The altered fatty acylcarnitines, amino acids and organic acids detected in tuberculosis patient urine

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SUMMARY

Mycobacterium tuberculosis is estimated to infect approximately one-third of the world's population, which can lead to an active, symptomatic disease called tuberculosis (TB), or to asymptomatic states, often referred to as latent TB infection. In 2015 alone, 10.4 million new TB cases were reported, resulting in an estimated 1.8 million deaths. Since the discovery of *M. tuberculosis* in 1882 by Robert Koch, a vast amount of genomics, proteomics and transcriptomics data have been generated, leading to our current understanding of *M. tuberculosis* and TB. Most of the data generated from studies used *M. tuberculosis* cultures; however, it is well-known that this organism's metabolism and growth in culture differs greatly from growth in the human host, where many different growth mechanisms and energy substrates are preferentially used. Furthermore, very little research to date has focused on the adaptations of *M. tuberculosis* to the host's defence mechanisms or growth environment, or for that matter, the host's adaptations or altered metabolic state in response to the infectious pathogen. This is important since the pathophysiology of *M. tuberculosis* is directly linked to its metabolism and complex physiology, and to that of the host. Additionally, this pathogen can utilise numerous growth substrates, either by scavenging this from the host or via *de novo* biosynthesis, in order to ensure its own survival.

Metabolomics has served well to expand the current knowledge of the disease and has contributed towards the improved diagnosis and treatment thereof, due to its unique capacity for identifying new disease biomarkers. Metabolomics is defined as the unbiased identification and quantification of the entire metabolome in a specific biological system, with the use of highly advanced analytical instruments, together with various statistical, computational and mathematical analyses. Metabolomics has enabled the identification of new metabolite markers in sputum, blood and urine from TB patients, describing novel *M. tuberculosis* metabolic pathways and host adaptations. Apart from their possible diagnostic applications, many of these new TB metabolite markers have contributed to the existing knowledge of the biology of the causative pathogen, including various underlying disease mechanisms related to *M. tuberculosis* drug resistance and virulence, as well as the mechanisms of TB drug action and related side-effects in the host. To date, however, very little data has been published on urine from TB patients, which can be considered an ideal sample matrix to identify markers associated with this host–pathogen interaction.

Considering this, in this investigation, a combined semi-targeted liquid and gas chromatography mass spectrometry metabolomics approach was used to compare the urinary fatty acylcarnitines, amino acids and selected organic acids of active TB patients with that of healthy individuals, in order to better characterise the TB-induced alterations to the

host metabolome. The generally elevated concentrations of the fatty acylcarnitines and amino acids are most likely due to TB-cachexia. However, the significantly elevated concentrations of arginosuccinate, asparate (and associated asparagine), ornithine (and associated proline and hydroxyproline) and glutamate (and associated glutamine) in particular, indicate a urea cycle abnormality, due to inhibition of N-acetylglutamate synthase by the accumulating propionyl-CoA, isovaleryl-CoA and methylmalonyl-CoA in TB patients. Furthermore, elevated propionylcarnitine, methylmalonate and methylcitrate in the TB patient urine are associated with a vitamin B_{12} deficiency, which deserves further investigation. Lastly, various metabolites indicative of lactic acidosis, ketoacidosis, oxidative stress and liver damage were identified in the urine of the TB patients.

Key words: metabolomics; tuberculosis; fatty acylcarnitines; amino acids; organic acids

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LIST OF ABBREVIATIONS

Abbreviation	Meaning	Abbreviation	Meaning
AcPIM	acyl phosphatidylinositol dimannoside	AG	arabinogalactan
ATP	adenosine triphosphate	BCG	Bacille Calmette-Guerin
BSTFA	N,O-Bis(trimethylsilyl) trifluoroacetamide	C_0	free carnitine
C_2	acetylcarnitine	C_3	propionylcarnitine
C_5	Isovalerylcarnitine / valerate	C_{10}	decanoylcarnitine
C_8	octanoylcarnitine	C_{14}	tetradecanoylcarnitine
C_{12}	dodecanoylcarnitine	C_{17}	heptadecanoate
C_{16}	palmitoylcarnitine	CTLA	cytotoxic T lymphocyte-associated protein
CD	cluster of differentiation	DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DAP	L-alanyl-D-iso-glutaminyl-meso-diaminopimelic acid	EMB	ethambutol
DOTS	directly observed treatment short-course	GC	gas chromatography
FAS	fatty acid synthase	HIV	human immonodeficiency virus
HCl	hydrocloric acid	IGRA	interferon gamma release assay
IFN-γ	interferon gamma	INH	isoniazid
IL	interleukin	LC	liquid chromatography
LAM	lipoarabinomannan	*M. bovis*	*Mycobacterium bovis*
LM	lipomannan	mAGP	mycolyl arabinogalactan-peptidogalycan complex

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Abbreviation	Meaning	Abbreviation	Meaning
M. tuberculosis	*Mycobacterium tuberculosis*	MDR-TB	multidrug-resistant tuberculosis
ManLAM	mannosylated lipoarabinomannan	MHC	major histocompatibility complex
MetH/E	methionine synthase	MutAB	methylmalonyl-CoA mutase
MS	mass spectrometry	NAGS	N-acetylglutamate synthase
Na_2SO_4	anhydrous sodium sulphate	NrdZ	ribonucleotide reductase
NMR	nuclear magnetic resonance	NWU	North-West University
PG	peptidoglycan	PKC	protein kinase C
PPD	purified protein derivative	PZA	pyrazinamide
QC	quality control	RIF	rifampicin
SP	surfactant protein	TB	tuberculosis
TCA	tricarboxylic acid	TGF-β	transforming growth factor beta
TLR	toll-like receptor	Th	T helper
TNF-α	tumour necrosis factor alpha	TMCS	trimethylchlorosilane
TST	tuberculin skin test	TOFMS	time-of-flight mass spectrometry
XDR-TB	extensively drug-resistant tuberculosis	WHO	World Health Organization

CHAPTER 1:
PREFACE

1.1 BACKGROUND AND MOTIVATION

Tuberculosis (TB) is a deadly, infectious disease resulting in a reported mortality of 1.5 million in 2015. Globally, approximately 10.4 million new TB cases are reported annually, of which 1.2 million are co-infected with the human immunodeficiency virus (HIV). Additionally, the suboptimal diagnostic and treatment approaches for TB are a major concern, contributing to rapid transfer and infection, disease progression and development of drug resistance. In 2015, 520 000 and 55 100 new cases of multidrug-resistant (MDR)-TB and extensively drug-resistant (XDR)-TB were reported globally, respectively. South Africa currently ranks sixth among the so-called 22 high-burden TB countries, ranks second with regards to the number of reported MDR-TB cases, and ranks number one for the amount of reported individuals with HIV/TB co-infection (Churchyard *et al.*, 2014; World Health Organization, 2016). The fact that 49 million deaths was reported globally from 2000 to 2015 due to both TB and MDR-TB, attests to the short comings of the current TB diagnostic and treatment approaches (World Health Organization, 2016). It is clear that there is an urgent need for new, sensitive, rapid and efficient diagnostic tests, not only to diagnose TB, but also to detect drug resistance and HIV co-infection, allowing for the timely treatment of TB and MDR-TB. The incomplete understanding of *M. tuberculosis* in the host, and its adaptations to the host's immune response, and vice versa, is currently one of the major limitations towards the development of more efficient diagnostic tests, vaccines and treatment approaches.

Considering the above, despite the fervent genomics, proteomics and transcriptomics research efforts to date since the discovery of the TB disease-causing pathogen, *M. tuberculosis*, in 1882 by Robert Koch, TB is still considered a global pandemic. Better elucidation of the disease mechanisms associated with TB, the pathogen's adaptations to the host immune response, and that of TB drugs, are still as relevant today as it was 135 years ago. Investigating this disease using an "omics" research perspective, the most recent of which being metabolomics, has served well to expand the current knowledge of the disease and the improved diagnosis and treatment thereof, due to its unique capacity for identifying new disease metabolite (bio)markers.

1.2 AIM AND OBJECTIVES

1.2.1 Aim

In this investigation, a combined semi-targeted liquid and gas chromatography mass spectrometry metabolomics approach was used to compare the urinary fatty acylcarnitines, amino acids and selected organic acids of active TB patients (n=31) and healthy individuals (n=29), to better characterise the TB-induced alterations to the host metabolome.

1.2.2 Objectives

Considering the above mentioned aim, the objectives of this study are to:

1. Compare the urinary fatty acylcarnitines of TB-positive and healthy control individuals, using a standardised, validated semi-targeted liquid chromatography mass spectrometry (LC-MS/MS) metabolomics approach.

2. Compare the urinary amino acids of TB-positive and healthy control individuals, using a standardised, validated semi-targeted amino acid extraction procedure followed by analysis using a gas chromatography mass spectrometry (GC-MS) metabolomics approach.

3. Compare specific urinary organic acids of TB-positive and healthy control individuals, using a standardised, validated semi-targeted organic acid extraction procedure followed by analysis using a two-dimensional gas chromatography time-of-flight mass spectrometry (GCxGC-TOFMS) metabolomics approach.

1.3 STRUCTURE OF DISSERTATION AND RESEARCH OUTPUTS

This dissertation complies with the requirements of the North-West University (NWU; Potchefstroom Campus), South Africa, for the completion of the degree Magister Scientiae (Biochemistry) in article format. Hence, a comprehensive literature overview (Chapter 2) and final conclusions (Chapter 5), along with a reference list at the end of each chapter are provided in accordance with these guidelines. The articles which emanated from this work are attached in Appendix B.

Chapter 1 (the current chapter) provides a brief background, and the aim and objectives of this study. The structure of the dissertation and the contributions made by all co-authors, co-workers and collaborators are also specified.

Chapter 2 provides a literature overview of the general concepts relevant to this investigation and metabolomics application to TB research, as a basis for better understanding this disease and the results/discussions in the following chapters.

Chapter 3 provides a brief explanation for using urine as an appropriate matrix in this investigation, and provides an overview/summary of the experimental design.

In Chapter 4, is the scientific paper that was written and submitted to *Clinical Infectious Diseases* (manuscript number: CID-88284) (see Appendix B). As stipulated by this journal, this chapter contains a structured Abstract, Introduction (literature background), Materials and Methods, Results and Discussion, and Conclusion section, and subsequently describes relevant scientific information pertaining to this investigation, in a concise manner.

Chapter 5 is the final conclusions considering all the results as a whole, in the context of the original aim, and also highlights future prospects.

Appendix A contains the patient descriptive/demographic and clinical information.

Appendix B contains the two scientific manuscripts to which the primary author contributed during this M.Sc. relating to the topic of investigation:

- Anthony, C., Luies, L. Mienie, J.L., Lindeque, J.Z., Ronacher, K., Walzl, G. & Loots, D. (2018). Detection of altered fatty acylcarnitines, amino acids and organic acids in tuberculosis patient urine. Submitted for publication to *Clinical Infectious Diseases* (manuscript number: CID-88284).
- Luies, L., Mienie, J., Motshwane, C., Ronacher, K., Walzl, G. & Loots, DT. (2017). Urinary metabolite markers characterising tuberculosis treatment failure. *Metabolomics*, 13 (10): 124.

1.4 AUTHOR CONTRIBUTIONS

The primary author/investigator is Christinah M.M. Anthony (née Motshwane). All co-authors, co-workers and collaborators, as well as their contributions made towards this work, are listed in Table 1.1. The following statement from the study promotors and primary author confirm their respective roles in this study, and give permission that the data generated and conclusions made may form part of this dissertation: I declare that my role in

this study, as indicated in Table 1.1, is a representation of my actual contribution, and I hereby give my consent that this work may be published as part of the M.Sc. dissertation of Christinah M. M. Anthony.

Prof. Du Toit Loots

Mrs. Christinah M.M. Anthony

Dr. J. Zander Lindeque

Dr. Laneke Luies

Table 1.1: The research team.

Co-author / co-worker / collaborator	Contribution
Mrs. Christinah M. M. Anthony (B.Sc. Hons. Biochemistry)	Responsible for project planning, data analyses and writing of this dissertation, as well as all other documentation and the publication associated with this study.
Prof. Du Toit Loots (Ph.D. Biochemistry)	Served as supervisor, and supervised all aspects of this study, including the project design, planning, writing of this dissertation, as well as all other documentation and the publication associated with this study.
Dr. J. Zander Lindeque (Ph.D. Biochemistry)	Served as co-supervisor, and supervised aspects relating to sample analysis, as well as all other documentation and the publication associated with this study.
Dr. Laneke Luies (Ph.D. Biochemistry)	Served as co-supervisor, and supervised writing of this dissertation. Performed the organic acid sample analysis used in Chapter 4 and assisted with writing of the publication.
Prof. L. Japie Mienie (Ph.D. Biochemistry)	Assisted with the interpretation of Chapter 4 and writing of the publication.
Mrs. Mari van Reenen (M.Sc. Statistics)	Assisted with statistical analysis of the data obtained in the study.
Potchefstroom Laboratory of Inborn Errors of Metabolism (PLIEM)	Determined the creatinine values of all patient collected urine samples.
Profs. Gerhard Walzl and Katharina Ronacher from the DST/NRF Centre of Excellence for Biomedical Tuberculosis Research/MRC Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University (Tygerberg), Cape Town, South Africa	Provided the patient urine samples used in this study.

1.5 REFERENCES

Churchyard, G., Mametja, L., Mvusi, L., Ndjeka, N., Hesseling, A., Reid, A., Babatunde, S. & Pillay, Y. 2014. Tuberculosis control in South Africa: Successes, challenges and recommendations. *SAMJ: South African Medical Journal*, 104(3):234-248.

World Health Organization. 2016. Global tuberculosis report 2016. Geneva, Switzerland (WHO Press).

CHAPTER 2:
LITERATURE STUDY

2.1 A BRIEF HISTORY OF TUBERCULOSIS

Despite fervent research efforts to date since the discovery of the tuberculosis (TB) disease-causing pathogen, *Mycobacterium tuberculosis*, TB is still considered a global pandemic, resulting in approximately 1.8 million deaths annually (World Health Organization, 2016). Over the past thirty years, TB has been a recrudescence even in countries where it was considered eradicated. This deadly pathogen belongs to the *Mycobacterium* genus, believed to have originated 150 million years ago (Daniel, 2006), and includes other genetically related *Mycobacterium* species known to cause TB in humans or other mammals, referred to as the *Mycobacterium tuberculosis* complex (MTBC). A schematic timeline of the history of the disease is given below in Figure 2.1. The MTBC is presumed to have originated in the Horn of Africa approximately 3 million years ago (Daniel, 2006; Gutierrez *et al.*, 2005), where it is believed to have coevolved with ancient hominids and have spread worldwide due to migration (Blouin *et al.*, 2012; Daniel, 2006). Ancient *M. tuberculosis* dates back approximately 40 000 years ago (Daniel, 2006), hence scientists have referred to TB as the first disease known to mankind. Throughout millennia TB has been known by various names across different cultures, including “phthisis”, “the white plague”, “romantic disease” and “consumption”, all of which reference its "drying" or "consuming" effect. The term “tuberculosis” first appeared in 1860, referring to the formation of tubercles/granulomas in infected individuals.

M. tuberculosis strains are classified into seven lineages, or spoligotype clades, each associated with specific geographical area, namely: Lineage 1, Indo-Oceanic (including the Manila family, East African-Indian, and some Manu/Indian strains); Lineage 2, East-Asian (including Beijing); Lineage 3, Delhi/Central-Asian Strains; Lineage 4, Euro-American (including the Latin American-Mediterranean, Ghana, Haarlem, X type, and T families); Lineage 5 and Lineage 6, West African 1 and 2, respectively (both of which correspond to *Mycobacterium africanum*); and the newly reported Lineage 7 (Brudey *et al.*, 2006; Comas *et al.*, 2013; Yimer *et al.*, 2015), which has been proposed as “Aethiops vetus” owing to its place of origin (Nebenzahl-Guimaraes *et al.*, 2016). These major lineages are predicted via spacer oligonucleotide typing (spoligotyping), which identifies polymorphisms occurring in direct repeat regions of the chromosomes in MTBC bacteria, which relate to a specific geographical pattern, allowing for strain discrimination (Sebban *et al.*, 2002).

Hershkovitz *et al*. 2008, used conventional PCR to examine bone samples showing evidence of typical TB infection. These human remains were from a woman and her infant buried at Atlit-Yam, a now submerged ancient Neolithic village in the Eastern Mediterranean, dating back over 9 000 years ago. DNA evidence was consistent with current genetic lineages, which was confirmed using high performance liquid chromatography detecting mycolic acids specific to the MTBC. These findings are the oldest evidence of human TB infection to date (Hershkovitz *et al.*, 2008; Hershkovitz *et al.*, 2015). Human TB infection was also confirmed in Neolithic bone remains discovered in Heidelberg (T'ao, 1942). In 2014, Bos *et al*. applied two independent dating techniques to analyse the mycobacterial genomes of three 1 000-year-old human skeletons from southern Peru, of the Chiribaya culture. These ancient strains are distinct from current human-adapted forms and were found to be closely correlated to those adapted to seals and sea lions, which are believed to have transmitted TB across the different continents (Bos *et al.*, 2014). This notion is supported by DNA evidence suggesting that animals contracted TB from humans, which may be linked to animal domestication and farming, since *Mycobacterium bovis* was found to be either descended from ancient *M. tuberculosis*, or have evolved independently. Furthermore, modern *M. tuberculosis* strains seem to have originated 20 000–15 000 years ago (Daniel, 2006; Sreevatsan *et al.*, 1997).

As illustrated in Figure 2.1, signs of early *M. tuberculosis* infection have been confirmed in Egypt (c. 3 000 BCE), India (c. 1290 BCE), China (c. 1 300 BCE) and South America (c. 500 CE) (Daniel, 2006; T'ao, 1942). A clinical description of TB was provided by Hippocrates, which included fever, a mucus-producing cough, colourless urine, loss of appetite and mental deliria (Daniel, 2006). Over the course of its existence, the exact nature of TB was the subject of heated debate as some believed physiological and inherited factors predispose individuals to TB, whilst others thought it was contagious (Saviola & Bishai, 2006). The infectious nature of TB was first proposed by Benjamin Marten, in 1720, suggesting that a microscopic organism was at fault. This was only demonstrated in 1868 by Jean Antoine Villemin, who inoculated rabbits with sputum obtained from infected humans; however, the exact etiological agent was still unknown (Daniel, 2006; Saviola & Bishai, 2006). Finally, on 24 March 1882, Robert Heinrich Hermann Koch became the first individual to isolate the slow-growing causal agent of TB, *M. tuberculosis* (also known as Koch's bacillus), by applying a new staining method to sputum collected from TB patients. Additionally, Koch was able to grow *M. tuberculosis* in pure culture, infect guinea pigs to observe their symptoms, and reisolate this pathogen from these guinea pigs. Shortly hereafter, Koch also demonstrated that *M. tuberculosis* was the sole cause of all disseminated forms of TB (Daniel, 2006; Koch, 1982). In 1890, Koch also announced his discovery of tuberculin as a means of immunisation and cure, however this was quickly discredited. Nevertheless, in 1907, Clemens Freiherr von Pirquet (who coined the terms "allergy" and "allergen") continued on the work of Koch, and found tuberculin was an effective diagnostic test for TB

when injected intracutaneously using a vaccination lancet. After his observations in healthy, asymptomatic children who reacted to tuberculin, he concluded that TB also exists in a latent form. In the following year, Charles Mantoux described the intracutaneous injection of tuberculin using a syringe and cannulated needle, and Florence Seibert developed purified protein derivative (PPD) in the 1930s, resulting in the tuberculin skin test used to detect *Mycobacterium* infection today (Daniel, 2006; Nayak & Acharjya, 2012).

In 1921, Albert Calmette and Camille Guérin used the first effective immunisation agent against TB on humans. This vaccine, called "BCG" (Bacille Calmette-Guérin), was developed from attenuated *M. bovis* (Daniel, 2006). However, the true medical revolution came with the discovery of streptomycin in 1944, as the first effective antibiotic against *M. tuberculosis*, followed by isoniazid (1951), pyrazinamide (1952), rifampicin (1957) and ethambutol (1962). Unfortunately, the first observations of drug resistance followed shortly, an occurrence which remains problematic at present, or perhaps even more so today due to the increase in MDR-TB strains over the last couple of years, especially in developing countries (Keshavjee & Farmer, 2012).

"If the importance of a disease for mankind is measured from the number of fatalities which are due to it, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera, and the like."

— Die Ätiologie der Tuberculose, famously presented by Robert Koch in 1882

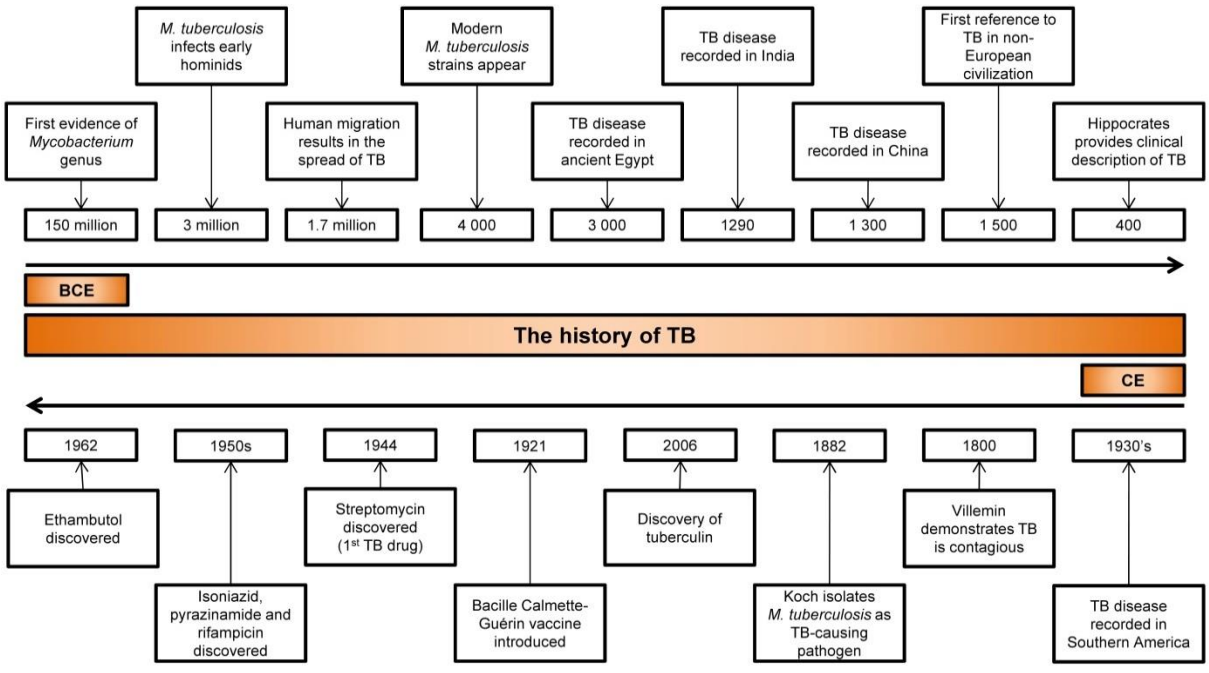

Figure 2.1: A Timeline of the history of TB, including its evolvement, spread and significant discoveries.

2.2 TUBERCULOSIS BACTERIOLOGY

M. tuberculosis is a small, rod-shaped airborne organism, measuring approximately 0.5 x 3 µm (Brennan, 2003). The cell wall of *M. tuberculosis* consists of (1) an outer layer (capsule), (2) a mycolyl arabinogalactan-peptidoglycan (mAGP) complex, and (3) an inner layer (see Figure 2.2) (Hett & Rubin, 2008). This thick, multi-layered *M. tuberculosis* envelope is mainly composed of various lipids (over 60%) that function as a permeability barrier, and is hence crucial for its survival within the host and also responsible for its virulence and persistence (Brennan & Nikaido, 1995).

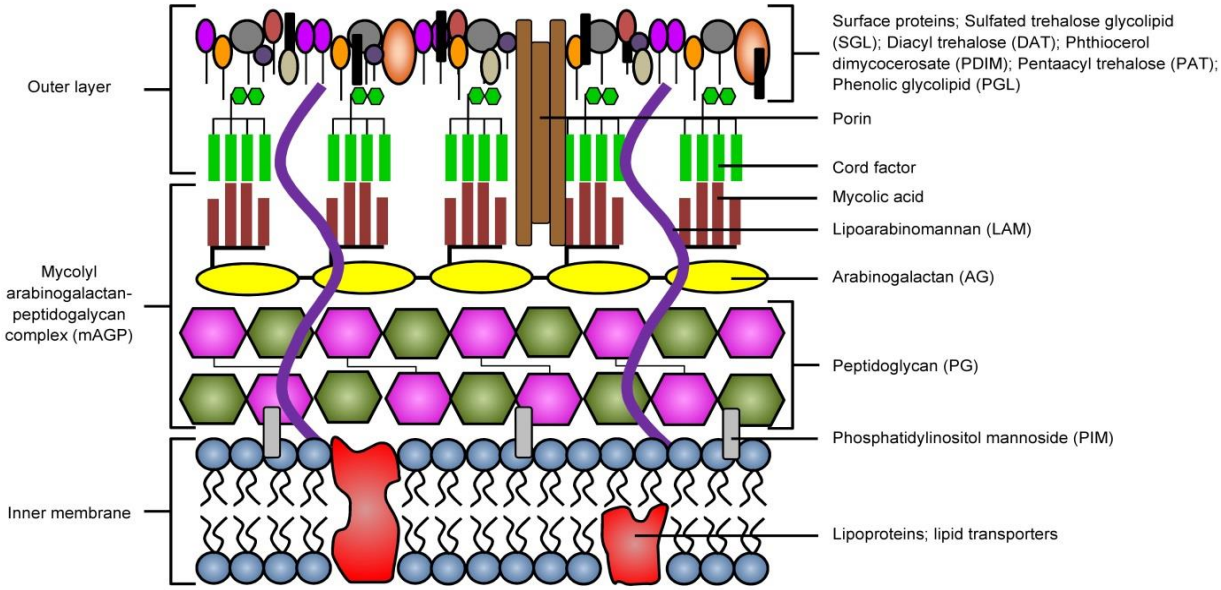

Figure 2.2: Simplified representation of the multi-layered M. tuberculosis cell wall.

Just outside the inner phospholipid layer, a number of peptidoglycan (PG) polymers are covalently bound to arabinogalactan (AG), which in turn is esterified with long-chainmycolic acids, forming the mAGP complex, which confers cell wall rigidity, resistance and impermeability (Brennan & Nikaido, 1995). PG has a mesh-like appearance and is considered the skeleton of the cell wall as it mainly contributes to cell wall rigidity, integrity and maintaining the shape, allowing the bacteria to resist osmotic pressure (Alderwick *et al.*, 2015; Van Heijenoort, 1998). PG contains peptides and glycan strands, which are composed of repeating N-acetylglucosamines regions, linked to N-acetylmuramic acid. The L-alanyl-D-iso-glutaminyl-meso-diaminopimelic acid (DAP) from one peptide chain, is linked to the terminal D-alanine residue from the L-alanyl-D-iso-glutaminyl-meso-DAP-D-alanine of another chain (Kotani *et al.*, 1970; Wietzerbin *et al.*, 1974). PG is surrounded by AG, which in turn is esterified to mycolic acids. The galactan found in AG is synthesized via galactofuranosyl transferase and is modified with long arabinan polymers (Makarov *et al.*,

2009), while the arabinan is modified by the addition of succinyl or non-acetylated galactosamine moieties (Crick *et al.*, 2001). These modifications are found mostly in pathogenic mycobacteria, and presumed to play a role in an accelerating the infection process (Kaur *et al.*, 2009). Mycolic acids comprise the majority of the cell wall structure and are responsible for the thick, waxy lipid coat that contributes to cell wall impermeability (Hett & Rubin, 2008). Mycolic acids are synthesized from β-hydroxylated α-alkyl-branched very long chain fatty acids (Kieser & Rubin, 2014) and have various functional groups attached. These mycolic acids produce three meromycolates (i.e. alpha-meroacids, methoxy-meroacids and keto-meroacids) with various levels of saturation, cyclopropanation and oxygenation, and are also considered important for virulence of the mycobacteria (Barry *et al.*, 2007). These mycolic acids are synthesized via two fatty acid synthase (FAS) systems, known as FAS-I (Boehringer *et al.*, 2013) and FAS-II (Gago *et al.*, 2011). FAS-I is involved in the condensation of medium-chain fatty acids (Asselineau *et al.*, 2002), whereas FAS-II is made up of interconnected protein complexes (Cantaloube *et al.*, 2011).

The outer layer (or capsule) is the first surface of the mycobacteria to interact with the host immune cells (i.e. macrophages and neutrophils), and hence it is considered the most important virulence factor for *M. tuberculosis* survival within its host (Stokes *et al.*, 2004). This layer is composed of free mycolic acids, including mycolic acid esters, lipomannan (LM), lipoarabinomannan (LAM) (Brennan, 2003), and various phospholipids including: phosphatidylinositol (PI), phosphatidylethanolamine (PE), cardiolipin, trehalose monomycolate and diacyl phosphatidyldimannoside (Bansal-Mutalik & Nikaido, 2014). Cord factors, also known as trehalose 6,6'-dimycolate or trehalose monomycolate, are esters of mycolic acids present in mycobacteria and contribute to bacterial virulence by inducing granulomatous reactions and preventing phagolysosomal fusion. The main function of the aforementioned cord factors, is the transfer of mycolic acids onto the arabinosyl units (Minnikin *et al.*, 2015). LM and LAM are long mannose polymer skeletons, the latter of whichis associated with pathogenic functionality, allowing for *M. tuberculosis* survival within host macrophages (Knechel, 2009). Additionally, LAM has also been shown to down-regulate the host immune response against *M. tuberculosis* via host protein kinase C (PKC) inhibition (Todar, 2009). Decreased PKC has been shown to exaggerated mortality in mice, not only resulting in an increased mycobacterial burden, but also leading to the uncontrolled proinflammatory cytokine responses, subsequently reducing alveolar macrophages, dendritic cells, and lipids (Parihar *et al.*, 2017). PI mannosides with up to four mannose residues, also present in the mycobacterial cell wall, are also thought to influence the interaction between the host's immune system and *M. tuberculosis* (Bansal-Mutalik & Nikaido, 2014). This polar lipid, together with PE and diphosphatidylglycerol, forms the basis of the membrane bilayer. Most mycobacteria have a family of four phosphatidylinositol mannosides (PIMs), which are comprised of mono- and diacyl phosphatidylinositol dimannosides ($AcPIM_2$ and Ac_2PIM_2,

respectively), as well as mono- and diacyl phosphatidylinositol hexamannosides ($AcPIM_6$ and Ac_2PIM_6, respectively). These PIMs act to form a bilayer environment of very low fluidity which slows drug influx, contributing to the general drug resistance phenotype of mycobacteria (Bansal-Mutalik & Nikaido, 2014; Minnikin *et al.*, 2015).

2.3 TUBERCULOSIS PATHOPHYSIOLOGY AND HOST IMMUNE RESPONSE

TB is transmitted when an individual with active TB coughs, sneezes, speaks, sings, and/or spits, resulting in the release of infectious aerosol droplets (usually 1–5 µm in diameter) containing live bacilli (Frieden & Driver, 2003; Knechel, 2009). Once inhaled into the respiratory tract of another individual, most of these droplets are trapped in mucus secreted by goblet cells as part of the host's mucociliary first-line of defence, tasked with preventing/blocking the entering of foreign matter. Hence, the invading bacteria are then be expelled via coughing up this mucus (Hurley, 2015). However, some bacilli bypass this defence and enter into the alveoli of the lungs, where they rapidly replicate. The host's innate immune response utilises four types of immune cells, namely: (1) macrophages (Armstrong & Hart, 1971; Guirado *et al.*, 2013; Srivastava *et al.*, 2014), (2) neutrophils (Ong *et al.*, 2015; Segal, 2005), (3) natural killer cells (Rothchild *et al.*, 2014) and (4) dendritic cells (Abraham & Medzhitov, 2011). This in turn triggers the host's non-specific innate second-line of defence via the activation of various mechanisms (Allen *et al.*, 2015), which include: (1) granulocyte or macrophage colony stimulating factors (Ballas *et al.*, 2012), (2) pattern recognition receptors (Baravalle *et al.*, 2011), (3) myeloid differentiation primary response proteins (MYD88) (Boussiotis *et al.*, 2000), (4) ligands (Kawai & Akira, 2010; Kleinnijenhuis *et al.*, 2011), (5) nucleotide oligomerization domain-like receptors (Kumar *et al.*, 2013; Oviedo-Boyso *et al.*, 2014), (6) Dectin-1 (Plato *et al.*, 2013), and (7) the complement receptors (Ferguson *et al.*, 2004). This host "pathogen recognition" immune response initiates phagocytosis of the infecting mycobacteria by alveolar host macrophages as a means to eradicate these bacteria, via the excretion of various enzymes and pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α) (Sasindran & Torrelles, 2011), interleukin (IL)-1 (Krumm *et al.*, 2014), IL-6 (Ilonidis *et al.*, 2005), IL-12 (Tung *et al.*, 2010), IL-17 (Khader & Cooper, 2008), IL-23 (Khader & Cooper, 2008) and interferon gamma (IFN-γ) (Sasindran & Torrelles, 2011; Simmons *et al.*, 2010). This host cell-mediated immune response results in the formation of a granuloma via T lymphocyte accumulation, restricting the replication and further spread of the bacilli (Cooper *et al.*, 2011). A granuloma is a collection of histiocytes, epithelioid cells, Langerhans giant cells and lymphocytes, and is usually necrotic. However, in response to this second-line of host defence against the invading organism, *M. tuberculosis* may respond by various processes which limits/subdue host-induced inflammation via an anti-inflammatory response preventing the production of

reactive oxygen and nitrogen species, as well as by increasing the pH of the *M. tuberculosis*-containing macrophages (Flynn & Chan, 2001). The host's innate immune response utilises four types of immune cells, namely: (1) macrophages (Armstrong & Hart, 1971; Guirado *et al.*, 2013; Srivastava *et al.*, 2014), (2) neutrophils (Ong *et al.*, 2015; Segal, 2005), (3) natural killer cells (Rothchild *et al.*, 2014) and (4) dendritic cells (Abraham & Medzhitov, 2011). The subsequent adaptive immune response activates, proliferates and creates very specific immune mechanisms for neutralising or eliminating the invading pathogen. Although this differs from the more general innate immune, there is a strong interaction or interdependency between the two immune responses, which is facilitated by the macrophages and dendritic cells. In order to better understand this section, a schematic outline of these processes is given in Figure 2.3.

When the host's mannose- and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptors recognise the mannosylated LAM (ManLAM) and PIMs expressed on the *M. tuberculosis* cell wall (as described in section 2.2), an anti-inflammatory host response may be activated (Geijtenbeek *et al.*, 2003; Lugo-Villarino *et al.*, 2011). These DC-SIGN receptors have the ability to suppress the host immune response via the acetylation of nuclear factor kappa beta (NF-κB) subunit p65 (Gringhuis *et al.*, 2007), however, only in the presence of simultaneous TLR stimulation (Konieczna *et al.*, 2015). Furthermore, host collectins (also known as collagen containing C-type lectins) function to eliminate pathogens via aggregation, complement activation, opsonisation, phagocytosis and by modulating both the inflammatory and adaptive immune response (Van de Wetering *et al.*, 2004). Surfactant protein (SP)-A, SP-D and mannose-binding lectin are collectins that specifically modulate the host inflammatory response, and are expressed in response to the sugar moieties expressed on the *M. tuberculosis* cell wall (Wright, 2005). SP-A and SP-D function to maintain the balance between the pro- and anti-inflammatory responses to *M. tuberculosis* (Sasindran & Torrelles, 2011). SP-A has been shown to have a good association/response with *M. tuberculosis* and can act as an opsonin, enhancing macrophage phagocytosis to some extent, and mediate inflammation by regulating the synthesis of reactive oxygen species and cytokine secretion (Torrelles *et al.*, 2008). Recent studies have also illustrated that SP-A may assist in the proliferation of *M. tuberculosis*. Following pathogen recognition however, *M. tuberculosis* is opsonised, which may result in the secretion of anti-inflammatory cytokines suppressing the host's cell-mediated immunity (Samten *et al.*, 2008), which in turn leads to increased mannose receptor expression, limiting phagosome maturation. SP-D, on the other hand, shows high affinity for *M. tuberculosis* ManLAM and PIMs, and initiates phagolysosomal fusion in order to limit intracellular bacterial growth. However, SP-D can also be manipulated by *M. tuberculosis*, reducing the capacity of host macrophage phagocytosis, subsequently increasing bacterial proliferation (Torrelles *et al.*, 2008). The anti-inflammatory cytokines IL-10 (Roilides *et al.*, 1997), IL-4 (Gibson *et al.*,

2003) and transforming growth factor beta (TGF-β) (Aung *et al.*, 2005) are associated with these processes. Hence, *M. tuberculosis* may avoid death by inhibiting phagolysosomal fusion, persisting in a slowly or non-replicating state (Sasindran & Torrelles, 2011).

The adaptive immune response is initiated via antigen presentation, followed by co-stimulation and cytokine production (Van Crevel *et al.*, 2002). Antigen presentation occurs via four mechanisms, namely (1) the major histocompatibility complex (MHC)-I molecules expressed on nucleated cells present mycobacterial proteins to antigen-specific CD8+ T cells and cytosolic antigens in the phagosome (Mazzaccaro *et al.*, 1996), (2) the MHC-II molecules present mycobacterial proteins to antigen-specific CD4+ T cells, which are processed in phagolysosomal compartments (Van Crevel *et al.*, 2002), (3) the non-polymorphic MHC-I molecules expressed (type-1 CD-1 [-a, b and c]) on macrophages and dendritic cells present mycobacterial lipoproteins to CD-1-restricted T cells, resulting in the activation more T cells in the early stages of infection, even before antigen presentation has matured (Van Crevel *et al.*, 2002), and (4) a mechanism involving the MHC-1b protein, however this process is not well understood (Lewinsohn *et al.*, 1998). These antigen presentation mechanisms, however, only lead to a T cell response in the presence of specific co-stimulatory signals. These co-stimulatory molecules are expressed on dendritic cells and macrophages, and bind to CD28 and cytotoxic T lymphocyte-associated protein (CTLA)-4 molecules on the T cells (Saha *et al.*, 1994). Antigen presentation is also regulated by cytokines, where pro-inflammatory cytokines stimulate MHC expression, and anti-inflammatory cytokines inhibit its expression (Van Crevel *et al.*, 2002). The cytokines produced by the macrophages and dendritic cells for this purpose include the type-1 cytokines; IL-12 (which induces IFN-γ production and drives T helper response), and IL-18 and IL-23 (which activate memory T cells) (Oppmann *et al.*, 2000). These play a crucial role in the host defence against mycobacteria, confirmed by the observation that a mutation in the genes coding for IL-12p40, IL-12Rβ1, IFNY-γ receptors 1 and 2 — all of which are involved in IFN-γ receptor signalling in macrophages and dendritic cells necessary for T cell stimulation — have been identified in patients with recurrent or fatal non-TB mycobacterial infection (Van Crevel *et al.*, 2002). Elevated concentrations of IL-10, resulting in a defective signal transduction, have also been identified in anergic TB patients (Boussiotis *et al.*, 2000).

If the innate immune response is overcome by *M. tuberculosis*, the subsequent adaptive immune response activates, proliferates and creates very specific immune mechanisms for neutralising or eliminating the invading pathogen (Alberts *et al.*, 2002). Although this differs from the more general innate immune, there is a strong interaction or interdependency between the two immune responses, which is facilitated by the macrophages and dendritic cells (Van Crevel *et al.*, 2002). In order to better understand this section, a schematic outline of these processes is given in Figure 2.3.

This cell-mediated adaptive immune response, activated during *M. tuberculosis* infection, is directed via the cluster of differentiation (CD) 4+ and CD8+ T cells. CD4+ T cells, also known as T helper (Th) cells, produce cytokines which activate CD8+ T cells, B cells and other antigen presenting cells. The various subgroups of Th cells include Th1, Th2 and Th17, each which is associated with a very specific cytokine response (Luckheeram *et al.*, 2012). The Th1 cells produce IL-12, IFN-γ (Herbst *et al.*, 2011), IL-2 and TNF-α (Trinchieri *et al.*, 2003) and hence serve to prevent disease. Th17 cells produce IL-17, IL-22 and IL-23, and function to regulate antimicrobial peptide production as well as the activation and recruitment of IFN-γ expressing T cells at mucosal sites, such as in the lungs (Khader *et al.*, 2007). Th2 cells regulate differentiation of antibody-secreting plasma cells and produce IL-4, IL-5, IL-9 and IL-23. However, these effector cells related to Th2 may actually enhance *M. tuberculosis* intracellular persistence (Potian *et al.*, 2011). This is thought to occur when the Th2 response is dominant, or when the Th1 response is suppressed, resulting in the activation of the alternative macrophage (M2) pathway, which promotes TB disease progression (Rook & Graham, 2007), collagen deposition and the formation of fibrosis in the inflamed lung tissue of TB patients (Harris *et al.*, 2007). This differs from the classical IFN-γ macrophage activation (M1) pathway, which allows for the production of pro-inflammatory cytokines and nitric oxide, in order to overcome the infection (Redente *et al.*, 2010). Regulatory T (T_{reg}) cells of the host, also known as suppressor T cells, not only modulate the immune system and maintain tolerance to self-antigens, but also abrogate autoimmune diseases. These cells suppress or down-regulate induction and proliferation of the effector T cells. *M. tuberculosis* can induce various types of T_{reg} cells, which restrict the host immune response as a countermeasure, enabling it to survive (Mendez *et al.*, 2004). Furthermore, T_{reg} cells may suppress antigen-specific IFN-γ production, subsequently inhibiting CD4+ T cells and the recruitment of CD8+ T cells (Periasamy *et al.*, 2011).

If the host is able to contain the *M. tuberculosis* in this state, it is referred to as latent TB, the non-infectious and asymptomatic state of the disease. However, 10% of these cases may progress into active TB, the highly infectious and symptomatic state of the disease, especially when the immune system becomes compromised. This may occur due to, for example, TB/HIV co-infection, malnutrition, smoking, alcoholism, enclosed air pollution, malignancy, silicosis, diabetes, renal failure and immune suppressing treatment as received by transplant patients (Jick *et al.*, 2006; Lönnroth & Raviglione, 2008). When this occurs, the granuloma becomes caseous and rupture, releasing the bacteria, which proliferate and result in an active disease state (Pawlowski *et al.*, 2012). The most common clinical symptoms associated with active TB include coughing with mucus discharge, weight loss, loss of appetite resulting in anorexia, fever, haemoptysis, chest pain and fatigue (Asch *et al.*, 1998).

Although TB mainly occurs in the lungs (i.e. pulmonary TB), the bacteria may also enter the blood stream via damaged vessels and propagate into various organs, where they proliferate. This is known as extra-pulmonary or disseminated TB and may affect the lymph nodes, bones and joints, urinary tract, intestinal tract, central nervous system, and various other organs (Swaminathan & Narendran, 2005).

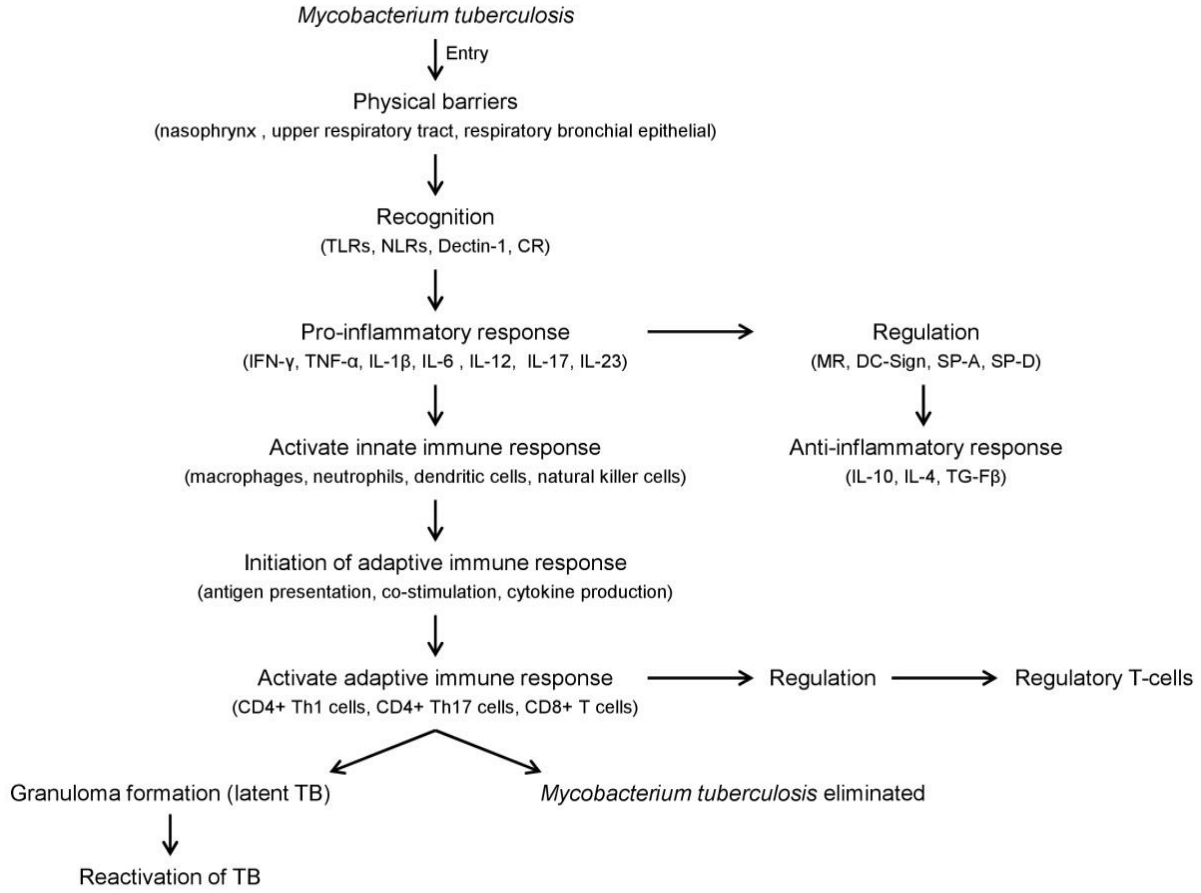

Figure 2.3: Host immune response induced by M. tuberculosis infection.

2.4 TUBERCULOSIS DIAGNOSTICS

In 2015, the World Health Organization (WHO) indicated that 10.4 million individuals were newly infected with *M. tuberculosis*, with 1.8 million deaths due to TB. The current goals of the “End TB Strategy” proposed by the WHO, aim to have TB reduced by 10% in 2020, with an additional 6.5% reduction by 2025. This strategy however, relies on the prompt diagnosis of infected individuals, with immediate treatment (World Health Organization, 2016). Currently, various techniques are used for diagnosing either latent or active TB, each with their own advantages and disadvantages, as will be discussed below.

The tuberculin skin test (TST) and interferon gamma release assay (IGRA) are the only two methods recommended by the WHO for diagnosing latent TB infection (World Health

Organization, 2016). The TST uses PPD, which are proteins obtained from heat-killed *M. tuberculosis* and other mycobacteria. During this test, PPD is intracutaneously injected into the forearm, causing an immune reaction if the patient is infected with *M. tuberculosis*, which is visible as a thickening of the skin at the site of injection, after 28 to 72 hours (González-Martín *et al.*, 2010). IGRAs, on the other hand, is an *in vitro* test based on T cell activity resulting in the release of IFN-γ, due to the presence of specific antigens related to the *M. tuberculosis* complex (Pai *et al.*, 2014). These antigens include early secreted antigenic target 6, culture filtrate protein 10, and TB7.7 (Rv2654), which are more specific to *M. tuberculosis* compared to the PPD used in the TST (Mahairas *et al.*, 1996). Although these methods are relatively easy to perform, they are not without disadvantages, which include an inability to distinguish between latent and active infection, low sensitivity and specificity compared to other techniques, as well as the occurrence of false-positive and false-negative results (Arend *et al.*, 2002; Menzies, 1999; Oztürk *et al.*, 2007). An IGRA is considered significantly more sensitive (±95%) than the TST, however, with a reduced specificity (±80%) comparatively (González-Martín *et al.*, 2010).

Currently, smear microscopy is the most commonly used technique for diagnosing active TB, and was first demonstrated by Robert Koch in 1882. During this acid-fast staining technique, sputum is smeared onto a plate, stained using a dye, heat dried, and treated with an acid-alcohol (known as the Ziehl-Neelsen staining method). Hereafter, the bacteria are either visibly red or appear as bacilli-shaped clear zones when viewed under a microscope (Trifiro *et al.*, 1990). This technique is considered inexpensive, quick and easy to perform (Singhal & Myneedu, 2015), however, it has a low sensitivity (62%), requires a large number of bacilli, cannot detect drug resistance, nor can it distinguish between different *Mycobacterium* species (Kivihya-Ndugga *et al.*, 2004). Considering this, bacteriological cultures are considered the gold standard for diagnosing active TB due to its high sensitivity and specificity of 98%, and requires only 10–100 bacteria/mL sample (Pfyffer, 2015). This technique relies on the growth of cultures using either solid or liquid media, each presenting with its own advantages and disadvantages. Although this method can detect drug resistance, it is considered time consuming due to the slow growth rate of *M. tuberculosis* (Caulfield & Wengenack, 2016).

Other approaches for diagnosing active TB include molecular techniques, serological/ immunological methods, and phage-based assays. Molecular techniques (i.e. nucleic acid amplification), such as the GeneXpert MTB/RIF assay, are based on the rapid detection of various *Mycobacterium* species via DNA amplification of regions specific to the MTBC. Molecular techniques are often used as a follow-up to confirm previous results, and offer a 100% sensitivity and specificity compared to smears, however it is less sensitive when compared to the current gold standard cultures (Pai *et al.*, 2003). The GeneXpert MTB/RIF

assay is a nearly fully automated cartridge-based amplification system that reduces cross-contamination. Not only is it easy to perform, it is faster (90 minutes) and more specific than cultures; however, it is comparatively more expensive, can only detect rifampicin resistance, and requires electricity, annual calibration and storage below 30ºC (Boehme *et al.*, 2011). Serological/immunological methods are based on the detection of host antibodies and other immune complexes in response to the infection. These methods lack accuracy, sensitivity and specificity compared to smears (Steingart *et al.*, 2011) since the various stages of *M. tuberculosis* infection each presents with its own immunological profile. Nevertheless, it is widely used in developing countries due its simplicity, speed and low cost (Olivier & Loots, 2011). Finally, due to its simplicity and speed, the phage-based assay is common in high TB prevalence countries, with a reported sensitivity and specificity of 75% and 98% respectively, compared to smears (Albert *et al.*, 2002; Park *et al.*, 2003). During this test, mycobacteriophages are infected with live *M. tuberculosis* present in a clinical sample, after which all the uninfected phages are removed. The remaining bacilli replicate, followed by amplification and are visualised as clear areas in a lawn of other host cells (Pai *et al.*, 2006). The only commercially available phage-based assay, the FASTPlaqueTB assay, has a reported sensitivity and specificity of 75% and 98% respectively, compared to smears (Albert *et al.*, 2002). Lastly, all the methods currently used which require sputum as a diagnostic sample, is problematic for children, and in patients co-infected with HIV since good sputum samples are very difficult to obtain from these individuals, hence a misdiagnosis or false negative result often occurs (Olivier & Loots, 2011). Recently, LAM detected in the urine of TB patients has also been considered for diagnostic purposes; however these results are disappointing in heterogeneous patient populations (Peter *et al.*, 2010) and unreliable in HIV co-infected patients (Paris *et al.*, 2017).

To date, no single diagnostic test exists with adequate sensitivity, specificity, speed, costs and simplicity. Considering this, a new TB diagnostic test is urgently needed, especially in developing countries with a high TB burden (Du Preez *et al.*, 2017).

2.5 TUBERCULOSIS VACCINATION

The only vaccine currently used against TB, is the BCG vaccine, which was developed in 1921 by using antigens isolated from an avirulent *M. bovis* strain. This vaccine is administered to all new-borns in high TB prevalence countries (with reports indicating an 85% coverage worldwide), in which case it is considered partially effective against severe paediatric TB; however, it is only effective in 50% of all adult cases (Moliva *et al.*, 2015; Orme, 2013).

Over the past decade, various efforts have been made to replace or improve on this vaccine, but thus far, newer candidates are either alternative forms of the *M. bovis* bacilli, and function to only boost previously vaccinated individuals, or provide the same level of immunity and protection provided by the original BCG vaccine, or were considered ineffective (Orme, 2005; Orme, 2013). Thirteen new vaccines are currently in clinical trials, including those in proof-of-concept field studies, while a number of others are in various stages of preclinical development (World Health Organization, 2016). A non-profitable organisation, Aeras Foundation (Rockville, Maryland, USA), has dedicated their resources to develop and test new TB vaccines and have discovered six new candidates, which include Aeras-402 (Abel *et al.*, 2010), M72 (Skeiky *et al.*, 2004), H56 (Lin *et al.*, 2012), ID93 (Baldwin *et al.*, 2012) Hybrid (Billeskov *et al.*, 2012), and MVA85A (Tameris *et al.*, 2013; Wilkie & McShane, 2014), the latter of which is currently in Phase II trials. Apart from these, other vaccines are used to simulate the immune system to target infection, are also under development (Orme, 2013). However, the results of the phase III trials will only be known within a few years (World Health Organization, 2015).

Considering this, there is an urgent need for new, safe and effective vaccines that protect against all forms of the TB disease (including drug-resistant strains), in all age groups, as well as in individuals co-infected with HIV.

2.6 TUBERCULOSIS TREATMENT

The aim of an anti-TB treatment regimen is to cure infected patients, prevent relapse, decrease the rate of transmission and prevent the development of drug resistance. Unfortunately, of the nearly 10.4 million new TB cases reported annually, only 85% are cured successfully. Furthermore, up to 520 000 MDR-TB cases are reported yearly (Falzon *et al.*, 2017), of which only 20% received treatment, and only 52% are cured. The average cost to treat an individual with drug-susceptible TB is up to 1000 US-dollar, while the treatment for drug-resistant cases may be as high as 20 000 US-dollar (World Health Organization, 2016).

TB drugs are classified as either first- or second-line drugs, used to treat drug-susceptible or drug-resistant cases respectively. Newly infected patients are given first-line anti-TB drugs when there is no indication of drug resistance. These drugs include isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB). Each of these drugs have its own mechanism of action. To summarise, INH is classified as a bactericidal drug, used to inhibit active growing *M. tuberculosis* bacilli (Carlton & Kreutzberg, 1966); RIF has powerful sterilising activity, and eliminates both active and semi-dormant *M. tuberculosis* via transcription inhibition; PZA, also a sterilising drug, eliminates semi-dormant *M. tuberculosis*

bacilli within host macrophages (Ahmad & Mokaddas, 2009; Gumbo *et al.*, 2009); and EMB (a bacteriostatic drug that is effective against both intra- and extracellular *M. tuberculosis* bacilli) adds additional coverage to combat the development of drug resistance (Ahmad & Mokaddas, 2009; Olivier & Loots, 2011).

First-line drugs are administered orally as stipulated by the WHO's Directly Observed Treatment Short-course (DOTS) program. DOTS is a six month treatment plan during which a combination of INH, RIF, PZA and EMB are administered for two months (initial phase), followed by the administration of only INH and RIF for four months (continuation phase) (Olivier & Loots, 2011). The DOTS program has a high success rate in patients infected with drug-susceptible *M. tuberculosis*. Unfortunately, several factors may lead to treatment failure in these cases, especially in third-world countries, including limited access to health providers, poor patient TB education/knowledge, and poverty, patient non-adherence (especially due to the associated side-effects), HIV- co-infection, as well as varying individual metabolic activity (De Villiers & Loots, 2013).

Second-line drugs, on the other hand, are used to treat drug-resistant *M. tuberculosis*, defined as resistance to both RIF and INH. These drugs are administered orally or via injection and include aminoglycosides (streptomycin, kanamycin, amikacin), polypeptides (capreomycin, viomycin), fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin, ofloxacin, gatifloxacin), as well as the newly approved delamanid and bedaquiline (Zumla *et al.*, 2013). For drug resistant cases, the WHO recommends the DOTS Plus program, effectively extending treatment to 24 months (Iseman, 1998). This regimen however, is less effective, more expensive, and may result in additional complications, such as hepatotoxicity. Although 21 new drugs are currently in Phase I trials, only bedaquiline and delamanid were recently approved for treating adults with MDR-TB (Brigden *et al.*, 2017).

Various side-effects are associated with the use of anti-TB drugs, often leading to patient non-adherence, the most common side-effect being anti-TB drug-induced hepatotoxicity or hepatitis. Other side-effects may manifest as: cutaneous (rash and/or skin irritation), nephrology (hyperuricemia, painful urination and kidney inflammation), abdominal (nausea, vomiting and cramps), respiratory issues (i.e. breathlessness), flu-like symptoms (fever, arthralgia, malaise and headaches) as well as sideroblastic anaemia, immunological reactions, or a colour change in bodily fluid (associated with RIF ingestion) (De Villiers & Loots, 2013).

2.7 IMMUNOMETABOLISM OF TUBERCULOSIS

M. tuberculosis has the ability to use various organic substrates, including carbohydrates, lipids (fatty acids and cholesterol), amino acids and organic acids, as a fuel/energy source for its central carbon metabolism (Baughn & Rhee, 2014). Previous studies have established that *M. tuberculosis* uses carbohydrates mainly for growth, and lipids for pathogenesis and persistence (Rhee *et al.*, 2011). However, the exact role/function of amino/organic acids has yet to be fully characterised. *M. tuberculosis'* metabolic adaptations are not only essential for pathogenesis and energy production during infection, but also for survival within the host. Thus, the ability of the pathogen to evolve and adapt according to its surroundings for survival, has led to the identification of various metabolic pathways that are not only beneficial for bacterial growth, but also those that affect the host's immune response (Shi *et al.*, 2016). A better understanding of all these metabolic and immunological processes, in both the pathogen and host, would undoubtedly result in improved diagnostic and treatment applications.

Immunometabolism, is the study of interactions between the bioenergetic pathways of metabolism and the immune cell function (Shi *et al.*, 2016), and illustrates how changes in the metabolism affects the host immune response. As previously mentioned, *M. tuberculosis* infection results in a host immune response, either leading to bacterial eradication or granuloma formation in an attempt to encapsulate the invading bacteria. This latent state of the infection is known as a M1 phenotype (Flynn *et al.*, 2011). However, if the host is unable to contain the infection, allowing *M. tuberculosis* to persist (active TB), there is an alteration in the polarisation of the macrophage and this is referred to as a M2 phenotype, which is associated with an anti-inflammatory response and an increased lipid metabolism, resulting in in foamy macrophages (Kim *et al.*, 2010). Previous studies suggest that instead of eliminating the infection, the macrophages start to play a key role in the expansion and dissemination of the infection.

Understanding why certain immune cells function in a particular way due to their metabolic fate has become an interesting topic in TB research in recent years. Under normal physiological conditions, immune cells are relatively quiescent and only activate in response to a pathological stimulus, which in turn allows for the activation different metabolic signatures, which are specific to the particular innate and/or adaptive immune response(s) (Ganeshan & Chawla, 2014). The innate immune response entails macrophage re-programming into the previously mentioned M1 phenotype. This response includes the activation of the IFN-γ and TLR ligands (such as lipopolysaccharides), which generate pro-inflammatory cytokines and Th1 adaptive immune cells. These M1 associated cells usually illustrate upregulated glycolysis, but decreased oxygen consumption. The M2

macrophages, however, form in response to the Th2 cytokines, scavenger receptors and anti-inflammatory activation. The metabolic profiles of these cells illustrate a more dormant nature where mitochondrial fatty acid oxidation (oxidative metabolism) provides a major carbon and energy source (Rodríguez-Prados *et al.*, 2010). Cellular metabolism therefore plays a key role in controlling the functioning of the immune cells. Nutrients such as glucose, glutamine and fatty acids which partake directly in the metabolic pathways of *M. tuberculosis* and the host were previously found to also determine the activity of immune regulators, including the mechanistic target of rapamycin complex 1, which is a key player in the immune response against TB. Methionine is also of particular interest since this amino acid is imported into cells, generating S-adenosylmethionine for epigenetic methylation of DNA and histones (Loftus & Finlay, 2016). Additionally, various ligation inhibitory receptors have been found to alter the cellular metabolism where receptors such as programmed death 1 and CTLA-4, expressed on human CD4 T cells, were found to inhibit aerobic glycolysis, but to the contrary, enhances fatty acid oxidation metabolism (Patsoukis *et al.*, 2015). Changes in the metabolic profile due to TB clearly has an influence in the host immune response against *M. tuberculosis*, and future studies on this topic could ultimately assist in better diagnostic and treatment approaches.

2.8 METABOLOMICS

Metabolomics is the latest addition to the “omics” revolution and is defined as the nonbiased identification and quantification of the total metabolome (all small compounds) of a specific biological system, utilising various analytical instrumentation as well as various statistical, computational and mathematical approaches (Du Preez *et al.*, 2017). Metabolomics is based on the principle that an external stimulus, such as *M. tuberculosis* infection, results in metabolic changes which are specific to the perturbation due to a pathophysiological incentive and/or a genetic alteration. Metabolomics is based on the analysis of an organism’s metabolic profile by assessing the end products of the perturbation that may have occurred in the metabolic pathway(s), giving clues to the overall physiological status (De Villiers & Loots, 2013).

As previously mentioned, metabolomics relies on using various highly specialised analytical instruments, including gas chromatography (GC), liquid chromatography (LC), and nuclear magnetic resonance (NMR), which are usually coupled to various different mass spectrometry (MS) techniques. Each of these instruments have their own advantages and disadvantages, and are chosen according to the aim(s) of an investigation (De Villiers & Loots, 2013). GC-MS, established in the 1950s, combines the separation capabilities of GC (determined by compound volatility) and the identification capabilities of MS. GC-MS is used

for both qualitative and quantitative analysis of the various metabolites in a complex mixture and has been widely considered as the "golden standard" for forensic substance identification, toxicology, trace analyses in the food industry, and in the pharmaceutical industry. The GC component uses the boiling point/volatility of compounds as an initial separation method, the polarity of the compounds as a second separation method and lastly the interaction of compounds with the capillary column, to obtain the best possible separation of a large number of compounds. The molecules are retained by the GC column and elute from the column at different times (i.e. retention times). The MS will then capture, ionise, accelerate, deflect, and detect the ionised molecules separately by breaking each molecule into ionised fragments and detecting these fragments using their mass-to-charge or time-of-flight ratios, depending on the type of MS used (Sneddon *et al.*, 2007). Some of the advantages of GC-MS include: (1) suitability for volatile and non-polar compounds, (2) well-established protocols and libraries for analysis and compound identification, (3) relatively lower running costs and (4) higher sensitivity and accuracy compared to other approaches. Additionally, the two dimensional GC-MS systems, such as GCxGC-TOFMS, allows for additional separation, higher sensitivity, a better dynamic range, more effective removal of matrix interferences, better deconvolution and hence, better compound detection and identification (De Villiers & Loots, 2013).

To compensate for the limitations of GC-MS, LC-MS was established in the mid-1990s and quickly became very popular due to its ability to analyse a broader spectrum of biological molecules, including larger, polar/ionic, thermally unstable and non-volatile compounds. LC-MS also has higher analytical sensitivity and specificity when compared to GC-MS (Pitt, 2009) and does not require derivatisation of highly polar compounds. The LC system consists of a mobile and stationary phase, and is used to separate all compounds in a sample based on its affinity for a specific polarity. A polarity gradient is set in the mobile phase that changes over specific time intervals. Hence, as the mobile phase polarity changes, the compounds with an affinity to the set polarity will separate from the sample mixture and migrate through the stationary phase, after which it will elute and be detected by the MS system (Grebe & Singh, 2011). However, LC-MS is not without its limitations, which include: (1) low chromatographic resolution, (2) inconsistent retention times, (3) higher signal-to-noise ratios, (4) isotope-based identification, and (5) higher running costs (De Villiers & Loots, 2013).

Metabolomics studies are either untargeted, semi-targeted or targeted. Untargeted approaches are the most common and refer to a comprehensive analysis of all measurable compounds in a sample. This approach provides a platform or basis for targeted or semi-targeted investigations or a confirmation of both. Untargeted approaches however, usually result in the detection of hundreds of variables, which require the use of various chemometric

techniques to narrow/focus the massive dataset generated into a smaller, significant and manageable dataset chunks. Other limitations include the time required for processing large datasets, the identification and characterisation of small metabolites, the coverage of the analytical platform used (which may be biased towards the detection of only high abundance molecules, such as in NMR), as well as the need for further validation procedures. In contrast, semi-targeted approaches are used to analyse a specific group of metabolites, such as amino acids, fatty acids, etc. This approach provides a thorough perspective of the metabolic enzymes, kinetics, end-products, and biochemical pathways these metabolites contribute to. Additionally, sample preparation for semi-targeted approaches can be optimised to remove the possibility of dominance of high abundance molecules. Nevertheless, all metabolomics studies should always include the use of internal standards (for normalisation and quantification) as well as quality controls to ensure that reliable results are obtained, and are both quantitative and qualitative (Roberts *et al.*, 2012).

Metabolomics has enabled the identification of new metabolite markers in sputum, blood and urine from TB patients, describing novel *M. tuberculosis* metabolic pathways and host adaptations. Apart from their possible diagnostic applications (Du Preez *et al.*, 2017), many of these new TB metabolite markers have contributed to the existing knowledge of the biology of the causative pathogen (Rhee *et al.*, 2011), including various underlying disease mechanisms related to *M. tuberculosis* drug resistance (Du Preez & Loots, 2012; Lahiri *et al.*, 2016; Loots, 2013; Loots, 2015) and virulence, such as the upregulation of numerous antioxidant pathways, the use of alternative energy substrates, a change in aconitase functionality towards mRNA binding and stability, and cell wall remodelling (Loots, 2014; Loots, 2015; Meissner-Roloff *et al.*, 2012; Rhee *et al.*, 2011). Additionally, the mechanisms of anti-TB drug action (Halouska *et al.*, 2007; Halouska *et al.*, 2012; Prosser & de Carvalho, 2013) and related side-effects in the host (Loots *et al.*, 2005; Luies & Loots, 2016) have also been elucidated. Furthermore, the use of metabolomic approaches have allowed for a better understanding the adaptations of *M. tuberculosis* to host defences and *vice versa*, resulting in the discovery of (1) the citramalate cycle in *M. tuberculosis*, which interacts with an upregulated glyoxylate cycle, (2) an increased use of fatty acids and glutamate by the pathogen, (3) an alternative hydrogen peroxide mechanism by the host to eliminate the infecting bacteria, (4) electron transport chain inhibition in host (Du Preez & Loots, 2013), and (5) a compromised insulin production, which may provide clues to better treatment approaches (Luies & Loots, 2016).

However, very little research to date has focused on the adaptations of *M. tuberculosis* to the host's defence mechanisms or growth environment, or for that matter, the host's adaptations or altered metabolic state in response to the infectious pathogen. This is important since the pathophysiology of *M. tuberculosis* is directly linked to its metabolism and complex

physiology (Mizrahi, 2012), and to that of the host, and this pathogen is able to utilise numerous growth substrates either by scavenging this from the host or via *de novo* biosynthesis, in order to ensure its own survival (Eisenreich *et al.*, 2010; Lee *et al.*, 2013). Additionally, very little data has been published on urine from TB patients, which can be considered an ideal sample matrix to identify markers associated with this host–pathogen interaction (Du Preez *et al.*, 2017).

Considering the above, it is clear that metabolomics can contribute to a better understanding of the TB disease state. The current semi-targeted investigation is novel in the sense that urine samples were analysed using various instrumental platforms to elucidate the underlying host-pathogen interactions and adaptations to better characterise TB.

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CHAPTER 3:
METHODOLOGY AND RESULTS

3.1 INTRODUCTION

Since the discovery of *M. tuberculosis* in 1882 by Robert Koch, a vast amount of genomics, proteomics and transcriptomics data has been generated, leading to our current understanding of *M. tuberculosis* and TB. The majority of these data have been generated from *M. tuberculosis* cultures; however, it is well-known that this organism's metabolism and growth in culture differs greatly from growth in the human host, where many different growth mechanisms and energy substrates are preferentially used. Sputum is the preferred sample matrix in most host-directed TB metabolomics studies to date, since this mucus-like biofluid is highly populated with *M. tuberculosis* bacilli in infected individuals. However, the use of sputum has limitations due to its complexities, such as its uneven consistency and viscosity, which require additional pre-processing steps (Du Preez *et al.*, 2017). Furthermore, sputum sampling is difficult in children and HIV-infected individuals. Considering this, blood samples (i.e. plasma or serum) have been investigated as an alternative patient sample matrix due to its homogeneous composition, however, the collection of this may be considered invasive and does not contain high concentrations of metabolites directly associated with *M. tuberculosis*. Alternatively, urine has become an attractive option considering its sterile nature, non-invasive means of collection, even viscosity, minimal sample preparation required to remove complex proteins/lipids, and large quantities can be collected in a timely fashion, which can easily be transported and/or analysed with minimal chance of contracting the disease. Urine will also contain high concentrations of those metabolites associated with the host's response to infection, making this media ideal for disease characterisation and the altered host response to TB (Du Preez *et al*., 2017; Emwas *et al*., 2015). However, despite these many advantages, urine cannot distinguish between susceptible and drug-resistant *M. tuberculosis* infection, and thus only indicate a general response due to TB disease. It is also important to note that any of these patient samples can be influenced by factors such as diet (Stella *et al.*, 2006), time of collection (Maher *et al.*, 2007), age and gender (Duarte & Gil, 2012; Slupsky *et al.*, 2007) as well as varying individual metabolic phenotypes (Gavaghan *et al.*, 2002). Hence, the metabolomics experimental design should not only include ways of eliminating analytical/experimental variation, but also biological variation.

3.2 EXPERIMENTAL DESIGN

The broad experimental design used to address the aim and objectives of this metabolomics investigation is illustrated in Figure 3.1.

During this metabolomics investigation, urine samples were collected from culture-confirmed active pulmonary TB patients (n=31) before any treatment commenced, as well as from healthy individuals (n=29) from the same geographical area. A summary with patient descriptive/demographic and clinical information is available in Appendix A. These patients were selected based on sample availability; however, a bigger sample cohort should be used to validate current findings.

To address the first objective, a validated fatty acylcarnitine methodology was used to extract aliquoted urine samples collected from the previously mentioned patients, followed by LC-MS/MS analysis. Hereafter, an amino acid extraction was performed on a second aliquot of the same patient cohort and analysed using a GC-MS approach, in accordance with achieving the goal set out in the second objective. To achieve the third objective, an organic acid extraction approach was applied to a third aliquot of the same patient collected urine samples, prior to GCxGC-TOFMS analysis. These methods have previously been validated with using quality control samples, and showed both good machine and analytical repeatability using relative concentrations and coefficient of variation values. Considering that validated methodologies were used, these data are acceptable for use to address the aims of this metabolomics investigation. Hence, the data was processed and cleaned prior to statistical analysis (comparing the experimental and control groups data), and the results obtained interpreted/discussed in the context of an altered fatty acylcarnitine, amino acid, and associated organic acid metabolic profile in TB-positive patients, considering the previously published literature on human metabolism and TB. More detail on these processes is given in Chapter 4.

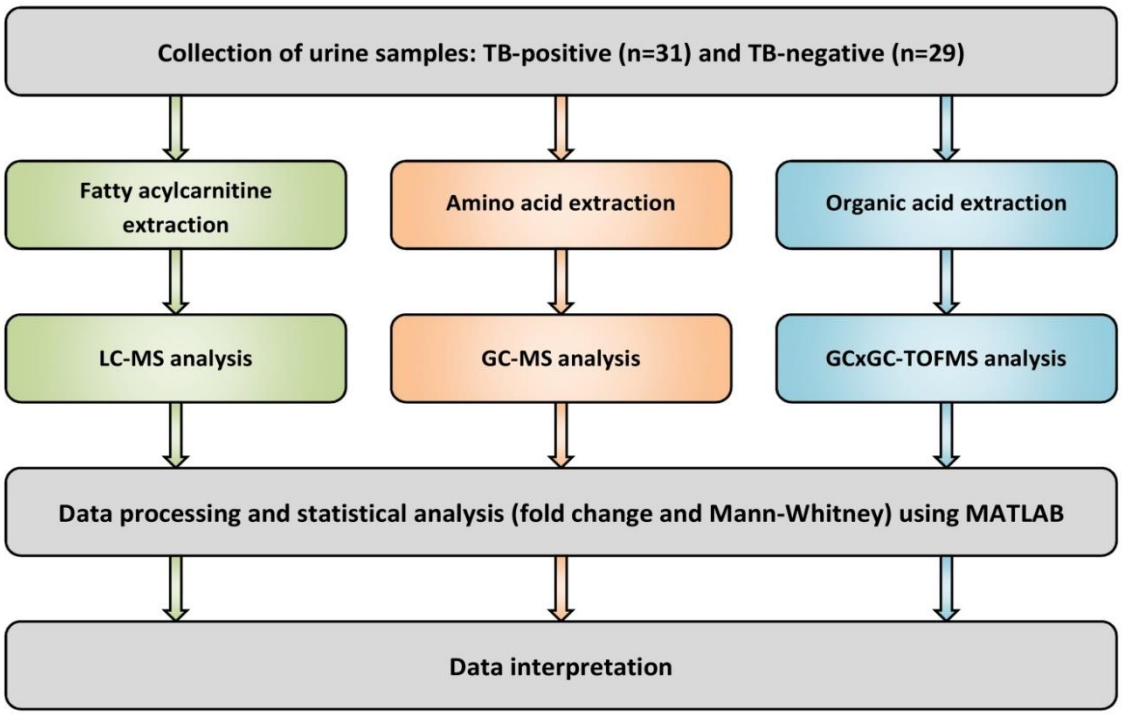

Figure 3.1: A schematic representation of the experimental design, used to address the study's aim.

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CHAPTER 4:
THE ALTERED FATTY ACYLCARNITINES, AMINO ACIDS AND ORGANIC ACIDS DETECTED IN TUBERCULOSIS PATIENT URINE

This chapter has been submitted for publication:

Anthony, C., Luies, L. Mienie, J.L., Lindeque, J.Z., Ronacher, K., Walzl, G. & Loots, D. (2018). Detection of altered fatty acylcarnitines, amino acids and organic acids in tuberculosis patient urine. Submitted for publication to *Clinical Infectious Diseases* (manuscript number: CID-88284).

4.1 ABSTRACT

Background: Despite fervent research efforts to date since the discovery of the tuberculosis (TB) disease-causing pathogen, *Mycobacterium tuberculosis*, in 1882 by Robert Koch, TB is still considered a global pandemic, resulting in an estimated 1.8 million deaths annually. Better elucidation of the mechanisms associated with TB, the pathogen's adaptations to the host immune response, and that of TB drugs, are still as relevant today as it was 135 years ago. Investigating this disease using an omics research perspective, the most recent of which being metabolomics, has served well to expand the current knowledge of the disease and the improved diagnosis and treatment thereof, due to its unique capacity for identifying new disease biomarkers.

Methods: In this investigation, a combined semi-targeted liquid and gas chromatography mass spectrometry metabolomics approach was used to compare the urinary fatty acylcarnitines, amino acids and selected organic acids of active TB patients to that of healthy individuals, to better characterise the TB-induced alterations to the host metabolome.

Results and Conclusion: A general increase in the fatty acylcarnitines and amino acids are most likely due to TB-induced cachexia. However, altered levels of various other individual or groups of specific metabolites in the TB patient urine, associated with a vitamin B_{12} deficiency, urea cycle abnormality, lactic acidosis, ketoacidosis, oxidative stress and liver damage in these patients, were also identified.

Keywords: metabolomics; tuberculosis; fatty acylcarnitines; amino acids; organic acids

4.2 INTRODUCTION

Infection with *M. tuberculosis* affects approximately one-third of the world's population, and can lead to active, symptomatic disease, called tuberculosis or to asymptomatic states, often referred to as latent infection. In 2015 alone, 10.4 million new TB cases were reported, resulting in an estimated 1.8 million deaths (World Health Organization, 2016). Since the discovery of *M. tuberculosis* in 1882 by Robert Koch, a vast amount of genomics, proteomics and transcriptomics data have been generated, leading to our current understanding of *M. tuberculosis* and TB. The majority of these data have been generated from studies using *M. tuberculosis* cultures; however, it is well-known that this organism's metabolism and growth in culture differs greatly from growth in the human host, where many different growth mechanisms and energy substrates are preferentially used (Du Preez *et al.*, 2017). Furthermore, very little research to date has focused on the adaptations of *M. tuberculosis* to the host's defence mechanisms or growth environment, or for that matter, the host's adaptations or altered metabolic state in response to the infectious pathogen. This is important since the pathophysiology of *M. tuberculosis* is directly linked to its metabolism and complex physiology (Mizrahi, 2012), and to that of the host. Additionally, this pathogen is able to utilize numerous growth substrates, either by scavenging this from the host or via *de novo* biosynthesis, in order to ensure its own survival (Eisenreich *et al.*, 2010; Lee *et al.*, 2013).

Metabolomics has enabled the identification of new metabolite markers in sputum, blood and urine from TB patients, describing novel *M. tuberculosis* metabolic pathways and host adaptations. Apart from their possible diagnostic applications, many of these new TB metabolite markers have contributed to the existing knowledge of the biology of the causative pathogen, including various underlying disease mechanisms related to *M. tuberculosis* drug resistance (Loots, 2014; Loots, 2015) and virulence (Rhee *et al.*, 2011), such as the upregulation of numerous antioxidant pathways, the use of alternative energy substrates, a change in aconitase functionality towards mRNA binding and stability, and cell wall remodelling (Loots, 2014; Loots, 2015; Meissner-Roloff *et al.*, 2012; Rhee *et al.*, 2011). Additionally, the mechanisms of TB drug action (Halouska *et al.*, 2007; Halouska *et al.*, 2012; Prosser & de Carvalho, 2013) and related side-effects in the host (Loots *et al.*, 2005; Luies & Loots, 2016) have also been elucidated. Furthermore, the use of metabolomic approaches have allowed for a better understanding of the adaptations of *M. tuberculosis* to host defences and *vice versa*, resulting in the discovery of (1) the citramalate cycle in *M. tuberculosis*, which interacts with an upregulated glyoxylate cycle, (2) an increased use of fatty acids and glutamate by the pathogen, (3) an alternative hydrogen peroxide mechanism by the host to eliminate the infecting bacteria, (4) electron transport chain inhibition in host

(Du Preez & Loots, 2013), and (5) a compromised insulin production, which may provide clues to better treatment approaches (Luies & Loots, 2016). To date, however, very little data has been published on urine from TB patients, which can be considered an ideal sample matrix to identify markers associated with this host–pathogen interaction (Du Preez *et al.*, 2017). Considering this, a combined semi-targeted liquid and gas chromatography mass spectrometry (LC-MS/MS and GC-MS, respectively) metabolomics approach was used to compare the urinary fatty acylcarnitines, amino acids and selected organic acids of active TB patients (n=31) to that of healthy individuals (n=29), to identify new metabolomics metabolite markers for TB.

4.3 MATERIALS AND METHODS

4.3.1 Clinical samples

Anonymised archived urine samples were obtained from the Faculty of Medicine and Health Sciences, DST/NRF Centre of Excellence for Biomedical Research, at the Stellenbosch University/MRC Centre for TB Research. These samples were transported frozen, on dry ice, to the NWU, Human Metabolomics, Laboratory of Infectious and Acquired Diseases Metabolomics, and immediately stored at -20ºC. These samples formed part of an observational cohort study of individuals with drug sensitive pulmonary TB, diagnosed using bacteriological cultures and smear microscopy, who were HIV-seronegative, not pregnant, and free from other diseases (Hesseling *et al.*, 2010). The samples selected for this investigation included 31 culture-confirmed active South African TB patients (18 males and 13 females, between the ages of 21 and 58), collected at time of diagnosis (i.e. prior to treatment administration). Additionally, urine samples were also collected from 29 age and gender matched healthy, TB-negative individuals from the same geographical area.

The investigation was conducted according to the Declaration of Helsinki and International Conference on Harmonisation Guidelines and ethically approved by the Ethics Committees of the North-West University, South Africa (reference number NWU-00127-11-A1) and Stellenbosch University Health Research Ethics Committee (reference number 99/039). All participants gave written informed consent for participation in the study.

4.3.1.1 Quality control samples

Quality control (QC) samples are used to ensure that the data obtained are reliable/valid since these are used to correct any potential batch effects (i.e. analytical drift in the analysed data). For the current investigation, a single, pooled QC sample was compiled by combining

0.5 mL of each urine sample into a new, clean vial. Hereafter, the sample was mixed, aliquoted into 1 mL vials, and storage at -20ºC until use. One QC aliquot was extracted and analysed per batch, in exactly the same manner as that of the patient samples (as described below in section 4.3.3), and injected in the beginning, middle and end of each analytical batch run comprising of 12 samples. The first QC sample injection was repeated five times prior to analysis in order equilibrate the instrument.

4.3.2 Chemicals and reagents

All fatty acylcarnitine isotopes [i.e. free carnitine (C_0), acetylcarnitine (C_2), propionylcarnitine (C_3), isovalerylcarnitine (C_5), octanoylcarnitine (C_8), decanoylcarnitine (C_{10}), dodecanoyl-carnitine (C_{12}), tetradecanoylcarnitine (C_{14}), palmitoylcarnitine (C_{16})] were kindly supplied by Dr. Herman J. ten Brink (Amsterdam, Netherlands). The butanolic hydrochloric acid (HCl), formic acid, 3-phenylbutyric acid, hexane and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Pyridine, HCl, anhydrous sodium sulphate (Na_2SO_4) and trimethylchlorosilane (TMCS) were purchased from Merck (Darmstadt, Germany). The ultra-pure water, acetonitrile, ethyl acetate and diethyl ether were from Honeywell Burdick & Jackson (Muskegon, Michigan, USA).

4.3.3 Sample analysis

Prior to the fatty acylcarnitines, amino acids and organic acids analyses, a creatinine determination was done on each urine sample (n=60) using a creatinine enzyme kit (Thermo Fisher Scientific, Massachusetts, USA; reference number 981845) and an Indiko Clinical Analyzer, Type 863 (Thermo Fisher Scientific). The creatinine values were used to normalise the volume of urine and/or reagents required for each analysis to ensure that all samples are equally concentrated and also for quantification purposes, where each compound detected was expressed as µmol/L creatinine.

4.3.3.1 Fatty acylcarnitine analysis

After the addition of 205 µL internal standard stock solution (made up using a combination of all the stable fatty acylcarnitine isotopes described above), all of the collected urine samples (100 µL) were deproteinated as described in Venter *et al.* (2015). Briefly, 30 µL of acetonitrile was added to each sample, followed by centrifugation (5 min at 12 000 g at 4ºC), in order to precipitate all proteins, after which the supernatant was decanted into a new tube without disturbing the protein pellet. This was followed by an overnight freezing step at -80ºC and

then evaporation to complete dryness under a steady stream of nitrogen at 37°C. Butylation and analysis of the samples were performed as described by Van Aardt *et al.* (2016). To summarise, 200 µL of 3N butanolic HCl was added to the dried residue and the samples were incubated for 60 min at 50°C. These butylated samples were then evaporated to dryness under a steady stream of nitrogen at 37°C and resuspended in a water:acetonitrile solution (50:50) (v/v) containing 0.1% formic acid.

The samples were analysed by injecting 1 µL into an Aligent 1290 series LC system coupled to a 6410 QQQ Mass Analyzer (Aligent Technologies, California, USA) consisting of a Micro Vacuum Degasser (G1379B), Binary pump SL (G1312B), preparative Autosampler HiP-ALS SL (G1367C), Thermostat ALS (G1330B) and Thermostatted Column Compartment SL (G1316B). The system was equipped with a C18 Zorbax SB-Aq column (150 mm x 2.1 mm x 3.5 µm) supplied by Agilent Technologies, which was kept at 30°C during the entire run. The chromatographic gradient started at 95% solvent A (water with 0.1% formic acid), with a flow rate of 0.2 mL/min, where it was maintained for 1.5 min. Hereafter, the gradient was increased to 18% solvent B (acetonitrile with 0.1% formic acid) over a period of 1.5 min, at which it was maintained for a further 2 min. The gradient was then increased to 100% solvent B over 10 min during which the flow was gradually increased to 0.3 mL/min and then kept constant at this flow for 3 min. The flow and gradient were then progressively decreased over a period of 2 min to 0.2 mL/min and 5% solvent B. A post-run continued for 8 min at 5% solvent B after every sample to ensure equilibration of the column, resulting in a total run time of 28 min per sample. The ESI source gas temperature was kept at 300°C, with a flow rate of 7.5 L/min, and nebuliser pressure was kept at 30 psi with capillary voltage at 3500 V (Van Aardt *et al.*, 2016). The nine most prevalent fatty acylcarnitines (C_0, C_2, C_3, C_5, C_8, C_{10}, C_{12}, C_{14} and C_{16}) were identified and quantified using the stable isotopes as a reference.

4.3.3.2 Amino acid analysis

An EZ:faast Kit (Separation, Randburg, South Africa) was used to extract the amino acids from the urine, and was performed according to the manufacturer's instruction manual. This kit provides all reagents required for sample preparation. To summarize, it consists of a solid phase extraction using a sorbet packed tip that binds the amino acids, thus allowing all other interfering metabolites to pass through the column. Thereafter, a liquid-liquid extraction is performed, followed by derivatization, after which after the sample was ready for GC-MS analysis.

Hereafter, a stable isotope mixture (Cambridge Isotope Laboratories, Inc., San Francisco, USA) was added for quantification purposes, followed by GC-MS analysis. To summarise, 1 µL of each extracted sample was randomly injected onto a HP 6890 Series GC (Hewlett-

Packard Company, North Carolina, USA), coupled to an Agilent 5973 Network: Mass Selective Detection MS (Aligent Technologies, California, USA), in splitless mode. A Zebron ZB-AAA GC Column (E16-000847) (10 m x 0.25 mm) was used for compound separation, using ultra-pure helium at a constant flow of 1.3 mL/min at a pressure of 0.37 psi as the carrier gas. The inlet temperature was kept at a constant 250°C for the entire run, and the initial oven temperature was 60°C, where it was held for 1 min, followed by an increase of 50°C/min to 110°C. The temperature was then increased at a rate of 20°C/min to 185°C, followed by a rate of 25°C/min to 235°C, and lastly at a rate of 30°C/min to a final temperature of 320°C, where it was held constant for an additional 0.5 min. The total run time per sample was 11.58 min. Peak detection and peak identification were done using Chemstation software (2011; Agilent Technologies MS Workstation, Version 7.0.0, USA).

4.3.3.3 Organic acid analysis

An organic acid analysis was performed as described in Luies and Loots (2016). Briefly, six drops of 5N HCl and a volume of internal standard stock solution (with a final concentration of 25 µmol phenylbutyric acid/mg urinary creatinine) were added to each urine sample. Hereafter, 6 mL of ethyl acetate was added, followed by mixing and centrifugation (3000 rpm for 3 min) to achieve a solvent phase separation. The organic phase was then aspirated into a second, clean Pyrex tube. Diethyl ether (3 mL) was added to the remaining aqueous phase, followed by mixing and centrifugation, after which the organic solvent was aspirated and added to the previously collected organic phase. Hereafter, approximately 3 g Na_2SO_4 was added to each sample, followed by a brief vortex and centrifugation step to pellet the Na_2SO_4. The combined organic phase was then decanted from the pellet and evaporated under a stream of nitrogen at 37°C, followed by derivatisation of the extract using BSTFA (22.6 µL/µmol creatinine), TMCS (4.5 µL/µmol creatinine) and pyridine (4.5 µL/µmol creatinine), at 60°C for 60 min.

The derivatised samples were analysed in a randomly order by injecting 1 µL of each, along with the necessary QC samples, into a Pegasus 4D GCxGC-TOFMS system (LECO Africa (Pty) Ltd, Johannesburg, South Africa), using a 1:12 split. The GCxGC system was equipped with a Restek Rxi-5Sil MS primary column (30 m x 0.25 mm x 0.25 µm) and Rxi-17 secondary column (0.9 m x 0.25 mm x 0.25 µm) for compound separation, using ultra-pure helium as the carrier gas at a constant flow of 1 mL/min. The injector temperature was held at a constant 280°C and cryomodulation was achieved using a hot pulse of nitrogen gas for 0.7 s, every 3 s. The temperature program for compound separation on the primary column was 55°C for 1 min, followed by an increase of 7°C/min to 285°C, at which it was held constant for 4 min. The temperature was then increased at a rate of 20°C/min to a final temperature of 305°C, where it was maintained for 1 min. The temperature ramp of the

secondary oven was identical to that of the primary oven, with the exception of a +5°C offset at all time points. To prevent the MS from detecting the solvent, the filament was switched off and no mass spectra were recorded for the first 8 min of each analytical run. The temperatures of the transfer line and ion source were maintained at 270°C and 200°C respectively, for the entire run, with a detector voltage set at 1600 V and the filament bias at -70 eV.

4.3.4 Statistical analysis

Data processing and statistical analysis were performed using MATLAB software (2012; The MathWorks Inc., Natick, Massachusetts, USA). Prior to statistical analysis, data collected from the three separate analytical instruments were subjected to a series of standardised metabolomics data processing steps, including a 50% zero filter (to eliminate compounds with extensive missing values over both groups) and a zero value replacement step (to compensate for machine detection limits), as described by Luies and Loots (2016). In order to identify significant compounds when comparing the TB-positive and healthy control groups, fold change values were calculated and Mann-Whitney tests were performed. Fold change examines the practical significance between two sets of samples, and an absolute fold change ≥ 2 is considered significant (Van den Berg *et al.*, 2006). Mann-Whitney *p*-values were evaluated at a 5% and 10% significance level (indicated by ** and * respectively in Tables 4.1–4.3), after correcting for multiple testing using the Bonferroni-Holm approach (Holm, 1979; McLaughlin & Sainani, 2014). Thus, only the threshold against which the *p*-values were compared to establish significance were adjusted, rather than the *p*-values themselves. It is important to note that some metabolites presented with large standard deviations, which can, at least in part, be ascribed to individual variation in terms of lifestyle confounders (e.g. diet), as well as the small groups used.

4.4 RESULTS AND DISCUSSION

TB is well-known to result in cachexia (Chang *et al.*, 2013), a dramatic loss of weight due to elevated lipolysis and a disproportionately high muscle protein degradation (Morley *et al.*, 2006). The generally elevated levels of fatty acylcarnitines (especially acetylcarnitine) observed in the TB patients suggests a general increase in lipid catabolism (Table 4.1). This elevated fatty acid breakdown in the TB patients was also confirmed by other fatty acid catabolism markers previously detected by Luies and Loots (2016). Similarly, the generally elevated levels of amino acids (Table 4.2) confirm disproportionate protein degradation.

Table 4.1: The fatty acylcarnitines detected in the urine of TB patients and healthy controls comparatively, using semi-targeted LC-MS/MS metabolomics.

Metabolite name	TB patients: Average concentration (µmol/L creatinine) (Standard error of the mean)	Healthy controls: Average concentration (µmol/L creatinine) (Standard error of the mean)	Fold change (absolute value)	Mann Whitney test (*p*-value)
Free Carnitine (C_0)	0.103 (0.041)	0.030 (0.019)	3.406	0.005**
Acetylcarnitine (C_2)	0.206 (0.123)	0.013 (0.005)	16.098	<0.001**
Propionylcarnitine (C_3)	0.095 (0.061)	0.019 (0.01)	5.094	<0.001**
Isovalerylcarnitine (C_5)	0.042 (0.019)	0.016 (0.013)	2.610	<0.001**
Octanoylcarnitine (C_8)	0.034 (0.011)	0.044 (0.038)	1.301	0.031
Decanoylcarnitine (C_{10})	0.114 (0.034)	0.134 (0.077)	1.178	0.212
Dodecanoylcarnitine (C_{12})	1.718 (0.568)	1.502 (1.115)	1.144	0.356
Tetradecanoylarnitine (C_{14})	7.268 (3.152)	4.551 (2.505)	1.597	0.053
Palmitoylcarnitine (C_{16})	107.61 (36.075)	89.248 (35.121)	1.206	0.500

** indicates a 5% significance, after correcting for multiple testing using the Bonferroni-Holm approach

Table 4.2: The amino acids detected in the urine of TB patients and healthy controls comparatively, using semi-targeted GC-MS metabolomics.

Metabolite name	TB patients: Average concentration (µmol/L creatinine) (Standard error of the mean)	Healthy controls: Average concentration (µmol/L creatinine) (Standard error of the mean)	Fold change (absolute value)	Mann Whitney test (*p*-value)
Alanine	29.916 (3.617)	23.220 (1.947)	1.288	0.161
Argininosuccinate	0.088 (0.010)	0.144 (0.017)	1.637	0.003*
Asparagine	15.267 (1.458)	11.112 (1.19)	1.374	0.004*
Aspartate	2.476 (0.336)	1.705 (0.499)	1.452	<0.001**
Cystathionine	1.236 (0.126)	1.592 (0.203)	1.287	0.183
Cysteine	20.192 (2.244)	15.501 (1.263)	1.303	0.097
Dopamine	0.101 (0.020)	0.077 (0.010)	1.314	0.267
Glutamate	4.66 (0.802)	1.787 (0.175)	2.608	<0.001**
Glutamine	64.855 (6.907)	37.289 (5.160)	1.714	<0.001**
Glycine	148.664 (23.873)	121.826 (33.673)	1.220	0.043
Glycine-Proline	0.45 (0.053)	0.570 (0.053)	1.267	0.032
Histidine	68.141 (10.646)	78.165 (9.043)	1.147	0.074
Hydroxylysine	0.424 (0.043)	0.308 (0.037)	1.377	0.011
Hydroxyproline	0.111 (0.048)	0.017 (0.004)	6.408	0.064
Isoleucine	2.030 (0.271)	1.295 (0.081)	1.567	0.005*
Leucine	4.656 (0.476)	3.246 (0.208)	1.435	0.003*
Lysine	12.802 (1.880)	12.407 (2.963)	1.016	0.076
Methionine	2.282 (0.270)	1.631 (0.156)	1.399	0.064
Ornithine	1.733 (0.241)	0.884 (0.098)	1.954	0.003*

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Metabolite name	TB patients: Average concentration (µmol/L creatinine) (Standard error of the mean)	Healthy controls: Average concentration (µmol/L creatinine) (Standard error of the mean)	Fold change (absolute value)	Mann Whitney test (*p*-value)
Phenylalanine	5.918 (0.381)	3.655 (0.339)	1.619	<0.001**
Pipecolate	0.230 (0.068)	0.057 (0.007)	4.034	<0.001**
Proline	2.095 (0.370)	0.743 (0.061)	2.818	<0.001**
Proline-hydroxyproline	20.653 (1.509)	22.134 (1.578)	1.072	0.292
Pyroglutamate	0.083 (0.023)	0.023 (0.003)	3.684	<0.001**
Serine	46.882 (4.618)	34.691 (3.002)	1.351	0.038
Threonine	14.028 (1.992)	12.542 (1.712)	1.119	0.292
Tryptophan	5.901 (0.583)	4.111 (0.325)	1.435	0.015
Tyrosine	10.036 (1.018)	7.197 (0.639)	1.394	0.005
Valine	5.895 (0.687)	3.932 (0.228)	1.499	0.002*
α-Aminoadipate	0.943 (0.093)	1.123 (0.153)	1.191	0.412
α-Aminobutyrate	1.298 (0.152)	1.561 (0.421)	1.209	0.471
α-Aminopimelate	0.007 (0.001)	0.007 (0.001)	1.074	0.239
γ-Aminobutyrate	0.049 (0.010)	0.045 (0.006)	1.105	0.465

** and * indicate a 5% and 10% significance respectively, after correcting for multiple testing using the Bonferroni-Holm approach

Interestingly however, several other specific fatty acylcarnitines, amino acids and organic acids detected indicate other abnormalities to the human metabolome due to TB. The significantly elevated levels of acetylcarnitine (indicative of acetyl-CoA accumulation), propionylcarnitine (indicative of propionyl-CoA accumulation), isovalerylcarnitine (indicative of isovaleryl-CoA accumulation) and various amino acids, including arginosuccinate, asparate (and associated asparagine), ornithine (and associated proline and hydroxyproline) and glutamate (and associated glutamine), are of particular interest considering that it strongly suggests a defective urea cycle in the TB patients, more specifically an inhibition of either carbamoyl phosphate synthetase or N-acetylglutamate synthase (NAGS) (Scaglia *et al.*, 2004), since orotic acid could not be detected (Caldovic *et al.*, 2010) (see Figure 4.1).

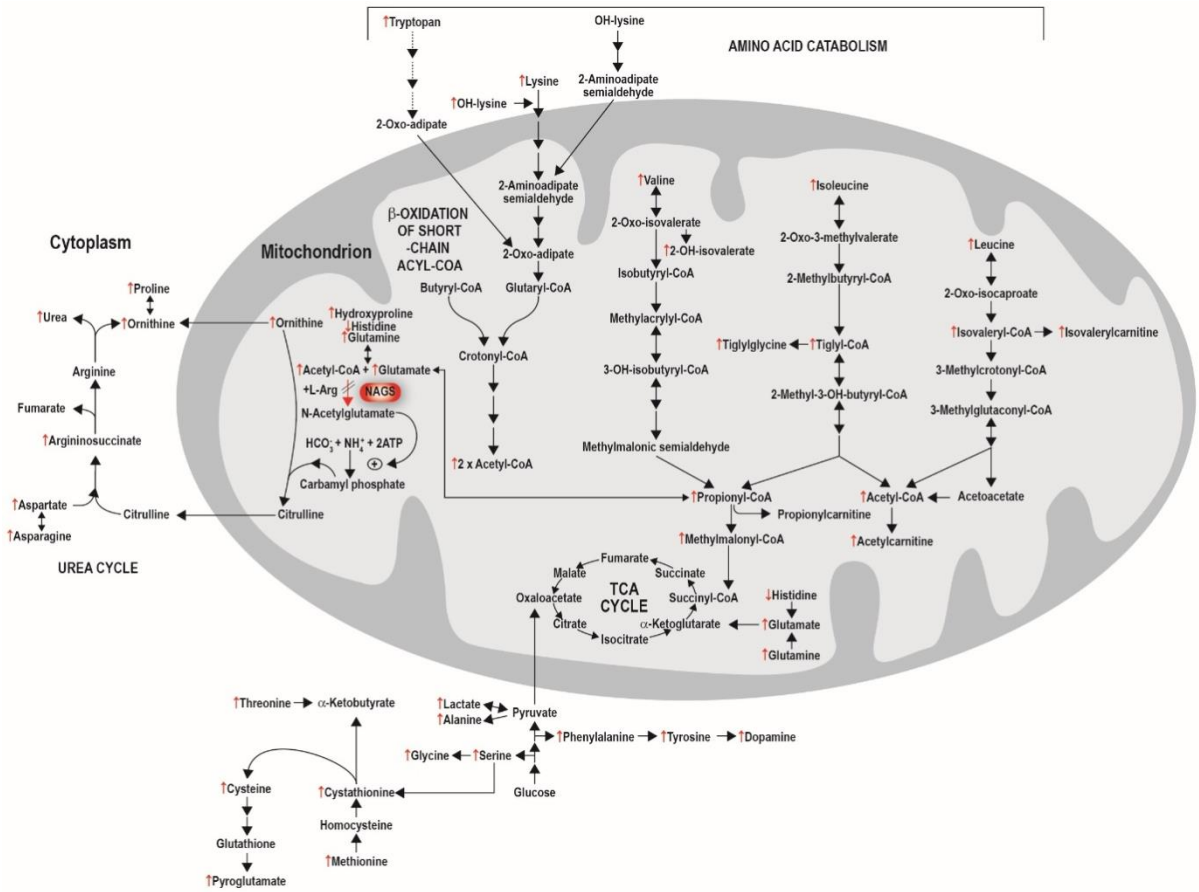

Figure 4.1: Abnormalities in the fatty acylcarnitines, amino acids and related organic acids detected in the urine of TB patients. Abbreviations: NAGS, N-acetylglutamate synthase; TCA, Tricarboxylic acid; ATP, adenosine triphosphate; CoA, coenzyme A.

The proposed mechanisms by which this occurs are due to the elevated propionyl-CoA and isovaleryl-CoA. Dercksen *et al.* (2014) indicated that propionyl-CoA and isovaleryl-CoA conjugates with glutamate, and the synthesized N-propionylglutamate and N-isovalerylglutamate respectively, directly inhibits NAGS. This further suggests inefficient removal of ammonia in a patient with active TB and may be an important survival mechanism for *M. tuberculosis*, since this pathogen requires ammonia to neutralise the acidic environment of the phagosome in order to survive within the host (Saviola, 2013). Further confirmation of the elevated propionyl-CoA, is the accompanying elevated concentrations of methylmalonate detected in the TB patients, which is also known to be an inhibitor of NAGS (Dercksen *et al.*, 2014). Perhaps of even greater interest, is the fact that elevated concentrations of methylmalonate (Table 4.3) in the urine are considered a particularly sensitive and definitive indicator for a vitamin B_{12} deficiency (Barness *et al.*, 1963; Green, 2011).

Table 4.3: The amino acid-associated organic acids detected in the urine of TB patients and healthy controls comparatively, using semi-targeted GCxGC-TOFMS metabolomics.

Metabolite name	TB patients: Average concentration (µmol/L creatinine) (Standard error of the mean)	Healthy controls: Average concentration (µmol/L creatinine) (Standard error of the mean)	Fold change (absolute value)	Mann Whitney test (*p*-value)
2-Hydroxyisovalerate	0.216 (0.117)	0.008 (0.003)	25.766	<0.001**
2-Hydroxyvalerate	163.613 (152.980)	1.825 (0.448)	89.381	<0.001**
2-Ketoglutarate	5.160 (4.305)	0.770 (0.314)	6.479	0.427
2-Ketovalerate	5.097 (4.021)	0.250 (0.149)	15.721	0.002**
2-Methyl-3-hydroxybutyrate	42.823 (36.828)	3.002 (0.352)	14.264	<0.001**
3-Hydroxy-2-methyl-butanoate	152.593 (114.431)	7.910 (0.850)	19.291	<0.001**
3-Methylglutarate	145.680 (132.298)	3.125 (0.382)	46.545	<0.001**
Lactate	124.647 (94.401)	10.817 (1.198)	11.523	<0.001**
Methylcitrate	7.086 (5.562)	0.773 (0.166)	9.033	0.003**
Methylmalonate	10.327 (3.712)	1.665 (0.169)	6.201	<0.001**
Methylsuccinate	30.958 (23.857)	1.926 (0.229)	16.075	<0.001**
Tiglylglycine	6.357 (3.780)	1.080 (0.124)	5.817	0.001**

** indicates a 5% significance, after correcting for multiple testing using the Bonferroni-Holm approach

Vitamin B_{12} is not only important for the human host, but is a key cofactor for the functioning of the following *M. tuberculosis* enzymes, essential to a vast majority of its metabolic and growth processes: (1) methionine synthase (MetH/E), (2) methylmalonyl-CoA mutase (MutAB) and (3) ribonucleotide reductase (NrdZ) (Gopinath *et al.*, 2013). As indicated in Figure 4.2, *M. tuberculosis* has the capacity to synthesize the citrate cycle intermediate succinyl-CoA from methylmalonyl-CoA via the vitamin B_{12}-dependent enzyme MutAB (Savvi *et al.*, 2008). Furthermore, *M. tuberculosis* can also use propionate as a carbon source for bacterial growth, but only when vitamin B_{12} is exogenously provided by the host in the form of cyanocobalamin (Eoh & Rhee, 2014; Savvi *et al.*, 2008). The interaction between the glyoxylate, citrate and methylcitrate cycles in this organism, supports an accelerated growth rate in the presence of heptadecanoate (C_{17}) and valerate (C_5), and further evidence for the above mentioned metabolic processes in *M. tuberculosis* is a significantly enhanced growth rate when growth media is supplemented with vitamin B_{12}(Savvi *et al.*, 2008). In the current investigation, the aforementioned propionylcarnitine, isovalerylcarnitine (the detoxification forms of the fatty acids propionate and isovalerate respectively), methylmalonate, and methylcitrate, were all significantly elevated in the TB patients when compared to that of the healthy controls. This, in the context of the other vitamin B_{12} related abnormalities described above, is indicative of a possible depletion of vitamin B_{12} in the host, most probably due to the scavenging of vitamin B_{12} by *M. tuberculosis* in order to use the host's propionate as an energy substrate for bacterial survival. Furthermore, 2-methylisocitrate, an intermediate of

the *M. tuberculosis* methylcitrate cycle, is a potent and very specific inhibitor of the NADP-isocitrate dehydrogenase in the host (Smith & Plaut, 1979), which reduces NADPH availability, in turn negatively influencing various host anabolic reactions (contributing to TB-cachexia), and glutathione disulfide synthesis (Ribas *et al.*, 2016) (contributing to oxidative stress) (Du Preez & Loots, 2013; Ghezzi, 2011; Luies & Loots, 2016).

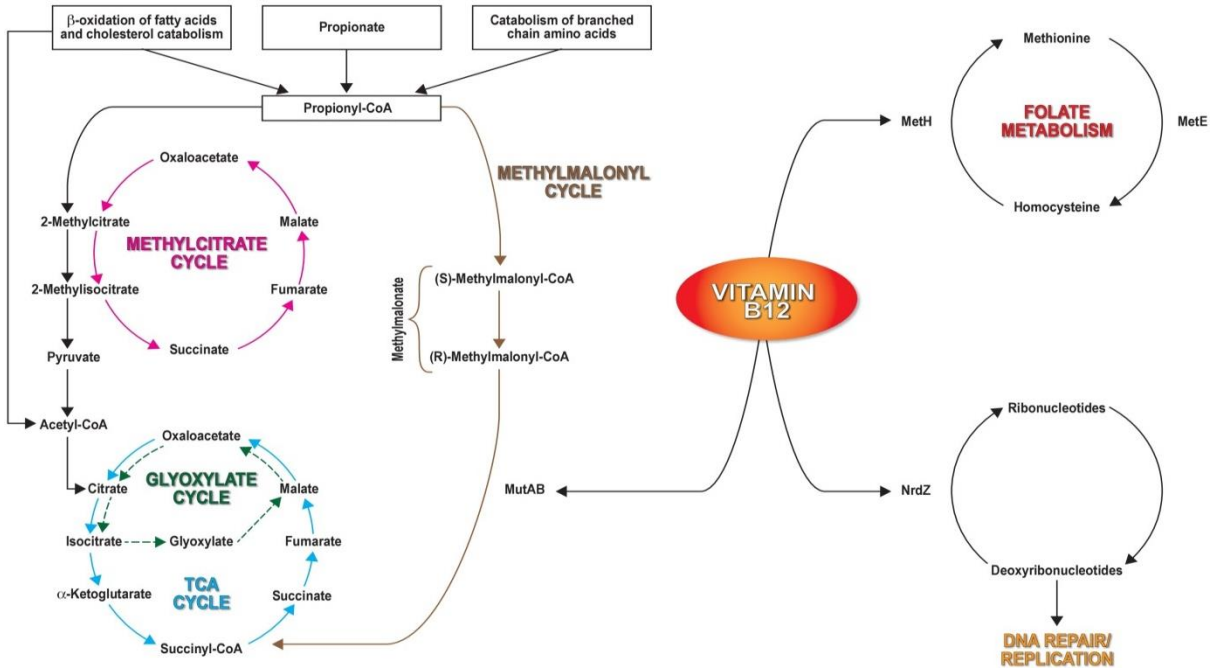

Figure 4.2: Vitamin B_{12} metabolism in M. tuberculosis. M. tuberculosis encodes methionine synthase (MetH/E) (involved in folate metabolism), ribonucleotide reductase (NrdZ) (for DNA repair and replication), and methylmalonyl-CoA mutase (MutAB) (involved in the methylmalonyl cycle), which all require vitamin B_{12} as a cofactor for its functionality.

M. tuberculosis also contains a class 1b ribonucleotide reductase (RNR), encoded by the mycobacterialgenes *NrdE* and *NrdF*, as well as a class 11 RNR, encoded by *NrdZ* (Dawes *et al.*, 2003). The latter is also vitamin B_{12}-dependent and catalyses the conversion of ribonucleotides to deoxyribonucleotides (see Figure 4.2), ensuring the cycling of nucleotides in the cell and replication of chromosomes (Szekeres *et al.*, 1997). Previous studies have indicated that *M. tuberculosis* expresses RNR in response to reduced oxygen, nitric oxide and carbon monoxide, i.e. during similar conditions as caused by the host immune response to *M. tuberculosis*, and is thus thought to be the controlling factor for maintaining latent infection (Kumar *et al.*, 2008; Shiloh *et al.*, 2008; Voskuil *et al.*, 2003). Considering this, an interesting hypothesis worth testing, would be whether or not *M. tuberculosis* transition from latent infection to active disease may be linked to a vitamin B_{12} deficiency in the host. To our knowledge, no studies have been done to date investigating the vitamin B_{12} status of untreated TB patients, however, the association between vitamin B_{12} and TB has been suggested previously (Chakraborty *et al.*, 2014; Toosi *et al.*, 2008), and folate deficiencies

(which is also vitamin B_{12}-dependent) in TB patients is a common occurrence (Evans & Attock, 1971).

As mentioned earlier, TB is associated with pronounced protein degradation (Chang *et al.*, 2013), which is proposed to occur via a number of different mechanisms, including: (1) the resulting inflammatory response due to *M. tuberculosis (Rajaram et al., 2011)*, (2) the association between TB and insulin resistance (Luies & Loots, 2016; Young *et al.*, 2009), (3) wasting due to the nutritional imbalance (and also linked with reduced insulin), resulting from the upregulation of leptin and ghrelin (Zheng *et al.*, 2013), the latter of which may also trigger (4) autophagy (Luies & Loots, 2016; Songane *et al.*, 2012). Furthermore, a general amino aciduria, as seen in this investigation, may be induced by several other metabolic and physiological occurrences, including lactic acidosis, ketoacidosis, hyperinsulinism, liver disease, and a vitamin B6 deficiency. Lactic acidosis is characterised by the accumulation of lactate, coupled with insufficient delivery of oxygen required for adequate aerobic metabolism (Rose *et al.*, 2011). In the current investigation, the elevated concentrations of alanine (although not significant), as well as the significantly elevated lactate, 2-hydroxyisovalerate (Landaas & Jakobs, 1977) and 2-hydroxyvalerate (Asano *et al.*, 1988) in the TB group, are strong indicators of lactic acidosis (Asano *et al.*, 1988; Landaas & Jakobs, 1977), which is a well-known occurrence in TB (Ntambwe & Maryet, 2012). The elevated concentrations of the branched chain amino acids (i.e. leucine, isoleucine and valine) in the TB group, also confirm previous associations between TB and abnormal insulin production/secretion (Luies & Loots, 2016) and/or diabetes (Felig *et al.*, 1970), as well as possible ketoacidosis (indicated by the elevated 2-hydroxyisovalerate (Landaas & Jakobs, 1977)) in these TB patients. Further confirmation of this are the significantly elevated concentrations of various organic acids associated with branched chain amino aciduria, including 2-ketovalerate (Hutton *et al.*, 1980), 2-methyl-3-hydroxybutyrate (Fernandes *et al.*, 2006), 3-hydroxy-2-methyl-butanoate (Sutton *et al.*, 2003), 3-methylglutarate (Manoli & Venditti, 2016), methylsuccinate (Newgard *et al.*, 2009), and tiglylglycine (Fernandes *et al.*, 2006) (see Table 4.3). Previously, Luies and Loots (2016) detected an abnormal organic acid profile (elevated phenylacetate and phenyllactate) indicative of elevated concentrations of phenylalanine and suggested that this is most likely due to the general amino aciduria induced by the proposed insulin abnormalities. However, Sonika and Kar (2012) have indicated a strong correlation between TB and liver disease, which is also a common occurrence in urea cycle defects (Nassogne *et al.*, 2005). Additionally, the liver and kidneys are the organs in the body containing the highest concentration of peroxisomes, which are well-known for their function in oxidative stress homeostasis and elevated concentrations of free radicals result in peroxisomal stress (Nordgren & Fransen, 2014). The latter is associated with elevations in pipecolate (Fernandes *et al.*, 2006), which was detected in the urine of the TB patient group, further confirming the associated metabolic picture described above.

4.5 CONCLUSION

This investigation was the first of its kind to use a multiple instrument, semi-targeted metabolomics approach to characterize the altered fatty acylcarnitines, amino acids and selected organic acids in the urine of TB patients. The generally elevated concentrations of the fatty acylcarnitines and amino acids are most likely due to TB-cachexia. However, the significantly elevated concentrations of arginosuccinate, asparate (and associated asparagine), ornithine (and associated proline and hydroxyproline) and glutamate (and associated glutamine) in particular, indicate a urea cycle abnormality, due to inhibition of NAGS by the accumulating propionyl-CoA, isovaleryl-CoA and methylmalonyl-CoA in TB patients. Furthermore, elevated propionylcarnitine, methylmalonate and methylcitrate in the TB patient urine are associated with a vitamin B_{12} deficiency, which deserves further investigation. Lastly, various metabolites indicative of lactic acidosis, ketoacidosis, oxidative stress and liver damage were identified in the urine of the TB patients.

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CHAPTER 5:
FINAL CONCLUSIONS AND FUTURE PROSPECTS

5.1 CONCLUDING SUMMARY

Although TB is believed to have originated nearly 40 000 years ago, and hence is often referred to as the first disease known to mankind, its causal agent, *M. tuberculosis*, was only isolated in 1882 by Robert Koch. This disease infects nearly a third of the global population and remains a global pandemic, resulting in an alarming 1.8 million deaths annually. Better elucidation of the disease mechanisms associated with TB, the pathogen's adaptations to the host immune response, and that of TB drugs, are still as relevant today as it was decades ago. Hence, investigating this disease using an omics research perspective may serve well to expand the current knowledge of the disease and the improved diagnosis and treatment thereof. To this end, metabolomics is the holistic, unbiased identification and quantification of the metabolome in a specific biological system, and has a unique capacity for identifying new disease metabolite (bio)markers in sputum, blood and urine from TB patients. Apart the possible diagnostic applications of such potential biomarkers, it may also contribute to the existing knowledge of *M. tuberculosis* biology and associated metabolic pathways, including various mechanisms related to bacterial drug resistance and virulence, as well as mechanisms relating to host adaptations, and drug action or the associated side-effects in the host.

To date, the majority of omics (i.e. genomics, proteomics, transcriptomics and metabolomics) data generated, have used *M. tuberculosis* isolates from cultures; however, it is well-known that this organism's metabolism, gene expression and growth mechanisms in culture differs greatly from when it's growing and surviving in the human host. Sputum has been the preferred sample matrix for TB metabolomics studies to date, mainly due to its high bacterial content and availability (since it is the preferred diagnostic sample matrix collected currently). However, it has various limitations with regards to its diagnostic and research applications, which include its complexity and variable viscosity, and hence require additional pre-processing steps. Furthermore, sputum sample collection in children and HIV-infected individuals is unreliable and invasive. This begs the question: "Would other sample matrixes (such and blood, breath or urine) collected from TB-positive patients, serve better for diagnostic and research applications?" Although the use of blood samples may serve as a good alternative to sputum due to its homogeneous composition, collection of such may be considered invasive, and the metabolite concentrations are comparatively less than in other sample types. Urine has become an attractive option as a research and diagnostic matrix for

identifying metabolite markers associated with host–pathogen adaptations and interactions. Furthermore, since it contains many host-derived intermediates and end-products of the metabolism in easily detectable concentrations, large amounts can be collected effortlessly in a non-invasive fashion, and it is generally considered to be sterile. To date, however, very little data has been published on the metabolome of urine collected from TB-positive patients.

As previously described in Chapter 4, and attached in Appendix B, this investigation was the first of its kind to use a multiple instrument, semi-targeted metabolomics approach to characterise the altered fatty acylcarnitines, amino acids and selected organic acids in the urine of TB patients. The generally elevated concentrations of the fatty acylcarnitines and amino acids are most likely due to TB-cachexia. However, the significantly elevated concentrations of arginosuccinate, asparate (and associated asparagine), ornithine (and associated proline and hydroxyproline) and glutamate (and associated glutamine) in particular, indicate a urea cycle abnormality, due to inhibition of NAGS by the accumulating propionyl-CoA, isovaleryl-CoA and methylmalonyl-CoA in TB patients. Furthermore, elevated propionylcarnitine, methylmalonate and methylcitrate in the TB patient urine are associated with a vitamin B_{12} deficiency, which deserves further investigation. Lastly, various metabolites indicative of lactic acidosis, ketoacidosis, oxidative stress and liver damage were identified in the urine of the TB patients.

5.2 FUTURE PROSPECTS

The profound ability of *M. tuberculosis* to adapt and evolve in response to its surrounding physiological conditions has made the eradication of TB impossible. A multi-instrumental approach, as used during this investigation, can better provide a holistic view of the TB disease state and its impact on the host metabolome.

Considering that the identities of the detected metabolites are rarely validated by comparison of their respective spectral patterns in biofluids with those of commercially available standard solutions, a first step in terms of future prospects would be to truly validate these markers using confirmatory tests in support of the metabolomics data presented here. Furthermore, the use of a bigger sample cohort is also crucial.

Additionally, future studies should aim to focus on the various altered metabolic pathways identified in this study, such as the abnormal urea cycle and the associated NAGS inhibition. Furthermore, the overwhelming evidence for the occurrence of a vitamin B_{12} deficiency in TB patients should be investigated as this could not only lead to better treatment options, but also elucidate the possible need for, or benefits of, vitamin B_{12} supplementation and an

improved treatment outcome. To date, no investigation has determined the vitamin B_{12} status of TB-positive individuals and how this relates to the conversion of a latent to and active TB disease state, nor disease progression or treatment outcome. The role of vitamin B12 in *M. tuberculosis* infection is definitely something that warrants further investigation. Future investigations should be conducted in TB patients to specifically illustrate the intracellular effect of vitamin B12, not only to observe various factors such as TB growth, but also to link these effects to the host metabolome and immune responses.

Additionally, this study motivates the integration of various omics approaches (i.e. systems biology), to better characterise TB, which might accelerate the development of new diagnostic and treatment initiatives. From the literature presented in this dissertation, it also becomes apparent that there is a strong association between immunology and metabolism. Hence, more metabolomics-derived metabolite markers could be interpreted in the context of this discipline, in order to better elucidate how TB interacts with its host and how it manipulates the host's immune system in order to ensure its survival.

APPENDIX A

Table A.1: Summary of patient descriptive/demographic and clinical information.

Patient ID:	Age:	Gender:	BMI:	Culture:	TTP:	Ziehl–Neelsen stain	Löwenstein–Jensen:	Does patient drink?	Does patient smoke?
5755	17	Female	22.1	Positive	3	1		No	No
5014	21	Female	22.5	Positive	2	3	1	No	No
4655	22	Female	19.3	Positive	8	1	1	Yes	Yes
5411	23	Male	18.0	Positive	1	3	1	No	No
4868	24	Male	18.8	Positive	5	1	1	No	No
5346	24	Female	16.6	Positive	6	1	1	No	No
3110	25	Female	18.3	Positive	5	1	1	No	No
4866	25	Female	21.1	Positive	7	1	1	No	No
5270	26	Female	17.5	Positive	4	2	1	No	No
5079	28	Female	21.5	Positive	11	0	1	No	No
5299	28	Female	18.1	Positive	1	3	1	No	No
5414	31	Male	17.0	Positive	4	1	1	No	No
5456	31	Male	18.5	Positive	6	1	1	No	No
2882	35	Female	21.2	Positive		1	2	Yes	Yes
4849	36	Female	18.1	Positive	5	1	1	No	No
3015	37	Male	19.4	Positive	3	1	1	Yes	Yes
5229	37	Male	15.8	Positive	1	2	1	No	No
4898	37	Female	18.5	Positive	3	2	1	No	No
4989	39	Male	16.3	Positive	14	0	1	No	No
4798	40	Male	20.5	Positive	9	1	1	No	Yes
2963	42	Male	15.0	Positive	3	1	2	No	Yes
5011	42	Male	15.9	Positive	3	1	1	No	No
4886	42	Female	17.5	Positive	4	1	1	No	No
2955	44	Female	16.6	Positive	12	1	1	Yes	Yes
5173	44	Male	17.7	Positive	5	1	1	No	No
4832	45	Female	18.6	Positive	7	1	1	No	No
5334	45	Female	22.0	Positive	12	0	1	No	No
5594	48	Male	19.8	Positive	11	0		Yes	No
4925	49	Male	20.8	Positive	9	1	1	No	No
5036	50	Male	20.5	Positive	2	3	1	No	No
5064	54	Male	16.4	Positive	9	0.3	1	No	No
3050	55	Female	20.1	Positive	2	1	1	Yes	Yes
2768	58	Male	17.7	Positive		0	0	Yes	No

APPENDIX B

The following have been submitted for publication:

- Anthony, C., Luies, L. Mienie, J.L., Lindeque, J.Z., Ronacher, K., Walzl, G. & Loots, D. (2018). Detection of altered fatty acylcarnitines, amino acids and organic acids in tuberculosis patient urine.

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Clinical Infectious Diseases

Detection of altered fatty acylcarnitines, amino acids and organic acids in tuberculosis patient urine

--Manuscript Draft--

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Abstract:	Background: Despite fervent research efforts to date since the discovery of the tuberculosis (TB) disease-causing pathogen, Mycobacterium tuberculosis, in 1882 by Robert Koch, TB is still considered a global pandemic, resulting in an estimated 1.8 million deaths annually. Better elucidation of the mechanisms associated with TB, the pathogen's adaptations to the host immune response, and that of TB drugs, are still as relevant today as it was 135 years ago. Investigating this disease using an omics research perspective, the most recent of which being metabolomics, has served well to expand the current knowledge of the disease and the improved diagnosis and treatment thereof, due to its unique capacity for identifying new disease biomarkers. Methods: In this investigation, a combined semi-targeted liquid and gas chromatography mass spectrometry metabolomics approach was used to compare the urinary fatty acylcarnitines, amino acids and selected organic acids of active TB patients to that of healthy individuals, in order to better characterize the TB-induced alterations to the host metabolome. Results and Conclusion: A general increase in the fatty acylcarnitines and amino acids are most likely due to TB-induced cachexia. However, altered levels of various other individual or groups of specific metabolites in the TB patient urine, associated with a vitamin B12 deficiency, urea cycle abnormality, lactic acidosis, ketoacidosis, oxidative stress and liver damage in these patients, were also identified.

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Detection of altered fatty acylcarnitines, amino acids and organic acids in tuberculosis patient urine

Abbreviated title: Altered urine profiles in TB patients

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Summary

LC-MS and GC-MS metabolomics were used to investigate the urinary fatty acylcarnitines, amino acids and organic acids in TB patients. Results indicate a urea cycle abnormality and vitamin B_{12} deficiency, in conjunction with lactic acidosis, ketoacidosis, oxidative stress and liver damage.

Abstract

Background: Despite fervent research efforts to date since the discovery of the tuberculosis (TB) disease-causing pathogen, *Mycobacterium tuberculosis*, in 1882 by Robert Koch, TB is still considered a global pandemic, resulting in an estimated 1.8 million deaths annually. Better elucidation of the mechanisms associated with TB, the pathogen's adaptations to the host immune response, and that of TB drugs, are still as relevant today as it was 135 years ago. Investigating this disease using an omics research perspective, the most recent of which being metabolomics, has served well to expand the current knowledge of the disease and the improved diagnosis and treatment thereof, due to its unique capacity for identifying new disease biomarkers.

Methods: In this investigation, a combined semi-targeted liquid and gas chromatography mass spectrometry metabolomics approach was used to compare the urinary fatty acylcarnitines, amino acids and selected organic acids of active TB patients to that of healthy individuals, in order to better characterize the TB-induced alterations to the host metabolome.

Results and Conclusion: A general increase in the fatty acylcarnitines and amino acids are most likely due to TB-induced cachexia. However, altered levels of various other individual or groups of specific metabolites in the TB patient urine, associated with a vitamin B_{12} deficiency, urea cycle abnormality, lactic acidosis, ketoacidosis, oxidative stress and liver damage in these patients, were also identified.

Keywords: metabolomics; tuberculosis; fatty acylcarnitines; amino acids; organic acids

1. Introduction

Infection with *Mycobacterium tuberculosis* affects approximately one-third of the world's population, and can lead to active, symptomatic disease, called tuberculosis or to asymptomatic states, often referred to as latent infection. In 2015 alone, 10.4 million new TB cases were reported, resulting in an estimated 1.8 million deaths [1]. Since the discovery of *M. tuberculosis* in 1882 by Robert Koch, a vast amount of genomics, proteomics and transcriptomics data have been generated, leading to our current understanding of *M. tuberculosis* and TB. The majority of these data have been generated from studies using *M. tuberculosis* cultures; however, it is well-known that this organism's metabolism and growth in culture differs greatly from growth in the human host, where many different growth mechanisms and energy substrates are preferentially used [2]. Furthermore, very little research to date has focused on the adaptations of *M. tuberculosis* to the host's defense mechanisms or growth environment, or for that matter, the host's adaptations or altered metabolic state in response to the infectious pathogen. This is important since the pathophysiology of *M. tuberculosis* is directly linked to its metabolism and complex physiology [3], and to that of the host. Additionally, this pathogen is able to utilize numerous growth substrates, either by scavenging this from the host or via *de novo* biosynthesis, in order to ensure its own survival [4, 5].

Metabolomics has enabled the identification of new biomarkers in sputum, blood and urine from TB patients, describing novel *M. tuberculosis* metabolic pathways and host adaptations. Apart from their possible diagnostic applications, many of these new TB biomarkers have contributed to the existing knowledge of the biology of the causative pathogen, including various underlying disease mechanisms related to *M. tuberculosis* drug resistance [6, 7] and virulence [8], such as the upregulation of numerous antioxidant pathways, the use of alternative energy substrates, a change in aconitase functionality towards mRNA binding and stability, and cell wall remodeling [6-9]. Additionally, the mechanisms of TB drug action [10-12] and related side-effects in the host [13, 14] have also been elucidated. Furthermore, the use of metabolomic approaches have allowed for a better understanding of the adaptations of *M. tuberculosis* to host defenses and *vice versa*, resulting in the discovery of (a) the citramalate cycle in *M. tuberculosis*, which interacts with an upregulated

glyoxylate cycle, (b) an increased use of fatty acids and glutamate by the pathogen, (c) an alternative hydrogen peroxide mechanism by the host to eliminate the infecting bacteria, (d) electron transport chain inhibition in host [15], and (e) a compromised insulin production, which may provide clues to better treatment approaches [14]. To date, however, very little data has been published on urine from TB patients, which can be considered an ideal sample matrix to identify markers associated with this host–pathogen interaction [2]. Considering this, a combined semi-targeted liquid and gas chromatography mass spectrometry (LC-MS and GC-MS, respectively) metabolomics approach was used to compare the urinary fatty acylcarnitines, amino acids and selected organic acids of active TB patients (n=31) to that of healthy individuals (n=29), in order to identify new metabolomics biomarkers for TB.

2. Materials and methods

2.1 Clinical samples

Anonymized archived urine samples were obtained from the Faculty of Medicine and Health Sciences, DST/NRF Centre of Excellence for Biomedical Research, at the Stellenbosch University/MRC Centre for TB Research. These samples were transported frozen, on dry ice, to the North-West University, Human Metabolomics, Laboratory of Infectious and Acquired Diseases Metabolomics, and immediately stored at -20ºC. These samples formed part of an observational cohort study of individuals with drug sensitive pulmonary TB, diagnosed using bacteriological cultures and smear microscopy, who were HIV-seronegative, not pregnant, and free from other diseases [16]. The samples selected for this investigation included 31 culture-confirmed active South African TB patients (18 males and 13 females, between the ages of 21 and 58), collected at time of diagnosis (i.e. prior to treatment administration). Additionally, urine samples were also collected from 29 age and gender matched healthy TB-negative individuals from the same geographical area.

2.2 Chemicals and reagents

All fatty acylcarnitine isotopes [i.e. free carnitine (C_0), acetylcarnitine (C_2), propionylcarnitine (C_3), isovalerycarnitine (C_5), octanoylcarnitine (C_8), decanoylcarnitine (C_{10}), dodecanoylcarnitine (C_{12}), tetradecanoylcarnitine (C_{14}), palmitoylcarnitine (C_{16})] were kindly supplied by Dr. Herman J. ten Brink (Amsterdam, Netherlands). The butanolic hydrochloric acid (HCl), formic acid, 3-phenylbutyric acid, hexane and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Pyridine, HCl, anhydrous sodium sulphate (Na_2SO_4) and trimethylchlorosilane (TMCS) were purchased from Merck (Darmstadt, Germany). The ultra-pure water, acetonitrile, ethyl acetate and diethyl ether were from Honeywell Burdick & Jackson (Muskegon, Michigan, USA).

2.3 Sample analysis

Prior to the fatty acylcarnitines, amino acids and organic acids analyses, a creatinine determination was done on each urine sample (n=60) using a creatinine enzyme kit (Thermo Fisher Scientific; reference number 981845) and an Indiko Clinical Analyzer, Type 863 (Thermo Fisher Scientific, Massachusetts, USA). The creatinine values were used to normalize the volume of urine and/or reagents required for each analysis and also for quantification purposes, where each compound detected was expressed as µmol/L creatinine.

2.3.1 Fatty acylcarnitines analysis

After the addition of 205 µL internal standard stock solution (made up using a combination of all the stable fatty acylcarnitine isotopes described above), all of the collected urine samples (100 µL) were deproteinated as described in Venter *et al*. (2015) [17]. Briefly, 30 µL of acetonitrile was added to each sample, followed by centrifugation (5 min at 12 000 g at 4ºC), in order to precipitate all proteins, after which the supernatant was decanted into a new tube without disturbing the protein pellet. This was followed by an overnight freezing step at -80ºC and then evaporation to complete dryness under a steady stream of nitrogen at 37°C. Butylation and analysis of the samples were performed as

described by Van Aardt *et al*. (2016) [18]. To summarize, 200 µL of 3N butanolic HCl was added to the dried residue and the samples were incubated for 60 min at 50°C. These butylated samples were then evaporated to dryness under a steady stream of nitrogen at 37°C and resuspended in a water:acetonitrile solution (50:50) (v/v) containing 0.1% formic acid.

The samples were analyzed by injecting 1 µL into an Aligent 1290 series LC system coupled to a 6410 QQQ mass analyzer (Aligent Technologies, California, USA) consisting of a Micro Vacuum Degasser (G1379B), Binary pump SL (G1312B), preparative Autosampler HiP-ALS SL (G1367C), Thermostat ALS (G1330B) and Thermostatted Column Compartment SL (G1316B). The system was equipped with a C18 Zorbax SB-Aq column (150 mm x 2.1 mm x 3.5 µm) supplied by Agilent Technologies, which was kept at 30°C during the entire run. The chromatographic gradient started at 95% solvent A (water with 0.1% formic acid), with a flow rate of 0.2 mL/min, where it was maintained for 1.5 min. Hereafter, the gradient was increased to 18% solvent B (acetonitrile with 0.1% formic acid) over a period of 1.5 min, at which it was maintained for a further 2 min. The gradient was then increased to 100% solvent B over 10 min during which the flow was gradually increased to 0.3 mL/min and then kept constant at this flow for 3 min. The flow and gradient were then progressively decreased over a period of 2 min to 0.2 mL/min and 5% solvent B. A post-run continued for 8 min at 5% solvent B after every sample to ensure equilibration of the column, resulting in a total run time of 28 min per sample. The ESI source gas temperature was kept at 300°C, with a flow rate of 7.5 L/min, and nebulizer pressure was kept at 30 psi with capillary voltage at 3500 V [18]. The nine most prevalent fatty acylcarnitines (C_0, C_2, C_3, C_5, C_8, C_{10}, C_{12}, C_{14} and C_{16}) were identified and quantified using the stable isotopes as a reference.

2.3.2 Amino acids analysis

An EZ:faast Kit (Separation, Randburg, South Africa) was used to extract the amino acids from the urine, and was performed according to the manufacturer's instruction manual. A stable isotope mixture (Cambridge Isotope Laboratories, Inc., San Francisco, USA) was added for quantification purposes, followed by GC-MS analysis. To summarize, 1 µL of each extracted sample was randomly

injected onto a HP 6890 Series GC (Hewlett-Packard Company, North Carolina, USA), coupled to an Agilent 5973 Network: Mass Selective Detection MS (Aligent Technologies, California, USA), in splitless mode. A Zebron ZB-AAA GC Column (E16-000847) (10 m x 0.25 mm) was used for compound separation, using ultra-pure helium at a constant flow of 1.3 mL/min at a pressure of 0.37 psi as the carrier gas. The inlet temperature was kept at a constant 250°C for the entire run, and the initial oven temperature was 60°C, where it was held for 1 min, followed by an increase of 50°C/min to 110°C. The temperature was then increased at a rate of 20°C/min to 185°C, followed by a rate of 25°C/min to 235°C, and lastly at a rate of 30°C/min to a final temperature of 320°C, where it was held constant for an additional 0.5 min. The total run time per sample was 11.58 min. Peak detection and peak identification were done using Chemstation software (2011; Agilent Technologies MS Workstation, Version 7.0.0, USA).

2.3.3 Organic acids analysis

An organic acid analysis was performed as described in Luies and Loots (2016) [14]. Briefly, six drops of 5N HCl and a volume of internal standard stock solution (with a final concentration of 25 µmol phenylbutyric acid/mg urinary creatinine) were added to each urine sample. Hereafter, 6 mL of ethyl acetate was added, followed by mixing and centrifugation (3000 rpm for 3 min) in order to achieve a solvent phase separation. The organic phase was then aspirated into a second, clean Pyrex tube. Diethyl ether (3 mL) was added to the remaining aqueous phase, followed by mixing and centrifugation, after which the organic solvent was aspirated and added to the previously collected organic phase. Hereafter, approximately 3 g Na_2SO_4 was added to each sample, followed by a brief vortex and centrifugation step in order to pellet the Na_2SO_4. The combined organic phase was then decanted from the pellet and evaporated under a stream of nitrogen at 37°C, followed by derivatization of the extract using BSTFA (22.6 µL/µmol creatinine), TMCS (4.5 µL/µmol creatinine) and pyridine (4.5 µL/µmol creatinine), at 60°C for 60 min.

The derivatized samples were analyzed in a random order by injecting 1 µL of each, along with the necessary quality control (QC) samples, into a Pegasus 4D GCxGC-TOFMS system (LECO Africa

(Pty) Ltd, Johannesburg, South Africa), using a 1:12 split. The GCxGC system was equipped with a Restek Rxi-5Sil MS primary column (30 m x 0.25 mm x 0.25 μm) and Rxi-17 secondary column (0.9 m x 0.25 mm x 0.25 μm) for compound separation, using ultra-pure helium as the carrier gas at a constant flow of 1 mL/min. The injector temperature was held at a constant 280°C and cryomodulation was achieved using a hot pulse of nitrogen gas for 0.7 s, every 3 s. The temperature program for compound separation on the primary column was 55°C for 1 min, followed by an increase of 7°C/min to 285°C, at which it was held constant for 4 min. The temperature was then increased at a rate of 20°C/min to a final temperature of 305°C, where it was maintained for 1 min. The temperature ramp of the secondary oven was identical to that of the primary oven, with the exception of a +5°C offset at all time points. To prevent the MS from detecting the solvent, the filament was switched off and no mass spectra were recorded for the first 8 min of each analytical run. The temperatures of the transfer line and ion source were maintained at 270°C and 200°C respectively, for the entire run, with a detector voltage set at 1600 V and the filament bias at -70 eV.

2.4 Statistical analysis

Data processing and statistical analysis were performed using MATLAB software (2012; The MathWorks Inc., Natick, Massachusetts, USA). Prior to statistical analysis, data collected from the three separate analytical instruments were subjected to a series of standardized metabolomics data processing steps, including a 50% zero filter and a zero value replacement step [14]. In order to identify significant compounds when comparing the TB-positive and TB-negative healthy control groups, fold change values were calculated and Mann-Whitney tests were performed. Fold change examines the practical significance between two sets of samples, and an absolute fold change ≥ 2 is considered significant [19]. Mann-Whitney *p*-values were evaluated at a 5% and 10% significance level (indicated by ** and * respectively in Tables 1–3), after correcting for multiple testing using the Bonferroni-Holm approach [20, 21]. Thus, only the threshold against which the *p*-values were compared to establish significance were adjusted, rather than the *p*-values themselves.

3. Results and Discussion

TB is well-known to result in cachexia [22], a dramatic loss of weight due to elevated lipolysis and a disproportionately high muscle protein degradation [23]. The generally elevated levels of fatty acylcarnitines (especially acetylcarnitine) observed in the TB patients suggests a general increase in lipid catabolism (Table 1). This elevated fatty acid breakdown in the TB patients was also confirmed by other fatty acid catabolism markers previously detected by Luies and Loots (2016) [14]. Similarly, the generally elevated levels of amino acids (Table 2) confirm disproportionate protein degradation.

Interestingly however, a number of other specific fatty acylcarnitines, amino acids and organic acids detected indicate other abnormalities to the human metabolome due to TB. The significantly elevated levels of acetylcarnitine (indicative of acetyl-CoA accumulation), propionylcarnitine (indicative of propionyl-CoA accumulation), isovalerylcarnitine (indicative of isovaleryl-CoA accumulation) and various amino acids, including arginosuccinate, asparate (and associated asparagine), ornithine (and associated proline and hydroxyproline) and glutamate (and associated glutamine), are of particular interest considering that it strongly suggests a defective urea cycle in the TB patients, more specifically an inhibition of either carbamoyl phosphate synthetase or N-acetylglutamate synthase (NAGS) [24], since orotic acid could not be detected [25] (see Figure 1).

The proposed mechanisms by which this occurs are due to the elevated propionyl-CoA and isovaleryl-CoA. Dercksen *et al*. (2014) [26] indicated that propionyl-CoA and isovaleryl-CoA conjugates with glutamate, and the synthesized N-propionylglutamate and N-isovalerylglutamate respectively, directly inhibits NAGS. This further suggests inefficient removal of ammonia in a patient with active TB and may be an important survival mechanism for *M. tuberculosis*, since this pathogen requires ammonia to neutralize the acidic environment of the phagosome in order to survive within the host [27]. Further confirmation of the elevated propionyl-CoA, is the accompanying elevated concentrations of methylmalonate detected in the TB patients, which is also known to be an inhibitor of NAGS [26]. Perhaps of even greater interest, is the fact that elevated concentrations of methylmalonate (Table 3)

in the urine are considered a particularly sensitive and definitive indicator for a vitamin B_{12} deficiency [28, 29].

Vitamin B_{12} is not only important for the human host, but is a key cofactor for the functioning of the following *M. tuberculosis* enzymes, essential to a vast majority of its metabolic and growth processes: (a) methionine synthase (MetH/E), (b) methylmalonyl-CoA mutase (MutAB) and (c) ribonucleotide reductase (NrdZ) [30]. As indicated in Figure 2, *M. tuberculosis* has the capacity to synthesize the citrate cycle intermediate succinyl-CoA from methylmalonyl-CoA via the vitamin B_{12}-dependent enzyme MutAB [31]. Furthermore, *M. tuberculosis* can also use propionate as a carbon source for bacterial growth, but only when vitamin B_{12} is exogenously provided by the host in the form of cyanocobalamin [31, 32]. The interaction between the glyoxylate, citrate and methylcitrate cycles in this organism, supports an accelerated growth rate in the presence of heptadecanoate (C17) and valerate (C5), and further evidence for the above mentioned metabolic processes in *M. tuberculosis* is a significantly enhanced growth rate when growth media is supplemented with vitamin B_{12} [31]. In the current investigation, the aforementioned propionylcarnitine, isovalerylcarnitine (the detoxification forms of the fatty acids propionate and isovalerate respectively), methylmalonate, and methylcitrate, were all significantly elevated in the TB patients when compared to that of the healthy controls. This, in the context of the other vitamin B_{12} related abnormalities described above, is indicative of a possible depletion of vitamin B_{12} in the host, most probably due to the scavenging of vitamin B_{12} by *M. tuberculosis* in order to use the host's propionate as an energy substrate for bacterial survival. Furthermore, 2-methylisocitrate, an intermediate of the *M. tuberculosis* methylcitrate cycle, is a potent and very specific inhibitor of the NADP-isocitrate dehydrogenase in the host [33], which reduces NADPH availability, in turn negatively influencing various host anabolic reactions (contributing to TB-cachexia), and glutathione disulfide synthesis [34] (contributing to oxidative stress) [14, 15, 35].

M. tuberculosis also contains a class 1b ribonucleotide reductase (RNR), encoded by the mycobacterial genes *NrdE* and *NrdF*, as well as a class 11 RNR, encoded by *NrdZ* [36]. The latter is also vitamin B_{12}-dependent and catalyzes the conversion of ribonucleotides to deoxyribonucleotides

(see Figure 2), ensuring the cycling of nucleotides in the cell and replication of chromosomes [37]. Previous studies have indicated that *M. tuberculosis* expresses RNR in response to reduced oxygen, nitric oxide and carbon monoxide, i.e. during similar conditions as caused by the host immune response to *M. tuberculosis*, and is thus thought to be the controlling factor for maintaining latent infection [38-40]. Considering this, an interesting hypothesis worth testing, would be whether or not *M. tuberculosis* transition from latent infection to active disease may be linked to a vitamin B_{12} deficiency in the host. To our knowledge, no studies have been done to date investigating the vitamin B_{12} status of untreated TB patients, however, the association between vitamin B_{12} and TB has been suggested previously [41, 42], and folate deficiencies (which is also vitamin B_{12}-dependent) in TB patients is a common occurrence [43].

As mentioned earlier, TB is associated with pronounced protein degradation [22], which is proposed to occur via a number of different mechanisms, including: (a) the resulting inflammatory response due to *M. tuberculosis* [44], (b) the association between TB and insulin resistance [14, 45], (c) wasting due to the nutritional imbalance (and also linked with reduced insulin), resulting from the upregulation of leptin and ghrelin [46], the latter of which may also trigger (d) autophagy [14, 47]. Furthermore, a general amino aciduria, as seen in this investigation, may be induced by a number of other metabolic and physiological occurrences, including lactic acidosis, ketoacidosis, hyperinsulinism, liver disease, and a vitamin B6 deficiency. Lactic acidosis is characterized by the accumulation of lactate, coupled with insufficient delivery of oxygen required for adequate aerobic metabolism [48]. In the current investigation, the elevated concentrations of alanine (although not significant), as well as the significantly elevated lactate, 2-hydroxyisovalerate [49] and 2-hydroxyvalerate [50] in the TB group, are strong indicators of lactic acidosis [49, 50], which is a well-known occurrence in TB [51]. The elevated concentrations of the branched chain amino acids (i.e. leucine, isoleucine and valine) in the TB group, also confirm previous associations between TB and abnormal insulin production/secretion [14] and/or diabetes [52], as well as possible ketoacidosis (indicated by the elevated 2-hydroxyisovalerate [49]) in these TB patients. Further confirmation of this are the significantly elevated concentrations of various organic acids associated with branched

chain amino aciduria, including 2-ketovalerate [53], 2-methyl-3-hydroxybutyrate [54], 3-hydroxy-2-methyl-butanoate [55], 3-methylglutarate [56], methylsuccinate [57], and tiglylglycine [54] (see Table 3). Previously, Luies and Loots (2016) [14] detected an abnormal organic acid profile (elevated phenylacetate and phenyllactate) indicative of elevated concentrations of phenylalanine and suggested that this is most likely due to the general amino aciduria induced by the proposed insulin abnormalities. However, Sonika and Kar (2012) [58] have indicated a strong correlation between TB and liver disease, which is also a common occurrence in urea cycle defects [59]. Additionally, the liver and kidneys are the organs in the body containing the highest concentration of peroxisomes, which are well-known for their function in oxidative stress homeostasis and elevated concentrations of free radicals result in peroxisomal stress [60]. The latter is associated with elevations in pipecolate [54], which was detected in the urine of the TB patient group, further confirming the associated metabolic picture described above.

4. Conclusion

This investigation was the first of its kind to use a multiple instrument semi-targeted metabolomics approach to characterize the altered fatty acylcarnitines, amino acids and selected organic acids in the urine of TB patients. The generally elevated concentrations of the fatty acylcarnitines and amino acids are most likely due to TB-cachexia. However, the significantly elevated concentrations of arginosuccinate, asparate (and associated asparagine), ornithine (and associated proline and hydroxyproline) and glutamate (and associated glutamine) in particular, indicate a urea cycle abnormality, due to inhibition of NAGS by the accumulating propionyl-CoA, isovaleryl-CoA and methylmalonyl-CoA in TB patients. Furthermore, elevated propionylcarnitine, methylmalonate and methylcitrate in the TB patient urine are associated with a vitamin B_{12} deficiency, which deserves further investigation. Lastly, various metabolites indicative of lactic acidosis, ketoacidosis, oxidative stress and liver damage were identified in the urine of the TB patients.

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FOOTNOTES PAGE

Author contribution

DTL conceptualized the study design; KR and GW provided all urine samples. CA, LL and ZL performed the GC and LC data analyses. DTL, LL, JM and CA interpreted the data, drafted the article and designed figures. All authors approved the final version to be submitted.

Conflict of interest

The authors declare that there are no conflicts of interest, and that this manuscript, and the work described therein, is unpublished and has not been submitted for publication elsewhere.

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Compliance with ethical requirements

The investigation was conducted according to the Declaration of Helsinki and International Conference on Harmonization Guidelines and ethically approved by the Ethics Committees of the North-West University, South Africa (reference number NWU-00127-11-A1) and Stellenbosch University Health Research Ethics Committee (reference number 99/039). All participants gave written informed consent for participation in the study.

Table and figure legends

Table 1: The fatty acylcarnitines detected in the urine of TB patients and healthy controls comparatively, using semi-targeted LC-MS metabolomics.

Table 2: The amino acids detected in the urine of TB patients and healthy controls comparatively, using semi-targeted GC-MS metabolomics.

438 Table 3: The amino acid-associated organic acids detected in the urine of TB patients and healthy
439 controls comparatively, using semi-targeted GCxGC-TOFMS metabolomics.

440 Figure 1: Abnormalities in the fatty acylcarnitines, amino acids and related organic acids detected in
441 the urine of TB patients. Abbreviations: NAGS, N-acetylglutamate synthase; TCA, Tricarboxylic
442 acid; ATP, adenosine triphosphate; CoA, coenzyme A.

443 Figure 2: Vitamin B_{12} metabolism in *M. tuberculosis*. *M. tuberculosis* encodes methionine synthase
444 (MetH/E) (involved in folate metabolism), ribonucleotide reductase (NrdZ) (for DNA repair and
445 replication), and methylmalonyl-CoA mutase (MutAB) (involved in the methylmalonyl cycle), which
446 all require vitamin B_{12} as a cofactor for its functionality.

Table 1: The fatty acylcarnitines detected in the urine of TB patients and healthy controls comparatively, using semi-targeted LC-MS metabolomics.

Metabolite name	**TB patients:** **Average concentration (µmol/L creatinine) (Standard error of the mean)**	**Healthy controls:** **Average concentration (µmol/L creatinine) (Standard error of the mean)**	**Fold change (absolute value)**	**Mann Whitney test (p-value)**
Free Carnitine (C_0)	0.103 (0.041)	0.030 (0.019)	3.406	0.005**
Acetylcarnitine (C_2)	0.206 (0.123)	0.013 (0.005)	16.098	<0.001**
Propionylcarnitine (C_3)	0.095 (0.061)	0.019 (0.01)	5.094	<0.001**
Isovalerylcarnitine (C_5)	0.042 (0.019)	0.016 (0.013)	2.610	<0.001**
Octanoylcarnitine (C_8)	0.034 (0.011)	0.044 (0.038)	1.301	0.031
Decanoylcarnitine (C_{10})	0.114 (0.034)	0.134 (0.077)	1.178	0.212
Dodecanoylcarnitine (C_{12})	1.718 (0.568)	1.502 (1.115)	1.144	0.356
Tetradecanoylarnitine (C_{14})	7.268 (3.152)	4.551 (2.505)	1.597	0.053
Palmitoylcarnitine (C_{16})	107.61 (36.075)	89.248 (35.121)	1.206	0.500

** indicates a 5% significance, after correcting for multiple testing using the Bonferroni-Holm approach.

Table 2: The amino acids detected in the urine of TB patients and healthy controls comparatively, using semi-targeted GC-MS metabolomics.

Metabolite name	**Active TB-positive patients:** Average concentration (µmol/L creatinine) (Standard error of the mean)	**TB-negative healthy controls:** Average concentration (µmol/L creatinine) (Standard error of the mean)	Fold change (absolute value)	Mann Whitney test (p-value)
Alanine	29.916 (3.617)	23.220 (1.947)	1.288	0.161
Argininosuccinate	0.088 (0.010)	0.144 (0.017)	1.637	0.003*
Asparagine	15.267 (1.458)	11.112 (1.19)	1.374	0.004*
Aspartate	2.476 (0.336)	1.705 (0.499)	1.452	<0.001**
Cystathionine	1.236 (0.126)	1.592 (0.203)	1.287	0.183
Cysteine	20.192 (2.244)	15.501 (1.263)	1.303	0.097
Dopamine	0.101 (0.020)	0.077 (0.010)	1.314	0.267
Glutamate	4.66 (0.802)	1.787 (0.175)	2.608	<0.001**
Glutamine	64.855 (6.907)	37.289 (5.160)	1.714	<0.001**
Glycine	148.664 (23.873)	121.826 (33.673)	1.220	0.043
Glycine-Proline	0.45 (0.053)	0.570 (0.053)	1.267	0.032
Histidine	68.141 (10.646)	78.165 (9.043)	1.147	0.074
Hydroxylysine	0.424 (0.043)	0.308 (0.037)	1.377	0.011
Hydroxyproline	0.111 (0.048)	0.017 (0.004)	6.408	0.064
Isoleucine	2.030 (0.271)	1.295 (0.081)	1.567	0.005*
Leucine	4.656 (0.476)	3.246 (0.208)	1.435	0.003*
Lysine	12.802 (1.880)	12.407 (2.963)	1.016	0.076
Methionine	2.282 (0.270)	1.631 (0.156)	1.399	0.064
Ornithine	1.733 (0.241)	0.884 (0.098)	1.954	0.003*
Phenylalanine	5.918 (0.381)	3.655 (0.339)	1.619	<0.001**

Pipecolate	0.230 (0.068)	0.057 (0.007)	4.034	<0.001**
Proline	2.095 (0.370)	0.743 (0.061)	2.818	<0.001**
Proline-hydroxyproline	20.653 (1.509)	22.134 (1.578)	1.072	0.292
Pyroglutamate	0.083 (0.023)	0.023 (0.003)	3.684	<0.001**
Serine	46.882 (4.618)	34.691 (3.002)	1.351	0.038
Threonine	14.028 (1.992)	12.542 (1.712)	1.119	0.292
Tryptophan	5.901 (0.583)	4.111 (0.325)	1.435	0.015
Tyrosine	10.036 (1.018)	7.197 (0.639)	1.394	0.005
Valine	5.895 (0.687)	3.932 (0.228)	1.499	0.002*
α-Aminoadipate	0.943 (0.093)	1.123 (0.153)	1.191	0.412
α-Aminobutyrate	1.298 (0.152)	1.561 (0.421)	1.209	0.471
α-Aminopimelate	0.007 (0.001)	0.007 (0.001)	1.074	0.239
γ-Aminobutyrate	0.049 (0.010)	0.045 (0.006)	1.105	0.465

** and * indicate a 5% and 10% significance respectively, after correcting for multiple testing using the Bonferroni-Holm approach.

Table 3: The amino acid-associated organic acids detected in the urine of TB patients and healthy controls comparatively, using semi-targeted GCxGC-TOFMS metabolomics.

Metabolite name	**Active TB-positive patients: Average concentration (µmol/L creatinine) (Standard error of the mean)**	**TB-negative healthy controls: Average concentration (µmol/L creatinine) (Standard error of the mean)**	**Fold change (absolute value)**	**Adjusted Mann Whitney test (p-value)**
2-Hydroxyisovalerate	0.216 (0.117)	0.008 (0.003)	25.766	<0.001**
2-Hydroxyvalerate	163.613 (152.980)	1.825 (0.448)	89.381	<0.001**
2-Ketoglutarate	5.160 (4.305)	0.770 (0.314)	6.479	0.427
2-Ketovalerate	5.097 (4.021)	0.250 (0.149)	15.721	0.002**
2-Methyl-3-hydroxybutyrate	42.823 (36.828)	3.002 (0.352)	14.264	<0.001**
3-Hydroxy-2-methyl-butanoate	152.593 (114.431)	7.910 (0.850)	19.291	<0.001**
3-Methylglutarate	145.680 (132.298)	3.125 (0.382)	46.545	<0.001**
Lactate	124.647 (94.401)	10.817 (1.198)	11.523	<0.001**
Methylcitrate	7.086 (5.562)	0.773 (0.166)	9.033	0.003**
Methylmalonate	10.327 (3.712)	1.665 (0.169)	6.201	<0.001**
Methylsuccinate	30.958 (23.857)	1.926 (0.229)	16.075	<0.001**
Tiglylglycine	6.357 (3.780)	1.080 (0.124)	5.817	0.001**

** indicates a 5% significance, after correcting for multiple testing using the Bonferroni-Holm approach.

Figure 1

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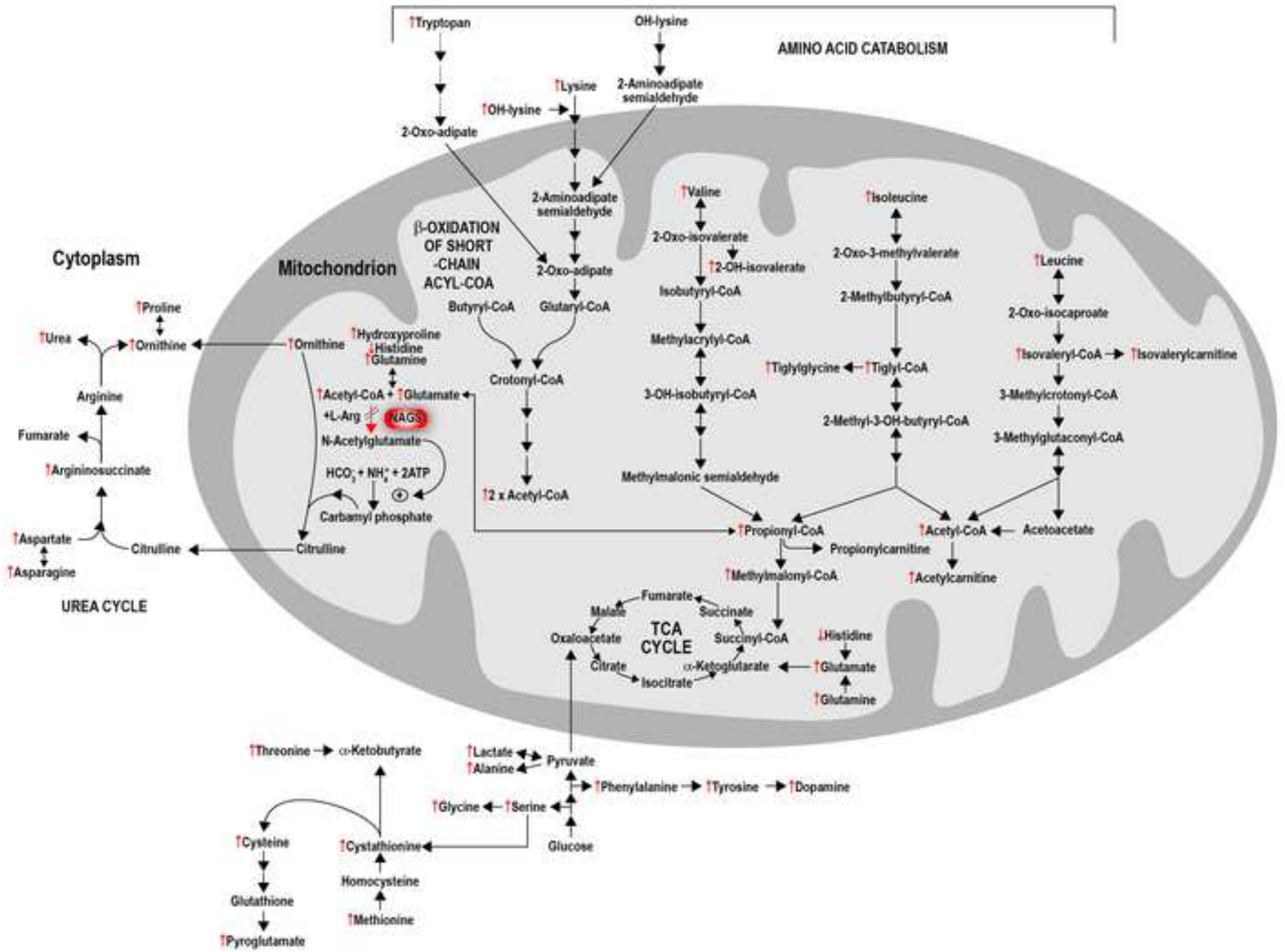

Figure 2

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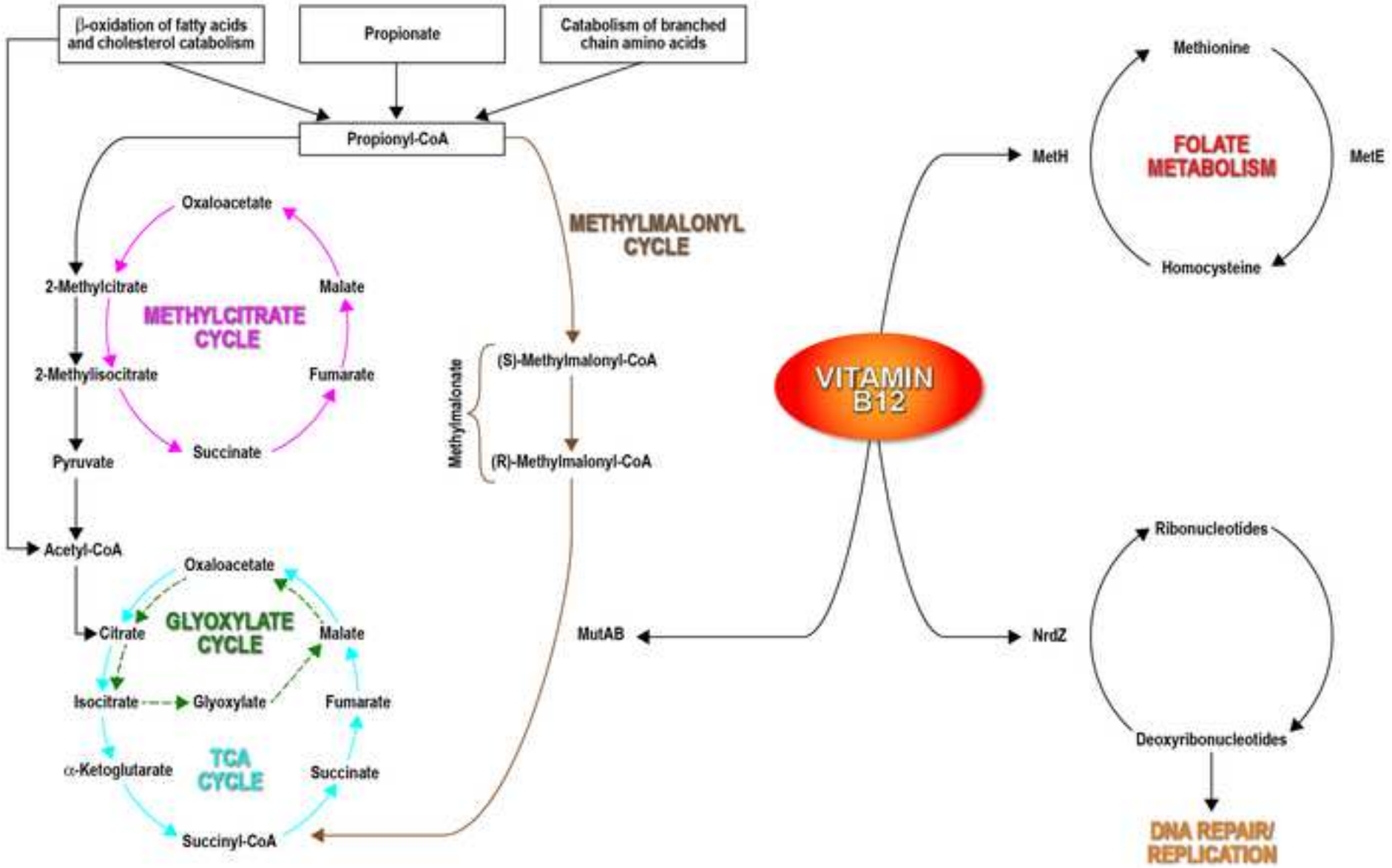

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ORIGINAL ARTICLE

Urinary metabolite markers characterizing tuberculosis treatment failure

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Abstract
Background Considering that approximately 15% of the nine million new tuberculosis (TB) cases reported per annum are not treated successfully, new, distinctive and specific biomarkers are needed to better characterize the biological basis of a poor treatment outcome.
Methods Urine samples from 41 active pulmonary TB patients were collected at baseline (time of diagnosis), during treatment (weeks 1, 2 and 4) and 2 weeks after treatment completion (week 26). These samples were divided into successful (cured) and unsuccessful (failed) treatment outcome groups and analyzed using a GCxGC-TOFMS metabolomics research approach.
Results The metabolite data collected showed clear differentiation of the cured and failed treatment outcome groups using the samples collected at the time of diagnosis, i.e. before any treatment was administered.
Conclusions The treatment failure group was characterized by an imbalanced gut microbiome, in addition to elevated levels of metabolites associated with abnormalities in the long-chain fatty acid β-oxidation pathway, accompanied by reduced L-carnitine and short-chain fatty acids, indicative of a mitochondrial trifunctional protein defect in particular. Furthermore, an altered amino acid metabolism was also observed in these patients, which confirms previous findings and associations to increased interferon gamma due to the host's immune response to *M. tuberculosis* and a compromised insulin secretion.

Keywords Biomarkers · *M. tuberculosis* · Metabolomics · Treatment failure · Tuberculosis · Urine

1 Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains the world's foremost cause of death from a single bacterial agent, which is alarming as it is considered curable. Nearly 10.4 million new TB cases are reported per

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annum, of which almost 15% are not treated successfully, resulting in approximately 1.5 million deaths globally (3 800 deaths a day) (World Health Organization 2016). TB treatment failure may be attributed to, amongst others: (a) irregular or inadequate anti-TB drug supplies to rural areas and third-world countries, (b) poor patient TB-education, (c) poor socio-economic circumstances, (d) the prolonged treatment duration, (e) treatment non-adherence, (f) anti-TB drug resistance, as well as (g) various biological/biochemical factors (De Villiers and Loots 2013).

The World Health Organization (WHO) recommends a 6-month multi-drug treatment regimen, in which a combination of four drugs [isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB)] are used for treating active TB. In patients with drug-susceptible TB, this regimen reportedly has a 1–4% failure rate, and only 7% of those patients with a successful treatment outcome relapse within 24 months (Dye et al. 2005). When considering that every active TB patient can potentially infect an additional 10–15 individuals per annum (World Health Organization 2016), it becomes evident that TB treatment failure and relapse are important considerations in achieving the millennium goals pertaining to the eradication of TB. Despite the many TB research efforts to date, the biological mechanisms associated with anti-TB drug response remains poorly understood. It is also unclear if certain patients have more efficient mechanisms for eliminating this disease and responding to the treatment thereof, and to what extent lifestyle and environmental factors may contribute to this (De Villiers and Loots 2013).

Early disease diagnosis and effective treatment protocols are the two primary objectives of TB control, aimed at reducing mortality and morbidity while also preventing the development of drug resistance (Antoine et al. 2007). The means to accurately predict prognosis from monitoring disease progression early in the treatment regimen would be regarded as a major breakthrough. This would instruct alternative treatment approaches in a timely fashion in order to reduce treatment failure and the development of drug-resistance (Walzl et al. 2008; Horne et al. 2010). Considering this, there is a need for new, sensitive and specific biomarkers, not only for use in the early prediction of treatment failure, but also for better characterizing and explaining the underlying mechanisms related to this occurrence, so that alternative treatment approaches can be developed. Once identified, these biomarkers need to be sufficiently validated for use as surrogate endpoints of treatment failure, early in the treatment regimen, ideally even before treatment begins. To date, no such urinary biomarkers have been identified with absolute certainty, hence the aim of this study was to use a two-dimensional gas chromatography time-of-flight mass spectrometry (GCxGC-TOFMS) metabolomics approach to identify biomarkers differentiating individuals with a successful (n = 26) and unsuccessful (n = 15) treatment outcome, as early as possible during the treatment regimen, which would also better characterize and explain the biological mechanisms related to TB treatment failure. Urine was selected as the preferred sample for addressing the above mentioned aim, since large quantities can easily be obtained and less complex sample preparation is required for analyses, as compared to sputum for instance. Furthermore, in recent years, the need for a holistic approach to metabolism has led to the development of urinary metabolomics for biomarker discovery in various diseases (Ryan et al. 2011; Mahapatra et al. 2014).

2 Methods

2.1 Clinical samples

Anonymized archived urine samples were procured from the Faculty of Medicine and Health Sciences, NRF/DST Centre of Excellence for Biomedical Research, at the Stellenbosch University/MRC Centre for TB Research, from where they were transported to the North-West University (NWU), Human Metabolomics: Infectious Disease Laboratory, for metabolomic analysis. These samples were selected from a prospective observational cohort study of individuals with active pulmonary TB, diagnosed using smear microscopy and bacteriological cultures (Hesseling et al. 2010). From these original samples, all treatment failure patients were included and matched by age, gender and extent of disease on chest X-rays to cured patients. Hence, the samples included were from 41 culture-confirmed active TB-positive South African patients (22 males and 19 females, between the age of 17 and 58) at baseline (time of diagnosis, thus prior to initiation of treatment), during the course of treatment with the directly observed treatment short-course (DOTS) strategy (weeks 1, 2 and 4) and 2 weeks after treatment completion (week 26). These patients had drug-susceptible TB, were human immunodeficiency virus (HIV)-seronegative, not pregnant, and with no other diseases (including diabetes, malignancy, lung cancer, chronic bronchitis and sarcoidosis). The samples were divided into successful (n = 26) and unsuccessful (n = 15) treatment outcome groups. According to the WHO, treatment failure can be defined as a patient whose sputum smear or culture is positive at month five or later during treatment (World Health Organization 2014).

2.2 Sample analysis

Creatinine values for all urine samples, including quality control (QC) samples, were determined using a creatinine enzyme kit (Thermo Scientific; reference number 981845)

and analyzed on an Indiko Clinical Analyzer, Type 863 (Thermo Scientific). These creatinine values are used to normalize metabolite concentrations, and to determine the volume of urine, internal standard, bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and pyridine needed for each extraction and derivatization. Organic acid extractions of the patient-collected urine samples were performed, analyzed on a Pegasus 4D GCxGC-TOFMS (LECO Africa (Pty) Ltd, Johannesburg, South Africa) along with the necessary QC samples, and processed to identify all compounds, following the methods described in Luies and Loots (2016) (Luies and Loots 2016).

2.3 Statistical data analysis

A standard metabolomics data clean-up procedure was applied before statistical data analysis. All metabolites were normalized relative to the internal standard by calculating the relative concentration of each, and a 50% zero filter was applied to each variable (Smuts et al. 2013) to eliminate those compounds with more than 50% zero values within both groups. Quantile equating was applied to correct for any batch effects (Wang et al. 2012), followed by a 50% quality control–coefficient of variation (QC–CV) filter. Lastly, all zero values were replaced with a value calculated as half of the lowest detected value present in the entire dataset, as these may be due to low abundance rather than being absent (Schoeman et al. 2012). GCxGC-TOFMS analysis yielded 782 compounds of which only 241 remained for further statistical analysis after the above mentioned data clean-up were completed. The data were analyzed via a variety of multi- and univariate statistical methods, using MetaboAnalyst (based on the statistical package "R"; version 2.10.0), which included principal components analysis (PCA) (Wold et al. 1987), partial least squares–discriminant analysis (PLS–DA) (Westerhuis et al. 2008), fold change (Du Preez and Loots 2013) and Mann–Whitney test (Pallant 2001).

3 Results and discussion

Using the GCxGC-TOFMS data generated, a PCA was done to determine at which time point individuals who responded to the TB treatment can be differentiated from those who were not cured from TB. The PCA scores plots show clear differentiation of the successful and unsuccessful treatment outcome groups, using the urine collected at time of diagnosis and again at week 26 (Fig. 1a, b), the latter of which can be expected since the cured individuals are TB culture negative, and those with a poor treatment outcome are still TB culture positive at this point in time (Luies and Loots 2016). No PCA differentiation was achieved for the other time intervals investigated (see supplementary material), most likely due to the effects of the anti-TB medication on the human metabolome, masking any underlying differences which

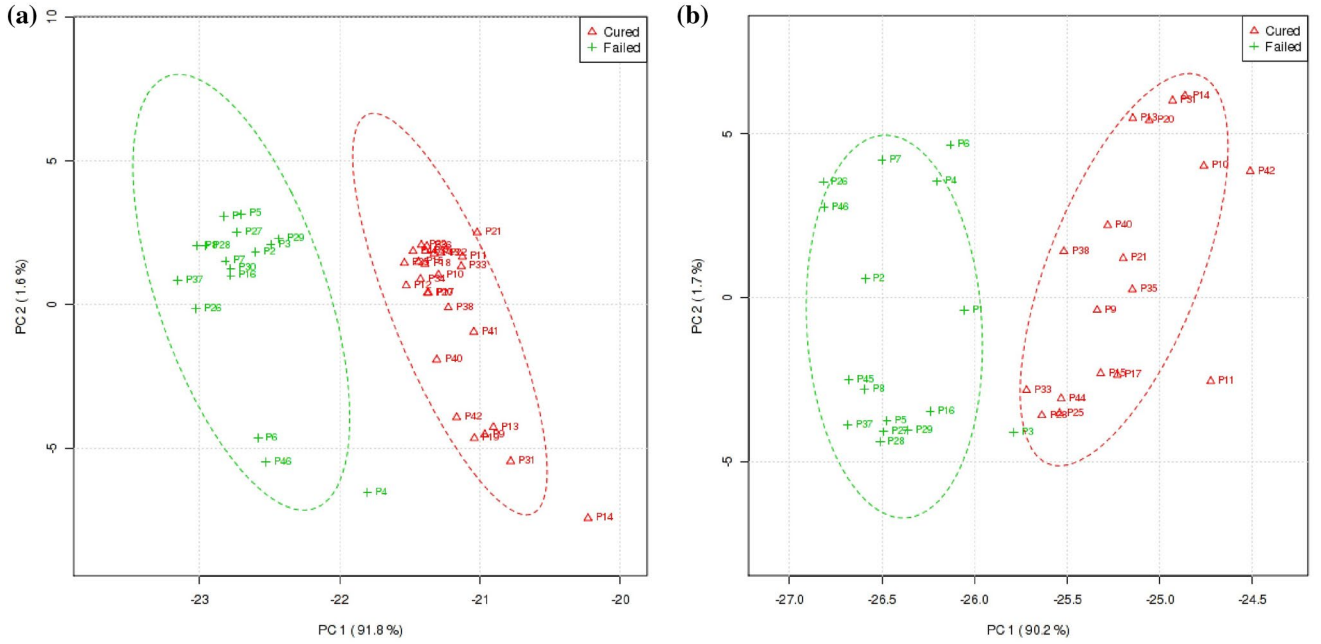

Fig. 1 Principal components analysis (PCA) scores plots of principal component 1 versus principal component 2 of the successful and unsuccessful treatment outcome groups. Clear differentiation of these two groups at **a** time of diagnosis and **b** week 26, due to variation in their underlying metabolite profiles. The variances accounted for are indicated in parenthesis

occur in the metabolism initially differentiating the successful and unsuccessful treatment outcome groups at time of diagnosis. For the differentiation achieved at time of diagnosis (Fig. 1a), the total amount of variance explained by the first two principal components (PCs) (R^2X cum) was 93.4%, of which PC1 accounted for 91.8%, and PC2 accounted for 1.6%. For the differentiation achieved at week 26 (Fig. 1b), the total amount of variance explained by the first two PCs (R^2X cum) was 91.9%, of which PC1 accounted for 90.2% and PC2 accounted for 1.7%.

Since the aim of this study was to identify biomarkers for the early prediction of treatment response and explain the mechanisms associated with treatment failure, only the PCA differentiation achieved at time of diagnosis was of further interest in this investigation. The separation of the groups at week 26 are mostly due to metabolic changes since one group is TB-positive and the other TB-negative, which was already investigated on a previous occasion (Luies and Loots 2016). Considering the above, a PLS–DA model was built for the time of diagnosis data and showed a modeling parameter R^2Y (cum) of 93.48%, indicative of the total explained variation of the response Y.

Clear separation of the successful and unsuccessful treatment outcome groups is remarkable considering the possible predictive value that these may have for identifying individuals who will or will not respond to conventional treatment, even before treatment commences. However, before this biosignature or the individual metabolite markers can be utilized for this purpose, further validation is needed using new subjects to ensure these findings can be generalized. In the context of this study, the biosignature/metabolite markers were used to better describe the biological mechanisms as to why certain individuals with drug-sensitive TB are not successfully treated. Subsequently, those compounds with a PLS–DA variable influence on the projection (VIP) > 1.0 (Smuts et al. 2013), or a fold change >|2|, or a Mann–Whitney with a P value < 0.05 (Du Preez and Loots 2013), were selected as the metabolite markers that best explained the variation between the analyzed groups. Of the 72 characteristic metabolite markers selected, 50 could be annotated using libraries compiled from previously injected standards, and are listed in Table 1.

When considering the metabolite markers best differentiating the successful and unsuccessful treatment outcome patient groups, the first important observation is the elevated levels of metabolites associated with tryptophan [quinolinic acid (Heyes et al. 1992)], phenylalanine [phenylacetic acid (Puri 2006), o-hydroxyphenylacetic acid (Taniguchi and Armstrong 1963)], and tyrosine [4-methylcatechol (Li et al. 2007), vanillylmandelic acid (Eisenhofer et al. 2004)] metabolism (see Fig. 2). The same metabolic flux and the resulting accumulation of these amino acids, were also previously reported by Luies and Loots (2016) in TB-positive patient urine, and explained by elevations in interferon gamma (IFN-γ) due to the host's immune response to *M. tuberculosis* and a compromised insulin secretion by the host (Luies and Loots 2016). Furthermore, markers directly associated with the presence of *M. tuberculosis* [ribitol (Silhavy et al. 2010)], a gut microbiota imbalance [2,3-butanediol (Hong 2011), 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (Shaw 2013), 3-hydroxy-3-(4-hydroxy-3-methoxyphenyl)propionic acid, 3-hydroxyhippuric acid (Gonthier et al. 2003), 4-hydroxybenzoic acid (Russell et al. 2013; Tomás-Barberán and Clifford 2000), 4-hydroxyphenyllactic acid (Russell et al. 2013), 5-hydroxymethyl-2-furoic acid (Jellum et al. 1973), benzoic acid (Russell et al. 2013), cis-4-hydroxycyclohexanecarboxylic acid (Kronick et al. 1983), citramalic acid (Du Preez and Loots 2013), furan-2,5-dicarboxylic acid (Pettersen and Jellum 1972; Jellum et al. 1973), furoylglycine (Pettersen and Jellum 1972), vanillic acid (Tomás-Barberán and Clifford 2000)], and DNA damage [2-deoxyribonic acid (Zhou and Greenberg 2012)] due to oxidative stress [parabanic acid (Marklund et al. 2000)], were also detected and correlate with those markers previously characterizing TB, as described by Luies and Loots (2016). Considering the fact that these markers are not only indicative of a general TB disease state (Luies and Loots 2016), but also more pronounced in the treatment failure group in this study, may indicate that these individuals who are unsuccessfully treated have an increased disease severity due to a microbiota imbalance and/or underlying host abnormality, which will be discussed in greater detail below.

The highly significant evidence from the almost three times elevated 3-hydroxy-3-(4-hydroxy-3methoxyphenyl) propionic acid and more than two times lower *cis*-4-hydroxycyclohexanecarboxylic acid in the treatment failure group, strongly testifies towards an imbalance in gut microbiota in the unsuccessfully treated patients, which is associated with a weaker immune system and inability to fight disease (Clemente et al. 2012). Both the innate and adaptive immune response systems have evolved to rely on microbiota interactions, which not only promote immune cell maturation but also influence the normal development of immune functions (Clemente et al. 2012). Additionally, gut microbiota is also well-known to interact with various drugs, influencing many factors relating to their absorption and subsequent plasma concentrations, and hence is considered an important contributor to xenobiotic/drug bioavailability and toxicity (Gonzalez et al. 2011).

Furthermore, altered levels of various dicarboxylic acids, acylcarnitines and 3-hydroxy fatty acids were detected in the urine of the treatment failure group. Lipid molecules are increasingly recognized as having the potential to shape the immune response to infectious pathogens (Kaushal 2012) since these are important bioactive mediators of cellular activity during pathophysiological

Table 1 The 50 urinary metabolite markers identified at time of diagnosis that best explain the variation detected between the successful and unsuccessful treatment outcome groups

Metabolite name	Successful treatment outcome: average concentration (mg/g creatinine) (standard error of the mean)	Unsuccessful treatment outcome: average concentration (mg/g creatinine) (standard error of the mean)	PLS–DA (VIP)	Fold change	Mann–Whitney test (*P* value)
Amino acid metabolites					
2-Ketovaleric acid	2.093 (0.428)	3.799 (1.302)	1.30	1.82	0.1318
2-Methyl-3-hydroxybutyric acid	3.777 (0.489)	5.487 (0.708)	2.30	1.45	0.0273
2-Piperidinecarboxylic acid (Pipecolic acid)	0.893 (0.230)	0.428 (0.104)	1.24	2.09	0.0197
3-Hydroxy-3-methylglutaric acid	9.437 (0.456)	12.179 (0.945)	1.08	1.29	0.0077
3-Hydroxyvaleric acid	7.691 (1.341)	8.896 (1.861)	1.05	1.16	0.3301
4-Methylcatechol	6.916 (1.150)	10.299 (1.854)	1.36	1.49	0.1022
Glyceric acid	0.268 (0.098)	0.356 (0.091)	1.14	1.33	0.0062
Methylsuccinic acid	3.912 (0.448)	6.581 (1.020)	2.60	1.68	0.0030
N-Tiglylglycine	0.834 (0.247)	1.567 (0.371)	1.91	1.88	0.0094
o-Hydroxyphenylacetic acid	3.093 (0.384)	5.293 (0.602)	2.50	1.71	0.0030
Oxalic acid	14.250 (1.308)	19.933 (2.116)	2.29	1.40	0.0191
Phenylacetic acid	0.305 (0.157)	0.903 (0.273)	2.14	2.97	0.0306
Quinolinic acid	5.311 (0.854)	9.448 (1.403)	5.50	1.78	0.0040
Vanillylmandelic acid	17.088 (0.878)	22.269 (1.830)	1.27	1.30	0.0040
M. tuberculosis cell wall components					
Ribitol	1.910 (0.627)	2.455 (0.523)	1.84	1.29	0.0708
Gut microbiota imbalance metabolites					
2,3-Butanediol	17.831 (4.351)	16.301 (5.137)	3.65	1.09	0.4450
3-(3-Hydroxyphenyl)-3-hydroxypropionic acid (HPHPA)	20.030 (3.029)	28.087 (4.093)	5.40	1.40	0.0553
3-Hydroxy-3-(4-hydroxy-3-methoxyphenyl)propionic acid	2.912 (0.575)	8.366 (2.431)	6.45	2.87	0.0474
3-Hydroxyhippuric acid	17.580 (1.716)	23.662 (2.525)	2.37	1.35	0.0289
4-Hydroxybenzoic acid	4.642 (0.663)	9.966 (1.754)	4.92	2.15	0.0027
4-Hydroxyphenyllactic acid	5.695 (1.526)	6.724 (1.720)	1.21	1.18	0.1485
5-Hydroxymethyl-2-furoic acid	17.563 (4.279)	21.994 (6.053)	4.46	1.25	0.1795
Benzoic acid	0.936 (0.275)	1.367 (0.251)	1.56	1.46	0.0229
cis-4-Hydroxycyclohexanecarboxylic acid	1.428 (0.610)	0.666 (0.190)	0.87	2.15	0.0306
Citramalic acid	8.700 (1.608)	10.742 (3.124)	1.66	1.23	0.4550
Furan-2,5-dicarboxylic acid	8.755 (2.540)	10.156 (3.446)	1.14	1.16	0.3032
Furoylglycine	7.466 (1.443)	10.421 (2.241)	3.39	1.40	0.1115
Vanillic acid	7.731 (0.876)	11.249 (1.230)	2.23	1.46	0.0082
DNA damage and oxidative stress markers					
2-Deoxyribonic acid	0.477 (0.113)	1.080 (0.245)	1.85	2.27	0.0169
Parabanic acid	2.984 (0.799)	3.584 (0.865)	2.68	1.20	0.0474
TCA and GABA metabolites					
3,4-Dihydroxybutyric acid	9.160 (0.825)	11.730 (0.89)	1.03	1.28	0.0108
4-Hydroxybutyric acid	0.305 (0.097)	0.544 (0.158)	1.68	1.79	0.0088
Citric acid	2.730 (0.838)	3.526 (1.619)	1.34	1.29	0.2691
Malic acid	4.579 (2.712)	5.085 (2.668)	3.13	1.11	0.4351

Table 1 (continued)

Metabolite name	Successful treatment outcome: average concentration (mg/g creatinine) (standard error of the mean)	Unsuccessful treatment outcome: average concentration (mg/g creatinine) (standard error of the mean)	PLS–DA (VIP)	Fold change	Mann–Whitney test (*P* value)
Dicarboxylic acids and 3-hydroxy fatty acids					
2,3-Dihydroxypentonic acid	4.632 (1.255)	6.118 (2.265)	2.56	1.32	0.3485
3,5-Dihydroxypentonic acid	3.956 (0.469)	5.698 (0.790)	1.29	1.44	0.0743
3-Hydroxydodecanedioic acid	4.946 (1.659)	2.313 (1.075)	2.27	2.14	0.2000
3-Hydroxysebacic acid	1.499 (0.386)	3.716 (1.140)	2.36	2.48	0.0582
3-Ketosebacic acid	2.419 (0.535)	6.451 (1.560)	6.96	2.67	0.0324
3-Methylhexanoic acid (3-methyladipic acid)	7.723 (0.800)	12.631 (1.637)	3.30	1.64	0.0058
cis,cis-4,7-Decadiene-1,10-dioic acid	9.523 (1.259)	12.113 (1.359)	1.19	1.27	0.0743
cis-4-Decene-1,10-dioic acid	1.403 (0.316)	2.561 (0.649)	1.74	1.83	0.0499
Heptanedioic acid (pimelic acid)	2.895 (0.434)	4.967 (0.684)	2.32	1.72	0.0024
Hexanedioic acid (adipic acid)	0.713 (0.192)	1.369 (0.443)	1.38	1.92	0.0816
Nonanedioic acid (azelaic acid)	4.596 (0.918)	6.465 (1.271)	2.01	1.41	0.0894
Octanedioic acid (suberic acid)	3.452 (0.595)	4.577 (0.394)	1.68	1.33	0.0169
Other					
1,2,3-Trihydroxybutane	1.962 (0.540)	2.842 (1.415)	1.22	1.45	0.4950
2-Methyl-1,2-dihydroxypropane	5.360 (1.768)	6.456 (2.985)	2.78	1.20	0.3672
4-Pentenoic acid	5.860 (0.898)	8.201 (1.289)	1.86	1.40	0.0611
Glucuronic acid	5.923 (0.442)	8.307 (0.757)	1.22	1.40	0.0062

**mg/g* milligram per gram, *PLS–DA* partial least squares–discriminant analysis, *VIP* variable influence on the projection

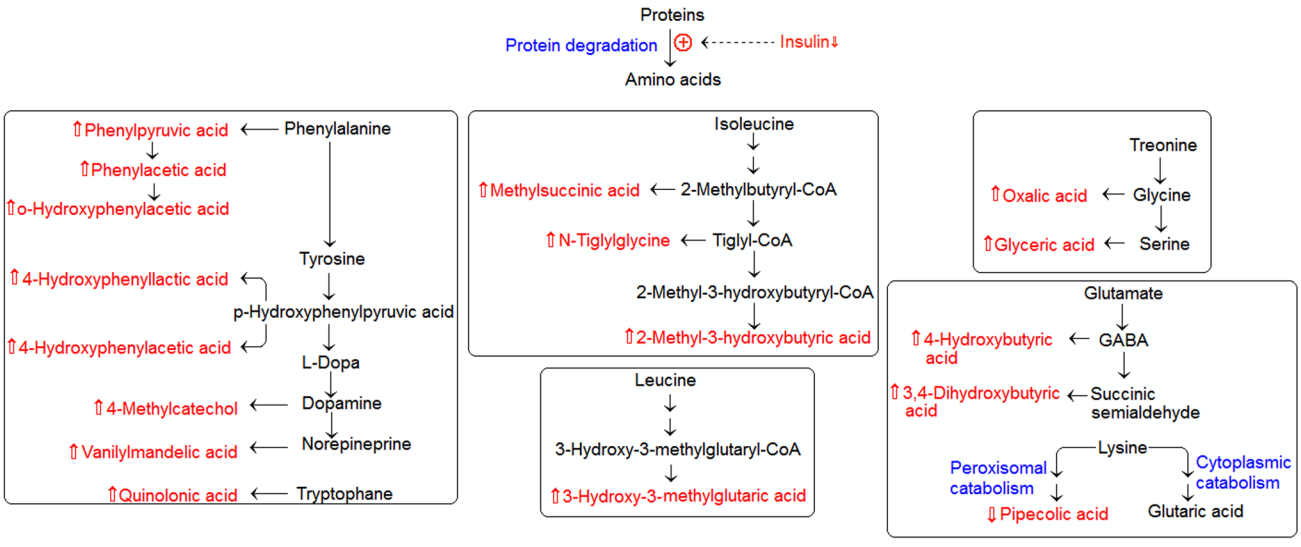

Fig. 2 Amino acid metabolism of the identified urinary metabolite markers. These markers are indicated as either increased (up arrow) or decreased (down arrow) in the treatment failure group comparetively

processes, and affect various activities including cell apoptosis, monocyte adhesion, platelet aggregation, and regulation of immune responses (Hasanally et al. 2014). Interestingly, however, the presence of these metabolites in urine are also used to distinguish between individuals with inherited defects of the following long-chain fatty acid (LCFA) transport-associated enzymes: (a) carnitine palmitoyltransferase 1 (CPT-1), (b) carnitine palmitoyltransferase 2 (CPT-2), (c) translocase (TL), (d) very-long-chain acyl-coenzyme A (CoA) dehydrogenase (VLCAD) or (e) any of the three enzymes making up the mitochondrial trifunctional protein (MTP); long chain 2-enoyl-Co A hydratase, long-chain 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase (Sim et al. 2002; Garg and Dasouki 2006). A CPT-1 deficiency results in reduced C_{16}–C_{18} acylcarnitines and increased free carnitine, a CPT-2, TL or VLCAD deficiency results in both elevated long chain acylcarnitines and dicarboxylic acids with reduced free carnitine, and a MTP deficiency (on any of its three associated enzymes) results in elevated long chain acylcarnitines, dicarboxylic acids and 3-hydroxy fatty acids with reduced free carnitine (Garg and Dasouki 2006). Since the organic acid extraction method used in this study was not suited for the extraction or detection of acylcarnitines, hence their absence in Table 1, we did a further ultra-performance liquid chromatography (UPLC) (Van Aardt et al. 2016) urinary acylcarnitine analysis (Venter et al. 2015), and found significantly elevated palmitoylcarnitine (C_{16}) in the urine of the treatment failure group comparatively (136.19 vs. 58.22 mmol/L; $P = 0.0095$). Additionally, although not statistically significant, the total short chain acylcarnitines were comparatively decreased in the treatment failure group (8.77 vs. 2.75 mmol/L; $P = 0.2298$). Free carnitine (C_0) was also decreased (0.64 vs. 2.58 mmol/L; $P = 0.4329$), as expected in such deficiencies, because these remain bound to the LCFAs and are unable to cross the mitochondrial membrane. Considering this diagnostic metabolite profile, a MTP deficiency or abnormality is suggested to occur in the treatment failure group (see Fig. 3). MTP mutations are estimated at a prevalence of approximately 1:75 000 (Garg and Dasouki 2006), and according to unpublished results of the Potchefstroom Laboratory for Inborn Errors of Metabolism (PLIEM), may be as high as 1 in 100 for a mutation in any of the seven enzyme/protein systems mentioned above. Additional evidence for this, is the serum carnitine deficiencies previously reported in 47.7% of all TB-positive patients (Hatamkhani et al. 2014), which are

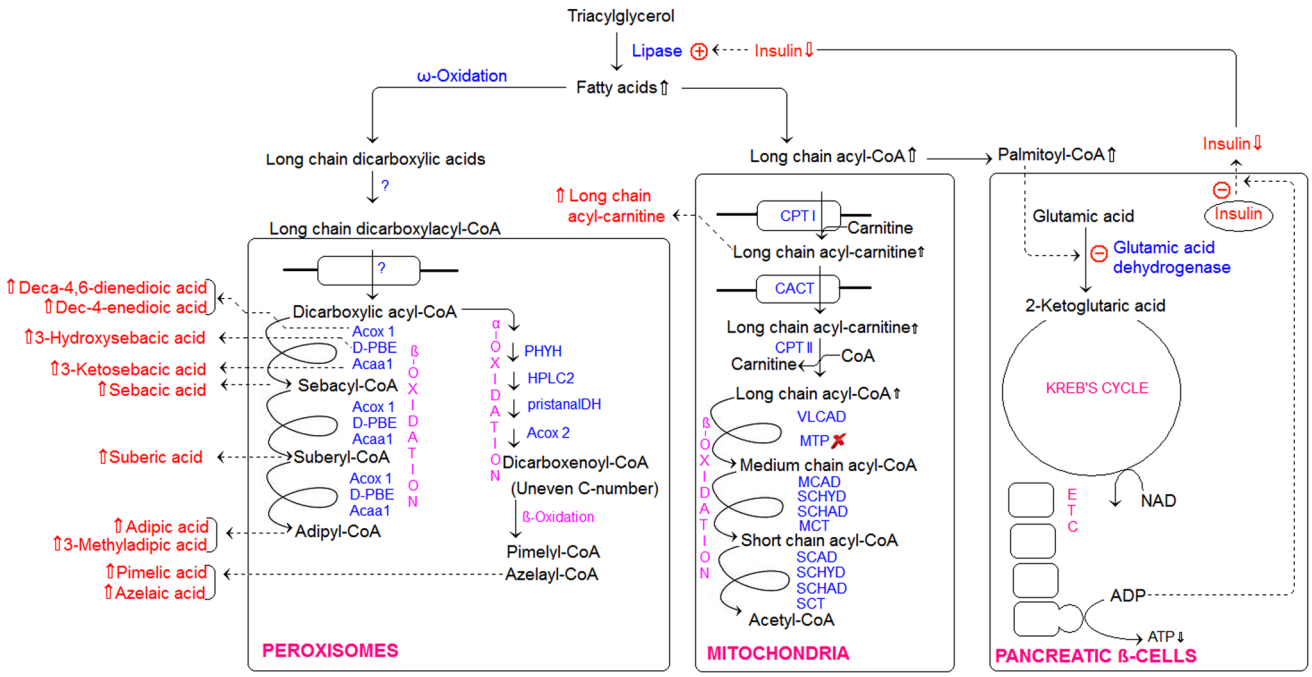

Fig. 3 Fatty acid oxidation of the identified urinary metabolite markers, which are either increased (up arrow) or decreased (down arrow) in the treatment failure group comparetively. *CPT I* carnitine palmitoyltranferase 1, *CPT II* carnitine palmitoyltransferase 2, *CACT* carnitine–acylcarnitine translocase, *VLCAD* very long chain acyl-CoA dehydrogenase, *MCAD* medium chain acyl-CoA dehydrogenase, *SCAD* short chain acyl-CoA dehydrogenase, *M/SCHAD* medium/short chain hydroxyacyl-CoA dehydrogenase, *MTP* mitochondrial trifunctional protein, *SCYD* short chain enoyl-CoA hydratase, *MCT* medium chain 3-ketoacyl-CoA thiolase, *SCT* medium chain 3-ketoacyl-CoA thiolase, *ACOX 1* acyl-CoA oxidase 1, *ACOX 2* acyl-CoA oxidase 2, *D-PBE* peroxisomal bifunctional enzyme, *ACAA1* 3-ketoacyl-CoA thiolase, *PHYH* Phytanoyl-CoA hydroxylase, *HPLC2* Unknown, *pistanalDH* pristanaldehyde dehydrogenase

also associated with these carnitine transporters (Flanagan et al. 2010). Furthermore, L-carnitine has been shown to play a significant role in T-cell-dependent antibacterial activity in the host, and enhance the immune response via reduced macrophage and lymphocyte malfunction in TB patients (Jirillo et al. 1993, 1991), hence a deficiency in L-carnitine, due to an underlying enzyme/protein system deficiency, may explain why these individuals did not respond to treatment. Confirmation of a reduced capacity for mitochondrial β-oxidation due to such a deficiency, is the elevated concentrations of fatty acids with odd numbered carbons, such as azelaic acid and heptanedioic (pimelic) acid in the treatment failure group, due to the above mentioned accumulating fatty acids undergoing peroxisomal α-oxidation prior to peroxisomal β-oxidation (Fig. 3) (Van Veldhoven 2010).

Accumulation of fatty acyl-CoA derivatives, due to the defective LCFA transport, can also contribute to a compromised insulin secretion (Luies and Loots 2016). Long-chain fatty acyl-CoA's are strong inhibitors of glutamate dehydrogenase (GDH) (Lai et al. 1994), and since this enzyme is responsible for the conversion of glutamate to α-ketoglutarate, and an elevated flux of the tricarboxylic acid (TCA) cycle, eventually resulting in insulin secretion (see Fig. 3) (Newsholme et al. 2006), suggests an additional means by which insulin secretion may be compromised in the treatment failure group. Further confirmation of this is the elevated levels of 3,4-dihydroxybutyric acid and 4-hydroxybutyric acid, indicating an increased metabolic flux in the gamma-aminobutyric acid (GABA) shunt pathway (Shinka et al. 2002), in the opposite direction of the glutamate to α-ketoglutarate reaction previously mentioned (see Fig. 2). Compromised insulin secretion has also been shown to result in the accumulation of various branched chain amino acids (BCAAs) (Lu et al. 2013), which explains the elevated leucine (3-hydroxyvaleric acid, 3-hydroxy-3-methylglutaric acid) and isoleucine (2-methyl-3-hydroxybutyric acid, methylsuccinic acid, N-tiglylglycine) metabolite intermediates observed in the treatment failure group comparatively.

Whatever the proposed mechanism, the patients in the treatment failure group show a reduced capacity to transport LCFA into the mitochondria for metabolism into short-chain fatty acids (SCFAs), which are well known for their antimicrobial effects [i.e. increase the fluidity of the bacterial cell wall and subsequently negatively influence cell wall integrity (Royce et al. 2013), and induce host defense peptide LL-37 (Mily et al. 2015)]. Considering this, apart from the reduced L-carnitine detected in the treatment failure group, the reduced SCFAs may be an additional explanation as to why these individuals may not be responding to the treatment and why the metabolite markers suggest elevated disease severity.

4 Conclusion

This study highlights the capacity of metabolomics to identify markers which predict a poor response to treatment, and also better characterize or propose previously unknown mechanisms resulting in TB treatment failure. The most significant observations in this metabolomics study were the elevated levels of those metabolites associated with an imbalance in the gut microbiome. Since this influence xenobiotic uptake and toxicity, the synchronous use of probiotics for optimizing the microbiome during first-line anti-TB treatment, may improve treatment outcome, and could be a topic of further investigation. Furthermore, it may be interesting to compare these patients to a cohort of healthy humans with a normal intestinal biosis in order to firmly allocate the microbiota dysbiosis to the treatment failure cohort. Similar experimental undertakings using a larger sample cohort could also give additional data supporting this hypothesis. Considering the fact that no PCA differentiation was achieved for the other time intervals investigated (see supplementary figure), it would be interesting to learn whether the microbiota in the successful and unsuccessful treatment outcome groups become equally disturbed or equally restored during treatment. The issue of whether the metabolites analyzed in the urine of these patients are due to an altered human host metabolism or whether they originate from the commensal intestinal bacteria can also be investigated in future using germ-free and conventionalized *M. tuberculosis*-infected laboratory animals. Lastly, another interesting observation was those metabolites associated with abnormalities in any of the three enzymes of the MTP complex in the treatment failure group. Since L-carnitine and SCFAs are also reduced in these individuals, and well known for their anti-mycobacterial properties, this metabolic pathway may explain why these individuals have an increased disease severity and/or a poor response to TB treatment.

Author contributions DTL conceptualized the study design; KR and GW provided all urine samples. LL performed the GC data acquisition/analysis and CM the UPLC data acquisition/analysis. LL, JM and DTL interpreted the data and drafted the article. All authors approved the final version to be submitted.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest, and that this manuscript, and the work described therein, is unpublished and has not been submitted for publication elsewhere.

Ethical approval Ethical approval for this investigation, conducted according to the Declaration of Helsinki and International Conference on Harmonization Guidelines, was obtained from the Ethics Committee of the North-West University, South Africa (reference number NWU-00127-11-A1), as well as from Stellenbosch University Health Research Ethics Committee (reference number 99/039) and Cape Town

City Health. All participants gave written informed consent for study participation and HIV testing.

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