosine metabolism (dopamine). The GABAergic activation of macrophages, ushers an increase in antimicrobial responses against mycobacteria *in vitro* and *in vivo* [49]. Spermine is a negative regulator of macrophage activation and, therefore, restrains the innate immune response [50]. Derailment of the aspartate pathway to eradicate persistent *M. tuberculosis* infection, resulted in an upregulation in the *lat* (*Rv3290c*) gene, which is involved in the conversion of lysine to 2-amino adipic acid semialdehyde [51]. This gene had previously been identified as a persistence related gene in

M. tuberculosis [52-54]. A by-product from lysine metabolism; cadaverine, functions in acid tolerance, glutathione metabolism, as well as membrane permeability and ribonucleic acid (RNA) synthesis in several bacteria [36; 55-57]. Dopamine directly affects cellular iron homeostasis and its exposure to macrophages increased the incorporation of non-transferrin bound iron into cells, subsequently promoting intracellular oxidative stress responses [58]. All the aforementioned amino acid metabolites were detected in significantly lower levels in the *mtp*-complement infection model compared to the WT infection model (Table 3.2). Of these metabolites, niacinamide was detected in the Δmtp infection model in elevated levels, when compared with WT infected host (Table 3.1). These findings suggest that the absence of the MTP adhesin does not induce significant changes in amino acid metabolism other than nicotinate and nicotinamide metabolism during infection. Overrepresentation of the MTP adhesin in complemented strain; however, results in comparatively more changes to amino acid metabolism during infection relative to the WT.

Synthesis of purine and pyrimidine nucleotides are important for various reasons, apart from the synthesis of nucleic acids as they drive most cellular reactions through the generation of energy [59]. Adenine, associated with nicotinate and nicotinamide metabolism, is involved in energy release [38; 39]. Additionally, 7-methylguanine found in the present study, previously detected in normal human biofluids by mass spectrometry, is indicative of depurination and deoxynucleic acid (DNA) methylation [60]. Pyrimidinedione, is involved in pyrimidine metabolism pathways [38; 39] and its scaffold or dihydroxypyrimidine is found in many biological active molecules [61]. A recent study explored the activity of Mg^{2+} chelating pyrimidinediones and reported a novel series of N-alkyl-5-hydroxypyrimidinone carboxamides showing antibacterial activity against *M. tuberculosis* [61]. The Δmtp infection model showed no significant changes to those metabolites involved in purine and pyrimidine metabolism; however, there was an overall reduction in metabolite levels in the *mtp*-complement infection model in comparison to the WT infection model (Figure 3.2). This finding indicates that the MTP adhesin itself does not influence purine and pyrimidine metabolism and its intermediates during infection, but the possible over-piliation in the complemented strain may result in purine and pyrimidine metabolism pathway inhibitions.

Table 3.1. Most significant metabolite changes between WT infected and Δmtp infected hosts selected as per multivariate, univariate and biological selection criteria together with their respective mean relative concentration and standard deviation, fold change with respect to the WT infection, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Category	Metabolite	Mean relative concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH- adjusted <i>p</i> -value
		Δmtp infected	WT infected				
Carbon metabolism	Lactic acid	6841.392 (3220.275)	539.340 (418.912)	12.683	2.135	3.376	<0.001
	Glyceric acid	193.749 (27.685)	94.497 (25.017)	2.050	1.302	3.072	<0.001
Fatty acid metabolism	5-Dodecenoic acid	13.759 (3.877)	2.968 (1.665)	4.636	2.382	3.649	<0.001
	Decanoic acid	22.989 (14.193)	6.497 (4.316)	3.539	1.860	1.763	0.009
	Dodecanoic acid	312.263 (36.131)	89.485 (22.661)	3.490	2.043	5.270	<0.001
	Eicosapentaenoic acid	573.840 (133.309)	260.182 (81.992)	2.206	2.594	2.643	<0.001
	13-Docosenoic acid	11.633 (3.823)	4.653 (3.936)	2.500	2.241	1.200	0.025
	Palmitelaidic acid	1495.601 (181.422)	685.646 (166.610)	2.181	2.592	3.465	<0.001
Amino acid metabolism	Niacinamide	130.183 (52.827)	51.065 (20.604)	2.549	1.276	2.149	0.003

WT infected: wildtype infected; Δmtp infected: *mtp*-gene deletion mutant infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg (BH) adjustment for false discovery rate; positive fold change = higher levels in Δmtp infected; negative fold change = higher levels in WT infected.

Table 3.2. Most significant metabolite changes between WT infected and *mtp*-complement infected hosts selected as per multivariate, univariate and biological selection criteria together with their respective mean relative concentration and standard deviation, fold change with respect to the WT infection, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Category	Metabolite	Mean relative concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH- adjusted <i>p</i> -value
		<i>mtp</i> -complement infected	WT infected				
Carbon metabolism	Citric acid	113.882 (55.036)	2.021 (1.202)	56.344	3.618	6.579	<0.001
	Fumaric acid	57.839 (37.124)	21.484 (3.858)	2.692	1.931	1.429	0.050
Amino acid metabolism	4-Aminobutanoic acid	35.004 (19.714)	81.018 (26.186)	-2.315	1.062	1.439	0.050
	2-Aminoadipic acid	0.777 (0.607)	2.045 (0.644)	-2.633	1.073	1.770	0.027
	Cadaverine	4.980 (4.151)	20.293 (8.124)	-4.075	1.647	2.013	0.012
	Dopamine	1.486 (1.027)	6.079 (4.832)	-4.092	1.067	1.328	0.050
	Spermine	1.380 (1.585)	6.589 (2.079)	-4.77	1.641	2.143	0.008
Purine and pyrimidine metabolism	Adenine	4.927 (1.974)	12.318 (3.889)	-2.500	2.041	2.441	0.005
	Pyrimidinedione	5.997 (4.079)	17.405 (4.969)	-2.902	1.464	1.479	0.048
	7-Methylguanine	1.88 (1.819)	6.549 (3.784)	-3.483	1.573	1.663	0.048

WT infected: wildtype infected; *mtp*-complement infected: complemented *mtp*-knockout strain infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg (BH) adjustment for false discovery rate; positive fold change = higher levels in complement infected; negative fold change = higher levels in WT infected.

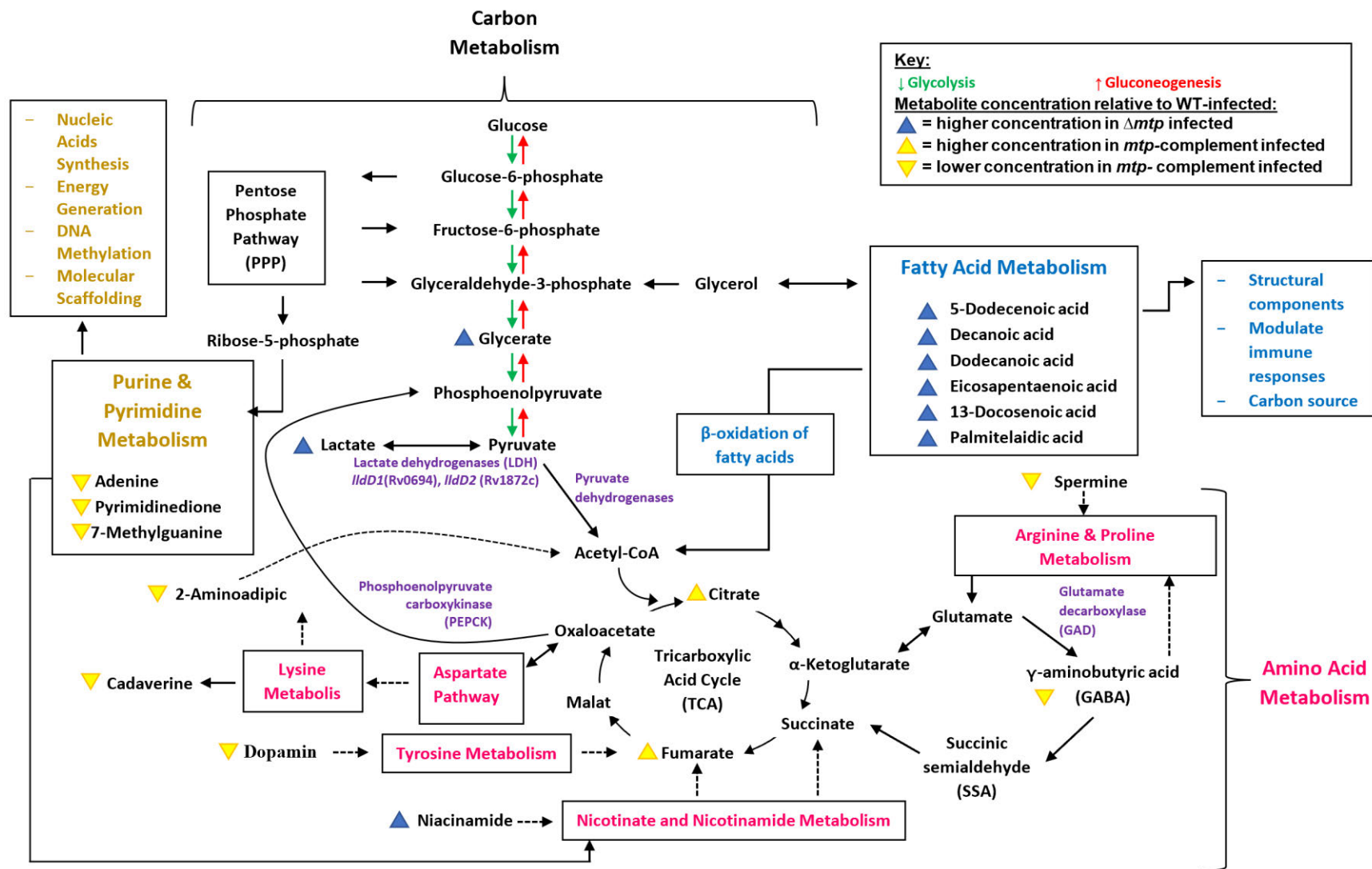


Figure 3.2. Metabolic pathway alterations in a THP-1 macrophage infection model using MTP-proficient and MTP-deficient strains of *M. tuberculosis*. Perturbations to carbon, fatty acid, amino acid and purine and pyrimidine metabolism between the Δmtp infection (blue arrows) and mtp -complement infection (yellow arrows) models relative to the WT infection model. Overall higher levels in the significantly detected metabolites in the Δmtp infected macrophages suggest an offset in structural components of *M. tuberculosis* and an attenuated ability to utilise energy sources, as well as modulate immune responses in the absence of MTP. Conversely, the mtp -complement infected macrophages, illustrated changes to amino acid metabolism and purine and pyrimidine metabolism indicating greater consumption of metabolites feeding into the TCA cycle relative to the WT.

3.5. Discussion

Several genomics/functional genomics [10; 11; 26], transcriptomics [13] and peptide studies [15] have provided evidence that the surface-located MTP adhesin [9] is a potentially suitable target as a TB diagnostic and vaccine candidate. A metabolomics study using MTP-proficient and MTP-deficient strains of *M. tuberculosis*, recently demonstrated MTP to influence the production of the pathogen's metabolome, in particular those metabolites associated with cell wall composition, fatty acid metabolism, and amino acid and peptidoglycan synthesis [23] all of which play important roles in *M. tuberculosis* pathogenesis. Moreover, MTP was demonstrated to contribute to metabolic changes in A549 epithelial host cells in which metabolite concentrations displayed “defective pathways” in the MTP-deficient *M. tuberculosis* infected host relative to the MTP-proficient *M. tuberculosis* infected host [62].

Due to its role as a physical barrier against respiratory infections and its abundance in the lung, alveolar epithelial cells (AEC) have a greater chance of encountering *M. tuberculosis* resident AMs in the initial stages of an infection [63]. However, as innate immune cells, macrophages characteristically provide a primary cellular niche for *M. tuberculosis* during early and chronic infection [64]. Given the different cellular functions of AECs and macrophages, and subsequently different metabolic baselines; the metabolic alterations resulting from infection with MTP-deficient *M. tuberculosis* will elicit metabolite signatures unique to each cell type. These unique metabolic profiles can be used independently or jointly to understand the effect MTP has on the pathogenicity of *M. tuberculosis* in an infection model. These metabolic alterations demonstrate co-adaptation of pathogen and host during infection, which potentially could be of value in the enhancement of current diagnostics and therapeutics. Therefore, the aim of the current study was to elucidate the role of MTP during host infection *in vitro* by ascertaining the alterations triggered by absence of the MTP adhesin on the metabolome of *M. tuberculosis* infected macrophages.

During the infection process, separation of metabolites from the intracellular pathogen and its host is challenging, as majority of metabolic processes are conserved in biological systems. Hence, there is a considerable overlap between host and pathogen metabolic pathways [65]. Metabolites that are commonly found between organisms include amino acids, central carbon metabolites and nucleotides [66], all of which were found to be significantly altered in the A549 epithelial cells [62] and the present study. While it was not possible to assign the detected metabolites to either pathogen or host by the untargeted GC×GC-TOFMS approach used in this study, the data generated provide insight into the effect MTP has on the metabolic pathways involved in adhesion and invasion of THP-1 macrophages with *M. tuberculosis* during infection.

By comparing the uninfected THP-1 macrophages with those infected with the three strains of *M. tuberculosis* used in the current study, changes in the metabolome of the biological system in response to infection were observed in amino acid, carbon, and fatty acid metabolism (Appendix C: Tables S3, S4 and S5). An in-depth analysis to determine the influence of the MTP adhesin on the metabolome signature of the infection models, showed significant group separation of the data in this study. The group variation seen when comparing the WT infected THP-1 macrophages with the Δmtp infected THP-1 macrophages (Figure 3.1a), was ascribed to the deletion of the *mtp* gene in the latter strain. The Δmtp infected macrophages demonstrated alterations in metabolites involved in composition of structural components of cells, indicating an attenuated ability of *M. tuberculosis* to utilise energy sources and modulate immune responses in THP-1 macrophages in the absence of MTP (Figure 3.2). The separation seen between the WT and *mtp*-complement infection samples (Figure 3.1b) was not ideal and may

have resulted from the combined perturbations brought about by knocking out of the *mtp* gene and the complementation of the same gene using an episomal plasmid vector. The lack of total restoration of the *mtp*-complement to the WT phenotype, is supported by a previous study which showed a reduction in adhesion and invasion levels of the Δmtp to THP-1 macrophages relative to the WT [11]. The complemented strain demonstrated increased piliation, and subsequently increased adhesion, when compared to WT with similar invasion levels [11]. The current data thus suggests that the density of piliation influences the adherence to THP-1 macrophages, which in turn, most likely resulted in the altered metabolic response seen during infection, when compared to that of the WT strain. The *mtp*-complement infected macrophages illustrated greater consumption of metabolites involved amino acid metabolism and purine and pyrimidine metabolism, which feed into the TCA cycle (Figure 3.2), thus explaining the higher levels of citric acid and fumaric acid relative to the WT.

Approximately 4% of the total metabolites detected in both the Δmtp infected macrophages and *mtp*-complement infected macrophages differed statistically and practically from the WT infected macrophages. It is important to note a large portion of metabolic alterations are due to the host's response to infection, and this would be common amongst the different infection groups. Hence, only the metabolic perturbations attributed to the absence/presence/overrepresentation of the MTP adhesin were emphasised in this study, as comparisons were made between infection groups using MTP-proficient and MTP-deficient strains of *M. tuberculosis*. Moreover, the small percentage of metabolic alterations are accounted for by the fact that *M. tuberculosis* will compensate for the lack of the MTP adhesin by utilising one of its other adhesins to function in host attachment, invasion and immune activation. Nonetheless, in the absence of MTP, the perturbed metabolome indicated pathway alterations, which potentially have biological implications during the first four hours of infection.

3.5.1. The MTP deletion alters glycolysis, PPP, and gluconeogenesis during infection of THP-1 macrophages

Central carbon metabolism (CCM) is responsible for universal energy production in all living organisms [67]. Glycerate is derived from the oxidation of glycerol and is known to support maximal *in vitro* growth rates of *M. tuberculosis* [68-70]. Furthermore, it has been demonstrated that *M. tuberculosis* can use lactate, a carbon substrate that is plentiful at the site of infection, as a sole carbon source for growth, *in vitro* [41]. In *M. tuberculosis*, the conversion of lactate, is strictly dependent on one of two potential dehydrogenases encoded by *lldD1* (Rv0694) and *lldD2* (Rv1872c) [71]. A previous study demonstrated that the replication of *M. tuberculosis* (H37Rv) $\Delta lldD2$ mutant was impaired in human macrophages, thereby indicating the critical role of lactate oxidation and gluconeogenesis in the intracellular growth of the pathogen [71]. In the Δmtp infected macrophages of the current study, glycerate and lactic acid were not utilised at the site of infection, thereby impeding intracellular growth of *M. tuberculosis*. In support of this finding, a previous functional genomics study demonstrated that the MTP adhesin facilitated replication of *M. tuberculosis* (V9124) during infection of macrophages, since the bacillary load of the Δmtp was lower than that of the WT at all time intervals, including four hours post infection [72].

The ^{13}C -tracing of the bioenergetic metabolism of *M. tuberculosis* infected human macrophages, including human monocyte-derived macrophages (hMDMs) and THP-1 macrophages, demonstrated a reduction in enrichment of the glycolytic metabolites as well as reductions in the TCA metabolites, including; citrate (citric acid), α -ketoglutarate (α -KG), fumarate (fumaric acid) and malate [73]. This indicated a decelerated flux through glycolysis

and the TCA cycle in *M. tuberculosis* infected host cells, compared to those uninfected, or infected with an attenuated strain [73]. When comparing the *mtp*-complement infected macrophages to the WT infected macrophages in the current study, the metabolic alterations suggests that the TCA cycle may have not been affected by MTP itself (Figure 3.2), but rather by the over-piliation resulting from complementation with an episomal plasmid containing an over-expressing *mtp* gene. Taken together, these data suggest a role for MTP in regulating carbon metabolism via glycolysis, PPP and gluconeogenesis pathways in an infection model.

3.5.2. Fatty acid metabolism is perturbed in the absence of MTP in a THP-1 macrophage infection model

Mycobacterium tuberculosis has been reported to shift its dependency from glucose to lipids in infected macrophages [42; 73]. Interestingly, it was demonstrated that the fatty acid associated pathway, methylcitrate cycle, operates in reverse, to allow optimal metabolism of lactate and pyruvate in *M. tuberculosis* [74]. An intermediate in the ALA metabolism, 5-dodecenoic acid, forms important structural components of cell membranes, thereby affecting the activity of various membrane bound enzymes and modulating inflammation [38; 39; 75-77]. Decanoic acid (capric acid), under limited concentrations was previously found to permit growth rates in *M. tuberculosis* (H37Rv), comparable to that of glycerol as the primary carbon source [78]. In contrast to the current study, 5-dodecenoic acid and decanoic acid were found in lower levels of A549 epithelial infection models in the absence of MTP [62] thus demonstrating that cells respond differently to infection based on their function. Dodecanoic acid (lauric acid), was previously found to inhibit growth of *M. tuberculosis* H37Ra and reduce both transcriptional and translational expressions of these genes in THP-1 differentiated macrophages following 24 hour incubation [79; 80]. Palmitelaidic acid is the trans fatty acid isomer of palmitoleic acid. Trans fatty acids were recognised to alter plasma lipids and lipoprotein phenotypes; however, the mechanisms involved are unknown [38; 39]. The biomarker eicosapentaenoic, an omega-3 fatty acid, found in mycobacterial cultures has been associated with increased growth stimulation of *M. tuberculosis* [81; 82]. It was demonstrated that a high omega-3 diet might be detrimental to mycobacteriosis during macrophage infection, in contrast to the decreased killing of *M. tuberculosis* in mice [82]. Furthermore, 13-docosenoic acid had been identified in cultured mycobacteria as a biomarker in many studies [83-85]. This metabolite is associated with the unique cell wall structure of *Mycobacterium* species [81]. Additionally, increased levels of docosenoic acid is strongly correlated with inflammation and immune activation [86].

Collectively, the significantly elevated levels of the aforementioned fatty acid metabolism intermediates in the THP-1 macrophages infected with the Δmtp strain indicate the importance of MTP in modulating fatty acid metabolism, and subsequently the mycolic acid constituents of the cell wall. This in turn may affect *M. tuberculosis* pathogenicity, as well as render it susceptible to anti-TB agents, suggesting a potential for curlicide/pilicide adjunctive therapy with existing anti-TB therapeutics.

3.5.3. Nicotinate and nicotinamide metabolism involved in amino acid metabolism are altered in the absence of MTP in a THP-1 macrophage infection model

Mycobacterium tuberculosis relies on amino acid uptake and degradation, in order to thrive as an intracellular pathogen [87]. Nicotinamide or niacinamide, is the amide version of vitamin B3 (niacin), possesses antioxidant, reprogramming and anti-inflammatory properties in human primary monocytes and monocyte-derived macrophages [88]. Additionally, the differential activity of nicotinamide on infected macrophages, adequately restricted attenuated strains, such as *Mycobacterium bovis* Bacille Calmette-Guérin (BCG), but not virulent strains such as *M. tuberculosis* [89]. Given that niacinamide was detected in the Δmtp infection model in elevated levels, when compared to WT infected host (Table 3.1), amino acid metabolism is only slightly migrated in the absence of MTP during infection.

A limitation in this study is that the *in vitro* conditions and findings may not accurately represent the natural environment and pathway alterations that occur during pulmonary infection. They do, however, provide insight into the infection process. Future research could include replication studies coupled with reverse transcription quantitative polymerase chain reaction (RT-qPCR) on *lldD1*(Rv0694), *lldD2* (Rv1872c) and PEPCCK, in order to determine if *mtp* regulates these genes, and if there is any correlation with the altered lactic acid levels detected. The importance of MTP in modulating fatty acid metabolism, and subsequently, cell wall constituents, motivates the exploration of *in vitro* antibiotic killing curves using isoniazid and rifampicin plus curlicide/pilicide treatment of Δmtp and WT strains. Furthermore, targeted ¹³C-labelling of *M. tuberculosis* can be used in conjunction with MS to trace metabolic interactions of pathogen and host, thereby overcoming the challenge of differentiating between host-derived and pathogen-derived metabolites [90].

3.6. Conclusion

Significant metabolic changes associated with an altered central carbon, fatty acid and amino acid metabolism, were induced by the absence of the MTP adhesin in a THP-1 macrophage infection model, as observed by an overall significantly higher level of the associated metabolites detected in the Δmtp infected macrophages relative to the WT infected macrophages. The perturbations resulting from the lack of the MTP adhesin in the Δmtp infection model indicate an altered host metabolic response to infection. Hence, the data support previous studies that promote the MTP adhesin is a virulence factor of note and a promising marker for therapeutic intervention. Anti-MTP monoclonal antibodies or a curlicide specifically developed to target MTP could be used as adjunctive therapy to enhance current TB treatment regimens thereby potentially reducing duration of treatment. In addition, MTP and its associated metabolites may be suitable for the development of non-invasive diagnostic targets.

References:

1. World Health Organization (2019). Global Tuberculosis Report 2019. *World Health Organisation* [Online]. Available: https://www.who.int/tb/publications/global_report/en/ [Accessed 2020].

2. Smith, I. (2003). *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clinical Microbiology Reviews*, 16(3), 463-496. <https://doi.org/10.1128/cmr.16.3.463-496.2003>.
3. Russell, D.G. (2001). *Mycobacterium tuberculosis*: Here today, and here tomorrow. *Nature Reviews. Molecular Cell Biology*, 2(8), 569-577. <http://dx.doi.org/10.1038/35085034>.
4. Pethe, K., Alonso, S., Biet, F., Delogu, G., Brennan, M.J., Loch, C., and Menozzi, F.D. (2001). The heparin-binding haemagglutinin of *M. tuberculosis* is required for extrapulmonary dissemination. *Nature*, 412(6843), 190-194. <https://doi.org/10.1038/35084083>.
5. Sia, J.K., Georgieva, M., and Rengarajan, J. (2015). Innate immune defenses in human tuberculosis: An overview of the interactions between *Mycobacterium tuberculosis* and innate immune cells. *Journal of Immunology Research*, 2015, 747543. <https://doi.org/10.1155/2015/747543>.
6. Pieters, J. (2008). *Mycobacterium tuberculosis* and the macrophage: Maintaining a balance. *Cell Host & Microbe*, 3(6), 399-407. <http://dx.doi.org/10.1016/j.chom.2008.05.006>.
7. Niemann, H.H., Schubert, W.D., and Heinz, D.W. (2004). Adhesins and invasins of pathogenic bacteria: A structural view. *Microbes and Infection*, 6(1), 101-112. <https://doi.org/10.1016/j.micinf.2003.11.001>.
8. Alberts, B., Johnson, A., Lewis, J., Walter, P., Raff, M., and Roberts, K. (2002). *Molecular Biology of the Cell 4th Edition: International Student Edition*. Routledge, Garland Science. New York.
9. Alteri, C.J., Xicohtencatl-Cortes, J., Hess, S., Caballero-Olín, G., Girón, J.A., and Friedman, R.L. (2007). *Mycobacterium tuberculosis* produces pili during human infection. *Proceedings of the National Academy of Sciences of the United States of America*, 104(12), 5145-5150. <https://doi.org/10.1073/pnas.0602304104>.
10. Naidoo, N., Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* pili (MTP), a putative biomarker for a tuberculosis diagnostic test. *Tuberculosis*, 94(3), 338-345. <http://dx.doi.org/10.1016/j.tube.2014.03.004>.
11. Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* pili promote adhesion to and invasion of THP-1 macrophages. *Japanese Journal of Infectious Diseases*, 67(6), 476-478. <https://doi.org/10.7883/yoken.67.476>.
12. Ramsugit, S., Pillay, B., and Pillay, M. (2016). Evaluation of the role of *Mycobacterium tuberculosis* pili (MTP) as an adhesin, invasin, and cytokine inducer of epithelial cells. *The Brazilian Journal of Infectious Diseases*, 20(2), 160-165. <http://dx.doi.org/10.1016/j.bjid.2015.11.002>.
13. Dlamini, M.T. (2016). *Whole transcriptome analysis to elucidate the role of mtp in gene regulation of pulmonary epithelial cells infected with Mycobacterium tuberculosis*. Masters dissertation University of KwaZulu-Natal, Durban, South Africa. <https://researchspace.ukzn.ac.za/items/71f0ed4e-aa63-4cca-8bf6-4d771326e616>.
14. Nyawo, G. (2016). *The role of Mycobacterium tuberculosis pili in pathogenesis: Growth and survival kinetics, gene regulation and host immune response, and in vitro growth kinetics*. Masters dissertation University of KwaZulu-Natal, Durban, South Africa. <https://researchspace.ukzn.ac.za/items/3628eab3-3e5f-47a6-9eb3-07c3f3c52ad7>.
15. Naidoo, N., Pillay, B., Bubb, M., Pym, A., Chiliza, T., Naidoo, K., Ndungu, T., Kasprovicz, V.O., and Pillay, M. (2018). Evaluation of a synthetic peptide for the detection of anti-*Mycobacterium tuberculosis* curli pili IgG antibodies in patients with pulmonary tuberculosis. *Tuberculosis* 109, 80-84. [10.1016/j.tube.2018.01.007](https://doi.org/10.1016/j.tube.2018.01.007).

16. Nalbantoglu, S. (2019). Metabolomics: Basic Principles and Strategies, in *Molecular Medicine*. IntechOpen: <https://www.intechopen.com/chapters/68486>.
17. Dunn, W.B. , and Ellis, D.I. (2005). Metabolomics: Current analytical platforms and methodologies. *TrAC Trends in Analytical Chemistry*, 24(4), 285-294. <http://dx.doi.org/10.1016/j.trac.2004.11.021>.
18. Patel, K.N., Patel, J.K., Patel, M.P., Rajput, G.C., and Patel, H.A. (2010). Introduction to hyphenated techniques and their applications in pharmacy. *Pharmaceutical Methods*, 1(1), 2-13. <https://doi.org/10.4103/2229-4708.72222>.
19. Cha, D., Cheng, D., Liu, M., Zeng, Z., Hu, X., and Guan, W. (2009). Analysis of fatty acids in sputum from patients with pulmonary tuberculosis using gas chromatography-mass spectrometry preceded by solid-phase microextraction and post-derivatization on the fiber. *Journal of Chromatography A*, 1216(9), 1450-1457. <https://doi.org/10.1016/j.chroma.2008.12.039>.
20. Meissner-Roloff, R.J., Koekemoer, G., Warren, R.M., and Loots, D.T. (2012). A metabolomics investigation of a hyper- and hypo-virulent phenotype of Beijing lineage *M. tuberculosis*. *Metabolomics*, 8, 1194-1203. <https://doi.org/10.1007/s11306-012-0424-6>.
21. Swanepoel, C.C. , and Loots, D.T. (2014). The use of functional genomics in conjunction with metabolomics for *Mycobacterium tuberculosis* research. *Disease Markers*, 2014, 124218. <https://doi.org/10.1155/2014/124218>.
22. Weiner, J., Maertzdorf, J., Sutherland, J.S., Duffy, F.J., Thompson, E., Suliman, S., McEwen, G., Thiel, B., Parida, S.K., Zyla, J., Hanekom, W.A., Mohny, R.P., Boom, W.H., Mayanja-Kizza, H., Howe, R., Dockrell, H.M., Ottenhoff, T.H.M., Scriba, T.J., Zak, D.E., Walzl, G., Kaufmann, S.H.E., and consortium, G.C. (2018). Metabolite changes in blood predict the onset of tuberculosis. *Nature Communications*, 9(1), 5208. <https://doi.org/10.1038/s41467-018-07635-7>.
23. Ashokcoomar, S., Reedoy, K.S., Senzani, S., Loots, D.T., Beukes Maasdorp, D., Van Reenen, M., Pillay, B., and Pillay, M. (2020). *Mycobacterium tuberculosis* curli pili (MTP) deficiency is associated with alterations in cell wall biogenesis, fatty acid metabolism and amino acid synthesis. *Metabolomics*, 16(9), 97. <https://doi.org/10.1007/s11306-020-01720-z>.
24. Gandhi, N.R., Moll, A., Sturm, A.W., Pawinski, R., Govender, T., Lalloo, U., Zeller, K., Andrews, J., and Friedland, G. (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet*, 368(9547), 1575-1580. 10.1016/s0140-6736(06)69573-1.
25. Bardarov, S., Bardarov, S., Pavelka, M.S., Sambandamurthy, V., Larsen, M., Tufariello, J., Chan, J., Hatfull, G., and Jacobs, W.R. (2002). Specialized transduction: An efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. *Microbiology (Reading)*, 148(Pt 10), 3007-3017. <https://doi.org/10.1099/00221287-148-10-3007>.
26. Ramsugit, S., Guma, S., Pillay, B., Jain, P., Larsen, M.H., Danaviah, S., and Pillay, M. (2013). Pili contribute to biofilm formation *in vitro* in *Mycobacterium tuberculosis*. *Antonie Van Leeuwenhoek*, 104(5), 725-735. <https://doi.org/10.1007/s10482-013-9981-6>.
27. Larsen, M.H., Biermann, K., Tandberg, S., Hsu, T., and Jacobs, W.R., Jr. (2007). Genetic manipulation of *Mycobacterium tuberculosis*. *Current Protocols in Microbiology*, Chapter 10, Unit 10A.12. <https://doi.org/10.1002/9780471729259.mc10a02s6>.

28. Beukes, D., Du Preez, I., and Loots, D.T. (2019). Total metabolome extraction from mycobacterial cells for GC-MS metabolomics analysis. *Methods in Molecular Biology*, 1859, 121-131. https://doi.org/10.1007/978-1-4939-8757-3_6.
29. Van Den Berg, R.A., Hoefsloot, H.C.J., Westerhuis, J.A., Smilde, A.K., and Van Der Werf, M.J. (2006). Centering, scaling, and transformations: Improving the biological information content of metabolomics data. *BMC Genomics*, 7, 142. <https://doi.org/10.1186/1471-2164-7-142>.
30. Benjamini, Y. , and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, 57(1), 289-300. <https://doi.org/10.1111/j.2517-6161.1995.tb02031.x>.
31. Fisher, R.A. (1992). The arrangement of field experiments. *Breakthroughs in Statistics*, 82-91. https://doi.org/10.1007/978-1-4612-4380-9_8.
32. Sullivan, G.M. , and Feinn, R. (2012). Using effect size-or why the *p* value is not enough. *Journal of Graduate Medical Education*, 4(3), 279-282. <https://doi.org/10.4300/jgme-d-12-00156.1>.
33. Clum, A., Huntemann, M., Bushnell, B., Foster, B., Foster, B., Roux, S., Hajek Patrick, P., Varghese, N., Mukherjee, S., Reddy, T.B.K., Daum, C., Yoshinaga, Y., O'Malley, R., Seshadri, R., Kyrpides Nikos, C., Elie-Fadrosh Emiley, A., Chen, I.M.A., Copeland, A., and Ivanova Natalia, N. (2021). DOE JGI Metagenome Workflow. *mSystems*, 6(3), 10.1128/msystems.00804-00820. 10.1128/msystems.00804-20.
34. Huntemann, M., Ivanova, N.N., Mavromatis, K., Tripp, H.J., Paez-Espino, D., Tennessen, K., Palaniappan, K., Szeto, E., Pillay, M., Chen, I.M.A., Pati, A., Nielsen, T., Markowitz, V.M., and Kyrpides, N.C. (2016). The standard operating procedure of the DOE-JGI Metagenome Annotation Pipeline (MAP v.4). *Standards in Genomic Sciences*, 11(1), 17. <https://doi.org/10.1186/s40793-016-0138-x>.
35. Akarachantachote, N., Chadcham, S., and Saithanu, K. (2014). Cut-off threshold of variable importance in projection for variable selection. *International Journal of Pure and Applied Mathematics*, 94(3), 307-322. <http://dx.doi.org/10.12732/ijpam.v94i3.2>.
36. Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., Li, Q., Shoemaker, B.A., Thiessen, P.A., Yu, B., Zaslavsky, L., Zhang, J., and Bolton, E.E. (2019). PubChem 2019 update: Improved access to chemical data. *Nucleic Acids Research*, 47(D1), D1102-D1109. <https://doi.org/10.1093/nar/gky1033>.
37. Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., and Morishima, K. (2017). KEGG: New perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Research*, 45(D1), D353-D361. <https://doi.org/10.1093/nar/gkw1092>.
38. Kanehisa, M. (2019). Toward understanding the origin and evolution of cellular organisms. *Protein Science*, 28(11), 1947-1951. <https://doi.org/10.1002/pro.3715>.
39. Kanehisa, M. , and Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*, 1, 27-30. <https://doi.org/10.1093/nar/28.1.27>.
40. Marrero, J., Rhee, K.Y., Schnappinger, D., Pethe, K., and Ehrt, S. (2010). Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for *Mycobacterium tuberculosis* to establish and maintain infection. *Proceedings of the National Academy of Sciences of the United States of America*, 107(21), 9819-9824. <https://doi.org/10.1073/pnas.1000715107>.
41. Billig, S., Schneefeld, M., Huber, C., Grassl, G.A., Eisenreich, W., and Bange, F.-C. (2017). Lactate oxidation facilitates growth of *Mycobacterium tuberculosis* in human macrophages. *Scientific Reports*, 7(1), 6484. <https://doi.org/10.1038/s41598-017-05916-7>.

42. Bloch, H. , and Segal, W. (1956). Biochemical differentiation of *Mycobacterium tuberculosis* grown *in vivo* and *in vitro*. *Journal of Bacteriology*, 72(2), 132-141. <https://doi.org/10.1128/jb.72.2.132-141.1956>.
43. McKinney, J.D., zu Bentrup, K.H., Muñoz-Elías, E.J., Miczak, A., Chen, B., Chan, W.-T., Swenson, D., Sacchettini, J.C., Jacobs, W.R., and Russell, D.G. (2000). Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature*, 406(6797), 735-738. <https://doi.org/10.1038/35021074>.
44. Schnappinger, D., Ehrt, S., Voskuil, M.I., Liu, Y., Mangan, J.A., Monahan, I.M., Dolganov, G., Efron, B., Butcher, P.D., Nathan, C., and Schoolnik, G.K. (2003). Transcriptional Adaptation of *Mycobacterium tuberculosis* within Macrophages: Insights into the Phagosomal Environment. *Journal of Experimental Medicine*, 198(5), 693-704. 10.1084/jem.20030846.
45. De Carvalho, L.P.S., Fischer, S.M., Marrero, J., Nathan, C., Ehrt, S., and Rhee, K.Y. (2010). Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalized co-catabolism of carbon substrates. *Chemistry & Biology*, 17(10), 1122-1131. <https://doi.org/10.1016/j.chembiol.2010.08.009>.
46. Kinsella, R.J., Fitzpatrick, D.A., Creevey, C.J., and McInerney, J.O. (2003). Fatty acid biosynthesis in *Mycobacterium tuberculosis*: Lateral gene transfer, adaptive evolution, and gene duplication. *Proceedings of the National Academy of Sciences of the United States of America*, 100(18), 10320-10325. <https://doi.org/10.1073/pnas.1737230100>.
47. Rhee, K.Y., De Carvalho, L.P., Bryk, R., Ehrt, S., Marrero, J., Park, S.W., Schnappinger, D., Venugopal, A., and Nathan, C. (2011). Central carbon metabolism in *Mycobacterium tuberculosis*: An unexpected frontier. *Trends Microbiology*, 19(7), 307-314. <http://dx.doi.org/10.1016/j.tim.2011.03.008>.
48. Dhakal, R., Bajpai, V.K., and Baek, K.H. (2012). Production of gaba (γ - aminobutyric acid) by microorganisms: A review. *Brazilian Journal of Microbiology*, 43(4), 1230-1241. <https://doi.org/10.1590/s1517-83822012000400001>.
49. Kim, J.K., Kim, Y.S., Lee, H.-M., Jin, H.S., Neupane, C., Kim, S., Lee, S.-H., Min, J.-J., Sasai, M., Jeong, J.-H., Choe, S.-K., Kim, J.-M., Yamamoto, M., Choy, H.E., Park, J.B., and Jo, E.-K. GABAergic signaling linked to autophagy enhances host protection against intracellular bacterial infections. *Nature Communications*, 2018. 9, 4184 DOI: <https://doi.org/10.1038/s41467-018-06487-5>.
50. Zhang, M., Wang, H., and Tracey, K.J. (2000). Regulation of macrophage activation and inflammation by spermine: A new chapter in an old story. *Critical Care Medicine*, 28(4 Suppl), N60-66. <https://doi.org/10.1097/00003246-200004001-00007>.
51. Hasenoehrl, E.J., Rae Sajorda, D., Berney-Meyer, L., Johnson, S., Tufariello, J.M., Fuhrer, T., Cook, G.M., Jacobs, W.R., and Berney, M. (2019). Derailing the aspartate pathway of *Mycobacterium tuberculosis* to eradicate persistent infection. *Nature Communications*, 10(1), 4215. 10.1038/s41467-019-12224-3.
52. Betts, J.C., Lukey, P.T., Robb, L.C., McAdam, R.A., and Duncan, K. (2002). Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Molecular Microbiology*, 43(3), 717-731. <https://doi.org/10.1046/j.1365-2958.2002.02779.x>.
53. Duan, X., Li, Y., Du, Q., Huang, Q., Guo, S., Xu, M., Lin, Y., Liu, Z., and Xie, J. (2016). *Mycobacterium* Lysine ϵ -aminotransferase is a novel alarmone metabolism related persister gene via dysregulating the intracellular amino acid level. *Scientific Reports*, 6, 19695. <https://doi.org/10.1038/srep19695>.

54. Keren, I., Minami, S., Rubin, E., and Lewis, K. (2011). Characterization and transcriptome analysis of *Mycobacterium tuberculosis* persisters. *mBio*, 2(3), e00100-00111. <https://doi.org/10.1128/mbio.00100-11>.
55. Kamio, Y., Itoh, Y., and Terawaki, Y. (1981). Chemical structure of peptidoglycan in *Selenomonas ruminantium*: Cadaverine links covalently to the D-glutamic acid residue of peptidoglycan. *Journal of Bacteriology*, 146(1), 49-53. <https://doi.org/10.1128/jb.146.1.49-53.1981>.
56. Neely, M.N. , and Olson, E.R. (1996). Kinetics of expression of the *Escherichia coli* cad operon as a function of pH and lysine. *Journal of Bacteriology*, 178(18), 5522-5528. <https://doi.org/10.1128/iai.00893-09>.
57. Samartzidou, H. , and Delcour, A.H. (1998). *E.coli* PhoE porin has an opposite voltage-dependence to the homologous *OmpF*. *The EMBO Journal*, 17(1), 93-100. <https://doi.org/10.1093/emboj/17.1.93>.
58. Dichtl, S., Haschka, D., Nairz, M., Seifert, M., Volani, C., Lutz, O., and Weiss, G. (2018). Dopamine promotes cellular iron accumulation and oxidative stress responses in macrophages. *Biochemical Pharmacology*, 148, 193-201. <https://doi.org/10.1016/j.bcp.2017.12.001>.
59. Angstadt, C.N. (1997). Purines and Pyrimidine Metabolism. *The University of Utah. NetBiochem* [Online]. Available: <https://library.med.utah.edu/NetBiochem/pupyr/pp.htm> [Accessed January 2024].
60. Chao, M.-R., Wang, C.-J., Yang, H.-H., Chang, L.W., and Hu, C.-W. (2005). Rapid and sensitive quantification of urinary N7-methylguanine by isotope-dilution liquid chromatography/electrospray ionization tandem mass spectrometry with on-line solid-phase extraction. *Rapid Communications in Mass Spectrometry*, 19(17), 2427-2432. <https://doi.org/10.1002/rcm.2082>.
61. Oh, S., Park, Y., Engelhart, C.A., Wallach, J.B., Schnappinger, D., Arora, K., Manikkam, M., Gac, B., Wang, H., Murgolo, N., Olsen, D.B., Goodwin, M., Sutphin, M., Weiner, D.M., Via, L.E., Boshoff, H.I.M., and Barry, C.E. (2018). Discovery and structure-activity-relationship study of N-alkyl-5-hydroxypyrimidinone carboxamides as novel antitubercular agents targeting decaprenylphosphoryl- β -d-ribose 2'-oxidase. *Journal of Medicinal Chemistry*, 61(22), 9952-9965. <https://doi.org/10.1021/acs.jmedchem.8b00883>.
62. Reedoy, K.S., Loots, D.T., Beukes, D., Reenen, M.V., Pillay, B., and Pillay, M. (2020). *Mycobacterium tuberculosis* curli pili (MTP) is associated with significant host metabolic pathways in an A549 epithelial cell infection model and contributes to the pathogenicity of *Mycobacterium tuberculosis*. *Metabolomics*, 16(11), 116. <http://dx.doi.org/10.1007/s11306-020-01736-5>.
63. Ryndak, M.B. , and Laal, S. (2019). *Mycobacterium tuberculosis* primary infection and dissemination: A critical role for alveolar epithelial cells. *Frontiers in Cellular and Infection Microbiology*, 9, 299-299. <http://dx.doi.org/10.3389/fcimb.2019.00299>.
64. McClean, C.M. , and Tobin, D.M. (2016). Macrophage form, function, and phenotype in mycobacterial infection: Lessons from tuberculosis and other diseases. *Pathogens and Disease*, 74(7), ftw068. <https://doi.org/10.1093/femspd/ftw068>.
65. Zimmermann, M., Kogadeeva, M., Gengenbacher, M., McEwen, G., Mollenkopf, H.J., Zamboni, N., Kaufmann, S.H.E., and Sauer, U. (2017). Integration of metabolomics and transcriptomics reveals a complex diet of *Mycobacterium tuberculosis* during early macrophage infection. *mSystems*, 2(4), e00057-00017. <https://doi.org/10.1128/mSystems.00057-17>.

66. Büscher, J.M., Czernik, D., Ewald, J.C., Sauer, U., and Zamboni, N. (2009). Cross-platform comparison of methods for quantitative metabolomics of primary metabolism. *Analytical Chemistry*, 81(6), 2135-2143. <https://doi.org/10.1021/ac8022857>.
67. Baughn, A.D. , and Rhee, K.Y. (2014). Metabolomics of central carbon metabolism in *Mycobacterium tuberculosis*. *Microbiology Spectrum*, 2(3). <https://doi.org/10.1128/microbiolspec.mgm2-0026-2013>.
68. Edson, N.L. (1951). The intermediary metabolism of the mycobacteria. *Bacteriology Reviews*, 15(3), 147-182. <https://doi.org/10.1128/br.15.3.147-182.1951>.
69. Long, E.R. (1922). The nutrition of acid-fast bacteria. *American Review of Tuberculosis*, 5(11), 857-8690096-0381.
70. Muñoz-Elías, E.J. , and McKinney, J.D. (2006). Carbon metabolism of intracellular bacteria. *Cellular Microbiology*, 8(1), 10-22. <https://doi.org/10.1111/j.1462-5822.2005.00648.x>.
71. Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E., Tekalia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., and Osborne, J. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, 393(6685), 537-544. <https://doi.org/10.1038/31159>.
72. Moodley, S. (2018). *The role of heparin binding haemagglutinin adhesin and curli pili on the pathogenicity of Mycobacterium tuberculosis*. Doctoral thesis, <https://researchspace.ukzn.ac.za/items/22f2cedb-2826-46ad-8a62-237a41b62d17>.
73. Cumming, B.M., Addicott, K.W., Adamson, J.H., and Steyn, A.J. (2018). *Mycobacterium tuberculosis* induces decelerated bioenergetic metabolism in human macrophages. *eLife*, 7, e39169. 10.7554/eLife.39169.
74. Serafini, A., Tan, L., Horswell, S., Howell, S., Greenwood, D.J., Hunt, D.M., Phan, M.-D., Schembri, M., Monteleone, M., Montague, C.R., Britton, W., Garza-Garcia, A., Snijders, A.P., VanderVen, B., Gutierrez, M.G., West, N.P., and De Carvalho, L.P.S. (2019). *Mycobacterium tuberculosis* requires glyoxylate shunt and reverse methylcitrate cycle for lactate and pyruvate metabolism. *Molecular Microbiology*, 112(4), 1284-1307. <https://doi.org/10.1111/mmi.14362>.
75. Wishart, D.S., Tzur, D., Knox, C., Eisner, R., Guo, A.C., Young, N., Cheng, D., Jewell, K., Arndt, D., Sawhney, S., Fung, C., Nikolai, L., Lewis, M., Coutouly, M.A., Forsythe, I., Tang, P., Shrivastava, S., Jeroncic, K., Stothard, P., Amegbey, G., Block, D., Hau, D.D., Wagner, J., Miniaci, J., Clements, M., Gebremedhin, M., Guo, N., Zhang, Y., Duggan, G.E., Macinnis, G.D., Weljie, A.M., Dowlatabadi, R., Bamforth, F., Clive, D., Greiner, R., Li, L., Marrie, T., Sykes, B.D., Vogel, H.J., and Querengesser, L. (2007). HMDB: The Human Metabolome Database. *Nucleic Acids Research*, 35(Issue suppl_1), D521-526. <https://doi.org/10.1093/nar/gkl923>.
76. Wishart, D.S., Knox, C., Guo, A.C., Eisner, R., Young, N., Gautam, B., Hau, D.D., Psychogios, N., Dong, E., Bouatra, S., Mandal, R., Sinelnikov, I., Xia, J., Jia, L., Cruz, J.A., Lim, E., Sobsey, C.A., Shrivastava, S., Huang, P., Liu, P., Fang, L., Peng, J., Fradette, R., Cheng, D., Tzur, D., Clements, M., Lewis, A., De Souza, A., Zuniga, A., Dawe, M., Xiong, Y., Clive, D., Greiner, R., Nazyrova, A., Shaykhutdinov, R., Li, L., Vogel, H.J., and Forsythe, I. (2009). HMDB: A knowledgebase for the human metabolome. *Nucleic Acids Research*, 37(Database issue), D603-610. <https://doi.org/10.1093/nar/gkn810>.
77. Wishart, D.S., Jewison, T., Guo, A.C., Wilson, M., Knox, C., Liu, Y., Djoumbou, Y., Mandal, R., Aziat, F., Dong, E., Bouatra, S., Sinelnikov, I., Arndt, D., Xia, J., Liu, P.,

- Yallou, F., Bjorndahl, T., Perez-Pineiro, R., Eisner, R., Allen, F., Neveu, V., Greiner, R., and Scalbert, A. (2013). HMDB 3.0-The Human Metabolome Database in 2013. *Nucleic Acids Research*, 41(Database issue), D801-807. <https://doi.org/10.1093/nar/gks1065>.
78. Youmans, A.S. , and Youmans, G.P. (1954). Studies on the metabolism of *Mycobacterium tuberculosis*. IV. The effect of fatty acids on the growth of *M. tuberculosis* var. *hominis*. *Journal of Bacteriology*, 67(6), 731-733. <https://doi.org/10.1128/jb.67.6.731-733.1954>.
 79. Muniyan, R. , and Gurunathan, J. (2016). Lauric acid and myristic acid from *Allium sativum* inhibit the growth of *Mycobacterium tuberculosis* H37Ra: *In silico* analysis reveals possible binding to protein kinase B. *Pharmaceutical Biology*, 54(12), 2814-2821. <https://doi.org/10.1080/13880209.2016.1184691>.
 80. Ong, M.H., Wong, H.K., Tengku-Muhammad, T.S., Choo, Q.C., and Chew, C.H. (2019). Pro-atherogenic proteoglycanase ADAMTS-1 is down-regulated by lauric acid through PI3K and JNK signaling pathways in THP-1 derived macrophages. *Current Molecular Biology Reports*, 46(3), 2631-2641. <https://doi.org/10.1007/s11033-019-04661-6>.
 81. Du Preez, I., Luies, L., and Loots, D.T. (2017). Metabolomics biomarkers for tuberculosis diagnostics: Current status and future objectives. *Biomarkers in Medicine*, 11, 179-194. <https://doi.org/10.2217/bmm-2016-0287>.
 82. Jordao, L., Lengeling, A., Bordat, Y., Boudou, F., Gicquel, B., Neyrolles, O., Becker, P.D., Guzman, C.A., Griffiths, G., and Anes, E. (2008). Effects of omega-3 and-6 fatty acids on *Mycobacterium tuberculosis* in macrophages and in mice. *Microbes and Infection*, 10(12-13), 1379-1386. <https://doi.org/10.1016/j.micinf.2008.08.004>.
 83. Schoeman, J.C., du Preez, I., and Loots du, T. (2012). A comparison of four sputum pre-extraction preparation methods for identifying and characterising *Mycobacterium tuberculosis* using GCxGC-TOFMS metabolomics. *Journal of Microbiological Methods*, 91(2), 301-311. <https://doi.org/10.1016/j.mimet.2012.09.002>.
 84. Olivier, I. , and Loots, D.T. (2012). A comparison of two extraction methods for differentiating and characterising various *Mycobacterium species* and *Pseudomonas aeruginosa* using GC-MS metabolomics. *African Journal of Microbiology Research*, 6, 3159-3172.
 85. Olivier, I. , and Loots, D.T. (2012). A metabolomics approach to characterise and identify various *Mycobacterium species*. *Journal of Microbiological Methods*, 88(3), 419-426. <https://doi.org/10.1016/j.mimet.2012.01.012>.
 86. Alqarni, A.M., Dissanayake, T., Nelson, D.J., Parkinson, J.A., Dufton, M.J., Ferro, V.A., and Watson, D.G. (2019). Metabolomic profiling of the immune stimulatory effect of eicosenoids on PMA-differentiated THP-1 cells. *Vaccines*, 7(4), 142. <https://doi.org/10.3390/vaccines7040142>.
 87. Gouzy, A., Poquet, Y., and Neyrolles, O. (2014). Amino acid capture and utilization within the *Mycobacterium tuberculosis* phagosome. *Future Microbiology*, 9(5), 631-637. <https://doi.org/10.2217/fmb.14.28>.
 88. Montserrat-de la Paz, S., Naranjo, M.C., Lopez, S., Abia, R., Muriana, F.J.G., and Bermudez, B. (2017). Niacin and its metabolites as master regulators of macrophage activation. *The Journal of Nutritional Biochemistry*, 39, 40-47. <https://doi.org/10.1016/j.jnutbio.2016.09.008>.
 89. Simmons, J.D., Peterson, G.J., Campo, M., Lohmiller, J., Skerrett, S.J., Tunaru, S., Offermanns, S., Sherman, D.R., and Hawn, T.R. (2019). Nicotinamide Limits Replication of *Mycobacterium tuberculosis* and Bacille Calmette-Guérin Within Macrophages. *The Journal of Infectious Diseases*. 10.1093/infdis/jiz541.

90. Beste, D.J., Noh, K., Niedenfuhr, S., Mendum, T.A., Hawkins, N.D., Ward, J.L., Beale, M.H., Wiechert, W., and McFadden, J. (2013). ¹³C-flux spectral analysis of host-pathogen metabolism reveals a mixed diet for intracellular *Mycobacterium tuberculosis*. *Chemistry and Biology*, 20(8), 1012-1021. <https://doi.org/10.1016/j.chembiol.2013.06.012>.

Chapter 3 revealed that *Mycobacterium tuberculosis* curli pili (MTP) influences the intertwined *M. tuberculosis* and THP-1 macrophage metabolomic profile during initial stages of infection. Metabolic intermediates involved in carbon, fatty acid and amino acid metabolism were differentially detected in MTP-proficient and MTP-deficient THP-1 macrophage infection models.

A comparison of the metabolite profiles of *M. tuberculosis* infected A549 epithelial cells and THP-1 macrophages is presented in Chapter 4 to determine the severity of impact that MTP has host cells. This chapter has been published as a journal article in *Comparative Immunology, Microbiology and Infectious Diseases*, (Elsevier) 2022 (DOI: <https://doi.org/10.1016/j.cimid.2022.101907>).

CHAPTER 4: *Mycobacterium tuberculosis* curli pili (MTP) facilitates a reduction of microbicidal activity of infected THP-1 macrophages during early stages of infection

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Ethics approval: The study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BE254/17 and BE255/17) attached in Appendix A.

Declaration of interest: The authors declare that they have no conflict of interest.

Authors contribution statement: Prof M. Pillay conceptualised and funded the study. Prof M. Pillay, Prof B. Pillay and Prof Loots designed the study. Ms Ashokcoomar conducted experiments, analysed and interpreted the data, and drafted the manuscript. Ms Reedoy conducted experiments. Ms Beukes processed the samples and performed GC×GC-TOFMS. Ms van Reenen conducted statistical bioinformatic analyses. All authors contributed to and approved the manuscript.

4.1. Abstract

Introduction: *Mycobacterium tuberculosis* (*M. tuberculosis*) curli pili (MTP) is a surface-located adhesin, which is involved in the initial point-of-contact between the pathogen and the host. Host-pathogen interaction is essential for establishing infection. *Mycobacterium tuberculosis* has the ability to infect various host lung cell types, which includes both the epithelial cells and macrophages; subsequent differences in their cellular function will be evident in their metabolic profiles.

Objective: This study aimed to compare the metabolic responses of these cell types to *M. tuberculosis* infection, with and without the presence of the MTP, in efforts to better elucidate the role of this adhesin in modulating metabolic pathways during infection. This may further contribute to the development of improved diagnostic and therapeutic interventions, much needed at present in order to improve control of the global tuberculosis (TB) epidemic.

Methods: This study used a two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-TOFMS) metabolomic approach to compare the metabolite profiles of A549 epithelial cells to that of THP-1 macrophages, infected with *M. tuberculosis*, in the presence and absence of MTP. Significant metabolites were identified using various univariate and multivariate statistical analysis.

Results: Totals of 44, 40, 50 and 34 metabolites were differentially detected when comparing the (a) uninfected A549 epithelial cells to uninfected THP-1 macrophages, (b) wildtype (WT) infected A549 epithelial cells to WT infected THP-1 macrophages, (c) Δmtp infected A549 epithelial cells to Δmtp infected THP-1 macrophages (d) *mtp*-complement infected A549 epithelial cells to *mtp*-complement infected THP-1 macrophages, respectively. These included metabolites that were involved in amino acid metabolism, fatty acid metabolism, general central carbon metabolism, and nucleic acid metabolism. In the absence of the *M. tuberculosis* MTP adhesin, the THP-1 macrophages predominantly displayed higher concentrations of amino acids and their metabolic intermediates, than the A549 epithelial cells. The deletion of MTP from *M. tuberculosis* in the host infection models potentially elicited a pro-inflammatory phenotype, particularly in the macrophage model.

Conclusion: In the presence of MTP, the metabolite profile changes indicate potential regulation of host defence mechanisms, accompanied by a reduction in microbicidal abilities of host cells. Hence MTP can be considered a virulence factor of *M. tuberculosis*. Therefore, blocking MTP interaction with the host may facilitate a faster pathogen clearance during the initial stages of infection, and potentially enhance current therapeutic interventions.

4.2. Introduction

Mycobacterium tuberculosis (*M. tuberculosis*), the causative agent of tuberculosis (TB), remains the second highest cause of illness and death by a single infectious agent after coronavirus disease of 2019 (COVID-19) [1]. The coronavirus pandemic has caused disruptions to TB health care, with a 21% reduction of TB treatment from 2019 to 2020, which presumably caused an additional half a million TB deaths [1]. In 2020, an estimated 9.9 million cases and 1.3 million deaths among human immunodeficiency virus (HIV)-negative people, were recorded globally [1]. Moreover, the burden of antimicrobial resistance remains, with proportions of 3-4% of new cases, and 18-21% of previously treated TB patients diagnosed with multidrug-resistant TB or rifampicin resistant TB (MDR/RR-TB) [1]. These concerns motivate the drive towards improved TB prevention, diagnostics and treatment regimens of late.

The surface location of adhesins promotes their use for developing effective diagnostic tests and more effective anti-TB agents, which could potentially disrupt the initial host-pathogen interaction. For years, mycobacteria were assumed to lack pili until evidence arose, which demonstrated *M. tuberculosis* curli pili (MTP) encoded by the open reading frame Rv3312A (*mtp*) [2], as one of many adhesins of *M. tuberculosis* [2-14]. Since then, MTP has been shown to bind to laminin, thereby making it an adherence factor responsible for mediating close interaction and colonisation with hosts [2] as well as involved in bacterial aggregation in biofilm formation [15].

The *mtp* gene has been demonstrated to be highly conserved amongst clinical strains [16]. A synthetic MTP peptide detected anti-MTP IgG antibodies in patients' serum samples with an accuracy of 97% [17]. Studies have highlighted that MTP acts as an adhesin and invasin to THP-1 macrophages [4] and A549 epithelial cells [5], modulates host immune responses post-infection in epithelial cells and BALB/C mice [18; 19], and affects both pathogen and host metabolic signatures [20-22].

Metabolomics has been widely utilised to identify new TB diagnostic biomarkers, to evaluate potential therapeutics, and gain insight into the biological mechanisms triggering TB onset and progression, *in vitro*, *in vivo* and in human patients [23-25]. Changes to a biological system, will subsequently result in alterations to the composition and concentration of the metabolites produced at that point in time [26]. Thus, characterising the metabolic alterations of the host as a result of infection, is critical to the discovery of new pathways essential for protection against the pathogen and the identification of host-directed therapies [24].

During infection, the responsibility to restore lung homeostasis falls upon both the alveolar macrophages (AMs) and lung epithelial cells (ECs), including the airway and alveolar EC (AECs). The AMs are considered the main immune cells in the lung and are responsible for phagocytosis of foreign particles and apoptotic cells, in addition to initiating and resolving inflammatory responses through a release of a plethora of immune mediators [27]. The AECs on the other hand, serve as a physical barrier, produce anti-microbial peptides, secrete a variety of immune mediators, and express anti-inflammatory membrane proteins which keep AMs in a quiescent state [27]. Understanding the differences in the functionality of these two cell types and their individual as well as combined response to *M. tuberculosis* infection, is beneficial to the fight against TB.

In an effort to improve current knowledge regarding the involvement of MTP in modulating host metabolic pathways during infection, the current study used a two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-TOFMS) metabolomics research approach to compare the metabolic profiles of *M. tuberculosis* infected A549 epithelial cells and THP-1 macrophages in the presence and absence of MTP.

4.3. Materials and methods

4.3.1. Ethics statement

The University of KwaZulu-Natal Biomedical Research Ethics Committee approved this study (BE254/17 and BE255/17). Bacterial culture, cell culture and infection experiments were conducted in a Biosafety Level 2+ (BSL 2+) laboratory at Medical Microbiology, University of KwaZulu-Natal, Durban South Africa. Metabolite extraction, derivatisation and analysis were conducted at the Centre for Human Metabolomics, North-West University, Potchefstroom, South Africa.

4.3.2. Bacterial strains, host cell lines and growth conditions

Three bacterial strains of *M. tuberculosis* were used for infections. These included *M. tuberculosis* wildtype (WT) V9124 from the F15/LAM4/KZN family [28], V9124 marked *mtp*-deletion mutant (Δmtp) and its *mtp*-complemented strain [15]. Bacterial strains were cultured in Middlebrook 7H9 broth or on 7H11 plates (Difco, Becton-Dickinson, Franklin Lakes, NJ, USA) supplemented as described by Ashokcoomar and Reedoy et al. (2020). Cultures were incubated at 37°C for 3-4 weeks (plates) or in a shaking incubator at 100 rpm for 7-8 days (broth).

The THP-1 monocytic cell line (ATCC TIB-202) and A549 human type II alveolar epithelial cell line (ATCC CCL-185) were cultured in L-glutamine supplemented Roswell Park Memorial Institute Medium (RPMI) 1640 medium (Lonza, BioWhittaker, Basel, Switzerland) and Eagle's Minimum Essential Medium (EMEM) (Lonza, Basel, Switzerland), respectively. Both RPMI and EMEM were supplemented with 10% fetal bovine serum (Biowest, Nuaille, France) to make complete cell culture media for growth of the respective cell lines. Host cell cultures were incubated in 75 cm² tissue culture flasks (Nest Biotechnology, Jiangsu, China) at 37°C in 5% CO₂ in a humidified incubator (Shel Lab CO₂ Incubator, Cornelius, OR, USA) until 70% to 90% confluency.

4.3.3. Preparation of host cell lines and bacterial inocula for infections

Twenty-four hours prior to each infection, viable (THP-1 monocytes were seeded at 9×10^5 cells/mL [21] and A549 epithelial cells were seeded at 5×10^5 cells/mL [22]. Host cell viability prior to infection was determined through cell counts using the trypan blue exclusion assay. THP-1 monocytes were differentiated into macrophages with the addition of phorbol 12-myristate 13-acetate (50 ng/mL) (Sigma-Aldrich, St. Louis, MO, USA). All flasks were incubated overnight at 37°C in 5% CO₂. Once differentiated, macrophages remained at a fixed concentration of 9×10^5 cells/mL since propagation is not possible. However, the A549 epithelial cells continued to replicate, thus additional cell counts were performed on the days of infection to determine the final concentration of A549 epithelial cells for accurate multiplicity of infection (MOI) calculations [21; 22]. Figure 4.1 outlines the sample allocation for *M. tuberculosis* infection of hosts, of which a total of 180 flasks were seeded.

Bacterial inocula for each strain were prepared by centrifugation (Mikro 200R, Hettich Zentrifugen, Tuttlingen, Germany) of log phase bacterial culture at $2\,000 \times g$ for 10 minutes. Bacterial pellets were resuspended in complete media for the respective host cell line infection to an optical density (OD_{600nm}) of approximately 1 (Lightwave II, Biochrom, Cambridge, UK), equivalent to 1×10^8 cells/mL [29].

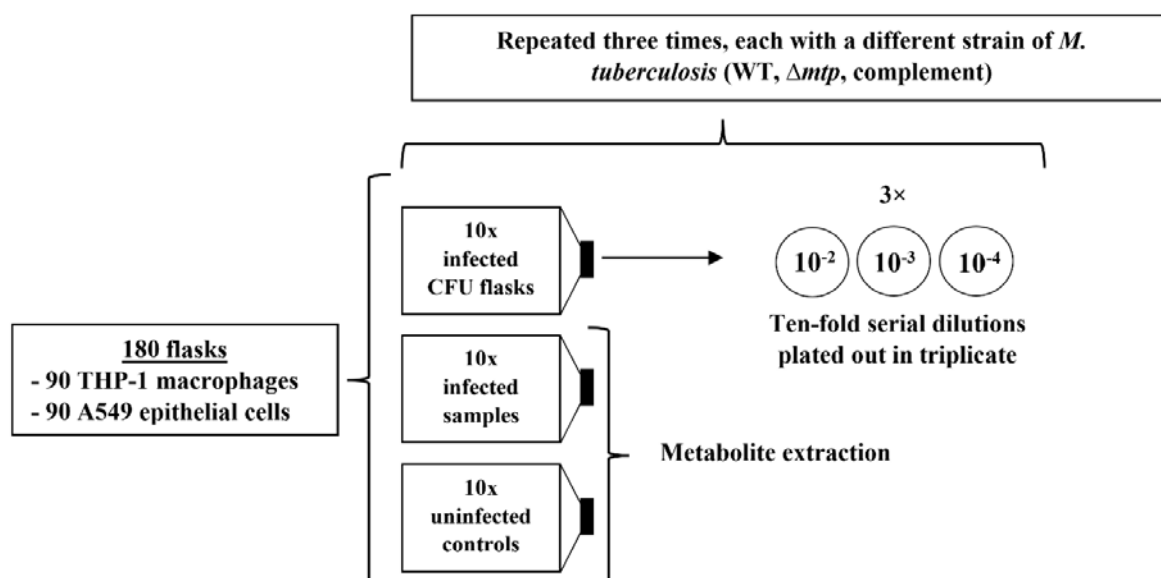


Figure 4.1. Workflow detailing infection of A549 epithelial cells and THP-1 macrophages with three strains of *M. tuberculosis*. A total of 180 flasks were seeded for experiments: 90 flasks for THP-1 macrophages and 90 flasks for A549 epithelial cells. For each of the 90 flasks, 30 flasks were assigned for each of the three bacterial strains (WT, Δmtp , and *mtp*-complement). For each bacterial strain, 10 flasks were used to determine CFUs and 20 flasks were used for metabolite extraction (10 flasks served as the uninfected controls, 10 flasks were the infection models). Thus, a total of 120 samples for the 12 sample groups underwent metabolite extraction. Only those samples with a minimum of 30 mg were used (116 samples); the rest were combined to make up the QCs, which were run through GC $\times$ GC-TOFMS along with the internal standard. WT: wildtype; Δmtp : *mtp*-deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain; CFUs: colony forming units; QCs: quality forming units; GC $\times$ GC-TOFMS: gas chromatography coupled with time-of-flight mass spectrometry.

4.3.4. Infection of host cell lines

All epithelial and macrophage monolayers were washed once with phosphate buffered saline (Sigma-Aldrich, St. Louis, MO, USA). The uninfected controls were resuspended in their respective complete cell culture media, whereas the flasks used as experimental and colony forming units (CFUs) samples were resuspended in complete media containing previously prepared bacterial inocula to achieve a MOI ratio of approximately 5:1. The A549 epithelial cells were incubated at 37°C in 5% CO₂ and 95% humidity (Shell Lab CO₂ Incubator, Cornelius, OR, USA) for two hours and the THP-1 macrophages were incubated under the same conditions for four hours [21; 22]. Infection time points were selected based on previous pilot studies performed on each cell line to determine the optimal durations of infection for metabolite extraction when investigating the MTP adhesin [21; 22].

Following the respective incubation times, the host cell monolayers were detached, washed and pelleted as described by Reedoy et al. (2020) [22] and Ashokcoomar et al. (2021) [21]. Host cell pellets were immediately frozen to halt additional metabolite turnover and stored at -80°C (Sigma-Aldrich, St. Louis, MO, USA) until metabolite extraction. The CFU flasks were lysed with 0.01% Triton X-100 (Millipore, Merck, Darmstadt, Germany) for 20 minutes, serially diluted and plated out in triplicate onto Middlebrook 7H11 plates, which were read after three weeks of incubation at 37°C (Thermo Fisher Scientific, Waltham, MA, USA) (Figure 4.1). The CFU readings allowed for the quantitation of invading bacteria and confirmation of reproducibility of the infection [21; 22].

4.3.5. Metabolite extraction and derivatisation

The wet cell masses of samples were weighed, followed by the extraction of various metabolite classes through a whole metabolome extraction method [30]. Metabolites were extracted in a MM400 mixer mill (Retsch GmbH & co. KG, Haan, Germany) using ultra-pure chloroform:methanol:water in a 1:3:1 ratio (Burdick & Jackson; Honeywell International Inc., Muskegon, MI, USA). Of the 120 samples, only 116 samples that contained a minimum of 30 mg each, were used for metabolite extraction. The other four with less than 30 mg, were THP-1 macrophage samples, namely, two from the uninfected group, one from the Δmtp infected group and one from the *mtp*-complement infected group. Of the 116 samples, 12 random samples (one from each of the 12 sample groups) were combined to make up 30 mg quality controls (QCs) samples for each group. This left a total of 104 samples: 54 samples in the A549 group and 50 in the THP-1 group.

Normalisation was performed using 0.49218 mg/mL 3-phenylbutyric acid (Sigma-Aldrich, St. Louis, MO, USA) as the internal standard. Derivatisation was carried out as previously described using methoxyamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), pyridine (Merck, Darmstadt, Germany), N,O-Bis(trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (Sigma-Aldrich, St. Louis, MO, USA) [21; 22].

4.3.6. GC×GC-TOFMS analysis

Experimental metabolite samples and 50 injections of QCs were randomised across three batches. Untargeted GC×GC-TOFMS was performed in a Pegasus 4D instrument (Leco Corporation, St. Joseph, MI, USA) using a three-split ratio on the Agilent 7890A GC (Agilent, Atlanta, GA) coupled to a time-of-flight mass spectrometer (Leco Corporation, St. Joseph, MI, USA) equipped with a Gerstel Multi-Purpose Sampler (Gerstel GmbH & co. KG, Eberhard-Gerstel- Platz 1, D-45473 Mülheim an der Ruhr). The primary and secondary columns were a Restek RTX-1ms (30 m, 250 μ m internal diameter, 0.25 μ M filament thickness) and a Restek RTX-17 (1.4 m, 250 μ m internal diameter, 0.25 μ M filament thickness) (LECO Africa), respectively. The transfer line temperature was held at 270 °C and the ion source temperature was 220 °C. The detector voltage was 1400 V and spectra between the range of 50 - 800 m/z were detected.

Leco Cooperation ChromaTOF software (version 4.51.6.0) was used to process the data, by which the 'spanning' tracking method was used to remove baselines and automatic smoothing was performed. A signal-to-noise ratio of 100 was used to detect the peaks with five apexing masses. Peak identities were determined by GC retention times and MS-generated mass fragmentation patterns. Spectra were matched to the National Institute of Standards and Technology (NIST) commercial libraries (main library and replicate library), as well as in house data to identify the metabolites. Statistical Compare was used for peak alignment and data matrix formation.

4.3.7. Statistical data analysis

Metabolites were identified with MATLAB (MATLAB with Statistics Toolbox (2019), version 9.5.0 (R2018b) software (Natick, Massachusetts: The MathWorks Inc) and Eigenvector PLS_Toolbox 8.7 (Wenatchee, WA: Eigenvector Research Inc. Software available at <http://www.eigenvector.com>). Statistical analysis was performed as previously reported [21; 22]. Briefly, pre-processing of data included the use of zero filters and zero value replacements,

in which metabolites with zero observations of 50% or more in all groups under consideration, were excluded and the remaining zeros were replaced by random numbers between the minimum and zero. The intermittent injection of QC samples throughout the GC×GC-TOFMS analysis ensured analytical repeatability, which was considered sufficient as the variability between experimental samples (biological variation) far exceeded the variability between QC samples (analytical variation) [21; 22]. Data were log transformed and auto scaled to improve normality and ensure that metabolite significance was not biased by differences in orders of magnitude, which do not imply biological importance [31].

Univariate analysis combined significance testing and effect sizes. The independent samples *t*-test was used to test for the statistical significance of difference between group means, with the adjusted $p \leq 0.05$ considered statistically significant [32]. The *p*-values were adjusted using the approach of Benjamini & Hochberg to correct for multiple testing by controlling the rate of false discovery (BH FDR) [33]. Cohen's *d*-value effect size was used to establish relevance of difference ($d \geq 0.8$ considered to be of practical importance) [34]. Though relative fold changes by definition do not account for variability around the mean, these were considered valid effect sizes due to the homogenous nature of sample groups, with fold change ≥ 2 considered noteworthy [35; 36]. Partial least squares discriminant analysis (PLS-DA) was the multivariate classification model employed to rank metabolites according to their discriminatory ability based on variable importance in projection (VIP) values. For stable PLS-DA models, metabolites with $VIP \geq 1$ were considered strong predictors [37]. Model stability was evaluated through leave-one-out cross validation of each model's predictive accuracy.

Only metabolites which met all four selection criteria, were further investigated for biological relevance through PubChem [38] and/or KEGG [39], as well as literature to allow for broad categorisation of these metabolites. Some metabolites were only detected in one cell type and not the other, hence some fold change calculations resulted in invalid numbers as a result of division by zero (as indicated in Tables 4.2 - 4.5). Data was reported for four comparison groups, namely, uninfected A549 epithelial cells versus uninfected THP-1 macrophages (Table 4.2), WT infected A549 epithelial cells versus WT infected THP-1 macrophages (Table 4.3), Δmtp infected A549 epithelial cells versus Δmtp infected THP-1 macrophages (Table 4.4), and *mtp*-complement infected A549 epithelial cells versus *mtp*-complement infected THP-1 macrophages (Table 4.5). Metabolites that could not be categorised into biological relevance or metabolites that were alternative derivatives of a metabolite, which was already in the comparison group tables, were listed in Appendix: D Tables S1 to S4.

4.4. Results

4.4.1. PLS-DA data validation illustrates adequate group separation between models

The predictive accuracy (R^2) and leave-one-out predictive accuracy (Q^2) for each of the four comparison groups were well above the cut-off values of 80% and 60%, respectively, proving model stability (Table 4.1). Therefore, all four PLS-DA models were considered valid as all displayed sufficient robustness against over fit (Figure 4.2). The comparison of A549 epithelial cells to THP-1 macrophages for the uninfected groups (Figure 4.2a) and infected groups (Figure 4.2b-d) showed distinctively different host cell metabolome profiles as seen by the separation of the ellipsoids for the respective figures.

Table 4.1. Validity of PLS-DA models indicated by the predictive accuracy and leave-one-out predictive accuracy for each comparison group.

Comparison	R² value (%)	Q² value (%)
Uninfected A549 versus Uninfected THP-1	99.688	98.140
WT infected A549 versus WT infected THP-1	99.690	98.615
Δmtp infected A549 versus Δmtp infected THP-1	98.920	96.129
mtp -complement infected A549 versus mtp -complement infected THP-1	99.816	99.077

PLS-DA: partial least squares discriminant analysis; WT infected: wildtype infected; Δmtp infected: mtp -deletion mutant infected; mtp -complement: complemented mtp -knockout strain infected; R²: predictive accuracy with a cut-off value of 80%; Q²: leave-one-out predictive accuracy with a cut-off value of 60%.

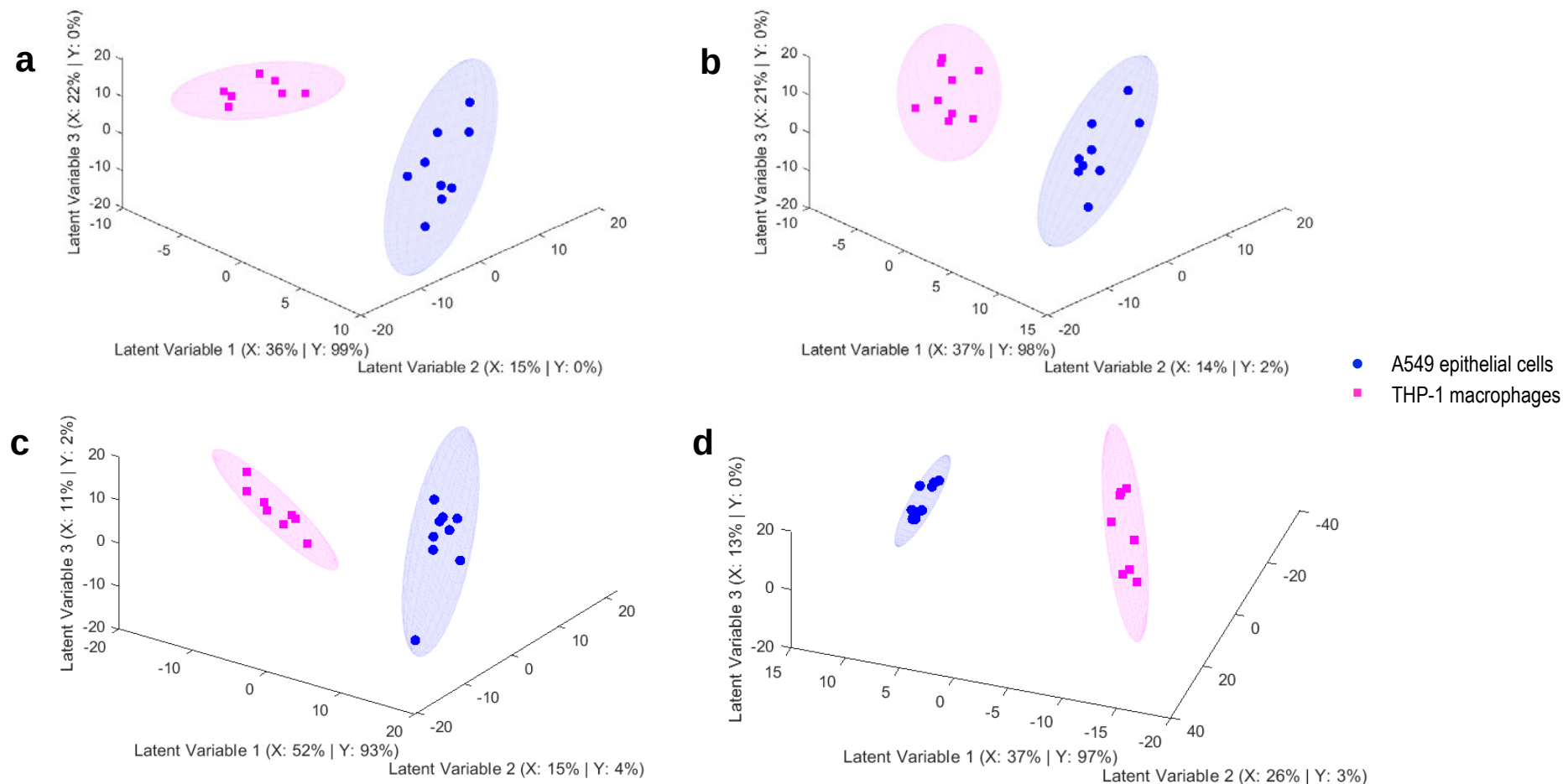


Figure 4.2. The 3D PLS-DA score plots for the four comparison groups. Models comparing (a) uninfected A549 epithelial cells to uninfected THP-1 macrophages, (b) WT infected A549 epithelial cells to WT infected THP-1 macrophages, (c) Δmtp infected A549 epithelial cells to Δmtp infected THP-1 macrophages (d) *mtp*-complement infected A549 epithelial cells to *mtp*-complement infected THP-1 macrophages. All four PLS-DA models validated (a-d) and their respective comparison groups showed adequate group separation within models. Ellipsoids represent 95% CI of score centroids of each group. Accuracy of group assignment (Y) and variation explained in measured metabolites (X), as achieved by each latent variable (LV), percentages are indicated along each axis. PLS-DA: partial least squares discriminant analysis; CI: confidence interval; WT: wildtype; Δmtp : *mtp*-deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain.

4.4.2. Shortlisting of significantly differentially detected metabolites between A549 epithelial cells and THP-1 macrophages revealed distinguishing host metabolic profiles

The number of metabolites that were shortlisted for their discriminatory ability between the two host cell types based on any of the selection criteria were: 228 when comparing uninfected A549 to THP-1 cells; 230 when comparing WT infected A549 to WT infected THP-1 cells; 230 when comparing Δmtp infected A549 to Δmtp infected THP-1 cells; and 230 when comparing *mtp*-complement infected A549 to *mtp*-complement infected THP-1 cells. Of these metabolites, only those that met all the statistical selection criteria (relative fold change ≥ 2 ; BH FDR p -value ≤ 0.05 ; Cohen's d -value effect size ≥ 0.8 ; and PLS-DA VIP ≥ 1) [32-35; 37] and were of biological relevance [38; 39] are listed in Tables 4.2 - 4.5. Hence, totals of 44, 40, 50 and 34 metabolites were reported as having the ability to differentiate between A549 epithelial cells and THP-1 macrophages for the uninfected A549 versus uninfected THP-1 comparison (Table 4.2), WT infected A549 versus WT infected THP-1 comparison (Table 4.3), Δmtp infected A549 versus Δmtp infected THP-1 comparison (Table 4.4) and *mtp*-complement infected A549 versus *mtp*-complement infected THP-1 comparison (Table 4.5), respectively. Furthermore, of these metabolites, 24, 22, 1, and 24 were detected in higher concentrations in the uninfected A549 versus uninfected THP-1 comparison (Table 4.2), WT infected A549 versus WT infected THP-1 comparison (Table 4.3), Δmtp infected A549 versus Δmtp infected THP-1 comparison (Table 4.4) and *mtp*-complement infected A549 versus *mtp*-complement infected THP-1 comparison (Table 4.5), respectively. Thus, the majority of the differentiating metabolites (55%, 55% and 71%) in the uninfected, WT infected, *mtp*-complement infected models were found in higher concentrations in the A549 epithelial cells relative to the THP-1 macrophages, respectively. This is in contrast to the Δmtp infected model, in which 98% of the differentially detected metabolites were observed to be in higher concentrations in the THP-1 macrophages relative to the A549 epithelial cells.

The detected differentiating metabolites in Tables 4.2 – 4.5 were grouped into four broad categories: amino acid metabolism, fatty acid metabolism, general carbon metabolism, and nucleic acid metabolism; however, some of these have important roles in other metabolic pathways, interlinking a vast range of common metabolic processes, irrespective of the cell line. Moreover, each cell line showed a characteristic metabolic profile due to their different functionalities in each of the four groups. Although some metabolites are detected in more than one model comparison, their concentrations vary and are specific to each model.

4.4.3. Metabolic profiles of uninfected A549 epithelial cells and THP-1 macrophages revealed baseline differences of metabolites associated with amino acid metabolism, fatty acid metabolism, carbon metabolism and nucleic acid metabolism

Of the 44 differentially detected metabolites when comparing uninfected A549 epithelial cells and THP-1 macrophages, 19 are involved in amino acid metabolism, eight in fatty acid metabolism, 12 in carbon metabolism, and five in nucleic acid metabolism. Metabolites associated with amino acid metabolism can be further categorised into metabolites associated with lysine metabolism such as those which play a role as a lysine derivative (6-aminocaproic acid), involved in lysine degradation (5-aminovaleric acid, glycine, and pentanedioic acid), biosynthesis (homoserine), or both biosynthesis and degradation (2-aminoadipic acid);

metabolites associated with cysteine and methionine metabolism (2-aminobutanoic acid, alanine, homoserine); valine, leucine and isoleucine biosynthesis (3-hydroxyisovaleric acid); phenylalanine metabolism (3-phenyllactic acid, 4-hydroxyphenyllactic acid, benzeneacetic acid); tyrosine metabolism (α -methyl-L-tyrosine), tryptophan metabolism (kynurenine); alanine, aspartate and glutamate metabolism (4-aminobutanoic acid, alanine); arginine biosynthesis (N-acetylglutamine); arginine and proline metabolism (4-aminobutanoic acid, 5-aminovaleric acid, putrescine, spermine); β -alanine metabolism (pantothenic acid, spermine); and glycine, serine and threonine metabolism (glycine, homoserine). The A549 epithelial cells predominantly showed higher concentrations of the metabolites involved in amino acid metabolism, with exception of those metabolites associated with phenylalanine, tyrosine, and tryptophan metabolism as well as arginine biosynthesis.

However, metabolites which play a role in fatty acid metabolism were predominantly detected in high concentrations in the THP-1 macrophage model. These metabolites can be classed as those involved in fatty acid degradation (1-hexadecanol, 5-dodecenoic acid); fatty acid biosynthesis (dodecanoic acid); butanoate metabolism (4-hydroxybutanoic acid); inflammatory responses (batyl alcohol); and cholesterol metabolism (lanosterol). The remaining fatty acid metabolites are involved in biotin synthesis (pimelic acid) and cell signalling (9-octadecenamide).

Differences in carbon metabolism between the cells lines were observed in glycerophospholipid metabolism (phosphorylethanolamine); amino sugar and nucleotide sugar metabolism and lipopolysaccharide biosynthesis (N-acetyl-D-glucosamine); carbohydrates involved in glycolysis/gluconeogenesis (D-fructopyranose, deoxyglucose, D-glucopyranuronic, lactic acid); fructose and mannose metabolism (L-sorbose, lactic acid); pentose phosphate pathway (PPP) (D-gluconic acid); citrate cycle/ tricarboxylic acid cycle (TCA cycle) (fumaric acid, malic acid); oxidative phosphorylation (OXPHOS) (phosphoric acid); and propanoate metabolism (propylene glycol).

Lastly, methyl α -D-ribofuranoside, pyrimidine, ribonic acid, oxalic acid (involved in purine metabolism), and uridine (involved in pyrimidine metabolism) were all found in higher concentrations in the A549 epithelial cell line. It is important to note that kynurenine and lanosterol were detected only in the THP-1 macrophages and pimelic acid was detected only in the A549 epithelial cells.

4.4.4. Metabolic profile differences between WT infected A549 epithelial cells and WT infected THP-1 macrophages revealed metabolome differences induced by infection with *M. tuberculosis*

Of the 40 differentially detected metabolites comparing the WT infected A549 epithelial cells and WT infected THP-1 macrophages, 17 are involved in amino acid metabolism, nine in fatty acid metabolism, 10 in carbon metabolism, and four in nucleic acid metabolism. Furthermore, seven of the metabolites (including 5'-methylthioadenosine (MTA), urea, adenine, 5,8,11-eicosatrienoic acid/mead acid, butanoic acid/ butyric acid, niacin and niacinamide) were not detected in the comparison of uninfected A549 epithelial cells versus uninfected THP-1 macrophages. Thus, the detection of these seven metabolites were as a result of infection with *M. tuberculosis*.

The WT infection models showed differences in amino acid metabolism as seen by higher concentrations of metabolites involved in cysteine and methionine metabolism (MTA), arginine biosynthesis and proline metabolism (urea) in the A549 epithelial cells compared to the THP-1 macrophages. Moreover, urea, interlinked to biotin, pyrimidine and purine

metabolism, and adenine, involved in nucleic acid metabolism, were found in higher concentrations in the THP-1 macrophages. Differences observed in fatty acid metabolism included fatty acid biosynthesis (5,8,11-eicosatrienoic acid/mead acid) and butanoate metabolism (butanoic acid) in which the A549 epithelial cells showed a higher concentration of the respective metabolites relative to the THP-1 macrophages. Lastly, differences in carbon metabolism were observed in nicotinate and nicotinamide metabolism, of which niacin was detected in higher concentrations in the A549 epithelial cells when compared to the THP-1 macrophages. This was in contrast to the lower concentration of niacinamide present in the A549 epithelial cells compared to the THP-1 macrophages. Additionally, although detected in slightly lower concentrations in the uninfected model, kynurenine and lanosterol were once again detected only in the THP-1 macrophages, indicative of its unique metabolic profile.

4.4.5. The absence of the MTP adhesin largely contributes to higher concentrations of amino acids in the THP-1 macrophage infection model relative to the A549 epithelial cell model

In the absence of the MTP adhesin, a total of the 50 differentially detected metabolites comparing Δmtp infected A549 versus Δmtp infected THP-1 macrophages revealed 28 to be involved in amino acid metabolism, eight in fatty acid metabolism, 11 in carbon metabolism, and three in nucleic acid metabolism. Apart from oxalic acid, all of the differentially detected metabolites were found in higher concentrations in the THP-macrophage model. Additionally, 25 of these metabolites were not detected in either the uninfected or WT infected models (including cadaverine, pipecolic acid, L-aspartic acid, L-cystine, L-methionine, L-isoleucine, L-valine, phenylalanine, L-tyrosine, L-tryptophan, dopamine, pyroglutamic acid, L-glutamine, L-alanine, N-acetylaspartic-acid, N-acetylglutamic-acid, L-hydroxyproline, N-acetylalanine, decanoic acid, tetradecanoic acid, ethanolamine, myo-inositol, scyllo-inositol, ribitol, and pseudouridine). Thus, 50% of the newly detected metabolites were as a result of the lack of MTP. The majority of the metabolites are involved in amino acid metabolism such as lysine degradation (cadaverine and pipecolic acid); cysteine and methionine metabolism (L-aspartic acid, L-cystine, and L-methionine); valine, leucine and isoleucine biosynthesis (L-isoleucine and L-valine); phenylalanine, tyrosine and tryptophan metabolism (phenylalanine, L-tyrosine, L-tryptophan, and dopamine); D-glutamine and D-glutamate metabolism (pyroglutamic acid and L-glutamine); alanine, aspartate and glutamate metabolism (L-alanine, N-acetylaspartic-acid, L-aspartic acid, L-glutamine); arginine biosynthesis (L-aspartic acid, N-acetylglutamic-acid, L-glutamine); arginine and proline metabolism (L-hydroxyproline); β -alanine metabolism (N-acetylalanine); and glycine, serine and threonine metabolism (L-aspartic acid, L-tryptophan).

Additional metabolic differences were observed in fatty acid metabolism, which included metabolites involved in fatty acid biosynthesis (decanoic acid and tetradecanoic acid). Moreover, carbon metabolism was altered as shown by differences in concentrations of metabolites involved in glycerophospholipid metabolism (ethanolamine); galactose metabolism (myo-inositol); inositol phosphate metabolism (myo-inositol and scyllo-inositol); which also involves scyllo-inositol; and pentose and glucuronate interconversions (ribitol). Lastly, nucleic acid metabolism exhibited differences in pyrimidine metabolism with the addition of pseudouridine. The metabolites 3-phenyllactic acid, benzeneacetic acid, kynurenine and lanosterol were detected only in the THP-1 macrophage model. The metabolites 3-phenyllactic acid, benzeneacetic acid, lanosterol were detected in the highest concentration in

this model compared to the uninfected and WT-proficient models; whereas the inverse was observed for kynurenine.

4.4.6. Complementation of Δmtp resulted in the fewest differentially detected metabolites when comparing *mtp*-complement infected A549 epithelial cells and *mtp*-complement infected THP-1 macrophages

Of the 34 differentially detected metabolites when comparing *mtp*-complement infected A549 epithelial cells and *mtp*-complement infected THP-1 macrophages, 17 are involved in amino acid metabolism, six in fatty acid metabolism, eight in carbon metabolism, and three in nucleic acid metabolism. This comparison revealed only five metabolites (asparagine, glyceric acid, citric acid, propanal, and guanosine) that were not previously seen in the uninfected, WT infected nor Δmtp infected models. Differences in metabolite concentrations were observed in alanine, aspartate and glutamate metabolism (asparagine); glycine, serine and threonine metabolism (glyceric acid); and alanine, aspartate and glutamate metabolism (citric acid) as part of amino acid metabolism. These metabolites interlink with carbon metabolism such as glycerophospholipid metabolism (glyceric acid), PPP (glyceric acid), and TCA cycle (citric acid). Carbon metabolism also showed differences in propanoate metabolism (propanal). Nucleic acid metabolism involved changes to purine metabolism with varying concentrations of guanosine. There was no additional difference in fatty acid metabolomic profiles of the host cell line for this infection model. Moreover, benzeneacetic acid and lanosterol were detected only in the THP-1 macrophage model. These results reveal that the *mtp*-complement infected hosts exhibited partial restoration of metabolic signatures due to the non-integrating plasmid used in the complementation process.

Table 4.2. Metabolites best characterising the variation between the uninfected A549 epithelial cells and THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Category	Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
		Uninfected A549 epithelial cells	Uninfected THP-1 macrophages				
Amino acid metabolism	2-Aminoadipic acid	6.395 (1.200)	2.574 (0.847)	2.485	1.297	3.012	<0.001
	6-Aminocaproic acid	4.641 (0.947)	0.990 (0.537)	4.688	1.941	4.19	<0.001
	Pentanedioic acid	6.773 (1.641)	1.804 (0.499)	3.754	2.009	4.664	<0.001
	2-Aminobutanoic acid	81.134 (24.510)	8.671 (2.596)	9.357	1.875	6.688	<0.001
	3-Hydroxyisovaleric acid	2.155 (1.683)	0.108 (0.287)	19.894	1.143	1.262	0.017
	3-Phenyllactic acid	0.159 (0.317)	124.383 (42.044)	-782.997	1.979	13.746	<0.001
	4-Hydroxyphenyllactic acid	4.639 (1.419)	54.399 (45.725)	-11.726	1.010	1.585	0.017
	Benzeneacetic acid	0.070 (0.209)	4.088 (1.361)	-58.789	1.805	5.205	<0.001
	α -Methyl-L-tyrosine	2.265 (1.273)	9.886 (4.592)	-4.364	1.198	1.81 9	0.008
	Kynurenine	0 (0)	2.708 (1.452)	#	2.574	2.344	0.004
	4-Aminobutanoic acid	93.556 (21.680)	11.813 (6.319)	7.920	2.141	4.129	<0.001
	Alanine	240.157 (148.973)	20.700 (9.485)	11.602	1.226	1.152	0.025
	N-Acetylglutamine	7.521 (9.187)	33.821 (29.449)	-4.497	1.234	1.220	0.046
	5-Aminovaleric acid	46.552 (14.077)	9.062 (3.408)	5.137	1.845	4.393	<0.001
	Putrescine	280.067 (59.862)	54.346 (27.316)	5.153	1.832	3.290	<0.001
	Spermine	15.103 (5.519)	5.001 (3.199)	3.020	1.349	2.051	0.004
	Pantothenic acid	20.039 (4.391)	59.444 (17.150)	-2.967	1.401	3.427	<0.001
	Glycine	5.316 (1.670)	1.766 (1.452)	3.010	1.517	1.648	0.013
	Homoserine	0.152 (0.234)	1.410 (0.767)	-9.259	1.022	1.881	0.007

PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages; * = fold change cannot be calculated as metabolite is present in only A549 epithelial cells.

Table 4.2 continued. Metabolites best characterising the variation between the uninfected A549 epithelial cells and THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Category	Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
		Uninfected A549 epithelial cells	Uninfected THP-1 macrophages				
Fatty acid metabolism	1-Hexadecanol	2.288 (2.260)	5.882 (1.956)	-2.571	1.264	1.555	0.007
	5-Dodecenoic acid	26.100 (7.001)	4.860 (2.023)	5.370	2.069	3.877	<0.001
	Dodecanoic acid	43.023 (8.606)	115.156 (30.046)	-2.677	1.149	3.283	<0.001
	Pimelic acid	0.654 (0.177)	0 (0)	*	1.607	3.612	<0.001
	4-Hydroxybutanoic acid	8.464 (3.715)	33.978 (7.628)	-4.015	1.283	3.244	<0.001
	9-Octadecenamide	60.571 (31.599)	29.541 (11.116)	2.050	1.135	1.281	0.039
	Batyl alcohol	4.240 (3.709)	28.759 (14.871)	-6.783	1.794	2.899	<0.001
	Lanosterol	0 (0)	64.982 (59.583)	#	1.757	2.002	0.007
Carbon metabolism	Phosphorylethanolamine	307.693 (169.843)	13.806 (7.498)	22.288	1.953	5.563	<0.001
	N-Acetyl-D-glucosamine	22.019 (8.674)	89.911 (36.540)	-4.083	1.034	2.808	0.001
	D-Fructopyranose	0.176 (0.529)	18.515 (14.075)	-105.066	1.865	2.106	0.005
	Deoxyglucose	29.915 (6.146)	13.788 (3.196)	2.170	1.209	3.391	<0.001
	D-Glucopyranuronic acid	18.872 (10.227)	0.274 (0.619)	68.752	2.273	3.961	<0.001
	L-Sorbose	56.321 (25.749)	1601.039 (603.147)	-28.427	1.579	7.035	<0.001
	Lactic acid	210.522 (117.525)	3856.177 (1604.205)	-18.317	1.667	5.896	<0.001
	D-Gluconic acid	42.060 (16.826)	350.739 (171.454)	-8.339	1.918	3.207	<0.001
	Fumaric acid	42.386 (28.245)	17.577 (5.207)	2.411	1.537	1.647	0.005
	Malic acid	50.461 (38.091)	22.646 (4.955)	2.228	1.240	1.221	0.020
	Phosphoric acid	13.475 (3.071)	33.355 (9.682)	-2.475	1.636	2.550	0.001
	Propylene glycol	0.869 (0.577)	8.00 (5.901)	-9.204	1.411	1.505	0.020

PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages; * = fold change cannot be calculated as metabolite is present in only A549 epithelial cells.

Table 4.2 continued. Metabolites best characterising the variation between the uninfected A549 epithelial cells and THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Category	Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
		Uninfected A549 epithelial cells	Uninfected THP-1 macrophages				
Nucleic acid metabolism	Methyl α -D-ribofuranoside	27.134 (11.817)	0.476 (0.310)	56.946	2.342	6.562	<0.001
	Pyrimidine	75.240 (18.704)	32.955 (8.047)	2.283	1.730	3.333	<0.001
	Ribonic acid	19.006 (6.107)	1.452 (0.677)	13.086	1.969	6.766	<0.001
	Oxalic acid	19.387 (25.170)	2.336 (1.229)	8.301	1.424	2.101	0.001
	Uridine	90.462 (23.166)	33.125 (8.755)	2.731	1.684	3.744	<0.001

PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages; * = fold change cannot be calculated as metabolite is present in only A549 epithelial cells.

Table 4.3. Metabolites best characterising the variation between the WT infected A549 epithelial cells and WT infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Category	Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
		WT infected A549 epithelial cells	WT infected THP-1 macrophages				
Amino acid metabolism	2-Aminoadipic acid	6.420 (0.690)	2.044 (0.644)	3.140	1.578	3.444	<0.001
	6-Aminocaproic acid	3.630 (0.959)	0.423 (0.339)	8.577	2.119	5.079	<0.001
	Pentanedioic acid	7.605 (2.182)	1.579 (0.320)	4.816	2.358	3.806	<0.001
	2-Aminobutanoic acid	82.387 (26.688)	7.000 (2.859)	11.770	2.118	5.840	<0.001
	5'-Methylthioadenosine	16.223 (5.746)	2.710 (1.379)	5.987	1.872	3.851	<0.001
	3-Hydroxyisovaleric acid	4.227 (3.183)	0.662 (0.504)	6.383	1.507	1.213	0.020
	3-Phenyllactic acid	0.104 (0.206)	87.924 (28.128)	-845.220	1.889	14.275	<0.001
	4-Hydroxyphenyllactic acid	4.244 (1.642)	44.941 (22.087)	-10.589	1.129	2.586	<0.001
	Benzeneacetic acid	0.035 (0.105)	3.903 (1.224)	-111.175	1.794	5.314	<0.001
	α -Methyl-L-tyrosine	2.943 (1.513)	11.508 (5.287)	-3.910	1.173	1.981	0.001
	Kynurenine	0 (0)	1.664 (0.160)	#	1.825	1.680	0.003
	Alanine	208.955 (108.846)	14.154 (7.409)	14.763	1.635	2.338	<0.001
	Urea	55.503 (22.440)	17.957 (16.772)	3.091	1.584	1.183	0.022
	5-Aminovaleric acid	47.396 (16.653)	6.727 (2.258)	7.045	2.157	4.575	<0.001
	Putrescine	298.338 (102.733)	48.989 (17.486)	6.091	1.948	4.408	<0.001
	Pantothenic acid	19.253 (5.161)	46.373 (13.751)	-2.409	1.161	2.794	<0.001
	Glycine	4.887 (2.368)	1.247 (0.884)	3.919	1.609	2.377	<0.001

WT infected: wildtype infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjustment for false discovery rate; positive fold change = higher levels in A549 epithelial cells; Negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages; * = fold change cannot be calculated as metabolite is present in only A549 epithelial cells.

Table 4.3 continued. Metabolites best characterising the variation between the WT infected A549 epithelial cells and WT infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Category	Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
		WT infected A549 epithelial cells	WT infected THP-1 macrophages				
Fatty acid metabolism	1-Hexadecanol	2.236 (1.794)	13.163 (2.831)	-5.887	2.349	3.271	<0.001
	5-Dodecenoic acid	21.972 (9.504)	2.968 (1.665)	7.404	2.321	3.227	<0.001
	Dodecanoic acid	35.143 (11.157)	89.485 (22.661)	-2.546	1.157	2.908	<0.001
	5,8,11-Eicosatrienoic acid	9.698 (3.901)	3.191 (1.787)	3.039	1.532	2.010	0.001
	Pimelic acid	0.759 (0.333)	0.101 (0.155)	7.491	1.634	1.999	0.001
	4-Hydroxybutanoic acid	9.834 (4.629)	33.393 (7.980)	-3.396	1.224	2.075	0.001
	Butanoic acid	1.214 (0.510)	0.378 (0.363)	3.210	1.310	1.771	0.002
	Batyl alcohol	3.928 (4.873)	30.771 (13.867)	-7.834	2.085	2.767	<0.001
	Lanosterol	0 (0)	60.533 (40.186)	#	1.874	3.142	<0.001
Carbon metabolism	Phosphorylethanolamine	269.488 (218.840)	10.061 (8.627)	26.785	2.064	3.967	<0.001
	D-Fructopyranose	0.205 (0.433)	12.650 (11.759)	-61.561	1.616	2.070	0.001
	Deoxyglucose	27.852 (7.891)	10.995 (2.308)	2.533	1.422	2.738	<0.001
	D-Glucopyranuronic acid	15.882 (9.334)	0.249 (0.748)	63.659	2.089	2.386	<0.001
	L-Sorbose	50.397 (27.620)	1112.534 (490.504)	-22.075	1.514	5.0969	<0.001
	D-Gluconic acid	44.155 (17.991)	283.150 (125.182)	-6.413	1.747	3.140	<0.001
	Phosphoric acid	13.577 (4.200)	35.765 (8.686)	-2.634	1.879	2.810	<0.001
	Propylene glycol	0.669 (0.582)	8.425 (4.279)	-12.585	1.744	3.155	<0.001
	Niacin	49.097 (13.990)	166.932 (43.635)	-3.400	1.001	3.991	<0.001
	Niacinamide	249.041 (102.351)	51.065 (20.604)	4.877	1.639	3.312	<0.001

WT infected: wildtype infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjustment for false discovery rate; positive fold change = higher levels in A549 epithelial cells; Negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages; * = fold change cannot be calculated as metabolite is present in only A549 epithelial cells.

Table 4.3 continued. Metabolites best characterising the variation between the WT infected A549 epithelial cells and WT infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Category	Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
		WT infected A549 epithelial cells	WT infected THP-1 macrophages				
Nucleic acid metabolism	Methyl α -D-ribofuranoside	25.105 (11.612)	0.348 (0.303)	72.087	2.347	5.362	<0.001
	Ribonic acid	19.092 (8.000)	0.915 (0.547)	20.863	2.187	5.349	<0.001
	Oxalic acid	12.061 (5.165)	2.977 (2.485)	4.051	1.199	1.814	0.002
	Adenine	6.154 (1.983)	12.318 (3.889)	-2.002	1.373	2.064	0.002

WT infected: wildtype infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjustment for false discovery rate; positive fold change = higher levels in A549 epithelial cells; Negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages; * = fold change cannot be calculated as metabolite is present in only A549 epithelial cells.

Table 4.4. Metabolites best characterising the variation between the Δmtp infected A549 epithelial cells and Δmtp infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH adjusted *p*-values.

Category	Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH- adjusted <i>p</i> -value
		Δmtp infected A549 epithelial cells	Δmtp infected THP-1 macrophages				
Amino acid metabolism	Cadaverine	1.499 (1.266)	31.998 (14.676)	-21.350	1.988	5.597	<0.001
	Pipecolic acid	13.908 (21.284)	70.508 (41.748)	-5.070	1.564	1.707	0.002
	5'-Methylthioadenosine	0.162 (0.324)	4.507 (1.420)	-27.889	1.120	4.669	<0.001
	L-Alanine	1507.353 (642.839)	4555.946 (1540.177)	-3.022	1.113	2.207	<0.001
	L-Aspartic acid	568.271 (218.263)	1899.314 (304.977)	-3.342	1.136	2.840	<0.001
	L-Cystine	4.369 (5.376)	123.931 (74.974)	-28.368	1.302	3.201	<0.001
	L-Methionine	373.349 (147.673)	1123.310 (185.693)	-3.009	1.607	2.741	<0.001
	L-Isoleucine	1470.561 (367.888)	3560.603 (325.045)	-2.421	1.151	1.063	0.025
	L-Valine	2007.646 (813.406)	4039.504 (553.281)	-2.012	1.221	1.008	0.031
	3-Phenyllactic acid	0 (0)	221.981 (42.926)	#	1.644	26.282	<0.001
	4-Hydroxyphenyllactic acid	0.080 (0.241)	161.840 (36.487)	-2017.150	1.850	20.514	<0.001
	Benzeneacetic acid	0 (0)	5.538 (2.218)	#	1.533	4.698	<0.001
	Phenylalanine	907.394 (271.773)	2072.944 (348.670)	-2.285	1.455	2.592	<0.001
	L-Tyrosine	1084.737 (395.464)	2258.748 (380.642)	-2.082	1.401	1.707	0.002
	L-Tryptophan	101.336 (72.419)	300.194 (105.379)	-2.962	1.148	1.027	0.030
	α -Methyl-L-tyrosine	2.000 (2.309)	20.772 (5.726)	-10.385	1.595	3.232	<0.001
	Dopamine	0.433 (0.990)	13.078 (8.772)	-30.236	1.664	3.285	<0.001
	Kynurenine	0 (0)	2.128 (1.211)	#	1.579	2.198	0.001
	Pyroglutamic acid	850.264 (228.117)	3570.255 (665.918)	-4.199	1.577	5.905	<0.001
	L-Glutamine	71.281 (30.888)	460.370 (148.677)	-6.459	1.578	2.484	<0.001
	4-Aminobutanoic acid	11.171 (4.594)	47.803 (15.291)	-4.2793	1.035	3.538	<0.001
	N-Acetylaspartic acid	15.303 (7.897)	104.711 (39.395)	-6.842	1.361	2.415	<0.001

Δmtp infected: *mtp*-deletion mutant infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages.

Table 4.4 continued. Metabolites best characterising the variation between the Δmtp infected A549 epithelial cells and Δmtp infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH adjusted *p*-values.

Category	Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH- adjusted <i>p</i> -value
		Δmtp infected A549 epithelial cells	Δmtp infected THP-1 macrophages				
Amino acid metabolism	N-Acetylglutamic acid	0.214 (0.339)	4.342 (2.206)	-20.331	1.280	3.392	<0.001
	N-Acetylglutamine	4.864 (5.701)	45.106 (29.848)	-9.273	1.078	1.519	0.009
	L-Hydroxyproline	11.974 (5.008)	132.144 (46.327)	-11.036	2.037	5.277	<0.001
	Pantothenic acid	7.362 (2.902)	59.688 (12.725)	-8.108	1.956	4.539	<0.001
	N-Acetylalanine	1.873 (1.635)	8.701 (4.526)	-4.645	1.082	1.500	0.009
	Ethanolamine	884.518 (480.229)	2482.635 (938.416)	-2.807	1.155	1.820	0.002
Fatty acid metabolism	1-Hexadecanol	1.914 (10.95)	7.697 (2.053)	-4.021	1.185	3.193	<0.001
	5-Dodecenoic acid	2.742 (1.209)	13.759 (3.877)	-5.017	1.313	4.428	<0.001
	Dodecanoic acid	25.592 (3.100)	312.263 (36.131)	-12.202	2.138	20.458	<0.001
	Decanoic acid	3.906 (2.291)	22.989 (14.193)	-5.885	1.309	2.355	<0.001
	Tetradecanoic acid	989.813 (256.145)	2586.149 (270.067)	-2.613	1.361	4.045	<0.001
	4-Hydroxybutanoic acid	2.923 (2.026)	32.430 (5.961)	-11.094	1.510	4.649	<0.001
	Batyl alcohol	2.561 (1.883)	31.468 (12.956)	-12.289	1.599	4.683	<0.001
	Lanosterol	0 (0)	94.325 (60.102)	#	1.553	3.559	<0.001

Δmtp infected: *mtp*-deletion mutant infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages.

Table 4.4 continued. Metabolites best characterising the variation between the Δmtp infected A549 epithelial cells and Δmtp infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH adjusted *p*-values.

Category	Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH- adjusted <i>p</i> -value
		Δmtp infected A549 epithelial cells	Δmtp infected THP-1 macrophages				
Carbon metabolism	N-Acetyl-D-glucosamine	5.674 (3.899)	96.440 (15.368)	-16.998	1.548	3.696	<0.001
	D-Fructopyranose	0.081 (0.243)	18.983 (11.433)	-234.188	1.509	3.610	<0.001
	Myo-Inositol	394.299 (98.898)	1804.071 (334.909)	-4.575	1.070	5.706	<0.001
	Scyllo-Inositol	2.754 (1.386)	18.398 (5.525)	-6.681	1.114	3.649	<0.001
	L-Sorbose	11.304 (7.773)	2438.477 (453.904)	-215.721	1.893	5.600	<0.001
	Lactic acid	54.731 (15.607)	6841.392 (3220.275)	-125.001	1.971	11.021	<0.001
	Ribitol	34.694 (42.643)	261.349 (232.300)	-7.533	1.269	2.270	<0.001
	D-Gluconic acid	28.226 (18.216)	391.298 (91.115)	-13.863	1.873	2.881	<0.001
	Propylene glycol	1.018 (1.076)	13.760 (7.506)	-13.513	1.453	3.378	<0.001
	Niacin	30.960 (5.507)	239.124 (46.547)	-7.724	1.164	9.873	<0.001
	Niacinamide	31.766 (11.381)	130.183 (52.826)	-4.098	1.021	3.199	<0.001
Nucleic acid metabolism	Oxalic acid	31.453 (32.490)	1.934 (1.592)	16.266	1.373	2.042	<0.001
	Adenine	5.670 (2.486)	15.206 (5.323)	-2.682	1.409	1.792	0.002
	Pseudouridine	35.820 (26.868)	88.143 (26.868)	-2.461	1.143	1.012	0.031

Δmtp infected: *mtp*-deletion mutant infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages.

Table 4.5. Metabolites best characterising the variation between the *mtp*-complement infected A549 epithelial cells and *mtp*-complement infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Category	Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH- adjusted <i>p</i> -value
		<i>mtp</i> -complement infected A549 epithelial cells	<i>mtp</i> -complement infected THP-1 macrophages				
Amino acid metabolism	2-Aminoadipic acid	5.179 (1.386)	0.777 (0.607)	6.670	1.8379	3.989	<0.001
	6-Aminocaproic acid	3.312 (1.275)	0.434 (0.569)	7.633	1.602	3.440	<0.001
	Pentanedioic acid	5.146 (0.863)	2.357 (2.118)	2.183	1.171	1.515	0.014
	2-Aminobutanoic acid	74.918 (28.558)	5.657 (5.797)	13.242	1.950	2.940	<0.001
	5'-Methylthioadenosine	9.855 (3.606)	1.819 (2.150)	5.416	1.430	2.634	<0.001
	L-Cystine	339.325 (67.798)	49.967 (43.309)	6.791	1.054	2.000	0.004
	3-Phenyllactic acid	0.303 (0.490)	137.671 (91.879)	-455.019	1.576	5.496	<0.001
	4-Hydroxyphenyllactic acid	4.928 (3.110)	269.397 (208.630)	-54.664	1.587	3.850	<0.001
	Benzeneacetic acid	0 (0)	9.735 (7.218)	#	2.127	2.945	<0.001
	Pyroglutamic acid	3791.504 (1126.552)	1549.601 (852.815)	2.447	1.222	1.956	0.003
	Alanine	248.951 (140.277)	14.204 (5.676)	17.527	1.298	1.973	0.002
	N-Acetylaspartic acid	123.515 (26.420)	37.305 (20.852)	3.311	1.037	2.228	0.002
	Asparagine	499.134 (141.459)	166.921 (180.847)	2.990	1.592	1.186	0.042
	Urea	27.785 (7.119)	8.736 (7.656)	3.180	1.148	1.344	0.025
	5-Aminovaleric acid	33.055 (10.967)	4.243 (2.326)	7.791	1.866	3.627	<0.001
	Spermine	11.324 (4.371)	1.380 (1.585)	8.206	1.786	3.064	<0.001
	Glycine	4.092 (0.831)	0.103 (0.290)	39.878	1.852	7.621	<0.001

mtp-complement: complemented *mtp*-knockout strain; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages.

Table 4.5 continued. Metabolites best characterising the variation between the *mtp*-complement infected A549 epithelial cells and *mtp*-complement infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Category	Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH- adjusted <i>p</i> -value
		<i>mtp</i> -complement infected A549 epithelial cells	<i>mtp</i> -complement infected THP-1 macrophages				
Fatty acid metabolism	1-Hexadecanol	2.523 (0.832)	16.382 (9.068)	-6.492	1.838	2.911	<0.001
	Dodecanoic acid	25.887 (5.447)	145.179 (59.545)	-5.608	1.624	3.717	<0.001
	Tetradecanoic acid	673.204 (120.947)	1752.900 (494.315)	-2.604	1.501	3.083	<0.001
	4-Hydroxybutanoic acid	6.171 (2.942)	167.409 (217.409)	-27.127	2.015	2.300	0.002
	Batyl alcohol	3.493 (2.605)	42.887 (34.898)	-12.278	1.701	2.010	0.003
	Lanosterol	0 (0)	58.411 (31.024)	#	1.564	4.222	<0.001
Carbon metabolism	Phosphorylethanolamine	207.967 (210.793)	41.405 (52.228)	5.023	1.297	1.389	0.021
	Glyceric acid	66.567 (20.740)	25.021 (15.804)	2.660	1.173	1.824	0.005
	Deoxyglucose	24.341 (5.799)	8.597 (3.615)	2.831	1.379	2.378	0.001
	D-Glucopyranuronic acid	14.191 (6.030)	0.164 (0.314)	86.785	1.740	4.416	<0.001
	Scyllo-Inositol	32.216 (10.847)	5.789 (7.074)	5.565	1.651	1.719	0.009
	Citric acid	3.175 (0.976)	113.883 (55.036)	-35.876	2.437	5.879	<0.001
	Propanal	1171.182 (242.598)	301.517 (319.432)	3.884	1.735	1.371	0.024
	Niacinamide	175.643 (70.691)	19.305 (13.379)	9.098	1.958	3.194	<0.001
Nucleic acid metabolism	Methyl α -D-ribofuranoside	26.532 (14.080)	0.449 (0.640)	59.135	1.888	5.148	<0.001
	Ribonic acid	22.904 (8.522)	0.434 (0.426)	52.763	2.120	7.309	<0.001
	Guanosine	39.175 (12.712)	3.102 (6.466)	12.630	1.340	2.941	<0.001

mtp-complement: complemented *mtp*-knockout strain; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages.

4.5. Discussion

The differentiation of metabolites from either the intracellular pathogen or the infected host model is highly challenging in an untargeted metabolic study. Therefore, the metabolic profiles reported in this study represents the synergistic effect from both pathogen and host within the infection models. Due to the functional differences between AECs and macrophages, baseline concentrations of metabolites, unique to each cell type, will vary considerably. By understanding and eliminating the differences in baseline metabolite concentrations, it is possible to determine the metabolic profile perturbations caused by infection of these two cell types with *M. tuberculosis*.

The surface-located MTP adhesin has been previously reported as an important contributor in host pathogen infection, including serving as an adhesin/invasin, and immune modulator in both A549 and THP-1 cells [2; 4; 15; 16; 20-22]. Additionally, MTP had no effect on macrophage and epithelial cell cytotoxicity levels at four hours and five days post-infection, while impacting the intracellular survival rate of MTP-proficient and deficient strains [40]. Therefore, it has been proposed to be a suitable biomarker for TB diagnostics and therapeutic interventions. The present study aimed to compare the metabolite profiles of A549 epithelial cells to that of THP-1 macrophages, infected with *M. tuberculosis* with and without MTP, in order to understand the mechanism/s employed by this adhesin in TB pathogenesis and which of the two cell types is more integrally involved in this process.

4.5.1. Baseline differences of host cell lines are attributed to the functional roles of each cell type

Amino acids such as arginine, cysteine, methionine, the three branched-chain amino acids (BCAAs; isoleucine, leucine and valine), phenylalanine, threonine, tryptophan, or lysine, enter the lungs through sodium-dependent and independent transporters as there is no *de novo* synthesis of these metabolites [41]. Amino acids are important for the integrity and function of epithelial barriers as they contribute to the synthesis of peptides, bioactive molecules and nucleic acids, which are essential for cell proliferation, immune response and maintaining cellular homeostasis [41]. Functional amino acids, such as arginine, glutamine, or glycine, activate nuclear factor erythroid 2-related factor 2 (Nrf2) survival signalling, in order to maintain the lung epithelial barrier [41], while inhibiting NRLP3 inflammasome in the absence of *M. tuberculosis*. Therefore, in the current study, concentrations of these amino acids and their intermediates in the A549 epithelial cells are an indication of maintenance of natural cell health and functions.

Fatty acids play an important role in the activation of macrophage polarisation states [42]. Classically activated (M1) macrophages exhibit microbicidal function generally induced by interferon (IFN)- γ and/or lipopolysaccharide (LPS) stimulation; whereas, alternatively activated (M2) macrophages exhibit healing, repairing and growth functions generally initiated by interleukin (IL)-4 or IL-13 stimulation, hence M2 macrophages are reported as immunomodulatory and poorly microbicidal [43; 44]. The immunosuppressive function of M2 macrophages allows them to have a high phagocytic capacity, produce extracellular matrix (ECM) components, angiogenic and chemotactic factors [45]. Thus, they serve as cellular reservoirs for many pathogens [45]. More importantly, M1 macrophages rely on aerobic glycolysis and M2 macrophages require the induction of fatty acid oxidation (FAO) and OXPHOS [46]. In the current study, the fatty acids detected in the uninfected macrophages point toward an immunoregulatory profile in the absence of *M. tuberculosis*.

In addition to this, the majority of the metabolites involved in central carbon metabolism (CCM) detected in this study were found in higher concentrations in macrophages relative to the A549

epithelial cells (Table 4.2). Given that CCM produces energy through glycolysis, gluconeogenesis, PPP, and the TCA cycle, which all require carbon substrates [47], elevated concentrations of these metabolites within the macrophages suggest, that they have lower energy demands than the A549 epithelial cells at baseline.

The comparatively higher concentrations of metabolites involved in purine and pyrimidine metabolism in the A549 epithelial cells compared to the THP-1 macrophages were expected, as the epithelial cell line is immortal. Hence, unlike the differentiated macrophages, the epithelial cells were actively growing at the time of cell harvesting, subsequently producing higher concentrations of metabolites involved in deoxynucleic acid (DNA) replication, ribonucleic acid (RNA) synthesis, and cellular bioenergetics [48].

Moreover, metabolites that were detected in either only A549 epithelial cells (pimelic acid) or THP-1 macrophages (kynurenine and lanosterol) are characteristic of natural cell functioning. Pimelic acid is naturally found in lung tissue lesions [49] and is an intermediate in the synthesis of biotin, which is key for maintaining the biochemical integrity of cells. Biotin is a cofactor needed for functioning of enzymes involved in carboxylation/decarboxylation reactions in fatty acid synthesis, amino acid metabolism, gluconeogenesis and TCA cycle [50]. Tryptophan is the essential amino acid, which fuels the synthesis of kynurenine (Kyn), which produces kynurenic acid and nicotinamide adenine dinucleotide (NAD⁺) [51]. The local and systemic balance between tryptophan and kynurenine directly affects metabolic and immune signalling pathways that drive an anti-inflammatory response [51; 52]. Lanosterol acts as an endogenous modulator of macrophage innate immune responses and its accumulation facilitates the increase in reactive oxygen species (ROS) and membrane fluidity, thereby potentiating phagocytosis to allow bacterial killing [53].

4.5.2. Host metabolic profiles suggest that MTP promotes moderate anti-inflammatory responses upon *M. tuberculosis* infection

The uninfected host cell metabolites listed in Table 4.2 served as a point of reference for the infection models. Various changes in the concentrations of these metabolites were observed within the host following infection. The significant metabolites that were not listed in Table 4.2, will be discussed in the sections below as they indicate the metabolic alterations of each cell type in response to *M. tuberculosis* infection in the presence of the MTP adhesin.

The metabolite, MTA, largely has a regulatory effect on tumorigenesis and is toxic to hematopoietic progenitor cells [54]. The MTA is rapidly converted to adenine and 5'-methylthioribose-1-phosphate, which can be redirected to purine and methionine pathways, respectively [55]. This metabolite has been demonstrated to suppress activation, differentiation, and effector function of antigen-specific immune cells without eliciting cell death of T cells [56]. Hence, the higher levels of MTA in epithelial cells, relative to the THP-1 macrophages, in the presence of the MTP adhesin (Table 4.3 and 4.5) indicate a possible prevention of LPS induced lethality, and suppression of circulating tumor necrosis factor- α (TNF- α), inducible nitric oxide synthase (iNOS) expression, and stimulation of interleukin (IL)-10 synthesis [57]. These collectively suggest a protective effect of MTP in favour of *M. tuberculosis*. Moreover, the constant secretion of low amounts of MTA in THP-1 macrophages (Table 4.3 and 4.5) leads to anti-proliferative effects in immune cells [56]. Interestingly, when MTP is absent in the host models (Table 4.4), the inverse is observed for relative mean concentrations of MTA, thus endorsing the immunomodulatory properties for MTA.

Urea was detected in the MTP-proficient (Table 4.3 and 4.5), but not in the MTP-deficient infection model (Table 4.4). Arginine can be metabolised either by inducible iNOS to nitric oxide and citrulline, or it can be hydrolysed to ornithine and urea by arginase [58]. Mycobacterial infection of macrophages facilitates the co-expression of iNOS and arginase [59-61]; therefore, macrophages do

not concisely fit within the classically activated or alternatively activated binary with respect to arginine utilisation in response to *M. tuberculosis* infection [62]. Additionally, asparagine (Table 4.5) functions as an amino acid exchange factor, plays a role in regulation of arginine import [63] and acts as an important source of nitrogen for the intracellular *M. tuberculosis*, thus allowing the pathogen to resist the host immune defence for survival within host cells [64]. Moreover, asparagine regulates the mammalian target of rapamycin complex 1 (mTORC1) activity, and subsequently, protein synthesis [63]. In the case of the epithelial cells, the urea cycle appears to be the favoured pathway for elimination of nitrogenous waste [62].

Adenine plays a dual role in cells as it is not only a fundamental building block of nucleic acids but it makes up part of adenosine triphosphate (ATP) and NAD⁺, which are used for energy generation [65]. The rapid production of ATP and other necessary metabolic intermediates supports pro-inflammatory effector functions. This is evidenced by increased lactate production or glycolytic enzyme expression in human and murine macrophages or lung tissue after *M. tuberculosis* infection [66]. Adenine was detected in both the WT infected (Table 4.3) and Δmtp infected (Table 4.4) host models, and thus, is reflective of the energy requirements of each cell type under these conditions. Compared to the Δmtp infected macrophages, WT infected macrophages had a lower adenine concentration (Table 4.3) suggesting a lower pro-inflammatory response and fewer energy associated molecules in the presence of MTP, as a protective mechanism for the pathogen. However, the A549 epithelial cells, displayed somewhat similar concentrations between the WT infected and Δmtp infected cells, indicating that the deletion of MTP from the model does not alter the energy requirements of the A549 epithelial cells, most likely due to its different functionality as compared to macrophages.

Guanine-based purines, such as the second messenger molecule, cyclic guanosine-monophosphate (cGMP), modulates calcium homeostasis, through activation and alteration of specific ion channels, association with protein kinases, and interaction with phosphodiesterases [67]. Guanosine was detected in higher concentrations in the *mtp*-complement infected A549 cells compared to the THP-1 macrophages (Table 4.5) indicating the metabolite's modulating effects on cellular growth, differentiation and survival through a reduction in apoptosis and inflammation [68] in the presence of overexpressed MTP. Moreover, since cGMP is associated with the release of TNF- α by activated macrophages [69], the low levels of guanosine in the *mtp*-complement infected THP-1 macrophages indicate an inhibited M1 macrophage polarisation in the presence of MTP.

The accumulation of unsaturated fatty acid, 5,8,11-eicosatrienoic acid, which is synthesised by the elongation and desaturation of oleic acid, has been previously associated with an essential fatty acid deficiency [70], in particular, a linoleic acid deficiency [71]. Moreover, conjugated-linoleic acid is associated with anti-*M. tuberculosis* activities [72] as omega 6 fatty acids promote airway inflammation by multiple pathways [73]. In the case of infected macrophages, fatty acids are characteristically imported and metabolised by *M. tuberculosis* to maintain infection [74]. The preferential utilisation of OXPHOS and FAO drives anti-inflammatory responses [75]. Therefore, the comparatively lower concentrations of 5,8,11-eicosatrienoic acid in the THP-1 macrophages, indicates an anti-inflammatory environment for *M. tuberculosis* to thrive in (Table 4.3). This is supported by the reduced concentrations of butanoic acid in the same cell line (Table 4.3). The presence of butyrate is associated with attenuated lung inflammation in ovalbumin (OVA)-challenged mice [76] and human peripheral blood mononuclear represented by a decrease in *M. tuberculosis*-induced proinflammatory cytokine responses, and an increase in IL-10 production [77].

Niacin and niacinamide/ nicotinamide are different forms of vitamin B3 [78], which are highly effective in preventing lung tissue damage [79]. More recently, it has been demonstrated that nicotinamide inhibited replication of attenuated Bacille Calmette-Guérin (BCG) but not *Mycobacterium bovis* or *M. tuberculosis* within macrophages [78]. Higher levels of niacinamide were

observed in WT infected and *mtp*-complement infected A549 epithelial cell model relative to the THP-1 macrophage model (Tables 4.3 and 4.5); however, in the absence of MTP, the macrophages accumulated this metabolite, in contrast to the lower concentrations in the A549 epithelial cells (Tables 4.4 and 4.5). An identical pattern for niacin was observed between the WT infected and Δmtp infected cell types, with the macrophages showing increased concentrations and the epithelial cells showing a decrease of the metabolite when the adhesin was removed. These metabolic profiles suggest the role of MTP in supporting growth of *M. tuberculosis* in both A549 epithelial cells and THP-1 macrophages in the presence of this adhesion, whereas the absence motivates cell death of A549 epithelial while inhibiting pathogen survival in macrophages.

Infection of the host cells with the *mtp*-complement revealed that the majority of the host metabolic concentrations returned to a level similar to the WT infected hosts. The host metabolic deviations from the WT infected profiles regarding carbon metabolism may be a knock-on effect of complement construction, subsequent to construction of the Δmtp . Overall, the WT infected and *mtp*-complement infected host profiles were comparable.

4.5.3. Removal of the MTP adhesin potentially facilitates infected THP-1 macrophages to efficiently remove the less virulent *M. tuberculosis* via pro-inflammatory systems

Macrophages exploit amino acid catabolism to sustain activation and maintenance of immune activity, mainly through mTOR signalling, nitric oxide production, immunomodulatory catabolites and metabolic competitors [80]. Increased intracellular levels of essential amino acids are known to enhance mTORC1 signalling pathway, thereby facilitating M1 polarisation of macrophages in mice [81] and preventing cell autophagy [82]. Moreover, transamination of amino acids allow them to enter the TCA cycle at one of many points, such as α -ketoglutarate, succinyl-CoA, fumarate, oxaloacetate, pyruvate, or acetyl-CoA [83]. This permits the use of the carbon skeletons of amino acids as fuel [83]. In the current study, the Δmtp infected macrophages displayed comparatively higher concentrations of 27 of the 28 amino acids when compared to the epithelial cells (Table 4.4). These findings indicate major, significant changes in the metabolic profile of THP-1 macrophages induced by the absence of MTP, with implications for changes to the TCA cycle, important for anabolic or energetic purposes and sustaining an inflammatory response [83]. The up to 2017.15-fold higher concentrations in these metabolites suggest higher pro-inflammatory responses in the macrophages when compared to epithelial cells in the absence of MTP. Contrastingly, despite not being significantly differentially detected in the other models (Tables 4.2, 4.3 and 4.5), the concentrations of the majority of the metabolites remained at a constant level for the A549 epithelial cells in the absence of MTP. This suggests that in the absence of the MTP adhesin, epithelial cells are not as predisposed as macrophages to metabolic alterations.

As intermediates of fatty acid synthesis, decanoic acid and tetradecanoic acid were detected in elevated concentrations in the Δmtp infected macrophages, relative to the Δmtp infected A549 epithelial cells (Table 4.4). These findings coincide with other studies, which demonstrated that upon macrophage infection with *M. bovis* BCG, *de novo* fatty acid synthesis is induced by the rate limiting enzyme, acetyl-CoA carboxylase (ACC1) [84]. Furthermore, it was demonstrated that ACC1 has no effect on systemic pro-inflammatory cytokine production in macrophages or T cell priming [84]. This suggest that fatty acid synthesis is dispensable for an intact innate response against mycobacteria [84]. Inositols, such as myo-inositol and its isomer, scyllo-inositol [85], exhibit tissue specific concentrations, which subsequently allows for various differential biological effects. They act as growth-promoting factors of mammalian cells, lipotropic factors, co-factors of enzymes, messenger

molecules in signal transduction and play a role in regulation of ROS production [86]. Pro-inflammatory macrophages exhibit higher levels of ROS and extracellular hydrogen peroxide compared to the anti-inflammatory macrophages [87]. In an attempt to digest lipoproteins, macrophages are known to create an acidic extracellular hydrolytic compartment by which acidification by vacuolar ATPase, enables aggregate catabolism through lysosomal acid hydrolases [88]. High levels of ribitol suggest a greater impact of the PPP pathway in acidic stress as compared to oxidative and iron stresses [89].

The accumulation of both inositols and ribitol in the Δmtp infected macrophages relative to the Δmtp infected A549 epithelial cells (Table 4.4) is an indication of an acidic pro-inflammatory environment within macrophages in the absence of MTP. Moreover, lactate, as a product of glycolysis, is a potential carbon source for *M. tuberculosis*. The comparatively higher lactate within the infected THP-1 macrophages in the absence of MTP (Table 4.4), indicates an inefficiency of *M. tuberculosis* in utilising lactate as an additional carbon source possibly resulting in intracellular growth retardation. Furthermore, higher levels of lactate indicates a glycolytic, pro-inflammatory phenotype of macrophages [90] thereby increasing the host capacity to kill *M. tuberculosis* [91] in the absence of MTP.

Pseudouridine is an intermediate in the pyrimidine metabolic pathway and is involved in RNA modification of stable noncoding RNAs (ncRNAs) [92]. It plays a critical role in the control of multiple stages of gene expression regulation [92]. Studies have found that pseudouridine concentrations were higher in patients treated with BCG compared to those that were not. This was explained by the upregulation of glucose transporter and enzyme encoding genes in BCG exposed lymphocytes, which are known to switch from OXPHOS to high aerobic glycolysis [93]. Therefore, in the present study, the perturbations to host metabolic signatures, in the absence of the MTP adhesin, mainly observed in the macrophages, suggests an attempt to prompt a cascade of pro-inflammatory responses to activate effective pathogen killing mechanisms.

In this study, an untargeted metabolomics approach was used to detect a broad spectrum of metabolites in an unbiased manner, in order to obtain a holistic interpretation of the various perturbations. A targeted approach such as one utilising carbon tracing of metabolites through cellular pathways, may now provide valuable information to confirm the deductions made from this study. Furthermore, more targeted metabolomics approaches may also be used. Additionally, this study was purely *in vitro*, therefore, these results do not represent the natural environment as many external factors will have to be considered when pathogen virulence and host defence mechanisms compete against each other.

Host responses to infection with *M. tuberculosis* are complicated, as they are influenced by virulence factors of the pathogen, as well as cell type and function, both elaborately regulated by molecular mechanisms that remain to be fully elucidated. Moreover, relative mean concentrations were compared between A549 epithelial cells and THP-1 macrophages, which have vastly different metabolite signatures at baseline, due to their very different functions. Consequently, if a metabolite plays an important role in metabolism but was not included as a significant metabolite, it is because they were found in similar concentrations in both host models.

Host cell viability after infection with the three strains of *M. tuberculosis* had not been determined for this study. However, the differences in the metabolic profiles are attributed to the different genetic makeup of the three strains of *M. tuberculosis*, rather than differential lysis of the host cells as supported by Ramsugit and Pillay in 2019 [40]. It is emphasised that this study only investigated early infection time points of either cell type; therefore, the metabolic signatures represent only a snapshot of the changes elicited by the MTP adhesin and does not represent the long-term effect of the lack of the adhesion on the host cells.

Nonetheless, this study does provide noteworthy insights into the diversities of baseline metabolite profiles of these cell lines, in addition to the initial changes induced by *M. tuberculosis* in the presence and absence of MTP. Cell polarisation states, selective metabolite synthesis/degradation pathways, gene regulation and immunological assays can also be further investigated in the presence and absence of MTP to confirm findings of this study.

4.6. Conclusion

This study was conducted in an effort to improve the current understanding of the role of MTP in modulating metabolic pathways of different host cell lines encountered during the initial stages of infection. The cumulative alterations to host metabolome profiles suggest that macrophages, compared to epithelial cells, advocate for the clearing of the pathogen through a more efficient pro-inflammatory response in the absence of MTP during *M. tuberculosis* infection. This contrasted with the epithelial cells, which did not elicit major changes in metabolome profiles in the absence of the MTP adhesin, as they are not biologically programmed to accelerate pathogen removal/killing from the site of infection. Holistically, the alterations to both macrophage and epithelial cell metabolic profiles suggests that the MTP adhesin plays a role in securing a protective environment for *M. tuberculosis* during initial stages of infection. The current data substantiates MTP as a virulence factor of note, thereby supporting its use as a biomarker for development of innovative diagnostic and therapeutic intervention.

References:

1. World Health Organization (2021). Impact of the COVID-19 pandemic on TB detection and mortality in 2020. *World Health Organization* [Online]. Available: <https://www.who.int/publications/m/item/impact-of-the-covid-19-pandemic-on-tb-detection-and-mortality-in-2020> [Accessed 8 October 2021].
2. Alteri, C.J., Xicohténcatl-Cortes, J., Hess, S., Caballero-Olín, G., Girón, J.A., and Friedman, R.L. (2007). *Mycobacterium tuberculosis* produces pili during human infection. *Proceedings of the National Academy of Sciences of the United States of America*, 104(12), 5145-5150. <https://doi.org/10.1073/pnas.0602304104>.
3. Pethe, K., Alonso, S., Biet, F., Delogu, G., Brennan, M.J., Locht, C., and Menozzi, F.D. (2001). The heparin-binding haemagglutinin of *M. tuberculosis* is required for extrapulmonary dissemination. *Nature*, 412(6843), 190-194. <https://doi.org/10.1038/35084083>.
4. Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* pili promote adhesion to and invasion of THP-1 macrophages. *Japanese Journal of Infectious Diseases*, 67(6), 476-478. <https://doi.org/10.7883/yoken.67.476>.
5. Ramsugit, S., Pillay, B., and Pillay, M. (2016). Evaluation of the role of *Mycobacterium tuberculosis* pili (MTP) as an adhesin, invasin, and cytokine inducer of epithelial cells. *The Brazilian Journal of Infectious Diseases*, 20(2), 160-165. <http://dx.doi.org/10.1016/j.bjid.2015.11.002>.
6. Be, N.A., Bishai, W.R., and Jain, S.K. (2012). Role of *Mycobacterium tuberculosis* pknD in the pathogenesis of central nervous system tuberculosis. *BMC Microbiology*, 12, 7. <https://doi.org/10.1186/1471-2180-12-7>.
7. Hickey, T.B.M., Thorson, L.M., Speert, D.P., Daffé, M., and Stokes, R.W. (2009). *Mycobacterium tuberculosis* Cpn60.2 and DnaK are located on the bacterial surface, where

- Cpn60.2 facilitates efficient bacterial association with macrophages. *Infection and Immunity*, 77(8), 3389-3401. <https://doi.org/10.1128/iai.00143-09>.
8. Kumar, S., Puniya, B.L., Parween, S., Nahar, P., and Ramachandran, S. (2013). Identification of novel adhesins of *M. tuberculosis* H37Rv using integrated approach of multiple computational algorithms and experimental analysis. *PloS One*, 8(7), e69790. <https://doi.org/10.1371/journal.pone.0069790>.
 9. Kinhikar, A.G., Vargas, D., Li, H., Mahaffey, S.B., Hinds, L., Belisle, J.T., and Laal, S. (2006). *Mycobacterium tuberculosis* malate synthase is a laminin-binding adhesin. *Molecular Microbiology*, 60(4), 999-1013. <https://doi.org/10.1111/j.1365-2958.2006.05151.x>.
 10. Brennan, M.J., Delogu, G., Chen, Y., Bardarov, S., Kriakov, J., Alavi, M., and Jacobs, W.R., Jr. (2001). Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. *Infection and Immunity*, 69(12), 7326-7333. <https://doi.org/10.1128/iai.69.12.7326-7333.2001>.
 11. Esparza, M., Palomares, B., García, T., Espinosa, P., Zenteno, E., and Mancilla, R. (2015). PstS-1, the 38-kDa *Mycobacterium tuberculosis* glycoprotein, is an adhesin, which binds the macrophage mannose receptor and promotes phagocytosis. *Scandinavian Journal of Immunology*, 81(1), 46-55. <https://doi.org/10.1111/sji.12249>.
 12. Diaz-Silvestre, H., Espinosa-Cueto, P., Sanchez-Gonzalez, A., Esparza-Ceron, M.A., Pereira-Suarez, A.L., Bernal-Fernandez, G., Espitia, C., and Mancilla, R. (2005). The 19-kDa antigen of *Mycobacterium tuberculosis* is a major adhesin that binds the mannose receptor of THP-1 monocytic cells and promotes phagocytosis of mycobacteria. *Microbial Pathogenesis*, 39(3), 97-107. <https://doi.org/10.1016/j.micpath.2005.06.002>.
 13. Ragas, A., Roussel, L., Puzo, G., and Rivi re, M. (2007). The *Mycobacterium tuberculosis* cell-surface glycoprotein apa as a potential adhesin to colonize target cells via the innate immune system pulmonary C-type lectin surfactant protein A. *The Journal of Biological Chemistry*, 282(8), 5133-5142. <https://doi.org/10.1074/jbc.m610183200>.
 14. Alteri, C. (2005). *Novel pili of Mycobacterium tuberculosis*. Doctoral thesis, The University of Arizona, United States of America. <https://repository.arizona.edu/handle/10150/195515?show=full>.
 15. Ramsugit, S., Guma, S., Pillay, B., Jain, P., Larsen, M.H., Danaviah, S., and Pillay, M. (2013). Pili contribute to biofilm formation *in vitro* in *Mycobacterium tuberculosis*. *Antonie Van Leeuwenhoek*, 104(5), 725-735. <https://doi.org/10.1007/s10482-013-9981-6>.
 16. Naidoo, N., Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* pili (MTP), a putative biomarker for a tuberculosis diagnostic test. *Tuberculosis*, 94(3), 338-345. <http://dx.doi.org/10.1016/j.tube.2014.03.004>.
 17. Naidoo, N., Pillay, B., Bubb, M., Pym, A., Chiliza, T., Naidoo, K., Ndungu, T., Kasproicz, V.O., and Pillay, M. (2018). Evaluation of a synthetic peptide for the detection of anti-*Mycobacterium tuberculosis* curli pili IgG antibodies in patients with pulmonary tuberculosis. *Tuberculosis* 109, 80-84. 10.1016/j.tube.2018.01.007.
 18. Dlamini, M.T. (2016). *Whole transcriptome analysis to elucidate the role of mtp in gene regulation of pulmonary epithelial cells infected with Mycobacterium tuberculosis*. Masters dissertation University of KwaZulu-Natal, Durban, South Africa. <https://researchspace.ukzn.ac.za/items/71f0ed4e-aa63-4cca-8bf6-4d771326e616>.
 19. Nyawo, G. (2016). *The role of Mycobacterium tuberculosis pili in pathogenesis: Growth and survival kinetics, gene regulation and host immune response, and in vitro growth kinetics*. Masters dissertation University of KwaZulu-Natal, Durban, South Africa. <https://researchspace.ukzn.ac.za/items/3628eab3-3e5f-47a6-9eb3-07c3f3c52ad7>.
 20. Ashokcoomar, S., Reedoy, K.S., Senzani, S., Loots, D.T., Beukes Maasdorp, D., Van Reenen, M., Pillay, B., and Pillay, M. (2020). *Mycobacterium tuberculosis* curli pili (MTP) deficiency

- is associated with alterations in cell wall biogenesis, fatty acid metabolism and amino acid synthesis. *Metabolomics*, 16(9), 97. <https://doi.org/10.1007/s11306-020-01720-z>.
21. Ashokcoomar, S., Loots, D.T., Beukes, D., Van Reenen, M., Pillay, B., and Pillay, M. (2021). *M. tuberculosis* curli pili (MTP) is associated with alterations in carbon, fatty acid and amino acid metabolism in a THP-1 macrophage infection model. *Microbial Pathogenesis*, 154, 104806. <https://doi.org/10.1016/j.micpath.2021.104806>.
 22. Reedoy, K.S., Loots, D.T., Beukes, D., Reenen, M.V., Pillay, B., and Pillay, M. (2020). *Mycobacterium tuberculosis* curli pili (MTP) is associated with significant host metabolic pathways in an A549 epithelial cell infection model and contributes to the pathogenicity of *Mycobacterium tuberculosis*. *Metabolomics*, 16(11), 116. <http://dx.doi.org/10.1007/s11306-020-01736-5>.
 23. Du Preez, I., Luies, L., and Loots, D.T. (2019). The application of metabolomics toward pulmonary tuberculosis research. *Tuberculosis*, 115, 126-139. <https://doi.org/10.1016/j.tube.2019.03.003>.
 24. Fernández-García, M., Rey-Stolle, F., Boccard, J., Reddy, V.P., García, A., Cumming, B.M., Steyn, A.J.C., Rudaz, S., and Barbas, C. (2020). Comprehensive examination of the mouse lung metabolome following *Mycobacterium tuberculosis* infection using a multiplatform mass spectrometry approach. *Journal of Proteome Research*, 19(5), 2053-2070. <https://doi.org/10.1021/acs.jproteome.9b00868>.
 25. Mirsaeidi, M., Banoei, M.M., Winston, B.W., and Schraufnagel, D.E. (2015). Metabolomics: Applications and promise in mycobacterial disease. *Annals of the American Thoracic Society*, 12(9), 1278-1287. <https://doi.org/10.1513/annalsats.201505-279ps>.
 26. Dunn, W.B. , and Ellis, D.I. (2005). Metabolomics: Current analytical platforms and methodologies. *TrAC Trends in Analytical Chemistry*, 24(4), 285-294. <http://dx.doi.org/10.1016/j.trac.2004.11.021>.
 27. Bissonnette, E.Y., Lauzon-Joset, J.-F., Debley, J.S., and Ziegler, S.F. (2020). Cross-talk between alveolar macrophages and lung epithelial cells is essential to maintain lung homeostasis. *Frontiers in Immunology*, 11(2734). 10.3389/fimmu.2020.583042.
 28. Gandhi, N.R., Moll, A., Sturm, A.W., Pawinski, R., Govender, T., Lalloo, U., Zeller, K., Andrews, J., and Friedland, G. (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet*, 368(9547), 1575-1580. [https://doi.org/10.1016/s0140-6736\(06\)69573-1](https://doi.org/10.1016/s0140-6736(06)69573-1).
 29. Larsen, M.H., Biermann, K., Tandberg, S., Hsu, T., and Jacobs, W.R., Jr. (2007). Genetic manipulation of *Mycobacterium tuberculosis*. *Current Protocols in Microbiology*, Chapter 10, Unit 10A.12. <https://doi.org/10.1002/9780471729259.mc10a02s6>.
 30. Beukes, D., Du Preez, I., and Loots, D.T. (2019). Total metabolome extraction from mycobacterial cells for GC-MS metabolomics analysis. *Methods in Molecular Biology*, 1859, 121-131. https://doi.org/10.1007/978-1-4939-8757-3_6.
 31. Van Den Berg, R.A., Hoefsloot, H.C.J., Westerhuis, J.A., Smilde, A.K., and Van Der Werf, M.J. (2006). Centering, scaling, and transformations: Improving the biological information content of metabolomics data. *BMC Genomics*, 7, 142. <https://doi.org/10.1186/1471-2164-7-142>.
 32. Fisher, R.A. (1992). The arrangement of field experiments. *Breakthroughs in Statistics*, 82-91. https://doi.org/10.1007/978-1-4612-4380-9_8.
 33. Benjamini, Y. , and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, 57(1), 289-300. <https://doi.org/10.1111/j.2517-6161.1995.tb02031.x>.

34. Sullivan, G.M. , and Feinn, R. (2012). Using effect size-or why the *p* value is not enough. *Journal of Graduate Medical Education*, 4(3), 279-282. <https://doi.org/10.4300/jgme-d-12-00156.1>.
35. Clum, A., Huntemann, M., Bushnell, B., Foster, B., Foster, B., Roux, S., Hajek Patrick, P., Varghese, N., Mukherjee, S., Reddy, T.B.K., Daum, C., Yoshinaga, Y., O'Malley, R., Seshadri, R., Kyrpides Nikos, C., Elo-Fadrosch Emiley, A., Chen, I.M.A., Copeland, A., and Ivanova Natalia, N. (2021). DOE JGI Metagenome Workflow. *mSystems*, 6(3), 10.1128/msystems.00804-00820. 10.1128/msystems.00804-20.
36. Huntemann, M., Ivanova, N.N., Mavromatis, K., Tripp, H.J., Paez-Espino, D., Tennessen, K., Palaniappan, K., Szeto, E., Pillay, M., Chen, I.M.A., Pati, A., Nielsen, T., Markowitz, V.M., and Kyrpides, N.C. (2016). The standard operating procedure of the DOE-JGI Metagenome Annotation Pipeline (MAP v.4). *Standards in Genomic Sciences*, 11(1), 17. <https://doi.org/10.1186/s40793-016-0138-x>.
37. Akarachantachote, N., Chadcham, S., and Saithanu, K. (2014). Cut-off threshold of variable importance in projection for variable selection. *International Journal of Pure and Applied Mathematics*, 94(3), 307-322. <http://dx.doi.org/10.12732/ijpam.v94i3.2>.
38. Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., Li, Q., Shoemaker, B.A., Thiessen, P.A., Yu, B., Zaslavsky, L., Zhang, J., and Bolton, E.E. (2020). PubChem in 2021: New data content and improved web interfaces. *Nucleic Acids Research*, 49(D1), D1388-D1395. <https://doi.org/10.1093/nar/gkaa971>.
39. Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., and Morishima, K. (2017). KEGG: New perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Research*, 45(D1), D353-D361. <https://doi.org/10.1093/nar/gkw1092>.
40. Ramsugit, S. , and Pillay, M. (2019). Curli pili affect the intracellular survival of *Mycobacterium tuberculosis*. *Journal of Infecting in Develpoing Countries*, 13(2), 179-180. <http://dx.doi.org/10.3855/jidc.9942>.
41. Chen, J., Jin, Y., Yang, Y., Wu, Z., and Wu, G. (2020). Epithelial dysfunction in lung diseases: Effects of amino acids and potential mechanisms. *Advances in Experimental Medicine and Biology*, 1265, 57-70. https://doi.org/10.1007/978-3-030-45328-2_4.
42. Batista-Gonzalez, A., Vidal, R., Criollo, A., and Carreño, L.J. (2020). New insights on the role of lipid metabolism in the metabolic reprogramming of macrophages. *Frontiers in Immunology*, 10(2993). <https://doi.org/10.3389/fimmu.2019.02993>.
43. Arora, S., Dev, K., Agarwal, B., Das, P., and Syed, M.A. (2018). Macrophages: Their role, activation and polarization in pulmonary diseases. *Immunobiology*, 223(4-5), 383-396. <https://doi.org/10.1016/j.imbio.2017.11.001>.
44. Orecchioni, M., Ghosheh, Y., Pramod, A.B., and Ley, K. (2019). Macrophage polarization: Different gene signatures in M1(LPS+) vs. classically and M2(LPS-) vs. alternatively activated macrophages. *Frontiers in Immunology*, 10, 1084. <https://doi.org/10.3389/fimmu.2019.01084>.
45. Röszer, T. (2015). Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. *Mediators of Inflammation*, 2015, 816460. <http://dx.doi.org/10.1155/2015/816460>.
46. Genoula, M., Franco, J.L.M., Maio, M., Dolotowicz, B., Ferreyra, M., Milillo, M.A., Mascarau, R., Moraña, E.J., Palmero, D., Matteo, M., Fuentes, F., López, B., Barrionuevo, P., Neyrolles, O., Cougoule, C., Lugo-Villarino, G., Vérollet, C., del Carmen Sasiain, M., and Balboa, L. (2020). Fatty acid oxidation of alternatively activated macrophages prevents foam cell formation, but *Mycobacterium tuberculosis* counteracts this process via HIF-1 α activation. *PLOS Pathogens*, 16(10), e1008929. <https://doi.org/10.1371/journal.ppat.1008929>.

47. Cumming, B.M. , and Steyn, A.J.C. (2015). Metabolic plasticity of central carbon metabolism protects mycobacteria. *Proceedings of the National Academy of Sciences*, 112(43), 13135-13136. <https://doi.org/10.1073/pnas.1518171112>.
48. Siddiqui, A. , and Ceppi, P. (2020). A non-proliferative role of pyrimidine metabolism in cancer. *Molecular Metabolism*, 35, 100962. <https://doi.org/10.1016/j.molmet.2020.02.005>.
49. Consden, R. , and Howard, A. (1957). Improved methods for detecting alpha, epsilon-diaminopimelic acid in lung lesions. *Journal of Clinical Pathology*, 10(2), 178-181. 10.1136/jcp.10.2.178.
50. Tong, L. (2013). Structure and function of biotin-dependent carboxylases. *Cellular and Molecular Life Sciences*, 70(5), 863-891. <https://doi.org/10.1007/s00018-012-1096-0>.
51. Sorgdrager, F.J.H., Naudé, P.J.W., Kema, I.P., Nollen, E.A., and Deyn, P.P. (2019). Tryptophan metabolism in inflammaging: From biomarker to therapeutic target. *Frontiers in Immunology*, 10, 2565. <https://doi.org/10.3389/fimmu.2019.02565>.
52. Minhas, P.S., Liu, L., Moon, P.K., Joshi, A.U., Dove, C., Mhatre, S., Contrepois, K., Wang, Q., Lee, B.A., Coronado, M., Bernstein, D., Snyder, M.P., Migaud, M., Majeti, R., Mochly-Rosen, D., Rabinowitz, J.D., and Andreasson, K.I. (2019). Macrophage *de novo* NAD⁺ synthesis specifies immune function in aging and inflammation. *Nature Immunology*, 20(1), 50-63. <https://doi.org/10.1038/s41590-018-0255-3>.
53. Araldi, E., Fernández-Fuertes, M., Canfrán-Duque, A., Tang, W., Cline, G.W., Madrigal-Matute, J., Pober, J.S., Lasunción, M.A., Wu, D., Fernández-Hernando, C., and Suárez, Y. (2017). Lanosterol modulates TLR4-mediated innate immune responses in macrophages. *Cell Reports*, 19(13), 2743-2755. <https://doi.org/10.1016/j.celrep.2017.05.093>.
54. Li, Y., Wang, Y., and Wu, P. (2019). 5'-Methylthioadenosine and cancer: Old molecules, new understanding. *Journal of Cancer*, 10(4), 927-936. <https://doi.org/10.7150/jca.27160>.
55. Angala, S.K., McNeil, M.R., Shi, L., Joe, M., Pham, H., Zuberogitia, S., Nigou, J., Boot, C.M., Lowary, T.L., Gilleron, M., and Jackson, M. (2017). Biosynthesis of the methylthioxylose capping motif of lipoarabinomannan in *Mycobacterium tuberculosis*. *ACS Chemical Biology*, 12(3), 682-691. <https://doi.org/10.1021/acscchembio.6b01071>.
56. Henrich, F.C., Singer, K., Poller, K., Bernhardt, L., Strobl, C.D., Limm, K., Ritter, A.P., Gottfried, E., Völkl, S., Jacobs, B., Peter, K., Mougiakakos, D., Dettmer, K., Oefner, P.J., Bosserhoff, A.-K., Kreutz, M.P., Aigner, M., and Mackensen, A. (2016). Suppressive effects of tumor cell-derived 5'-deoxy-5'-methylthioadenosine on human T cells. *Oncoimmunology*, 5(8), e1184802. <https://doi.org/10.1080/2162402x.2016.1184802>.
57. Hevia, H., Varela-Rey, M., Corrales, F.J., Berasain, C., Martínez-Chantar, M.L., Latasa, M.U., Lu, S.C., Mato, J.M., García-Trevijano, E.R., and Avila, M.A. (2004). 5'-methylthioadenosine modulates the inflammatory response to endotoxin in mice and in rat hepatocytes. *Hepatology*, 39(4), 1088-1098. <https://doi.org/10.1002/hep.20154>.
58. Rath, M., Müller, I., Kropf, P., Closs, E.I., and Munder, M. (2014). Metabolism via arginase or nitric oxide synthase: Two competing arginine pathways in macrophages. *Frontiers in Immunology*, 5, 532. <http://dx.doi.org/10.3389/fimmu.2014.00532>.
59. El Kasmi, K.C., Qualls, J.E., Pesce, J.T., Smith, A.M., Thompson, R.W., Henao-Tamayo, M., Basaraba, R.J., König, T., Schleicher, U., Koo, M.S., Kaplan, G., Fitzgerald, K.A., Tuomanen, E.I., Orme, I.M., Kanneganti, T.D., Bogdan, C., Wynn, T.A., and Murray, P.J. (2008). Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nature Immunology*, 9(12), 1399-1406. <https://doi.org/10.1038/ni.1671>.
60. Mattila, J.T., Ojo, O.O., Kepka-Lenhart, D., Marino, S., Kim, J.H., Eum, S.Y., Via, L.E., Barry, C.E., 3rd, Klein, E., Kirschner, D.E., Morris, S.M., Jr., Lin, P.L., and Flynn, J.L. (2013). Microenvironments in tuberculous granulomas are delineated by distinct populations

- of macrophage subsets and expression of nitric oxide synthase and arginase isoforms. *Journal of Immunology*, 191(2), 773-784. <https://doi.org/10.4049/jimmunol.1300113>.
61. Qualls, J.E., Subramanian, C., Rafi, W., Smith, A.M., Balouzian, L., DeFreitas, A.A., Shirey, K.A., Reutterer, B., Kernbauer, E., Stockinger, S., Decker, T., Miyairi, I., Vogel, S.N., Salgame, P., Rock, C.O., and Murray, P.J. (2012). Sustained generation of nitric oxide and control of mycobacterial infection requires argininosuccinate synthase 1. *Cell Host & Microbe*, 12(3), 313-323. <https://doi.org/10.1016/j.chom.2012.07.012>.
 62. Crowther, R.R. , and Qualls, J.E. (2021). Metabolic regulation of immune responses to *Mycobacterium tuberculosis*: a spotlight on L-arginine and L-tryptophan metabolism. *Frontiers in Immunology*, 11(628432). <https://doi.org/10.3389/fimmu.2020.628432>.
 63. Krall, A.S., Xu, S., Graeber, T.G., Braas, D., and Christofk, H.R. (2016). Asparagine promotes cancer cell proliferation through use as an amino acid exchange factor. *Nature Communications*, 7, 11457. <https://doi.org/10.1038/ncomms11457>.
 64. Gouzy, A., Larrouy-Maumus, G., Bottai, D., Levillain, F., Dumas, A., Wallach, J.B., Caire-Brandli, I., de Chastellier, C., Wu, T.-D., Poincloux, R., Brosch, R., Guerquin-Kern, J.-L., Schnappinger, D., Sório de Carvalho, L.P., Poquet, Y., and Neyrolles, O. (2014). *Mycobacterium tuberculosis* exploits asparagine to assimilate nitrogen and resist acid stress during infection. *PLoS Pathogens*, 10(2), e1003928. <https://doi.org/10.1371/journal.ppat.1003928>.
 65. Haag, F., Adriouch, S., Braß, A., Jung, C., Möller, S., Scheuplein, F., Bannas, P., Seman, M., and Koch-Nolte, F. (2007). Extracellular NAD and ATP: Partners in immune cell modulation. *Purinergic Signalling*, 3(1-2), 71-81. <https://doi.org/10.1007/s11302-006-9038-7>.
 66. Vrieling, F., Kostidis, S., Spaink, H.P., Haks, M.C., Mayboroda, O.A., Ottenhoff, T.H.M., and Joosten, S.A. (2020). Analyzing the impact of *Mycobacterium tuberculosis* infection on primary human macrophages by combined exploratory and targeted metabolomics. *Scientific Reports*, 10(1), 7085. <https://doi.org/10.1038/s41598-020-62911-1>.
 67. Belluardo, N., Mudò, G., Di Liberto, V., Frinchi, M., Condorelli, D.F., Traversa, U., Ciruela, F., Ciccarelli, R., Di Iorio, P., and Giuliani, P. (2021). Investigating the role of guanosine on human neuroblastoma cell differentiation and the underlying molecular mechanisms. *Frontiers in Pharmacology*, 12, 658806. <https://doi.org/10.3389/fphar.2021.658806>.
 68. Jiang, S., Bendjelloul, F., Ballerini, P., D'Alimonte, I., Nargi, E., Jiang, C., Huang, X., and Rathbone, M.P. (2007). Guanosine reduces apoptosis and inflammation associated with restoration of function in rats with acute spinal cord injury. *Purinergic Signalling*, 3(4), 411-421. <https://doi.org/10.1007/s11302-007-9079-6>.
 69. Lin, S.H., Chuang, H.Y., Ho, J.C., Lee, C.H., and Hsiao, C.C. (2018). Treatment with TNF- α inhibitor rectifies M1 macrophage polarization from blood CD14⁺ monocytes in patients with psoriasis independent of STAT1 and IRF-1 activation. *Journal of Dermatological Science*, 91(3), 276-284. <https://doi.org/10.1016/j.jdermsci.2018.05.009>.
 70. Mead, J.F. , and Slaton, W.H., Jr. (1956). Metabolism of essential fatty acids. III. Isolation of 5,8,11-eicosatrienoic acid from fat-deficient rats. *The Journal of Biological Chemistry*, 219(2), 705-709. [https://doi.org/10.1016/S0021-9258\(18\)65729-1](https://doi.org/10.1016/S0021-9258(18)65729-1).
 71. Kinoshita, Y., Yoshizawa, K., Hamazaki, K., Emoto, Y., Yuri, T., Yuki, M., Shikata, N., Kawashima, H., and Tsubura, A. (2014). Mead acid inhibits the growth of KPL-1 human breast cancer cells *in vitro* and *in vivo*. *Oncology Reports*, 32(4), 1385-1394. <https://doi.org/10.3892/or.2014.3390>.
 72. Choi, W.H. (2016). Evaluation of anti-tubercular activity of linolenic acid and conjugated-linoleic acid as effective inhibitors against *Mycobacterium tuberculosis*. *Asian Pacific Journal of Tropical Medicine*, 9(2), 125-129. <https://doi.org/10.1016/j.apjtm.2016.01.021>.

73. Rutting, S., Xenaki, D., Lau, E., Horvat, J., Wood, L.G., Hansbro, P.M., and Oliver, B.G. (2018). Dietary omega-6, but not omega-3, polyunsaturated or saturated fatty acids increase inflammation in primary lung mesenchymal cells. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 314(6), L922-L935. 10.1152/ajplung.00438.2017.
74. Nazarova, E.V., Montague, C.R., Huang, L., La, T., Russell, D., and VanderVen, B.C. (2019). The genetic requirements of fatty acid import by *Mycobacterium tuberculosis* within macrophages. *eLife*, 8, e43621. <https://doi.org/10.7554/elife.43621>.
75. Shim, D., Kim, H., and Shin, S.J. (2020). *Mycobacterium tuberculosis* infection-driven foamy macrophages and their implications in tuberculosis control as targets for host-directed therapy. *Frontiers in Immunology*, 11, 910. <https://doi.org/10.3389/fimmu.2020.00910>.
76. Vieira, R.d.S., Castoldi, A., Basso, P.J., Hiyane, M.I., Câmara, N.O.S., and Almeida, R.R. (2019). Butyrate attenuates lung inflammation by negatively modulating Th9 cells. *Frontiers in Immunology*, 10, 67. <https://doi.org/10.3389/fimmu.2019.00067>.
77. Lachmandas, E., Van Den Heuvel, M., C.N.A., Damen, M.S.M.A., Cleophas, M.C.P., Netea, M.G., and Van Crevel, R. (2016). Diabetes mellitus and increased tuberculosis susceptibility: The role of short-chain fatty acids. *Journal of Diabetes Research*, 2016, 6014631. <https://doi.org/10.1155/2016/6014631>.
78. Simmons, J.D., Peterson, G.J., Campo, M., Lohmiller, J., Skerrett, S.J., Tunaru, S., Offermanns, S., Sherman, D.R., and Hawn, T.R. (2020). Nicotinamide limits replication of *Mycobacterium tuberculosis* and Bacille Calmette-Guérin within macrophages. *Journal of Infectious Diseases*, 221(6), 989-999. <https://doi.org/10.1093/infdis/jiz541>.
79. Nagai, A., Matsumiya, H., Hayashi, M., Yasui, S., Okamoto, H., and Konno, K. (1994). Effects of nicotinamide and niacin on bleomycin-induced acute injury and subsequent fibrosis in hamster lungs. *Experimental Lung Research*, 20(4), 263-281. <https://doi.org/10.3109/01902149409064387>.
80. Viola, A., Munari, F., Sánchez-Rodríguez, R., Scolaro, T., and Castegna, A. (2019). The metabolic signature of macrophage responses. *Frontiers in Immunology*, 10, 1462. <https://doi.org/10.3389/fimmu.2019.01462>.
81. Andaloussi, A.E., Haloul, M.A., Muhamuda, K., Tominello, T., Wells, J.Z., and Ismail, N. (2019). mTORC1-mediated macrophage polarization into M1 contributes to *Ehrlichia*-induced sepsis. *Journal of Immunology*, 202(1_Supplement), 190.163. <https://doi.org/10.4049/jimmunol.202.Supp.190.63>.
82. Muranen, T., Iwanicki, M.P., Curry, N.L., Hwang, J., DuBois, C.D., Coloff, J.L., Hitchcock, D.S., Clish, C.B., Brugge, J.S., and Kalaany, N.Y. (2017). Starved epithelial cells uptake extracellular matrix for survival. *Nature Communications*, 8, 13989. <https://doi.org/10.1038/ncomms13989>.
83. Diskin, C. , and Pålsson-McDermott, E.M. (2018). Metabolic modulation in macrophage effector function. *Frontiers in Immunology*, 9(270). <https://doi.org/10.3389/fimmu.2018.00270>.
84. Stüve, P., Minarrieta, L., Erdmann, H., Arnold-Schrauf, C., Swallow, M., Guderian, M., Krull, F., Hölscher, A., Ghorbani, P., Behrends, J., Abraham, W.-R., Hölscher, C., Sparwasser, T.D., and Berod, L. (2018). *De novo* fatty acid synthesis during Mycobacterial infection is a prerequisite for the function of highly proliferative T cells, but not for dendritic cells or macrophages. *Frontiers in Immunology*, 9(495). <https://doi.org/10.3389/fimmu.2018.00495>.
85. López-Gamero, A.J., Sanjuan, C., Serrano-Castro, P.J., Suárez, J., and Rodríguez de Fonseca, F. (2020). The biomedical uses of inositols: A nutraceutical approach to metabolic dysfunction in aging and neurodegenerative diseases. *Biomedicines*, 8(9), 295. <https://doi.org/10.3390/biomedicines8090295>.

86. Chhetri, D.R. (2019). Myo-inositol and its derivatives: Their emerging role in the treatment of human diseases. *Frontiers in Pharmacology*, 10(1172). <https://doi.org/10.3389/fphar.2019.01172>.
87. Griess, B., Mir, S., Datta, K., and Teoh-Fitzgerald, M. (2020). Scavenging reactive oxygen species selectively inhibits M2 macrophage polarization and their pro-tumorigenic function in part, via Stat3 suppression. *Free Radical Biology & Medicine*, 147, 48-60. 10.1016/j.freeradbiomed.2019.12.018.
88. Haka, A.S., Grosheva, I., Chiang, E., Buxbaum, A.R., Baird, B.A., Pierini, L.M., and Maxfield, F.R. (2009). Macrophages create an acidic extracellular hydrolytic compartment to digest aggregated lipoproteins. *Molecular Biology of the Cell*, 20(23), 4932-4940. <https://doi.org/10.1091/mbc.e09-07-0559>.
89. Rizvi, A., Shankar, A., Chatterjee, A., More, T.H., Bose, T., Dutta, A., Balakrishnan, K., Madugulla, L., Rapole, S., Mande, S.S., Banerjee, S., and Mande, S.C. (2019). Rewiring of metabolic network in *Mycobacterium tuberculosis* during adaptation to different stresses. *Frontiers in Microbiology*, 10, 2417. <http://dx.doi.org/10.3389/fmicb.2019.02417>.
90. Kiran, D. , and Basaraba, R.J. (2021). Lactate metabolism and signaling in tuberculosis and cancer: A comparative review. *Frontiers in Cellular and Infection Microbiology*, 11, 624607. <https://doi.org/10.3389/fcimb.2021.624607>.
91. Ó Maoldomhnaigh, C., Cox, D.J., Phelan, J.J., Mitermite, M., Murphy, D.M., Leisching, G., Thong, L., O'Leary, S.M., Gogan, K.M., McQuaid, K., Coleman, A.M., Gordon, S.V., Basdeo, S.A., and Keane, J. (2021). Lactate alters metabolism in human macrophages and improves their ability to kill *Mycobacterium tuberculosis*. *Frontiers in Immunology*, 12. <https://doi.org/10.3389/fimmu.2021.663695>.
92. Zhao, Y., Dunker, W., Yu, Y.-T., and Karijovich, J. (2018). The role of noncoding RNA pseudouridylation in nuclear gene expression events. *Frontiers in Bioengineering and Biotechnology*, 6(8), 8. <https://doi.org/10.3389/fbioe.2018.00008>.
93. Kühtreiber, W.M., Tran, L., Kim, T., Dybala, M., Nguyen, B., Plager, S., Huang, D., Janes, S., Defusco, A., Baum, D., Zheng, H., and Faustman, D.L. (2018). Long-term reduction in hyperglycemia in advanced type 1 diabetes: The value of induced aerobic glycolysis with BCG vaccinations. *NPJ Vaccines*, 3, 23. <https://doi.org/10.1038/s41541-018-0062-8>.

Chapter 4 investigated and compared the effect of *Mycobacterium tuberculosis* curli pili (MTP) on the metabolome signatures of two functionally different cell types. THP-1 macrophages were demonstrated to be more responsive to MTP than the A549 epithelial cells.

The impact of MTP on carbon flux and cellular energetics of *M. tuberculosis* infected THP-1 macrophages were investigated in Chapter 5. This chapter has been submitted to “*The International Journal of Biochemistry and Cell Biology*” (Elsevier) is under review for publication (BC-D-25-00033).

CHAPTER 5: *Mycobacterium tuberculosis* curli pili (MTP) reduces oxygen consumption rate of THP-1 macrophages during early infection.

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Ethics approval: The study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC/00002915/2021) attached in Appendix A.

Declaration of interest: The authors declare that they have no conflict of interest.

Author contribution statement: Prof Pillay conceptualised the study. Prof Pillay, and Prof Steyn funded the study. Prof Pillay, Prof Steyn, Dr Cumming, and Ms Ashokcoomar designed the study. Ms Ashokcoomar and Ms Naidoo conducted experiments. Ms Baig and Mr Truebody provided technical assistance. Dr Mackenzie edited the draft manuscript. Ms Ashokcoomar analysed and interpreted the data, drafted and edited the manuscript. All authors contributed to and approved the manuscript.

5.1. Abstract

Introduction: *Mycobacterium tuberculosis* (*M. tuberculosis*) interacts with the host alveoli, and once first contact is established, bacterial clearance may take many months due to drug resistance and ineffectual treatment regimens. Hence, the development of improved therapeutics that focus on the host-pathogen interaction is essential, particularly those prioritising prevention.

Objective: This study aimed to elucidate the effect of *M. tuberculosis* curli pili (MTP), a surface-located pathogen adhesin, on the bioenergetic and metabolomic profiles of THP-1 macrophages during initial stages of infection.

Methodology: Differentiated THP-1 macrophages were infected for four hours with wildtype (WT), Δmtp , or *mtp*-complement strains of *M. tuberculosis*. Thereafter, bioenergetic profiles and metabolic flux were determined through cell mitochondrial stress test (CMST) and glycolytic rate assay (GRA) performed in an extracellular flux analyser. The metabolic flux of uninfected and *M. tuberculosis* infected THP-1 macrophages were determined through $^{13}\text{C}_6$ -glucose tracing liquid chromatography with tandem mass spectrometry. Statistical analysis highlighted differences/similarities amongst the THP-1 macrophage groups.

Results: The CMST revealed that the Δmtp infected THP-1 macrophages mimicked the higher oxygen consumption rate (OCR) for basal respiration, adenosine triphosphate (ATP) production, maximal respiration and spare respiratory capacity of the uninfected THP-1 macrophages, relative to the WT and *mtp*-complement infected THP-1 macrophages. Hence the similar modulation of respiratory parameters of the Δmtp infected THP-1 macrophages indicated a favourable outcome to THP-1 macrophages in the absence of MTP. The GRA showed the highest compensatory glycolytic rate in the Δmtp infected THP-1 macrophages. Given that increased glycolysis in macrophages is indicative of a pro-inflammatory response, the data suggests that the macrophages are more proficient at launching an immune response if the adhesin is absent. This implies that MTP dampens the immune response to allow for survival and replication of the pathogen during early infection of THP-1 macrophages. As expected, carbon tracing metabolomics revealed that *M. tuberculosis* infection caused the redirection of carbon from the tricarboxylic acid cycle to glycolysis, in addition to an increased flux through pentose phosphate pathway. However, in the Δmtp infected THP-1 macrophages, the total metabolite abundance was lower, similar to the uninfected THP-1 macrophages.

Conclusion: The findings suggest that the decrease in OCR of the aforementioned respiratory parameters of differentiated THP-1 macrophages are attributed to the MTP adhesin of *M. tuberculosis*. Furthermore, in the absence of MTP, the increased glycolytic capability of the macrophages potentially signals a healthy host immune response to *M. tuberculosis* infection. This will potentially facilitate a prompt clearance of the intracellular pathogen before it establishes a successful infection, implying that the presence of MTP facilitates the survival of the pathogen during the early stages until infection is established. These findings support the growing evidence that the MTP adhesin is an important virulence factor and if the interaction between pathogen and host is interrupted/stopped, the host will be more adept to readily clear the infection.

5.2. Introduction

Primarily infecting pulmonary macrophages, *Mycobacterium tuberculosis* (*M. tuberculosis*) remains the top cause of disease from a single infectious agent, which results in prolonged and recurring illness. This causative agent of tuberculosis (TB) was responsible for 10.8 million incident cases and 1.25 million deaths in 2023 [1]. Current TB treatment regimens range from four to nine months, which is exacerbated by the persistence of drug-resistant strains and coexisting medical conditions

[1]. With the long and ineffectual TB regimen, patients are more likely to stop treatment, leading to further medical complications, development of increased resistance, and transmission to other individuals. Hence, improved treatment and prevention is essential in the global fight against TB.

The aerosolised *M. tuberculosis* makes contact with the resident alveolar macrophages (AMs) and interstitial macrophages (IMs) in the lungs, resulting in robust host innate immune responses involved in the early clearance of *M. tuberculosis* [2]. However, *M. tuberculosis* has the unique ability to evade host killing and preferentially establish a niche within macrophages [2]. During immune suppression or hyperactivation, *M. tuberculosis* virulence factors can favour the pathogen during an active infection, causing an upset in macrophage homeostasis [2]. The dissemination of *M. tuberculosis* is concealed by the functional and morphological heterogeneity of macrophages as well as their plasticity, which helps to determine the direction of macrophage activation *in vitro/in vivo*. Hence, depending on the activation status, pro-inflammatory macrophages rely on glycolysis to meet energy demands, whilst anti-inflammatory macrophages mainly rely on oxidative phosphorylation (OXPHOS) to meet energy demands [3]. These two major pathways are employed by cells to meet energy demands through the generation of adenosine triphosphate (ATP) and the switch between the pathways can provide insights on how the cells adapt to changes in the environment [4].

The host-pathogen interaction is crucial for the establishment of infection. *Mycobacterium tuberculosis* has multiple surface-located adhesins that are vital to the initial attachment and invasion of the host [5]. Compelling evidence has shown that one such adhesin is *M. tuberculosis* curli pili (MTP) encoded by an open reading frame designated as *Rv3312A (mtp)* [6]. Furthermore, MTP has been demonstrated as a virulence factor of note since it reacts with immunoglobulin G (IgG) antibodies in patient sera, promotes adhesion to and invasion of THP-1 macrophages [7], modulate host immune responses post-infection [8; 9], and the *mtp* gene is unique to *M. tuberculosis* complex strains and is highly conserved amongst clinical isolates [10]. More recently, alterations in carbon, fatty acid, and amino acid metabolism were demonstrated to be modulated by MTP in *M. tuberculosis* and its infection models [11; 12]. However, there is little to no information with regards to the effect of MTP on the macrophage carbon flux and respiratory profiles during *M. tuberculosis* infection.

Changes to energy metabolism can be elucidated by metabolic flux analysis. Targeted tandem mass spectrometry is used for the identification and quantitation of specific metabolites, with the use of a non-radioactive stable isotope carbon-13 (¹³C)-glucose in central carbon metabolism and related pathways [13]. The stable isotopic tracer is used to quantify biochemical networks and assess metabolic pathway flux [14], which contributes to the understanding of cell growth, maintenance and responses to environmental changes.

Extracellular flux analysers facilitate the non-invasive, real-time detection and monitoring of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of cells. Infection with *M. tuberculosis* facilitates the rewiring of energy metabolism of differentiated THP-1 macrophages and human monocyte derived macrophages (hMDMs), which is influenced by differential pathogenicity and live mycobacterial burdens [4]. This is evidenced by studies that have combined bioenergetics, targeted metabolomics and infection models, to demonstrate that *M. tuberculosis* infected macrophages decrease flux through glycolysis, pentose phosphate pathway (PPP) and tricarboxylic acid (TCA) cycle [4; 15]. Contrastingly, another study displayed that *M. tuberculosis* infected macrophages exhibited a dose-dependent increase in glycolysis [16].

The use of bioenergetic analysis and targeted tandem mass spectrometry on MTP-deficient and MTP-proficient live *M. tuberculosis* infected THP-1 macrophages, has not been studied previously. Hence, this study aimed to investigate the impact of MTP on the bioenergetic metabolism of *M. tuberculosis* infected THP-1 macrophages and the breakdown/incorporation of carbon labelled glucose in the absence and presence of MTP during the initial stages of infection. The insights from this host-pathogen interplay during early stages of infection can enrich the current understanding of the role of

MTP in the modulation of macrophage metabolism, thereby, contributing to the development of improved TB treatment and prevention strategies.

5.3. Materials and methods

5.3.1. Ethics statement

The Biomedical Research Ethics Committee of University of KwaZulu-Natal approved the study (BREC/00002915/2021). Experiments were conducted in a Biosafety Level 3 (BSL 3) laboratory and a cell culture laboratory at Africa Health Research Institute (AHRI), and a BSL2+ laboratory at Medical Microbiology, University of KwaZulu-Natal (UKZN), Durban, South Africa.

5.3.2. *Mycobacterium tuberculosis* and THP-1 macrophages growth conditions and infection procedure

The wildtype (WT) strain used in this study was the drug-susceptible *M. tuberculosis* V9124 [17], its *mtp* deletion mutant (Δmtp) previously constructed via specialised transduction [18; 19] and the insertion of an allelic exchange substrate (kindly donated by W. R. Jacobs, Jr., AECOM). The *mtp*-complemented strain was previously constructed by electrotransformation of the deletion mutant with the pMV261 plasmid containing *mtp* [18].

All mycobacterial strains were cultured in Middlebrook 7H9 broth (Difco, Becton-Dickinson, Franklin Lakes, NJ, USA) supplemented with 10% (v/v) oleic albumin dextrose catalase (Becton-Dickinson, Franklin Lakes, NJ, USA), 0.5% (v/v) glycerol (Sigma-Aldrich, St. Louis, MO, USA) and 0.05% tween-80 (Sigma-Aldrich, St. Louis, MO, USA). Additionally, the Δmtp was cultured in broth with the addition of hygromycin B ($75 \mu\text{g mL}^{-1}$) and the *mtp*-complement was cultured in broth with additional kanamycin ($30 \mu\text{g mL}^{-1}$). When required, cultures were grown on 7H11 plates (Difco, Becton-Dickinson, Franklin Lakes, NJ, USA) similarly supplemented as described above with the exception of tween-80. Bacterial cultures were incubated in a shaking incubator (I-26 Shaking Incubator, New Brunswick Scientific, Canada) at 100 rpm for 7-8 days (broth) or for 3-4 weeks (plates) at 37°C.

The THP-1 monocytic cell line (ATCC TIB-202) was cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 medium with L-glutamine (GIBCO, Grand Island, New York, USA), which was additionally supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), and 25 mM D-glucose (Sigma-Aldrich, St. Louis, MO, USA) to make complete cell culture media. Cells were cultured in T75 cm² tissue culture flasks (Nest Biotechnology, Jiangsu, China) and incubated at 37°C with 5% CO₂ in a humidified incubator (Shel Lab CO₂ Incubator, Cornelius, OR, USA) until 70% to 90% confluency. The THP-1 monocytes were terminally differentiated into macrophage monolayers with phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 25 nM for 24 hours for each experiment. Differentiated macrophages were then allowed to rest for 24 hours in complete media without PMA. The THP-1 macrophages were seeded at a density of 100 000 cells per well in XF96 cell culture plates (Agilent, Santa Clara, CA) and 3×10^6 cells per well in six well plates (Lasec, Cape Town, RSA) for the extracellular flux experiments and the targeted metabolomics experiments, respectively. The THP-1 macrophage monolayers were infected with *M. tuberculosis* strains 48 hours post seeding. The multiplicity of infection (MOI) ratio of approximately 5:1 of bacteria to host was used to infect host cells for four hours at 37°C in 5% CO₂ and 95% humidity (Appendix E: Tables S1.1-S1.3). For both the bioenergetics and

metabolomics experiments, four sample groups were used: uninfected THP-1 macrophages, WT infected THP-1 macrophages, Δmtp infected THP-1 macrophages, and *mtp*-complement infected THP-1 macrophages.

5.3.3. Extracellular flux analysis via cell mitochondrial stress test (CMST) and glycolytic rate assay (GRA)

Extracellular flux analysis non-invasively measures the OCR and extracellular proton release by ECAR in the immediate cell culture medium [20]. This enables real-time detection of cellular respiration and acidification, thereby providing information surrounding the two major energy generation pathways: OXPHOS through mitochondrial respiration, and glycolysis. These two pathways form the basis of bioenergetic metabolism as they both produce ATP.

In this study, the Seahorse XF96 Extracellular Flux Analyzer (Agilent, Santa Clara, CA) was utilised to determine the effect of MTP on mitochondrial respiration and glycolysis of *M. tuberculosis* infected THP-1 macrophages. A minimum of three independent experiments were conducted per assay. Briefly, XF96 cartridges were hydrated overnight, and the drug ports were loaded with the relevant drug at 10× the final concentrations for the respective assays. All media used in the assays were adjusted to pH 7.4 and pre-warmed to 37°C (Thermo Fisher Scientific, Waltham, MA, USA). Differentiated THP-1 macrophages were independently infected with the three *M. tuberculosis* strains. In the case of the uninfected THP-1 macrophages, the respective media was used to substitute the *M. tuberculosis* inoculum. The XF96 cell culture plate was de-gassed for 30 minutes in a 37°C incubator preceding three washes with XF assay medium prior to adding 180 mL of XF assay medium for the experimental runs. For each OCR or ECAR reading, the Seahorse XF96 Extracellular Flux Analyzer was set for 3 minutes of mixing and 4 minutes of measurement [4].

Mitochondrial respiration rate was determined with the CMST, which examined the effect of the three mycobacterial infections on the key respiratory parameters in the host cells. After the initial four OCR measurements, three OCR measurements were taken after each injection of a respiratory modulating drug. These included the first injection of oligomycin (at a final concentration of 1.5 µM), the second injection of carbonilcyanide *p*-triflouromethoxyphenylhydrazine (FCCP) (at a final concentration of 1 µM), and the third injection of rotenone with antimycin A (at final concentrations of 0.5 µM each) [21].

Glycolysis of the mycobacterial infected macrophages was determined by the GRA. Following the four initial measurements of ECAR and OCR, three subsequent readings were performed after injections of rotenone with antimycin A (at final concentrations of 0.5 µM each) and 2-deoxy-D-glucose (2-DG) (at a final concentration of 50 mM). Glycolytic proton efflux rate (glycoPER) was calculated as glycoPER = proton efflux rate (PER) – mitochondrial PER (mitoPER) [21].

The XF96 cell culture plates were formalin fixed, followed by cell counts under 4× magnification with a DAPI filter in an Agilent BioTek Cytation 5 (Agilent, Santa Clara, CA). These cell counts were used to normalise the extracellular flux data sets. Additionally, extra cell culture plates were seeded, infected with the same innocula, washed with phosphate buffered saline (PBS) and lysed with 0.25% Triton X-100 (Millipore, Merck, Darmstadt, Germany). These lysates were used to plate out ten-fold serial dilutions to assess colony forming units (CFUs) of each infection to ensure reproducibility of adhesion and invasion of *M. tuberculosis* (Appendix E: Table S1.1-S1.2).

5.3.4. Metabolite extraction and targeted liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

The six well plates that were seeded with the macrophages and infected with mycobacteria as described above were used for the targeted metabolomics experiment (Appendix E: Table S1.3). After infection, the standard cell culture media was removed and replaced with media containing a stable $^{13}\text{C}_6$ -glucose isotope. This labelled media was prepared identically to the complete cell culture media, except for the replacement of regular glucose with 4.5 g/L of $^{13}\text{C}_6$ -glucose (Syntho Inc, Ontario, Canada). The THP-1 macrophages were then incubated (Shell Lab CO₂ Incubator, Cornelius, OR, USA) for eight hours to allow sufficient incorporation of the radiolabelled $^{13}\text{C}_6$ -glucose. Following the incubation at 37°C with 5% CO₂ in a humidified incubator, the supernatants of each well were collected along with the PBS used to wash the cells three times. The supernatants were centrifuged (Mikro 200R, Hettich Zentrifugen, Tuttlingen, Germany) to check if there was significant cell detachment from the plate. No pellet was observed, which indicated minimal cell detachment. Therefore, only the cells in the plate were processed for metabolite extraction.

Ice-cold methanol (Sigma-Aldrich, St. Louis, MO, USA) was added to each well to halt the metabolism of the cells. An equal volume of ice-cold water containing internal standards of D₄-alanine and D₄-succinate (Sigma-Aldrich, St. Louis, MO, USA) (final concentration of 100 µg/mL) were added to each well which was then cell scraped. The cell suspensions were collected, briefly vortexed (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged (Thermo Fisher Scientific, Waltham, MA, USA) at 3000×g (4°C) for 5 minutes to remove the cell debris. The supernatants containing the extracted metabolites were transferred to Spin-X centrifuge tube filters (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged (Mikro 200R, Hettich Zentrifugen, Tuttlingen, Germany) at 20 000×g (4°C) for 10 minutes. All samples were then lyophilised in a SpeedVac (Thermo Fisher Scientific, Waltham, MA, USA) at 35°C for approximately 24 hours and the lyophilised metabolites were stored at –80 °C (Sigma-Aldrich, St. Louis, MO, USA) until the metabolites were prepared for LC-MS/MS. Each lyophilised sample was reconstituted in 100 µL of distilled water. From this, 10 µL were used for bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA, USA), which was used to normalise the metabolomic data sets (Appendix E: Table S2 and Figure S1), 30 µL were added to 70 µL of acetonitrile in a mass spectroscopy snap ring vial (Separations Scientific, Roodepoort, GP, ZA) for amino acid analysis, and 60 µL of sample were transferred into mass spectroscopy snap ring vials for metabolite analysis.

An aqueous 0.1% formic acid isocratic mobile phase program was used to chromatographically separate small organic molecules on an Aminex HPX-87 column (300×7.8 mm) (Bio-Rad, Hercules, CA, USA) with a sample injection volume of 20 µL and a flow rate of 400 µL/minute. A gradient elution program was used to chromatographically separate amino acids on an ethylene bridged hybrid (BEH) Amide column (2.1×100 mm, 1.7 µM) (Waters, Milford, MA, USA) with a sample injection volume of 1 µL and a flow rate of 200 µL/minute. The mobile phase solutions comprised A (Aqueous 0.1% formic acid) and B (acetonitrile, 0.1% formic acid) with the gradient program: 0–0.2 min, 99% of B; 0.2–24 minutes, a curvilinear decrease from 99 – 30% of B; 24–25 minutes, 30% of B; 25.1–35 minutes, 99% of B. An UltiMate 3000 ultra-high pressure liquid chromatography (UPLC) instrument (Dionex, Sunnyvale, CA, USA) was used for LC-MS/MS. Mass spectrometry was performed in negative and positive modes to monitor organic molecules and amino acids, respectively. A scan range of 50 – 750 m/z was used to acquire the ion chromatograms on a Q Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with selective ion monitoring.

5.3.5. Software and data analysis

The Wave Desktop 2.6.3.5 Software, available from the Agilent website (<https://www.agilent.com/en/products/cell-analysis/software-download-for-wave-desktop>), was used to normalise the OCR and ECAR values from the extracellular flux analysis to the cell count numbers. The Wave software has the capability to export the data into the XF Report Generators for calculation of the parameters from the specific assays including the CMST and GRA.

The Skyline Desktop 23.1 Software (https://skyline.ms/wiki/home/software/Skyline/page.view?name=SkylineInstall_64_23-1) was used to select organic molecules/amino acid peaks in the chromatograms from the LC-MS/MS analysis. For each THP-1 macrophage group, the total abundance of the detected compounds was calculated as area under the curve (AUC) normalised to protein concentrations (BCA assays) and internal standards prior to being uploaded onto MetaboAnalyst 6.0 (<https://www.metaboanalyst.ca/>) to perform pathway analysis. Additionally, the carbon flux for each THP-1 macrophage group was determined by dividing the total abundance of labelled compound by the total abundance of the compound (labelled and unlabelled). All data was exported into GraphPad Prism Version 8.0 for statistical analysis. Data was analysed by one-way analysis of variance (ANOVA) and Tukey's test with statistical significance considered with an adjusted p -value ≤ 0.05 .

5.4. Results

5.4.1. Bioenergetic metabolism of *M. tuberculosis* Δmtp infected THP-1 macrophages resembles that of the uninfected THP-1 macrophages

The CMST was used to determine fundamental parameters of infected THP-1 macrophages mitochondrial function through the detection of OCR with the aid of respiration modulating drugs and built-in injection ports [20; 21]. The respiratory parameters investigated were basal respiration, ATP-linked respiration, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and proton leak. Measurements of OCR were performed before, during and after injection strategies and is illustrated as respiratory profiles (Figure 5.1a).

The cellular energetic demand of the THP-1 macrophages at rest are represented as basal respiration (Figure 5.1b) [22; 23]. The relative ability of the THP-1 macrophages to meet the energetic needs of the cells by the production of mitochondrial ATP are illustrated as ATP production (Figure 5.1c) [22; 23]. The maximum rate of respiration by the THP-1 macrophages during a high energy demand period are depicted by maximal respiration (Figure 5.1d) [22; 23]. Moreover, the theoretical ability of THP-1 macrophages to respond to changes in energetic demand is a reflection of cellular fitness and flexibility, which is represented as spare respiratory capacity (Figure 5.1e) [22; 23]. All four of these respiratory parameters showed a significant ($p < 0.001$) decrease in OCR for the WT infected THP-1 macrophages compared to the uninfected, Δmtp infected and *mtp*-complement infected THP-1 macrophages (Figure 5.1b-e). Hence, by knocking out *mtp* from the pathogen, the Δmtp infected THP-1 macrophages have similar basal respiration, ATP production, maximal respiration and spare respiratory capacity to that of the uninfected macrophages. This is indicated by the lack of significant differences between these two groups. Although significantly different to WT infected macrophages, the OCR readings for ATP production (Figure 5.1c), maximal respiration (Figure 5.1d) and spare respiratory capacity (Figure 5.1e) of the *mtp*-complement infected THP-1 macrophages lie between the WT infected and the Δmtp infected THP-1 macrophages. This indicates only partial restoration of

the phenotype, potentially attributed to the non-integrating plasmid during complementation of the *M. tuberculosis*.

Interestingly, proton leak (Figure 5.1f) of the three *M. tuberculosis* infected THP-1 macrophages was significantly lower ($p < 0.001$) than uninfected macrophages, with the *mtp*-complement infected THP-1 macrophages also being significantly different to the WT infected ($p = 0.019$) and Δmtp infected THP-1 macrophages ($p = 0.034$). Additionally, the non-mitochondrial respiration of all three *M. tuberculosis* infected THP-1 macrophages was significantly higher ($p < 0.001$) than uninfected macrophages (Figure 5.1g). Moreover, a shift from an energetic profile (uninfected THP-1 macrophages and Δmtp infected THP-1 macrophages) toward a quiescent profile was observed for the WT infected THP-1 macrophages (Appendix E: Figure S2). Data suggest that in the absence of MTP during early stages of *M. tuberculosis* infection, the THP-1 macrophages retain the energetic profile. Therefore, the presence of MTP on *M. tuberculosis* results in an overall decrease of OCR of THP-1 macrophages relative to the uninfected healthy THP-1 macrophages.

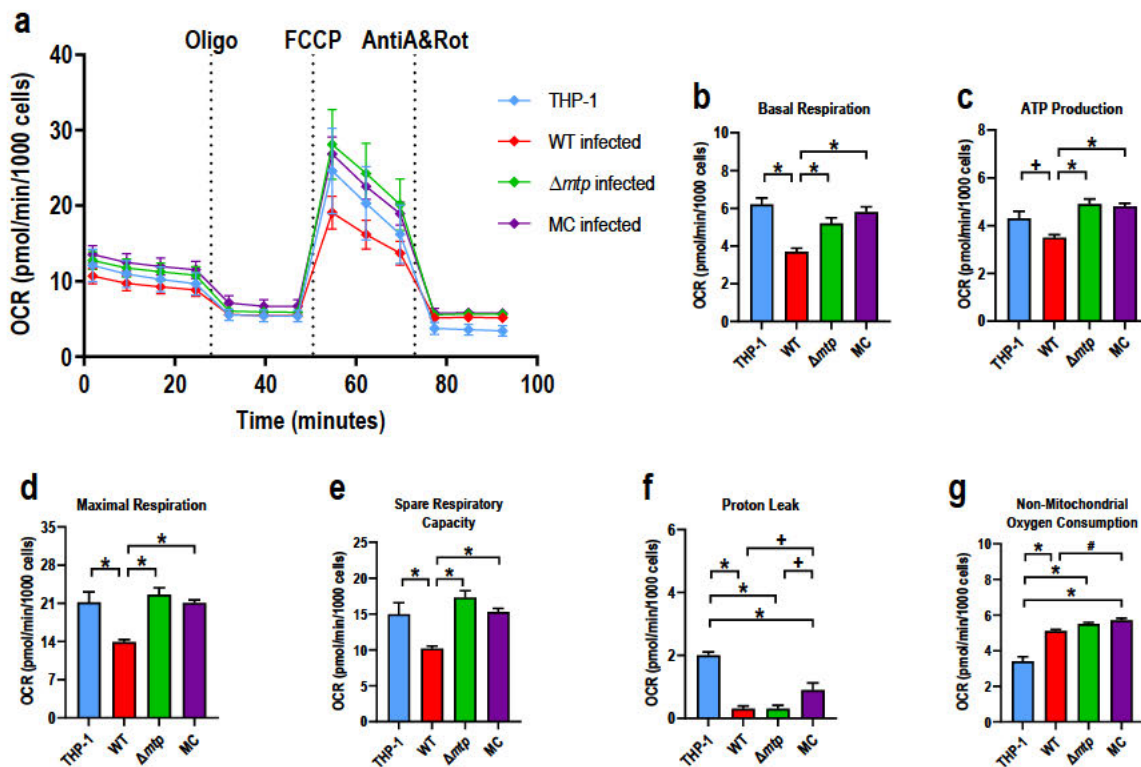


Figure 5.1. Uninfected THP-1 macrophages and Δmtp *M. tuberculosis* infected THP-1 macrophages display a similar OCR for basal respiration, ATP production, maximal respiration and spare respiratory capacity. (a) Respiratory profiles (OCR) were generated from the extracellular flux analysis performed on differentiated THP-1 macrophages after a four-hour infection with *M. tuberculosis* at a MOI of 5. Experiments were performed on uninfected THP-1 macrophages (THP-1), WT infected THP-1 macrophages (WT), Δmtp infected THP-1 macrophages (Δmtp), and *mtp*-complement infected THP-1 macrophages (MC). Respiratory parameters that were investigated included (b) basal respiration, (c) ATP production, (d) maximal respiration, (e) spare respiratory capacity, (f) proton leak, and (g) non-mitochondrial respiration. Profiles and respiratory parameters are representative of three independent experiments, normalised via Cytation5 per 1000 cells and shown as the mean $\pm$ SEM (n = minimum of 7 biological replicates). Data was analysed by one-way ANOVA and Tukey's test for multiple comparisons to determine the significance of bioenergetic parameters. The adjusted p -values are represented as; *, $p \leq 0.001$; #, $p \leq 0.005$; +, $p \leq 0.05$.

5.4.2. *Mycobacterium tuberculosis* curli pili reduces the ability of *M. tuberculosis* infected THP-1 macrophages to meet energy demands via glycolysis during early infection

Through the detection of medium acidification or PER, with OCR and ECAR measurements, it is possible to determine the glycoPER of the THP-1 macrophages (Figure 5.2a). The detected PERs account for the contribution of extracellular acidification derived from both mitochondrial and TCA cycle activity. During the process of glycolysis within the cytoplasm, glucose is converted into pyruvate and then into lactate, thus extruding protons into the extracellular media affecting the ECAR. The media is further acidified and hydrated by the production of CO₂ from mitochondrial TCA cycle. Therefore, by detecting and inhibiting mitochondrial respiration and acidification, the mitochondrial derived proton efflux rate (mitoPER) can be used to calculate the glycoPER from the total PER [21; 24].

The basal glycolytic rates ($p \leq 0.001$) and compensatory glycolytic rates (p range from ≤ 0.05 to 0.001) were significantly higher in all three *M. tuberculosis* infected THP-1 macrophages relative to the uninfected cells, regardless of the presence/absence of MTP (Figure 5.2b and 5.2c). This is expected since *M. tuberculosis* infection of macrophages causes an increase in glycolysis, which is associated with the production of pro-inflammatory cytokines [25-28]. However, the Δmtp infected THP-1 macrophages had the highest compensatory glycolytic rate, indicating a potentially greater ability to facilitate a pro-inflammatory response to the infection relative to the WT and *mtp*-complement infected macrophages.

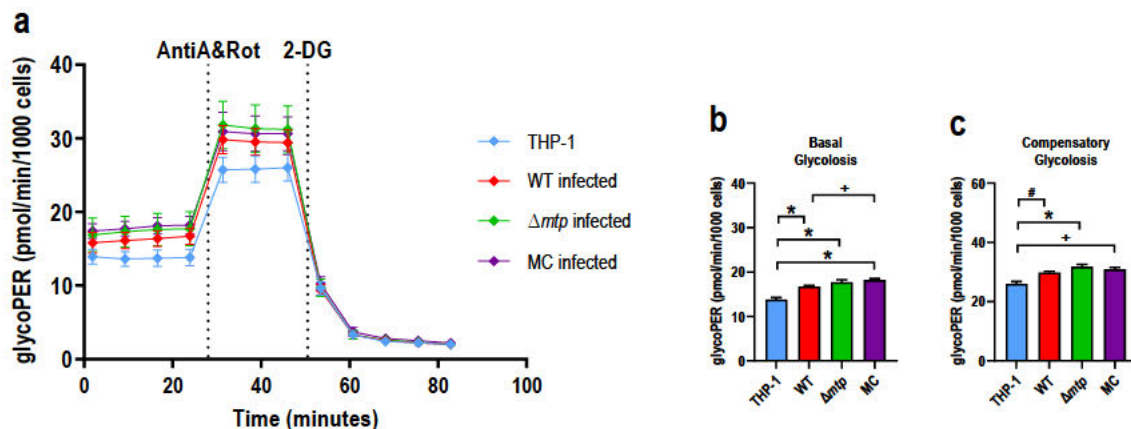


Figure 5.2. *M. tuberculosis* infection of THP-1 macrophages results in an increase of glycoPER. (a) GlycoPER profiles were generated from the extracellular flux analysis performed on differentiated THP-1 macrophages after a four-hour infection with *M. tuberculosis* at a MOI of 5. Experiments were performed on uninfected THP-1 macrophages (THP-1), WT *M. tuberculosis* infected THP-1 macrophages (WT), *M. tuberculosis* Δmtp infected THP-1 macrophages (Δmtp), and *mtp*-complemented *M. tuberculosis* infected THP-1 macrophages (MC). The glycoPER parameters that were investigated included (b) basal glycoPER and (c) compensatory glycoPER, which both illustrate a significant increase in the glycolytic rate of the THP-1 macrophages, following a *M. tuberculosis* infection. Glycolytic profiles and parameters are a representative of three independent experiments and were normalised via Cytation5 per 1000 cells and shown as the mean $\pm$ SEM (n = minimum of 7 biological replicates). Data was analysed by one-way ANOVA and Tukey's test for multiple comparisons to determine the significance of bioenergetic parameters. The adjusted p -values are represented as; *, $p \leq 0.001$; #, $p \leq 0.005$; +, $p \leq 0.05$.

5.4.3. *Mycobacterium tuberculosis* curli pili does not affect carbon flux through glycolysis, TCA cycle and PPP of *M. tuberculosis* infected THP-1 macrophages

Differentially significant organic molecules and amino acids are illustrated in Figure 5.3 and broken down into three major pathways: glycolysis/gluconeogenesis (Figure 5.3a), PPP (Figure 5.3b), and TCA cycle (Figure 5.3c). Interestingly, the Δmtp infected THP-1 macrophages induced a significant reduction of the total abundance of pathway intermediates, in contrast to the WT infected THP-1 macrophages (Appendix E: Table S3). These intermediates include fructose 6-phosphate, fructose 1,6-bisphosphate, lactate, and dihydroxyacetone phosphate from glycolysis (Figure 5.3a), sedoheptulose 7-phosphate and ribose from PPP (Figure 5.3b), citrate/isocitrate, α -ketoglutarate, succinate, fumarate, malate, glutamate, glutamine, aspartate, asparagine and arginine from TCA cycle (Figure 5.3c). Majority of the compounds were present in similar lower levels between the Δmtp infected THP-1 macrophages and uninfected THP-1 macrophages, relative to the greater abundance of compounds observed in the WT and *mtp*-complement infected macrophages (Figure 5.3).

To understand the effect of MTP on ^{13}C -incorporation into intermediates of glycolysis (Figure 5.3a), PPP (Figure 5.3b), and TCA cycle (Figure 3c) of *M. tuberculosis* infected THP-1 macrophages, the flux of the total labelled isotopologues were compared amongst the four THP-1 macrophage groups (Appendix E: Table S4). *Mycobacterium tuberculosis* infection caused an increase in flux through glycolysis and PPP. Fructose 6-phosphate, fructose 1,6-bisphosphate, and phosphoenolpyruvate of glycolysis had an increased carbon flux in response to infection with *M. tuberculosis* (Figure 5.3a). Additionally, the *M. tuberculosis* infection of THP-1 macrophages caused an increase carbon flux of ribose and sedoheptulose 7-phosphate in PPP (Figure 5.3b). These carbon incorporation profiles of the infected THP-1 macrophages indicate that the cells appear to be undergoing a glycolytic reprogramming by increasing flux through glycolysis and PPP (Figure 5.3a and 5.3b). The increased flux through glycolysis, visualised as a high glucose uptake observed in the first two intermediates (fructose 6-phosphate, fructose 1,6-bisphosphate) of glycolysis of *M. tuberculosis* infected macrophages, is postulated to be employed for the rapid generation of ATP [29].

The opposite was observed for the labelling patterns of the TCA cycle, in which *M. tuberculosis* infection of THP-1 macrophages was met by a decrease in flux of ^{13}C -incorporation (Figure 5.3c). Lower flux was observed in TCA intermediates like citrate/isocitrate, α -ketoglutarate, succinate, and malate (Figure 5.3c). The diversion of carbon from TCA cycle toward glycolysis [30] indicates a switch from OXPHOS to glycolysis in attempts to favour lactate as an energy source [30; 31]. Moreover, amino acids that are linked to TCA cycle, such as glutamate, aspartate, asparagine and arginine all displayed a lower flux of ^{13}C -incorporation (Figure 5.3c). Holistically, the LC-MS/MS data revealed that the presence/absence of MTP has no significant effect on *M. tuberculosis* infected THP-1 macrophages.

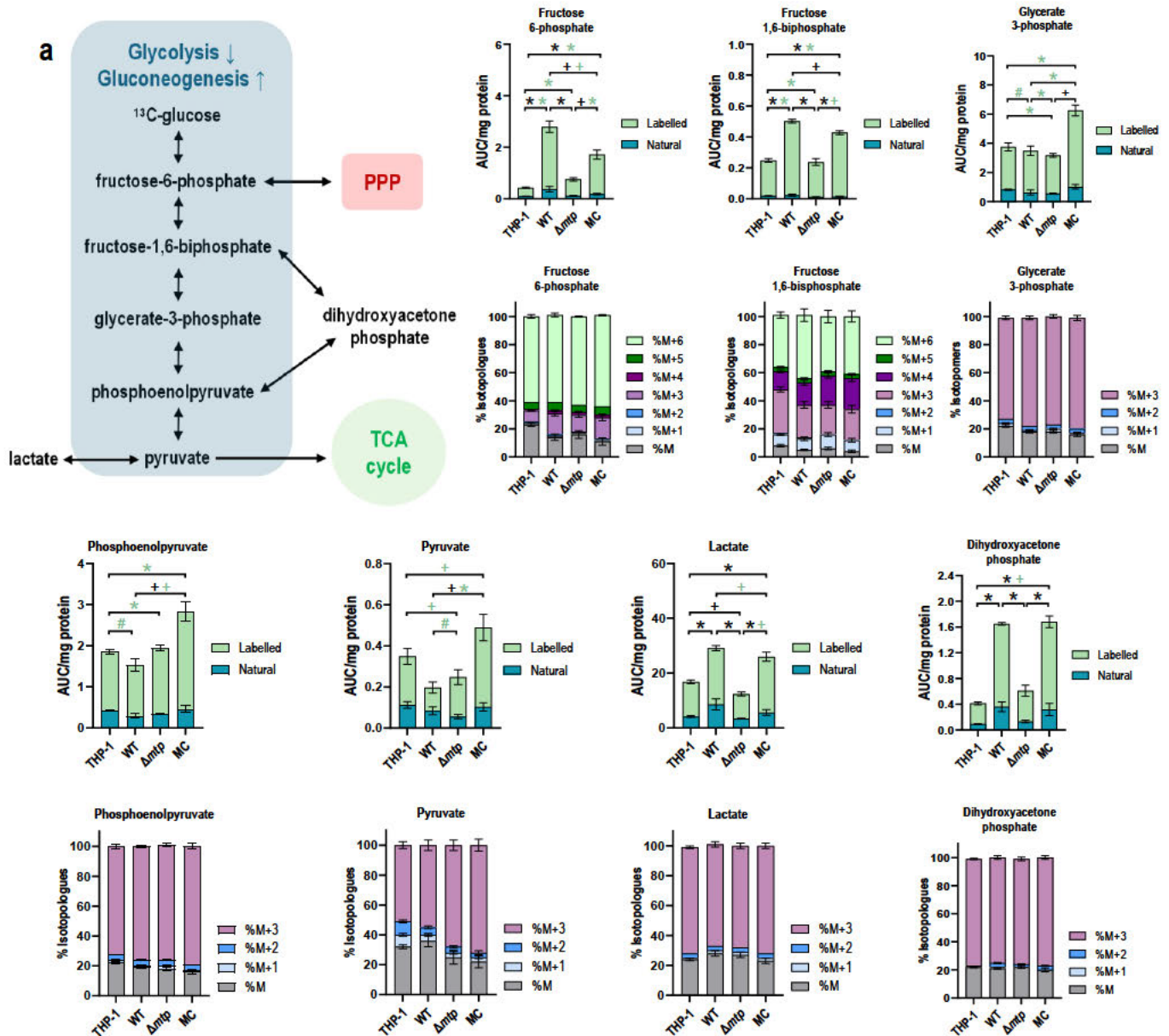


Figure 5.3. Total abundance and isotopologue distribution of incorporated $^{13}\text{C}_6$ -glucose into glycolysis/gluconeogenesis, PPP and TCA cycle of THP-1 macrophages. Experiments were performed on uninfected THP-1 macrophages (THP-1), WT infected THP-1 macrophages (WT), Δmtp infected THP-1 macrophages (Δmtp), and mtp -complemented infected THP-1 macrophages (MC). An MOI of 5 for each *M. tuberculosis* strain was used to infect the THP-1 macrophages for four hours and followed by eight hours of incubation with labelled media to perform $^{13}\text{C}_6$ -glucose tracing amongst different infection groups. On the total abundance graphs, there are two sets of statistical significance indicated: the difference between absolute abundance (black symbols) (Appendix E: Table S3), and the difference between carbon flux (Appendix E: Table S4). Statistical differences amongst the THP-1 macrophages, for each compound involved in (a) glycolysis/gluconeogenesis, (b) PPP, and (c) TCA cycle are illustrated. The isotopologue graphs illustrate the percentage of ^{13}C atoms incorporations. Data are representative of three independent experiments and shown as the mean $\pm$ SEM ($n = 5$ biological replicates). Data was analysed by one-way ANOVA and Tukey's test for multiple comparisons to determine the significance of metabolic flux for each compound. The adjusted p -values are represented as; *, $p \leq 0.001$; #, $p \leq 0.005$; +, $p \leq 0.05$.

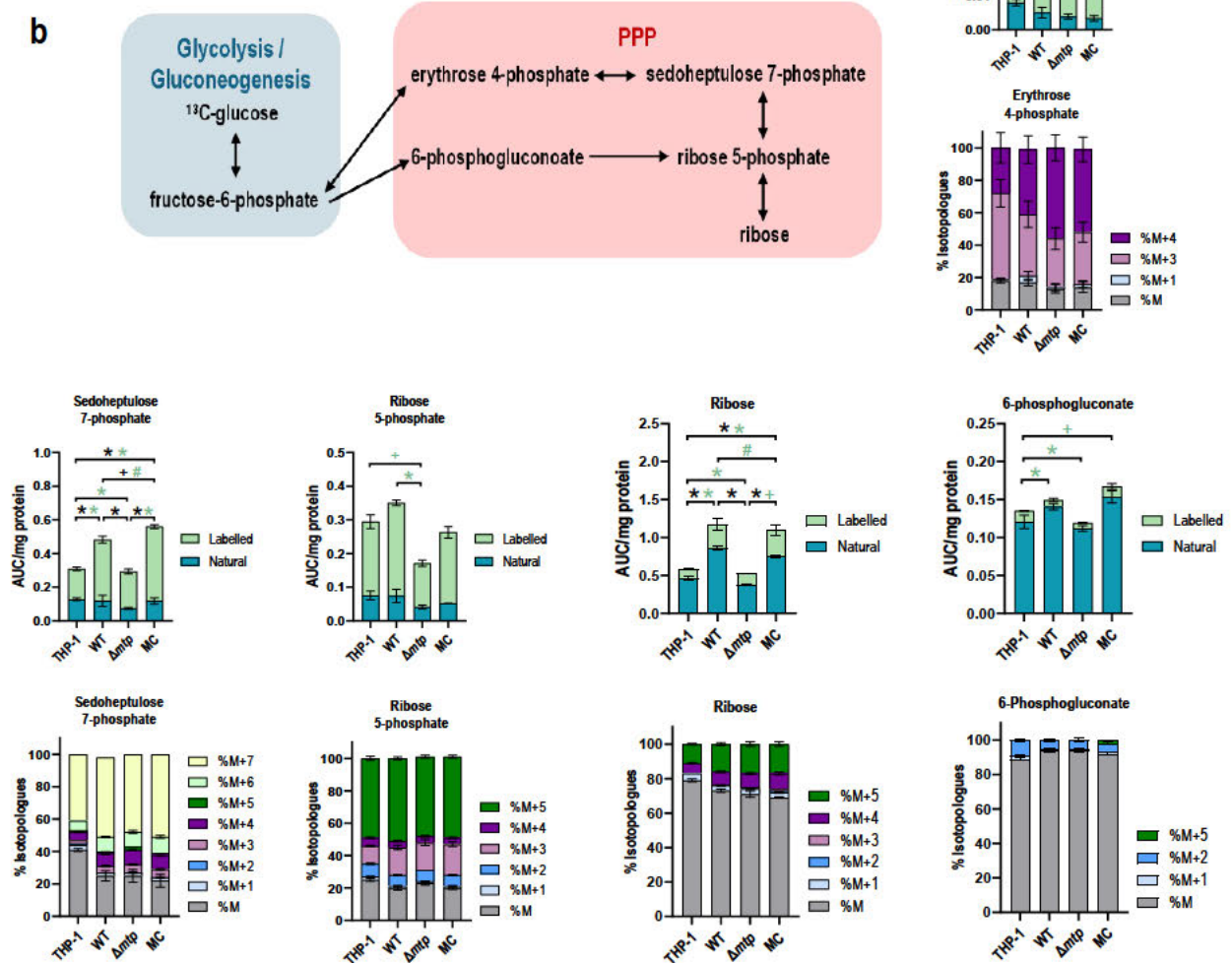


Figure 5.3 continued. Total abundance and isotopologue distribution of incorporated $^{13}\text{C}_6$ -glucose into glycolysis/gluconeogenesis, PPP and TCA cycle of THP-1 macrophages. Experiments were performed on uninfected THP-1 macrophages (THP-1), WT infected THP-1 macrophages (WT), Δmtp infected THP-1 macrophages (Δmtp), and mtp -complemented infected THP-1 macrophages (MC). An MOI of 5 for each *M. tuberculosis* strain was used to infect the THP-1 macrophages for four hours and followed by eight hours of incubation with labelled media to perform $^{13}\text{C}_6$ -glucose tracing amongst different infection groups. On the total abundance graphs, there are two sets of statistical significance indicated: the difference between absolute abundance (black symbols) (Appendix E: Table S3), and the difference between carbon flux (Appendix E: Table S4). Statistical differences amongst the THP-1 macrophages, for each compound involved in (a) glycolysis/ gluconeogenesis, (b) PPP, and (c) TCA cycle are illustrated. The isotopologue graphs illustrate the percentage of ^{13}C atoms incorporations. Data are representative of three independent experiments and shown as the mean $\pm$ SEM ($n = 5$ biological replicates). Data was analysed by one-way ANOVA and Tukey's test for multiple comparisons to determine the significance of metabolic flux for each compound. The adjusted p -values are represented as; *, $p \leq 0.001$; #, $p \leq 0.005$; +, $p \leq 0.05$.

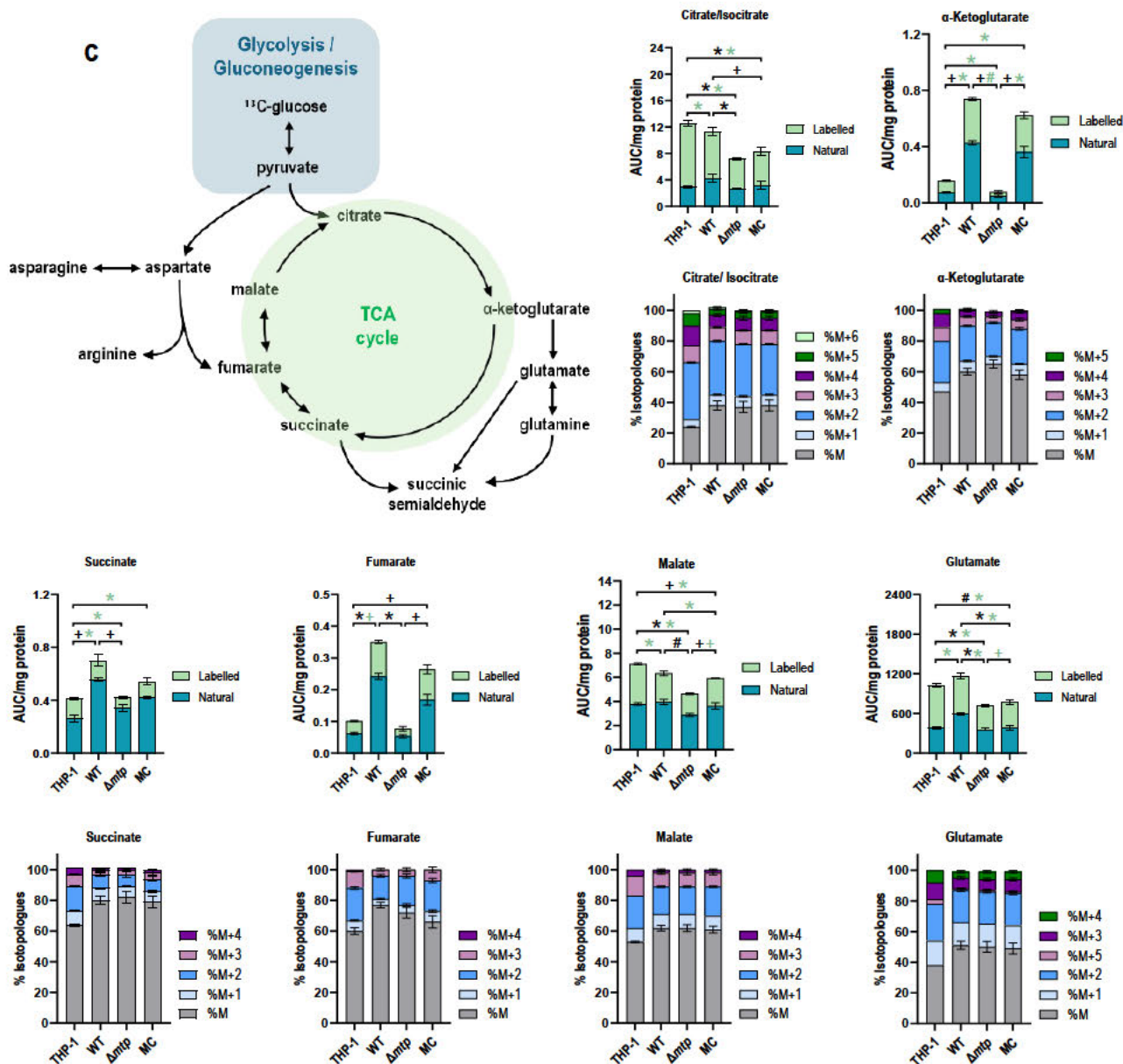


Figure 5.3 continued. Total abundance and isotopologue distribution of incorporated $^{13}\text{C}_6$ -glucose into glycolysis/gluconeogenesis, PPP and TCA cycle of THP-1 macrophages. Experiments were performed on uninfected THP-1 macrophages (THP-1), WT infected THP-1 macrophages (WT), Δmtp infected THP-1 macrophages (Δmtp), and mtp -complemented infected THP-1 macrophages (MC). An MOI of 5 for each *M. tuberculosis* strain was used to infect the THP-1 macrophages for four hours and followed by eight hours of incubation with labelled media to perform $^{13}\text{C}_6$ -glucose tracing amongst different infection groups. On the total abundance graphs, there are two sets of statistical significance indicated: the difference between absolute abundance (black symbols) (Appendix E: Table S3), and the difference between carbon flux (Appendix E: Table S4). Statistical differences amongst the THP-1 macrophages, for each compound involved in (a) glycolysis/ gluconeogenesis, (b) PPP, and (c) TCA cycle are illustrated. The isotopologue graphs illustrate the percentage of ^{13}C atoms incorporations. Data are representative of three independent experiments and shown as the mean $\pm$ SEM ($n = 5$ biological replicates). Data was analysed by one-way ANOVA and Tukey's test for multiple comparisons to determine the significance of metabolic flux for each compound. The adjusted p -values are represented as; *, $p \leq 0.001$; #, $p \leq 0.005$; +, $p \leq 0.05$.

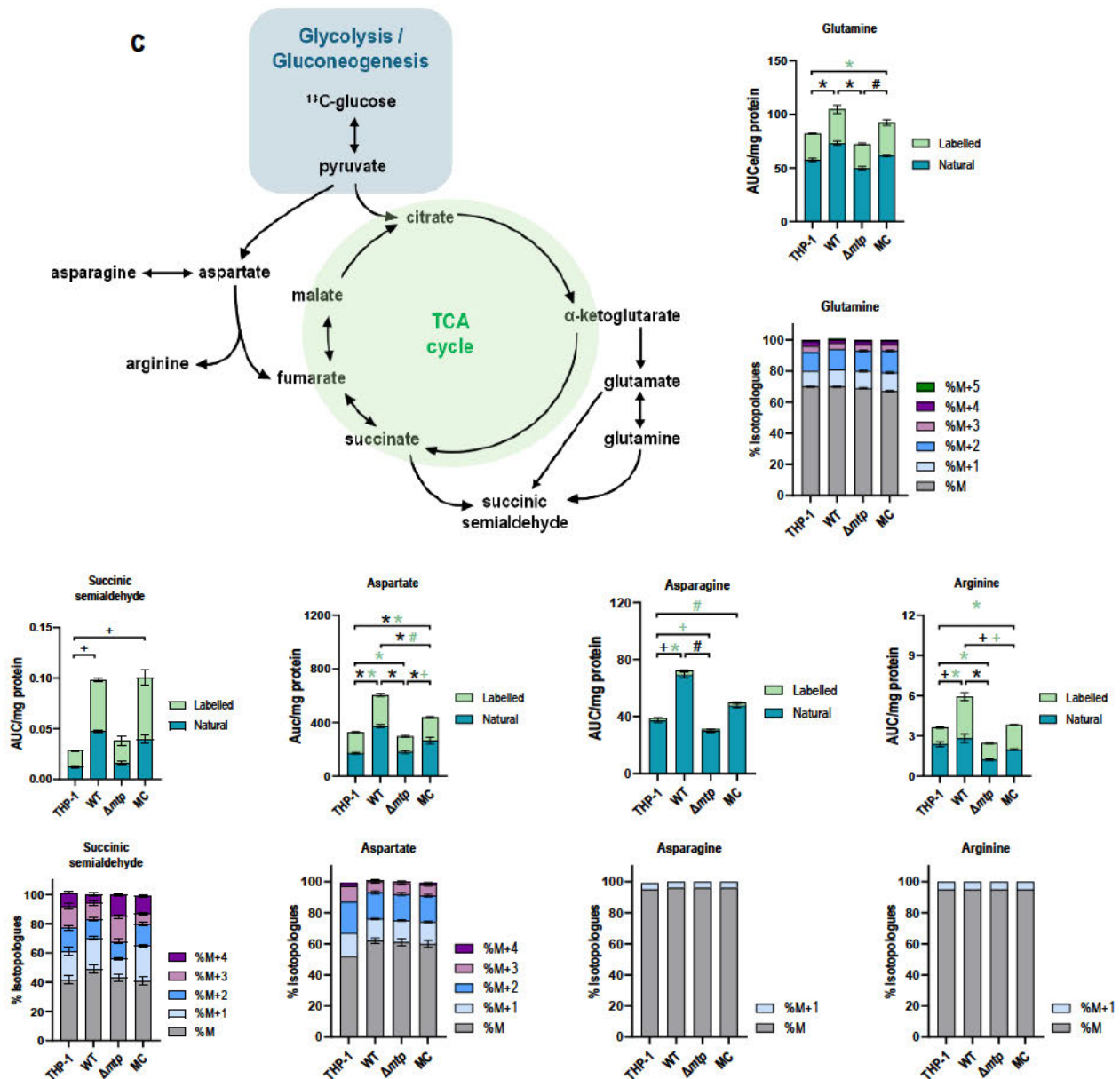


Figure 5.3 continued. Total abundance and isotopologue distribution of incorporated $^{13}\text{C}_6$ -glucose into glycolysis/gluconeogenesis, PPP and TCA cycle of THP-1 macrophages. Experiments were performed on uninfected THP-1 macrophages (THP-1), WT infected THP-1 macrophages (WT), Δmtp infected THP-1 macrophages (Δmtp), and mtp -complemented infected THP-1 macrophages (MC). An MOI of 5 for each *M. tuberculosis* strain was used to infect the THP-1 macrophages for four hours and followed by eight hours of incubation with labelled media to perform $^{13}\text{C}_6$ -glucose tracing amongst different infection groups. On the total abundance graphs, there are two sets of statistical significance indicated: the difference between absolute abundance (black symbols) (Appendix E: Table S3), and the difference between carbon flux (Appendix E: Table S4). Statistical differences amongst the THP-1 macrophages, for each compound involved in (a) glycolysis/ gluconeogenesis, (b) PPP, and (c) TCA cycle are illustrated. The isotopologue graphs illustrate the percentage of ^{13}C atoms incorporations. Data are representative of three independent experiments and shown as the mean $\pm$ SEM ($n = 5$ biological replicates). Data was analysed by one-way ANOVA and Tukey's test for multiple comparisons to determine the significance of metabolic flux for each compound. The adjusted p -values are represented as; *, $p \leq 0.001$; #, $p \leq 0.005$; +, $p \leq 0.05$.

5.5. Discussion

The impact of MTP on THP-1 macrophage bioenergetic and metabolomic profiles during early stages of infection with *M. tuberculosis* were investigated by extracellular flux analysis using the CMST and GRA assays, and targeted metabolomics via carbon labelled experiments, respectively. The CMST demonstrated that MTP decreases the OCR of respiratory parameters such as basal respiration, ATP production, maximal respiration and spare respiratory capacity of *M. tuberculosis* infected THP-1 macrophages. The GRA revealed that *M. tuberculosis* infection of THP-1 macrophages caused an increased glycoPER; however, MTP potentially plays a role in regulating the glycolytic capacity of these macrophages during early infection. Moreover, targeted metabolomics illustrated that regardless of the presence or absence of MTP, *M. tuberculosis* infection of THP-1 macrophages causes an increased flux of carbon into glycolysis and PPP, which is met with a reduced flux into TCA cycle.

5.5.1. Macrophage plasticity determines whether *M. tuberculosis* infection triggers a pro-inflammatory or anti-inflammatory response

Macrophages are the first responders of host innate defence mechanisms, and have crucial roles in tissue maintenance, repair, homeostasis, and inflammation regulation [32; 33]. Subsequent to the inhalation of *M. tuberculosis*, the innate immune responders confront the intracellular pathogen, which attempts to hijack the host cells and slow down their metabolic functions [34-37]. *Mycobacterium tuberculosis* is one of the most successful intracellular pathogens due to its ability to adapt to the environment [38; 39]. Furthermore, the evolution of a number of mechanisms to counter the host immune response renders macrophages a suitable niche for the survival of the pathogen [40]. Macrophages are known to have metabolic plasticity, which impacts pathology [2]. Customarily, the fate and function of macrophages are determined by polarisation, which results in distinct metabolic profiles and activation types of pro-inflammatory and anti-inflammatory macrophages.

During *M. tuberculosis* infection, pro-inflammatory macrophages like IMs clear or restrain the intracellular infection [41]. In addition, pro-inflammatory macrophages impede *M. tuberculosis* proliferation and actively increase aerobic glycolysis to utilise more glucose to meet increased energy demand [42]. The upregulation of glycolysis and PPP confers at least two advantages to these activated macrophages; the rapid generation of ATP, and generation of metabolic intermediates to a pro-inflammatory phenotype [43]. Inflammation is promoted by the generation of nicotinamide adenine dinucleotide (NADH), which aids the reprogramming of inflammatory gene expression by serving as fuel for reactive oxygen species (ROS) generation through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme family [43]. Additionally, the pro-inflammatory macrophages reroute carbon flux away from the TCA cycle toward lactate production from pyruvate [44]. This preference supports the prompt cellular turnover that results in bioenergetic and biosynthetic intermediates that allow for ROS production thereby generating an anti-mycobacterial oxidative burst [45]. This aids in activation of pro-inflammatory genes and the downstream effectors, subsequently restricting growth of *M. tuberculosis* [46].

In contrast, anti-inflammatory macrophages such as AMs, support the persistence and proliferation of *M. tuberculosis* as they favour a more cost-ineffective profile [47]. This is achieved through mitochondrial OXPHOS, fatty acid oxidation (FAO) and glutamine metabolism to meet cellular energy demands [46].

5.5.2. *Mycobacterium tuberculosis* curli pili alters THP-1 macrophage bioenergetic metabolism to establish *M. tuberculosis* infection

In the present study, the CMST findings demonstrated that the OCR parameters such as basal respiration, ATP-linked respiration, spare respiratory capacity, and maximal respiration of the uninfected THP-1 macrophages and the Δmtp infected THP-1 macrophages were similar (Figure 5.1). Contrastingly, these parameters were found to be the lowest in the WT infected THP-1 macrophages. This is supported by the downregulation of enzymes involved in OXPHOS during *M. tuberculosis* infection in a murine model [30]. Regardless of the *M. tuberculosis* strain used in the current study, the THP-1 macrophages had elevated non-mitochondrial respiration and reduced proton leak. Therefore, it was deduced that the MTP adhesin plays a role only in modulating OXPHOS related respiratory parameters of THP-1 macrophages.

The results from the GRA revealed that MTP has no significant effect on the higher glycolytic rates observed proceeding *M. tuberculosis* infection of THP-1 macrophages (Figure 5.2). A recent study highlighted that the pro-inflammatory macrophage response to *M. tuberculosis* infection was brought about by driving glycolysis to allow for the appropriate cytokine production, antigen presentation and containment of the pathogen [48]. Additionally, the Δmtp infected THP-1 macrophages displayed the highest compensatory glycolytic rate, which is associated with an increased potential of the host to mount an inflammatory response, much like that of a pro-inflammatory macrophage. Thus, Δmtp resembles the attenuated *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) strain during infection, as both increase the glycoPER of macrophages, consequentially increasing the glycolytic contribution to ATP production [4]. Hence, it is proposed that inactivation of MTP will cause an appropriate and healthy macrophage metabolic response to eradicate the infection.

5.5.3. Total abundance of metabolites is influenced by MTP to facilitate modulation of immune response

The use of LC-MS/MS to detect the breakdown/incorporation of $^{13}\text{C}_6$ -glucose within THP-1 macrophages demonstrated that MTP significantly affected the total abundance of metabolites involved in glycolysis, TCA and PPP, but not the carbon flux through these pathways (Figure 5.3). The secretion of lactate is often used as a measure of glycolysis with higher levels being indicative of a switch to aerobic glycolysis [49]. Lactate was found in lower total levels in the Δmtp infected THP-1 macrophages and had no significant difference in flux compared to the other *M. tuberculosis* THP-1 macrophage infection groups (Figure 5.3a). This contradicted previous findings from untargeted metabolomics using the same model in which lactate was found in excess in the Δmtp infected THP-1 macrophages [11]. This difference emphasises the importance of variation in metabolism seen in different snapshots in time. The difference in lactate production may be explained by the use of samples that were snap frozen shortly after the four hour infection in the previous study [11] compared to the additional eight hours used to allow the appropriate carbon labelling in the current study,

Moreover, lactate has a compelling immunomodulatory effect. When *M. tuberculosis* stimulates a robust shift of macrophages towards aerobic glycolysis, the increased production and secretion of lactate as a by-product occurs in conjunction with a pro-inflammatory response [50]. Studies have reported an immediate increase of glycolysis in hMDMs upon exposure to *M. tuberculosis*, but OXPHOS remained stable [50]. Additionally, CFUs of lactate treated, *M. tuberculosis* infected hMDMs showed a significant reduction in growth of virulent *M. tuberculosis* compared to untreated macrophages [50]. However, lactate was reported to have a negative feedback effect on macrophages,

and potentially cause a glycolytic shift [50]. Another study demonstrated the crucial role of monocarboxylate transporter 4 (Mct4) for the extracellular transport of lactate [51]. The expression of Mct4 in macrophages and TB patients was promoted by *M. tuberculosis* infection and this protein facilitated the survival of the pathogen in macrophages and mice [51]. Other carbon tracing studies have also reported the reduction of lactate secretion following the eight hours of labelling with the use of stable isotopes and LC-MS/MS [4]. They reported a shift from a reduction of mitochondrial dependency on glucose to enhanced dependency on endogenous fatty acids in macrophages used for bacterial survival under conditions of stress [4]. The data presented in the current study substantiates the notion that as infection progresses, availability of valuable carbon sources is limited, so adaptation and flexibility of cells to the environment must occur swiftly and effectively. Hence, it is proposed that in the absence of MTP, glycolysis is initially ramped up and OXPHOS remains stable, resulting in the accumulation of lactate. This causes the macrophage to restrict nutrient supply to the pathogen by restricting intracellular lactate accumulation potentially through Mct4 subsequently facilitating *M. tuberculosis* killing.

Additionally, in the current study activity of TCA was reduced while PPP was increased for all *M. tuberculosis* infected THP-1 macrophages (Figure 5.3b and 5.3c). These findings were supported by a recent study, which reported relatively lower TCA cycle fluxes compared to glycolysis and PPP in *M. tuberculosis* infected and uninfected THP-1 macrophages [15]. Moreover, TCA cycle derived amino acids like aspartate, glutamate and glutamine fluxes differed between uninfected and *M. tuberculosis* infected macrophages [15]. Macrophage amino acid fluxes are known to be reprogrammed upon *M. tuberculosis* infection, with glutamine and arginine metabolism being important in regulation of macrophage polarisation [15; 52; 53]. The downregulation of TCA cycle flux may represent a protective host response in the priming of macrophages to release ROS and other pro-inflammatory molecules [26]. Although an increased flux through PPP was previously associated in live virulent *M. tuberculosis* infected macrophages, a decreased flux through this pathway was seen in dead *M. tuberculosis* infected and BCG infected THP-1 macrophages [4]. In immune cells, a higher flux of carbon through PPP was observed during an inflammatory response [54; 55]. Hence, successfully virulent *M. tuberculosis* may restrict flux through PPP flux as an immune evasion mechanism since activated macrophages increase PPP activity [56; 57].

Holistically, results from this study suggests that the Δmtp infected THP-1 macrophages display more of a pro-inflammatory phenotype during the first few hours of infection in comparison to the WT infected THP-1 macrophages. Hence, MTP confers a disadvantage to the host during early infection to enable survival and replication of the pathogen, thus supporting observations from our previous studies [7; 8; 10-12; 18; 58].

5.5.4. MTP impacts a chain of events to aid *M. tuberculosis* during infection

In an attempt to kill the pathogen via ROS and defensins, pro-inflammatory macrophages induce an exaggerated pro-inflammatory response, which disrupts tissue homeostasis and intactness [59; 60]. In an effort to counter this, the macrophages typically switch from a pro-inflammatory to an anti-inflammatory state to dampen excessive inflammation [61] but end up serving as a niche for *M. tuberculosis*. This subdues immune surveillance and clearance of the pathogen in addition to *M. tuberculosis* ability to subvert continuous innate cell resistance by masking its pathogen-associated molecular patterns (PAMPs) [62]. These events protect the pathogen from killing and facilitate its smooth passage to the distal regions of the lungs, causing a chronic infection [61]. This plastic behaviour is an important mechanism of virulence majorly influenced by the distinct microenvironment, caused by extensive metabolic reshuffling [2].

Therefore, in the absence of the MTP adhesin, the first responders like AMs and IMs are speculated not to be subjected to large bacterial loads by *M. tuberculosis* given the pathogen's reduced ability to adhere to and invade these immune cells [7]. Blocking/hindering the MTP adhesin may additionally result in *M. tuberculosis* detection and clearance by immune cells as excessive inflammation is less likely to be triggered. This is due to the similar increased OXPHOS (basal respiration, ATP production, maximal respiration and spare respiratory capacity), and lower total metabolite abundance seen in the uninfected and Δmtp infected THP-1 macrophages. Apart from this, the similar carbon flux seen between the Δmtp infected and WT infected THP-1 macrophages indicate activation of the host's response to infection by *M. tuberculosis*. However, *M. tuberculosis* is at a disadvantage as the macrophage limits nutrient availability and has a greater ability to increase its glycolytic bacterial killing abilities. These interesting findings can be used to explore development of promising adjunctive therapies for TB prevention and treatment.

The findings presented in the current study should be interpreted with caution as the complex nature of human cell types and their network interactions complicate the understanding and discernment of outcomes to environmental changes [60]. The *in vitro* findings may be an imprecise representation of the host internal environment given the array of host genetic variability [63]. Moreover, mixed macrophage phenotypes, which express both pro-inflammatory and anti-inflammatory markers have been reported and they contain permissive and controller subpopulations [64]. The microenvironmental conditioning and immunological stimulus present in different tissues also directs macrophage diversity. Hence, macrophages are better categorised based on the effector molecules they produce such as interferon IFN- γ , IL-4, or IL-10 [65]. These effector molecules among others like IL-6 and IL-12 were detected in higher concentrations in the supernatants of WT infected THP-1 macrophages relative to uninfected THP-1 macrophages during three time points [66]. Therefore, it is no longer a simple concept of the dimorphic macrophage heterogeneity [2; 67]. The delineation of macrophage subtypes to that of protective or pathogenic is rather complex. Nonetheless, the data presented in the current study contribute to a better understanding of the effects of MTP on host bioenergetic metabolism and metabolomics.

5.6. Conclusion

A reduction of host respiratory parameters was observed in the WT infected THP-1 macrophages. Decrease in OXPHOS of WT infected differentiated THP-1 macrophages can be partly attributed to the MTP adhesin present on the surface of *M. tuberculosis*. Therefore, by inhibiting/blocking of the MTP interaction with the host, the immune cells retain their typical energy demands and outputs to commission a healthy immune response. Additionally, high compensatory glycolytic capacity potentially facilitates the prompt clearance of the intracellular pathogen before it establishes a successful infection. The findings from this study demonstrate that MTP facilitates survival of *M. tuberculosis* during the early stages of macrophage infection, hence, it is an important virulence factor of *M. tuberculosis* and if interaction between the pathogen and host is interrupted/stopped, the host will be better equipped to clear an *M. tuberculosis* infection.

References:

1. World Health Organization (2024). Global tuberculosis report. *World Health Organization* [Online]. Available: <https://www.who.int/publications/i/item/9789240101531> [Accessed October 2024].
2. Ahmad, F., Rani, A., Alam, A., Zarin, S., Pandey, S., Singh, H., Hasnain, S.E., and Ehtesham, N.Z. (2022). Macrophage: A cell with many faces and functions in tuberculosis. *Frontiers in Immunology*, 13, 747799. <https://doi.org/10.3389/fimmu.2022.747799>.
3. Fraternali, A., Brundu, S., and Magnani, M. (2015). Polarization and repolarization of macrophages. *Journal of Clinical & Cellular Immunology*, 6(2), 1-10. <https://doi.org/10.4172/2155-9899.1000319>.
4. Cumming, B.M., Addicott, K.W., Adamson, J.H., and Steyn, A.J. (2018). *Mycobacterium tuberculosis* induces decelerated bioenergetic metabolism in human macrophages. *Elife*, 7, e39169. <https://doi.org/10.7554/elife.39169>.
5. Menozzi, F.D., Reddy, V.M., Cayet, D., Raze, D., Debie, A.S., Dehouck, M.P., Cecchelli, R., and Locht, C. (2006). *Mycobacterium tuberculosis* heparin-binding haemagglutinin adhesin (HBHA) triggers receptor-mediated transcytosis without altering the integrity of tight junctions. *Microbes and Infection*, 8(1), 1-9. <https://doi.org/10.1016/j.micinf.2005.03.023>.
6. Alteri, C.J., Xicohtencatl-Cortes, J., Hess, S., Caballero-Olín, G., Girón, J.A., and Friedman, R.L. (2007). *Mycobacterium tuberculosis* produces pili during human infection. *Proceedings of the National Academy of Sciences of the United States of America*, 104(12), 5145-5150. <https://doi.org/10.1073/pnas.0602304104>.
7. Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* pili promote adhesion to and invasion of THP-1 macrophages. *Japanese Journal of Infectious Diseases*, 67(6), 476-478. <https://doi.org/10.7883/yoken.67.476>.
8. Nyawo, G. (2016). *The role of Mycobacterium tuberculosis pili in pathogenesis: Growth and survival kinetics, gene regulation and host immune response, and in vitro growth kinetics*. Masters dissertation University of KwaZulu-Natal, Durban, South Africa. <https://researchspace.ukzn.ac.za/items/3628eab3-3e5f-47a6-9eb3-07c3f3c52ad7>.
9. Dlamini, M.T. (2016). *Whole transcriptome analysis to elucidate the role of mtp in gene regulation of pulmonary epithelial cells infected with Mycobacterium tuberculosis*. Masters dissertation University of KwaZulu-Natal, Durban, South Africa. <https://researchspace.ukzn.ac.za/items/71f0ed4e-aa63-4cca-8bf6-4d771326e616>.
10. Naidoo, N., Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* pili (MTP), a putative biomarker for a tuberculosis diagnostic test. *Tuberculosis*, 94(3), 338-345. <http://dx.doi.org/10.1016/j.tube.2014.03.004>.
11. Ashokcoomar, S., Loots, D.T., Beukes, D., Van Reenen, M., Pillay, B., and Pillay, M. (2021). *M. tuberculosis* curli pili (MTP) is associated with alterations in carbon, fatty acid and amino acid metabolism in a THP-1 macrophage infection model. *Microbial Pathogenesis*, 154, 104806. <https://doi.org/10.1016/j.micpath.2021.104806>.
12. Ashokcoomar, S., Reedoy, K.S., Senzani, S., Loots, D.T., Beukes Maasdorp, D., Van Reenen, M., Pillay, B., and Pillay, M. (2020). *Mycobacterium tuberculosis* curli pili (MTP) deficiency is associated with alterations in cell wall biogenesis, fatty acid metabolism and amino acid synthesis. *Metabolomics*, 16(9), 97. <https://doi.org/10.1007/s11306-020-01720-z>.
13. Walvekar, A., Rashida, Z., Maddali, H., and Laxman, S. (2018). A versatile LC-MS/MS approach for comprehensive, quantitative analysis of central metabolic pathways. *Wellcome Open Research*, 3, 122. <https://doi.org/10.12688/wellcomeopenres.14832.1>.

14. Allen, D.K. , and Young, J.D. (2020). Tracing metabolic flux through time and space with isotope labeling experiments. *Current Opinion in Biotechnology*, 64, 92-100. <https://doi.org/10.1016/j.copbio.2019.11.003>.
15. Borah Slater, K., Moraes, L., Xu, Y., and Kim, D. (2023). Metabolic flux reprogramming in *Mycobacterium tuberculosis*-infected human macrophages. *Frontiers in Microbiology*, 14, 1289987. <https://doi.org/10.3389/fmicb.2023.1289987>.
16. Tazoll, S.C. (2022). *Mycobacterium tuberculosis* infection of human macrophages: Effects on cellular metabolism and lipid droplet composition. Doctoral thesis, Universität zu Lübeck, Germany. <https://www.zhb.uni-luebeck.de/epubs/ediss2723.pdf>.
17. Gandhi, N.R., Moll, A., Sturm, A.W., Pawinski, R., Govender, T., Lalloo, U., Zeller, K., Andrews, J., and Friedland, G. (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet*, 368(9547), 1575-1580. [https://doi.org/10.1016/s0140-6736\(06\)69573-1](https://doi.org/10.1016/s0140-6736(06)69573-1).
18. Ramsugit, S., Guma, S., Pillay, B., Jain, P., Larsen, M.H., Danaviah, S., and Pillay, M. (2013). Pili contribute to biofilm formation *in vitro* in *Mycobacterium tuberculosis*. *Antonie Van Leeuwenhoek*, 104(5), 725-735. <https://doi.org/10.1007/s10482-013-9981-6>.
19. Bardarov, S., Bardarov, S., Pavelka, M.S., Sambandamurthy, V., Larsen, M., Tufariello, J., Chan, J., Hatfull, G., and Jacobs, W.R. (2002). Specialized transduction: An efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. *Microbiology (Reading)*, 148(Pt 10), 3007-3017. <https://doi.org/10.1099/00221287-148-10-3007>.
20. Agilent (2024). Seahorse real-time cell metabolic analysis. [Online]. Available: <https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-analyzers> [Accessed April 2024].
21. Cumming, B.M., Reddy, V.P., and Steyn, A.J.C. (2020). The analysis of *Mycobacterium tuberculosis*-induced bioenergetic changes in infected macrophages using an extracellular flux analyzer. *Methods in Molecular Biology*, 2184(Methods in Molecular Biology), 161-184. https://doi.org/10.1007/978-1-0716-0802-9_12.
22. Marchetti, P., Fovez, Q., Germain, N., Khamari, R., and Kluza, J. (2020). Mitochondrial spare respiratory capacity: Mechanisms, regulation, and significance in non-transformed and cancer cells. *FASEB Journal*, 34(10), 13106-13124. <https://doi.org/10.1096/fj.202000767R>.
23. Chacko, Balu K., Kramer, Philip A., Ravi, S., Benavides, Gloria A., Mitchell, T., Dranka, Brian P., Ferrick, D., Singal, Ashwani K., Ballinger, Scott W., Bailey, Shannon M., Hardy, Robert W., Zhang, J., Zhi, D., and Darley-Usmar, Victor M. (2014). The bioenergetic health index: A new concept in mitochondrial translational research. *Clinical Science*, 127(6), 367-373. <https://doi.org/10.1042/cs20140101>.
24. Bailey, J.D., Diotallevi, M., Nicol, T., McNeill, E., Shaw, A., Chuaiphichai, S., Hale, A., Starr, A., Nandi, M., Stylianou, E., McShane, H., Davis, S., Fischer, R., Kessler, B.M., McCullagh, J., Channon, K.M., and Crabtree, M.J. (2019). Nitric oxide modulates metabolic remodeling in inflammatory macrophages through TCA cycle regulation and itaconate accumulation. *Cell Reports*, 28(1), 218-230.e217. <https://doi.org/10.1016/j.celrep.2019.06.018>.
25. Kumar, R., Singh, P., Kolloli, A., Shi, L., Bushkin, Y., Tyagi, S., and Subbian, S. (2019). Immunometabolism of phagocytes during *Mycobacterium tuberculosis* infection. *Frontiers in Molecular Biosciences*, 6, 105. <https://doi.org/10.3389/fmolb.2019.00105>.
26. Howard, N.C. , and Khader, S.A. (2020). Immunometabolism during *Mycobacterium tuberculosis* Infection. *Trends in Microbiology*, 28(10), 832-850. <https://doi.org/10.1016/j.tim.2020.04.010>.

27. Shi, L., Eugenin, E.A., and Subbian, S. (2016). Immunometabolism in Tuberculosis. *Frontiers in Immunology*, 7, 150. <https://doi.org/10.3389/fimmu.2016.00150>.
28. Li, X., Shen, H., Zhang, M., Teissier, V., Huang, E.E., Gao, Q., Tsubosaka, M., Toya, M., Kushioka, J., Maduka, C.V., Contag, C.H., Chow, S.K.-H., Zhang, N., and Goodman, S.B. (2023). Glycolytic reprogramming in macrophages and MSCs during inflammation. *Frontiers in Immunology*, 14, 1199751. <https://doi.org/10.3389/fimmu.2023.1199751>.
29. Vazquez, A., Liu, J., Zhou, Y., and Oltvai, Z.N. (2010). Catabolic efficiency of aerobic glycolysis: The Warburg effect revisited. *BMC Systems Biology*, 4, 58. <https://doi.org/10.1186/1752-0509-4-58>.
30. Shi, L., Salamon, H., Eugenin, E.A., Pine, R., Cooper, A., and Gennaro, M.L. (2015). Infection with *Mycobacterium tuberculosis* induces the Warburg effect in mouse lungs. *Scientific Reports*, 5, 18176. <https://doi.org/10.1038/srep18176>.
31. Krawczyk, C.M., Holowka, T., Sun, J., Blagih, J., Amiel, E., DeBerardinis, R.J., Cross, J.R., Jung, E., Thompson, C.B., Jones, R.G., and Pearce, E.J. (2010). Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood*, 115(23), 4742-4749. [10.1182/blood-2009-10-249540](https://doi.org/10.1182/blood-2009-10-249540).
32. Wynn, T.A., Chawla, A., and Pollard, J.W. (2013). Macrophage biology in development, homeostasis and disease. *Nature*, 496(7446), 445-455. <https://doi.org/10.1038/nature12034>.
33. Gordon, S., Plüddemann, A., and Martinez Estrada, F. (2014). Macrophage heterogeneity in tissues: Phenotypic diversity and functions. *Immunology Reviews* 262(1), 36-55. <https://doi.org/10.1111/imr.12223>.
34. Hingley-Wilson, S.M., Sambandamurthy, V.K., and Jacobs, W.R., Jr. (2003). Survival perspectives from the world's most successful pathogen, *Mycobacterium tuberculosis*. *Nature Immunology*, 4(10), 949-955. <https://doi.org/10.1038/ni981>.
35. Marrero, J., Rhee, K.Y., Schnappinger, D., Pethe, K., and Ehrt, S. (2010). Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for *Mycobacterium tuberculosis* to establish and maintain infection. *Proceedings of the National Academy of Sciences of the United States of America*, 107(21), 9819-9824. <https://doi.org/10.1073/pnas.1000715107>.
36. McKinney, J.D., zu Bentrup, K.H., Muñoz-Elías, E.J., Miczak, A., Chen, B., Chan, W.-T., Swenson, D., Sacchettini, J.C., Jacobs, W.R., and Russell, D.G. (2000). Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature*, 406(6797), 735-738. <https://doi.org/10.1038/35021074>.
37. Singh, V., Jamwal, S., Jain, R., Verma, P., Gokhale, R., and Rao, K.V. (2012). *Mycobacterium tuberculosis*-driven targeted recalibration of macrophage lipid homeostasis promotes the foamy phenotype. *Cell Host Microbe*, 12(5), 669-681. <https://doi.org/10.1016/j.chom.2012.09.012>.
38. Eoh, H. (2014). Metabolomics: A window into the adaptive physiology of *Mycobacterium tuberculosis*. *Tuberculosis*, 94(6), 538-543. <https://doi.org/10.1016/j.tube.2014.08.002>.
39. Chai, Q., Zhang, Y., and Liu, C.H. (2018). *Mycobacterium tuberculosis*: An adaptable pathogen associated with multiple human diseases. *Frontiers in Cellular and Infection Microbiology*, 8, 158. <https://doi.org/10.3389/fcimb.2018.00158>.
40. Cambier, C.J., Falkow, S., and Ramakrishnan, L. (2014). Host evasion and exploitation schemes of *Mycobacterium tuberculosis*. *Cell*, 159(7), 1497-1509. <https://doi.org/10.1016/j.cell.2014.11.024>.
41. Chakarov, S., Lim, H.Y., Tan, L., Lim, S.Y., See, P., Lum, J., Zhang, X.M., Foo, S., Nakamizo, S., Duan, K., Kong, W.T., Gentek, R., Balachander, A., Carbajo, D., Bleriot, C., Malleret, B., Tam, J.K.C., Baig, S., Shabeer, M., Toh, S.E.S., Schlitzer, A., Larbi, A., Marichal, T., Malissen, B., Chen, J., Poidinger, M., Kabashima, K., Bajenoff, M., Ng, L.G.,

- Angeli, V., and Ginhoux, F. (2019). Two distinct interstitial macrophage populations coexist across tissues in specific subtissular niches. *Science*, 363(6432), eaau0964. <https://doi.org/10.1126/science.aau0964>.
42. Mould, K.J., Barthel, L., Mohning, M.P., Thomas, S.M., McCubbrey, A.L., Danhorn, T., Leach, S.M., Fingerlin, T.E., O'Connor, B.P., Reisz, J.A., D'Alessandro, A., Bratton, D.L., Jakubzick, C.V., and Janssen, W.J. (2017). Cell origin dictates programming of resident versus recruited macrophages during acute lung injury. *American Journal of Respiratory Cell and Molecular Biology*, 57(3), 294-306. <https://doi.org/10.1165/rcmb.2017-0061oc>.
 43. Erlich, J.R., To, E.E., Luong, R., Liong, F., Liong, S., Oseghale, O., Miles, M.A., Bozinovski, S., Brooks, R.D., Vlahos, R., Chan, S., O'Leary, J.J., Brooks, D.A., and Selemidis, S. (2022). Glycolysis and the pentose phosphate pathway promote LPS-Induced NOX2 oxidase- and IFN- β -dependent inflammation in macrophages. *Antioxidants*, 11(8), 1488. <https://doi.org/10.3390/antiox11081488>.
 44. Shi, L., Jiang, Q., Bushkin, Y., Subbian, S., and Tyagi, S. (2019). Biphasic dynamics of macrophage immunometabolism during *Mycobacterium tuberculosis* infection. *mBio*, 10(2). <https://doi.org/10.1128/mbio.02550-18>.
 45. Thiriot, J.D., Martinez-Martinez, Y.B., Endsley, J.J., and Torres, A.G. (2020). Hacking the host: Exploitation of macrophage polarization by intracellular bacterial pathogens. *Pathogens and Disease*, 78(1), ftaa009. <https://doi.org/10.1093/femspd/ftaa009>.
 46. Huang, L., Nazarova, E.V., Tan, S., Liu, Y., and Russell, D.G. (2018). Growth of *Mycobacterium tuberculosis* in vivo segregates with host macrophage metabolism and ontogeny. *J Exp Med*, 215(4), 1135-1152. 10.1084/jem.20172020.
 47. Williams, M. , and Svedberg, F.R. (2021). Does tissue imprinting restrict macrophage plasticity? *Nature Immunology*, 22(2), 118-127. 10.1038/s41590-020-00849-2.
 48. Hackett, E.E., Charles-Messance, H., O'Leary, S.M., Gleeson, L.E., Muñoz-Wolf, N., Case, S., Wedderburn, A., Johnston, D.G.W., Williams, M.A., Smyth, A., Ouimet, M., Moore, K.J., Lavelle, E.C., Corr, S.C., Gordon, S.V., Keane, J., and Sheedy, F.J. (2020). *Mycobacterium tuberculosis* limits host glycolysis and IL-1 β by restriction of PFK-M via microRNA-21. *Cell Reports*, 30(1), 124-136.e124. <https://doi.org/10.1016/j.celrep.2019.12.015>.
 49. Lamprecht, D.A., Finin, P.M., Rahman, M.A., Cumming, B.M., Russell, S.L., Jonnala, S.R., Adamson, J.H., and Steyn, A.J. (2016). Turning the respiratory flexibility of *Mycobacterium tuberculosis* against itself. *Nature Communications*, 7, 12393. <https://doi.org/10.1038/ncomms12393>.
 50. Ó Maoldomhnaigh, C., Cox, D.J., Phelan, J.J., Mitermite, M., Murphy, D.M., Leisching, G., Thong, L., O'Leary, S.M., Gogan, K.M., McQuaid, K., Coleman, A.M., Gordon, S.V., Basdeo, S.A., and Keane, J. (2021). Lactate alters metabolism in human macrophages and improves their ability to kill *Mycobacterium tuberculosis*. *Frontiers in Immunology*, 12. <https://doi.org/10.3389/fimmu.2021.663695>.
 51. Huang, Y., Li, R., Chen, S., Wang, Q., Han, Z., Liang, Y., Liang, Y., Li, Q., Lin, L., Wen, Q., Zhou, C., Zhou, X., and Ma, L. (2024). Monocarboxylate transporter 4 facilitates *Mycobacterium tuberculosis* survival through NF-kB p65-mediated interleukin-10 production. *iScience*, 27(8), 110238. <https://doi.org/10.1016/j.isci.2024.110238>.
 52. Kelly, B. , and Pearce, E.L. (2020). Amino assets: How amino acids support immunity. *Cell Metabolism*, 32(2), 154-175. <https://doi.org/10.1016/j.cmet.2020.06.010>.
 53. Gauthier, T. , and Chen, W. (2022). Modulation of macrophage immunometabolism: A new approach to fight infections. *Frontiers in Immunology*, 13, 780839. <https://doi.org/10.3389/fimmu.2022.780839>.

54. Corcoran, S.E. , and O'Neill, L.A. (2016). HIF1 α and metabolic reprogramming in inflammation. *Journal of Clinical Investigation*, 126(10), 3699-3707. <https://doi.org/10.1172/jci84431>.
55. Haschemi, A., Kosma, P., Gille, L., Evans, Charles R., Burant, Charles F., Starkl, P., Knapp, B., Haas, R., Schmid, Johannes A., Jandl, C., Amir, S., Lubec, G., Park, J., Esterbauer, H., Bilban, M., Brizuela, L., Pospisilik, J.A., Otterbein, Leo E., and Wagner, O. (2012). The sedoheptulose kinase CARKL irects macrophage polarization through control of glucose metabolism. *Cell Metabolism*, 15(6), 813-826. <https://doi.org/10.1016/j.cmet.2012.04.023>.
56. Jha, A.K., Huang, S.C., Sergushichev, A., Lampropoulou, V., Ivanova, Y., Loginicheva, E., Chmielewski, K., Stewart, K.M., Ashall, J., Everts, B., Pearce, E.J., Driggers, E.M., and Artyomov, M.N. (2015). Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity*, 42(3), 419-430. <https://doi.org/10.1016/j.immuni.2015.02.005>.
57. Hackett, E.E. , and Sheedy, F.J. (2020). An army marches on Its stomach: Metabolic intermediates as antimicrobial mediators in *Mycobacterium tuberculosis* infection. *Frontiers in Cellular and Infection Microbiology*, 10, 446. <https://doi.org/10.3389/fcimb.2020.00446>.
58. Ramsugit, S., Pillay, B., and Pillay, M. (2016). Evaluation of the role of *Mycobacterium tuberculosis* pili (MTP) as an adhesin, invasin, and cytokine inducer of epithelial cells. *The Brazilian Journal of Infectious Diseases*, 20(2), 160-165. <http://dx.doi.org/10.1016/j.bjid.2015.11.002>.
59. Lastrucci, C., Bénard, A., Balboa, L., Pingris, K., Souriant, S., Poincloux, R., Al Saati, T., Rasolofo, V., González-Montaner, P., Inwentarz, S., Moraña, E.J., Kondova, I., Verreck, F.A., Sasiain Mdel, C., Neyrolles, O., Maridonneau-Parini, I., Lugo-Villarino, G., and Cougoule, C. (2015). Tuberculosis is associated with expansion of a motile, permissive and immunomodulatory CD16(+) monocyte population via the IL-10/STAT3 axis. *Cell Research*, 25(12), 1333-1351. <https://doi.org/10.1038/cr.2015.123>.
60. Liu, Y.C., Zou, X.B., Chai, Y.F., and Yao, Y.M. (2014). Macrophage polarization in inflammatory diseases. *International Journal of Biological Sciences*, 10(5), 520-529. <https://doi.org/10.7150/ijbs.8879>.
61. Sheedy, F.J. , and Divangahi, M. (2021). Targeting immunometabolism in host defence against *Mycobacterium tuberculosis*. *Immunology*, 162(2), 145-159. <https://doi.org/10.1111/imm.13276>.
62. Cambier, C.J., Takaki, K.K., Larson, R.P., Hernandez, R.E., Tobin, D.M., Urdahl, K.B., Cosma, C.L., and Ramakrishnan, L. (2014). Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. *Nature*, 505(7482), 218-222. <https://doi.org/10.1038/nature12799>.
63. Simmons, J.D., Stein, C.M., Seshadri, C., Campo, M., Alter, G., Fortune, S., Schurr, E., Wallis, R.S., Churchyard, G., Mayanja-Kizza, H., Boom, W.H., and Hawn, T.R. (2018). Immunological mechanisms of human resistance to persistent *Mycobacterium tuberculosis* infection. *Nature Reviews. Immunology*, 18(9), 575-589. <https://doi.org/10.1038/s41577-018-0025-3>.
64. Trombetta, A.C., Soldano, S., Contini, P., Tomatis, V., Ruaro, B., Paolino, S., Brizzolara, R., Montagna, P., Sulli, A., Pizzorni, C., Smith, V., and Cutolo, M. (2018). A circulating cell population showing both M1 and M2 monocyte/macrophage surface markers characterizes systemic sclerosis patients with lung involvement. *Respiratory Research*, 19(1), 186. <https://doi.org/10.1186/s12931-018-0891-z>.
65. Murray, P.J., Allen, J.E., Biswas, S.K., Fisher, E.A., Gilroy, D.W., Goerdts, S., Gordon, S., Hamilton, J.A., Ivashkiv, L.B., Lawrence, T., Locati, M., Mantovani, A., Martinez, F.O., Mege, J.L., Mosser, D.M., Natoli, G., Saeij, J.P., Schultze, J.L., Shirey, K.A., Sica, A., Suttles,

- J., Udalova, I., van Genderachter, J.A., Vogel, S.N., and Wynn, T.A. (2014). Macrophage activation and polarization: Nomenclature and experimental guidelines. *Immunity*, 41(1), 14-20. <https://doi.org/10.1016/j.immuni.2014.06.008>.
66. Moodley, S. (2018). *The role of heparin binding haemagglutinin adhesin and curli pili on the pathogenicity of Mycobacterium tuberculosis*. Doctoral thesis, <https://researchspace.ukzn.ac.za/items/22f2cedb-2826-46ad-8a62-237a41b62d17>.
67. Nahrendorf, M. , and Swirski, F.K. (2016). Abandoning M1/M2 for a network model of macrophage function. *Circulation Research*, 119(3), 414-417. <https://doi.org/10.1161/circresaha.116.309194>.

Chapter 5 illustrated that *Mycobacterium tuberculosis* infection of THP-1 macrophages caused changes to carbon flux. The *M. tuberculosis* curli pili (MTP) did not influence carbon flux; however, it did influence total metabolite concentrations. Moreover, bioenergetic analysis revealed that MTP decreases oxidative phosphorylation and enables the lower compensatory glycolysis of the *M. tuberculosis* infected THP-1 macrophage host.

In Chapter 6, gene expression levels were investigated in pure bacterial culture as well as in intracellular bacteria isolated from THP-1 macrophages to determine if MTP plays a role in regulating intermediary metabolism in different environments. Chapter 6 has been accepted for publication in the “*Journal of Medical Microbiology*” (Microbiology Society) and is currently in press (JMM-D-24-00682).

CHAPTER 6: Differential expression of genes associated with lipid import, β -oxidation and lactate oxidation induced by *Mycobacterium tuberculosis* curli pili (MTP) in broth culture compared to intracellular bacilli within THP-1 macrophages

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Ethics approval: The study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC/00002915/2021) attached in Appendix A.

Declaration of interest: Both authors declare that they have no conflict of interest.

Author contribution: Prof Pillay conceptualised, designed and funded the study. Ms Ashokcoomar designed the experimental protocol, conducted experiments, analysed the data and drafted the manuscript. Both authors contributed to and approved the manuscript.

6.1. Abstract

Introduction: The adhesin, *Mycobacterium tuberculosis* curli pili, assists the pathogen in attachment, invasion and disease progression. Previously, this adhesin was demonstrated to contribute to cell wall functions and fatty acid metabolism of the pathogen and affects total metabolite abundance in central carbon metabolism and fatty acid metabolism of the host. The accumulation/depletion of metabolites are reliant on gene expression of proteins involved in the import, transport and breakdown of substrates.

Gap statement: *Mycobacterium tuberculosis* curli pili has not been investigated in relation to genes involved in import/transport/breakdown of substrates.

Aim: This study aimed to investigate the possible regulatory role of MTP in modulating metabolic changes of the pathogen in different microenvironments.

Methods: Ribonucleic acid (RNA) was harvested from bacterial broth cultures of adhesin-proficient and adhesin-deficient *M. tuberculosis*. These strains were also used to infect differentiated THP-1 macrophages for four hours prior to isolation of intracellular bacteria, RNA, and reverse transcription real-time quantitative polymerase chain reaction. The expression levels of selected genes involved in fatty acid transport (*lucA*, *mce1D*, *mceG*, *Rv2799*, *Rv0966c*, *omamB*), β -oxidation (*fadA5*, *fadB*), lactate oxidation (*lldD1*, *lldD2*) and gluconeogenic carbon flow (*pckA*) were analysed by absolute quantification.

Results: The gene expression levels of *lucA*, *mce1D* and *pckA* were significantly lower, and that of *Rv2799*, *Rv0966c*, *mceG*, *fadA5* and *lldD2* were significantly higher, in the adhesin-proficient cultured bacterial strains relative to the Δmtp strain. The intracellular adhesin-proficient bacteria displayed significantly higher gene expression levels of *Rv2799* and significantly lower gene expression levels of *Rv0966c*, *fadA5*, *lldD1* and *pckA* relative to the Δmtp strain. Interestingly, during early infection, the intracellular Δmtp displayed significantly increased expression of *omamB*, *mceG*, *fadB*, *lldD1*, and *lldD2* relative to the broth culture. This trend was inversed in the wildtype models.

Conclusion: *Mycobacterium tuberculosis* curli pili is significantly associated with the regulation of genes involved in lipid transport, β -oxidation, and lactate oxidation.

6.2. Introduction

Despite the advancements made in vaccination, diagnosis and treatment of tuberculosis (TB) over the last 142 years, the longstanding battle to eradicate *Mycobacterium tuberculosis* (*M. tuberculosis*) continues. Of the 10.8 million people who fell ill with TB in 2023, 55% were adult males, 33% were adult females and 12% were children [1]. Drug resistance to first-line rifampicin (RR-TB) and isoniazid, known as multidrug-resistant TB (MDR-TB) requires treatment with second-line drugs. In 2023, 3.7% of incident cases were estimated to be MDR/RR-TB, of which only 44% were diagnosed and treated [1]. Hence, intensified research and innovation are urgently required to help meet The End TB Strategy targets set for 2030 and 2035 [1].

The lack of suitable and accurate biomarkers impedes development of rapid and effective diagnostics and therapeutics that are essential to address the burden of TB. Essential to microbial pathogenesis, extracellular surface molecules/appendages known as adhesins, are considered valuable biomarkers [2]. Adhesins facilitate the initial interaction between pathogen and host, subsequently, permitting invasion of the host during infections. *Mycobacterium tuberculosis* curli pili (MTP) has been evidenced as a prominent adhesin of *M. tuberculosis*. This adhesin is encoded by the open reading frame *Rv3312A* (*mtp*), and is not arranged as part of an operon or cluster with curli/pili associated genes [3]. Instead, *mtp* is located between intermediary metabolism associated genes [4], is highly conserved amongst clinical isolates, and unique to *M. tuberculosis* complex strains [5]. Numerous

genomics/functional genomics [5-8], transcriptomics [9-12], biomarker evaluation [5; 13] and functional metabolomic studies [14-16] have provided evidence that MTP is a potentially suitable target as a TB diagnostic and vaccine candidate.

As a result of the metabolic alterations observed in the MTP-deficient strain of *M. tuberculosis*, as well as its infected host during early stages of infection, it was proposed that MTP influences the production of metabolites associated with cell composition, carbon metabolism, fatty acid metabolism, and amino acid metabolism [14-17]. These metabolic changes are a result of perturbations in gene regulation of various membrane bound enzymes or genes involved in β -oxidation, lipid transport/metabolism, and gluconeogenic flow [14; 15]. *Mycobacterium tuberculosis* has the ability to utilise multiple nutrient sources such as sugars, fats and other available carbon rich compounds [18-20], hence, an understanding of the function and regulation of the array of genes involved in nutrient assimilation may further elucidate the mechanisms of TB pathogenesis.

The uptake and transport of fatty acids and cholesterol during *M. tuberculosis* infection is mediated by mammalian cell entry (Mce) proteins [21]. Additionally, the genome of *M. tuberculosis* encompasses four unlinked *mce* loci (*mce1-mce4* operons), which are responsible for the transport of lipids into *M. tuberculosis* [22]. The recently identified *Rv2799*, *Rv0966c*, *Rv0200/omamB*, *Rv0172/mce1D*, and *Rv0655/mceG* were shown to be involved in fatty acid import and the putative components of the Mce fatty acid transporters [23]. Moreover, lipid uptake coordinator A (*Rv3723/lucA*) was demonstrated to play an important role in *M. tuberculosis* virulence as it facilitates the import of both fatty acids and cholesterol [22; 23].

Once transported into the cell, fatty acids and cholesterol need to be broken down before incorporation into the tricarboxylic acid (TCA) cycle and repurposed within the cell. There are over 100 genes involved in β -oxidation of fatty acids to produce acetyl-coenzyme A (acetyl-CoA) in *M. tuberculosis*, many of which are annotated as non-essential *in vitro*, possibly due to extensive gene redundancy [24]. The multi-catalytic enzyme, FadB, catalyses both the hydration and the 3-hydroxy dehydrogenation steps in the β -oxidation cycle [25]. The FadB protein forms a trifunctional complex with the thiolase FadA (FadA–FadB complex), which mediates three of the four reaction steps in β -oxidation [26]. The β -oxidation of cholesterol is catalysed by acetyl-CoA acetyltransferase (FadA5) responsible for thiolysis of acetoacetyl CoA [27].

Intracellular growth of *M. tuberculosis* is brought about by biomass formation through gluconeogenesis. The gluconeogenic carbon flow is mediated by phosphoenolpyruvate carboxykinase (PEPCK), encoded by *pckA* (*Rv0211*) and is considered crucial for the survival of *M. tuberculosis* in macrophages and in mice [28]. Moreover, given that macrophages respond to infection by switching from pyruvate oxidation to pyruvate reduction, lactate is plentiful at the site of infection and is exploited as a carbon substrate by *M. tuberculosis* [29]. In *M. tuberculosis*, the conversion of lactate, is dependent on one of two potential L-lactate dehydrogenases. These enzymes are encoded by *lldD1* (*Rv0694*) and *lldD2* (*Rv1872c*) and were demonstrated to play a crucial role in lactate oxidation of this intracellular pathogen [30].

The regulation of the above-mentioned genes could have potentially altered the metabolic profiles reported previously in cultured MTP-deficient *M. tuberculosis*, which displayed an accumulation of fatty acids, cell wall constituents and carbon metabolism intermediates, relative to WT *M. tuberculosis* [15]. Moreover, the MTP-deficient *M. tuberculosis* infected THP-1 macrophages had higher concentrations of lactate, glyceric acid and fatty acids relative to the WT infected macrophages [14]. Hence, it was postulated that MTP plays a significant role in regulation of genes, both in pure bacterial culture and in intracellular bacteria harvested from infected THP-1 macrophages. Therefore, in this study, reverse transcription quantitative polymerase chain reactions (RT-qPCR) was used to investigate the role of MTP on the regulation of genes, which modulate lipid metabolism, carbon metabolism, and cellular import/transport functions in bacterial culture and intracellular bacteria

isolated from infected THP-1 macrophages. The data presented provides further insight into the metabolomic alterations observed in the previous studies [14; 15].

6.3. Materials and methods

6.3.1. Ethics statement and laboratory facilities

The Biomedical Research Ethics Committee of the University of KwaZulu-Natal approved this study (BREC/00002915/2021). The experimental work was conducted in a Biosafety Level 2+ (BSL 2+) laboratory at Medical Microbiology, University of KwaZulu-Natal, Durban South Africa.

6.3.2. Bacterial strains and growth conditions

Mycobacterium tuberculosis V9124 from the F15/LAM4/KZN family [31], was used as the WT strain in this study. The marked *mtp*-deletion mutant (Δmtp) was constructed through specialised transduction of the WT by deleting and replacing 188-bp region of the *mtp* gene with a $\gamma\delta$ res-sacB-hyg- $\gamma\delta$ res cassette [7; 32]. This deletion mutant was used to construct its *mtp*-complement strain via electrotransformation with pMV261 [7]. These strains were confirmed to either possess or lack *mtp* prior to experimental assays.

The strains were cultured in Middlebrook 7H9 broth (Difco, Becton-Dickinson, Franklin Lakes, NJ, USA) supplemented with 10% (v/v) oleic albumin dextrose catalase (Becton–Dickinson, Franklin Lakes, NJ, USA), 0.5% (v/v) glycerol (Sigma-Aldrich, St. Louis, MO, USA) and 0.05% tween-80 (Sigma-Aldrich, St. Louis, MO, USA). Prior to experiments, the Δmtp was cultured in media supplemented with hygromycin B (75 μ g/mL) and the *mtp*-complement was cultured with hygromycin B (75 μ g/mL) and kanamycin (30 μ g/mL). Cultures for colony forming units (CFUs) were grown on Middlebrook 7H11 plates (Difco, Becton-Dickinson, Franklin Lakes, NJ, USA) similarly supplemented as described above with the exception of tween-80. All cultures were incubated at 37°C for 3-4 weeks (plates) or in a shaking incubator (I-26 Shaking Incubator, New Brunswick Scientific, Canada) at 100 rpm for 7-8 days (broth).

The three bacterial strains were cultured to late log phase with an OD_{600nm} of approximately 1 (0.8 to 1 range), equivalent to 1×10^8 cells/mL [33] in 50 mL of supplemented 7H9 (Appendix F: Table S1). Thereafter, cultured bacteria were pelleted at 4000 $\times g$ for 15 minutes at 4°C (Mikro 200R, Hettich Zentrifugen, Tuttlingen, Germany). The pellets were resuspended in 1 mL TriZol reagent (Ambion, Life Technologies, USA) and transferred into O-ring tubes for storage at -80°C until ribonucleic acid (RNA) was extracted. This experiment was conducted three times, with three replicates per strain.

6.3.3. Host cell lines, growth conditions and infection with *M. tuberculosis*

Roswell Park Memorial Institute Medium (RPMI) 1640 medium with L-glutamine (Lonza, BioWhittaker, Basel, Switzerland) was supplemented with 10% fetal bovine serum (Biowest, Nuaille, France) to produce complete cell culture media for growth of THP-1 monocytic cell line (ATCC TIB-202). Cells were cultured in T75 cm² tissue culture flasks (Nest Biotechnology, Jiangsu, China) and incubated at 37°C in 5% CO₂ in a humidified incubator (Shel Lab CO₂ Incubator, Cornelius, OR, USA) until 70% to 90% confluency.

A total of three biological assays were performed, each with 24 seeded flasks. Forty-eight hours prior to infection, THP-1 monocytes were seeded at a density of 1.25×10^6 cells/mL in a total of 25 mL and were differentiated into macrophages with phorbol 12-myristate 13-acetate (50 ng/mL) (Sigma-Aldrich, St. Louis, MO, USA). The media was changed 24 hours later, and macrophages were allowed to rest for 24 hours, prior to infection.

Bacterial inocula were prepared to an OD_{600nm} of approximately 1 for each *M. tuberculosis* strain so that a multiplicity of infection (MOI) ratio of approximately 5:1 bacteria to host was achieved (Appendix F: Table S2). Macrophage monolayers were rinsed once with phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA), and complete cell culture media was replaced before infection with the respective bacterial inocula. The flasks were incubated at 37°C in 5% CO₂ and 95% humidity (Shell Lab CO₂ Incubator, Cornelius, OR, USA) for four hours of infection (based off the previous pilot study in Chapter 3). Ten-fold serial dilutions of bacterial inocula were plated onto Middlebrook 7H11 agar plates to confirm the MOI (Appendix F: Table S2). Following the four hour infection, the monolayers were washed with PBS and lysed with 0.25% Triton X-100 (Millipore, Merck, Darmstadt, Germany) for 20 min. Lysates were pelleted and rinsed with PBS before combining the six replicates of each *M. tuberculosis* strain into two samples for storage in TriZol reagent as described above (three flasks into one sample). Of the 24 flasks, six were used for plating out ten-fold serial dilutions for the determination of CFUs to ascertain reproducibility of infection as well as calculate the invasion of *M. tuberculosis* (Appendix F: Tables S3, S4 and S5).

6.3.4. Extraction of RNA, DNase treatment and complementary deoxynucleic acid (cDNA) conversion

RNA extractions were performed according to Larsen et al., 2007, with the use of a Precellys 24 lysis and homogenisation machine (Bertin Technologies, Montigny-le-Bretonneux, France) and the addition of an extra 70% ethanol (Sigma-Aldrich, St. Louis, MO, USA) wash. RNA concentrations and purities were evaluated via optical density readings and denaturing gels for bacterial culture samples (Appendix F: Table S6 and Figure S1) and intracellular bacterial samples (Appendix F: Table S7 and Figure S2). A single sample for each strain from each biological assay was selected for DNase treatment and cDNA synthesis. The RNA was standardised to 100 µg/µL prior to DNase treatment (Thermo Fisher Scientific, Waltham, MA, USA) and converted to cDNA with the high-capacity cDNA reverse transcription kit (Roche Applied Sciences, Penzberg, Germany) as per the manufacturer's instructions. The cDNA quality, concentration and purity for bacterial culture samples (Appendix F: Table S8 and Figure S3) and intracellular bacterial samples (Appendix F: Table S9 and Figure S4) were considered prior to RT-qPCR.

6.3.5. Real time quantitative PCR

Primer efficiencies were tested, and PCR conditions were optimised for all primer sets prior to experiments. The Ssoadvanced Universal SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA) was used to perform RT-qPCR using a total reaction volume of 10 µL, in a 7500 RT-qPCR Detection System (Applied Biosystems, Foster City, CA, USA). Cycling conditions were as follows for a total of 40 cycles; holding stage at 95°C for 30 seconds, cycling stage at 95°C for 5 seconds with specific annealing temperatures for 30 seconds (Appendix F: Table S10). Melt curve analysis was measured as continuous fluorescence set at 95°C for 15 seconds, 60°C for 1 minute, 95°C for 30 seconds, and 60°C for 15 seconds. The resulting gene expression data was normalised using 16S ribosomal RNA

(16S rRNA) and was analysed by absolute quantification using the standard curve method. Three biological replicates and four technical replicates were performed for each strain and gene.

6.3.6. Data analysis

The standard curve parameters for each gene were used to calculate absolute transcript numbers for each run with the equation: $y = mx + c$, in which y was the cycle threshold (C_t) value, m was the slope, c was the y-intercept, and x was the transcript number. The housekeeping gene (16S rRNA) was used to normalise the individual transcript numbers (ratio = gene transcript number/16S rRNA transcript number). The resulting data was tested for normality by a Shapiro-Wilk test in which parametric data will have a p -value ≥ 0.05 . For multiple comparisons, the normalised data was used to conduct one-way analysis of variance (ANOVA) with Tukey's test for correcting for multiple comparisons (post hoc analysis) for each gene. This was done to compare the effect of the absence/presence of MTP on gene expression among *M. tuberculosis* strains from bacterial cultures (Figure 6.1) and intracellular *M. tuberculosis* from infected THP-1 macrophages (Figure 6.2) in which the p -values ≤ 0.05 were considered significant. Additionally, cultured bacterial and intracellular bacterial gene expression were compared for each gene by parametric unpaired t -test with statistical significance considered when the $p \leq 0.05$ (Figure 6.3). All the statistical analyses were performed on GraphPad Prism version 8.0.

6.4. Results

6.4.1. *Mycobacterium tuberculosis* curli pili upregulates the genes associated with uptake and breakdown of lipids and lactate during late log growth phase of *M. tuberculosis* in liquid culture

The absolute transcript numbers of cultured bacteria were determined through RT-qPCR and the expression of all 11 genes were statistically significantly different between the WT and Δmtp strains (Figure 6.1). The gene expressions of *lucA* ($p = 0.005$), *mce1D* ($p = 0.003$) and *pckA* ($p < 0.001$) were significantly higher in Δmtp than the WT and *mtp*-complement, but similar between the latter two strains. This indicates that under optimum growth conditions, during late log phase of *M. tuberculosis* growth, MTP down regulates *lucA*, *mce1D*, and *pckA*. This is expected to affect the putative components of fatty acid transporters and the rate of gluconeogenesis, by resulting in a reduction of flow through both these processes in the WT and *mtp*-complement.

However, the gene expressions of *Rv2799* ($p < 0.001$), *Rv0966c* ($p < 0.001$), *mceG* ($p = 0.002$), *fadA5* ($p = 0.007$), and *lldD2* ($p < 0.001$) were significantly lower in Δmtp than the WT and *mtp*-complement, but similar in the latter two strains. Therefore, in the presence of MTP these putative components of the fatty acid transporters are active along with fatty acid oxidation (FAO) during exponential growth of *M. tuberculosis*. Hence, *M. tuberculosis* readily uses lipids and lactate during growth.

Although *omamB* expression was significantly lower in Δmtp than the WT ($p < 0.001$), there was no significant difference between Δmtp and *mtp*-complement. Moreover, the *mtp*-complement was significantly different to the WT for *omamB* ($p < 0.001$). The expressions of *fadB* and *lldD1* were significantly different for all three strains, with the lowest seen in the Δmtp and the highest in the WT. The *mtp*-complement displayed gene expression levels between that of the Δmtp and WT for *fadB* and *lldD1*. Majority of the gene expression for WT and *mtp*-complement were similar, except for

omamB. Overall, the *mtp*-complement displayed gene expression levels between expression profiles of the WT and Δmtp . From these gene expression trends, it is deduced that complementation of *mtp* by a non-integrating plasmid resulted in partial restoration of MTP. Furthermore, MTP played a role in upregulating eight of the 11 genes investigated in the cultured bacterial model, as is visualised by the WT displaying higher gene expression for *Rv2799*, *Rv0966c*, *omamB*, *mceG*, *fadA5*, *fadB*, *lldD1* and *lldD2* relative to Δmtp .

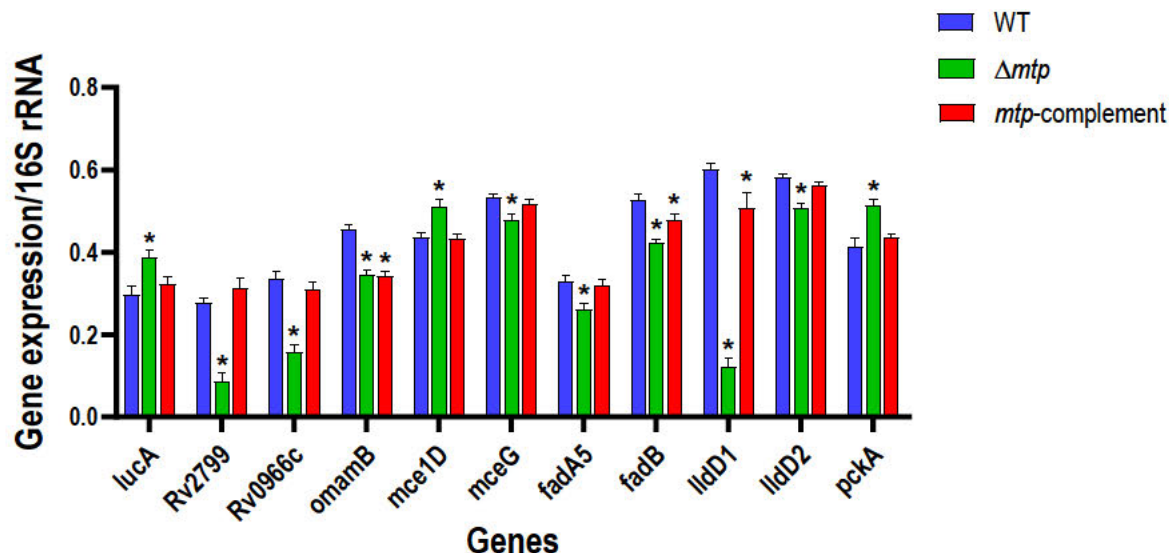


Figure 6.1. Bacterial gene expression levels of the three mycobacterial strains grown in Middlebrook 7H9 broth culture. Absolute quantification of gene expression was determined through RT-qPCR of the bacterial strains (WT V9124; *mtp*-deletion mutant; and *mtp*-complemented strain). Three biological assays and four technical repeats were conducted for each of the 11 selected genes (*lucA*, *Rv2799*, *Rv0966c*, *omamB*, *mce1D*, *mceG*, *fadA5*, *fadB*, *lldD1*, *lldD2*, and *pckA*). Gene expression levels were normalised to the housekeeping 16S rRNA for each gene of interest and was represented by standard error mean (SEM) bars. Data was analysed by one-way ANOVA and Tukey's test with statistical significance considered when the adjusted $p \leq 0.05$ (indicated by * to denote significance relative to WT). Significant differences between WT and Δmtp were seen for *lucA* ($p = 0.005$), *Rv2799* ($p < 0.001$), *Rv0966c* ($p < 0.001$), *omamB* ($p < 0.001$), *mce1D* ($p = 0.003$), *mceG* ($p = 0.002$), *fadA5* ($p = 0.007$), *fadB* ($p < 0.001$), *lldD1* ($p < 0.001$), *lldD2* ($p < 0.001$), *pckA* ($p < 0.001$). Significant differences between WT and *mtp*-complement were seen for *omamB* ($p < 0.001$), *fadB* ($p < 0.034$), and *lldD1* ($p < 0.040$).

6.4.2. *Mycobacterium tuberculosis* curli pili downregulates the genes involved in uptake and breakdown of lipids and lactate of intracellular *M. tuberculosis* during early infection

Ten of the 11 genes investigated displayed statistically significant differences between the WT and Δmtp strains (Figure 6.2). The expression of four genes, *Rv0966c* ($p = 0.020$), *fadA5* ($p < 0.001$), *lldD1* ($p = 0.001$), and *pckA* ($p = 0.002$) were significantly higher in Δmtp than WT and *mtp*-complement strains, but similar for the latter two strains. The gene expression of *Rv2799* ($p < 0.001$) was significantly lower in Δmtp than the WT and *mtp*-complement, while no significant difference was observed between the latter two strains. The gene expressions of *lucA*, *omamB*, *mce1D*, *fadB* and *lldD2* were significantly higher in Δmtp and *mtp*-complement than the WT. For *lucA* and *omamB* the

lowest gene expression was seen in the WT, followed by Δmtp and then *mtp*-complement with the highest gene expressions. For *mce1D*, *fadB*, and *lldD2* the lowest gene expression was seen in the WT, followed by *mtp*-complement and then Δmtp . The gene expression of *mceG* was not significantly different amongst all three strains; however, the highest gene expression was observed in the Δmtp followed by *mtp*-complement and then WT.

The data revealed partial restoration of MTP via complementation. Relative to the WT, the intracellular Δmtp displayed a higher expression for all the investigated genes, with the exception of *Rv2799*. Holistically, this data indicates that MTP plays a role in the downregulation of these genes in intracellular *M. tuberculosis* during the initial infection process.

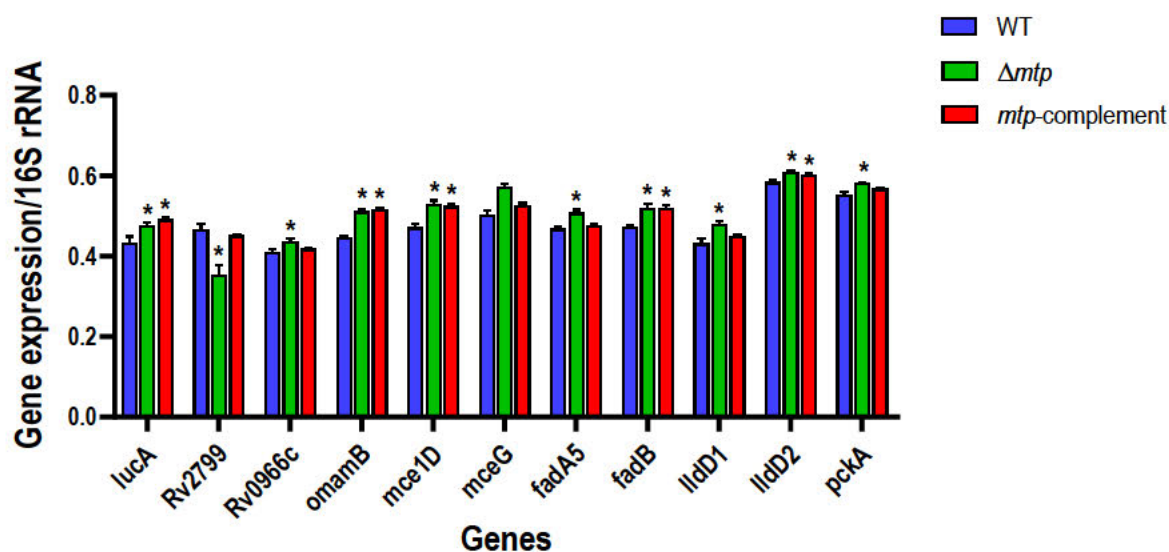


Figure 6.2. Intracellular gene expression levels of the three mycobacterial strains during early infection of THP-1 macrophages. Absolute quantification of gene expression was determined through RT-qPCR of the intracellular bacterial strains isolated from THP-1 macrophage infection model (WT V9124; *mtp*-deletion mutant; and *mtp*-complemented strain). Three biological assays and four technical repeats were conducted for each of the 11 selected genes (*lucA*, *Rv2799*, *Rv0966c*, *omamB*, *mce1D*, *mceG*, *fadA5*, *fadB*, *lldD1*, *lldD2*, and *pckA*). Gene expression levels were normalised to the housekeeping 16S rRNA for each gene of interest and were represented by standard error mean (SEM) bars. Data was analysed by one-way ANOVA and Tukey's test with statistical significance considered when the adjusted $p \leq 0.05$ (indicated by * to denote significance relative to WT). Significant differences between WT and Δmtp were seen for *lucA* ($p = 0.011$), *Rv2799* ($p < 0.001$), *Rv0966c* ($p = 0.020$), *omamB* ($p < 0.001$), *mce1D* ($p < 0.001$), *fadA5* ($p < 0.001$), *fadB* ($p = 0.002$), *lldD1* ($p = 0.001$), *lldD2* ($p = 0.005$), *pckA* ($p = 0.002$). Significant differences between WT and *mtp*-complement were seen for *lucA* ($p = 0.001$), *omamB* ($p < 0.001$), *mce1D* ($p < 0.001$), *fadB* ($p = 0.001$), and *lldD2* ($p = 0.030$).

6.4.3. *Mycobacterium tuberculosis* curli pili regulates the expression of genes involved in fatty acid import and lactate oxidation of intracellular *M. tuberculosis*

When viewed separately, the expression levels of 7 of 11 genes (*Rv0966c*, *omamB*, *mceG*, *fadA5*, *fadB*, *lldD1* and *lldD2*) showed an inverse trend between the two models in the Δmtp groups. However, to evaluate changes to gene expression during the initial stages of infection, cultured bacterial gene expression was compared to the intracellular gene expression for each strain (Figure 6.3). The data revealed that the intracellular bacteria displayed a significantly higher expression of *lucA* ($p < 0.001$), *Rv2799* ($p < 0.001$), *Rv0966c* ($p = 0.001$), *fadA5* ($p < 0.001$), and *pckA* ($p < 0.001$) in all three strains relative to the cultured bacterial counterparts. These results indicate that the genes encoding these putative components of lipid transporters, which facilitate the import of both fatty acids and cholesterol, as well as β -oxidation and gluconeogenic carbon flow are not dependent on the MTP adhesin. Rather, the upregulation of these genes is likely triggered by the entry of *M. tuberculosis* into the THP-1 macrophage environment to allow the pathogen to acquire the necessary nutrients. Although there is no statistical evidence, the change in expression levels of these genes in Δmtp appear to have an altered change in expression levels between the two models (which either exhibit the largest change or smallest change), relative to the MTP-proficient strains.

Significant differences in the expression of *omamB*, *mce1D*, *mceG*, *fadB*, *lldD1*, and *lldD2* were observed between cultured and intracellular bacteria. Significantly lower expression of *fadB* was observed in the intracellular WT ($p = 0.002$) compared to the broth grown WT. In contrast, significantly higher *fadB* was expressed by the intracellular Δmtp ($p < 0.001$) and intracellular *mtp*-complement ($p = 0.021$) strains compared to their respective cultured bacterial groups. Genes such as *omamB* ($p < 0.001$), and *lldD2* ($p < 0.001$) were expressed at significantly higher levels in the intracellular Δmtp and *mtp*-complement strains, while no significant difference was exhibited by the WT in both models. The WT ($p = 0.040$) and *mtp*-complement ($p < 0.001$) demonstrated significant differences in *mce1D* expression, with a higher gene expression in the intracellular group, whereas the trend seen in Δmtp differed from the WT and *mtp*-complement as it showed no difference in *mce1D* expression between the bacterial culture and intracellular bacteria. The intracellular WT expressed lower levels of *mceG* and *lldD1* ($p = 0.011$ and $p < 0.001$, respectively). In contrast, significantly higher expression of both these genes was displayed by the intracellular Δmtp ($p < 0.001$ for both), with the *mtp*-complement showing no statistical difference between the bacterial groups. Thus, the findings demonstrate that in the presence of MTP, *mceG*, and *lldD1* are downregulated, while *mce1D* is upregulated in intracellular *M. tuberculosis*. This indicates that MTP regulates the expression of the lactate dehydrogenase gene, *lldD1*, and fatty acid import genes, *mce1D* and *mceG*, of *M. tuberculosis* during the first four hours of infection.

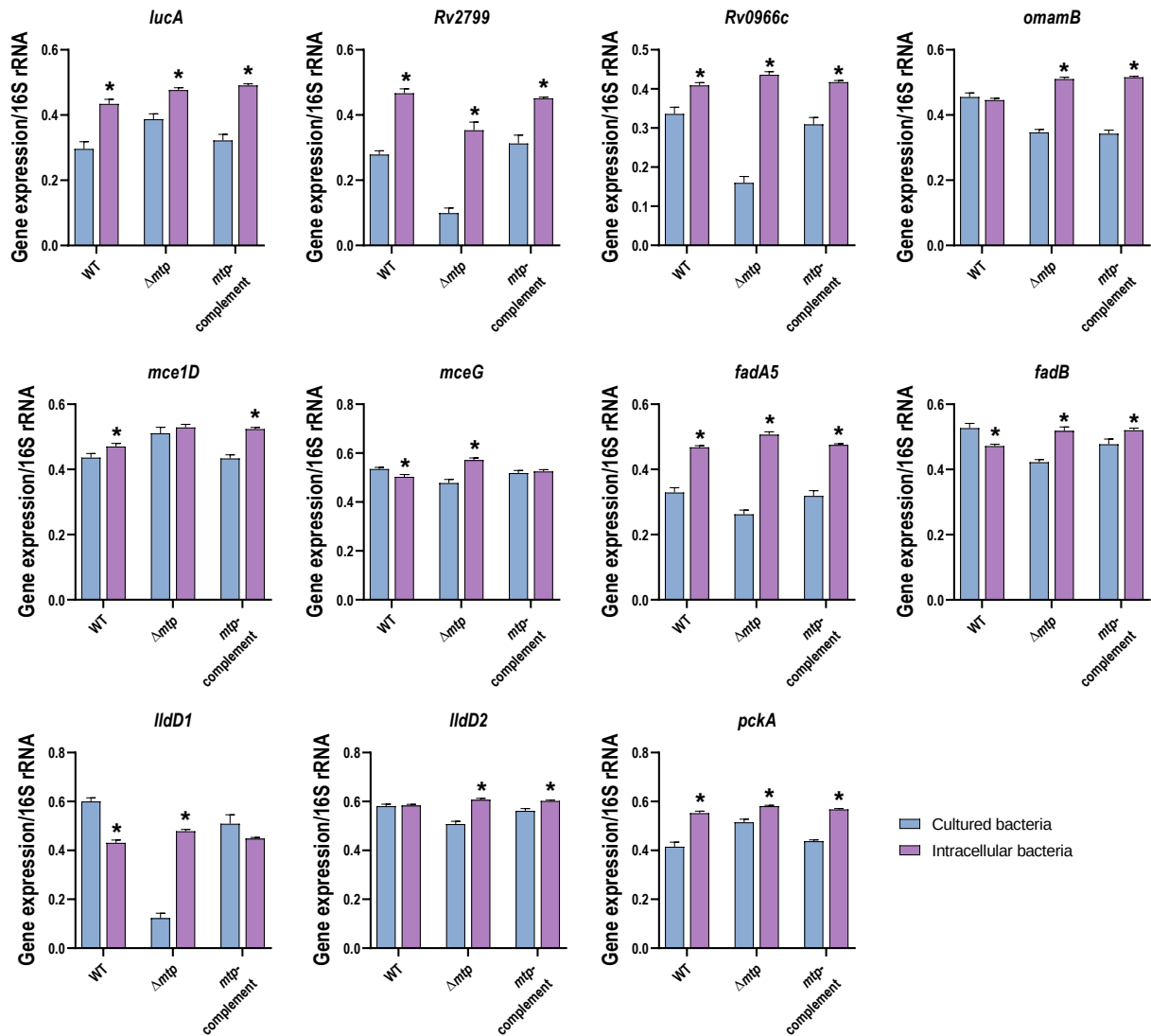


Figure 6.3. Gene expression comparison between cultured *M. tuberculosis* and intracellular *M. tuberculosis* from THP-1 macrophage infections. Absolute quantification of gene expression was determined through RT-qPCR of the cultured bacterial strains and bacterial RNA isolated from intracellular bacteria (WT V9124; *mtp*-deletion mutant; and *mtp*-complement). Three biological assays and four technical repeats were conducted for each of the 11 selected genes (*lucA*, *Rv2799*, *Rv0966c*, *omamB*, *mce1D*, *mceG*, *fadA5*, *fadB*, *lldD1*, *lldD2*, and *pckA*). Gene expression levels were normalised to the housekeeping 16S rRNA for each gene of interest and is represented by standard error mean (SEM) bars. Data was analysed by parametric unpaired *t*-test with statistical significance considered when the $p \leq 0.05$ (indicated by * to denote significance between models of the same strain). Significant differences between cultured WT and intracellular WT were seen for *lucA* ($p < 0.001$), *Rv2799* ($p < 0.001$), *Rv0966c* ($p = 0.001$), *mce1D* ($p = 0.040$), *mceG* ($p = 0.011$), *fadA5* ($p < 0.001$), *fadB* ($p = 0.002$), *lldD1* ($p < 0.001$), and *pckA* ($p < 0.001$). Significant differences between Δmtp and intracellular Δmtp were seen for *lucA* ($p < 0.001$), *Rv2799* ($p < 0.001$), *Rv0966c* ($p < 0.001$), *omamB* ($p < 0.001$), *mceG* ($p < 0.001$), *fadA5* ($p < 0.001$), *fadB* ($p < 0.001$), *lldD1* ($p < 0.001$), *lldD2* ($p < 0.001$), and *pckA* ($p < 0.001$). Significant differences between *mtp*-complement and intracellular *mtp*-complement were seen for *lucA* ($p < 0.001$), *Rv2799* ($p < 0.001$), *Rv0966c* ($p < 0.001$), *omamB* ($p < 0.001$), *mce1D* ($p < 0.001$), *fadA5* ($p < 0.001$), *fadB* ($p = 0.021$), *lldD2* ($p = 0.001$), *pckA* ($p < 0.001$).

6.5. Discussion

The unique and conserved MTP adhesin has been motivated as a potential target for TB treatment strategies [5]. Numerous *M. tuberculosis* virulence studies have revealed that MTP not only acts as an adhesin and invasin to host cells [8; 34], but also modulates host immune responses and metabolism during infection [9; 11; 14-17]. Given that *mtp* is situated amongst intermediary metabolism genes [4], clear differences in metabolite concentrations of pure bacterial culture and infection models were proposed to be attributed to the presence or absence of MTP [14; 15; 17]. Untargeted metabolomics had previously demonstrated that Δmtp displayed an accumulation of intermediates of fatty acid metabolism in both late log phase bacterial culture and THP-1 macrophage infection models [14; 15; 17]. Additionally, perturbations were observed for metabolites such as lactate and intermediates of central carbon metabolism [14].

In the current study, expression levels of *M. tuberculosis* genes involved in lipid transport, gluconeogenic carbon flow, β -oxidation and lactate oxidation were evaluated by absolute quantification using the standard curve method for RT-qPCR. *Mycobacterium tuberculosis* was isolated from broth culture during late log phase of growth and from cell lysates of THP-1 macrophages following four hours of infection. The results highlighted the differential gene expression firstly, among the three different cultures of *M. tuberculosis* strains (WT, Δmtp and *mtp*-complement) (Figure 6.1), secondly, among intracellular bacteria of the three strains (Figure 6.2), and thirdly, between bacteria grown in culture medium and intracellular bacteria (Figure 6.3). Most of the genes investigated were downregulated in the exponentially growing MTP-deficient *M. tuberculosis* relative to the MTP-proficient strains. Contrastingly, most of these genes were upregulated in the MTP-deficient intracellular *M. tuberculosis*. At a glance, MTP appears to be more influential on gene expression levels of *M. tuberculosis* grown in broth culture than that extracted from the intracellular space (Appendix F: Figure S5). Therefore, a comparison of the gene expression levels between these two models was statistically evaluated and revealed that MTP is significantly associated with the regulation of the intracellular pathogen's genes involved with lipid import (*mce1D* and *mceG*), and lactate oxidation (*lldD1*).

Mycobacterium tuberculosis is a proficient pathogen as it quickly adapts to changes in its microenvironment and is capable of surviving on a plethora of nutrients. Although *M. tuberculosis* can survive extracellularly, much of its life cycle is committed to growing intracellularly, where nutrient availability is known to fluctuate [35; 36]. Moreover, *M. tuberculosis* is adept at co-catabolising different carbon sources [37]. The acquisition and assimilation of multiple carbon sources such as sugars, amino acids, host-derived fatty acids, and cholesterol, provide the pathogen with sources of nitrogen, carbon and energy during host infection [38].

6.5.1. Lipid import via Mce1 transport protein and β -oxidation of fatty acids modulated by MTP alters pathogenicity of intracellular *M. tuberculosis*

Cholesterol is known to accumulate at the site of infection and is essential for mycobacterial entry into macrophages [39]. Accumulation of cholesterol within macrophages occurs as a result of *M. tuberculosis* triggering the host to: increase the uptake and enhance *de novo* cholesterol synthesis; inhibit the degradation of cholesterol esters; and impede cholesterol efflux [40]. The accumulation of cholesterol promotes the formation of lipid droplets and foam cells, ultimately facilitating the persistent survival of the pathogen [40]. Moreover, when varying nutrient availability is encountered during infection, *M. tuberculosis* heavily relies on its adaptability to utilise host lipids for growth and structural integrity of the mycobacterial cell wall by the formation of complex lipids [22]. This subsequently provides protection against host immune responses and antibiotics.

Each multi-protein Mce transporter is known to import different lipids, with Mce1 chiefly transporting fatty acids, and Mce4 dedicated to importing cholesterol [22]. The shared protein, LucA, interacts with both Mce1 and Mce4 subunits, thus, facilitating the co-ordinated import of lipids [22]. Mycobacterial Mce transporters comprise cytoplasmic ATPase (MceG), and multiple Mce-associated membrane (Mam) and orphaned Mam (Omam) proteins [41]. These complex import proteins are integral to *M. tuberculosis* pathogenicity and their regulation determines the outcome of infection. Therefore, in this study, *lucA* and genes encoding putative components the Mce1 transporter (*Rv2799*, *Rv0966c*, *mce1D*, *mceG*, and *omamB*) were investigated in the absence/presence of MTP.

Cultured and intracellular Δmtp had higher expression of *lucA* relative to the WT (Figure 6.1 and 6.2). When bacterial culture and intracellular Δmtp were compared, *lucA* exhibited the smallest difference in expression levels between the two models (Figure 6.3). Studies have demonstrated that mutations in *lucA* lead to impaired growth of *M. tuberculosis* in environments where cholesterol was the primary carbon source [22]. Hence, MTP regulates the expression of *lucA* in response to the change in environment allowing the pathogen to adequately adapt to the available nutrients.

Of the five genes associated with putative components of the Mce1 fatty acid transporter, four (*Rv2799*, *Rv0966c*, *omamB* and *mceG*) were expressed at lower levels in the culture of Δmtp relative to the WT (Figure 6.1). These findings suggest that the lack of MTP results in the potential reduction of fatty acid import in bacterial culture. Moreover, lower expression levels were also observed in both β -oxidation genes investigated (*fadA5* and *fadB*) in the bacterial culture of Δmtp (Figure 6.1), suggesting that MTP plays a role in FAO. Hence MTP facilitates the upregulation of genes involved in fatty acid importer Mce1 and the FadA–FadB complex involved in β -oxidation within *M. tuberculosis*.

Moreover, the reduced expression of fatty acid import and oxidation genes coupled with the previously reported fatty acid accumulation in Δmtp culture [15], is proposed to additionally be influenced by *de novo* fatty acid synthesis. Bacterial *de novo* fatty acid synthesis is achieved by type I fatty acid synthase (FAS-I) and type II fatty acid synthase (FAS-II). The multifunctional enzyme, FAS-I, initiates *de novo* synthesis of medium-chain fatty acids from acetyl-CoA, while FAS-II is responsible for their elongation into longer-chain fatty acids such as mycolic acids, which critically contribute to the structural integrity and impermeability of the mycobacterial cell envelope [42–44]. Regulation of *M. tuberculosis* fatty acid synthases is complex and involves various transcriptional regulators such as the transcriptional repressor Mce3R [45] and the essential activator FasR [46]. Therefore, further investigation of *de novo* fatty acid regulation and synthesis is required to confirm if *M. tuberculosis* potentially overcompensates for the lack of import and oxidation of extracellular lipids in the absence of MTP. Currently, data suggest that MTP aids *M. tuberculosis* to import and breakdown fatty acids to meet growth requirements.

Interestingly, the higher expression levels of *Rv0966c*, *omamB*, *mce1D*, *fadA5* and *fadB* (Figure 6.2) of intracellular Δmtp indicates that the previously detected accumulation of lipids within the Δmtp infected THP-1 macrophages mainly encompassed the host derived fatty acids [14]. This deduction is supported by studies, which demonstrated that under healthy conditions, *M. tuberculosis* oxygen consumption was one tenth of the macrophage respiration [47]. Therefore, the contribution of *M. tuberculosis* derived metabolites to the differential concentrations detected were negligible in comparison to its influence on host activities and the microenvironment [14].

The understanding that *M. tuberculosis* reprograms host metabolism to guarantee a continuous supply of essential lipids for its long-term persistence *in vivo* has recently been questioned [48]. Evidence from immunological and microbiological studies have given rise to the new concept that macrophages metabolically adapt to *M. tuberculosis* infection by accumulating fatty acids in the cytoplasm. This potentiates antimycobacterial host responses and influences *M. tuberculosis* to transition into fat-saving survival mode [48]. Therefore, the previously observed higher fatty acid levels in the Δmtp

infected THP-1 macrophage [14] together with the higher expression of intracellular *M. tuberculosis* lipid import and oxidation genes (Figure 6.2) indicate that when MTP is absent, the macrophage gravitates toward an antimicrobial response.

6.5.2. The rate of lactate oxidation and gluconeogenic carbon flow are regulated by MTP

The evolutionary significance of the putative lactate dehydrogenase encoded by *lldD1* is underscored by its conservation in mycobacterial species and its sequence homology to other lactate dehydrogenases [49]. Additionally, *lldD1* aids in the synthesis of the redox cofactor, mycofactocin, which is important for metabolism [50]. Mycofactocin is essential for *M. tuberculosis* growth in the presence of lactate [50]. Thus, *lldD1* is not merely a redundant gene, but is a vital component of the metabolic machinery adapted by the pathogen to facilitate metabolic flexibility within the hostile environment of the host [50].

Although demonstrated to be responsible for lactate oxidation [29], the multifunctional *lldD2* is indispensable to *M. tuberculosis*. This gene confers resistance to oxidative stress in addition to metabolic elasticity and virulence within the intracellular niche [51]. Oxidative stress is imposed on *M. tuberculosis* by the host in its efforts to clear out infection. In doing so, *lldD2* manages and maintains the redox balance, which is vital for *M. tuberculosis* survival against reactive oxygen species generated by macrophages in a pro-inflammatory response [51]. The expression of genes involved in cell wall lipid metabolism and virulence systems, such as ESX-1, is also influenced by *lldD2* [51].

The lactate dehydrogenases (*lldD1* and *lldD2*) in the Δmtp displayed the lowest gene expression levels for bacterial culture (Figure 6.1) and showed the highest gene expression levels in the intracellular bacteria (Figure 6.2). This demonstrates that the mutant did not utilise much of the available lactate in culture; but attempted to use the excessively available lactate during infection. Macrophages generate lactate to modulate immune responses [52]. Hence, the disproportionately available lactate previously detected in Δmtp infected THP-1 macrophages [14] is an indication of the macrophage's response to infection with Δmtp and not an indication of the inability of *M. tuberculosis* to use lactate as a carbon source in the absence of MTP. Therefore, MTP potentially controls lactate oxidation in *M. tuberculosis* to maintain expression levels of *lldD2* or downregulate expression of *lldD1*, particularly within the host to avoid effector cell detection.

Given that *pckA* is crucial for gluconeogenesis and anaplerotic pathways, studies have shown that upregulation of this gene was found in the presence of fatty acids such as palmitate [53]. This reflects the pivotal function of *pckA* in the pathogen's adaptability to fluctuating nutrient availability during infection. Furthermore, *pckA* was described as a virulence factor since the absence of PEPCCK led to impaired gluconeogenesis and subsequently affected energy production and survival of bacteria [53]. This gene is also involved in resistance to oxidative stress and facilitates the synthesis of key metabolites that are necessary for mycolic acid and lipid synthesis, which contribute to bacterial structural integrity [54; 55].

In the current study, the highest levels of *pckA* expression were observed in the Δmtp for both bacterial culture (Figure 6.1) and intracellular bacteria (Figure 6.2). Furthermore, an increase in intracellular bacterial gene expression of *pckA* was observed in all bacterial strains with Δmtp displaying the smallest change in expression (Figure 6.3) indicative of the bacterium's fitness. These findings demonstrate that gluconeogenic carbon flow is not dependent on the presence of MTP. Rather, the rate of *M. tuberculosis* gluconeogenic carbon flow is influenced by MTP during exponential growth and replication.

6.5.3. *Mycobacterium tuberculosis* curli pili shapes the extent of cultured and intracellular bacterial gene regulation

The analysis of the pure bacterial culture and intracellular bacterial gene expression levels revealed that the change in environment resulted in an overall trend of an atypical response of Δmtp compared to that of the WT and *mtp*-complement (Figure 6.3). The gene expression levels were either drastically increased or minimally changed in the mutant strain. This indicates that MTP may additionally be associated with maintaining the bacterial metabolic regulation in order to ensure infection of THP-1 macrophages, and thus validates previous findings [8; 14; 34; 56] of MTP as a virulence factor of *M. tuberculosis*. While data from this study is restricted to a four-hour timepoint, previous studies have demonstrated the virulence attributes of MTP from the time of infected to 21-day post infection [10; 11; 56].

This study was limited to three bacterial strains and the complemented strain used in this study only displayed partial transcriptomic restoration given the nature of its construct. A select few genes were investigated, hence the data presented here is merely a preview of the transcriptomic alterations caused by the MTP or the lack thereof. The changes seen in gene expression level for the two models used in this study may not be representative of a larger and more complex pathogen-host system and interactions. Nevertheless, the information presented here provides new knowledge on the adaptability of *M. tuberculosis* to exploit different strategies to survive and succeed as an intracellular pathogen.

6.6. Conclusion

Removal of the MTP from *M. tuberculosis* culminates into transcriptional alterations observed in bacterial culture, as well as intracellular bacteria isolated from infected THP-1 macrophages. Adjustments to lipid import, β -oxidation, and lactate oxidation of *M. tuberculosis* indicated that MTP may be associated with the modulation of multiple functions during early infection. Therefore, MTP potentially acts as a modulator of genes that contribute to metabolism and metabolic flexibility of *M. tuberculosis*. This study provided evidence that corroborated previous metabolomic outcomes and highlighted the bacterial molecular mechanisms influenced by MTP. In conclusion, MTP significantly contributes to the success of *M. tuberculosis* as an intracellular pathogen. By shifting focus of TB intervention strategies toward intercepting the MTP interaction with the host, it may be possible to offset *M. tuberculosis* pathogenicity and confer an advantage to the host.

References:

1. World Health Organization (2024). Global tuberculosis report. *World Health Organization* [Online]. Available: <https://www.who.int/publications/i/item/9789240101531> [Accessed October 2024].
2. Govender, V.S., Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* adhesins: Potential biomarkers as anti-tuberculosis therapeutic and diagnostic targets. *Microbiology*, 160(Pt 9), 1821-1831. <https://doi.org/10.1099/mic.0.082206-0>.
3. Alteri, C.J., Xicohténcatl-Cortes, J., Hess, S., Caballero-Olín, G., Girón, J.A., and Friedman, R.L. (2007). *Mycobacterium tuberculosis* produces pili during human infection. *Proceedings of the National Academy of Sciences of the United States of America*, 104(12), 5145-5150. <https://doi.org/10.1073/pnas.0602304104>.

4. Alteri, C. (2005). *Novel pili of Mycobacterium tuberculosis*. Doctoral thesis, The University of Arizona, United States of America. <https://repository.arizona.edu/handle/10150/195515?show=full>.
5. Naidoo, N., Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* pili (MTP), a putative biomarker for a tuberculosis diagnostic test. *Tuberculosis*, 94(3), 338-345. <http://dx.doi.org/10.1016/j.tube.2014.03.004>.
6. Govender, V.S. (2018). *Investigating the in vitro roles played by the major adhesins HBHA and MTP in the pathogenesis of M. tuberculosis, in a novel double gene knock-out mutant strain*. Doctoral thesis, University of KwaZulu-Natal, Durban, South Africa. <https://researchspace.ukzn.ac.za/items/8ecb8b6d-8381-4847-83d2-d9725e209b2f>.
7. Ramsugit, S., Guma, S., Pillay, B., Jain, P., Larsen, M.H., Danaviah, S., and Pillay, M. (2013). Pili contribute to biofilm formation *in vitro* in *Mycobacterium tuberculosis*. *Antonie Van Leeuwenhoek*, 104(5), 725-735. <https://doi.org/10.1007/s10482-013-9981-6>.
8. Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* pili promote adhesion to and invasion of THP-1 macrophages. *Japanese Journal of Infectious Diseases*, 67(6), 476-478. <https://doi.org/10.7883/yoken.67.476>.
9. Dlamini, M.T. (2016). *Whole transcriptome analysis to elucidate the role of mtp in gene regulation of pulmonary epithelial cells infected with Mycobacterium tuberculosis*. Masters dissertation University of KwaZulu-Natal, Durban, South Africa. <https://researchspace.ukzn.ac.za/items/71f0ed4e-aa63-4cca-8bf6-4d771326e616>.
10. Moodley, S. (2018). *The role of heparin binding haemagglutinin adhesin and curli pili on the pathogenicity of Mycobacterium tuberculosis*. Doctoral thesis, <https://researchspace.ukzn.ac.za/items/22f2cedb-2826-46ad-8a62-237a41b62d17>.
11. Nyawo, G. (2016). *The role of Mycobacterium tuberculosis pili in pathogenesis: Growth and survival kinetics, gene regulation and host immune response, and in vitro growth kinetics*. Masters dissertation University of KwaZulu-Natal, Durban, South Africa. <https://researchspace.ukzn.ac.za/items/3628eab3-3e5f-47a6-9eb3-07c3f3c52ad7>.
12. Naidoo, T.J. (2021). *The role of Mycobacterium tuberculosis curli pili (MTP) and heparin-binding hemagglutinin adhesin (HBHA) on global in vitro bacterial transcriptomics*. Masters dissertation University of KwaZulu-Natal, Durban, South Africa. <https://researchspace.ukzn.ac.za/items/a554ee5c-b4be-4a16-81a5-792a4fb0f6a3>.
13. Naidoo, N., Pillay, B., Bubb, M., Pym, A., Chiliza, T., Naidoo, K., Ndung'u, T., Kasproicz, V.O., and Pillay, M. (2018). Evaluation of a synthetic peptide for the detection of anti-*Mycobacterium tuberculosis* curli pili IgG antibodies in patients with pulmonary tuberculosis. *Tuberculosis*, 109, 80-84. <https://doi.org/10.1016/j.tube.2018.01.007>.
14. Ashokcoomar, S., Loots, D.T., Beukes, D., Van Reenen, M., Pillay, B., and Pillay, M. (2021). *M. tuberculosis* curli pili (MTP) is associated with alterations in carbon, fatty acid and amino acid metabolism in a THP-1 macrophage infection model. *Microbial Pathogenesis*, 154, 104806. <https://doi.org/10.1016/j.micpath.2021.104806>.
15. Ashokcoomar, S., Reedoy, K.S., Senzani, S., Loots, D.T., Beukes Maasdorp, D., Van Reenen, M., Pillay, B., and Pillay, M. (2020). *Mycobacterium tuberculosis* curli pili (MTP) deficiency is associated with alterations in cell wall biogenesis, fatty acid metabolism and amino acid synthesis. *Metabolomics*, 16(9), 97. <https://doi.org/10.1007/s11306-020-01720-z>.
16. Ashokcoomar, S., Reedoy, K.S., Loots, D.T., Beukes, D., Van Reenen, M., Pillay, B., and Pillay, M. (2022). *M. tuberculosis* curli pili (MTP) facilitates a reduction of microbicidal activity of infected THP-1 macrophages during early stages of infection. *Comparative Immunology, Microbiology and Infectious Diseases*, 90-91, 101907. <https://doi.org/10.1016/j.cimid.2022.101907>.

17. Reedoy, K.S., Loots, D.T., Beukes, D., Reenen, M.V., Pillay, B., and Pillay, M. (2020). *Mycobacterium tuberculosis* curli pili (MTP) is associated with significant host metabolic pathways in an A549 epithelial cell infection model and contributes to the pathogenicity of *Mycobacterium tuberculosis*. *Metabolomics*, 16(11), 116. <http://dx.doi.org/10.1007/s11306-020-01736-5>.
18. Bloch, H. , and Segal, W. (1956). Biochemical differentiation of *Mycobacterium tuberculosis* grown *in vivo* and *in vitro*. *Journal of Bacteriology*, 72(2), 132-141. <https://doi.org/10.1128/jb.72.2.132-141.1956>.
19. McKinney, J.D., Höner zu Bentrop, K., Muñoz-Elías, E.J., Miczak, A., Chen, B., Chan, W.T., Swenson, D., Sacchettini, J.C., Jacobs, W.R., Jr., and Russell, D.G. (2000). Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature*, 406(6797), 735-738. 10.1038/35021074.
20. VanderVen, B.C., Fahey, R.J., Lee, W., Liu, Y., Abramovitch, R.B., Memmott, C., Crowe, A.M., Eltis, L.D., Perola, E., Deininger, D.D., Wang, T., Locher, C.P., and Russell, D.G. (2015). Novel inhibitors of cholesterol degradation in *Mycobacterium tuberculosis* reveal how the bacterium's metabolism is constrained by the intracellular environment. *PLoS Pathogens*, 11(2), e1004679. <https://doi.org/10.1371/journal.ppat.1004679>.
21. Ekiert, D.C., Bhabha, G., Isom, G.L., Greenan, G., Ovchinnikov, S., Henderson, I.R., Cox, J.S., and Vale, R.D. (2017). Architectures of lipid transport systems for the bacterial outer membrane. *Cell*, 169(2), 273-285.e217. <https://doi.org/10.1016/j.cell.2017.03.019>.
22. Nazarova, E.V., Montague, C.R., La, T., Wilburn, K.M., Sukumar, N., Lee, W., Caldwell, S., Russell, D.G., and VanderVen, B.C. (2017). Rv3723/LucA coordinates fatty acid and cholesterol uptake in *Mycobacterium tuberculosis*. *eLife*, 6, e26969. <https://doi.org/10.7554/elife.26969>.
23. Nazarova, E.V., Montague, C.R., Huang, L., La, T., Russell, D., and VanderVen, B.C. (2019). The genetic requirements of fatty acid import by *Mycobacterium tuberculosis* within macrophages. *eLife*, 8, e43621. <https://doi.org/10.7554/elife.43621>.
24. Williams, K.J., Boshoff, H.I., Krishnan, N., Gonzales, J., Schnappinger, D., and Robertson, B.D. (2011). The *Mycobacterium tuberculosis* β -oxidation genes *echA5* and *fadB3* are dispensable for growth *in vitro* and *in vivo*. *Tuberculosis*, 91(6), 549-555. <https://doi.org/10.1016/j.tube.2011.06.006>.
25. Venkatesan, R. , and Wierenga, R.K. (2013). Structure of mycobacterial β -oxidation trifunctional enzyme reveals its altered assembly and putative substrate channeling pathway. *ACS Chemical Biology*, 8(5), 1063-1073. <https://doi.org/10.1021/cb400007k>.
26. Cox, J.A.G., Taylor, R.C., Brown, A.K., Attoe, S., Besra, G.S., and Fütterer, K. (2019). Crystal structure of *Mycobacterium tuberculosis* FadB2 implicated in mycobacterial β -oxidation. *Acta Crystallographica. Section D, Structural Biology*, 75(Pt 1), 101-108. <https://doi.org/10.1107/s2059798318017242>.
27. Kendall, S.L., Burgess, P., Balhana, R., Withers, M., ten Bokum, A., Lott, J.S., Gao, C., Uhia-Castro, I., and Stoker, N.G. (2010). Cholesterol utilization in mycobacteria is controlled by two TetR-type transcriptional regulators: *kstR* and *kstR2*. *Microbiology*, 156(5), 1362-1371. <https://doi.org/10.1099/mic.0.034538-0>.
28. Marrero, J., Rhee, K.Y., Schnappinger, D., Pethe, K., and Ehrt, S. (2010). Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for *Mycobacterium tuberculosis* to establish and maintain infection. *Proceedings of the National Academy of Sciences of the United States of America*, 107(21), 9819-9824. <https://doi.org/10.1073/pnas.1000715107>.

29. Billig, S., Schneefeld, M., Huber, C., Grassl, G.A., Eisenreich, W., and Bange, F.-C. (2017). Lactate oxidation facilitates growth of *Mycobacterium tuberculosis* in human macrophages. *Scientific Reports*, 7(1), 6484. <https://doi.org/10.1038/s41598-017-05916-7>.
30. Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E., Tekaiia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., and Osborne, J. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, 393(6685), 537-544. <https://doi.org/10.1038/31159>.
31. Gandhi, N.R., Moll, A., Sturm, A.W., Pawinski, R., Govender, T., Lalloo, U., Zeller, K., Andrews, J., and Friedland, G. (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet*, 368(9547), 1575-1580. [https://doi.org/10.1016/s0140-6736\(06\)69573-1](https://doi.org/10.1016/s0140-6736(06)69573-1).
32. Bardarov, S., Bardarov, S., Pavelka, M.S., Sambandamurthy, V., Larsen, M., Tufariello, J., Chan, J., Hatfull, G., and Jacobs, W.R. (2002). Specialized transduction: An efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. *Microbiology (Reading)*, 148(Pt 10), 3007-3017. <https://doi.org/10.1099/00221287-148-10-3007>.
33. Larsen, M.H., Biermann, K., Tandberg, S., Hsu, T., and Jacobs, W.R., Jr. (2007). Genetic manipulation of *Mycobacterium tuberculosis*. *Current Protocols in Microbiology*, Chapter 10, Unit 10A.12. <https://doi.org/10.1002/9780471729259.mc10a02s6>.
34. Ramsugit, S., Pillay, B., and Pillay, M. (2016). Evaluation of the role of *Mycobacterium tuberculosis* pili (MTP) as an adhesin, invasin, and cytokine inducer of epithelial cells. *The Brazilian Journal of Infectious Diseases*, 20(2), 160-165. <http://dx.doi.org/10.1016/j.bjid.2015.11.002>.
35. Grosset, J. (2003). *Mycobacterium tuberculosis* in the extracellular compartment: An underestimated adversary. *Antimicrobial Agents and Chemotherapy*, 47(3), 833-836. <https://doi.org/10.1128/aac.47.3.833-836.2003>.
36. Huang, L., Nazarova, E.V., Tan, S., Liu, Y., and Russell, D.G. (2018). Growth of *Mycobacterium tuberculosis* in vivo segregates with host macrophage metabolism and ontogeny. *J Exp Med*, 215(4), 1135-1152. 10.1084/jem.20172020.
37. De Carvalho, L.P., Fischer, S.M., Marrero, J., Nathan, C., Ehrt, S., and Rhee, K.Y. (2010). Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalized co-catabolism of carbon substrates. *Chemical Biology*, 17(10), 1122-1131. <https://doi.org/10.1016/j.chembiol.2010.08.009>.
38. Borah, K., Mendum, T.A., Hawkins, N.D., Ward, J.L., Beale, M.H., Larrouy-Maumus, G., Bhatt, A., Moulin, M., Haertlein, M., Strohmeier, G., Pichler, H., Forsyth, V.T., Noack, S., Goulding, C.W., McFadden, J., and Beste, D.J.V. (2021). Metabolic fluxes for nutritional flexibility of *Mycobacterium tuberculosis*. *Molecular Systems Biology*, 17(5), e10280. <https://doi.org/10.15252/msb.202110280>.
39. Gatfield, J. , and Pieters, J. (2000). Essential role for cholesterol in entry of mycobacteria into macrophages. *Science*, 288(5471), 1647-1650. <https://doi.org/10.1126/science.288.5471.1647>.
40. Chen, Z., Kong, X., Ma, Q., Chen, J., Zeng, Y., Liu, H., Wang, X., and Lu, S. (2024). The impact of *Mycobacterium tuberculosis* on the macrophage cholesterol metabolism pathway. *Frontiers in Immunology*, 15, 1402024. <https://doi.org/10.3389/fimmu.2024.1402024>.
41. Rank, L., Herring, L.E., and Braunstein, M. (2021). Evidence for the mycobacterial Mce4 transporter being a multiprotein complex. *Journal of Bacteriology*, 203(10), e00685-00620. <http://dx.doi.org/10.1128/jb.00685-20>.

42. Takayama, K., Wang, C., and Besra, G.S. (2005). Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis*. *Clinical Microbiology Reviews*, 18(1), 81-101. <https://doi.org/10.1128/cmr.18.1.81-101.2005>.
43. Salzman, V., Mondino, S., Sala, C., Cole, S.T., Gago, G., and Gramajo, H. (2010). Transcriptional regulation of lipid homeostasis in mycobacteria. *Molecular Microbiology*, 78(1), 64-77. <https://doi.org/10.1111/j.1365-2958.2010.07313.x>.
44. Bhatt, A., Molle, V., Besra, G.S., Jacobs, W.R., Jr., and Kremer, L. (2007). The *Mycobacterium tuberculosis* FAS-II condensing enzymes: Their role in mycolic acid biosynthesis, acid-fastness, pathogenesis and in future drug development. *Molecular Microbiology*, 64(6), 1442-1454. <https://doi.org/10.1111/j.1365-2958.2007.05761.x>.
45. Santangelo, M.P., Klepp, L., Nuñez-García, J., Blanco, F.C., Soria, M., García-Pelayo, M.D.C., Bianco, M.V., Cataldi, A.A., Golby, P., Jackson, M., Gordon, S.V., and Bigi, F. (2009). Mce3R, a TetR-type transcriptional repressor, controls the expression of a regulon involved in lipid metabolism in *Mycobacterium tuberculosis*. *Microbiology (Reading)*, 155(Pt 7), 2245-2255. <https://doi.org/10.1099/mic.0.027086-0>.
46. Mondino, S., Vázquez, C.L., Cabruja, M., Sala, C., Cazenave-Gassiot, A., Blanco, F.C., Wenk, M.R., Bigi, F., Cole, S.T., Gramajo, H., and Gago, G. (2020). FasR regulates fatty acid biosynthesis and is essential for virulence of *Mycobacterium tuberculosis*. *Frontiers in Microbiology*, 11, 586285. <https://doi.org/10.3389/fmicb.2020.586285>.
47. Cumming, B.M., Addicott, K.W., Adamson, J.H., and Steyn, A.J. (2018). *Mycobacterium tuberculosis* induces decelerated bioenergetic metabolism in human macrophages. *Elife*, 7, e39169. <https://doi.org/10.7554/elife.39169>.
48. Laval, T., Chaumont, L., and Demangel, C. (2021). Not too fat to fight: The emerging role of macrophage fatty acid metabolism in immunity to *Mycobacterium tuberculosis*. *Immunological Reviews*, 301(1), 84-97. <https://doi.org/10.1111/imr.12952>.
49. Haft, D.H. (2011). Bioinformatic evidence for a widely distributed, ribosomally produced electron carrier precursor, its maturation proteins, and its nicotinoprotein redox partners. *BMC Genomics*, 12, 21. <https://doi.org/10.1186/1471-2164-12-21>.
50. Krishnamoorthy, G., Kaiser, P., Constant, P., Abu Abed, U., Schmid, M., Frese, C.K., Brinkmann, V., Daffé, M., and Kaufmann, S.H.E. (2021). Role of premycofactocin synthase in growth, microaerophilic adaptation, and metabolism of *Mycobacterium tuberculosis*. *mBio*, 12(4), e0166521. <https://doi.org/10.1128/mbio.01665-21>.
51. Stanley, S., Wang, X., Liu, Q., Kwon, Y.Y., Frey, A.M., Hicks, N.D., Vickers, A.J., Hui, S., and Fortune, S.M. (2023). Ongoing evolution of the *Mycobacterium tuberculosis* lactate dehydrogenase reveals the pleiotropic effects of bacterial adaption to host pressure. *PLoS Pathogens*, 20(2), e1012050. <https://doi.org/10.1371/journal.ppat.1012050>.
52. Manosalva, C., Quiroga, J., Hidalgo, A.I., Alarcón, P., Anseoleaga, N., Hidalgo, M.A., and Burgos, R.A. (2022). Role of lactate in inflammatory processes: Friend or foe. *Frontiers in Immunology*, 12, 808799. <https://doi.org/10.3389/fimmu.2021.808799>.
53. Gutka, H.J., Rukserree, K., Wheeler, P.R., Franzblau, S.G., and Movahedzadeh, F. (2011). glpX gene of *Mycobacterium tuberculosis*: Heterologous expression, purification, and enzymatic characterization of the encoded fructose 1,6-bisphosphatase II. *Applied Biochemistry and Biotechnology*, 164(8), 1376-1389. <https://doi.org/10.1007/s12010-011-9219-x>.
54. Forrellad, M.A., Vázquez, C.L., Blanco, F.C., Klepp, L.I., García, E.A., Rocha, R.V., Luciana, V., Bigi, M.M., Gutierrez, M.G., and Bigi, F. (2019). Rv2617c and P36 are virulence factors of pathogenic mycobacteria involved in resistance to oxidative stress. *Virulence*, 10(1), 1026-1033. <https://doi.org/10.1080/21505594.2019.1693714>.

55. Leblanc, C., Prudhomme, T., Tabouret, G., Ray, A., Burbaud, S., Cabantous, S., Mourey, L., Guilhot, C., and Chalut, C. (2012). 4'-Phosphopantetheinyl transferase PptT, a new drug target required for *Mycobacterium tuberculosis* growth and persistence *in vivo*. *PLoS Pathogens*, 8(12), e1003097. <https://doi.org/10.1371/journal.ppat.1003097>.
56. Ramsugit, S. , and Pillay, M. (2019). Curli pili affect the intracellular survival of *Mycobacterium tuberculosis*. *Journal of Infecting in Develpoing Countries*, 13(2), 179-180. <http://dx.doi.org/10.3855/jidc.9942>.

Chapter 6 displayed that *Mycobacterium tuberculosis* curli pili is involved in the differential expression of genes associated with lipid import, β -oxidation and lactate oxidation of *M. tuberculosis* in broth culture and THP-1 macrophage intracellular bacilli. Additionally, this chapter provided evidence that corroborated previous metabolomic outcomes.

Chapter 7 synthesises the important findings from Chapters 2, 3, 4, 5, and 6 with limitations, future recommendations and conclusions.

CHAPTER 7: Synthesis of research findings

A thorough review of the relevant literature is described in Chapter 1: The steadily rising tuberculosis (TB) case numbers, from the estimated 10.1 million in 2020 to 10.8 million in 2024, reported by the World Health Organization (WHO) remains a major public health concern [1]. This rise in TB rates was seen despite the monumental efforts made by countries, like South Africa, that were previously classified as high TB burdened countries, to meet the WHO End TB Strategy milestones for 2025 and targets for 2030 and 2035 [1]. Additionally, the causative agent of TB, *Mycobacterium tuberculosis* (*M. tuberculosis*), has the unique ability to evade host immunity and has expertly adapted mechanisms to resist anti-TB drugs [2]. Hence, the management of transmission, delays in TB diagnosis, and ineffectual TB therapies are areas that require urgent improvement in order to reverse the upward trend. Given the numerous challenges faced with drug resistance, long-term TB treatment regimens and host genetic and environmental factors that influence disease outcomes at a later stage in infection [3], research expanding on *M. tuberculosis* pathogenicity during early stages of infection may benefit the search for novel biomarkers to support development of improved prevention, diagnostics and treatment.

Bacterial adhesins as biomarkers appear to be promising as these molecules are easily accessible and have immunogenic characteristics [4]. Recent reports suggest that the surface-located *M. tuberculosis* curli pili (MTP) may be well suited for use as a notable biomarker for the improvement of anti-TB strategies [5-8].

7.1. A review of MTP as a biomarker for the design of rapid diagnostic tests and improved therapeutics

Adhesins play a role in initial host-pathogen interaction as these structures are externally exposed proteinaceous molecules [9], which serve as virulence factors in *M. tuberculosis* pathogenicity [10]. The MTP adhesin, encoded by *Rv3312A* (*mtp*), which is located between intermediary metabolism associated genes, was demonstrated to bind to laminin *in vitro* [6; 11]. This adhesin contributed to biofilm production, most likely facilitating survival through antibiotic tolerance within this niche [12]. The MTP adhesin has been shown to be unique to *M. tuberculosis* complex strains [13], and acts not only as an adhesin, but an invasin to THP-1 macrophages [8], as well as epithelial cells [7]. In addition, MTP has been demonstrated to minimally modulate the host immune response, probably as a survival mechanism to facilitate establishment of early infection [7]. A synthetic MTP peptide showed 97% accuracy in detecting anti-MTP antibodies in patients' serum [5]. Unpublished transcriptome studies post infection revealed significant regulation by MTP of the host immune response in epithelial cells [14] and mice [15]. The MTP adhesin has been implicated to facilitate intracellular replication of wildtype (WT) *M. tuberculosis* (V9124) during infection of macrophages [16]. The MTP adhesin does not affect host cell cytotoxicity in macrophages and epithelial cells, indicating that this adhesin unlike other pili, does not impact host cell death pathways [17]. The significantly lower intracellular survival rate of the MTP-deficient mutant strain in macrophages relative to the WT strain is in keeping with reports that pili promote bacterial resistance to killing by macrophages [17]. This collective body of evidence suggests that MTP is a suitable biomarker for the design of TB diagnostics and therapeutics.

In response to adhesion, bacteria alter both their own and their hosts' physiology to favour their persistence [18]. Despite much research elucidating *M. tuberculosis* metabolism, to date, the interlinked host-pathogen metabolism in response to adhesins, particularly MTP, as well as its effect on the *M. tuberculosis* metabolism has not been documented. This motivated the need to investigate

the molecular mechanisms of MTP in the regulation of *M. tuberculosis* cell surface associated functions and metabolism as well as the influence of MTP in host metabolism and bioenergetics during early stages of infection.

7.2. Metabolic profiling revealed that MTP influences cell wall biogenesis, fatty acid metabolism and amino acid synthesis of *M. tuberculosis*

Section 7.2 synthesises Chapter 2: *M. tuberculosis* curli pili (MTP) deficiency is associated with alterations in cell wall biogenesis, fatty acid metabolism and amino acid synthesis. Ashokcoomar, S., Reedoy, K. S., Senzani, S., Loots, D. T., Beukes, D., van Reenen, Pillay, B., and Pillay, M. Original research article published in *Metabolomics*, 2020.

Untargeted two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-TOFMS) of pelleted wet cell mass of pure bacterial WT *M. tuberculosis* V9124, its Δmtp deletion mutant and its *mtp*-complemented strain was performed to fingerprint each of these strains. The *M. tuberculosis* metabolites were identified and categorised according to their biological involvement in cellular structures or functions and metabolic pathways. The *mtp*-complement exhibited partial restoration of WT phenotype due to the nature of the complement construct. Once MTP-deficient and MTP-proficient strains were compared to each other, differential metabolite concentrations were used as an indication of metabolic changes brought about by the presence/absence of the adhesin. The WT metabolomic profile differed statistically from the Δmtp profile. The elevated mean concentrations of metabolites in the Δmtp relative to the WT demonstrated that in the absence of MTP, *M. tuberculosis* shows a decreased utilisation of carbohydrates for cell wall biogenesis and functions and alters its fatty acids and amino acid metabolism. Moreover, reverse transcription quantitative polymerase chain reaction (RT-qPCR) performed on selected genes (*glf*, *glmU*, *fadD32*, *fadE5* and *glpK*) validated metabolic data.

The WT used in this study was previously demonstrated to be faster growing [15] and therefore, will utilise metabolites involved in energy production for cell wall synthesis and growth more readily than the slower growing Δmtp , a characteristic which is reminiscent of hypo-virulent strains [19; 20]. In the current study, 11 significant metabolites were collectively categorised to be involved in cell wall biogenesis and growth of bacterial cultures. Ten of these metabolites were increased in the Δmtp . This suggested that the lack of the MTP adhesin resulted in a reduced ability of *M. tuberculosis* to utilise these metabolites for biological processes.

Unique glycolipids located in mycobacterial cell envelopes are considered crucial for maintaining membrane structure [21]. Moreover, fatty acids accumulate in mycobacterial globules, which function as reservoirs for carbon energy source [22]. Fatty acid metabolism accounted for eight significant metabolites, seven of which were detected in higher concentrations in the Δmtp relative to the WT. This was indicative of the weakened ability of *M. tuberculosis* to utilise lipids in synthesising lipid-rich cell envelopes or a dysfunction of fatty acid metabolism in the absence of MTP.

Seven of the nine metabolites involved in amino acid and protein synthesis had a higher relative concentration in the WT compared to Δmtp . Essential amino acids are important for synthesis of nucleotides and proteins, which facilitates the proper functioning of cellular processes and maintenance of structures [23; 24]. Furthermore, amino acid synthesis in *M. tuberculosis* confers an advantage to the pathogen during nutrient starvation in the host [25; 26]. Therefore, the perturbations to amino acid metabolism observed in Δmtp , indicates that the lack of MTP hinders amino acid and protein synthesis. Hence MTP is postulated to play a role in the regulation of cell wall biogenesis, fatty acid metabolism and amino acid synthesis of *M. tuberculosis* and could further influence its interaction with the THP-1 macrophages. However, this remained to be investigated.

7.3. During initial stages of THP-1 macrophage infection, MTP alters carbon, fatty acid and amino acid metabolism

Section 7.3 synthesises Chapter 3: *M. tuberculosis* curli pili (MTP) is associated with alterations in carbon, fatty acid and amino acid metabolism in a THP-1 macrophage infection model. Ashokcoomar, S., Loots, D. T., Beukes, D., van Reenen, Pillay, B., and Pillay, M. Original research article published in *Microbial Pathogenesis*, 2021.

It is challenging to separate pathogen and host metabolites during infection as there is a large overlap between metabolic pathways [27]. Studies involving infection models indicated that the significant pathways engaged during bacterial infection include amino acid metabolism [25; 28; 29], carbohydrate uptake and utilisation [30], gluconeogenesis [31], lipid-based fuelling of central carbon metabolism (CCM) [32] and mycobacterial lipid uptake and breakdown [33-35]. An in-depth analysis of *M. tuberculosis* infected THP-1 macrophages was performed using GC×GC-TOFMS. This global metabolomic approach revealed the combined metabolic alterations to the host-pathogen metabolome as a result of the absence and presence of MTP in the *M. tuberculosis* strains used. Results revealed that both the Δmtp infected THP-1 macrophages and the *mtp*-complemented infected THP-1 macrophages displayed a 4% variance in the metabolome from the WT infected THP-1 macrophages. The variation observed between the WT infected and *mtp*-complement infected THP-1 macrophages was not ideal and was speculated to be a result of knocking out *mtp* and complementation with an overexpressing episomal plasmid vector. The lack of total phenotypic restoration may be attributed to the over expression of *mtp* observed in the *mtp*-complement [8; 12]. Subsequently the density of piliation influences the rate of adhesion to THP-1 macrophages and consequently alters the metabolic response. The *mtp*-complement infected THP-1 macrophages displayed greater consumption of metabolites involved in amino acid and purine and pyrimidine metabolism, which feed into the tricarboxylic (TCA) cycle, thus, explaining the higher levels of citric acid and fumaric acid relative to the WT infected THP-1 macrophages.

All nine of the detected metabolites involved in carbon, fatty acid, and amino acid metabolism were found in significantly elevated concentrations in the Δmtp infected THP-1 macrophages relative to the WT infected macrophages. Of particular interest was the 13 times higher concentration of lactate in the Δmtp infected THP-1 macrophages. Therefore, it was deduced that MTP affects carbon metabolism through alterations in glycolysis, pentose phosphate pathway (PPP), and gluconeogenesis pathways. In macrophages, *M. tuberculosis* is known to switch from glucose dependency to lipids for energy [36]. The accumulation of fatty acid intermediates demonstrated potential perturbations in the flow of fatty acids into CCM of the host thus influencing *M. tuberculosis* co-metabolism of available substrates [37]. Moreover, MTP plays a minor role in regulating the intertwined host-pathogen amino acid metabolism, as was observed with the accumulation of niacinamide in the Δmtp infected THP-1 macrophages. Differential activity of this metabolite is known to restrict replication of attenuated strains of mycobacteria, such as *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) [38].

Holistically, data demonstrated that MTP elicits metabolic alterations to THP-1 macrophage CCM, fatty acid and amino acid metabolism. However, macrophages are not the only cells that face the threat of *M. tuberculosis* invasion. Therefore, other cellular models should be evaluated to truly understand the impact MTP has on the host during initial stages of infection. Additionally, investigation of the flux through these pathways will provide more detailed insight into the differential metabolomic profiles observed.

7.4. *Mycobacterium tuberculosis* curli pili manipulates THP-1 macrophage metabolism to a larger extent compared to A549 epithelial cell metabolism, in attempts to reduce microbiocidal killing during early *M. tuberculosis* infection

Section 7.4 synthesises Chapter 4: *M. tuberculosis* curli pili (MTP) facilitates a reduction of microbiocidal activity of infected THP-1 macrophages during early stages of infection. Ashokcoomar, S., Reedoy, K. S., Loots, D. T., Beukes, D., van Reenen, Pillay, B., and Pillay, M. Original research article published in *Comparative Immunology, Microbiology and Infectious Diseases*, 2022.

Mycobacterium tuberculosis infects both alveolar epithelial cells and macrophages. Infection of these host cells with MTP-proficient and MTP-deficient *M. tuberculosis* was previously evidenced with the aid of GC×GC-TOFMS [39; 40]. The consequential changes to individual metabolic signatures revealed that MTP persuades nucleic acid synthesis, amino acid metabolism, glutathione metabolism, oxidative stress, and lipid metabolism in *M. tuberculosis* infected A549 epithelial cells [40], whereas, carbon, fatty acid and amino acid metabolism were effected in *M. tuberculosis* infected THP-1 macrophages [39]. These two separate studies gave rise to the question of how these cell types comparatively react to *M. tuberculosis* infection and MTP. Therefore, an investigation into which of these cells is more responsive to MTP, or lack thereof, was conducted. In this study, a GC×GC-TOFMS approach was used to reveal totals of 44, 40, 50 and 34 differentially detected metabolites between A549 epithelial cells and THP-1 macrophages when respectively comparing their uninfected, WT infected, Δmtp infected, and *mtp*-complement infected metabolic signatures. The range of significant metabolites identified, were broadly categorised into those that are involved in amino acid metabolism, fatty acid metabolism, general CCM, and nucleic acid metabolism.

Results revealed that 55% of the uninfected, 55% of the WT infected, 71% of the *mtp*-complement infected A549 epithelial cell metabolites were detected at elevated concentrations, relative to the THP-1 macrophages. Contrastingly, 98% of the differentially detected metabolites in the Δmtp infected THP-1 macrophage were observed at elevated concentrations, relative to the A549 epithelial cell counterpart. At baseline, the relative metabolomic profiles displayed appropriate concentrations of metabolites for their cellular roles in either healthy maintenance and function of the physical barrier or as immunoregulatory cells [41; 42].

Interestingly, in the *M. tuberculosis* infected cells, MTP had an overall protective effect in favour of the pathogen during initial stages of infection in both cell models. In the Δmtp infected cells, the major perturbations observed in the macrophages advocated for pathogen clearance potentially via efficient pro-inflammatory responses, whereas the epithelial cells did not elicit drastic metabolic changes in the absence of MTP as they were not biologically programmed to accelerate pathogen removal/killing.

Cumulatively, the data suggested that the THP-1 macrophages were predominantly more responsive to MTP than the A549 epithelial cells, potentially driving response to infection. Furthermore, MTP plays a role in the regulation of host defence mechanisms to aid pathogen survival. This can be confirmed by investigating the flux of carbon through pathways like glycolysis or elucidating the effect of MTP on energy generation pathways to confirm macrophage polarity.

7.5. *Mycobacterium tuberculosis* curli pili does not affect carbon flux, but decreases oxidative phosphorylation of *M. tuberculosis* infected THP-1 macrophages

Section 7.5 synthesises Chapter 5: *M. tuberculosis* curli pili reduces oxygen consumption rate of THP-1 macrophages during early infection. Ashokcoomar, S., Naidoo, T. J., Cumming B. M., Baig,

Z., Truebody, B., Mackenzie, J.S., Steyn, A. J.C., and Pillay, M. Original research article submitted to *The International Journal of Biochemistry and Cell Biology* (10/1/2025).

Thus far, untargeted metabolomics was used to investigate *M. tuberculosis* and its infected hosts' metabolic signatures associated with MTP. The combination of targeted liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and extracellular flux analysers has been used by other research groups to discern *M. tuberculosis* survival and pathogenic strategies [36; 43; 44]. Therefore, a similar approach was taken to investigate the effect MTP has on THP-1 macrophage carbon flux and cellular energetics. Differentiated THP-1 macrophages were infected for four hours with WT, Δmtp , or *mtp*-complement strains of *M. tuberculosis* prior to determination of bioenergetic profiles through cell mitochondrial stress test (CMST) and glycolytic rate assay (GRA). Furthermore, metabolic flux of macrophage groups was determined by $^{13}\text{C}_6$ -glucose tracing and LC-MS/MS.

Bioenergetic results from the CMST revealed that the Δmtp infected THP-1 macrophages mimicked the higher oxygen consumption rate (OCR) for specific respiratory parameters. The MTP adhesin influenced basal respiration, ATP production, maximal respiration and spare respiratory capacity of THP-1 macrophages. Data generated from the GRA demonstrated that although infection with *M. tuberculosis* triggers an increase in glycolytic rates, MTP slightly suppresses compensatory glycolytic rates in THP-1 macrophages to facilitate pathogen survival. Surprisingly, tracing of carbon incorporation into essential pathways such as glycolysis, TCA cycle, PPP, revealed that MTP does not encourage changes to carbon flux of THP-1 macrophages as *M. tuberculosis* infected macrophages all showed similar trends in carbon flux. Instead, MTP affected the total concentrations of metabolites involved in the aforementioned pathways, with the metabolomic profile of Δmtp infected THP-1 macrophages resembling the uninfected THP-1 macrophages. Once more, *mtp*-complemented *M. tuberculosis* displayed partial restoration of phenotype and functionality to the WT *M. tuberculosis* as their effects on the host cell were mostly similar. Lower lactate concentrations detected in the Δmtp infected THP-1 macrophages after carbon labelling experiments contradicted the previously detected level observed, thus emphasising the quick metabolic adjustment of both host and pathogen in response to environmental changes.

Lowered mitochondrial OCR and increased glycolysis in macrophages are associated with pro-inflammatory responses [45]. Moreover, as a consequence of *M. tuberculosis* infection, macrophages redirect carbon from TCA cycle to glycolysis, and in some instances, this is met with increased flux through PPP [46; 47], as observed in this study. Therefore, data revealed that macrophages are more proficient at launching a healthy immune response if MTP is absent, thus implying that MTP dampens the immune response to allow for survival and replication of *M. tuberculosis* in THP-1 macrophages. The observed changes to various host-pathogen carbon utilisation and energy generation pathways caused by MTP should be further validated by gene expression studies, which will determine the exact mechanisms by which *M. tuberculosis* regulates the uptake and breakdown of available carbon sources for its replication and survival.

7.6. *Mycobacterium tuberculosis* curli pili differentially regulates expression of genes involved in lipid import, β -oxidation and lactate oxidation of *M. tuberculosis*

Section 7.6 synthesises Chapter 6: Differential expression of genes associated with lipid import, β -oxidation and lactate oxidation induced by *M. tuberculosis* curli pili (MTP) in broth culture compared to intracellular bacilli within THP-1 macrophages. Ashokcoomar, S., and Pillay, M. Original research article accepted for publication in the *Journal of Medical Microbiology* (6/3/2025).

The transport, accumulation and depletion of metabolites are reliant on gene expression of proteins and their components involved in these processes [48]. *Mycobacterium tuberculosis* is well defined as a successful pathogen due to its ability to co-metabolise a plethora of available nutrient sources

and its proficiency to adapt to different microenvironments [47; 49]. Hence, elucidating molecular mechanisms by which *M. tuberculosis* regulates nutrient acquisition is important for enhancing current understanding of the pathogen's virulence, particularly in the presence/absence of MTP.

To determine the validity of claims made from the untargeted and targeted metabolomics experiments, the effect of MTP on gene expression levels was evaluated for two *M. tuberculosis* models. These included a pure bacterial cultured model, and an intracellular bacterial model whereby *M. tuberculosis* was isolated from infected THP-1 macrophages following a four-hour infection. Both models used the same three strains of *M. tuberculosis* (WT, Δmtp , *mtp*-complement) from which RNA was extracted for RT-qPCR of selected genes involved in fatty acid transport (*lucA*, *mce1D*, *mceG*, *Rv2799*, *Rv0966c*, *omamB*), β -oxidation (*fadA5*, *fadB*), lactate oxidation (*lldD1*, *lldD2*) and gluconeogenic carbon flow (*pckA*) [30; 50-54].

Results illustrated that the gene expression levels in Δmtp were significantly different to the WT and *mtp*-complement strains, the latter two displaying similar gene expression levels to each other, for most of the genes investigated in the two models. Relative gene expression levels of late log phase cultured *M. tuberculosis* revealed the MTP plays a significant role in the regulation of genes involved in the typical utilisation of lipids and lactate during exponential growth. Genes involved in lipid uptake and breakdown as well as lactate oxidation were significantly downregulated in the MTP-proficient intracellular *M. tuberculosis*. Therefore, lipid import via Mce1 transport protein and β -oxidation of fatty acids is modulated by MTP to alter the pathogenicity of intracellular *M. tuberculosis*. Additionally, when cultured bacteria were compared to intracellular bacteria, trends in expression levels of *lldD1*, *mceD1* and *mceG* between MTP-proficient and MTP-deficient strains were notably different. This was an indication that MTP regulates the expression of genes involved in fatty acid import and lactate oxidation of intracellular *M. tuberculosis* to shape the outcome of infection in favour of the pathogen.

Collectively, MTP is influential to the transcriptome of *M. tuberculosis* as it is involved in modulating multiple functions during early infection. Therefore, MTP contributes to metabolism and metabolic flexibility of the pathogen.

7.7. Limitations of study and recommendations for future work

Metabolomics studies are sensitive and can quickly and accurately identify metabolites across a range of molecular compounds and samples. However, they are hindered by the confines of current reference libraries. Therefore, in efforts to adequately detect metabolites, annotations of unidentified metabolites should be made, and libraries should be constantly expanding to include all cell types and metabolite classes.

Metabolomic samples are time sensitive, hence, can only provide information for a snapshot of the infection process. Therefore, reproducibility of data is reliant on efficient sample extraction, preparation, and processing. Moreover, this study only elaborated on a four-hour infection. Future work should introduce more time points in order to determine the long-term effect of MTP on host and pathogen metabolomes.

The use of $^{13}\text{C}_6$ -glucose as a tracer in targeted metabolomics could only provide insight into glucose breakdown during infection. Future studies can incorporate other labelled compounds such as lactate and fatty acids to outline the specific molecular mechanisms involved in the assimilation of these nutrient sources. This can be coupled with gene expression studies using an array of genes involved in their respective process, for example *M. tuberculosis* genes involved in *de novo* synthesis of fatty acids. This will provide more detailed understanding of *M. tuberculosis* metabolism, flexibility and pathogenicity.

This study was restricted to *M. tuberculosis* V9124 from the F15/LAM4/KZN family and THP-1 monocytic cell line under controlled conditions. Although results are informative, they may not accurately represent the complexity of the natural environment of an infection, thus making it difficult to extrapolate the findings to all clinical strains of *M. tuberculosis* and human metabolomes.

From the motivations provided in this study, a closer look into *in vitro* antibiotic killing curves using first line anti-TB drugs in addition to a MTP curlicide/pilicide treatment of *M. tuberculosis* strains is recommended. Anti-MTP monoclonal antibodies or a curlicide specifically developed to target MTP could be tested for use as adjunctive therapy to enhance current TB treatment regimens.

7.8. Conclusions

This study provided evidence that MTP is a virulence factor of *M. tuberculosis*. This surface-located adhesin doubles as a means of attachment for the pathogen and a modulator of metabolism. Contributing to the structural integrity and metabolomic fingerprint of *M. tuberculosis*, MTP, particularly affects cell wall biosynthesis, fatty acid and amino acid metabolism of the bacteria in culture media under optimum conditions. Additionally, during infection of its preferential macrophage host, *M. tuberculosis* employs MTP in the regulation of bacterial genes involved in lactate oxidation, β -oxidation and lipid import to secure its chances of survival and improve its flexibility in hostile and quickly changing host microenvironments. Furthermore, MTP plays a role in the manipulation of macrophage polarity by tipping the scales toward a dampened pro-inflammatory response in efforts to evade host killing mechanisms. Hence, to give the host an advantage, anti-TB strategies should shift focus toward interception of MTP interaction with the host in attempts to offset *M. tuberculosis* pathogenicity. This could potentially be achieved by the development and implementation of small molecule inhibitors or anti-body-based therapies. Therefore, this study supports previous research substantiating MTP adhesin as a notable virulence factor with potential as a biomarker for development of novel therapeutic intervention and diagnostics.

References:

1. World Health Organization (2024). Global tuberculosis report. *World Health Organization* [Online]. Available: <https://www.who.int/publications/i/item/9789240101531> [Accessed October 2024].
2. Alsayed, S.S.R. , and Gunosewoyo, H. (2023). Tuberculosis: Pathogenesis, current treatment regimens and new drug targets. *International Journal of Molecular Sciences*, 24(6), 5202. <http://dx.doi.org/10.3390/ijms24065202>.
3. Guha, P., Dutta, S., Murti, K., Charan, J.K., Pandey, K., Ravichandiran, V., and Dhingra, S. (2024). The integration of omics: A promising approach to personalized tuberculosis treatment. *Medicine in Omics*, 12, 100033. <https://doi.org/10.1016/j.meomic.2024.100033>.
4. Stones, D.H. , and Krachler, A.-M. (2015). Fatal attraction: How bacterial adhesins affect host signaling and what we can learn from them. *International Journal of Molecular Sciences*, 16(2), 2626-2640. <https://doi.org/10.3390/ijms16022626>.
5. Naidoo, N., Pillay, B., Bubb, M., Pym, A., Chiliza, T., Naidoo, K., Ndung'u, T., Kasproicz, V.O., and Pillay, M. (2018). Evaluation of a synthetic peptide for the detection of anti-*Mycobacterium tuberculosis* curli pili IgG antibodies in patients with pulmonary tuberculosis. *Tuberculosis*, 109, 80-84. <https://doi.org/10.1016/j.tube.2018.01.007>.
6. Alteri, C.J., Xicohténcatl-Cortes, J., Hess, S., Caballero-Olín, G., Girón, J.A., and Friedman, R.L. (2007). *Mycobacterium tuberculosis* produces pili during human infection. *Proceedings*

- of the National Academy of Sciences of the United States of America, 104(12), 5145-5150. <https://doi.org/10.1073/pnas.0602304104>.
7. Ramsugit, S., Pillay, B., and Pillay, M. (2016). Evaluation of the role of *Mycobacterium tuberculosis* pili (MTP) as an adhesin, invasin, and cytokine inducer of epithelial cells. *The Brazilian Journal of Infectious Diseases*, 20(2), 160-165. <http://dx.doi.org/10.1016/j.bjid.2015.11.002>.
 8. Ramsugit, S. , and Pillay, M. (2014). *Mycobacterium tuberculosis* pili promote adhesion to and invasion of THP-1 macrophages. *Japanese Journal of Infectious Diseases*, 67(6), 476-478. <https://doi.org/10.7883/yoken.67.476>.
 9. Niemann, H.H., Schubert, W.D., and Heinz, D.W. (2004). Adhesins and invasins of pathogenic bacteria: A structural view. *Microbes and Infection*, 6(1), 101-112. <https://doi.org/10.1016/j.micinf.2003.11.001>.
 10. Klemm, P. , and Schembri, M.A. (2000). Bacterial adhesins: Function and structure. *International Journal of Medical Microbiology*, 290(1), 27-35. [https://doi.org/10.1016/s1438-4221\(00\)80102-2](https://doi.org/10.1016/s1438-4221(00)80102-2).
 11. Alteri, C. (2005). *Novel pili of Mycobacterium tuberculosis*. Doctoral thesis, The University of Arizona, United States of America. <https://repository.arizona.edu/handle/10150/195515?show=full>.
 12. Ramsugit, S., Guma, S., Pillay, B., Jain, P., Larsen, M.H., Danaviah, S., and Pillay, M. (2013). Pili contribute to biofilm formation *in vitro* in *Mycobacterium tuberculosis*. *Antonie Van Leeuwenhoek*, 104(5), 725-735. <https://doi.org/10.1007/s10482-013-9981-6>.
 13. Naidoo, N., Ramsugit, S., and Pillay, M. (2014). *Mycobacterium tuberculosis* pili (MTP), a putative biomarker for a tuberculosis diagnostic test. *Tuberculosis*, 94(3), 338-345. <http://dx.doi.org/10.1016/j.tube.2014.03.004>.
 14. Dlamini, M.T. (2016). *Whole transcriptome analysis to elucidate the role of mtp in gene regulation of pulmonary epithelial cells infected with Mycobacterium tuberculosis*. University of KwaZulu-Natal, Durban.
 15. Nyawo, G. (2016). *The role of Mycobacterium tuberculosis pili in pathogenesis: growth and survival kinetics, gene regulation and host immune response, and in vitro growth kinetics*. University of KwaZulu-Natal, Durban.
 16. Moodley, S. (2018). *The role of heparin binding haemagglutinin adhesin and curli pili on the pathogenicity of Mycobacterium tuberculosis*. Doctoral thesis, <https://researchspace.ukzn.ac.za/items/22f2cedb-2826-46ad-8a62-237a41b62d17>.
 17. Ramsugit, S. , and Pillay, M. (2019). Curli pili affect the intracellular survival of *Mycobacterium tuberculosis*. *Journal of Infecting in Develpoing Countries*, 13(2), 179-180. <http://dx.doi.org/10.3855/jidc.9942>.
 18. Stones, D.H. , and Krachler, A.M. (2016). Against the tide: the role of bacterial adhesion in host colonization. *Biochemical Society Transactions*, 44(6), 1571-1580. 10.1042/BST20160186.
 19. Meissner-Roloff, R.J., Koekemoer, G., Warren, R.M., and Loots, D.T. (2012). A metabolomics investigation of a hyper- and hypo-virulent phenotype of Beijing lineage *M. tuberculosis*. *Metabolomics*, 8(6), 1194-1203. 10.1007/s11306-012-0424-6.
 20. Swanepoel, C.C. , and Loots, D.T. (2014). The use of functional genomics in conjunction with metabolomics for *Mycobacterium tuberculosis* research. *Disease Markers*, 2014, 124218. <https://doi.org/10.1155/2014/124218>.
 21. Sancho-Vaello, E., Albesa-Jové, D., Rodrigo-Unzueta, A., and Guerin, M.E. (2017). Structural basis of phosphatidyl-myo-inositol mannosides biosynthesis in mycobacteria. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1862(11), 1355-1367. <https://doi.org/10.1016/j.bbalip.2016.11.002>.

22. Schaefer, C.M., Lu, R., Nesbitt, N.M., Schiebel, J., Sampson, N.S., and Kisker, C. (2015). FadA5 a thiolase from *Mycobacterium tuberculosis*: A steroid-binding pocket reveals the potential for drug development against tuberculosis. *Structure*, 23(1), 21-33. <https://doi.org/10.1016/j.str.2014.10.010>.
23. Csonka, L.N. (1989). Physiological and genetic responses of bacteria to osmotic stress. *Microbiology Reviews*, 53(1), 121-147. <https://doi.org/10.1128/mr.53.1.121-147.1989>.
24. Zhang, L., Alfano, J.R., and Becker, D.F. (2015). Proline metabolism increases katG expression and oxidative stress resistance in *Escherichia coli*. *Journal of Bacteriology*, 197(3), 431-440. <https://doi.org/10.1128/JB.02282-14>.
25. Zhang, Y.J., Reddy, M.C., Ioerger, T.R., Rothchild, A.C., Dartois, V., Schuster, B.M., Trauner, A., Wallis, D., Galaviz, S., Huttenhower, C., Sacchettini, J.C., Behar, S.M., and Rubin, E.J. (2013). Tryptophan biosynthesis protects mycobacteria from CD4 T-cell-mediated killing. *Cell*, 155(6), 1296-1308. <https://doi.org/10.1016/j.cell.2013.10.045>.
26. Zelante, T., Fallarino, F., Bistoni, F., Puccetti, P., and Romani, L. (2009). Indoleamine 2,3-dioxygenase in infection: The paradox of an evasive strategy that benefits the host. *Microbes and Infection*, 11(1), 133-141. <https://doi.org/10.1016/j.micinf.2008.10.007>.
27. Zimmermann, M., Kogadeeva, M., Gengenbacher, M., McEwen, G., Mollenkopf, H.J., Zamboni, N., Kaufmann, S.H.E., and Sauer, U. (2017). Integration of metabolomics and transcriptomics reveals a complex diet of *Mycobacterium tuberculosis* during early macrophage infection. *mSystems*, 2(4), e00057-00017. <https://doi.org/10.1128/mSystems.00057-17>.
28. Gouzy, A., Larrouy-Maumus, G., Wu, T.-D., Peixoto, A., Levillain, F., Lugo-Villarino, G., Guerquin-Kern, J.-L., de Carvalho, L.P.S., Poquet, Y., and Neyrolles, O. (2013). *Mycobacterium tuberculosis* nitrogen assimilation and host colonization require aspartate. *Nature Chemical Biology*, 9(11), 674-676. <https://doi.org/10.1038/nchembio.1355>.
29. Gouzy, A., Larrouy-Maumus, G., Bottai, D., Levillain, F., Dumas, A., Wallach, J.B., Caire-Brandli, I., de Chastellier, C., Wu, T.-D., Poincloux, R., Brosch, R., Guerquin-Kern, J.-L., Schnappinger, D., S rio de Carvalho, L.P., Poquet, Y., and Neyrolles, O. (2014). *Mycobacterium tuberculosis* exploits asparagine to assimilate nitrogen and resist acid stress during infection. *PLoS Pathogens*, 10(2), e1003928. <https://doi.org/10.1371/journal.ppat.1003928>.
30. Marrero, J., Rhee, K.Y., Schnappinger, D., Pethe, K., and Ehrt, S. (2010). Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for *Mycobacterium tuberculosis* to establish and maintain infection. *Proceedings of the National Academy of Sciences of the United States of America*, 107(21), 9819-9824. <https://doi.org/10.1073/pnas.1000715107>.
31. Trujillo, C., Blumenthal, A., Marrero, J., Rhee, K.Y., Schnappinger, D., and Ehrt, S. (2014). Triosephosphate isomerase is dispensable in vitro yet essential for *Mycobacterium tuberculosis* to establish infection. *MBio*, 5(2), e00085. <https://doi.org/10.1128/mbio.00085-14>.
32. Munoz-Elias, E.J., Upton, A.M., Cherian, J., and McKinney, J.D. (2006). Role of the methylcitrate cycle in *Mycobacterium tuberculosis* metabolism, intracellular growth, and virulence. *Molecular Microbiology*, 60(5), 1109-1122. <https://doi.org/10.1111/j.1365-2958.2006.05155.x>.
33. Pandey, A.K. , and Sassetti, C.M. (2008). Mycobacterial persistence requires the utilization of host cholesterol. *Proceedings of the National Academy of Sciences*, 105(11), 4376-4380. <https://doi.org/10.1073/pnas.0711159105>.

34. Chang, J.C., Miner, M.D., Pandey, A.K., Gill, W.P., Harik, N.S., Sasseti, C.M., and Sherman, D.R. (2009). *Igr* genes and *Mycobacterium tuberculosis* cholesterol metabolism. *Journal of Bacteriology*, 191(16), 5232-5239. <https://doi.org/10.1128/jb.00452-09>.
35. Lee, W., VanderVen, B.C., Fahey, R.J., and Russell, D.G. (2013). Intracellular *Mycobacterium tuberculosis* exploits host-derived fatty acids to limit metabolic stress. *The Journal of Biological Chemistry*, 288(10), 6788-6800. <https://doi.org/10.1074/jbc.m112.445056>.
36. Cumming, B.M., Addicott, K.W., Adamson, J.H., and Steyn, A.J. (2018). *Mycobacterium tuberculosis* induces decelerated bioenergetic metabolism in human macrophages. *Elife*, 7, e39169. <https://doi.org/10.7554/elife.39169>.
37. De Carvalho, L.P., Fischer, S.M., Marrero, J., Nathan, C., Ehrt, S., and Rhee, K.Y. (2010). Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalized co-catabolism of carbon substrates. *Chemical Biology*, 17(10), 1122-1131. <https://doi.org/10.1016/j.chembiol.2010.08.009>.
38. Simmons, J.D., Peterson, G.J., Campo, M., Lohmiller, J., Skerrett, S.J., Tunaru, S., Offermanns, S., Sherman, D.R., and Hawn, T.R. (2020). Nicotinamide limits replication of *Mycobacterium tuberculosis* and Bacille Calmette-Guérin within macrophages. *Journal of Infectious Diseases*, 221(6), 989-999. <https://doi.org/10.1093/infdis/jiz541>.
39. Ashokcoomar, S., Loots, D.T., Beukes, D., Van Reenen, M., Pillay, B., and Pillay, M. (2021). *M. tuberculosis* curli pili (MTP) is associated with alterations in carbon, fatty acid and amino acid metabolism in a THP-1 macrophage infection model. *Microbial Pathogenesis*, 154, 104806. <https://doi.org/10.1016/j.micpath.2021.104806>.
40. Reedoy, K.S., Loots, D.T., Beukes, D., Reenen, M.V., Pillay, B., and Pillay, M. (2020). *Mycobacterium tuberculosis* curli pili (MTP) is associated with significant host metabolic pathways in an A549 epithelial cell infection model and contributes to the pathogenicity of *Mycobacterium tuberculosis*. *Metabolomics*, 16(11), 116. <http://dx.doi.org/10.1007/s11306-020-01736-5>.
41. Genoula, M., Franco, J.L.M., Maio, M., Dolotowicz, B., Ferreyra, M., Milillo, M.A., Mascarau, R., Moraña, E.J., Palmero, D., Matteo, M., Fuentes, F., López, B., Barrionuevo, P., Neyrolles, O., Cougoule, C., Lugo-Villarino, G., Vérollet, C., del Carmen Sasiain, M., and Balboa, L. (2020). Fatty acid oxidation of alternatively activated macrophages prevents foam cell formation, but *Mycobacterium tuberculosis* counteracts this process via HIF-1 α activation. *PLOS Pathogens*, 16(10), e1008929. <https://doi.org/10.1371/journal.ppat.1008929>.
42. Chen, J., Jin, Y., Yang, Y., Wu, Z., and Wu, G. (2020). Epithelial dysfunction in lung diseases: Effects of amino acids and potential mechanisms. *Advances in Experimental Medicine and Biology*, 1265, 57-70. https://doi.org/10.1007/978-3-030-45328-2_4.
43. Lamprecht, D.A., Finin, P.M., Rahman, M.A., Cumming, B.M., Russell, S.L., Jonnala, S.R., Adamson, J.H., and Steyn, A.J. (2016). Turning the respiratory flexibility of *Mycobacterium tuberculosis* against itself. *Nature Communications*, 7, 12393. <https://doi.org/10.1038/ncomms12393>.
44. Mackenzie, J.S., Lamprecht, D.A., Asmal, R., Adamson, J.H., Borah, K., Beste, D.J.V., Lee, B.S., Pethe, K., Rousseau, S., Krieger, I., Sacchettini, J.C., Glasgow, J.N., and Steyn, A.J.C. (2020). Bedaquiline reprograms central metabolism to reveal glycolytic vulnerability in *Mycobacterium tuberculosis*. *Nature Communications*, 11(1), 6092. <https://doi.org/10.1038/s41467-020-19959-4>.
45. Fraternali, A., Brundu, S., and Magnani, M. (2015). Polarization and repolarization of macrophages. *Journal of Clinical & Cellular Immunology*, 6(2), 1-10. <https://doi.org/10.4172/2155-9899.1000319>.

46. Howard, N.C. , and Khader, S.A. (2020). Immunometabolism during *Mycobacterium tuberculosis* Infection. *Trends in Microbiology*, 28(10), 832-850. <https://doi.org/10.1016/j.tim.2020.04.010>.
47. Borah Slater, K., Moraes, L., Xu, Y., and Kim, D. (2023). Metabolic flux reprogramming in *Mycobacterium tuberculosis*-infected human macrophages. *Frontiers in Microbiology*, 14, 1289987. <https://doi.org/10.3389/fmicb.2023.1289987>.
48. Qin, H., Niu, T., and Zhao, J. (2019). Identifying multi-omics causers and causal pathways for complex traits. *Frontiers in Genetics*, 10, 110. <https://doi.org/10.3389/fgene.2019.00110>.
49. Huang, L., Nazarova, E.V., Tan, S., Liu, Y., and Russell, D.G. (2018). Growth of *Mycobacterium tuberculosis* in vivo segregates with host macrophage metabolism and ontogeny. *Journal of Experimental Medicine*, 215(4), 1135-1152. <https://doi.org/10.1084/jem.20172020>.
50. Ekiert, D.C., Bhabha, G., Isom, G.L., Greenan, G., Ovchinnikov, S., Henderson, I.R., Cox, J.S., and Vale, R.D. (2017). Architectures of lipid transport systems for the bacterial outer membrane. *Cell*, 169(2), 273-285.e217. <https://doi.org/10.1016/j.cell.2017.03.019>.
51. Nazarova, E.V., Montague, C.R., La, T., Wilburn, K.M., Sukumar, N., Lee, W., Caldwell, S., Russell, D.G., and VanderVen, B.C. (2017). Rv3723/LucA coordinates fatty acid and cholesterol uptake in *Mycobacterium tuberculosis*. *eLife*, 6, e26969. <https://doi.org/10.7554/elife.26969>.
52. Nazarova, E.V., Montague, C.R., Huang, L., La, T., Russell, D., and VanderVen, B.C. (2019). The genetic requirements of fatty acid import by *Mycobacterium tuberculosis* within macrophages. *eLife*, 8, e43621. <https://doi.org/10.7554/elife.43621>.
53. Cox, J.A.G., Taylor, R.C., Brown, A.K., Attoe, S., Besra, G.S., and Fütterer, K. (2019). Crystal structure of *Mycobacterium tuberculosis* FadB2 implicated in mycobacterial β -oxidation. *Acta Crystallographica. Section D, Structural Biology*, 75(Pt 1), 101-108. <https://doi.org/10.1107/s2059798318017242>.
54. Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E., Tekaiia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., and Osborne, J. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, 393(6685), 537-544. <https://doi.org/10.1038/31159>.

APPENDICES

Appendix A: BREC approval

This study is a conversion of a Masters degree to a Doctoral degree, therefore, there are two University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC) reference numbers assigned to Ms S. Ashokcoomar: Masters (BE254/17) and PhD (BREC/00002915/2021). Chapters 2 and 4 are additionally associated with BREC reference number (BE255/17) assigned to the co-author Ms K. S. Reedoy.



Amended letter
04 February 2020

Ms S Ashokcoomar (213503221)
Discipline of Medical Microbiology and Infection Control
School of Laboratory Medicine and Medical Sciences
213503221@stu.ukzn.ac.za

Dear Ms Ashokcoomar

Title: The role of *Mycobacterium tuberculosis* HBHA and MTP in host and pathogen metabolite production in an infection model of THP-1 macrophages.

Degree: Public Service Module (PBNL6SL)

BREC Ref No: BE254/17

New Title: GCxGC-TOFMS elucidates the role of *M. tuberculosis* curi pili (MTP) in host and pathogen metabolomics in a THP-1 macrophage infection model

We wish to advise you that your correspondence received on 09 January 2020 submitting and application for Amendments to change the title to the above and other amendments for the above study has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee.

The committee will be notified of the above approval at its next meeting to be held on 10 March 2020

Yours sincerely


Professor V Rambiritch
Chair: Biomedical Research Ethics Committee

cc supervisor: taylor@ukzn.ac.za

cc postgraduate administrator: arumugamd@ukzn.ac.za

13 December 2024

Ms Shinese Ashokcoomar (213503221)
School of Laboratory Medicine & Medical Science
Medical School

Dear Ms Ashokcoomar

Protocol reference number: BREC/00002915/2021

Project title: The molecular mechanisms of *M. tuberculosis* curli pili (MTP) in regulation of cell wall associated functions, fatty acid metabolism, carbon metabolism and bioenergetics of the pathogen during host infection

Degree: PhD

New Title: The molecular mechanisms of *Mycobacterium tuberculosis* curli pili (MTP) in regulation of fatty acid metabolism, carbon metabolism, and bioenergetics of the pathogen and host during infection

We wish to advise you that your correspondence received on 09 December 2024 submitting an application for amendments listing 3 amendments including a change of title to the new title above has been noted and approved by a subcommittee of the Biomedical Research Ethics Committee.

The committee will be advised of the above at its next meeting to be held on 11 February 2025.

Yours sincerely



.....
Ms. A Marimuthu
(for) Prof T Hardcastle
Deputy Chair: Biomedical Research Ethics Committee



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07 February 2020

Ms KS Reedoy (213505224)
Medical Microbiology and Infection control
1st Floor DDMRI Building
NRMSM
213505224@stu.ukzn.ac.za

Dear Ms Reedoy

Title: Metabolomic profiling to elucidate the in vitro of Mycobacterium tuberculosis HBHA and MTP in an epithelial cell model of infection.

Degree: MMedSc

BREC Ref No: BE255/17

New Title: M. Tuberculosis pili (MTP) modulates pathogen and host metabolomic changes in an A549 epithelial cell model of infection

We wish to advise you that your correspondence received on 09 January 2020 submitting and application for Amendments to change the title to the above and other amendments for the above study has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee.

The committee will be notified of the above approval at its next meeting to be held on 10 March 2020 .

Yours sincerely

Professor V Rambiritch
Chair: Biomedical Research Ethics Committee

cc supervisor: taylor@ukzn.ac.za

cc postgraduate administrator: arumugamd@ukzn.ac.za

Appendix B: Supplementary information for Chapter 2

Polymerase chain reaction (PCR) confirmation of bacterial strains

InstaGene Matrix (BioRad, Hercules, CA, USA) was used to perform total deoxyribonucleic acid (DNA) extraction for all strains. This method was selected based on standardizing the DNA extraction for the inclusion of both genomic and plasmid DNA given how the mutant and complemented strains were constructed. Therefore, crude DNA from each strain was used to perform conventional PCR reactions using the specific primers and their respective annealing temperatures reported in Table S1. The expected PCR product of 300 base pairs (bp) and 731 bp was observed in Figure S1a and b, respectively. This indicated the presence of the hygromycin resistance (*hyg^R*) cassette and allelic exchange substrate (AES) in both Δmtp (lane 4) and *mtp*-complement samples (lane 5), respectively. The wildtype (WT) (lane 2) in Figure S1a and b shows no banding which was expected, as it lacks the *hyg^R* cassette and AES, respectively. The expected PCR product of 436 bp was seen in Figure S1c for the WT (lane 2) and *mtp*-complement (lane 5) samples indicative of the presence of the *mtp* gene which is absent in the Δmtp (lane 4). All negative controls (Figure S1a, b and c; lane 5) were clear of any contaminants.

Table S1. PCR target and primer sequences used for confirmation of three strains.

PCR target	Primers	Annealing temperature (°C)	PCR product size bp
Region of <i>hyg^R</i> cassette	Hyg-F: 5'-acccccattccgaggtctt-3' Hyg-R: 5'-ccggaaggcgttgagatgca-3'	55	300
Upstream AES	<i>mtp</i> -LL: 5'- tttttttgcataaattgctcacgattcatgccggtgag- 3' Universal uptag 5'-gatgtctcactgaggtctct-3'	50	731
<i>mtp</i> gene	<i>mtp</i> -261F: 5'-ttttaagatctcatgtaccggttcgcgtg-3' <i>mtp</i> -261R: 5'-tttttaagcttggcacgggagcgtaaatctg-3'	60	436

hyg^R: hygromycin resistance; AES: allelic exchange substrate; *mtp*: gene encoding MTP.

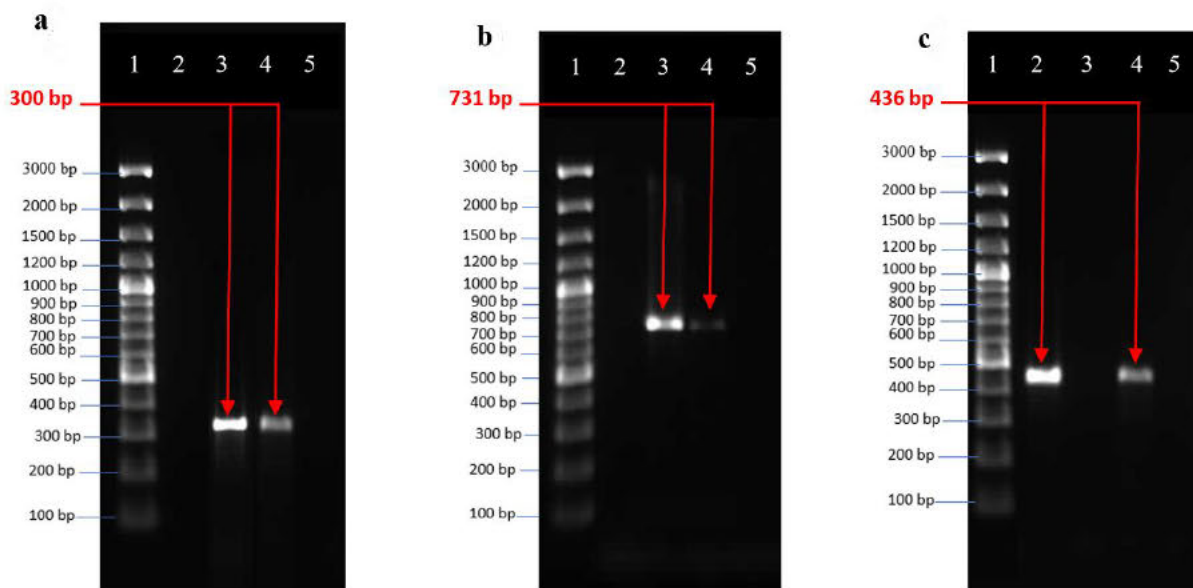


Figure S1. Gel electrophoresis images of PCR products confirming bacterial strains run at 70 V for 3 hours on a 1.5% agarose gel using 100 bp marker. (a) PCR targeting a region of hygromycin resistance (*hyg^R*) cassette, (b) PCR targeting upstream allelic exchange substrate (AES), and (c) PCR targeting the *mtp* gene. The molecular weight marker from ACE BioLabs (100 bp DNA ladder, 100-3000bp) was loaded in lane 1, WT (V9124) was loaded in lane 2, Δmtp was loaded in lane 3, *mtp*-complement was loaded in lane 4, and the negative control is enabled in lane 5. Expected band sizes were (a) 300 bp, (b) 731 bp, and (c) 436 bp.

Bacterial colony forming units (CFU) counts and significance values

Broth cultures were revived, grown to an OD_{600nm} of 1, and plated out to confirm that the *M. tuberculosis* cultures at this optical density were equivalent to approximately 1×10^8 CFU/mL. The cultures were 10-fold serially diluted to determine CFU/mL for each strain (Table S2). Dilutions were prepared in Eppendorf tubes (Merck, Darmstadt, Germany) by adding 100 μ L of OD_{600nm} 1 bacterial suspensions to 900 μ L of 7H9 broth. A volume of 100 μ L was plated onto 7H11 agar plates. Plates were sealed in CO₂ permeable plastic bags and incubated at 37°C for 3 weeks and colonies counted thereafter. The CFU count was calculated using the formula: CFU/mL = (average number colonies $\times$ dilution factor) / (volume plated out (mL)). GraphPad Prism version 8.0 software (GraphPad Software, La Jolla, CA, USA) was used to assess the significance levels between combinations of the three strains using non-parametric, unpaired Mann Whitney testing. All the *p*-values were above 0.05 hence the differences between the CFU/mL values were non-significant (Table S3). This confirms that any observations detected in the experimental results were attributed to the deletion of *mtp* and not from any other variation in experimental conditions. Hence, OD_{600nm} of 1 cultures of each strain from the same stocks were used for experimental samples and pellet collection using two levels of standardisation: initial inoculum standardisation, and subsequent OD_{600nm} standardisation using a range of 0.95 – 1.10. From the metabolomics data obtained, excellent separation was observed between the strains, indicating that the resulting variation was due to group structure differences, i.e. metabolome differences, thereby validating the standardisation techniques used in this study.

Table S2. CFUs for each strain done in triplicate.

Strain	Dilution	Colonies			CFU/mL
		Plate 1	Plate 2	Plate 3	
WT	neat	Lawn	Lawn	Lawn	-
	10 ⁻¹	Lawn	Lawn	Lawn	-
	10 ⁻²	Lawn	Lawn	Lawn	-
	10 ⁻³	TMTC	TMTC	TMTC	-
	10 ⁻⁴	>200	>200	>200	-
	10 ⁻⁵	151	118	156	1.41×10 ⁸
<i>Δmtp</i>	neat	Lawn	Lawn	Lawn	-
	10 ⁻¹	Lawn	Lawn	Lawn	-
	10 ⁻²	Lawn	Lawn	Lawn	-
	10 ⁻³	TMTC	TMTC	TMTC	-
	10 ⁻⁴	>200	>200	>200	-
	10 ⁻⁵	85	94	121	1.00×10 ⁸
<i>mtp</i> -complement	neat	Lawn	Lawn	Lawn	-
	10 ⁻¹	Lawn	Lawn	Lawn	-
	10 ⁻²	Lawn	Lawn	Lawn	-
	10 ⁻³	TMTC	TMTC	TMTC	-
	10 ⁻⁴	>200	>200	>200	-
	10 ⁻⁵	82	137	122	1.14×10 ⁸

TMTC: too much to count; WT: wildtype; *Δmtp*: *mtp*-gene knockout mutant; *mtp*-complement: complemented *mtp*-knockout strain.

Table S3. Significance testing between the three *M. tuberculosis* strains.

Strain comparison	<i>p</i> -value	Significance
WT and <i>Δmtp</i>	0.200	None
<i>Δmtp</i> and <i>mtp</i> -complement	0.700	None
WT and <i>mtp</i> -complement	0.400	None

WT: wildtype; *Δmtp*: *mtp*-gene knockout mutant; *mtp*-complement: complemented *mtp*-knockout strain.

Inoculum standardisation and wet pelleted cell mass

Each bacterial strain was revived, passaged and grown to an OD_{600nm} of approximately 1, from which bacterial cultures were used to prepare the standardised bacterial inoculum of OD_{600nm} = 0.015 in a total volume of 40 mL. This inoculum was used for a total of 10 replicates per strain. After the standardised flasks were incubated, final OD_{600nm} readings were taken (Table S4). From these samples 22 mL were used to collect the wet pelleted cell mass for metabolomics extraction (Table S4) and 17 mL was used for ribonucleic acid (RNA) extraction (Table S5).

Table S4. Final OD_{600nm} readings and respective pelleted wet cell mass of each sample.

		Replicates									
		1	2	3	4	5	6	7	8	9	10
WT	OD _{600nm}	1.10	1.10	1.07	1.01	1.07	1.06	1.06	0.98	1.03	1.10
	Weight (mg)	46.9	39.0	35.8	47.7	44.3	43.4	50.2	50.2	45.0	55.6
<i>Δmtp</i>	OD _{600nm}	0.97	1.03	1.10	1.10	1.08	1.08	1.09	1.05	1.07	1.08
	Weight (mg)	43.5	42.5	43.9	41.3	41.6	42.6	40.6	44.6	43.8	43.6
<i>mtp</i>- complement	OD _{600nm}	1.10	1.06	1.09	0.98	1.09	1.06	1.03	0.97	1.08	1.02
	Weight (mg)	55.2	40.4	44.0	38.9	47.1	44.6	56.6	53.1	42.3	38.4

WT: wildtype; *Δmtp*: *mtp*-gene knockout mutant; *mtp*-complement: complemented *mtp*-knockout strain.

Quality assurance for GC×GC-TOFMS analysis

To ensure analytical repeatability a quality control (QC) sample was injected intermittently throughout the GC×GC-TOFMS analysis. Principal component analysis (PCA), an unsupervised parametric projection approach used to summarise variation, depicted in the resulting scores plot (Figure S2). Scores represent samples (QC or grouped) as projected onto principal components (PC) or latent variables (LV), which were constructed as linear combinations of metabolites. The analysis was considered successful as the variability between experimental samples far exceeded the variability between QC sample analyses (Figure S2). Given that samples were replicates and the relatively small group sizes, outlier removal could not be justified.

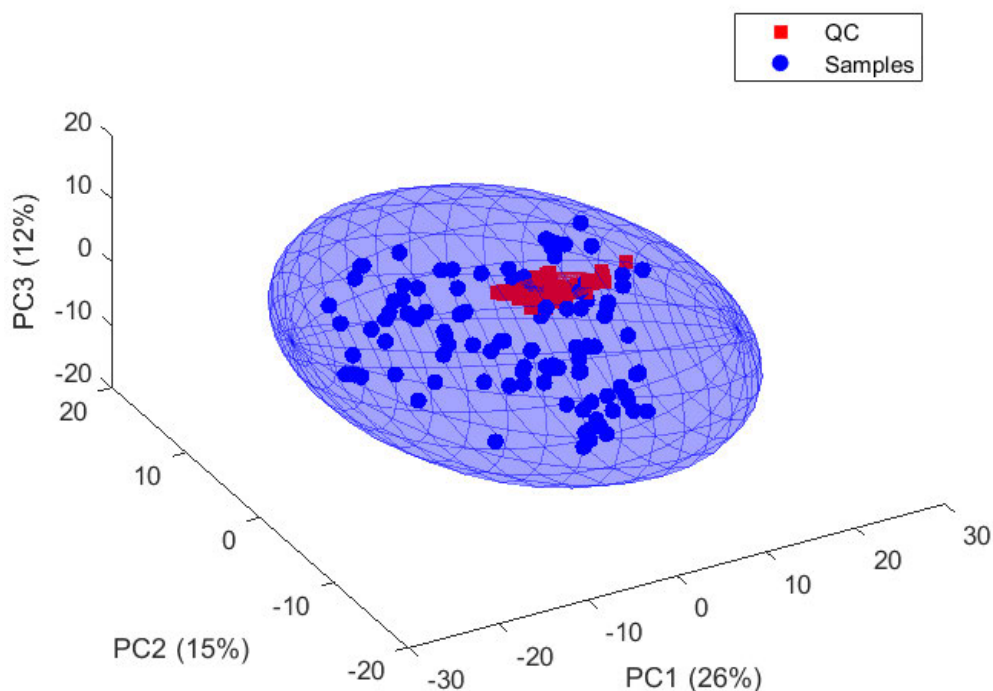


Figure S2. Quality assurance PCA scores plots. Projections of the QC samples (red) relative to the experimental samples (blue) with ellipses represent a 90% CI (confidence intervals) for experimental samples and 50% CI for QC injections.

PCA score plots and partial least squares discriminant analysis (PLS-DA) comparing the strains used in the study

The unsupervised PCA models generated from comparisons of WT to Δmtp (Figure S3a), WT to *mtp*-complemented (Figure S3c) and Δmtp to *mtp*-complemented (Figure S3e) strains illustrate good separation of data. The separation was reiterated by the supervised PLS-DA models generated from comparisons of WT to Δmtp (Figure S3b), WT to *mtp*-complemented (Figure S3d), and Δmtp to *mtp*-complemented (Figure S3f) strains.

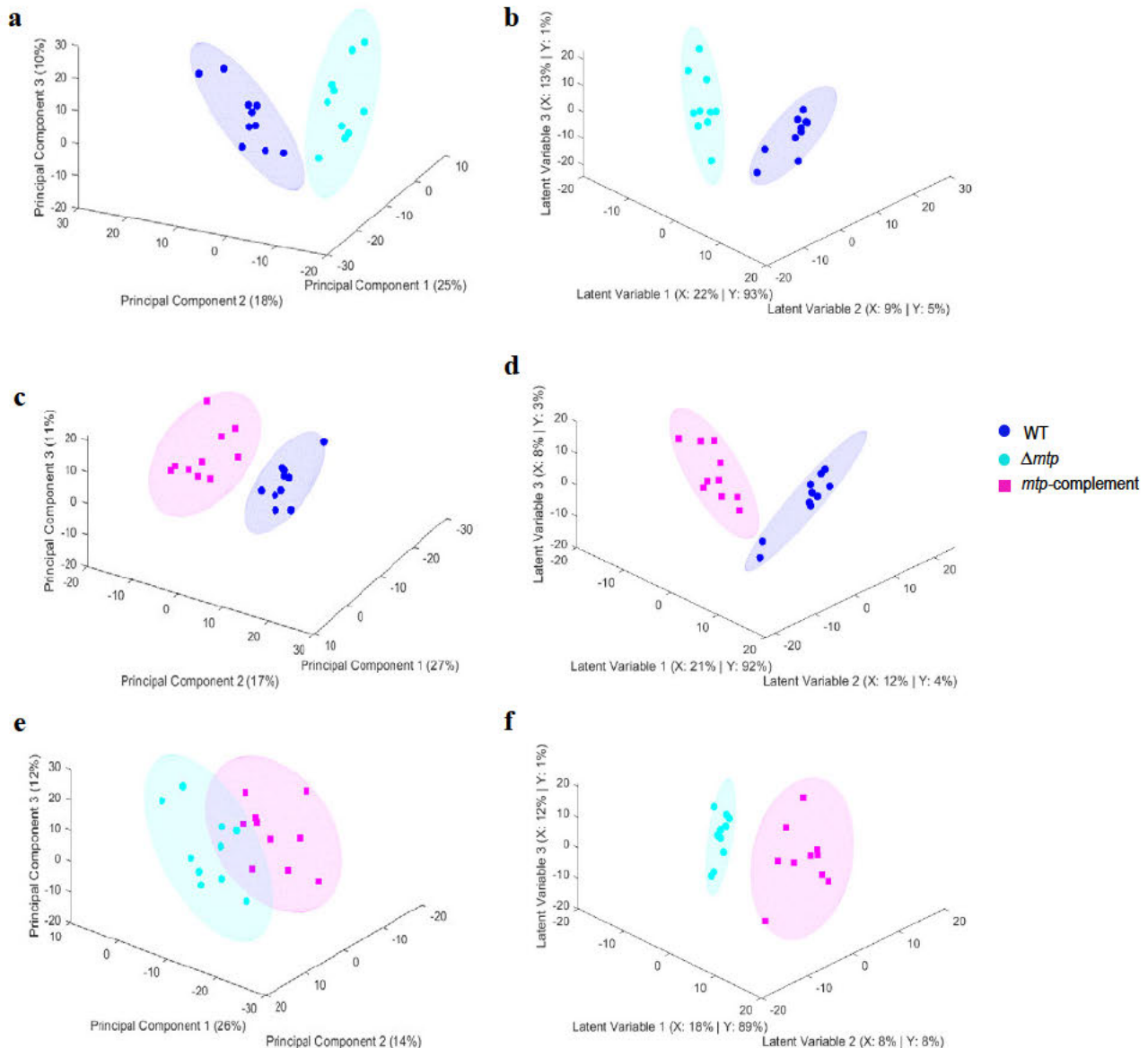


Figure S3. PCA and PLS-DA score plots comparing the bacterial strains. The PCA comparing *M. tuberculosis* strains are illustrated in (a) WT to Δmtp , (c) WT to *mtp*-complement, and (e) Δmtp to *mtp*-complement. The PLS-DA comparing *M. tuberculosis* strains are illustrated in (b) WT to Δmtp , (d) WT to *mtp*-complement, and (f) Δmtp to *mtp*-complement. WT metabolites (royal blue) are separated from those of Δmtp (cyan) and complement (pink) in their respective models. All ellipses represent a 90% CI.

RNA concentrations and integrity

RNA extraction was performed on all three strains from the same broth cultures used for pellet collection (Table S4). The concentration and purity ratios of the RNA are depicted in Table S5. Highlighted in green are the samples that were chosen for subsequent reverse transcription quantitative PCR (RT-qPCR) analysis based on yield and purity. This was confirmed by running all the samples on denaturing 3-(N-morpholino) propanesulfonic acid (MOPS) gels. The best samples that are denoted with green asterisks (Figure S4).

Table S5. Concentrations and purity ratios obtained after RNA extraction.

	Conc. (ng/ μ L)	A _{260/} 280	A _{260/} 230	Conc. (ng/ μ L)	A _{260/} 280	A _{260/} 230	Conc. (ng/ μ L)	A _{260/} 280	A _{260/} 230
Replicate	WT			Δmtp			<i>mtp</i> -complement		
1	288.0	1.94	2.26	208.6	1.88	2.19	34.3	1.86	0.85
2	300.5	1.92	2.13	209.9	1.96	2.06	55.5	1.87	1.19
3	250.0	1.91	2.10	197.0	1.89	2.14	86.5	1.84	1.45
4	484.4	1.89	2.20	318.3	1.92	2.01	88.6	1.88	1.48
5	238.0	1.88	2.16	298.3	2.01	2.12	48.5	1.84	1.27
6	329.7	1.90	2.28	239.1	1.89	2.26	43.5	1.89	1.35
7	635.8	1.99	2.26	287.9	2.02	2.11	539.4	2.02	2.22
8	656.3	1.99	2.19	414.9	1.93	2.01	688.4	1.98	2.24
9	469.3	1.92	2.20	348.7	1.93	2.12	617.7	1.97	2.20
10	267.3	1.92	2.29	561.5	1.96	2.00	688.5	1.99	2.14

Conc.: concentration; A_{260/280} or A_{260/230}: ratio of absorbance; WT: wildtype; Δmtp : *mtp*-gene knockout mutant; *mtp*-complement: complemented *mtp*-knockout strain.

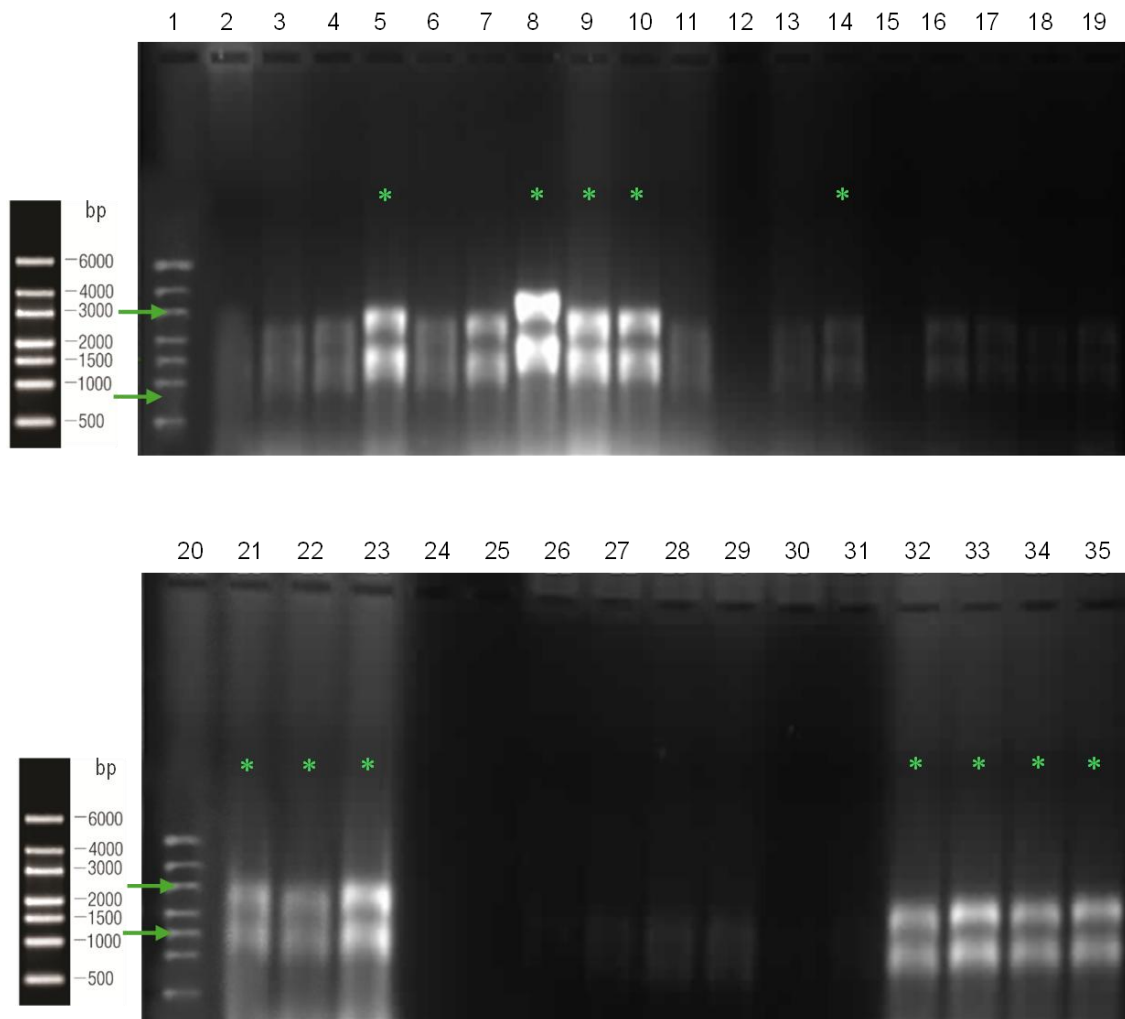


Figure S4. Denaturing MOPS gel of RNA extracted from three strains of *M. tuberculosis* and run at 70 V for 3 hours on a 1.5% agarose gel. The molecular weight marker was RiboRuler high range RNA ladder (lanes 1 and 20). No sample was loaded in lane 12, 24 and 25. Lanes 2 – 11 correspond to the WT samples from Table S5, where samples loaded in lanes 5, 8, 9 and 10 were used for RT-qPCR. Lanes 13 – 19 and 21 – 23 correspond to the Δmtp samples from Table S5, where samples loaded in lanes 14, 21, 22 and 23 were used for RT-qPCR. Lanes 26 – 35 correspond to the *mtp*-complement samples from Table S5, where samples loaded in lanes 32, 33, 34 and 35 were used for RT-qPCR.

Primer efficiencies

Table S6. Primer sequences, efficiency percentages and R² values for each primer set.

Gene	Primer sequence	Primer efficiency	R ²
<i>glf</i>	Forward primer: 5'-cgtttgacctttcgtcgt-3' Reverse primer: 5'-ccgtactgtggacctcgat-3'	101.239	0.970
<i>glmU</i>	Forward primer: 5'-gcaccaggatcacgaagtc-3' Reverse primer: 5'-gtggctggcatgtacggtct-3'	91.569	0.945
<i>fadD32</i>	Forward primer: 5'-ctttccaccgaacgagacg-3' Reverse primer: 5'-agtagagggcgccgaagaag-3'	101.599	0.974
<i>fadE5</i>	Forward primer: 5'-cagaagaagtggcggtcct-3' Reverse primer: 5'-ccagcaccaggtggaagatg-3'	135.435	0.994
<i>glpK</i>	Forward primer: 5'-taccaaccaactgagacga-3' Reverse primer: 5'-cttgccgccagagaaataag-3'	117.581	0.991
<i>16S rRNA</i>	Forward primer: 5'-cctacgggaggcagcagt-3' Reverse primer: 5'-cgtttacggcgtggactac-3'	175.01	0.983

glf: UDP-galactopyranose mutase (*Rv3809c*); *glmU*: bifunctional N-acetyltransferase/ uridylyltransferase (*Rv1018c*); *fadD32*: acyl-CoA synthetase (*Rv3801c*); *fadE5*: acyl-CoA dehydrogenase (*Rv0244c*); *glpK*: glycerol kinase (*Rv3696c*); 16S rRNA: 16S ribosomal ribonucleic acid.

Spearman correlation plots

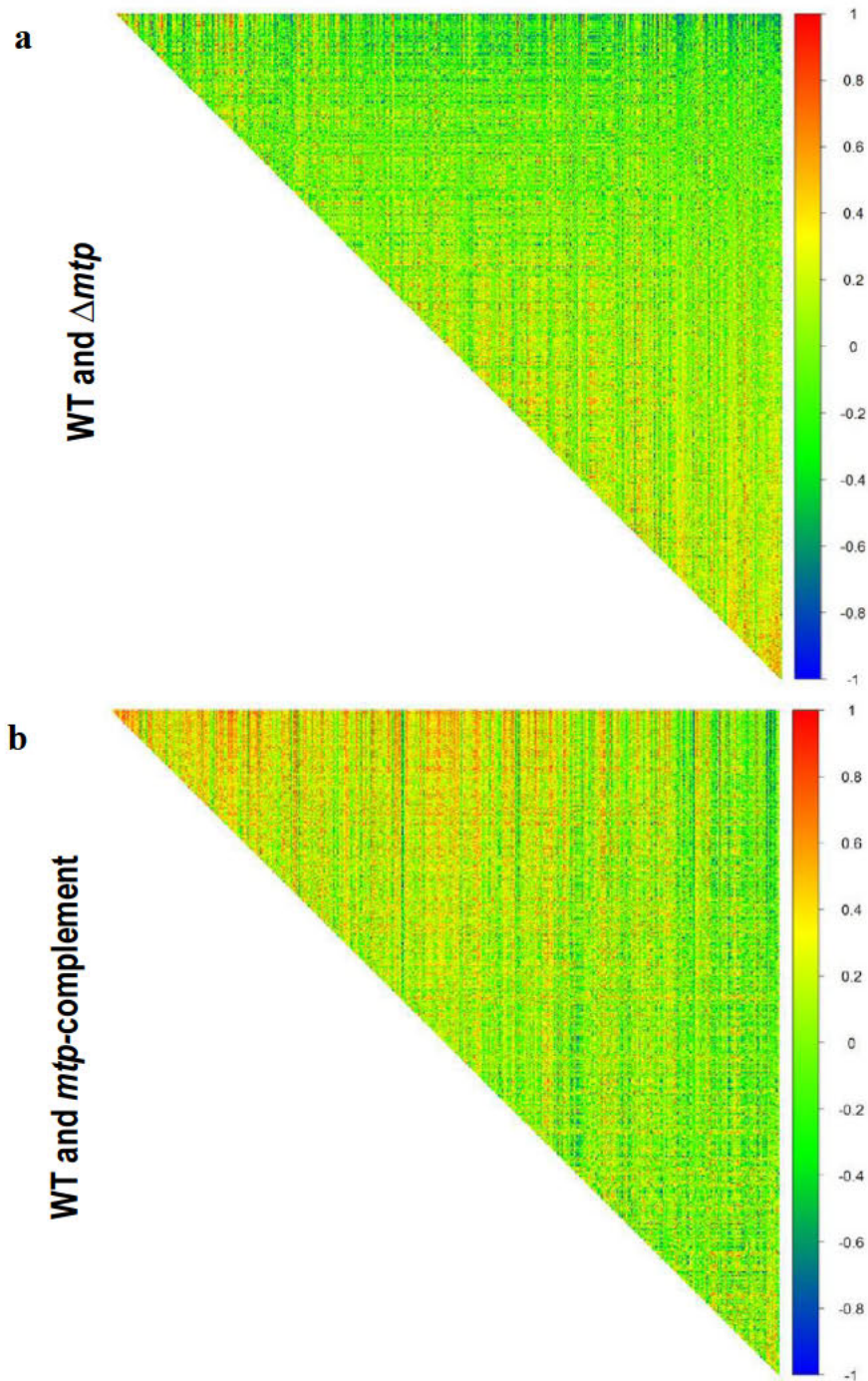


Figure S5. Spearman correlation plots comparing the bacterial strains. (a) WT and Δmtp strains showing a poor degree of correlation with the abundance of green colouration. (b) WT and mtp -complement strains showing a greater degree of correlation as observed by the more evident orange/red colouration. Low correlation values, closer to 0, are associated with the green colouring and high correlation values, closer to 1 or -1, are associated with the red or dark blue colouring, respectively.

Additional metabolites detected through GC×GC-TOFMS

Statistically and biologically significant metabolites that met all selection criteria but were not supported by literature and analysis of metabolic metabolites and pathways using PubChem and/or KEGG, are indicated here. A total of 23 additional metabolites were detected in significantly varied concentrations when comparing the WT and Δmtp (Table S7). The majority of the detected metabolites were found in higher concentrations in the Δmtp relative to the WT; six of the 23 metabolites, were however found in lower concentrations. A total of 42 additional metabolites were determined to be significantly different when comparing the WT and *mtp*-complement, (Table S8). Some of the metabolites seen here (Table S8) were identical to those detected for the Δmtp and WT comparison. Although these metabolites are biologically significant, they were excluded from Table 2.2 due to the fold change not being as different as the fold change seen in the WT and Δmtp comparison. This indicated that the concentrations of these metabolites found in the *mtp*-complement were closer to that of the WT thereby showing partial functional restoration. Hence these metabolites were excluded from Table 2.2 as they are not exclusive to the *mtp*-complement metabolic profile. Table S8 shows 22 metabolites were found in higher concentrations in the *mtp*-complement strain and 20 metabolites were found in lower concentrations relative to the WT. A total of 17 additional metabolites were detected in significantly varied concentrations when comparing the Δmtp and *mtp*-complement (Table S9), all of which were detected in lower concentrations in the *mtp*-complement relative to the Δmtp .

Table S7. Significant metabolites with unknown function between *M. tuberculosis* WT and Δmtp strains selected as per multivariate and univariate selection criteria together with their respective mean relative concentration and standard deviation, fold change with respect to the WT, PLS-DA VIP value, Cohen's *d*-value as effect size and *t*-test *p*-values.

Metabolites	Mean relative concentration and standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	<i>p</i> -value
	WT	Δmtp				
3-Nonanol	4.08E-04 (1.42E-04)	1.13E-03 (2.89E-04)	2.765	5.326	2.489	<0.001
3,5-Di-tert-butyl-4-trimethylsiloxy toluene	5.33E-04 (1.98E-04)	1.14E-03 (3.10E-04)	2.136	5.263	1.954	<0.001
2,4,6-Cycloheptatrien-1-one	4.75E-04 (1.74E-04)	1.37E-03 (4.17E-04)	2.888	5.138	2.149	<0.001
1-Trifluorosilyltridecane	3.66E-04 (2.45E-04)	1.46E-03 (5.17E-04)	3.987	5.009	2.117	<0.001
N-Trimethylsilylcyclohexylamine	1.73E-04 (1.50E-04)	5.39E-04 (1.80E-04)	3.117	4.423	2.028	<0.001
3,6,9,12-Tetraoxa-2,13-disilatetradecane	2.41E-03 (1.04E-03)	1.12E-03 (3.94E-04)	-2.148	2.681	1.245	0.003
Silane	1.91E-04 (1.73E-04)	5.24E-04 (2.67E-04)	2.746	2.084	1.247	0.005
Tetracosan-1-ol trimethylsilyl ether	1.42E-04 (6.31E-05)	2.86E-04 (1.36E-04)	2.010	1.775	1.059	0.010
Ethylbis(trimethylsilyl)amine	1.04E-04 (4.56E-05)	4.36E-05 (2.27E-05)	-2.377	1.415	1.317	0.003
Octadec-9Z-enol trimethylsilyl ether	5.97E-05 (1.36E-05)	4.06E-04 (1.87E-04)	6.800	1.073	1.849	<0.001
Camphoric acid	2.34E-04 (8.14E-05)	5.83E-05 (2.16E-05)	-4.013	7.550	2.160	<0.001
5-Nonanol	6.15E-05 (3.07E-05)	2.12E-04 (6.04E-05)	3.442	6.199	2.488	<0.001
2-O-Glycerol- α -D-galactopyranoside	1.58E-04 (5.70E-05)	5.07E-04 (6.32E-05)	-3.214	5.786	5.528	<0.001
α -D-(+)-Talopyranose	1.94E-04 (9.21E-05)	8.22E-04 (2.64E-04)	4.238	1.924	2.384	<0.001
E-7-Tetradecenol	6.64E-05 (1.60E-05)	3.63E-04 (2.08E-04)	5.456	1.553	1.425	0.002
Glycerol glycoside	9.45E-04 (3.01E-04)	2.20E-03 (2.64E-04)	2.328	5.806	4.169	<0.001
Propanoic acid	3.17E-03 (9.80E-04)	1.25E-03 (2.95E-04)	-2.532	5.591	1.956	0.001
Benzoic acid	8.43E-05 (3.85E-05)	2.41E-04 (2.97E-05)	2.856	5.225	1.689	<0.001
2-Deoxy-2-ribose	4.81E-05 (2.64E-05)	9.95E-05 (1.77E-05)	2.069	4.180	1.946	0.001
2-Monopalmitoylglycerol	1.62E-04 (4.18E-05)	3.32E-04 (1.06E-04)	2.044	1.789	1.593	0.005
Pyrrole-2-carboxylic acid	1.61E-04 (5.59E-05)	6.64E-05 (3.19E-05)	-2.430	1.688	1.699	0.003
Salicylic acid	1.08E-04 (1.11E-04)	4.20E-04 (7.85E-05)	3.883	1.612	2.812	<0.001
α -D-Glucopyranoside	8.14E-04 (3.36E-04)	1.72E-03 (3.06E-04)	2.110	1.531	2.688	<0.001

WT: wildtype; Δmtp : *mtp*-gene knockout mutant; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; positive fold change = higher concentration in Δmtp ; negative fold change = higher concentration in WT.

Table S8. Significant metabolites with unknown function between *M. tuberculosis* WT and *mtp*-complement strains selected as per multivariate and univariate selection criteria together with their respective mean relative concentration and standard deviation, fold change with respect to the WT, PLS-DA VIP value, Cohen's *d*-value as effect size and *t*-test *p*-values.

Metabolites	Mean relative concentration and standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	<i>p</i> -value
	WT	<i>mtp</i> -complement				
2-O-Glycerol- α -d-galactopyranoside	1.58E-04 (5.70E-05)	3.47E-04 (1.17E-04)	2.197	1.802	1.619	0.005
3-Hydroxyisovaleric acid	4.43E-04 (2.48E-04)	1.66E-04 (1.01E-04)	-2.664	3.082	1.116	0.034
cis-13-Docosenoic acid	7.37E-05 (4.00E-05)	4.11E-04 (2.17E-04)	5.583	4.773	1.556	0.007
D-Fructose	1.95E-03 (6.86E-04)	4.79E-03 (1.33E-03)	2.459	3.651	2.134	0.001
D-Galactose	1.84E-03 (8.58E-04)	5.09E-03 (2.25E-03)	2.771	1.663	1.444	0.009
D-Glucose	2.20E-03 (1.15E-03)	6.47E-03 (3.28E-03)	2.947	1.150	1.306	0.015
D-Manose	3.87E-03 (2.13E-03)	7.75E-03 (3.18E-03)	2.006	1.030	1.223	0.028
Docosanoic acid	3.37E-04 (1.16E-04)	8.61E-04 (2.03E-04)	2.553	3.224	2.584	<0.001
α -D-Glucopyranoside	8.14E-04 (3.36E-04)	1.93E-03 (7.57E-04)	2.372	2.411	1.478	0.008
2-Monopalmitoylglycerol	1.62E-04 (4.18E-05)	3.32E-04 (1.12E-04)	2.044	1.875	1.513	0.007
3,6,10,13-Tetraoxa-2,14-disilapentadecane	1.89E-04 (5.76E-05)	7.14E-05 (2.61E-05)	-2.648	3.980	2.044	0.002
1,8-cis-Undecadien-5-yne 3,7-bis-trimethylsilyl ether	1.45E-03 (5.35E-04)	7.10E-04 (1.94E-04)	-2.043	6.366	1.386	0.011
9-Tetradecenoic acid	1.89E-04 (2.61E-05)	5.76E-05 (2.61E-05)	-2.648	3.980	2.044	0.002
α -D-(+)-Talopyranose	5.58E-04 (3.35E-04)	0.002 (3.52E-04)	2.713	1.671	2.272	<0.001
Allonic acid	2.78E-04 (1.75E-04)	8.07E-04 (1.74E-04)	2.903	4.777	3.021	<0.001
Butanoic acid	2.61E-02 (8.90E-03)	1.02E-01 (4.35E-02)	3.886	2.640	1.778	0.004
Camphoric acid	2.34E-04 (8.48E-05)	5.33E-05 (1.86E-05)	-4.395	8.274	0.222	0.003
Erythrofuranose	2.93E-03 (1.01E-03)	1.06E-03 (1.80E-04)	-2.766	6.351	1.848	0.003
Lyxofuranose	1.87E-01 (7.41E-02)	5.54E-02 (5.22E-03)	-3.378	6.172	1.911	0.003
Tagatose	1.16E-03 (3.71E-04)	3.10E-03 (6.84E-04)	2.688	4.547	2.851	<0.001
D-Erythro-2-Pentulose	7.60E-05 (3.79E-05)	2.27E-05 (1.33E-05)	-3.350	3.207	1.409	0.010

WT: wildtype; *mtp*-complement: complemented *mtp*-knockout strain; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; positive fold change = higher concentration in *mtp*-complement; Negative fold change = higher concentration in WT.

Table S8 continued. Significant metabolites with unknown function between *M. tuberculosis* WT and *mtp*-complement strains selected as per multivariate and univariate selection criteria together with their respective mean relative concentration and standard deviation, fold change with respect to the WT, PLS-DA VIP value, Cohen's *d*-value as effect size and *t*-test *p*-values.

Metabolites	Mean relative concentration and standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	<i>p</i> -value
	WT	<i>mtp</i> -complement				
D-Erythrotetrofuranose	7.73E-05 (3.43E-05)	1.90E-05 (9.01E-05)	-4.074	8.120	1.701	0.005
7-Tetradecenol	6.64E-05 (1.60E-05)	3.94E-04 (1.55E-04)	5.933	1.967	2.118	0.002
Ethylbis(trimethylsilyl)amine	1.04E-04 (4.54E-05)	2.89E-05 (8.47E-06)	-3.590	2.246	1.369	0.006
Glycoside	2.55E-03 (3.63E-03)	7.43E-03 (4.00E-03)	2.909	1.567	1.223	0.048
Hexacosanoic acid	1.63E-03 (4.69E-04)	4.59E-03 (1.83E-03)	2.807	4.298	1.613	0.006
L-Aspartic acid	1.39E-02 (4.55E-03)	6.67E-03 (4.37E-03)	-2.089	3.363	1.604	0.012
Levoglucosan	2.12E-03 (6.84E-04)	8.63E-04 (1.68E-04)	-2.460	4.462	1.844	0.003
Meso-Erythritol	1.63E-04 (6.98E-05)	4.44E-04 (1.67E-04)	2.716	2.592	1.675	0.005
Methylcitric acid	9.54E-04 (3.78E-04)	4.29E-04 (4.59E-04)	-2.225	3.242	1.390	0.012
Myo-Inositol	6.61E-05 (4.32E-05)	1.35E-04 (2.84E-05)	2.045	2.406	1.601	0.006
N-Acetyl-D-glucosamine	1.38E-04 (4.23E-05)	3.74E-04 (1.90E-04)	2.706	1.541	1.239	0.020
Octadec-9Z-enol trimethylsilyl ether	5.97E-05 (1.36E-05)	6.31E-04 (3.42E-04)	10.558	3.025	1.668	0.006
Phosphorimidic acid	3.22E-04 (1.29E-04)	1.59E-04 (3.35E-05)	-2.027	3.875	1.267	0.017
Propanetriol	8.56E-04 (2.70E-04)	4.07E-04 (1.20E-04)	-2.076	2.250	1.625	0.005
Propanoic acid	5.18E-03 (1.39E-03)	2.08E-03 (1.38E-03)	-2.490	6.450	2.228	0.002
Pyrimidine	5.14E-04 (2.35E-04)	1.98E-04 (1.35E-04)	-2.593	1.017	1.348	0.015
Silane	1.53E-04 (9.88E-05)	1.12E-03 (7.42E-04)	7.367	3.422	1.311	0.015
Tagatofuranose	8.91E-04 (6.02E-04)	1.25E-04 (2.24E-04)	-7.103	4.928	1.272	0.017
Tetracosanoic acid	5.12E-04 (1.23E-04)	1.46E-03 (5.17E-04)	2.850	5.021	1.832	0.003
Trimethylsilyl 2,2-dimethyl-3,6,9,12-tetraoxa-2-silatetradecan-14-oate	1.83E-04 (6.01E-05)	6.83E-05 (3.70E-05)	-2.684	3.990	1.916	0.002
Tryptophan	4.01E-03 (1.42E-03)	1.59E-03 (1.28E-03)	-2.520	3.744	1.703	0.007

WT: wildtype; *mtp*-complement: complemented *mtp*-knockout strain; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; positive fold change = higher concentration in *mtp*-complement; Negative fold change = higher concentration in WT.

Table S9. Significant metabolites with unknown function between *M. tuberculosis* Δmtp and *mtp*-complement strains selected as per multivariate and univariate selection criteria together with their respective mean relative concentration and standard deviation, fold change with respect to the Δmtp , PLS-DA VIP value, Cohen's *d*-value as effect size and *t*-test *p*-values.

Metabolites	Mean relative concentration and standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	<i>p</i> -value
	Δmtp	<i>mtp</i> -complement				
1,2-Bis(trimethylsiloxy)ethane	8.60E-05 (3.11E-05)	3.80E-05 (2.92E-05)	-2.261	1.740	1.540	0.018
16-Trimethylsilyloxy-9-octadecenoic acid	1.88E-05 (6.81E-04)	8.11E-04 (2.54E-04)	-2.315	2.202	1.565	0.007
1-Trifluorosilyltridecane	1.459E-03 (5.17E-04)	4.495E-04 (3.48E-04)	-3.247	2.461	1.955	0.002
2,4,6-Cycloheptatrien-1-one	1.45E-03 (4.73E-04)	5.09E-04 (1.87E-04)	-2.852	2.634	1.990	0.002
3,6,10,13-Tetraoxa-2,14-disilapentadecane	1.45E-03 (4.73E-04)	5.09E-04 (1.87E-04)	-2.827	2.316	1.997	0.002
3-Nonanol	1.14E-03 (2.98E-04)	4.95E-04 (1.41E-04)	-2.293	2.400	2.151	0.001
5-(Methylthio)-Salicylic acid	4.20E-04 (7.85E-05)	4.72E-05 (2.47E-05)	-8.901	1.762	4.752	<0.001
5-Nonanol	2.33E-04 (8.98E-05)	7.17E-05 (4.03E-05)	-3.253	2.661	1.799	0.003
6-Dimethyl(trimethylsilyl)silyloxytetradecane	1.51E-04 (5.22E-05)	6.01E-05 (3.27E-05)	-2.522	1.429	1.752	0.005
Alanine, phenyl-, trimethylsilyl ester	1.85E-03 (7.95E-04)	6.79E-04 (6.48E-04)	-2.672	1.319	1.430	0.019
Erythrofuranose	2.19E-03 (2.86E-04)	1.06E-03 (1.80E-04)	-2.074	1.807	3.978	<0.001
Lyxofuranose	1.37E-01 (1.66E-02)	5.54E-02 (5.22E-03)	-2.471	1.856	5.083	<0.001
D-Allofuranose	2.15E-04 (9.06E-05)	4.21E-05 (1.21E-05)	-5.108	1.724	1.910	0.003
D-Erythrotetrofuranose	1.54E-03 (3.23E-04)	7.10E-04 (2.40E-04)	-2.303	1.541	2.377	<0.001
N,N-Bis(2-chloroethyl)-N-trimethylsilylamine	5.67E-05 (2.77E-05)	1.98E-05 (1.11E-05)	-2.858	1.089	1.316	0.018
N-Trimethylsilylcyclohexylamine	5.38E-04 (1.81E-04)	1.34E-04 (1.36E-04)	-4.641	2.735	2.240	0.001
Silane	2.70E-04 (1.52E-04)	6.96E-05 (3.39E-05)	-3.871	2.574	1.320	0.018

Δmtp : *mtp*-gene knockout mutant; *mtp*-complement: complemented *mtp*-knockout strain; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; positive fold change = higher concentration in *mtp*-complement; negative fold change = higher concentration in Δmtp .

Appendix C: Supplementary information for Chapter 3

Pilot study determining incubation time (four hours) used for infection

Aim and purpose of pilot: The primary aim of this pilot was to determine the optimal incubation period for infection given that the study focussed on the metabolic alterations caused in the absence of the MTP adhesin which plays a role in the initial host-pathogen interaction. Generally, infection time of one to four hours is used. It takes approximately one hour post infection for adhesion and two hours post infection for invasion to occur. After these processes, host cells should have ample time for its metabolome to reflect the infection. Conversely, a long waiting period may not accurately represent the metabolome alterations at the time of infection as metabolites constantly adjust with changes in the environment as time elapses. Hence, two and four hours were selected to be investigated.

Methods: Preparation of bacterial strain and cell culture was performed as detailed in the main text with the following specifications: THP-1 macrophages were seeded at 1×10^6 cells/mL in 30 mL complete cell culture media. A total of 16 flasks were seeded; eight flasks was used per infection time point. Of the eight flasks, three were infected and used to determine CFU/mL, three flasks were infected and used for metabolite extraction and two flasks were used as uninfected control for metabolite extraction. For both selected time points, 1.5 mL of WT *M. tuberculosis* (V9124) bacterial inoculum approximately OD_{600nm} of 1 was used to achieve a MOI of approximately 5 for infected flasks. Additionally, 30 mL complete media was added to the uninfected control flasks. All flasks were placed into an incubator at 37°C in 5% CO₂ (Shel Lab CO₂ Incubator, Cornelius, OR, USA) at the same time. Following two hours of infection, the eight flasks that were allocated to the two-hour time point were removed and pellets were processed for metabolite extraction and cell lysates were plated out as indicated in the main text. The remaining eight flasks were incubated for an additional two hours to make the total infection duration 4 hours. Thereafter, these flasks were removed, and pellets were processed for metabolite extraction and cell lysates were plated out as indicated in the main text. The metabolites were analysed via untargeted GC×GC-TOFMS and statistical analysis was performed as indicated in main text.

Results and Conclusions: Metabolic analysis revealed a total of 961 and 980 metabolites detected at two hours and four hours post infection, respectively. The four-hour time point displayed a higher number of significantly altered mean relative concentrations between the uninfected controls and infected samples. Hence, a larger number of metabolites were detected at this time point. There was no statistically significant difference between the CFU/mL between the two incubation periods; therefore, any metabolic alterations observed were attributed to the duration of infection as all other conditions were kept constant. The CFU/mL for both the incubation times was approximately $x \times 10^5$ CFU/mL (Table S1), thus infection was reproducible for both time points. However, because a less prominent grouping of metabolites and separation of experimental treatments was observed between the metabolic profiles of the uninfected control and infected samples at two hours (Fig. S1a) compared to four hours (Figure S1b), the latter incubation period was considered superior for the purpose of this study.

Table S1. Colonies counted for each time point in triplicate and calculated CFUs for pilot infection

Time point (hours post infection)	Flask	Dilution	Number of Colonies			CFU/mL
			Replicate 1	Replicate 2	Replicate 3	
2	1	10 ⁻²	>200	>200	>200	-
		10 ⁻³	38	34	33	3.5×10 ⁵
		10 ⁻⁴	7	3	5	-
	2	10 ⁻²	>200	>200	>200	-
		10 ⁻³	37	50	40	4.2×10 ⁵
		10 ⁻⁴	9	8	3	-
	3	10 ⁻²	>200	>200	>200	-
		10 ⁻³	44	46	73	5.4×10 ⁵
		10 ⁻⁴	5	7	3	-
4	1	10 ⁻²	>200	>200	>200	-
		10 ⁻³	40	33	37	3.7×10 ⁵
		10 ⁻⁴	2	5	7	-
	2	10 ⁻²	>200	>200	>200	-
		10 ⁻³	75	46	67	6.3×10 ⁵
		10 ⁻⁴	7	6	7	-
	3	10 ⁻²	>200	>200	>200	-
		10 ⁻³	53	52	41	4.9×10 ⁵
		10 ⁻⁴	5	9	6	-

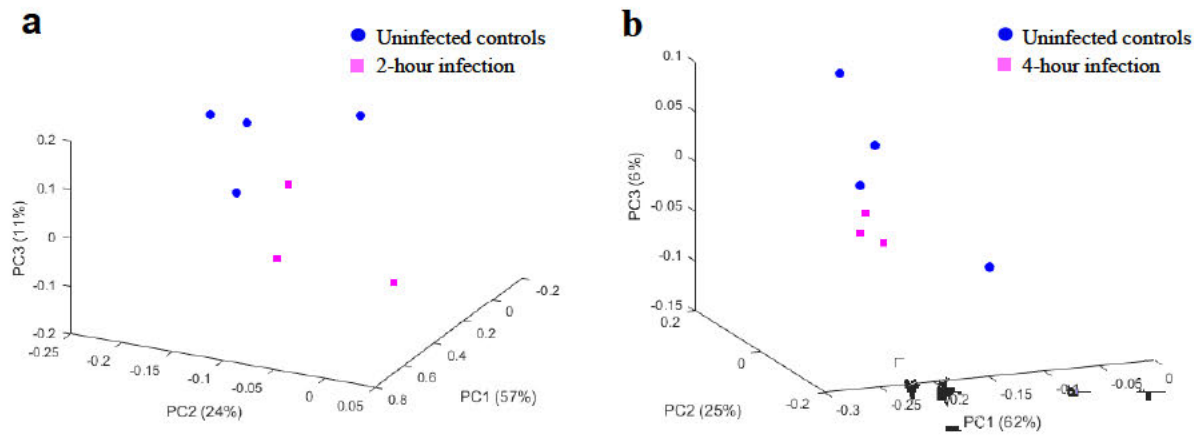


Figure S1. 3D PCA comparing time points for THP-1 infected with *M. tuberculosis*. For both two-group comparisons namely, (a) uninfected versus infected after a two-hour incubation and (b) uninfected versus infected after a four hour incubation, good separation is observed between the naturally grouping samples groups. There was a more distinct separation in b, indicating a greater difference in metabolome profiles between uninfected hosts and infected hosts incubated for four hours of infection. Note, confidence intervals could not be generated as the groups were too small. Uninfected controls are depicted in royal blue and infected samples are depicted in pink for the respective infection duration time point. The percentage of the overall variation in x is explained by each component indicated in brackets along each axis.

CFUs and MOI confirmation

GraphPad Prism version 8 software (GraphPad Software, La Jolla, CA, USA) was used to assess significance levels of CFUs and MOI differences between combinations of the three strains used during infection. Non-parametric, unpaired Mann Whitney test with ($\alpha = 5\%$) was used to establish significance. For individual WT infection, Δmtp infection and *mtp*-complement infection, CFU/mL were reported as reproducible (not significantly different $p > 0.999$) across all 10 replicates for each of the three strains (CFUs in Figure S2). There were statistically significant differences ($p < 0.0001$) for all CFU comparisons (WT and Δmtp , Δmtp and *mtp*-complement, and WT and *mtp*-complement). Given that the adhesin was knocked out of the Δmtp , a lower number of invading bacteria (CFUs in Figure S2) is expected for the mutant (average of 8.8×10^5 CFU/mL) compared to the WT (average of 1.3×10^6 CFU/mL), a comparison which was the main focus of this study. Unexpectedly, the number of invading bacteria in *mtp*-complemented strain (average of 1.8×10^5 CFU/mL) was lower than the WT (1.3×10^6 CFU/mL). This is explained by the difference in MOI used for the *mtp*-complement (5.1) compared to the WT (6) (Figure S2). This study aimed for an approximate MOI of 5, therefore a range of 0.9 between the two MOIs was less than ideal and is postulated to have caused the lower CFU/mL observed in the *mtp*-complement strain (Table S2). However, statistical analysis was additionally performed on the MOIs to reveal that there were no significant differences between the MOIs (WT and Δmtp with $p = 0.4000$; Δmtp and complement with $p = 0.1000$; and WT and complement with $p > 0.9999$), confirming the differences observed in the infection study are characteristic of alterations to the *mtp* gene.

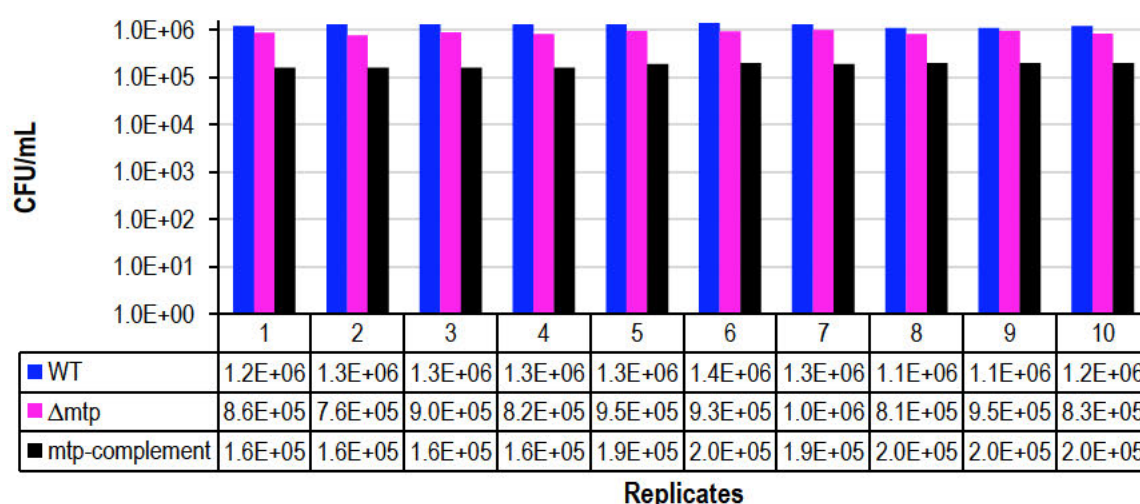


Figure S2. Bar graph depicting infection reproducibility per *M. tuberculosis* infection model through the number of invading bacteria. Ten replicates were used to determine the mean CFUs/mL for each of the three strains of *M. tuberculosis* used for infection of THP-1 macrophages. The means were 1.3×10^6 CFU/mL (standard deviation of 9.2×10^4), 8.8×10^5 CFU/mL (standard deviation of 7.2×10^4), 1.8×10^5 CFU/mL (standard deviation of 1.8×10^4) for the WT, Δmtp and *mtp*-complement, respectively. There were statistically significant differences for the number of invading bacteria between the WT and Δmtp , Δmtp and *mtp*-complement, and WT and *mtp*-complement ($p < 0.0001$ for all). Contrastingly, there was no significant difference ($p > 0.999$) in CFUs/mL between the 10 replicates within each of the infection groups (WT infected, Δmtp infected and *mtp*-complement infected), thus each of the infection groups were reproducible.

Table S2. Inoculum OD_{600nm} readings and respective volumes used for infections with their associated MOI.

Strain	Inoculum OD _{600nm}	Inoculum volume (mL)	Dilution	Number of Colonies			CFU /mL	MOI
				Rep 1	Rep 2	Rep 3		
WT	1.03	1	10 ⁻⁴	>200	>200	>200	-	-
			10 ⁻⁵	90	118	114	1.1×10 ⁸	6
			10 ⁻⁶	18	9	7	-	-
Δmtp	1.03	1.1	10 ⁻⁴	>200	>200	>200	-	-
			10 ⁻⁵	96	87	95	9.3×10 ⁷	5.7
			10 ⁻⁶	9	9	10	-	-
<i>mtp</i> -complement	1.04	0.8	10 ⁻⁴	>200	>200	>200	-	-
			10 ⁻⁵	118	113	112	1.1×10 ⁸	5.1
			10 ⁻⁶	10	11	16	-	-

Rep: replicate; WT: wildtype; Δmtp : *mtp*-gene deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain; CFU/mL: colony forming unit per millilitre = (average number of colonies × dilution factor)/ (volume plated out in mL); MOI; multiplicity of infection = (number of bacterial cells)/ (number of host cells) = (average CFU/mL × volume of inoculum)/(number of cells seeded × total volume).

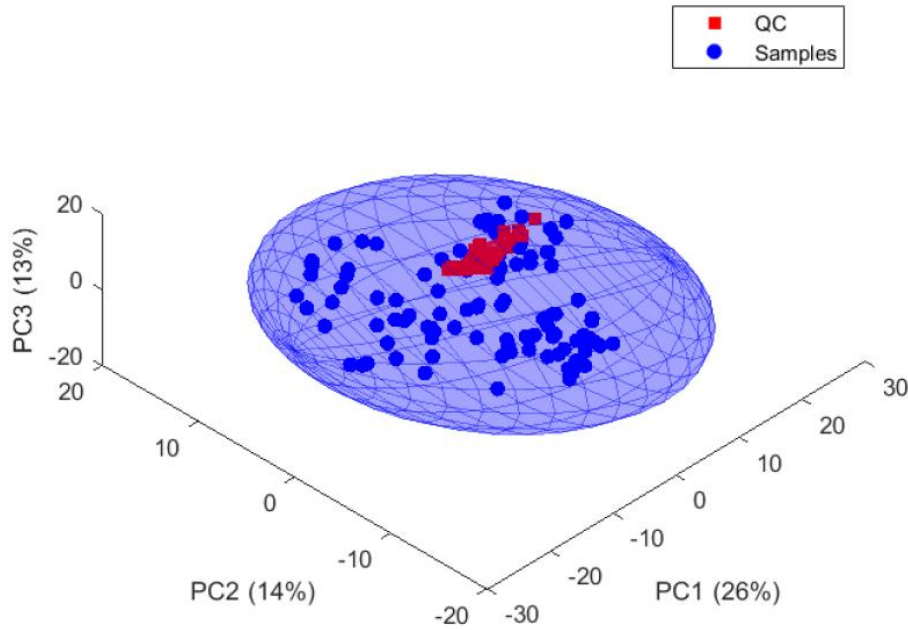


Figure S3. Quality assurance PCA scores plot. Projection of the QC samples (red) which were intermittently injected throughout the GC×GC-TOFMS analysis relative to the experimental samples (blue). The ellipsoid represents a 90% CI (confidence intervals) for experimental samples and 50% CI for QC injections. The quality assurance was successful as the variability between experimental samples far exceeded the variability between QC samples.

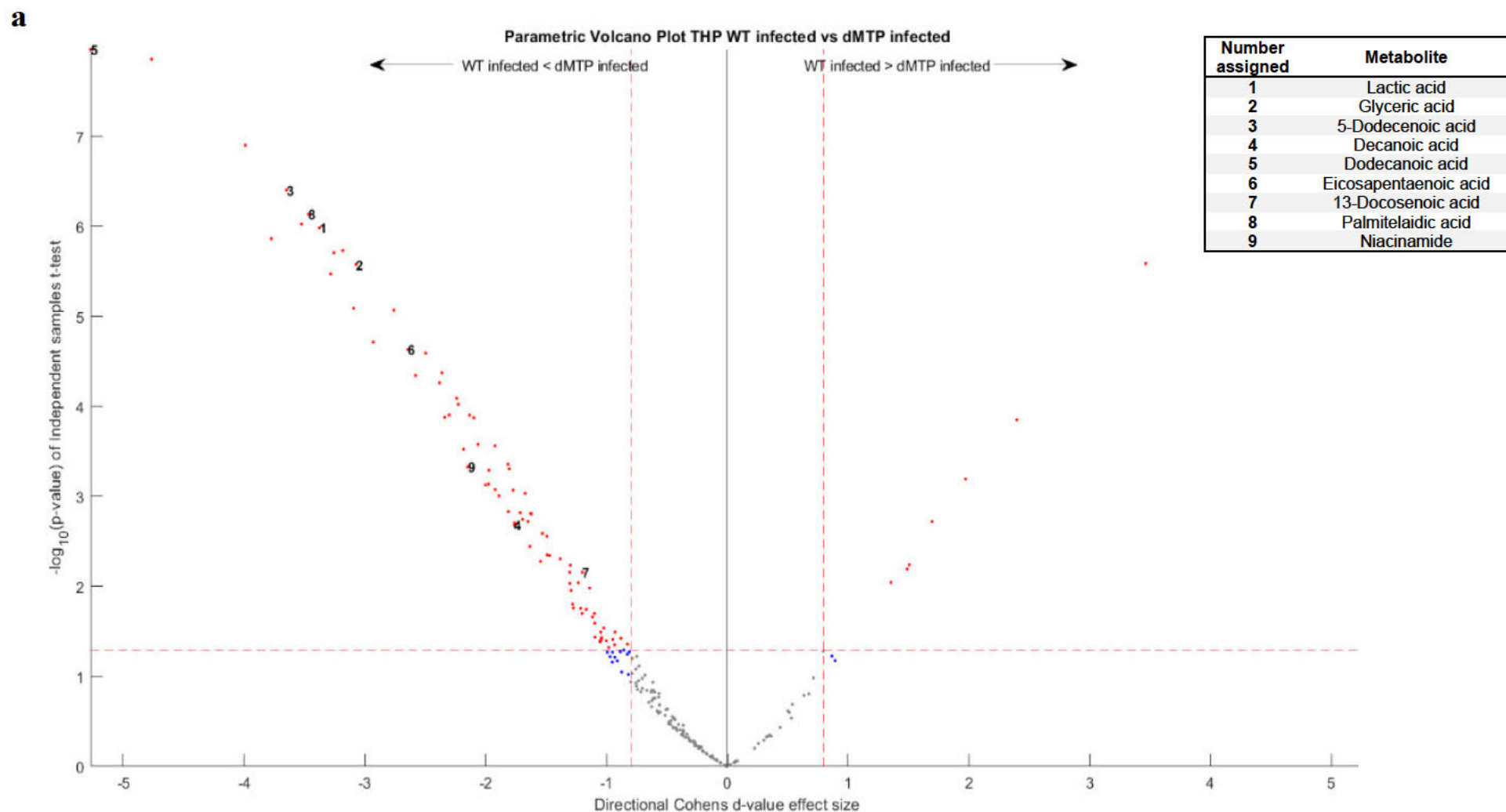


Figure S4. Volcano plots illustrating metabolites of practical significance, statistical and practical significance or neither. (a) Volcano plot comparing WT infected versus Δmtp infected. The blue dots represent metabolites with practical significance and fall beyond the vertical thresholds (representing Cohen's d -values of effect size = 0.8). The red dots represent metabolites which are statistically as well as practically significantly different between groups and are separated by the horizontal threshold (representing 5% significance level). The black dots represent metabolites with neither practical nor statistical significance.

b

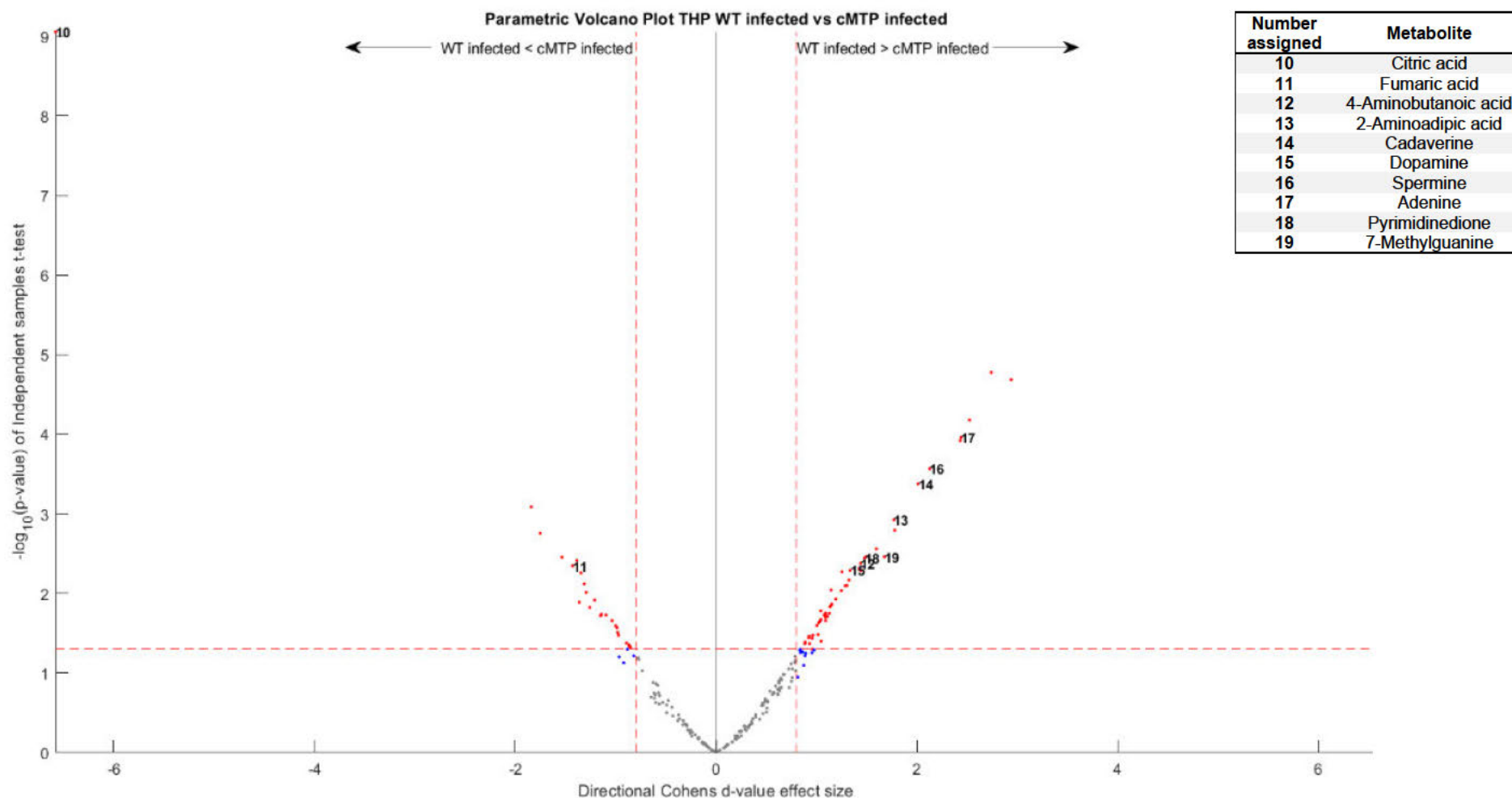


Figure S4 continued. Volcano plots illustrating metabolites of practical significance, statistical and practical significance or neither. (b) Volcano plot comparing WT infected versus *mtp*-complement infected. The blue dots represent metabolites with practical significance and fall beyond the vertical thresholds (representing Cohen's *d*-values of effect size = 0.8). The red dots represent metabolites which are statistically as well as practically significantly different between groups and are separated by the horizontal threshold (representing 5% significance level). The black dots represent metabolites with neither practical nor statistical significance.

c

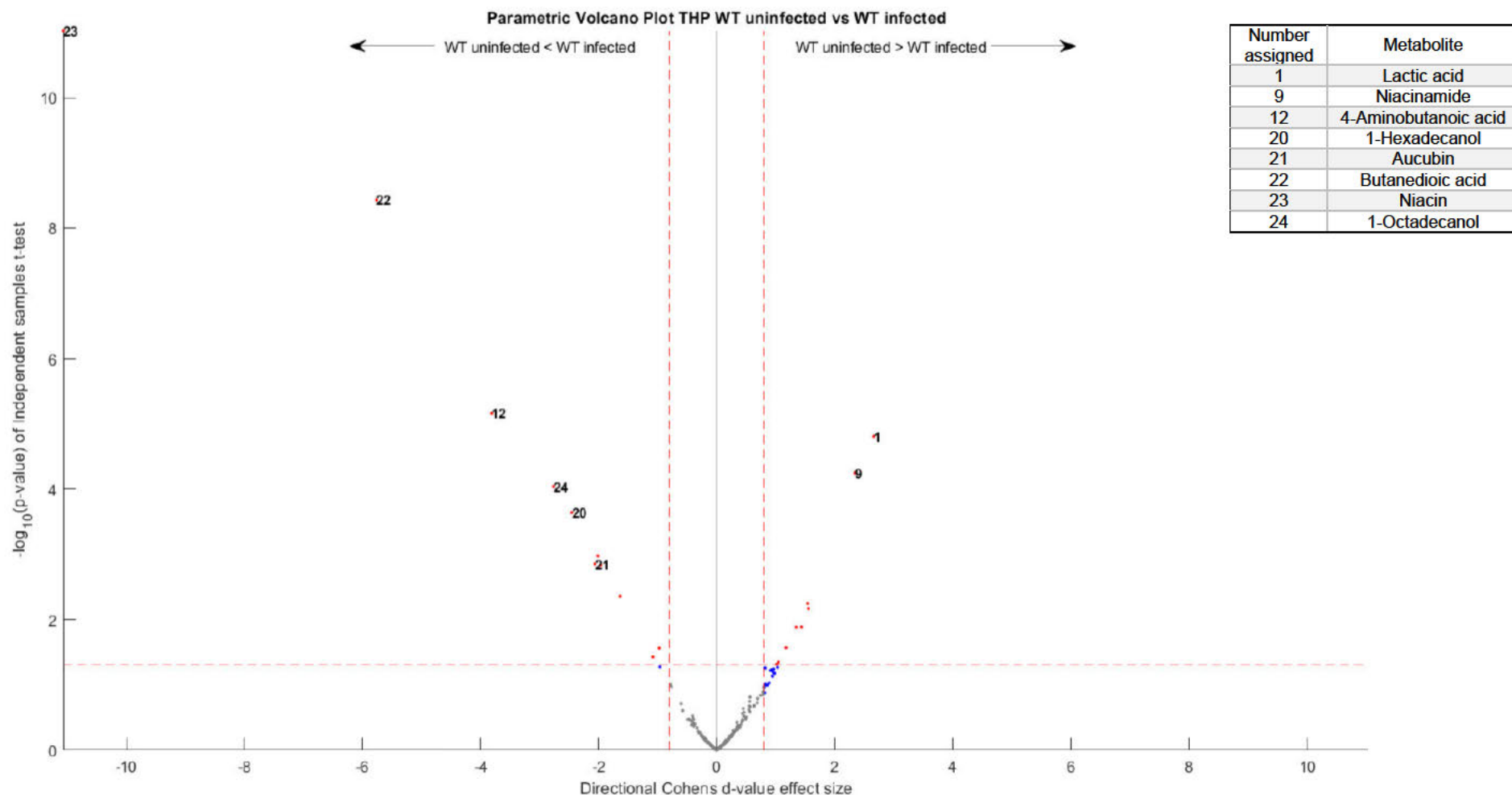


Figure S4 continued. Volcano plots illustrating metabolites of practical significance, statistical and practical significance or neither. (c) Volcano plot comparing the WT uninfected versus WT infected. The blue dots represent metabolites with practical significance and fall beyond the vertical thresholds (representing Cohen's *d*-values of effect size = 0.8). The red dots represent metabolites which are statistically as well as practically significantly different between groups and are separated by the horizontal threshold (representing 5% significance level). The black dots represent metabolites with neither practical nor statistical significance.

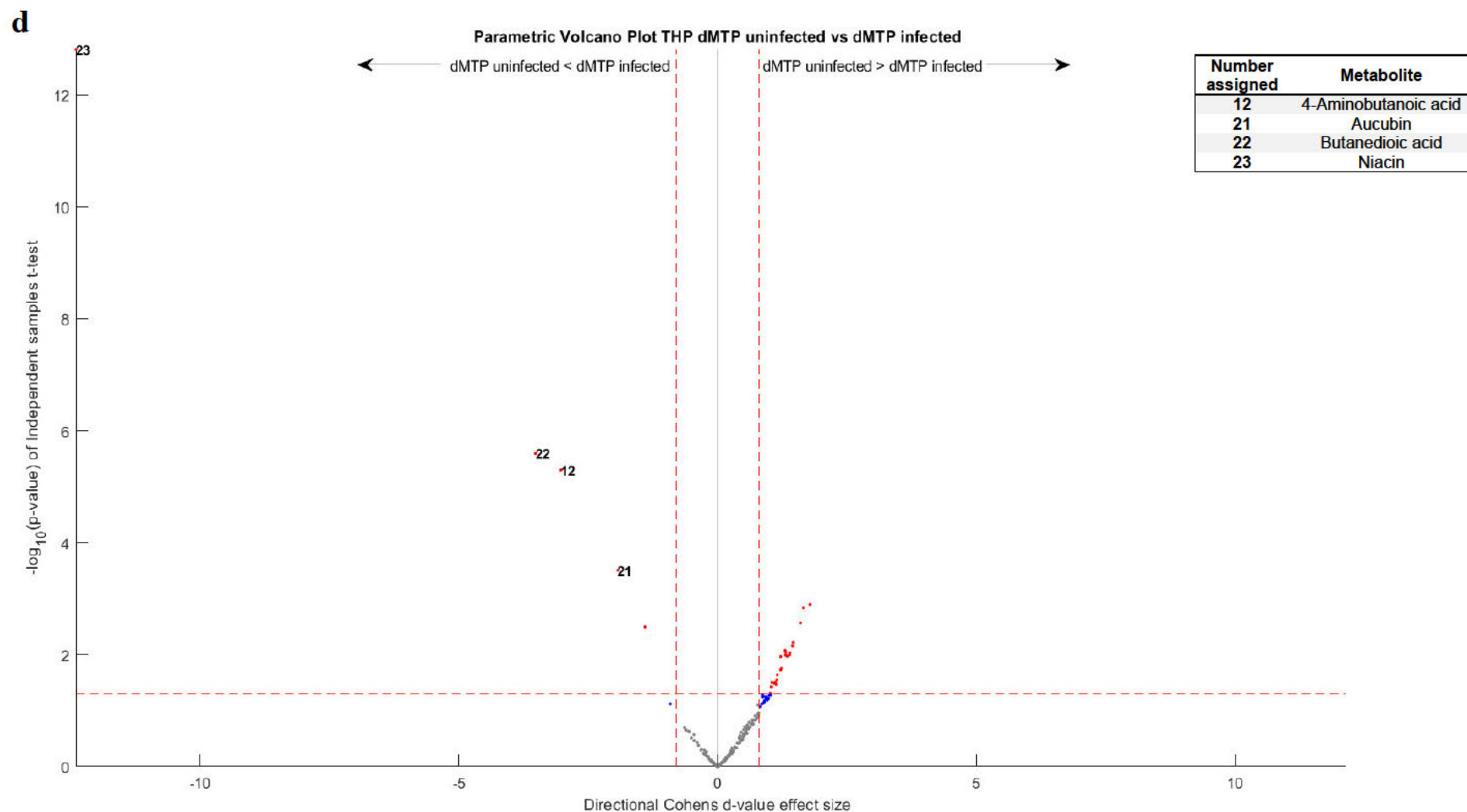


Figure S4 continued. Volcano plots illustrating metabolites of practical significance, statistical and practical significance or neither. (d) Volcano plot comparing Δmtp uninfected versus Δmtp infected. The blue dots represent metabolites with practical significance and fall beyond the vertical thresholds (representing Cohen's d -values of effect size = 0.8). The red dots represent metabolites which are statistically as well as practically significantly different between groups and are separated by the horizontal threshold (representing 5% significance level). The black dots represent metabolites with neither practical nor statistical significance.

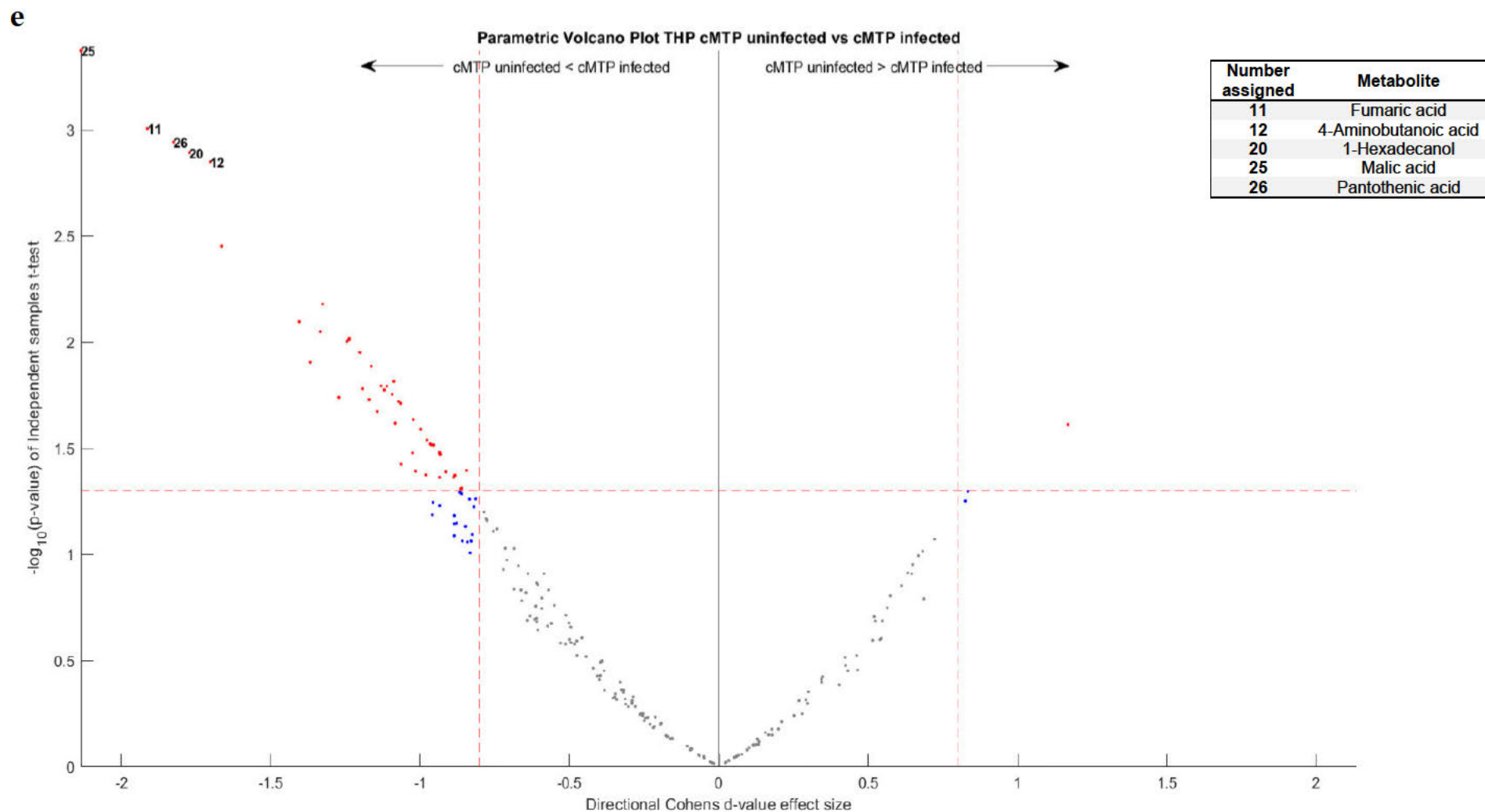


Figure S4 continued. Volcano plots illustrating metabolites of practical significance, statistical and practical significance or neither. (e) Volcano plot comparing *mtp*-complement uninfected versus *mtp*-complement infected. The blue dots represent metabolites with practical significance and fall beyond the vertical thresholds (representing Cohen's *d*-values of effect size = 0.8). The red dots represent metabolites which are statistically as well as practically significantly different between groups and are separated by the horizontal threshold (representing 5% significance level). The black dots represent metabolites with neither practical nor statistical significance.

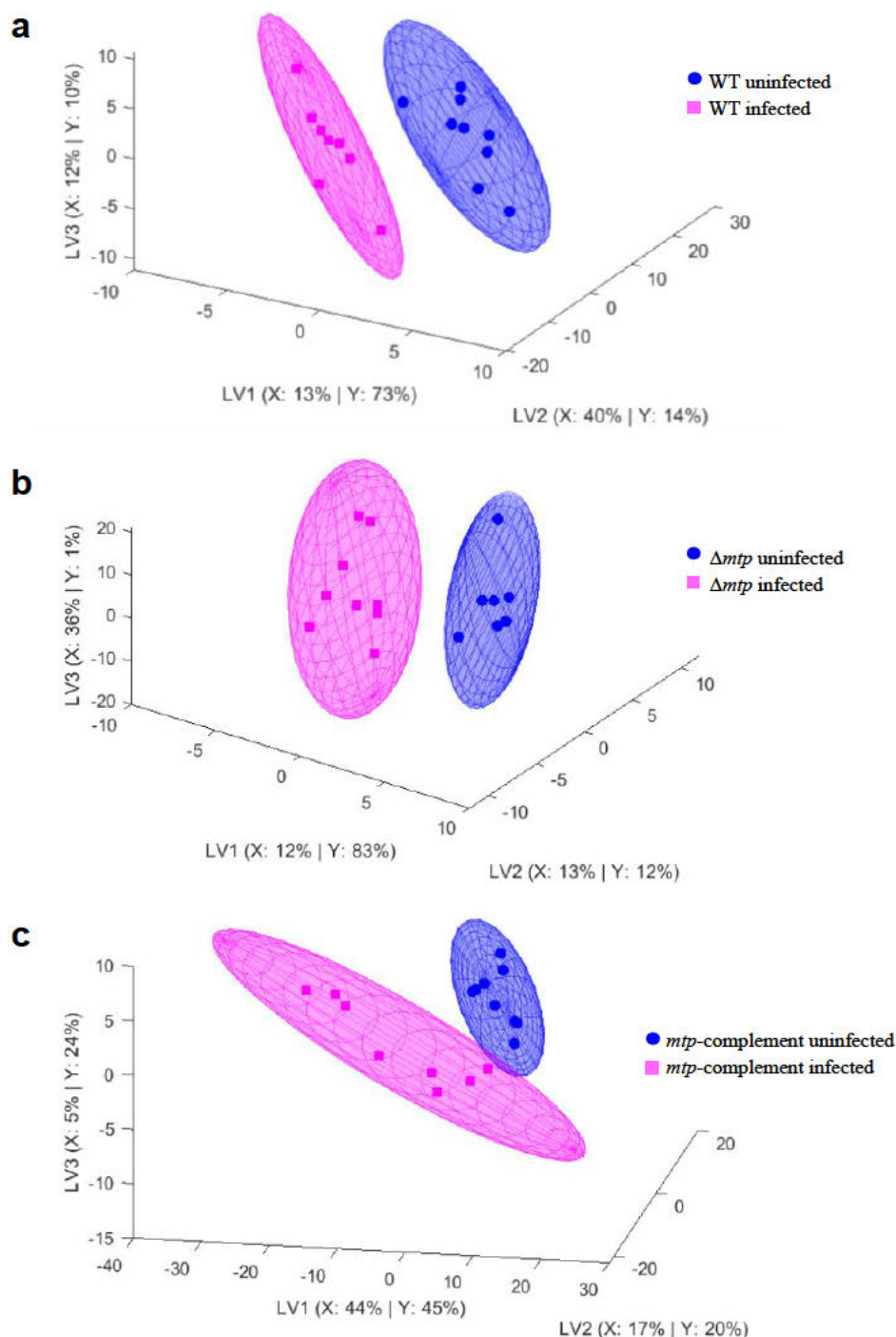


Figure S5. The 3D PLS-DA score plots of various group comparisons. (a) WT uninfected macrophages to WT infected macrophages ($R^2 = 96.19\%$; $Q^2 = 76.45\%$), (b) Δmtp uninfected macrophages to Δmtp infected macrophages ($R^2 = 96.43\%$; $Q^2 = 62.92\%$), (c) *mtp*-complement uninfected macrophages to *mtp*-complement infected macrophages ($R^2 = 88.50\%$; $Q^2 = 8.21\%$) in which the uninfected macrophages are represented in royal blue and the respective infected macrophages are represented in pink. The R^2 = proportion of variance in the group labels explained by the model; Q^2 = proportion of variance in the group labels explained by the model given the leave-one-out cross validation. Ellipsoids represent 95% CI of score centroids of each group. Accuracy of group assignment (Y) and variation explained in measured metabolites (X), as achieved by each latent variable (LV), percentages are indicated along each axis.

Table S3. Most significant metabolite changes between WT uninfected host and *M. tuberculosis* WT infected host categorised by biological significance and selected as per univariate selection criteria together with their respective mean relative concentration and standard deviation, fold change with respect to the WT uninfected, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Metabolite	Mean relative concentration with standard deviation		Fold change	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
	WT infected	WT uninfected			
1-Hexadecanol	166.932 (43.635)	9.257 (0.959)	18.033	2.454	0.007
4-Aminobutanoic acid	27.123 (6.888)	3.840 (6.416)	7.063	3.811	<0.001
Aucubin	81.018 (26.186)	11.813 (6.319)	6.859	2.066	0.033
Butanedioic acid	0.662 (0.504)	0.108 (0.287)	6.114	5.763	<0.001
Lactic acid	8.774 (2.243)	1.715 (0.490)	5.116	2.661	<0.001
Niacin	3.066 (0.814)	1.151 (0.492)	2.663	11.069	<0.001
Niacinamide	13.163 (2.831)	5.882 (1.956)	2.238	2.342	0.003
1-Octadecanol	1.565 (2.809)	0.772 (2.0433)	2.027	2.763	<0.001

WT: wildtype; positive fold change = higher levels in WT infected; negative fold change = higher levels in WT uninfected.

Table S4. Most significant metabolite changes between Δmtp uninfected host and *M. tuberculosis* Δmtp infected host categorised by biological significance and selected as per univariate selection criteria together with their respective mean relative concentration and standard deviation, fold change with respect to the Δmtp uninfected, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Metabolite	Mean relative concentration with standard deviation		Fold change	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
	Δmtp infected	Δmtp uninfected			
Niacin	239.124 (46.547)	18.126 (3.513)	13.192	12.402	<0.001
Aucubin	10.360 (2.932)	1.945 (3.809)	5.327	1.929	0.018
4-Aminobutanoic acid	47.804 (15.291)	12.412 (5.299)	3.851	3.030	<0.001
Butanedioic acid	5.679 (1.215)	2.373 (0.655)	2.394	3.522	<0.001

Δmtp infected: *mtp*-gene deletion mutant infected; Δmtp uninfected: *mtp*-gene deletion mutant uninfected; positive fold change = higher levels in Δmtp -infected; negative fold change = higher levels in Δmtp -uninfected.

Table S5. Most significant metabolite changes between *mtp*-complement uninfected host and *M. tuberculosis* *mtp*-complement infected host categorised by biological significance and selected as per univariate selection criteria together with their respective mean relative concentration and standard deviation, fold change with respect to the *mtp*-complement uninfected, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Metabolite	Mean relative concentration with standard deviation		Fold change	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
	<i>mtp</i> -complement infected	<i>mtp</i> -complement uninfected			
4-Aminobutanoic acid	35.004 (19.714)	9.541 (3.672)	3.669	1.703	0.062
Malic acid	64.924 (35.727)	17.977 (12.245)	3.612	2.136	0.062
Pantothenic acid	28.937 (17.550)	8.471 (3.527)	3.416	1.825	0.062
Fumaric acid	57.839 (37.124)	18.059 (11.165)	3.203	1.913	0.062
1-Hexadecanol	16.382 (9.068)	5.619 (2.570)	2.915	1.773	0.062

mtp-complement infected: complemented *mtp*-knockout strain infected; *mtp*-complement uninfected: complemented *mtp*-knockout strain uninfected; positive fold change = higher levels in complement-infected; negative fold change = higher levels in complement-uninfected.

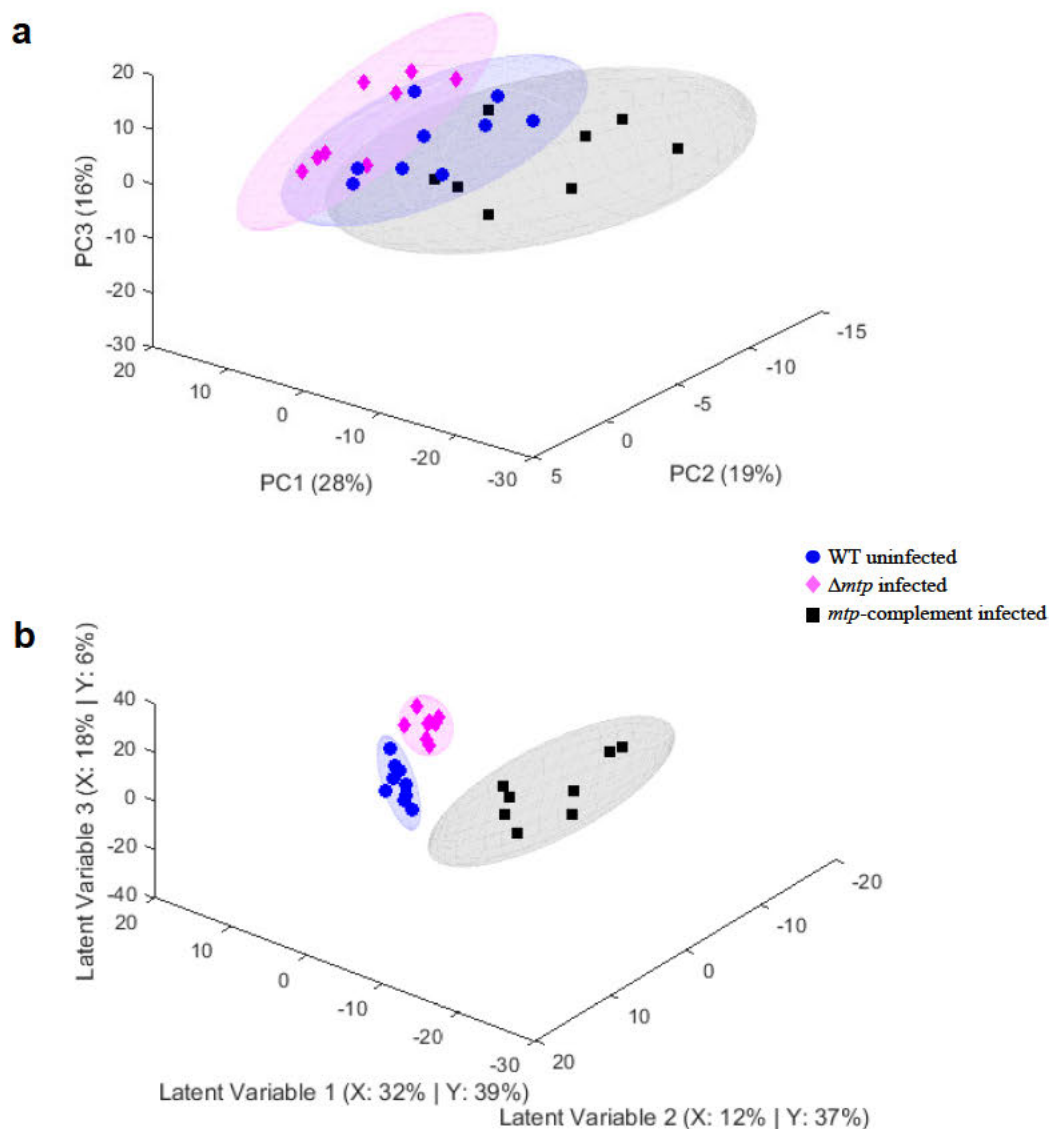


Figure S6. The 3D PCA and 3D PLS-DA scores plots for all three infection groups. (a) The PCA depicts the unsupervised grouping and separation of all three infection groups. Each of the infection groups (WT infected in blue; Δmtp infected in pink and *mtp*-complement infected in black) are naturally grouped with some overlap of the ellipsoids. This study used stringent selection criteria, one of which included PLS-DA VIP values, hence, (b) the PLS-DA depicts supervised grouping and separation of all three infection groups. Each of the infection groups are distinctively clustered and well separated from one another. However, this model did not validate as $R^2 = 82.21\%$ and $Q^2 = 47.91\%$, therefore this comparison was unsuitable for further interpretation. Hence the individual PLS-DA comparisons were reported in the main text. The R^2 = proportion of variance in the group labels explained by the model; Q^2 = proportion of variance in the group labels explained by the model given the leave-one-out cross validation. Ellipsoids represent 95% CI of score centroids of each group. Accuracy of group assignment (Y) and variation explained in measured metabolites (X), as achieved by each latent variable (LV), percentages are indicated along each axis.

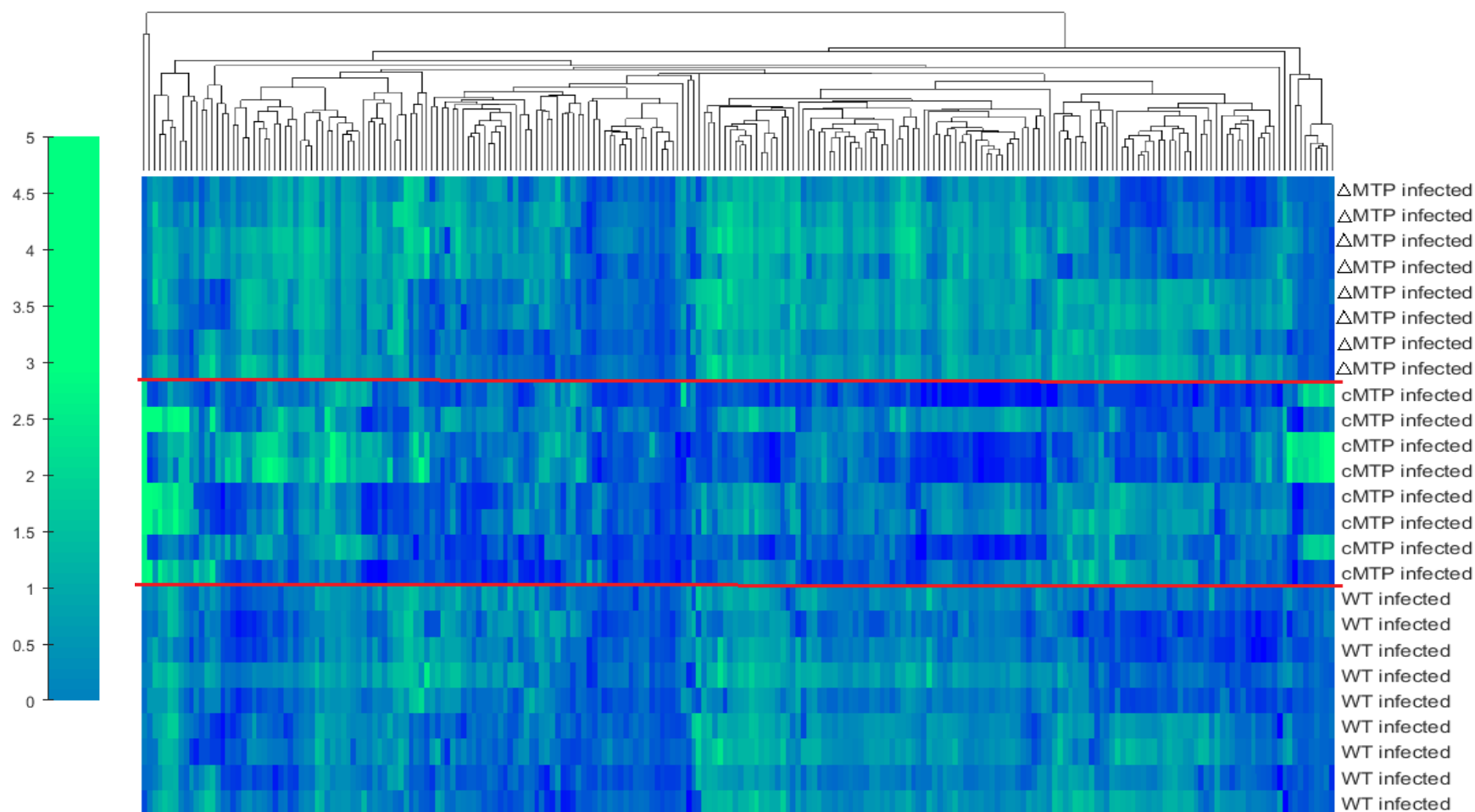


Figure S7. Heatmap depicting all metabolites detected for each infection sample. The entire data set is visually summarized to show the alterations of the metabolic profiles brought about by each of the infection groups. The rows represent individual samples pertaining to an infection group and the columns represent metabolites. The two horizontal red lines separate the three experimental infection groups (Δ MTP = *mtp*-mutant infected; cMTP = *mtp*-complement infected; WT = wildtype infected). The change in metabolite levels observed are represented by the gradient colour bar to the left of the figure, in which green represents greater mean relative concentration differences than the blue. The dendrogram at the top of the figure clusters metabolites hierarchically. However, since the data is untransformed, this is more like represented groups of metabolites with similar values. Overall, only a small percentage of the metabolome showed alterations between the infection groups.

Appendix D: Supplementary information for Chapter 4

Table S1. Significant metabolite alterations between uninfected A549 epithelial cells and THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values

Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
	Uninfected A549 epithelial cells	Uninfected THP-1 macrophages				
1-(2-Methoxy-1-methylethoxy)-2-propanol	0.476 (0.600)	27.490 (25.225)	-57.780	2.034	3.665	<0.001
1-Monopalmitylglycerol bis-trimethylsilyl ether	7.704 (3.237)	26.732 (8.553)	-3.470	1.616	3.373	<0.001
2-(Methylamino)ethanol	92.944 (51.561)	14.610 (10.046)	6.362	1.320	1.708	0.009
2,5-Bis((trimethylsilyl)oxy)pyrazine	0.373 (0.541)	3.058 (2.599)	-8.188	1.536	1.375	0.028
Alanine, 3TMS derivative	402.225 (150.146)	17.254 (10.622)	23.312	2.106	5.333	<0.001
Acedoben, 2TMS derivative	0 (0)	2.948 (1.170)	#	2.066	3.212	<0.001
Allonic acid, γ -lactone, 4TMS derivative	8.205 (6.593)	64.467 (31.884)	-7.857	1.530	2.357	<0.001
Glyceryl-glycoside, TMS ether	0 (0)	37.704 (17.965)	#	1.895	6.233	<0.001
Isoserine, 3TMS derivative	15.197 (2.376)	0 (0)	*	2.338	16.909	<0.001
meso-Erythritol, 4TMS derivative	49.350 (8.712)	17.007 (5.047)	2.902	1.521	3.510	<0.001
Pentanedioic acid, 2-[(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	2.219 (2.412)	11.454 (2.635)	-5.163	1.390	2.618	<0.001
Phosphoethanolamine, N,O,O-TMS	549.477 (295.854)	59.797 (24.405)	9.189	1.385	2.591	<0.001
Phosphoric acid, 2-(methoxyimino)-3-[(trimethylsilyl)oxy]propyl bis(trimethylsilyl) ester	47.570 (8.715)	15.578 (3.054)	3.054	1.084	2.499	0.002
Pyrazine, 3,6-dihydro-2,5-bis-(trimethylsiloxy)	1.996 (1.995)	5.244 (2.494)	-2.628	1.137	1.236	0.031
S-[2-[N,N-Dimethylamino]ethyl]N,N-dimethylcarbamoyl thiocarbohydroximate	1020.361 (312.659)	239.548 (164.796)	4.260	2.109	2.134	0.004
Tridecanoic acid, TMS derivative	3.286 (1.193)	7.425 (1.627)	-2.260	1.1107	2.462	<0.001
Trimethylsilyl 2-amino-4-oxo-4-pyrrol2-[(trimethylsilyl)amino]phenylmorphobutanoate	0 (0)	9.482 (3.353)	#	2.449	5.022	<0.001

PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages; * = fold change cannot be calculated as metabolite is present in only A549 epithelial cells.

Table S2. Significant metabolite alterations between WT infected A549 epithelial cells and WT infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
	WT infected A549 epithelial cells	WT infected THP-1 macrophages				
1-(2-Methoxy-1-methylethoxy)-2-propanol, TMS derivative	0.498 (0.803)	22.589 (12.186)	-45.372	1.946	3.137	<0.001
1-Monopalmitylglycerol bis-trimethylsilyl ether	6.503 (2.986)	25.124 (6.323)	-3.864	1.805	3.645	<0.001
1-Octadecanol, TMS derivative	0.839 (0.488)	3.066 (0.814)	-3.654	1.849	3.311	<0.001
2-(Methylamino)ethanol, N,O-bis(trimethylsilyl)	93.639 (44.182)	5.647 (4.977)	16.582	2.111	3.416	<0.001
Alanine, 3TMS derivative	308.968 (232.880)	12.634 (8.024)	30.107	2.220	4.643	<0.001
Acedoben, 2TMS derivative	0 (0)	2.070 (0.806)	#	1.594	3.172	<0.001
Allonic acid, γ -lactone, 4TMS derivative	8.894 (1.712)	48.582 (18.660)	-5.462	1.335	2.300	<0.001
Aucubin, hexakis(trimethylsilyl) ether	1.014 (0.340)	27.123 (6.888)	-26.759	1.3556	11.364	<0.001
Carbonic acid, 2-dimethylaminoethyl ethyl ester	4.715 (3.995)	14.378 (4.927)	-3.049	1.276	1.5823	0.004
Glyceryl-glycoside TMS ether	0.211 (0.424)	41.081 (13.673)	-194.505	1.958	10.533	<0.001
Isoserine, 3TMS derivative	14.482 (4.314)	0 (0)	*	2.275	9.775	<0.001
meso-Erythritol, 4TMS derivative	47.440 (12.962)	14.089 (3.768)	3.367	1.706	4.265	<0.001
Phosphoethanolamine, N,O,O-TMS	447.512 (217.015)	42.910 (15.444)	10.429	1.548	3.997	<0.001
Pyrazine, 3,6-dihydro-2,5-bis-(trimethylsiloxy)	1.877 (2.236)	5.598 (2.640)	-2.983	1.265	1.265	0.033
S-[2-[N,N-Dimethylamino]ethyl]N,N-dimethylcarbamoyl thiocarbohydroximate	956.945 (408.358)	222.828 (121.673)	4.295	2.016	2.576	<0.001
Tridecanoic acid, TMS derivative	2.672 (1.032)	6.849 (1.814)	-2.563	1.265	2.657	<0.001
Trimethylsilyl 2-amino-4-oxo-4-pyrrol2-[(trimethylsilyl)amino]phenylmorphobutanoate	0 (0)	7.455 (2.346)	#	2.231	5.842	<0.001

WT infected: wildtype infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages; * = fold change cannot be calculated as metabolite is present in only A549 epithelial cells.

Table S3. Significant metabolite alterations between Δmtp infected A549 epithelial cells and Δmtp infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH- adjusted <i>p</i> -value
	Δmtp infected A549 epithelial cells	Δmtp infected THP-1 macrophages				
1-(2-Methoxy-1-methylethoxy)-2-propanol, TMS derivative	1.411 (1.020)	45.677 (30.710)	-32.368	1.532	3.288	<0.001
1-Monopalmitin, 2TMS derivative	16.933 (5.084)	42.716 (11.315)	-2.523	1.069	3.087	<0.001
1-Monopalmitylglycerol bis-trimethylsilyl ether	5.668 (2.900)	30.737 (8.001)	-5.423	1.567	4.693	<0.001
2-(2-Methoxyethoxy)acetic acid, TMS derivative	3.032 (1.125)	12.246 (5.626)	-4.039	1.213	2.291	<0.001
2-Amino-1-methyl-1H-imidazol-4-ol	20.947 (7.901)	756.834 (280.830)	-36.130	1.651	8.999	<0.001
3-Amino-2-piperidone, 2TMS derivative	56.900 (40.659)	258.933 (87.964)	-4.551	1.320	1.616	0.003
4-Methyl-5-thiazoleethanol, trimethylsilyl ether	3.847 (1.434)	11.681 (2.505)	-3.036	1.417	3.306	<0.001
7-(Trimethylsilyl)-2,6-bis[(trimethylsilyl)oxy]-7H-purine	63.232 (29.380)	405.593 (60.929)	-6.414	1.677	2.051	0.001
9H-Purin-6-ol, 2TMS derivative	682.304 (249.204)	2234.21 (298.521)	-3.275	1.747	2.081	0.001
9-Tetradecenoic-Acid	21.302 (4.019)	62.146 (21.303)	-2.917	1.282	2.435	<0.001
Acedoben, 2TMS derivative	0 (0)	4.133 (1.389)	#	1.834	4.716	<0.001
Allonic acid, γ -lactone, 4TMS derivative	0.650 (0.697)	79.927 (18.686)	-123.019	2.028	10.470	<0.001
Glycerol-glycoside TMS ether	0.102 (0.305)	138.765 (40.065)	-1366.580	1.898	14.514	<0.001
L-(+)-Threose, tris(trimethylsilyl) ether, methyloxime (syn)	9.419 (2.200)	55.875 (16.412)	-5.932	1.375	6.057	<0.001
Lanthionine, N,N',O,O'-tetrakis(trimethylsilyl)	1.674 (1.681)	17.434 (14.928)	-10.416	1.109	2.013	0.001
L-Cysteine, 3TMS derivative	244.865 (116.430)	1265.835 (257.091)	-5.170	1.257	2.663	<0.001
Methane, (methylsulfinyl)(methylthio)	2.108 (2.574)	30.071 (11.987)	-14.267	1.751	3.873	<0.001
Monomyristoylglycerol	0.668 (0.459)	3.295 (1.115)	-4.936	1.545	3.487	<0.001
N-(Trimethylsilyl)hexanamide	28.431 (28.150)	85.328 (33.478)	-3.001	1.284	1.116	0.020
N-Acetylaspartic-Acid-(diTMS)	4.568 (3.256)	29.356 (17.980)	-6.427	1.279	2.678	<0.001
Pentitol, 3-desoxy-tetrakis-O-(trimethylsilyl)	0.115 (0.229)	9.443 (3.854)	-81.904	2.006	4.957	<0.001

Δmtp infected: *mtp*-deletion mutant infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages.

Table S3 continued. Significant metabolite alterations between Δmtp infected A549 epithelial cells and Δmtp infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH- adjusted <i>p</i> -value
	Δmtp infected A549 epithelial cells	Δmtp infected THP-1 macrophages				
Pyrazine, 3,6-dihydro-2,5-bis-(trimethylsiloxy)	2.116 (2.163)	7.868 (2.151)	-3.718	1.206	1.646	0.002
Pyrimidinedione	0.410 (0.563)	12.086 (4.656)	-29.494	1.700	4.725	<0.001
Pyroglutamic acid, TMS derivative	270.484 (84.157)	1687.985 (402.564)	-5.131	1.891	5.288	<0.001
Timonacic, 2TMS derivative	16.950 (11.973)	131.134 (92.684)	-7.737	1.166	1.965	0.001
Tridecanoic acid, TMS derivative	2.737 (0.484)	17.222 (2.701)	-6.292	1.819	10.903	<0.001
Trimethylsilyl 2-amino-4-oxo-4-pyrrol2- [(trimethylsilyl)amino]phenylmorphobutanoate	0 (0)	8.801 (1.649)	#	1.761	11.585	<0.001

Δmtp infected: *mtp*-deletion mutant infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; # = fold change cannot be calculated as metabolite is present in only THP-1 macrophages.

Table S4. Significant metabolite alterations between *mtp*-complement infected A549 epithelial cells and *mtp*-complement infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
	<i>mtp</i> -complement infected A549 epithelial cells	<i>mtp</i> -complement infected THP-1 macrophages				
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	4.041 (1.827)	8.229 (2.429)	-2.036	1.075	1.647	0.007
1-Monopalmitylglycerol bis-trimethylsilyl ether	5.909 (1.557)	64.961 (56.643)	-10.948	2.054	1.494	0.016
1-Octadecanol, TMS derivative	0.712 (0.143)	4.533 (3.216)	-6.365	1.923	1.575	0.013
2-(Methylamino)ethanol, N,O-bis(trimethylsilyl)	109.388 (52.949)	5.745 (5.496)	19.040	1.792	3.502	<0.001
9-Tetradecenoic-Acid	22.704 (8.634)	67.072 (47.414)	-2.954	1.395	1.546	0.013
Alanine, 3TMS derivative	427.909 (267.899)	10.084 (7.288)	42.435	2.054	4.754	<0.001
Aucubin, hexakis(trimethylsilyl) ether	3.836 (1.513)	294.575 (336.437)	-76.788	1.243	1.500	0.016
Dibutyl phthalate	159.585 (28.973)	358.321 (130.057)	-2.248	1.321	2.139	0.002
Glycine, 3TMS derivative	5476.993 (2164.826)	1266.508 (1908.166)	4.324	1.768	1.567	0.013
Heptadecanoic acid & TMS derivative	115.728 (22.219)	309.946 (155.653)	-2.678	1.776	2.000	0.003
Isoleucine, 3TMS derivative	16.13 (4.906)	0 (0)	*	1.930	7.635	<0.001
Lanthionine, N,N',O,O'-tetrakis(trimethylsilyl)	19.006 (8.029)	5.235 (6.304)	3.630	1.073	1.635	0.009
L-Cysteine, 3TMS derivative	1529.579 (264.424)	636.173 (669.378)	2.404	1.238	1.180	0.043
meso-Erythritol, 4TMS derivative	52.528 (15.293)	13.325 (6.456)	3.942	1.638	3.0175	<0.001
N-Acetylaspartic-Acid-(diTMS)	46.953 (29.493)	11.168 (7.930)	4.204	1.157	2.110	0.003
Palmitelaidic acid, & TMS derivative	592.704 (114.455)	1507.076 (572.007)	-2.543	1.809	2.178	0.002
Pentanedioic acid, 2-[(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	2.281 (1.638)	15.044 (13.551)	-6.596	1.039	1.520	0.013
Phosphoric acid, 2-(methoxyimino)-3-[(trimethylsilyl)oxy]propyl bis(trimethylsilyl) ester	31.416 (7.110)	11.058 (11.124)	2.841	1.241	1.200	0.041

mtp-complement infected: complemented *mtp*-knockout strain infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; * = fold change cannot be calculated as metabolite is present in only A549 epithelial cells.

Table S4 continued. Significant metabolite alterations between *mtp*-complement infected A549 epithelial cells and *mtp*-complement infected THP-1 macrophages as indicated by relative mean concentrations, standard deviations, fold changes, PLS-DA VIP value, Cohen's *d*-value as effect size and BH-adjusted *p*-values.

Metabolite	Relative mean concentration with standard deviation (ng/mg cells)		Fold change	PLS-DA VIP	Cohen's <i>d</i> -value	BH-adjusted <i>p</i> -value
	<i>mtp</i> -complement infected A549 epithelial cells	<i>mtp</i> -complement infected THP-1 macrophages				
S-[2-[N,N-Dimethylamino]ethyl]N,N-dimethylcarbamoyl thiocarbohydroximate	869.790 (156.707)	330.471 (182.929)	2.632	1.186	1.671	0.009
Tridecanoic acid, TMS derivative	2.198 (0.557)	12.222 (5.944)	-5.561	1.772	3.208	<0.001
Triethylamine, 2-chloro-2'-hydroxy-, O-TMS	8.073 (6.017)	0.896 (1.472)	9.014	1.480	2.838	<0.001

mtp-complement infected: complemented *mtp*-knockout strain infected; PLS-DA VIP: partial least squares discriminatory analysis variable in projection value; BH-adjusted: Benjamini & Hochberg adjusted for false discovery rate; positive fold change = higher levels in A549 epithelial cells; negative fold change = higher levels in THP-1 macrophages; * = fold change cannot be calculated as metabolite is present in only A549 epithelial cells.

Appendix E: Supplementary information for Chapter 5

Confirmation of MOI through CFUs of *M. tuberculosis*

For all THP-1 macrophage infections, the optical density (OD_{600nm}) of *M. tuberculosis* were taken. Inoculums of the *M. tuberculosis* were standardised to an OD_{600nm} of 1 for the infections. This was done by pelleting the bacteria and resuspending it in the appropriate media used for the experimental assay. The MOI ratio of approximately 5:1 of *M. tuberculosis* to host was used for all infections, which proceeded for four hours at 37°C in 5% CO₂ and 95% humidity (Shel Lab CO₂ Incubator, Cornelius, OR, USA). For both the bioenergetics and metabolomic experiments, four sample groups were used: uninfected THP-1 macrophages, wildtype V9124 infected THP-1 macrophages (WT), Δmtp infected THP-1 macrophages (Δmtp) and *mtp*-complement infected THP-1 macrophages. The media/ inoculums that were used for infections were plated out (10 μ L) to determine CFUs to confirm the respective MOI.

CFU/mL = [(average number of colony forming units $\times$ dilution factor)/ volume plated].

MOI = [(CFU/mL $\times$ volume of inoculum in mL)/number of host cells per well].

For the cell mitochondrial stress test (CMST) and glycolytic rate assay (GRA) infections 5 μ L of inoculum was used to infect 100 000 cells per well. For the liquid chromatography tandem mass spectrometry (LC-MS/MS) infections 150 μ L of inoculum was used to infect 3 000 000 cells per well. The uninfected groups had no CFUs to report as there was not *M. tuberculosis* in the samples. The *M. tuberculosis* infection groups are reported below (Table S1.1 - S1.3). Additionally, extra cell culture plates were seeded, infected with the same inoculums, washed with phosphate buffered saline (PBS) and lysed with 0.25% Triton X-100 (Millipore, Merck, Darmstadt, Germany). These lysates were used to plate out ten-fold serial dilutions to assess CFUs (Table S1.1 - S1.3) of each infection to ensure reproducibility of infection and invasion of *M. tuberculosis*.

Table S1.1. MOI and CFU for CMST.

	<i>M. tuberculosis</i> strains and adjusted inoculums used for infection		
	WT	Δmtp	<i>mtp</i> -complement
OD _{600nm} prior to adjustment	0.82	0.82	0.76
Dilution used for count	10 ⁻⁴	10 ⁻⁴	10 ⁻⁴
Average CFU	101	88	110
CFU/mL	1.0 $\times$ 10 ⁸	8.8 $\times$ 10 ⁷	1.1 $\times$ 10 ⁸
MOI	5.03	4.42	5.50
Lysates after four hours of infection			
Dilution used for count	10 ⁻¹	10 ⁻¹	10 ⁻¹
Average CFU	49	46	53
CFU/mL	4.9 $\times$ 10 ⁴	4.6 $\times$ 10 ⁴	5.3 $\times$ 10 ⁴

WT: wildtype; Δmtp : *mtp*-gene deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain; CFU/mL: colony forming unit per millilitre = (average number of colonies $\times$ dilution factor)/ (volume plated out in mL); MOI; multiplicity of infection = (number of bacterial cells)/ (number of host cells) = (average CFU/mL $\times$ volume of inoculum)/(number of cells seeded $\times$ total volume).

Table S1.2. MOI and CFU for GRA.

	<i>M. tuberculosis</i> strains and adjusted inoculums used for infection		
	WT	Δmtp	<i>mtp</i> -complement
OD _{600nm} prior to adjustment	0.81	0.87	0.72
Dilution used for count	10 ⁻⁴	10 ⁻⁴	10 ⁻⁴
Average CFU	111	111	110
CFU/mL	1.1×10 ⁸	1.1×10 ⁸	1.1×10 ⁸
MOI	5.55	5.56	5.03
Lysates after four hours of infection			
Dilution used for count	10 ⁻¹	10 ⁻¹	10 ⁻¹
Average CFU	46	49	53
CFU/mL	5.6×10 ⁴	5.1×10 ⁴	5.3×10 ⁴

WT: wildtype; Δmtp : *mtp*-gene deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain; CFU/mL: colony forming unit per millilitre = (average number of colonies × dilution factor)/ (volume plated out in mL); MOI; multiplicity of infection = (number of bacterial cells)/ (number of host cells) = (average CFU/mL × volume of inoculum)/(number of cells seeded × total volume).

Table S1.3. MOI and CFU for LC-MS/MS.

	<i>M. tuberculosis</i> strains and adjusted inoculums used for infection		
	WT	Δmtp	<i>mtp</i> -complement
OD _{600nm} prior to adjustment	0.80	0.85	0.83
Dilution used for count	10 ⁻⁴	10 ⁻⁴	10 ⁻⁴
Average CFU	95	101	96
CFU/mL	9.5×10 ⁷	1.0×10 ⁸	9.6×10 ⁷
MOI	4.75	5.00	4.80
Lysates after four hours of infection			
Dilution used for count	10 ⁻¹	10 ⁻¹	10 ⁻¹
Average CFU	49	50	49
CFU/mL	4.9×10 ⁴	5.0×10 ⁴	4.9×10 ⁴

WT: wildtype; Δmtp : *mtp*-gene deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain; CFU/mL: colony forming unit per millilitre = (average number of colonies × dilution factor)/ (volume plated out in mL); MOI; multiplicity of infection = (number of bacterial cells)/ (number of host cells) = (average CFU/mL × volume of inoculum)/(number of cells seeded × total volume).

Bicinchoninic acid (BCA) assay for normalisation of LC-MS/MS data

Protein concentrations of each sample was determined with the BCA assay. Ten µL per sample was used to measure absorbance at 540 nm (Table S2). The sample blank reading was removed from the individual sample readings and this value was used to calculate the protein concentration per sample in mg/mL (Table S2) extrapolated from the generated line of best fit with the standards µg/mL (Figure S1). The equation for the line of best fit: $y = 0.0008x + 0.0003$ with an R^2 of 0.9956.

Table S2. Individual absorbance readings after removal of blank reading and extrapolate protein concentrations from the BCA assay.

Sample	Absorbance (540 _{nm})	Protein concentration (mg/mL)
Uninfected THP-1 replicate 1	0.062	0.70
Uninfected THP-1 replicate 2	0.056	1.21
Uninfected THP-1 replicate 3	0.097	1.08
Uninfected THP-1 replicate 4	0.087	1.20
Uninfected THP-1 replicate 5	0.096	1.23
WT infected THP-1 replicate 1	0.015	0.18
WT infected THP-1 replicate 2	0.02	0.25
WT infected THP-1 replicate 3	0.058	0.72
WT infected THP-1 replicate 4	0.056	0.70
WT infected THP-1 replicate 5	0.073	0.91
Δmtp infected THP-1 replicate 1	0.01	0.57
Δmtp infected THP-1 replicate 2	0.046	0.77
Δmtp infected THP-1 replicate 3	0.062	0.90
Δmtp infected THP-1 replicate 4	0.072	0.75
Δmtp infected THP-1 replicate 5	0.06	0.93
<i>mtp</i> -complement infected THP-1 replicate 1	0.026	0.32
<i>mtp</i> -complement infected THP-1 replicate 2	0.019	0.23
<i>mtp</i> -complement infected THP-1 replicate 3	0.071	0.88
<i>mtp</i> -complement infected THP-1 replicate 4	0.064	0.80
<i>mtp</i> -complement infected THP-1 replicate 5	0.06	0.68

WT: wildtype; Δmtp : *mtp*-gene deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain.

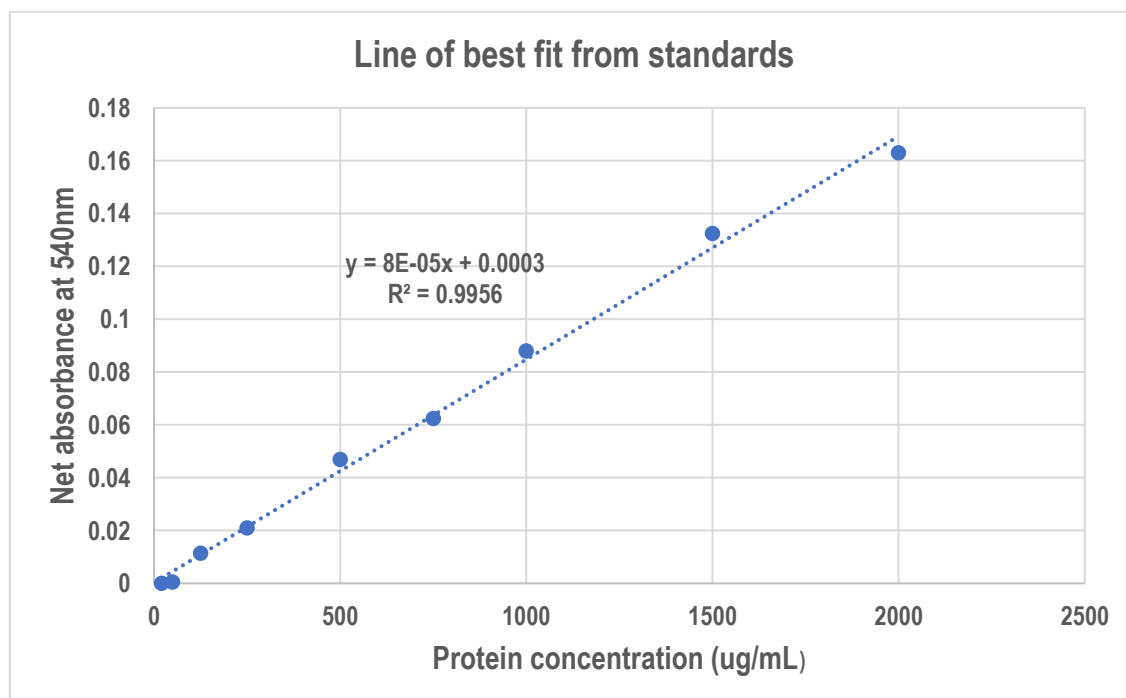


Figure S1. Line of best fit for the BCA assay. The equation for the line of best fit was used to calculate the protein concentration per sample. This was used to normalize the metabolomic data after it had been normalized to the internal standards.

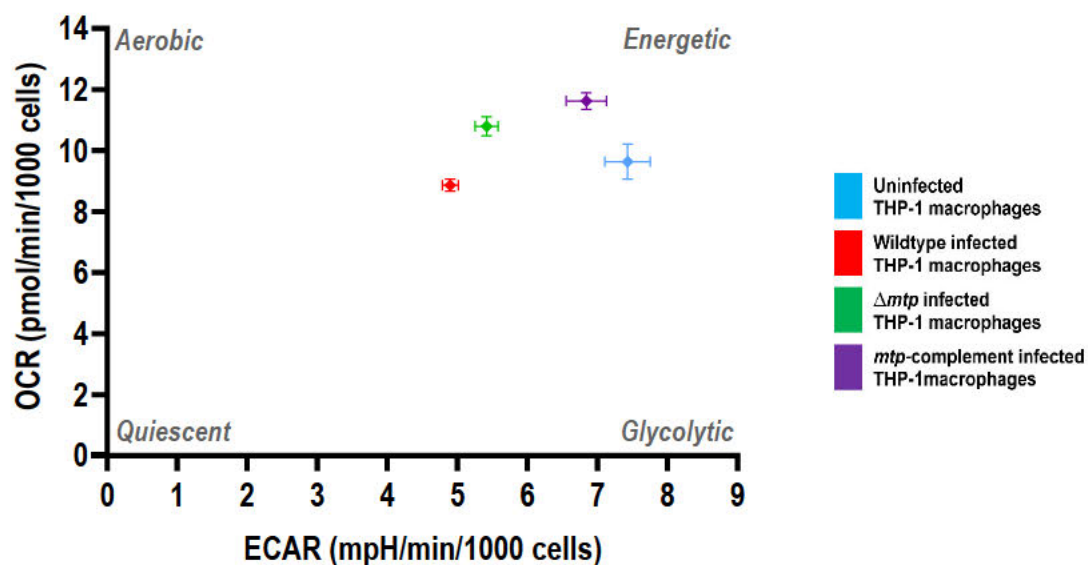


Figure S2. Shift of energy phenotypes of THP-1 macrophages in response to *M. tuberculosis* infection. Basal OCR and ECAR measurements from the respiratory assay (Figure 5.1) before addition of oligomycin were plotted to generate phenograms of the THP-1 macrophages. The *mtp*-complement infected THP-1 macrophages have the most glycolytic and energetic profile amongst the *M. tuberculosis* infected macrophages. Experiments were performed on four THP-1 macrophage groups; uninfected THP-1 macrophages (THP-1), WT infected THP-1 macrophages (WT), Δmtp infected THP-1 macrophages (Δmtp), and *mtp*-complemented (MC) infected THP-1 macrophages. The energetic profiles are a representative of three independent experiments and were normalized via Cytation5 per 1000 cells and shown as the mean $\pm$ SEM (n = minimum of 7 biological replicates).

Statistical Analysis of $^{13}\text{C}_6$ -glucose incorporation by one-way analysis of variance (ANOVA)

Data was exported into GraphPad Prism Version 8.0 for statistical analysis. Data was analysed by one-way ANOVA and Tukey's test with statistical significance considered with an adjusted p -value ≤ 0.05 . The p -values for total abundance (Table S3) and the carbon flux (Table S4) for each compound is detailed below. The total abundance data included natural and labelled normalized area under curve (AUC), and the carbon flux data used AUC to calculate the percentage of total labelled normalized area from total normalized area.

Table S3. ANOVA adjusted *p*-values from posthoc Tukeys multiple comparisons test for total abundance of each compound and comparison group.

Compound	Uninfected vs WT infected THP-1 macrophages	Uninfected vs <i>Δmtp</i> infected THP-1 macrophages	Uninfected vs <i>mtp</i>-complement infected THP-1 macrophages	WT infected vs <i>Δmtp</i> infected THP-1 macrophages	WT infected vs <i>mtp</i>-complement infected THP-1 macrophages	<i>Δmtp</i> infected vs <i>mtp</i>- complement infected THP-1 macrophages
Glycerate 3-phosphate	-	-	-	-	-	0.032
6-Phosphogluconate	-	-	-	-	-	-
α-Ketoglutaric acid	0.022	-	-	0.009	-	0.032
Citrate/Isocitrate	-	0.000	0.001	0.001	0.011	-
Dihydroxyacetone phosphate	0.000	-	0.000	0.000	-	0.000
Erythrose 4-phosphate	0.035	-	-	-	-	-
Fructose 1,6-bisphosphate	0.000	-	0.000	0.000	0.015	0.000
Fructose 6-phosphate	0.000	-	0.001	0.000	0.007	0.016
Fumarate	0.001	-	0.021	0.000	-	0.008
Glycerol 3-phosphate	-	-	0.009	-	0.001	0.007
Glyoxylate	0.000	-	0.013	0.004	0.038	-
L-lactate	0.000	0.008	0.000	0.000	-	0.000
Phosphoenolpyruvate	-	-	-	-	0.028	-
Pyruvate	-	-	-	-	0.023	-
Ribose	0.000	-	0.000	0.000	-	0.000
Ribose 5-phosphate	-	0.026	-	0.001	-	-
Sedoheptulose 7-phosphate	0.000	-	0.000	0.000	0.013	0.000

WT: wildtype; *Δmtp*: *mtp*-gene deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain; vs: versus.

Table S3 continued. ANOVA adjusted *p*-values from posthoc Tukeys multiple comparisons test for total abundance of each compound and comparison group.

Compound	Uninfected vs WT infected THP-1 macrophages	Uninfected vs Δmtp infected THP-1 macrophages	Uninfected vs <i>mtp</i>-complement infected THP-1 macrophages	WT infected vs Δmtp infected THP-1 macrophages	WT infected vs <i>mtp</i>-complement infected THP-1 macrophages	Δmtp infected vs <i>mtp</i>- complement infected THP-1 macrophages
Succinate	0.023	-	-	0.027	-	-
Succinic semialdehyde	0.046	-	0.039	-	-	-
Malate	-	0.000	0.045	0.003	-	0.024
Arginine	0.006	-	-	0.000	0.013	-
Asparagine	0.014	-	-	0.003	-	-
Aspartate	0.000	-	0.000	0.000	0.000	0.000
Cysteine	-	-	0.002	-	0.000	0.002
Glutamate	-	0.000	0.003	0.000	0.000	-
Glutamine	0.001	-	-	0.000	-	0.002
Glycine	0.000	-	-	0.000	0.000	-
Proline	-	-	-	0.021	-	-
Serine	0.000	-	0.000	0.000	0.000	0.000

WT: wildtype; Δmtp : *mtp*-gene deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain.

Table S4. ANOVA adjusted *p*-values from posthoc Tukeys multiple comparisons test for carbon flux each compound and comparison group.

Compound	Uninfected vs WT infected THP-1 macrophages	Uninfected vs Δmtp infected THP-1 macrophages	Uninfected vs <i>mtp</i> -complement infected THP-1 macrophages	WT infected vs Δmtp infected THP-1 macrophages	WT infected vs <i>mtp</i> -complement infected THP-1 macrophages	Δmtp infected vs <i>mtp</i> - complement infected THP-1 macrophages
Glycerate 3-phosphate	0.002	0.000	0.000	0.000	0.000	-
6-Phosphogluconate	0.000	0.000	0.012	-	-	-
α -Ketoglutaric acid	0.000	0.000	0.000	0.004	-	0.001
Citrate/Isocitrate	0.000	0.000	0.000	-	-	-
Dihydroxyacetone phosphate	-	-	0.022	-	-	-
Erythrose 4-phosphate	-	-	-	-	-	-
Fructose 1,6-bisphosphate	0.000	0.000	0.000	-	-	0.020
Fructose 6-phosphate	0.000	0.000	0.000	-	0.037	0.000
Fumarate	0.024	-	-	-	-	-
Glycerol 3-phosphate	0.000	0.000	0.000	-	-	-
Glyoxylate	0.001	0.010	0.018	-	-	-
L-lactate	-	-	-	-	0.019	0.042
Phosphoenolpyruvate	0.002	0.000	0.000	-	0.013	-
Pyruvate	-	0.05	0.013	0.002	0.001	-
Ribose	0.000	0.000	0.000	-	0.002	0.045
Ribose 5-phosphate	-	-	-	-	-	-

WT: wildtype; Δmtp : *mtp*-gene deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain; vs: versus.

Table S4 continued. ANOVA adjusted *p*-values from posthoc Tukeys multiple comparisons test for carbon flux each compound and comparison group.

Compound	Uninfected vs WT infected THP-1 macrophages	Uninfected vs Δmtp infected THP-1 macrophages	Uninfected vs <i>mtp</i>-complement infected THP-1 macrophages	WT infected vs Δmtp infected THP-1 macrophages	WT infected vs <i>mtp</i>-complement infected THP-1 macrophages	Δmtp infected vs <i>mtp</i>- complement infected THP-1 macrophages
Sedoheptulose 7- phosphate	0.000	0.000	0.000	-	0.002	0.001
Succinate	0.000	0.000	0.000	-	-	-
Succinic semialdehyde	-	-	-	-	-	-
Malate	0.000	0.000	0.000	-	0.001	0.007
Arginine	0.000	0.000	0.000	-	0.031	-
Asparagine	0.001	0.008	0.002	-	-	-
Aspartate	0.000	0.000	0.000	-	0.004	0.042
Cysteine	0.000	-	-	0.000	0.000	-
Glutamate	0.000	0.000	0.000	0.001	0.000	0.006
Glutamine	-	-	0.000	-	0.000	0.001
Glycine	0.000	0.015	0.001	0.004	-	-
Proline	0.000	0.000	0.000	-	0.000	0.000
Serine	-	0.021	0.031	-	-	-

WT: wildtype; Δmtp : *mtp*-gene deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain; vs: versus.

Appendix F: Supplementary information for Chapter 6

Table S1. Average OD_{600nm} reading of cultured *M. tuberculosis* strains for the three biological assays.

Strain	Biological assay 1 (OD _{600nm})	Biological assay 2 (OD _{600nm})	Biological assay 3 (OD _{600nm})
WT	0.824	0.820	0.813
Δmtp	0.821	0.802	0.898
<i>mtp</i> -complement	0.855	0.840	0.876

WT: wildtype V9124; Δmtp : *mtp*-deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain.

Table S2. MOI of the three biological assays for the THP-1 macrophage infections.

Strain	OD _{600nm}	Dilution	Rep 1	Rep 2	Rep 3	Average	CFU/ mL	MOI
Biological assay 1 WT	1.009	10 ⁻²	Lawn	Lawn	Lawn	-	-	-
		10 ⁻³	>200	>200	>200	-	-	-
		10 ⁻⁴	104	122	107	111.00	1.1×10 ⁸	5.28
		10 ⁻⁵	23	19	17	-	-	-
Biological assay 1 <i>Δmtp</i>	1.036	10 ⁻²	Lawn	Lawn	Lawn	-	-	-
		10 ⁻³	>200	>200	>200	-	-	-
		10 ⁻⁴	124	122	113	119.67	1.2×10 ⁸	5.744
		10 ⁻⁵	42	22	31	-	-	-
Biological assay 1 <i>mtp</i> - complement	1.012	10 ⁻²	Lawn	Lawn	Lawn	-	-	-
		10 ⁻³	>200	>200	>200	-	-	-
		10 ⁻⁴	109	98	89	98.67	9.9×10 ⁷	4.74
		10 ⁻⁵	12	28	9	-	-	-
Biological assay 2 WT	1.016	10 ⁻²	Lawn	Lawn	Lawn	-	-	-
		10 ⁻³	>200	>200	>200	-	-	-
		10 ⁻⁴	101	97	106	101.33	1.0×10 ⁸	4.86
		10 ⁻⁵	11	9	7	-	-	-
Biological assay 2 <i>Δmtp</i>	1.006	10 ⁻²	Lawn	Lawn	Lawn	-	-	-
		10 ⁻³	>200	>200	>200	-	-	-
		10 ⁻⁴	121	113	90	108.00	1.1×10 ⁸	5.18
		10 ⁻⁵	8	12	15	-	-	-
Biological assay 2 <i>mtp</i> - complement	1.009	10 ⁻²	Lawn	Lawn	Lawn	-	-	-
		10 ⁻³	>200	>200	>200	-	-	-
		10 ⁻⁴	107	99	85	97.00	9.7×10 ⁷	4.66
		10 ⁻⁵	10	17	9	-	-	-
Biological assay 3 WT	0.940	10 ⁻²	Lawn	Lawn	Lawn	-	-	-
		10 ⁻³	>200	>200	>200	-	-	-
		10 ⁻⁴	109	98	82	96.33	9.6×10 ⁷	4.62
		10 ⁻⁵	5	8	14	-	-	-
Biological assay 3 <i>Δmtp</i>	1.002	10 ⁻²	Lawn	Lawn	Lawn	-	-	-
		10 ⁻³	>200	>200	>200	-	-	-
		10 ⁻⁴	117	120	111	116.00	1.2×10 ⁸	5.57
		10 ⁻⁵	20	15	14	-	-	-
Biological assay 3 <i>mtp</i> - complement	1.017	10 ⁻²	Lawn	Lawn	Lawn	-	-	-
		10 ⁻³	>200	>200	>200	-	-	-
		10 ⁻⁴	124	98	86	102.67	1.0×10 ⁸	4.93
		10 ⁻⁵	12	8	9	-	-	-

Rep: replicate; WT: wildtype; *Δmtp*: *mtp*-deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain.

$$CFU/mL = \frac{\text{average number of colonies} \times \text{dilution factor}}{\text{volume plated out in mL (0.01)}}$$

$$MOI = \frac{\text{Total number of bacterial cells}}{\text{Total number of THP-1 cells}} = \frac{(CFU/mL \times \text{total volume of inoculum in mL (1.5 mL)})}{(\text{Number of cells (1.25} \times 10^6) \times \text{volume (25 mL)})}$$

Table S3. CFUs of the biological assay 1 of the THP-1 macrophage infections.

Strain	Dilution	Rep 1	Rep 2	Rep 3	Average	CFU/mL	Average CFU/mL
Biological assay 1 WT 1	Neat	Lawn	Lawn	Lawn	-	-	1.2×10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	120	117	125	120.67	1.2×10 ⁶	
	10 ⁻³	11	10	12	-	-	
Biological assay 1 WT 2	Neat	Lawn	Lawn	Lawn	-	-	1.2×10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	128	114	115	119.00	1.2×10 ⁶	
	10 ⁻³	17	12	18	-	-	
Biological assay 1 Δmtp 1	Neat	Lawn	Lawn	Lawn	-	-	1.1×10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	98	107	102	102.33	1.0×10 ⁶	
	10 ⁻³	3	6	8	-	-	
Biological assay 1 Δmtp 2	Neat	Lawn	Lawn	Lawn	-	-	1.1×10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	99	114	119	110.67	1.1×10 ⁶	
	10 ⁻³	9	16	13	-	-	
Biological assay 1 <i>mtp</i> -complement 1	Neat	Lawn	Lawn	Lawn	-	-	1.2×10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	127	114	105	115.33	1.2×10 ⁶	
	10 ⁻³	19	10	11	-	-	
Biological assay 1 <i>mtp</i> -complement 2	Neat	Lawn	Lawn	Lawn	-	-	1.2×10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	118	124	117	119.67	1.2×10 ⁶	
	10 ⁻³	18	14	12	-	-	

Rep: replicate; WT: wildtype; Δmtp : *mtp*-deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain.

$$CFU/mL = \frac{(\text{average number of colonies} \times \text{dilution factor})}{\text{volume plated out in mL (0.01)}}$$

Table S4. CFUs of the biological assay 2 of the THP-1 macrophage infections.

Strain	Dilution	Rep 1	Rep 2	Rep 3	Average	CFU/mL	Average CFU/mL
Biological assay 2 WT 1	Neat	Lawn	Lawn	Lawn	-	-	1.2 x 10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	116	115	120	117.00	1.2 x 10 ⁶	
	10 ⁻³	11	12	12	-	-	
Biological assay 2 WT 2	Neat	Lawn	Lawn	Lawn	-	-	1.2 x 10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	130	103	115	116.00	1.2 x 10 ⁶	
	10 ⁻³	11	16	8	-	-	
Biological assay 2 <i>Δmtp</i> 1	Neat	Lawn	Lawn	Lawn	-	-	1.0 x 10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	97	98	104	99.33	9.9 x 10 ⁵	
	10 ⁻³	3	4	9	-	-	
Biological assay 2 <i>Δmtp</i> 2	Neat	Lawn	Lawn	Lawn	-	-	1.1 x 10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	99	114	119	110.67	1.1 x 10 ⁶	
	10 ⁻³	9	16	13	-	-	
Biological assay 2 <i>mtp</i> -complement 1	Neat	Lawn	Lawn	Lawn	-	-	1.3 x 10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	127	128	132	129.00	1.3 x 10 ⁶	
	10 ⁻³	9	10	14	-	-	
Biological assay 2 <i>mtp</i> -complement 2	Neat	Lawn	Lawn	Lawn	-	-	1.3 x 10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	128	124	136	129.33	1.3 x 10 ⁶	
	10 ⁻³	19	11	12	-	-	

Rep: replicate; WT: wildtype; *Δmtp*: *mtp*-deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain.

$$CFU/mL = \frac{(\text{average number of colonies} \times \text{dilution factor})}{\text{volume plated out in mL (0.01)}}$$

Table S5. CFUs of the biological assay 3 of the THP-1 macrophage infections.

Strain	Dilution	Rep 1	Rep 2	Rep 3	Average	CFU/mL	Average CFU/mL
Biological assay 3 WT 1	Neat	Lawn	Lawn	Lawn	-	-	1.2 x 10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	118	126	125	123.00	1.2 x 10 ⁶	
	10 ⁻³	9	10	15	-	-	
Biological assay 3 WT 2	Neat	Lawn	Lawn	Lawn	-	-	1.2 x 10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	128	111	121	120.00	1.2 x 10 ⁶	
	10 ⁻³	7	11	19	-	-	
Biological assay 3 <i>Δmtp</i> 1	Neat	Lawn	Lawn	Lawn	-	-	1.0 x 10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	96	101	92	96.33	9.6 x 10 ⁵	
	10 ⁻³	2	16	8	-	-	
Biological assay 3 <i>Δmtp</i> 2	Neat	Lawn	Lawn	Lawn	-	-	1.1 x 10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	99	117	103	106.33	1.1 x 10 ⁶	
	10 ⁻³	9	14	12	-	-	
Biological assay 3 <i>mtp</i> -complement 1	Neat	Lawn	Lawn	Lawn	-	-	1.3 x 10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	130	126	132	129.33	1.3 x 10 ⁶	
	10 ⁻³	14	10	12	-	-	
Biological assay 3 <i>mtp</i> -complement 2	Neat	Lawn	Lawn	Lawn	-	-	1.2 x 10 ⁶
	10 ⁻¹	>200	>200	>200	-	-	
	10 ⁻²	124	120	117	120.33	1.2 x 10 ⁶	
	10 ⁻³	19	7	14	-	-	

Rep: replicate; WT: wildtype; *Δmtp*: *mtp*-deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain.

$$CFU/mL = \frac{(\text{average number of colonies} \times \text{dilution factor})}{\text{volume plated out in mL (0.01)}}$$

Table S6. Nanodrop readings of cultured bacterial RNA for three biological assays in triplicate for the three strains of *M. tuberculosis*.

Biological assay	Sample	Concentration (ng/ μ L)	A _{260/280}	A _{260/230}
1	WT 1	2047.0	1.69	1.57
	WT 2	1631.8	1.61	1.46
	*WT 3	1927.4	1.72	1.61
	Δmtp 1	1194.8	1.72	1.62
	* Δmtp 2	1423.4	1.80	1.72
	Δmtp 3	906.1	1.73	1.66
	* <i>mtp</i> -complement 1	2708.1	1.86	1.79
	<i>mtp</i> -complement 2	2210.0	1.84	1.81
	<i>mtp</i> -complement 3	1633.1	1.73	1.64
2	WT 1	1352.5	1.59	1.40
	*WT 2	1402.8	1.54	1.35
	WT 3	1406.5	1.63	1.49
	Δmtp 1	1141.2	1.53	1.35
	* Δmtp 2	1191.2	1.44	1.25
	Δmtp 3	1237.4	1.41	1.18
	<i>mtp</i> -complement 1	3885.2	1.75	1.65
	<i>mtp</i> -complement 2	2976.7	1.66	1.52
	* <i>mtp</i> -complement 3	2381.4	1.46	1.25
3	WT 1	1112.6	1.60	1.47
	*WT 2	1078.9	1.50	1.33
	WT 3	1326.0	1.44	1.24
	* Δmtp 1	1335.6	1.64	1.51
	Δmtp 2	1073.8	1.62	1.49
	Δmtp 3	906.1	1.44	1.25
	<i>mtp</i> -complement 1	1550.1	1.73	1.63
	* <i>mtp</i> -complement 2	1971.3	1.77	1.72
	<i>mtp</i> -complement 3	2001.1	1.83	1.81

A_{260/280} or A_{260/230}: ratio of absorbance; WT: wildtype; Δmtp : *mtp*-deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain; *: samples selected for DNase treatment and cDNA synthesis.

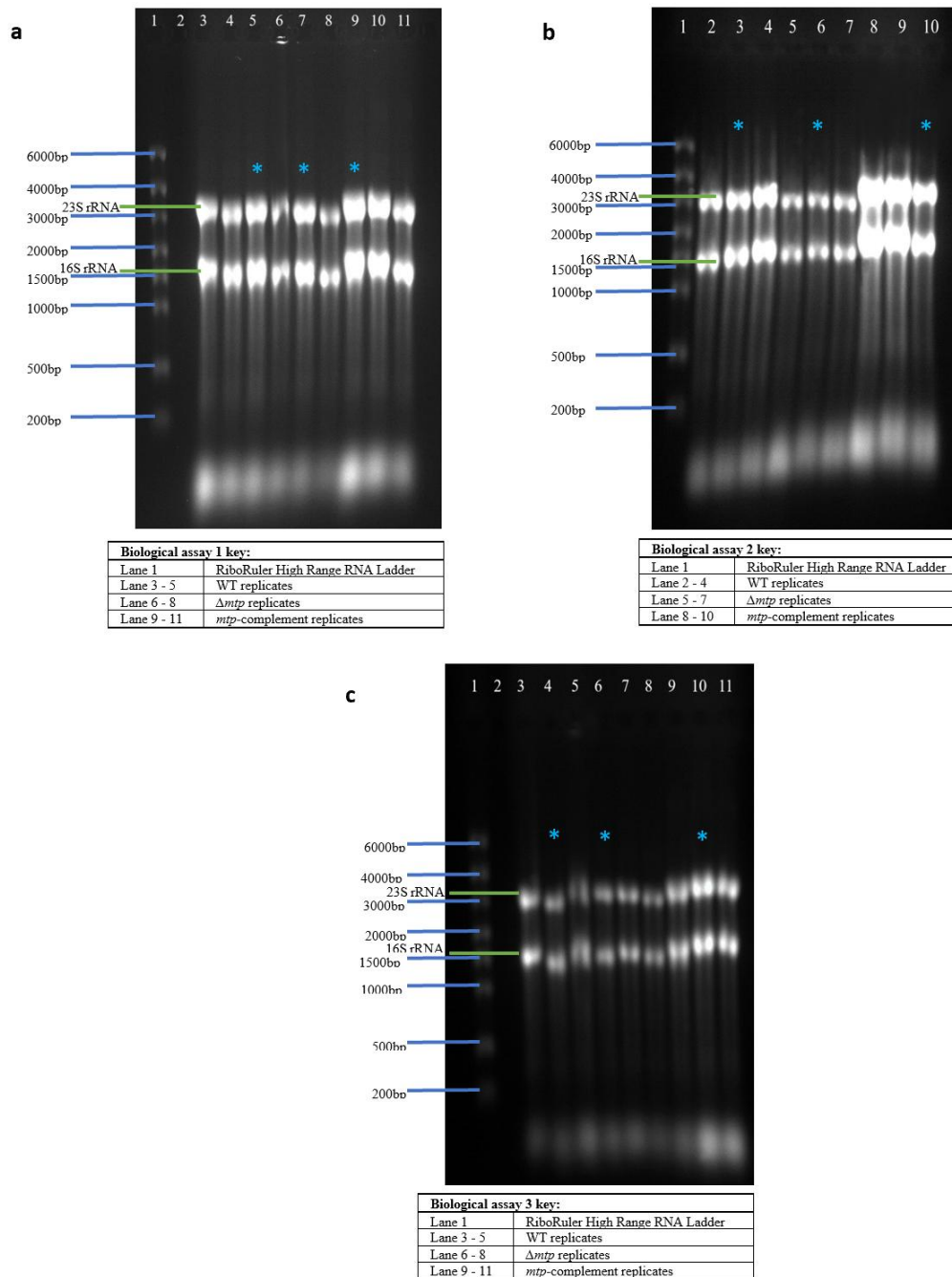


Figure S1. Denaturing MOPS gel of all pure cultured bacterial samples from the three biological assays. All three MOPS gels were made with 1.5% agarose and run at 60 V for two hours. (a) Biological assay 1, with the molecular weight marker (RiboRuler High Range RNA Ladder) in the first lane, followed by the replicates of each strain of *M. tuberculosis* (WT, Δmtp , *mtp*-complement) in sequential order. (b) Biological assay 2, with the molecular weight marker in the first lane, followed by the replicates of each strain of *M. tuberculosis* (WT, Δmtp , *mtp*-complement) in sequential order. (c) Biological assay 3, with the molecular weight marker in the first lane, followed by the replicates of each strain of *M. tuberculosis* (WT, Δmtp , *mtp*-complement) in sequential order. All samples showed two bands (23S rRNA, approximately 3000 bp and 16S ribosomal RNA, approximately 1500 bp). *, samples selected for DNase treatment and cDNA conversion.

Table S7. Intracellular bacterial RNA concentrations and purity ratios for all three biological assays.

Biological assay	Sample	RNA concentration (ng/uL)	A _{260/280}	A _{260/230}
1	WT 1	2273.5	1.88	2.17
	WT 2	2105.3	1.85	2.14
	Δmtp 1	2213.2	1.90	2.15
	Δmtp 2	2202.7	1.89	2.18
	<i>mtp</i> -complement 1	3566.9	1.95	2.17
	<i>mtp</i> -complement 2	3485.9	1.80	2.10
2	WT 1	2081.2	1.95	2.19
	WT 2	2071.2	1.90	2.16
	Δmtp 1	2224.5	1.95	2.17
	Δmtp 2	2201.8	1.90	2.13
	<i>mtp</i> -complement 1	2344.0	1.95	2.11
	<i>mtp</i> -complement 2	2332.2	1.93	2.10
3	WT 1	2316.1	1.92	2.09
	WT 2	2349.9	1.92	2.12
	Δmtp 1	2921.5	2.02	2.20
	Δmtp 2	2891.2	2.02	2.21
	<i>mtp</i> -complement 1	1343.5	2.00	2.18
	<i>mtp</i> -complement 2	1363.1	1.99	2.20

A_{260/280} or A_{260/230}: ratio of absorbance; WT: wildtype; Δmtp : *mtp*-deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain.

$$CFU/mL = \frac{(\text{average number of colonies} \times \text{dilution factor})}{\text{volume plated out in mL (0.01)}}$$

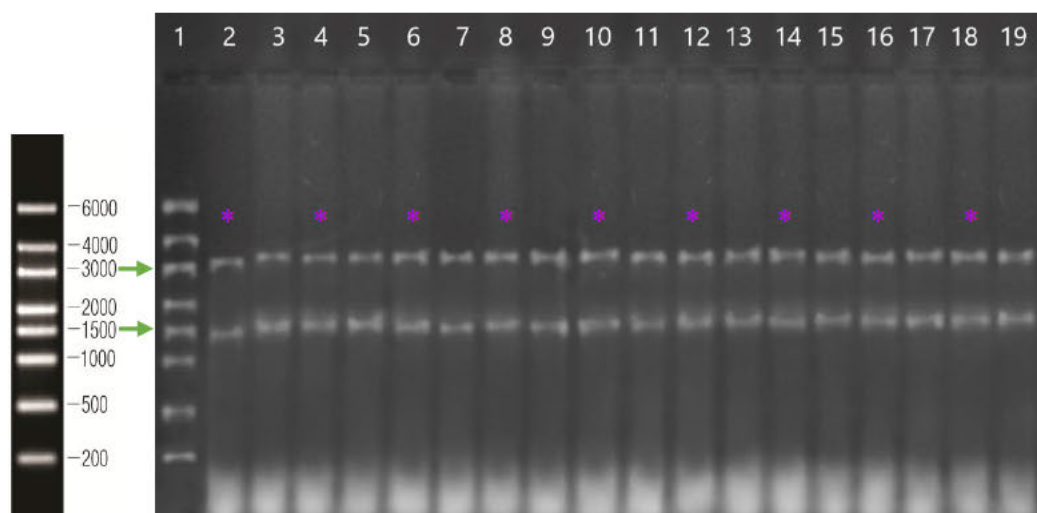


Figure S2. Denaturing MOPS gel of all strains in duplicate for the three biological assays of intracellular bacteria. The MOPS gel was comprised 1.5% agarose and was run at 70 V for 3 hours. RiboRuler high range RNA ladder was used as the molecular weight marker in lane 1. Two bands were expected around 3000bp (23s rRNA) and 1500bp (16s rRNA). Biological assay 1 is shown in lanes 2-7; biological assay 2 is shown in lanes 8-13 and biological assay 3 is shown in lanes 14-19. Lanes 2 and 3, 8 and 9, and 14 and 15 have RNA samples for WT duplicate for the respective biological assay; lanes 4 and 5, 10 and 11, and 16 and 17 have RNA samples for Δmtp duplicate for the respective biological assay; and lanes 6 and 7, 12 and 13, and 18 and 19 have RNA samples for *mtp*-complement duplicate for the respective biological assay. *, samples selected for DNase treatment and cDNA conversion.

Table S8. Concentration and purities of cDNA from the selected cultured bacterial samples after DNase treatment and cDNA synthesis for all three biological assays used for 16S rRNA PCR confirmation and RT-qPCR.

Sample	cDNA concentration (ng/ μ L)	A _{260/280}	A _{260/230}
Biological assay 1: WT 3	2577.1	1.78	2.21
Biological assay 2: WT 2	2634.6	1.77	2.19
Biological assay 3: WT 2	1927.6	1.80	2.22
Biological assay 1: Δmtp 2	2652.4	1.78	2.22
Biological assay 2: Δmtp 2	2726.8	1.76	2.18
Biological assay 3: Δmtp 1	1796.8	1.81	2.24
Biological assay 1: <i>mtp</i> -complement 1	2649.3	1.77	2.21
Biological assay 2: <i>mtp</i> -complement 3	2672.4	1.77	2.18
Biological assay 3: <i>mtp</i> -complement 2	1637.7	1.81	2.26

A_{260/280} or A_{260/230}: ratio of absorbance; WT: wildtype; Δmtp : *mtp*-deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain.

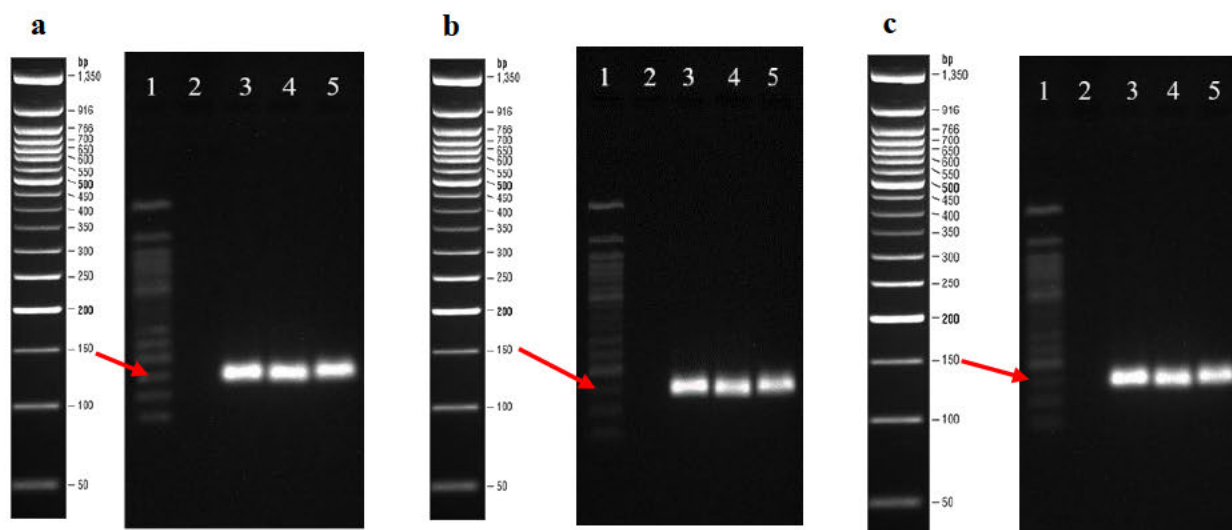


Figure S3. 16S rRNA PCR confirmation gels for cultured bacteria. All samples were run on 1.5% agarose gels for 90 minutes at 60 V. Each biological assay was run on separate gels. The expected product size of the 16S rRNA target was 151 bp, which was present for each strain in each biological assay. The molecular weight marker (Quick-Load Purple 50 bp DNA ladder) is in lane 1 of all gels. Lane 3 is the WT, lane 4 is the Δmtp , and lane 5 is the *mtp*-complement for (a) biological assay 1, (b) biological assay 2, and (c) biological assay 3, respectively.

Table S9. Concentration and purities of cDNA from the selected intracellular bacterial samples after DNase treatment and cDNA synthesis for all three biological assays used for 16s rRNA PCR confirmation and RT-qPCR.

Sample	cDNA concentration (ng/ μ L)	A _{260/280}	A _{260/230}
Biological assay 1: WT 1	2348.5	1.83	2.30
Biological assay 2: WT 1	2136.1	1.83	2.30
Biological assay 3: WT 1	2423.8	1.82	2.28
Biological assay 1: Δmtp 1	1642.4	1.84	2.32
Biological assay 2: Δmtp 1	2261.0	1.83	2.29
Biological assay 3: Δmtp 1	2039.2	1.83	2.30
Biological assay 1: <i>mtp</i> -complement 1	2278.8	1.82	2.28
Biological assay 2: <i>mtp</i> -complement 1	2044.4	1.83	2.30
Biological assay 3: <i>mtp</i> -complement 1	2259.7	1.83	2.29

A_{260/280} or A_{260/230}: ratio of absorbance; WT, wildtype Δmtp : *mtp*-deletion mutant; *mtp*-complement: complemented *mtp*-knockout strain.

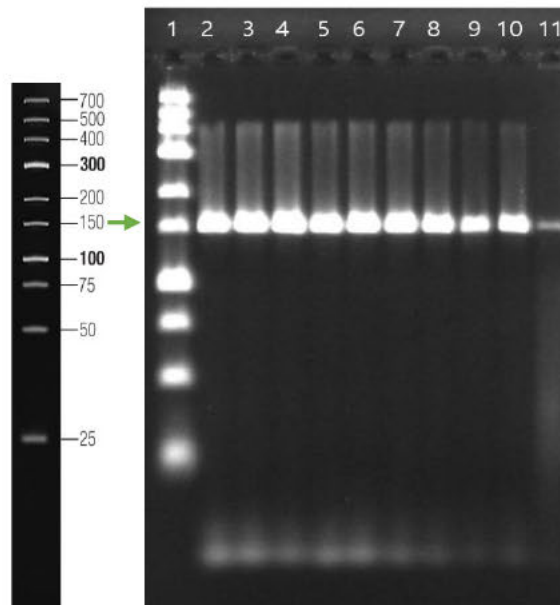


Figure S4. 16s ribosomal RNA confirmation of intracellular bacteria from THP-1 macrophage infection. The 3% agarose gel was run at 90 V for 75 minutes. GeneRuler low range DNA ladder was used as the molecular weight marker in lane 1. The expected product size is 151 bp. WT, Δmtp and *mtp*-complement form biological assay 1 is depicted in lanes 2, 3 and 4, respectively. WT, Δmtp and *mtp*-complement form biological assay 2 is depicted in lanes 5, 6 and 7, respectively. WT, Δmtp and *mtp*-complement form biological assay 3 is depicted in lanes 8, 9 and 10, respectively. A positive bacterial control was included in lane 11.

Table S10. Primer sequences, product sizes and annealing temperatures.

Gene name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
<i>Rv3723/lucA</i>	cgggtggtattcactttgct	cgacaggtagaacccaaga	234	61
<i>Rv2799</i>	aggggggtgtcgtgttcacc	gacgtcgaacgggatgtctc	235	60
<i>Rv0966c</i>	ctatgcggcaaccacctacc	gtcagcttcttcggcacgtt	171	61
<i>Rv0200/omamB</i>	tggtggtgttcgatgtcctg	acgacgcaacagccagaagt	158	61
<i>Rv0172/mce1D</i>	ggcaagggttaagcaaatcaa	ggtaacctgtcggtgaact	189	59
<i>Rv0655/mceG</i>	tgacatcgatcatggagaagc	gatcagctggctcaggtagg	196	60
<i>Rv0860/fadB</i>	gtcaaccaacgtgatgaacg	ggcttaccgaatgtctccaa	237	60
<i>Rv3546/fadA5</i>	catctgattgccgggttgat	actgggtcggcaggtcgata	154	60
<i>Rv0694/lldD1</i>	gggggtactgtgatgggattg	tcattcttctggggatcttg	228	59
<i>Rv1872c/lldD2</i>	accctgtctcagttcaaca	gaaactcgatgtcgcggaac	194	60
<i>Rv0211/pckA</i>	ccgagaagcacaagaactcc	acagaacggcaccacataca	206	61
16S rRNA	ggcgtgcttaacacatgcaa	catgcacccgtgtgtctat	151	60
<i>MTB000019/rrs</i>				

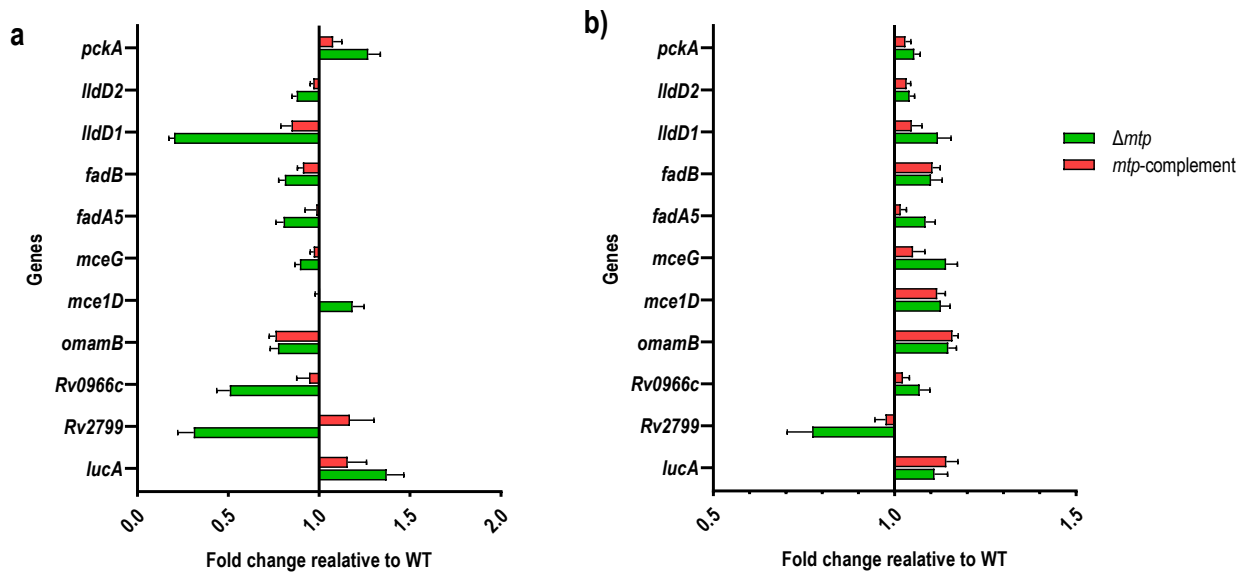


Figure S5. Fold changes of gene expression levels relative to the WT groups. The absolute quantification of gene expression was determined through RT-qPCR of the (a) cultured bacterial strains, and (b) intracellular bacterial strains isolated from THP-1 macrophages (WT V9124; Δmtp -deletion mutant; and *mtp*-complemented strain). Three biological assays and four technical repeats were conducted for each of the 11 selected genes (*lucA*, *Rv2799*, *Rv0966c*, *omamB*, *mce1D*, *mceG*, *fadA5*, *fadB*, *lldD1*, *lldD2*, and *pckA*). Gene expression levels were normalized to the housekeeping 16S rRNA for each gene of interest. Fold change is relative to the respective WT group for (a) and (b) and were represented by standard error mean (SEM) bars. A fold change of 1 denotes that gene expression levels were identical to the WT group. Fold changes lower than 1 denoted that the gene expression levels were lower than the WT group, and fold changes higher than 1 denoted that the gene expression levels were higher than the WT group.

Appendix G: Permissions to reproduce figures and tables



College of Health Sciences
School of Laboratory Medicine and Medical Sciences
Medical Microbiology

To whom it may concern

Dear Sir/Madam

2 December 2024

Permission granted to Ms Shinese Ashokcoomar for the use of a modified version of our previously published Table and figure in her thesis: “The molecular mechanisms of *Mycobacterium tuberculosis* curli pili (MTP) in regulation of fatty acid metabolism, carbon metabolism, and bioenergetics of the pathogen and host during infection”

I hereby grant Ms Shinese Ashokcoomar, my PhD student permission to reproduce modifications of the following published Table and Figure from publications in which I am the Corresponding/Senior Author.

The original figure “Fig. 2 The requirement of *mtp* in piliation. Transmission electron micrographs of negatively-stained *M. tuberculosis* V9124 wild-type (a), Δmtp mutant (b), and *mtp*-complemented (c) strains. Black arrows point to pili fibres. Scale bars 0.2 μm ” as it appeared in the published manuscript titled: “Pili contribute to biofilm formation in vitro in *Mycobacterium tuberculosis*” is as follows:

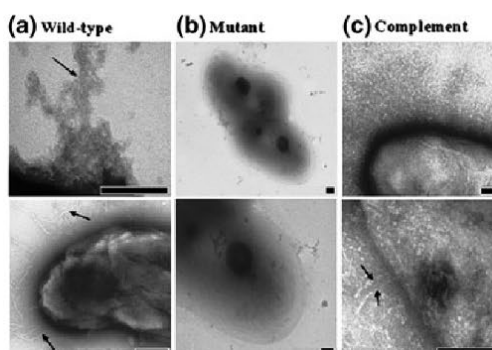


Fig. 2 The requirement of *mtp* in piliation. Transmission electron micrographs of negatively-stained *M. tuberculosis* V9124 wild-type (a), Δmtp mutant (b), and *mtp*-complemented (c) strains. Black arrows point to pili fibres. Scale bars 0.2 μm

The modified figure that Ms Ashokcoomar intends to reproduce is as follows:

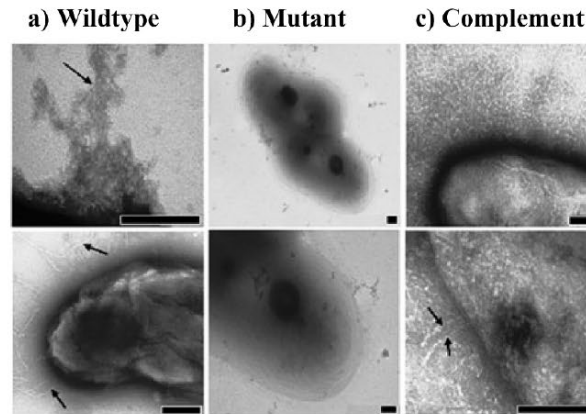


Figure 1.3. Electron micrographs of negatively stained *M. tuberculosis* strains with and without MTP pili. a) WT *M. tuberculosis* V9124 with MTP, b) Δmtp mutant without MTP, and c) *mtp*-complemented strain with MTP. Black arrows point to pili fibres. Scale bars 0.2 μm (Ramsugit et al., 2013).

The original Table “Table 1. A list of the currently known *Mycobacterium tuberculosis* adhesins” as it appeared in the publication titled: “Identification of *Mycobacterium tuberculosis* adherence mediating components: A review of key methods to confirm adhesin function” is as follows:

Table 1. A list of the currently known *Mycobacterium tuberculosis* adhesins

Adhesin	Gene (s)	Mediates adhesion to	Reference (s)
19-kDa antigen	<i>Rv3763</i>	Monocytes and macrophages	(24, 25)
Alanine- and proline-rich antigen (Apa)	<i>Rv1860</i>	Pulmonary surfactant protein A and macrophages	(24, 35)
Antigen 85 complex	<i>Rv0129c</i> , <i>Rv1886c</i> , <i>Rv3803c</i> , and <i>Rv3804c</i>	Fibronectin and macrophages	(17, 24)
Cpn60.2 molecular chaperone	<i>Rv0440</i>	Macrophages	(16, 36)
Curli pili	<i>Rv3312A</i>	Laminin, <i>M. tuberculosis</i> , macrophages, and epithelial cells	(13, 14, 19, 28)
DnaK molecular chaperone	<i>Rv0350</i>	Macrophages	(36)
Early secreted antigen ESAT-6	<i>Rv3875</i>	Laminin	(37)
Glutamine synthetase A1	<i>Rv2220</i>	Fibronectin	(23)
Glyceraldehyde-3-phosphate dehydrogenase	<i>Rv1436</i>	Possibly fibronectin (as occurs in group A streptococci)	(38)
Heparin-binding hemagglutinin adhesin	<i>Rv0475</i>	<i>M. tuberculosis</i> and epithelial cells	(12, 27)
Laminin-binding protein	<i>Rv2986c</i>	Laminin	(20)
L,D-transpeptidase	<i>Rv0309</i>	Fibronectin and laminin	(18)
Malate synthase	<i>Rv1837c</i>	Fibronectin, laminin, and epithelial cells	(21)
Membrane protein	<i>Rv2599</i>	Collagen, fibronectin, and laminin	(18)
<i>Mycobacterium</i> cell entry-1 protein	<i>Rv0169</i>	Epithelial cells	(26)
N-acetylmuramoyl-L-alanine amidase	<i>Rv3717</i>	Fibronectin and laminin	(18)
PE-PGRS proteins	<i>Rv1759c</i>	Fibronectin	(22)
	<i>Rv1818c</i>	<i>M. tuberculosis</i> and macrophages	(11)
Protein kinase D	<i>Rv0931c</i>	Brain endothelia and laminin	(15)
PstS-1 (38-kDa antigen)	<i>Rv0934</i>	Macrophages	(24)
Type IV pili	<i>Rv3654c-Rv3660c</i>	Possibly macrophages and epithelial cells	(39)

The modified table that Ms Ashokcoomar intends to reproduce is as follows:

Table 1.2. Currently known *M. tuberculosis* adhesins and their associated binding structure (Ramsugit and Pillay, 2016).

Adhesin	Mediates adhesion to
19-kDa antigen	Monocytes and macrophages
Alanine- and proline-rich antigen (<u>Apa</u>)	Pulmonary surfactant protein A and macrophages
Antigen 85 complex	Fibronectin and macrophages
Cpn60.2 molecular chaperone	Macrophages
Curli pili (MTP)	Laminin, macrophages and epithelial cells
<u>DnaK</u> molecular chaperone	Macrophages
Early secreted antigen ESAT-6	Laminin
Glutamine synthetase A1	Fibronectin
Glyceraldehyde-3-phosphate dehydrogenase	Possibly fibronectin (as occurs in group A streptococci)
Heparin-binding hemagglutinin adhesin	Epithelial cells
Laminin-binding protein	Laminin
<u>L,D</u> -transpeptidase	Fibronectin and laminin
Malate synthase	Fibronectin, laminin, and epithelial cells
Membrane protein	Collagen, fibronectin, and laminin
<i>Mycobacterium</i> cell entry-1 protein	Epithelial cells
N-acetylmuramoyl-L-alanine amidase	Fibronectin and laminin
PE-PGRS proteins	Fibronectin and macrophages
Protein kinase D	Brain endothelia and laminin
PstS-1 (38-kDa antigen)	Macrophages
Type IV pili	Possibly macrophages and epithelial cells

Sincerely



Yours faithfully

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Appendix H: Turnitin report

The similarity reports generated by Turnitin includes the abstract, introduction, Chapter 1, Chapter 5, Chapter 6, and Chapter 7. The report could not include Chapters 2, 3, and 4 since they are already published at the time of the Turnitin submission.

PhD Thesis: The molecular mechanisms of Mycobacterium tuberculosis curli pili (MTP) in regulation of fatty acid metabolism, carbon metabolism, and bioenergetics of the pathogen and host during infection

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