



Urinary metabolomic profiling for characterization, diagnosis and treatment response in paediatric Tuberculous Meningitis

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LIST OF ABBREVIATIONS

¹ H-NMR	Proton nuclear magnetic resonance
2HAA	2-Hydroxyphenylacetic acid
2HGA	2-Hydroxyglutaric acid
2KGA	2-Ketoglutaric acid
2MBG	2-methylbutyrylglycine
2ODA	2-octenedioic acid
3HGA	3-hydroxyglutaric acid
3HIA	3-Hydroxyisovaleric acid
3HPA	3-Hydroxypropionic acid
3MCG	3-Methylcrotonylglycine
3MGA	3-methylglutaconic acid
4HHA	4-Hydroxyhippuric acid
4HPAA	4-Hydroxyphenylacetic acid
5HHA	5-Hydroxyhexanoic acid
5HIAA	5-Hydroxyindoleacetic acid
6-PG	6-Phosphogluconate
AA	Aconitic acid
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BCAAs	Branched chain amino acids
BCAT1	Branched-chain amino acid transferase-1
BCAT2	Branched-chain amino acid transferase-2
BGA	Brain-gut axis
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAG	Diacylglycerol
DHAP	Dihydroxyacetone phosphate
EA	Ethylmalonic acid
ENS	Enteric nervous system
FDR	False discovery rate
GA	Glucaric acid
GC-MS	Gas chromatography mass spectrometry
GIT	Gastrointestinal tract
GLN	Glutamine
GLU	Glutamate
GLYT1	Glycine transporter1
GLYT2	Glycine transporter2
GSH	Glutathione
HA	Homovanillic acid
HG	Hexanoylglycine
HMDB	Human Metabolome Database
HOCl	Hypochlorous acid
HREC	Health Research Ethics Committee
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IG	Isobutyrylglycine
IZN	Isoniazid
KatG	Catalase-peroxidase

LAM	Lipoarabinomannan
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
<i>M. tb</i>	<i>Mycobacterium tuberculosis</i>
MCA	Methylcitric acid
MCA	Methylcitric acid
MPO	Myeloperoxidase
MSA	Methylsuccinic acid
NAAA	N-acetylaspatic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NAGS	N-Acetyl glutamate synthase
NAP	N-acetylphenylalanine
NAT	N-acetyltyrosine
P-5-P	Pyroxidal-5-phosphate (vitamin B ₆)
PAG	Phenylacetylglutamine
PCA	Principal component analysis
PEP	Phosphoenolpyruvate
PLS-DA	Partial least squares discriminant analysis
PYR	Pyrazinamide
QC	Quality control
R5P	Ribulose-5-phosphate
RIF	Rifampicin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TB	Tuberculosis
TBM	Tuberculous meningitis
TCA	Tricarboxylic acid cycle
TG	Tiglylglycine
UDP-GlcNAc	Uridine diphosphate-N-acetylglucosamine
VA	Vanillactic acid
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
α-KG	α-ketoglutarate

Preface

My journey into the realm of science began as a child. Growing up in a rural community, I have always been fascinated by how a single mosquito bite can transcend into illness and fever. The rapid onset of uncontrollable shivering accompanied by various symptoms puzzled me, sparking amazement and a desire to unravel the underlying principles governing this phenomenon. Over time, I came to the realisation that the phenomenon was rooted in scientific principles. The intricate chemical interactions within the human body system captivated me. This early captivation served as a catalyst for a constant commitment to exploring the intricacies of biology and to delve into the elaborate structures of living organisms. Motivated by an aspiration to alleviate the suffering of individuals afflicted by infectious diseases in rural areas, I embarked on a scientific journey that ultimately led me to the realm of biochemistry.

Particularly within the realm of biochemistry, I have come to realise that the intricate chemical signals present in the body's metabolic network play a crucial role in comprehending prevalent global health issues such as diabetes mellitus, obesity, malnutrition, and atherosclerotic cardiovascular disease. These findings have further fuelled my interest in pursuing an M.Sc. focused on metabolic syndrome, delving deep into the complexities of metabolic disorders in humans that are associated with various metabolic conditions. While encountering obstacles along the way, my perseverance and curiosity fuelled my progress, helped me achieve set objectives, and produced promising outcomes. The quest to unravel the underlying mechanisms underlying these phenomena not only provided a sense of assurance but also served as my personal drive. These transformative encounters have ultimately propelled me towards embarking on a challenging PhD expedition often likened to a marathon rather than a sprint. Having gained in-depth knowledge in the field of omics, particularly metabolomics, propelled me to understand why a single inhaled droplet containing the pathogen - *Mycobacterium tuberculosis* (*M. tb*) - can ultimately damage the human brain. To understand the mechanisms underlying the metabolic impact after the pathogen enters the human brain, resulting in tuberculous meningitis (TBM), the mechanisms of which are largely unexplored, led to my desire to pursue this current investigation. This disease, prevalent in infants and young children, often culminates in death. My collaborations with an extensive team of TBM experts were orchestrated. At the NWU (South Africa), and the Vrije University (VU) in The Netherlands, in collaboration with Stellenbosch University (South Africa), and a NUFFIC-NRF Doctoral Scholarship, ensured the successful completion of this investigation. The thesis has been written with satisfaction and dedication, following the prescribed article format specified by NWU and endorsed through discussions aligning with the VU's thesis criteria in Amsterdam. Finally, I return all the glory to God Almighty for the wisdom, strength, blessings throughout this project, and grace which has been instrumental in completing this endeavour successfully. To my dear family, especially my beloved wife (Abisola), for being a constant support system, their love and belief in me have been a constant source of motivation. I am truly grateful for their presence in my life.

"Those with great determination and resilience shape history. Some people witness history unfold, while others are unaware of the historical events happening around them."

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Summary

The primary hypothesis formulated for this investigative study is: 'Can urinary metabolic profiles of paediatric cases of tuberculous meningitis (TBM) provide sufficient metabolic information to characterise and diagnose TBM, and monitor treatment response, thereafter?' Secondly, if a brain-gut axis exists, then chronic central nervous system (CNS) bacterial infection(s) should theoretically be reflected in the urine. The premise here is that chronic CNS bacterial infection(s) will affect the gut microbiome and that altered metabolism in both the CNS and the gut will release metabolites into the blood that are filtered (kidneys) and excreted in the urine. Upon extensive deliberation and contemplation regarding the elucidation of the hypothesis. Specific objectives became clear for this study:

- (I) Characterise the urinary metabolic profile of the patient with TBM to comprehensively understand the aetiology, pathogenesis, clinical manifestations, epidemiology, and prognosis of the disease.
- (II) Identify metabolite profiles that characterise the three stages of severity of TBM and potential biosignatures for early diagnosis of the disease, which may lead to a better outcome.
- (III) Monitor the patients' responses to treatment and identify possible prognostic markers to predict a successful treatment outcome.

The structure of this thesis follows.

Chapter 1 is a literature overview of existing knowledge of *Mycobacterium tuberculosis* (*M. tb*), with a focus on the brain-gut axis exposed to chronic bacterial infections in the central nervous system (CNS), underscoring the significance of the brain-gut axis within the realm of chronic bacterial infections in the CNS with concise step-by-step pathological aspects of *M. tb* in term of CNS infection. A brief description of the existing literature provides a complete overview of chronic bacterial infections of the CNS with a central focus on tuberculous meningitis (TBM), as well as the impact of bacterial infection on the gut microbiome and the potential application of urinary metabolic profiling for the diagnosis of *M. tb* infections. Infection with *M. tb* leads to perturbed metabolism in both the CNS and gut and will release metabolites into the blood that are filtered (by the kidneys) and excreted in the urine. Identifying the gap in the literature: no non-invasive (urinary) method of diagnosis of TBM (problem statement), showed that the association between the brain-intestinal connection and bacterial infections of the CNS is relatively scarce in the current literature. The intricate relationships with the gut microbiota are still inadequately elucidated. Metabolomics as a scientific method of choice was selected to fill this gap. But which metabolic markers should we use for such purposes? If a brain-gut axis for CNS infection exists, then metabolic markers of such chronic CNS infection(s) should theoretically be reflected in the urine. From this, the biological research question focuses primarily on individuals with TBM, while also addressing the repercussions of bacterial infection on the gut microbiome. A prognostic metabolic model for TBM within the brain is suggested, using myeloperoxidase (MPO), interferon-gamma (IFN- γ), and vascular endothelial growth factor (VEGF), predicting related downstream metabolic indicators that can be detected in urine. Increased concentrations of MPO and IFN- γ in cerebrospinal fluid (CSF) are associated with TBM. The fundamental principles of biochemistry are used to postulate a prognostic metabolic model for TB and to

investigate the potential of urinary metabolic profiling in the diagnosis and surveillance of this chronic bacterial infection. Urinary metabolic profiling could serve as a valuable tool for the detection and monitoring of CNS infections, as well as for the design of new therapeutic approaches for the treatment of TBM according to the prognostic metabolic model, especially in resource-constrained settings where access to advanced diagnostic tools can be limited.

Chapter 2 describes the use of an untargeted proton nuclear magnetic resonance (^1H -NMR) metabolomics approach to identify characteristic metabolic markers that differentiate severe cases of paediatric TBM from controls, characterizing the urinary metabolic profile of TBM. This is pivotal for better understanding the underlying disease mechanisms, formulating effective prevention and treatment modalities, and facilitating further research. The study compared the metabolic profiles of urine samples collected from the most severe TBM form (stage 3, $n=13$) to an age-matched non-neurological control group ($n=44$). The outcome: twenty-nine urinary metabolites were identified as candidates that characterised the advanced TBM. These metabolites were categorized into six dysregulated metabolic pathways: upregulated tryptophan catabolism, perturbation of amino acid metabolism, increased energy production, disrupted gut microbiota metabolism, ketoacidosis, and increased nitrogen excretion. This research offers novel biological insights into urinary metabolite biosignatures that can differentiate paediatric TBM patients from control subjects, serving as potential non-invasive metabolic markers for early differential diagnosis and treatment follow-up.

Chapter 3 focuses on the urinary gut metabolites associated with TBM and its treatment, highlighting the potential of identifying novel biomarkers for TBM diagnosis and treatment monitoring. Using a semi-targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach to investigate metabolites associated with gut metabolism. Urine samples from a control group ($n = 40$) were compared against an experimental group ($n = 35$) with confirmed TBM, which were subdivided into TBM stage 1 ($n = 8$), stage 2 ($n = 11$), and stage 3 ($n = 16$). Seventy-eight selected compounds of microbiome origin were evaluated. These metabolites were also measured at different time points during the 6-month TBM treatment period. Eight unique (statistically significant at time point 1) urinary metabolites associated with altered gut metabolism were identified in the TBM patients when compared to the control group: 2-methylbutyrylglycine, 3-hydroxypropionic acid, 3-methylcrotonylglycine, 4-hydroxyhippuric acid, isobutyrylglycine, phenylacetylglutamine, as well as 5-hydroxyindoleacetic acid (5HIAA), 5-hydroxyhexanoic acid, and methylcitric acid (identified at time points: 3, 4 and 5). These metabolites were linked to alterations in tryptophan metabolism, fatty acid metabolism, and *M. tb* metabolism, respectively. These metabolites showed a constant elevated level for TBM stage 1 across the entire treatment period. This highlighted the importance of gut metabolism and identifying corresponding microbial metabolites for improved management of TBM patients. The investigation also found that these metabolites are associated with an altered host metabolome, caused by *M. tb*, and the breakdown of *M. tb* cell wall components caused by anti-TB medication and/or dysbiosis commonly associated with TBM.

Chapter 4 This chapter identifies and describes urinary biomarkers in the urine of patients with TBM using proton nuclear magnetic resonance (^1H -NMR) metabolomics, for possible diagnostic applications. This

investigation compared urine from 32 patients with TBM (stratified into stages 1, 2 and 3) against that from 39 controls and found four metabolites with good diagnostic potential for severe TBM (stage 2 and 3), but not for mild TBM (stage 1). The comparison between (stage 1) TBM and severe (stages 2 and 3) TBM was unsuccessful. Our multivariate metabolic model could successfully classify severe but not mild TBM. Five significant biological metabolites were identified: 1-methylnicotinamide, 3-hydroxyisovaleric acid, 5-aminolevulinic acid, N-acetylglutamine, and methanol, with the four of these metabolites (excluding methanol: ROC analysis revealed that methanol lacked diagnostic sensitivity) showing good diagnostic potential for severe TBM. These markers have not yet been established as definitive diagnostic tools because validation studies are needed. However, they may serve as candidate biomarkers for TBM diagnosis. Additionally, 14 unknown compounds were identified as important for differentiating TBM patients from controls, but further research is needed to validate these biomarkers and determine their clinical utility.

Chapter 5, this investigation covers the semi-targeted approach using gas chromatography-mass spectrometry (1D GC-MS) for the semi-targeted metabolomics analysis of alkanes and other volatile organic compounds (VOCs) in the urine of paediatric patients with TBM. This investigation compared all volatile underivatized compounds present in the urine of 27 confirmed cases of paediatric TBM over a 6-month treatment period with 13 control groups. The results showed that four alkanes (pentadecane, 5,7-dimethyl-undecane, 4,7-dimethyl-undecane and 2,6-dimethyl-undecane), three alkenes (2,5-dimethyl-2-hexene, 4,4-dimethyl-1-pentene and 3-methoxy-1-pentene), and three other VOCs of biological interest were identified as statistically significant. These volatile compounds remained perturbed during the TBM treatment. This new systemic metabolic information on *M. tb* in the host highlighted the role of alkanes and VOCs in the potential persistence of *M.tb* in the host.

Ultimately, **Chapter 6** functions as a comprehensive discourse on the collective outcomes and impacts derived from this investigation, as well as a contemplation of the objectives originally proposed before the study commenced. Subsequently, there is a succinct examination of inquiries that emerged throughout the thesis project, along with recommendations for potential avenues for advancement after this doctoral research. Furthermore, Chapter 6 critically examines the accomplishments of the dissertation within the framework of pertinent biological and clinical dimensions associated with TBM, thereby addressing the specified objectives. The questions culminate with insights regarding the constraints of the current study and potential future directions.

Keywords: tuberculous meningitis (TBM); metabolomics; urine; paediatric, proton nuclear magnetic resonance (¹H NMR) spectroscopy; gut-microbiome; liquid chromatography–tandem mass spectrometry (LC-MS); biomarkers, gas chromatography-mass spectrometry (GC-MS).

Format: This thesis is articulated in a manuscript format and adheres to the stipulations established by North-West University, South Africa, as well as Vrije Universiteit, Amsterdam. Consequently, the ensuing comprehensive, peer-reviewed articles constitute a component of the dissertation.

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Table of contents

LIST OF ABBREVIATIONS.....	III
PREFACE.....	V
ACKNOWLEDGEMENTS.....	VII
SUMMARY.....	IX
TABLE OF CONTENTS	XIII
LIST OF TABLES.....	XIX
LIST OF FIGURES	XX
GENERAL BACKGROUND ON TUBERCULOSIS	2
1.1 Introduction	2
1.2 Epidemiology and incidence of TBM in South Africa	2
1.3 Pathogenesis and transmission of TBM	3
1.4 Clinical characteristics of TBM and challenges in TBM diagnosis.....	5
1.5 The facultative nature of <i>Mycobacterium tuberculosis</i> orchestrates its survival in the host ...	6
1.6 The burden of TBM and filling the gaps.....	10
1.7 Metabolomics.....	11
1.8 Urinary metabolomics	12
1.9 The biological questions of the study.....	12
1.10 Objectives of this thesis.....	13

PART 1 OVERVIEW OF BRAIN-TO-GUT AXIS EXPOSED TO CHRONIC CNS BACTERIAL INFECTION(S) AND A PREDICTIVE URINARY METABOLIC PROFILE OF A BRAIN INFECTED BY MYCOBACTERIUM TUBERCULOSIS.....	23
Abstract.....	24
2.2 Introduction	25
2.3 Brain–Gut Concept.....	26
2.4 Bacterial infections of the CNS And their effect on The Brain–Gut Axis.....	29
2.5 Urine reflects dysbiosis within bacterial CNS infection(s)	32
2.6 Validated 3-marker CSF immunological signature of TBM	36
2.7 Vascular endothelial growth factor (VEGF).....	36
2.8 Myeloperoxidase	39
2.9 Interferon- γ	40
2.10 Proposed predictive metabolic model of TBM in the brain based upon IFN- γ , MPO and VEGF	42
2.11 Conclusion	46
PART 2 URINARY METABOLIC CHARACTERIZATION OF ADVANCED TUBERCULOUS MENINGITIS CASES IN A SOUTH AFRICAN PAEDIATRIC POPULATION	68
3.1 Abstract.....	69
3.2 Introduction.....	70
3.3 Materials and methods	71
3.3.1 Sample origin and selection criteria.....	71
3.3.2 Sample transport, storage and handling	73
3.3.3 Sample preparation and ^1H NMR analysis	73
3.3.4 Data pre-processing and statistical analysis.....	73
3.4 Results.....	74
3.5 Discussion.....	80

3.5.1 Upregulated tryptophan catabolism	80
3.5.2 Altered vitamin B3 metabolism	84
3.5.3 Perturbation of amino acid metabolism.....	85
3.5.4 Metabolic burst energy metabolites	89
3.5.5 Disrupted gut microbiota metabolism.....	90
3.5.6 Ketoacidosis	92
3.5.7 Increased nitrogen excretion.....	93
3.6 Urinary metabolic map of TBM.....	94
3.7 Limitations of the study and TB medications	95
3.8 Conclusion	95

PART 3 URINARY MARKERS OF MYCOBACTERIUM TUBERCULOSIS AND DYSBIOSIS IN PAEDIATRIC TUBERCULOUS MENINGITIS CASES UNDERGOING TREATMENT 108

4.1 Abstract.....	110
4.2 Introduction	111
4.3 Materials and methods	112
4.3.1 Sampling.....	112
4.3.2 Sample handling and storage.....	113
4.3.3 Chemicals and standards	113
4.3.4 Sample preparation and LC–MS/MS analysis	113
4.3.5 Statistical analysis	114
4.4 Results.....	115
4.4.1 Quality assurance.....	115
4.4.2 Initial urinary profile.....	115
4.4.3 Longitudinal urinary profile during TBM treatment	117
4.5 Discussion.....	120

4.5.1 Metabolic map and link to previous TB marker studies.....	120
4.5.2 3-Hydroxypropionic acid (3HPA) – host immune response, gut microbiome and <i>M. tb</i> metabolism	123
4.5.3 Prolonged immune response-related protein catabolism to treatment and induced <i>M. tb</i> death	124
4.5.4 Differences in tryptophan metabolism between disease severity and time.....	125
4.5.5 Disturbances in fatty acid metabolism	126
4.5.6 Perturbed tyrosine–phenylalanine metabolism.....	127
4.5.7 Urinary biomarkers for <i>M. tb</i>	128
4.6 Limitations of this study.....	128
4.7 Conclusions	128

PART 4 THE DIAGNOSTIC POTENTIAL OF URINE IN PAEDIATRIC PATIENTS UNDERGOING INITIAL TREATMENT FOR TUBERCULOUS MENINGITIS 136

5.1 Introduction	139
5.2 Materials and methods	140
5.2.1 Sampling.....	140
5.2.2 Sample collection	141
5.2.3 Sample transport, storage, and handling	141
5.2.4 Sample preparation and ¹ H NMR analysis.....	141
5.2.5 Data pre-processing	143
5.2.6 Statistical analysis	143
5.2.7 Ethics approval and consent to participate	144
5.3 Results and discussion	146
5.3.1 Quality assurance.....	146
5.3.2 Qualitative overview: multivariate statistics.....	146
5.3.3 Quantitative statistical evaluation.....	146
5.3.4 Correlations	147
5.3.5 Evaluation of diagnostic markers for TBM	147

5.3.6 Biological context for the four TBM diagnostic metabolites	149
5.3.7 TBM stage differentiation	151
5.3.8 Unknown compounds—Biological or medication related?	152
5.3.9 Speculation of the identities of unknowns	152
5.4 Conclusions	155

PART 5 EXPLORATORY INVESTIGATION OF URINARY ALKANES AND OTHER VOLATILE ORGANIC COMPOUNDS IN PAEDIATRIC PATIENTS WITH TUBERCULOUS MENINGITIS

6.1 Introduction	170
6.2 Materials and methods.....	171
6.2.1 Sampling and ethics	171
6.2.2 Sample handling, storage, and transportation	172
6.2.3 Sample preparation and GC-TOFMS analysis	172
6.2.4 Data pre-processing	173
6.2.5 Statistical analysis	173
6.3 Results.....	174
6.3.1 Alkanes.....	175
6.3.2 Other volatile organic compounds (VOCs)	177
6.4 Discussion.....	180
6.4.1 Alkanes.....	180
6.4.2 2-Pyrrolidinone	181
6.4.2 2-Butenoic acid.....	182
6.4.3 3-Heptanone.....	183
6.5 Conclusions	183

PART 6 ANALYSIS OF THE CENTRAL CONCLUSIONS OF THIS THESIS AND POTENTIAL FUTURE DIRECTIONS FOR THIS RESEARCH.....

7.1 The first goal of the thesis: gain biological insight into the pathogenesis of TBM	192
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7.2 Fulfilling the second goal of the thesis: Identify metabolite profiles that characterise the three stages of TBM and potential biosignatures for an earlier differential diagnosis.....194

7.3 Limitations.....197

7.4 Future perspectives.....198

ANNEXURE A: 202

ANNEXURE B: 208

ANNEXURE C: 222

ANNEXURE D: 226

List of Tables

Table 2.1 Main findings from studies describing the role of microbiota in the pathogenesis of neurodegenerative/psychiatric diseases.	27
Table 2.2 Summary of CSF VEGF concentrations in different types of meningitis.	38
Table 2.3 Performance of IGRAs on CSF from TBM cases as a stand-alone diagnostic tool.....	42
Table 3.1 Demographic, clinical, laboratory and imaging findings of 13 paediatric patients diagnosed with advanced tuberculous meningitis.	72
Table 3.2 Data of the quantified (concentration: $\mu\text{mol}/\text{mmol}$ creatinine) important metabolites (n=28) that characterize TBM. A p-value ≤ 0.05 indicates statistical significance, and a Cohen's d effect size ≥ 0.6 indicates practical significance.	87
Table 5.1 Demographic and clinical characteristics of the TBM patients in this study stratified into stages 1, 2 and 3.	142
Table 5. 2 Quantitative statistically significant results (achieved 3 of 4 demarcated critical cut-off criteria of controls compared to TBM stages 1, 2 and 3).	153
Table 5. 3 Statistically and practically significant bins of the reduced data set based on the Wilcoxon t test with an FDR p value < 0.01 and Hedge's effect size g value > 2.0	157
Table 5.4 Diagnostic sensitivity of the five urinary metabolic markers of TBM at specified cut-off concentrations. The specificity for all was 97.5%, with a 95% confidence interval of 87%-100%.	160
Table 6.1 Statistically significant (FDR p-value < 0.05) compounds in the baseline paediatric TBM patients when compared to the control group. Hedge's effect sizes are also given as g-values, with a medium effect size identified as $0.2 < g \leq 0.8$, and a large effect size identified as $g > 0.8$	175
Table 6.2 Four urinary alkanes over TBM treatment points compared to controls. Statistical significance indicated by FDR p-value < 0.05 . Hedge's effect sizes indicate a medium effect size at $0.2 < g \leq 0.8$ and a large effect size at $g > 0.8$. The groups are shown as controls (C), one month of TB treatment (1), two months of TB treatment (2), three months of TB treatment (3), four months of TB treatment (4), five months of TB treatment (5), and six months of TB treatment (6).	177
Table 6.3 Quantitative measures for the other statistically significant VOCs over TBM treatment points compared to controls. Statistical significance indicated by FDR p-value < 0.05	179
Table S3.1 Quantitative statistical data of the 137 ^1H -NMR spectral bins, with metabolite annotations, that were identified as important based upon the rule: VIP comp 1 and comp 2 > 1.0 OR p-value FDR ≤ 0.05 and absolute fold change ≥ 2.0	204
Table S4.1 Demographics of experimental study cohort affirmed as definite TBM, organized into stages (1, 2 and 3).....	212
Table S4.2 Quantitative data indicating 30 metabolites at T1 that are of statistical and practical importance [PLS-DA VIP value (component 1 AND 2) > 1.0 AND ANOVA Kruskal-Wallis adjusted p-value < 0.01 AND effect size d-value > 0.8]. For Effect size conditional highlighting: $d < 0.8$ = red dot; $0.8 \leq d \leq 1.0$ = yellow dot; $d > 1.0$ = green dot.....	213
Table S4.3 Seventy-eight (78) targeted compounds of interest used in this study, including standards/isotopes.....	214
Table S4.4 Multiple reaction monitoring (MRM's) values and expected retention times of compounds of interest.....	218
Table S4. 5 Binary gradient conditions and experimental parameters of LC-MS/MS.....	221

List of Figures

Figure 1.1 The aerosolised transmission of <i>M. tb</i> from adult to child, and dissemination within the child to the brain where TB granulomas develop, expected immune response that constitute the formation granulomas. Illustrated through a schematic diagram.	5
Figure 1.2 An illustration of the metabolic modifications of <i>M. tb</i> in the host and their impact on disease pathogenesis.	8
Figure 2.1 Model predicting the downstream metabolic effects of VEGF, IFN- γ and MPO in the brain, and the expected attendant urinary profile, in a TBM patient.	44
Figure 3.1 Schematic diagram indicating sequence of steps of experimental design of ¹ H NMR metabolomics and statistical analyses performed.	75
Figure 3.2 PCA scores plots. (A) (Left): Quality controls (QCs) and all cases—20 QCs, 44 controls and 13 TBM cases at initial treatment (TBM), with 95% CI ellipses.	77
Figure 3.3 Supervised PLS-DA scores plot of 40 controls and 13 TBM cases at initial treatment (TBM), with 95% CI ellipses. The total explained variance from component 1 and component 2 is 47.8%.	78
Figure 3.4 Volcano plot showing univariate statistically significant bins. Thresholds of absolute fold change ≥ 2.0 (log ₂) and p-value ≤ 0.05 (log ₁₀) are given as dotted lines.	79
Figure 3.5 Box plots of the relative concentration ($\mu\text{mol}/\text{mmol}$ creatinine) of seven urinary metabolites of Perturbation of amino acids.	81
Figure 3.6 Box plots of the relative concentration ($\mu\text{mol}/\text{mmol}$ creatinine) of eight urinary metabolites of Gut microbiota perturbation.	82
Figure 3.7 Box plots of the relative concentration ($\mu\text{mol}/\text{mmol}$ creatinine) of seven urinary metabolites of perturbed energy metabolism.	83
Figure 3.8 Box plots of the relative concentration ($\mu\text{mol}/\text{mmol}$ creatinine) of six urinary metabolites identified as being part of ketoacidosis, altered vitamin B metabolism and increase nitrogen excretion.	84
Figure 3.9 The metabolic pathways characterizing the changed urinary metabolic profiles in TBM stage 3 patients.	94
Figure 4.1 PCA scores plot of initial urinary profile for all 75 metabolites analysed at T1. PCA 1 & 2 explained 48% of the total variation.	116
Figure 4.2 Venn diagram of significant metabolites across all six time points of TBM treatment.	118
Figure 4.3 Violin plots of eight significant metabolites at T1.	119
Figure 4.4 Linear regression plots with error plots across all six TBM treatment points (T1–T6), for control, TBM_S1, TBM_S2 and TBM_S3, of eight metabolites found to be significant only at T1.	121
Figure 4.5 Branched-chain amino acid (BCAA) catabolism.	122
Figure 4.6 Microbial-specific (blue background) bi-cycle of acetyl-CoA, propionyl-CoA and carbon fixation via the microbial cycle of hydroxypropionic acid and hydroxybutyric acid. Both these microbial cycles rely upon the intermediary 3HPA, which is elevated and associated with dysbiosis during TBM.	125
Figure 5.1 Schematic study design illustrating the experimental workflow – from (left to right): sampling, sample collection, sample preparation, ¹ H-NMR analysis, and statistical analysis.	145

Figure 5.2 PCA score plots of (A) Controls vs. all three TBM stages, (B) Controls vs. Stage 1 TBM, (C) Controls vs. Stage 2 TBM, (D) Controls vs. Stage 3 TBM.....	149
Figure 5.3 Random Forest outputs supporting the OPLSDA results.	151
Figure 5. 4 Plot showing correlations with an r value greater than 0.6 and a p value < 0.05 for all important quantified variables in Table 5.2 for all the TBM cases.	156
Figure 5.5 PCA score plots of revised data sets of (LEFT) controls vs mild TBM, showing some overlap, and (RIGHT) controls vs severe TBM, showing complete natural differentiation between the groups.	157
Figure 5.6 Violin plots depicting the five significant metabolites (1-methylnicotinamide, 3-hydroxyisovaleric acid, 5-aminolevulinic acid, N-acetylglutamine, and methanol) in mild and severe TBM.	158
Figure 5.7 ROC curves of 1-methylnicotinamide, 3-hydroxyisovaleric acid, 5-aminolevulinic acid, N-acetylglutamine and methanol. These five ROC curves indicate that each metabolite has diagnostic potential.....	159
Figure 6.1 Schematic study design depicting the experimental workflow.	174
Figure 6.2 Box plots of the four urinary alkanes identified as showing significant changes across the longitudinal TBM data set.	176
Figure 6.3 Box plots of six urinary compounds identified as showing significant changes across the longitudinal TBM data set.	178
Figure S3.1 Four outliers identified in control group using PCA with 95% CI.	202
Figure S3.2 Hierarchical cluster analysis results shown as dendrograms (Euclidean distance measure, and clustering algorithm using Ward's method) All cases of controls (n=40, in red) and TBM cases (n=13, in green).	203
Figure S4.1 A PCA of all the batches(n=19). Notably, one case (014_T3) stood out as an outlier, and was removed from analysis.	209
Figure S4.2 PCA showing that the samples of the QC.	210
Figure S4.3 PCA of the three TBM stages at T1 shows no characteristic grouping nor differentiation between the stages.....	211
Figure S5.1 PCA scores plot of all patients and QC samples.	223
Figure S5.2 PCA scores plot of all the TBM stages showing no differentiation.	224
Figure S5.3 Above: 1D and 2D JRES NMR spectra showing a doublet at 1.55 ppm (above right) and quartet at 5.19 ppm (above left).	225

CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THESIS

GENERAL BACKGROUND ON TUBERCULOSIS

1.1 Introduction

Tuberculosis (TB), a disease that emanates from a single pathogenic organism – *Mycobacterium tuberculosis* (*M. tb*), is the second leading cause of death from infectious diseases since COVID-19 became the leading cause in 2020 (WHO, 2023a), and has been described since ancient times (over 70,000 years) by scientists as “the white plague, phthisis, and scrofula (the King’s Evil)” (Patrascu et al., 2023, Elizabeth and Angelo, 2015). The World Health Organisation's (WHO) annual Global TB Report 2023 indicated that in 2022 approximately 7.5 million new cases of TB were diagnosed and officially reported worldwide (WHO, 2023b). Before the COVID-19 pandemic, the number of recovered TB cases was 7.1 million people in 2019, 16% above the 2021 reported cases and 28% above that of 2020 reported cases. This is the highest number since the global TB surveillance began in the mid-1990's (WHO, 2023b). Children (aged <15) comprised 12% of those who contracted TB in 2022. Globally, TB results in a detrimental health burden, with an estimated 1.3 million deaths from the disease worldwide reported in 2022 (95% uncertainty interval (UI): 1.18-1.43 million). Additionally, an estimated 183,000 (95% uncertainty interval (UI): 164,000-202,000) of the global number of TB fatalities in 2022 were children, representing 16% of the total TB fatalities in that year (WHO, 2023b). Africa suffers from one of the highest burdens of TB worldwide. The African region witnessed the occurrence of the second-highest count of new TB cases, accounting for 23% of the global total number of new TB cases in 2022 (WHO, 2023a). Furthermore, Africa has the second highest number of paediatric TB cases worldwide, and sub-Saharan Africa has the highest percentage of paediatric cases in any region of the world (Seddon and Shingadia, 2014, Lamb and Starke, 2017). Challenges such as resource constraints, conflict, and political instability, have hindered progress in achieving the WHO targets for halting the rising incidence rates of TB, particularly in various African regions (van Toorn and Solomons, 2014). South Africa is highlighted as one of 30 countries globally with a high TB burden (Dlatu et al., 2022). In 2022, approximately 54,200 people succumbed to TB in South Africa, while around 280,000 contracted the disease, resulting in an infection rate of 468 per 100,000 people (Spotlight, 2023). The incidence rate in the Western Cape region of South Africa was recorded as among the highest in the country (Dlatu et al., 2022).

1.2 Epidemiology and incidence of TBM in South Africa

Extrapulmonary TB accounts for 16% of all TB cases reported worldwide (Barichello et al., 2023), including TBM – associated with high mortality and morbidity (Solomons, 2023). The most lethal form of TB is TBM, which manifests itself as an extrapulmonary TB that spreads to the central nervous system, invading the brain meninges (Slane and Unakal, 2023; Seddon et al., 2019). Children are particularly vulnerable to contracting TBM because of their still underdeveloped immune systems. Paediatric TBM is a serious threat to children's health and can cast a long and devastating shadow over the lives of children, particularly in regions burdened by TB (Saal et al., 2023). The morbidity and mortality of paediatric TBM in South Africa are high, with an estimated 20% death rate, and about half of the patients die during treatment or survive, but not without serious neurological disabilities (Solomons, 2023; Garg, 2010; Rohlwink et al., 2016). The highest prevalence of TBM is in children aged between 2 and 4 years, during

the phase when their brains are still developing (Van Well et al., 2009; Wolzak et al., 2012; Huynh et al., 2022). Age is considered to be the most important risk factor for developing TBM and is common in children aged 0 to 4 years, in populations with high TB prevalence; with children under 2 years of age with untreated TB typically developing TBM (Philip et al., 2015, Thwaites et al., 2000). Children are particularly vulnerable in the Western Cape province of South Africa, TBM is the most common form of paediatric meningitis (Wolzak et al., 2012). Most commonly observed in children, TBM often manifests as a form of subacute meningitis, thereby posing a challenge in its differentiation from other forms of meningoencephalitis (Mezochow et al., 2017, Bahr et al., 2019). The time between infection and illness in children can be short, while it is typically longer in adults. Currently, there is a lack of information on the global impact of paediatric TBM, mainly due to the common occurrence of nonspecific clinical symptoms and the limited availability of accurate diagnostic methods (du Preez et al., 2022, Dodd et al., 2021). Consequently, children affected by TBM often receive a delayed diagnosis or unfortunately remain undiagnosed, leading to fatal outcomes (du Preez et al., 2022). Due to these unique disease characteristics in children a different approach is needed to diagnose these paediatric patients (Lamb and Starke, 2017). Considering all the above, TBM remains a significant etiological factor that contributes to neurologic impairment in children residing in countries with limited resources. This condition exerts considerable pressure on both families and healthcare systems (Graham and Donald, 2014), and a better understanding of the disease, and the timely diagnosis of TBM in paediatric patients is of utmost importance to mitigate mortality and functional impairment.

1.3 Pathogenesis and transmission of TBM

The pathogenesis of TBM, like other forms of TB, is influenced by various factors. The Commission on Social Determinants of Health (CSDH) defines structural determinants of health as the conditions that create or reinforce social stratification. These social determinants are among the key risk factors for TB (Lienhardt, 2001). These factors include amongst others, poverty, overcrowding, illiteracy, and malnutrition (Garg, 1999, Garg, 2010). TBM can be characterised as a lethal condition that manifests in children due to the early dissemination of the *M. tb* bacilli through the blood (Patrascu et al., 2023, Seddon et al., 2019). The primary mode of transmission of *M. tb* to a healthy individual is through the inhalation of infected aerosols (Lamb and Starke, 2017). The transmission routes in children are more likely to be infected with *M. tb* through close contact with infected adults in their immediate environment. Transmission from child to child is rare. Infections in children usually occur at home, but outbreaks have been reported in schools, daycare homes, churches, and on school buses (Lamb and Starke, 2017, Martinez et al., 2019, Millership et al., 2009). After inhaling the aerosolised *M. tb*, the host immune response either eliminates or contains the microorganism. However, if this mechanism fails, it may lead to the spread of bacilli through the bloodstream to other organs of the host, including the meninges that enclose the brain (Jain et al., 2006, Krishnan et al., 2010, Dodd et al., 2021).

As previously mentioned, the primary entry portal of *M. tb* in the host is to the lungs in most cases, which is the first site of infection (Moule and Cirillo, 2020). Sometimes, the inhaled pathogen can be eliminated in the upper respiratory tract; however, if it reaches the terminal air spaces of the alveoli of the lungs, the

pathogen resides and is engulfed by resident alveolar macrophages and recruited neutrophils. Once in the lungs, the pathogenic bacteria proliferate within these alveolar macrophages (Slane and Unakal, 2023, O'Garra et al., 2013, Sasindran and Torrelles, 2011, Rock et al., 2008). The pathogen harmlessly resides within the phagosome macrophage of the infected host and establishes an intracellular niche conducive to its replication (Russell, 2007). The host's subsequent mechanism against *M. tb* bacilli is apoptosis of the infected macrophages. Virulent strains of *M. tb* have developed multiple mechanisms to evade and manipulate host immune responses, resulting in extended survival and proliferation within the host macrophages (Garg, 2010; Lee et al., 2009; Porcelli and Jacobs Jr, 2008). One of the characteristics of pathogenic *M. tb* is its ability to prevent normal acidification of the phagosomes. The further prevention of phagolysosomal fusion enables the infecting bacilli to replicate in the inactivated macrophages, which have limited antimicrobial defence mechanisms (Russell, 2011, Warner, 2015, Smith, 2003). Stimulated macrophages of the immune system aggregate at the site of the infection. The infected macrophages, which act as an isolated habitat for *M. tb*, obstruct the access of activated accumulating antibodies, resulting in the formation of granulomas (Cresswell et al., 2019, Sholeye et al., 2022). The immune cells within these granulomatous structures attempt to control the infection (Sholeye et al., 2022), and this altered metabolic activity, via altered bioenergetics and biosynthetic processes, which are typically necessary to effectively eliminate pathogen, in the case of *M. tb* infection, unfortunately results in the confinement of the infecting pathogen within the immune cell (Kaplan et al., 2003; Petersen and Smith, 2013; Phelan et al., 2018). *M. tb* continues to replicate within this granuloma, resulting in the development of lesions within the centre of the granuloma. The interior of the granuloma subsequently becomes necrotic with caseous-like material and hypoxic (Via et al., 2008; Sholeye et al., 2022). Eruption of these granulomas results in the infectious bacilli escaping into the bloodstream and via the circulatory system spreading to other parts of the body (Huynh et al., 2022). The circulating *M. tb* has different mechanisms to cross the blood-brain barrier (BBB), infecting the meninges. Most of the granulomatous inflammation occurs in the subarachnoid space (leptomeninges) and to a much lesser extent in the superficial brain parenchyma (adjacent to the pia mater). Mostly, this superficial intraparenchymal component is just a transpial or perivascular extension (Virchow-Robin spaces) of a leptomeningeal granuloma. Zaharie et al. (2020) state that the development and rupture of these early granulomas can give rise to TBM progression to its more advanced stages of disease. *M. tb* disease in the brain leads to a variety of severe clinical complications, such as hydrocephalus, infarction, and tuberculoma. Neuroinflammation (and associated increased intracranial pressure) and neuronal injury are two of the major causes of death or disability in TBM. Figure 1.1 shows an illustrated schematic of the route of *M. tb* infection from adult to child, into the brain, and the subsequent development of granulomas and TBM.

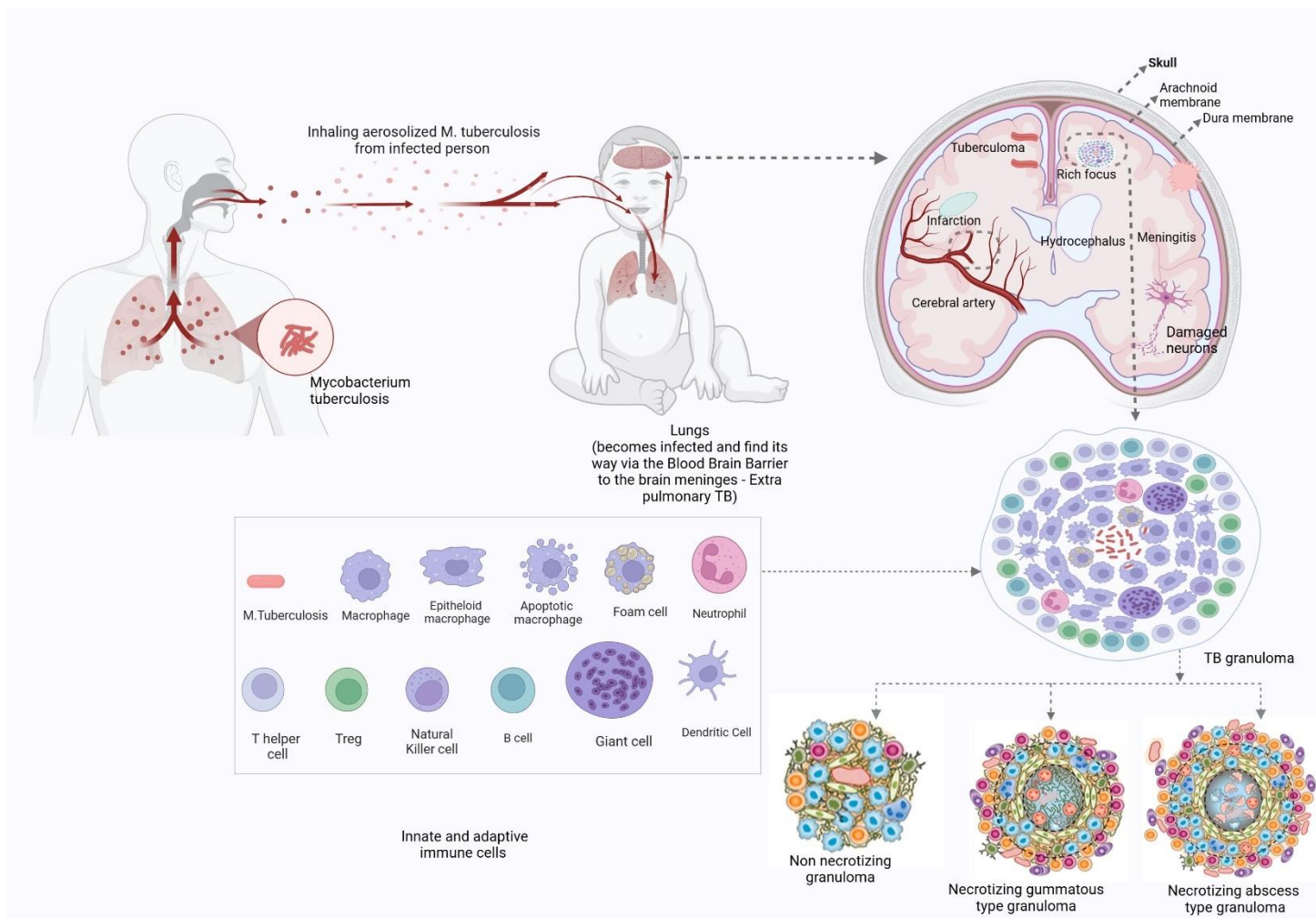


Figure 1.1 The aerosolised transmission of *M. tb* from adult to child and dissemination within the child to the brain where TB granulomas develop, expected immune response that constitutes the formation granulomas. Illustrated through a schematic diagram.

1.4 Clinical characteristics of TBM and challenges in TBM diagnosis

The early diagnosis of TBM in children is problematic, especially in low- and middle-income countries due to various socioeconomic reasons (Seid et al., 2023, Solomons, 2023, Dodd et al., 2021). Furthermore, children affected by TBM frequently exhibit nonspecific clinical manifestations, and the characteristic rigidity of the neck associated with meningitis is often lacking in the initial phases of the disease (Van Toorn et al., 2012; Thwaites and Hien, 2005; du Preez et al., 2022). Typically, the clinical manifestation of paediatric TBM is associated with vague and non-specific symptoms including irritability, inadequate feeding, fever, seizures, vomiting, coughing, lethargy, weight loss, and failure to grow (Solomons, 2023; Török, 2015). Furthermore, the diagnosis is especially difficult in infants/toddlers, who may struggle to express specific complaints verbally. The onset of symptoms also varies and may be acute, subacute, or gradual (Daniel et al., 2019, Checkley et al., 2008, Lammie et al., 2009). Unlike adults, children do not necessarily show the classic signs of meningitis; however, the disease progresses rapidly, complicating both the diagnosis and management of the disease (Garg, 2010; Dodd et al., 2021; Handryastuti et al., 2023). The presentation of hydrocephalus, for example, one of the most severe neurological symptoms caused by TBM, is a common occurrence in children rather than adults. Hydrocephalus also manifests

more severely within TBM patients in comparison to hydrocephalus from different forms of bacterial meningitis (Mezochow et al., 2017, Rajshekhar, 2009). Survivors of childhood TBM also suffer from long-term complications of neurocognitive and neurological impairments after successful eradication of the infectious organism (Saal et al., 2023), since shortly after the onset of TBM, a significant number of children experience reduced physical brain development and psychological growth and, and the resulting mental retardation subsequently restricting their normal (re)integration into society (Igbokwe et al., 2023, Rohlwink et al., 2016, Wait et al., 2002). In children, once the clinical manifestations of advanced neurological impairment become evident, it is often too late to revert the condition (Solomons, 2023). The delay in neurodevelopment is an important long-term consequence of TBM in children, and the elevated probability of unfavourable consequences is mortality (Saal et al., 2023; Igbokwe et al., 2023; Wait et al., 2002). Furthermore, predicting the outcome of childhood TBM is challenging, due to various pathological mechanisms and variations in the child host's immunological response (Mason et al., 2016a). Of the 17 studies done on paediatric TBM (n=872 children in total) 12 have indicated sequelae in 5–100% of cases (Igbokwe et al., 2023). TBM progression is typically classified into three unique stages (I, IIa, IIb, and III), based on the level of consciousness and various neurological deficits, linked to short- and long-term outcomes. In a study done in 548 children with TBM, normal outcomes were found in all 14 children eventually diagnosed with stage I TBM and children with stage III disease, had a 4.8 times higher risk of poor outcome compared to those with stage I or II disease (Van Well et al., 2009; Van Toorn et al., 2012). Considering this, even though the burden of paediatric TB has become more widely known over the past few decades, a better characterisation of paediatric TBM is still required, in addition to the subsequent development of more sensitive and targeted diagnostic methods (Dewandel et al., 2023).

1.5 The facultative nature of *Mycobacterium tuberculosis* orchestrates its survival in the host

Elucidating the disease mechanisms of paediatric TBM is imperative to understanding the complexities of *M. tb*, especially in the brain. *M. tb* is well known to be metabolically flexible, with an extraordinary ability to sense and adapt to the changing host environment during persistent infection, making it extremely capable of outcompeting its host for nutrients, by modifying its, and that of the host's, gene expression (Beatrice, 2013; Kumar et al., 2011). This bacterium effectively persists and thrives within the host, by scavenging the host's resources via pathways crucial in its evolutionary development as a pathogen (Mishra and Surolia, 2018). Its ability to co-metabolise various carbon substrates within the host cells also contributes to its resilience (Figure 1.2). The central carbon metabolism of *M. tb* plays a pivotal role in its physiological processes and pathogenicity (Rhee et al., 2011; Baughn and Rhee, 2014; Xu et al., 2022). A comprehensive investigation has confirmed the presence of various key enzymes involved in the central carbon metabolic pathways, which include glycolysis, the pentose phosphate pathway (PPP), the tricarboxylic acid cycle (TCA), and the glyoxylate shunt in *M. tb* (Cole et al., 1998). *M. tb* also contains genes for ATP synthesis via aerobic oxidative phosphorylation (electron transport chain, cytochrome b reductase, cytochrome c oxidase) and anaerobic phosphorylation (nitrate reductase, nitrite reductase, fumarate reductase) (Cole et al., 1998; Sohaskey and Wayne, 2003). Thus, to adapt to fluctuating nutrient availability, *M. tb* can easily utilise various sources of carbon and nitrogen to grow, replicate, and survive

(Gouzy et al., 2014; Borah et al., 2019). From the standpoint of *M. tb* metabolism, it is hypothesised that the virulent mycobacterium uses glucose and triacylglycerides as their primary carbon sources during the initial stages of replication under aerobic conditions. Subsequently, due to the scarcity of glucose in the macrophage environment, a transition occurs towards the utilisation of lipids (Boshoff and Barry 3rd, 2005; Eoh and Rhee, 2013). As the infection progresses and there is an escalation in immune activation and host-induced stress on *M. tb*, the normal process of aerobic respiration becomes increasingly restricted. Consequently, the bacterium adapts its metabolism to ensure its survival within this host-induced hypoxic environment by stopping its growth (Boshoff and Barry 3rd, 2005; Watanabe et al., 2011; Eoh and Rhee, 2013; Gouzy et al., 2014). The ability to switch between oxygen-rich and hypoxic conditions makes it highly challenging to combat this bacterium (Warner, 2015; Muñoz-Elías et al., 2006; Beste and McFadden, 2010a). Due to the aforementioned adaptable nature of *M. tb* in the host, and the largely variable symptoms of the host, determining the precise metabolic status of bacilli during various stages of the infection cycle *in vivo* continues to present a formidable diagnostic challenge (Rock et al., 2008; Beste and McFadden, 2010a; Beste and McFadden, 2010b). Hence, this facultative nature of the metabolism of *M. tb* has been the subject of intense research (Muñoz-Elías et al., 2006; Borah et al., 2019). It was determined that *M. tb* utilises the host to acquire specific essential substrates that it is incapable of producing on its own, enabling it to effectively ensure its survival during the infection process (Rhee, 2013). Consequently, this process induces an inherent imbalance within the host organism's homeostasis; thus, disrupting the overall host equilibrium and resulting in a significant depletion in the essential macronutrients that are necessary for the optimal functionality of the host's biological system (Parbhoo et al., 2022; Beatrice, 2013; Park et al., 2021).

Attention regarding this must be paid to the amino acids, since they serve as a crucial nitrogen supply to the host's immune cells, required to combat the *M. tb* infection, in addition to acting as a nitrogen source for *M. tb* metabolic processes (Amalia et al., 2022; Shin et al., 2011). These amino acids actively facilitate the host's intracellular metabolic pathways via adenosine triphosphate (ATP) synthesis, various nucleotide intermediates, and maintaining a redox balance (Amalia et al., 2022). The primary amino acids responsible for providing nitrogen for *M. tb* growth include aspartate, glutamate, asparagine, and glutamine, all present within human macrophages where *M. tb* can persist. These amino acids are actively transferred from the cytoplasm of the host's immune cells into *M. tb* (Gouzy et al., 2014; Shin et al., 2011). Furthermore, the unique metabolic capacity of *M. tb*, allows it to continuously produce these amino acids, allowing it to survive periods of nutrient deprivation by the host, as a defensive strategy to inhibit *M. tb* growth (Mishra and Surolia, 2018). However, the three amino acids, namely asparagine, tryptophan, and arginine, appear to be the primary amino acids the host targets for this (Mishra and Surolia, 2018). Their presence/absence, and that of their derivatives and metabolic intermediates, has a significant impact on the behaviour of the pathogen, as well as the immune response of the host (Mishra and Surolia, 2018). Studies have shown that under conditions of pathogenesis and survival within the host, there is an increase in the expression of genes involved in the biosynthesis of these amino acids. RelA, a gene responsible for sensing amino acid levels, can detect deprivation of nutrients, along with CarD, a vital transcription regulator found in *M. tb*. This detection leads to the activation of genes involved in amino

acid biosynthesis in *M. tb* during episodes of hypoxia and amino acid starvation induced by the host (Primm et al., 2000; Traxler et al., 2008; Avarbock et al., 2005; Kaur et al., 2018).

Protein kinase G (PknG), a serine/threonine kinase found in *M. tb* on the other hand, plays a significant role in the regulation of both its carbon and nitrogen metabolism. This is achieved through the phosphorylation of the glycogen accumulation regulator (GarA), which subsequently controls the tricarboxylic acid (TCA) cycle and glutamate synthesis (Burastero et al., 2022, Rieck et al., 2017, Cowley et al., 2004). The competition between the host and the pathogen for survival encompasses a wide range of metabolites (Mishra and Surolia, 2018). The *M. tb* also upregulates its lipid metabolism within the macrophages, most likely due to the absence of available carbohydrates in these immune-activated phagosomes. Moreover, the enzyme isocitrate lyase exhibits significant induction *in vivo* and was seen to be upregulated in all macrophage disease models. Isocitrate lyase serves as a critical component of the glyoxylate shunt, effectively harnessing fatty acids as a source of energy (Beatrice, 2013; Daniel et al., 2011). Furthermore, most recently, *M. tb* was discovered to also have a citramalic acid cycle, allowing it to utilise pyruvate as a substrate for its central carbon metabolism (du Preez and Loots, 2013). Furthermore, its capacity to utilise other alternate carbon sources, such as branched-chain fatty acids, branched-chain amino acids, and cholesterol, leads to the production of propionyl CoA. *M. tb* has two unique primary mechanisms for utilising propionate: 1) the methylcitric acid cycle, using isocitrate lyase, and 2) the methylmalonyl pathway, which employs a vitamin B12-dependent methylmalonyl-CoA mutase (Savvi et al., 2008; Muñoz-Elías et al., 2006; Warner, 2015).

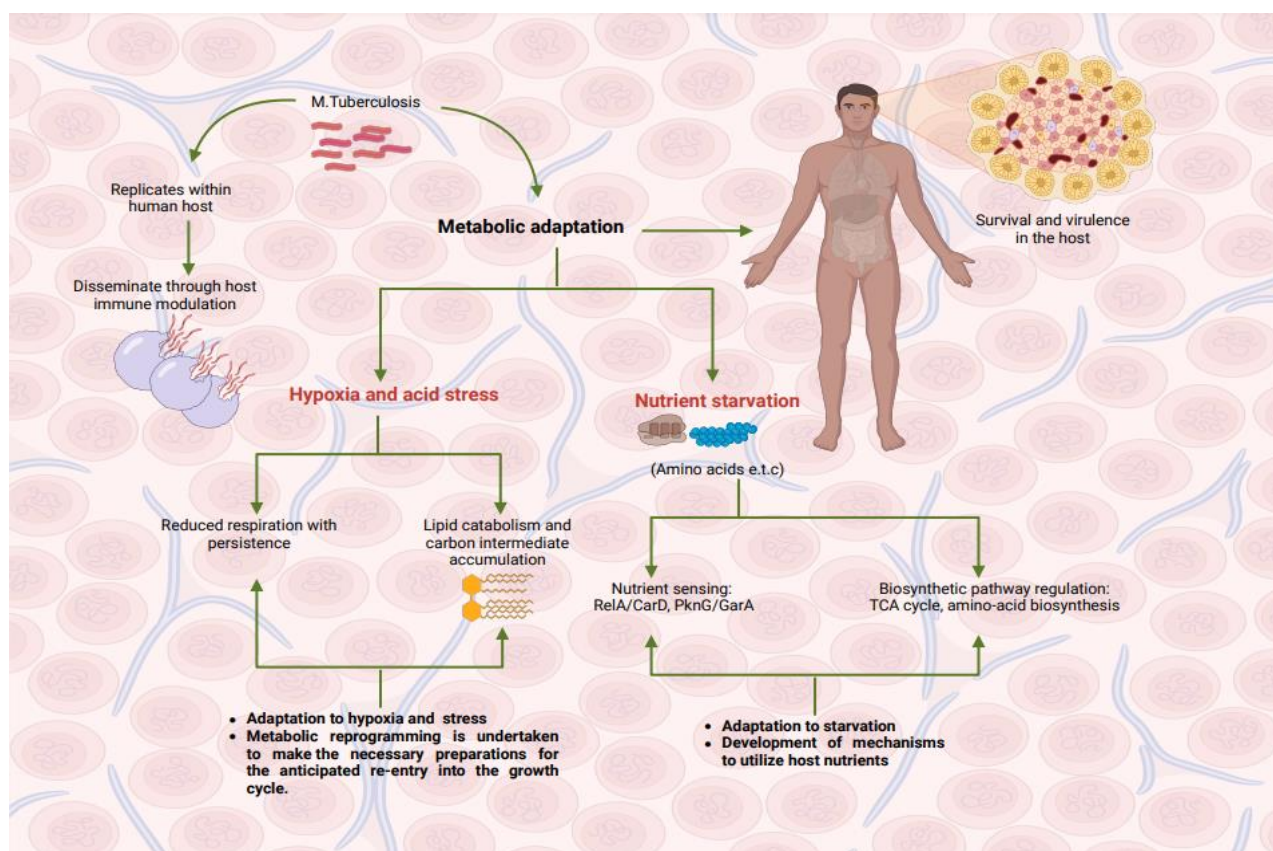


Figure 1.2 An illustration of the metabolic modifications of *M. tb* in the host and their impact on disease pathogenesis.

RelA, an amino acid sensor gene, CarD an essential transcription regulator in *Mycobacterium tuberculosis*, PknG, a mycobacterial serine/threonine-protein kinase, GarA, protein GarA regulates the tricarboxylic acid cycle and glutamate synthesis, TCA, tricarboxylic acid cycle.

Considering the latter, *M. tb* can obtain vitamin B12 directly from the host cell (Gopinath et al., 2013). This scavenging mechanism also includes the acquisition of iron, thought to be a major contributor to the high prevalence of anaemia among paediatric TB patients (Gopinath et al., 2013, Song et al., 2010, Pepperell, 2022, Young et al., 2015). Various vitamin B12-dependent enzymes play a crucial role in many fundamental biochemical pathways in *M. tb*, a bacterium that is known to encode three B12-dependent enzymes (Gopinath et al., 2013). namely 1) the aforementioned methylmalonyl-coenzyme A (CoA) mutase, 2) MutAB, and 3) metH-encoded methionine synthase, and have demonstrated their dependence on B12 for their activity within the host system (Savvi et al., 2008; Warner et al., 2007). Vitamins act as cofactors/coenzymes in enzyme reactions and many exhibit antibacterial properties. In the host, vitamin A serves to repress the transcription of a coat protein produced by *M. tb* that hampers the fusion of phagolysosomes, and its deficiency has been strongly linked to an increased susceptibility to TB. The active form of vitamin D stimulates antimycobacterial activity in macrophages *in vitro*, thus inhibiting the growth of *M. tb* by stimulating superoxide bursts that facilitate phagolysosome fusion (Weiss and Schaible, 2015; Martineau et al., 2008; Martineau et al., 2007; Rook et al., 1986; Bahloul et al., 2022). Furthermore, various mycobacterial mechanisms for perceiving and combating internal or external stress via redox buffering systems, such as thioredoxins, mycothiol, ergothioneine, and protein thiols, are aimed towards neutralising oxidative harm caused by reactive nitrogen species (RNS) and reactive oxygen species (ROS), via the enzymes superoxide dismutase (SOD) and catalase-peroxidase (KatG), which are capable of rendering superoxides inactive. The early upregulation of SOD and KatG during infection suggests an escalation in oxidative damage caused by superoxides (Kumar et al., 2011; Beatrice, 2013; Amalia et al., 2022). Iron is essential for the survival of *M. tb*, confirmed by the observation that the bacillus struggles to replicate and persist in low-iron environments (Phelan et al., 2018; Skaar, 2010; Beatrice, 2013). Within the host, iron functions in the electron transport chain, and for oxygen transport, and storage (Gray and Winkler, 1996; Perutz, 1990; Doherty, 2007). Subsequently, *M. tb* has developed various pathways to scavenge iron, including the utilisation of host heme and non-heme iron through *M. tb* siderophore-dependent iron and heme uptake pathways (Chao et al., 2019; Cassat and Skaar, 2013). Furthermore, the secretion of small molecules with iron chelating properties additionally helps to facilitate iron scavenging, transport, and uptake by *M. tb* (Saha et al., 2013; Miethke and Marahiel, 2007; Wilson et al., 2016). The limited availability of free iron and heme iron in the host poses a significant challenge to the pathogen (Chao et al., 2019). Within the macrophage phagosome, *M. tb* has very low levels of available free iron at its disposal, despite the relatively high levels of iron present in the macrophage cytosol (Ratledge, 2004; Lane et al., 2015). To overcome this hurdle, *M. tb* siderophores have been proposed to facilitate iron diffusion across the phagosomal membrane (Luo et al., 2005). With the subsequent increased availability of iron, *M. tb* growth and replication are significantly promoted and aggravating *M. tb* infection (Gangaidzo et al., 2001; Nairz et al., 2010; Weiss and Schaible, 2015).

1.6 The burden of TBM and filling the gaps.

A better characterisation of TBM, and ultimately improved diagnostic tools and treatment approaches, are still required, especially so in children. Sensitive and specific diagnostic tools for paediatric TBM are still lacking despite the increased global awareness of its burden (Dewandel et al., 2023). In South Africa, access to medical facilities is limited for many children, especially in rural and informal urban areas, compounding an already delayed diagnosis (Solomons, 2023). In addition, diagnosis of TBM in children relies mainly on a physician's suspicion, followed by the administration of empirical anti-tuberculous therapy (Solomons, 2023). In most cases, TBM is diagnosed through various clinical observations, and abnormalities in CSF, much of the time with no further confirmation through microbiological testing (Graham and Donald, 2014). An accurate prognosis and disease diagnosis for TBM largely depends on the stage of the disease, (van Toorn and Solomons, 2014). Existing diagnostic techniques for TBM are considered invasive, complex, and time-consuming and the gold standard is the culture of *M. tb* from CSF (Handryastuti et al., 2023). The paucibacillary nature of TBM and the low availability of CSF also in especially children makes the presence of *M. tb* in children with suspected TBM challenging (Huynh et al., 2022). It is imperative to note that this process necessitates the performance of a lumbar puncture, which subsequently requires expertise, instrumentation, and medical facilities to obtain. It is also crucial to acknowledge that even with the most advanced laboratory resources currently available, the diagnostic tests at present (such as smear microscopy, molecular tests including Xpert MTB/RIF, or CSF culture) exhibit only a moderate level of sensitivity, which often leads to false negative outcomes (Glimåker et al., 2015; Richie and Josephson, 2015; Bahr et al., 2019; Seddon et al., 2019). The combination of these diagnostic challenges, coupled with the rapid deterioration of health in the absence of treatment, results in a significant number of individuals succumbing to TBM without the disease ever being properly identified (Seddon et al., 2019; Dodd et al., 2021). In Africa, particularly in South Africa, managing childhood TBM is difficult and complex, necessitating a multidisciplinary strategy including better public health and more research on the topic (Solomons, 2023). The primary questions that persist today regarding this are: (1) what is the best way to diagnose paediatric TBM?; (2) what is the fastest and optimal treatment strategy for this disease?; (3) how can we mitigate the rate of disease progression in a population and reduce neurological morbidity among paediatric survivors? The answers to these questions all start by better understanding the disease, and further application to improving the aforementioned.

The true global burden of childhood TB which progresses to TBM in children, remains unknown (Van Toorn, 2015). Biomarkers, which can encompass a wide range of measurable entities, whether they be molecules, genes, or physical characteristics, serve as an invaluable tool within the realm of medical research and diagnostic procedures. Biomarkers provide valuable insights and serve as a qualitative or quantitative indicator, specifically pertaining to a particular disease state or any other physiological state, elucidating the intricate workings and intricacies of an organism (Huss, 2015; Yu et al., 2023; Strimbu and Tavel, 2010). Currently, no clear biomarkers nor biosignature exists for TBM; let alone one from patient samples collected non-invasively, such as urine. Hence, the elucidation of a non-invasive biosignature for early TBM detection is of the highest priority. The development of affordable, accurate diagnostic tests for TBM in resource-poor settings remains a priority.

1.7 Metabolomics

Metabolomics can be defined as the identification and quantification of the small molecule metabolic products and intermediates (the metabolome) of a biological system (cell, tissue, organ, biofluid or organism), at a specific point in time, and changes in metabolite concentrations in response to genetic or environmental perturbation(s) (Smolinska et al., 2012; Lajis et al., 2017). Within the field of infectious diseases, the gaps in our comprehension of microbial metabolism have become especially apparent in the sphere of TB research (Rhee, 2013). However, a primary element contributing to this challenge is our limited understanding of the resulting metabolic changes of *M. tb* and the resulting host response to infection and disease. Even though over a century has passed since Koch's initial identification of *M. tb* being the causative agent for TB despite all the genomics, proteomics and transcriptomics data collected, and all the subsequent diagnostic techniques and treatment approaches TB is still considered a global epidemic today. This is largely driven by an inadequate understanding of the disease, especially from a metabolomics research perspective (Nathan, 2009; Rhee et al., 2011; Rhee, 2013). Metabolites are vital constituents of biological systems and highly informative about the organism's physiological state; serving as biomarkers of disease and monitoring therapeutic response (Goodacre, 2010). Understanding the perturbation in terms of these metabolic pathways/networks assists the researcher in understanding the metabolic dynamics associated with the disease/abnormality (Smolinska et al., 2012). Metabolomics approaches have to date been used with great success in several areas of biomedical research, including to determine the metabolic consequences of various genetic modifications, cancer diagnostics, inborn errors of metabolism, diagnosing neurological diseases and toxicology studies (Blasco et al., 2010; Beger et al., 2006; Ellis et al., 2007; Gowda et al., 2008). A study by van der Greef et al. (2004) describes using metabolomics as a prospective tool for clinical setup to detect early metabolic perturbations in a biological system before the disease symptoms are present. However, the complexity of the metabolome requires more than a single analytical technique to provide sufficient, comprehensive characterisation (Dunn and Ellis, 2005). Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the techniques most commonly used in metabolite profiling and analysis of complex biofluids (Gowda et al., 2008). These techniques are well-established complementary analytical methods for generating metabolomics profiles (Smolinska et al., 2012). Liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) have higher sensitivity and better resolution than NMR, and both detect metabolites that are present in concentrations well below the detection limit of NMR (Smolinska et al., 2012). NMR, however, allows for a more robust and broader view of the metabolome, allowing holistic detection of metabolites, as well as being highly specific and far easier to perform with regards to the sample preparation steps prior to analysis — devoid of chemical alteration via derivatization which is typically required for MS analyses (Mason et al., 2016a; Fiehn, 2016; Barderas et al., 2011). The applications of these analytical techniques for metabolomics, especially those related to infectious human diseases (Loots, 2016), have brought unique and novel insight into the underlying pathogenesis of diseases, and subsequently the identification of various metabolic biomarkers for use in improved disease diagnosis (Duarte et al., 2014) and treatment. In Africa, many infectious diseases relevant to its society have been studied, such as malaria (Li et al., 2008), schistosomiasis (Wang et al., 2004), TB (Chen et al., 2013), HIV (Hewer et al., 2006) and human African trypanosomiasis (Chen et al., 2013; Wang et al., 2004;

Li et al., 2008; Wang et al., 2008; Hewer et al., 2006), highlighting the uniqueness and reliability of these metabolomics techniques.

1.8 Urinary metabolomics

Urinary metabolomics has successfully been used to elucidate the metabolic changes caused by specific diseases or treatments (Di Poto et al., 2021), and is considered an excellent biofluid for non-targeted metabolomics research, offering a comprehensive evaluation of metabolic profiles (Olivier et al., 2023), since it contains a plethora of metabolic information, large volumes can be collected non-invasively from a subject, is non-viscous, contains fewer protein complexes and lipids than other biofluids, and is considered to be sterile of the infectious organism (Emwas et al., 2015; Bouatra et al., 2013; Gupta-Wright et al., 2016). Subsequently, in a clinical research context, it has been utilised with great success to find biomarkers for diseases or adverse health states (Sequeira-Antunes and Ferreira, 2023). Urinary metabolomic profiling in pulmonary TB patients has also been shown to be an efficient means towards better diagnosing and monitoring an individual's response to treatment (Luies et al., 2017). Mason et al. (2016b) illustrated holistically the metabolic complexity of TBM and provided proof-of-concept that a putative biosignature of urinary metabolites can be defined for non-invasive diagnosis and prognosis of paediatric TBM patients. Until now, there have been indications that methylcitric acid might serve as a potential marker of the disease. From the literature, we know that *M. tb* utilises a specialised methylcitric acid cycle involving propionyl-CoA for alternative energy production (Mason et al., 2016b). Since the discovery of TBM, research has been ongoing towards developing a better diagnostic test, but no studies have yet explored the diagnostic accuracy for TBM in paediatrics using urine. There is compelling evidence indicating the presence of several previously undiscovered biochemical markers, which harbour valuable data that could aid in the better detection of TBM from patient-collected urine, particularly in paediatric cases. Such findings are poised to instigate a shift in the prevailing paradigm surrounding TBM, prompting further scrutiny from both researchers and clinicians towards delving deeper into metabolomics as a means of enhancing our understanding of the disease and ultimately better diagnostic accuracy and prognostic outcomes.

1.9 The biological questions of the study

The most prominent challenges that currently exist towards paediatric TBM are a thorough understanding of the disease, inadequate approaches to the prevention, and treatment of the disease, the fact that childhood deaths and sequelae of TBM constitute a crucial burden for society due to the prolonged course of the disease, and accurate and timely diagnosis and the monitoring and prediction of treatment outcomes. As previously mentioned, the early detection of paediatric TBM is imperative to facilitate efficient treatment and mitigate the associated risk of death. However, diagnosing TBM in children early has proved to be an arduous task due to the vague nature of their symptoms, which can be atypical or completely absent. Furthermore, many of the current diagnostic techniques produce inconclusive results, such as negative or equivocal smear and culture findings, despite the patient showing clinical manifestations of TBM. This indicates that there is still much knowledge to be gained and there are still many untapped metabolic data sources to be explored.

The gold standard for diagnosing TBM requires CSF through a lumbar puncture - an invasive sample collection method. Currently, available CSF assays are often not sufficient for a definitive diagnosis of TBM. Most cases of TBM are diagnosed on clinical grounds without bacteriological confirmation of CSF (Garg, 2019). CSF collected by lumbar puncture and sent for culturing for *M. tb* is positively identified in fewer than 50% of all reported TBM cases, which means that the current laboratory-based diagnostic approaches often cannot be confirmed alone by microbiological assays. Therefore, there is a need to investigate an alternative biological matrix for the identification of novel TBM biomarkers.

1.10 Objectives of this thesis

The focus of this research study is to accurately diagnose TBM using urine at the earliest curable stages of this disease in children, to better characterise the disease, and to evaluate treatment response. Thus, the specific research objectives of this study were:

- (1) To characterise the urinary metabolic profile of TBM in children.
- (2) To identify metabolite profiles characterising the three stages of TBM in children and identify potential biosignatures for earlier differential diagnosis of these three stages.
- (3) To determine if metabolites (biomarkers) could be used to evaluate the treatment response; namely, do the identified metabolic markers of TBM resolve during treatment over 6 months.

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**PART 1 OVERVIEW OF BRAIN-TO-GUT AXIS EXPOSED TO CHRONIC CNS
BACTERIAL INFECTION(S) AND A PREDICTIVE URINARY METABOLIC PROFILE OF A
BRAIN INFECTED BY MYCOBACTERIUM TUBERCULOSIS**

CHAPTER 2



Overview of Brain-to-Gut Axis Exposed to Chronic CNS Bacterial Infection(s) and a Predictive Urinary Metabolic Profile of a Brain Infected by *Mycobacterium tuberculosis*

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Abstract

A new paradigm in neuroscience has recently emerged – the brain–gut axis (BGA). The contemporary focus in this paradigm has been gut → brain (“bottom-up”), in which the gut-microbiome, and its perturbations, affects one’s psychological state-of-mind and behavior, and is pivotal in neurodegenerative disorders. The emerging brain → gut (“top-down”) concept, the subject of this review, proposes that dysfunctional brain health can alter the gut-microbiome. Feedback of this alternative bidirectional highway subsequently aggravates the neurological pathology. This paradigm shift, however, focuses upon non-communicable neurological diseases (progressive neuroinflammation). What of infectious diseases, in which pathogenic bacteria penetrate the blood–brain barrier and interact with the brain, and what is this effect on the BGA in bacterial infection(s) that cause chronic neuroinflammation? Persistent immune activity in the CNS due to chronic neuroinflammation can lead to irreversible neurodegeneration and neuronal death. The properties of cerebrospinal fluid (CSF), such as immunological markers, are used to diagnose brain disorders. But what of metabolic markers for such purposes? If a BGA exists, then chronic CNS bacterial infection(s) should theoretically be reflected in the urine. The premise here is that chronic CNS bacterial infection(s) will affect the gut-microbiome and that perturbed metabolism in both the CNS and gut will release metabolites into the blood that are filtered (kidneys) and excreted in the urine. Here we assess the literature on the effects of chronic neuroinflammatory diseases on the gut-microbiome caused by bacterial infection(s) of the CNS, in the context of information attained via metabolomics-based studies of urine. Furthermore, we take a severe chronic neuroinflammatory infectious disease – tuberculous meningitis (TBM), caused by *Mycobacterium tuberculosis*, and examine three previously validated CSF immunological biomarkers – vascular endothelial growth factor, interferon-gamma and myeloperoxidase – in terms of the expected changes in normal brain metabolism. We then model the downstream metabolic

2.2 Introduction

A new paradigm in neuroscience has emerged in recent years – the brain–gut axis (BGA) – involving bidirectional communication between the brain and gut. This implicates a variety of pathways, including the enteric nervous system (ENS), central nervous system (CNS), gastrointestinal tract (GIT), endocrine system/GI hormones, and immune response, all integrated to orchestrate the bidirectional feedback loop of the BGA. As averred by Hippocrates, the Greek physician acknowledged by many as the father of modern medicine, “*All disease starts in the gut.*” The gut-microbiome is made up of innumerable microbes, which function in a mutualistic relationship with the human host (Collins et al., 2012; Zhu et al., 2017). Currently, scientific evidence supports the notion that homeostatic imbalance is initiated in the gut-microbiome, mediated by several microbe-derived molecules, in the gut– brain (“bottom-up”) direction of communication (Foster and Neufeld, 2013; Martin et al., 2018). Stable gut microbiota are essential for normal gut physiology and contribute to appropriate signaling along the BGA (Forsythe et al., 2010; Cryan and Dinan, 2012; Schroeder and Bäckhed, 2016). Over the past decade, however, neuroscience research on the BGA has focused on how perturbations in the gut-microbiome affect the brain in a feedback loop, centered on the premise of “*you are what you eat*” and “*gut feelings*” (Moos et al., 2016; Sherwin et al., 2016; Zmora et al., 2019). Considering the bottom-up motif, particularly its perturbations in the gut-microbiome, can have a clear and direct effect on the host’s psychological state-of-mind (depression, anxiety, bipolar disorder), behavior (autism) and also in the pathogenesis and/or progression of various neurodegenerative diseases (Alzheimer’s, Parkinson’s, and multiple sclerosis). These disorders associated with the bottom-up direction of communication have been succinctly and meticulously detailed in many topical research reviews (Mayer et al., 2014; Konturek et al., 2015; Powell et al., 2017; Zhu et al., 2017; Martin et al., 2018; Ambrosini et al., 2019). Perturbations of the BGA associated with non-communicable neurological diseases – to what degree, the precise mechanism involved, and their appropriate therapy – are not yet well understood. Many studies on the role of microbiota in the pathogenesis of neurodegenerative/psychiatric diseases exist, however, and their main findings are summarized in **Table 1**.

The focus of this review is on the brain–gut (“top down”) direction of the BGA. In particular, perturbations of brain metabolism induced by invading bacteria and, as a consequence, gut dysbiosis. Within the contemporary paradigm of a perturbed BGA, most of the relevant research centers on noncommunicable neurological diseases, synonymous with a slow, gradual progression of neuroinflammation. However, the link between the brain–gut concept and CNS bacterial infection(s) is less prevalent in the literature, and hence the focus of this review. The most recent and comprehensive review of the BGA was by Cryan et al. (2019). However, only a very small section, amounting to half a page, discusses infections and the brain, even though bacterial penetration of the blood–brain barrier (BBB), and subsequent infection, leads to a cascade of events within the brain, modulating a feedback effect on the host gut-microbiome (Dando et al., 2014; Bauer et al., 2016; Martin et al., 2018). Bacterial infection(s) of the CNS induce an inflammatory response via glia mediators, pivotal to establishing communication between the host’s immune system and the brain (DiSabato et al., 2016) and, ultimately, generating sustained feedback on the BGA (Geyer et al., 2019).

As a proof of a novel concept for the BGA, we use three previously validated immunological CSF markers of tuberculous meningitis (TBM) – vascular endothelial growth factor (VEGF), interferon-gamma (IFN- γ), and myeloperoxidase (MPO) – to model/predict the metabolic changes, and are the basis for postulating a metabolic cascade, expected within the brain of a TBM patient. It is well known that important diagnostic and prognostic information related to alterations in metabolic cascades and disruption of homeostasis can be characterized through metabolite profiling of urine (An and Gao, 2015; Emwas et al., 2015). Hence, logic dictates that if the BGA exists then the impact of chronic CNS bacterial infection(s) (such as TBM) should be reflected in the host's urine.

2.3 Brain–Gut Concept

According to the brain–gut (“top-down”) concept, the brain can alter the community structure and function of the gut-microbiome in a bidirectional interaction feedback loop, characterized by continuous communication between the CNS and the GIT (Zhu et al., 2017; Karol and Agata, 2019). The GIT is a highly complex organ involved in multiple dynamic physiological processes, while interacting with the gut microbiome – an extensive and diverse community of bacteria (Parker et al., 2018). The brain nerves (e.g., vagus nerve), which control unconscious tasks, run from the brainstem to the gut, maintaining the physical bidirectional communication between the CNS and intestinal wall. The brain-to-gut signaling pathway affects host–bacteria interactions in the GIT by influencing

Table 2.1 | Main findings from studies describing the role of microbiota in the pathogenesis of neurodegenerative/psychiatric diseases.

Disorders		Main findings	References
Neurodegenerative			
Parkinson's disease (PD)	(i)	Gut microbiota influence the activity of enteric neurons, affecting cellular α -synuclein (α -syn) secretion, characterized by the accumulation and aggregation of α -syn in the substantia nigra (SN).	(Braak et al., 2003). (Mulak and Bonaz, 2015). (Nair et al., 2018).
	(ii)	Gastrointestinal dysfunction is present in ~80% of PD patients.	(Fitzgerald et al., 2019,
	(iii)	α -Synucleinopathy is suggested to be an early indicator of PD pathology.	Scheperjans et al., 2015,
	(iv)	The vagal nerve, which serves as channel for α -syn from the ENS to the CNS, is crucial for the communication between gut microbiota and the brain.	Ulusoy et al., 2013). (Lebouvier et al., 2009).
	(v)	Pathological hallmarks of PD are a loss of dopaminergic neurons in the SN and the presence of cytoplasmic eosinophilic inclusions termed Lewy bodies (LBs).	(Lebouvier et al., 2009).
	(vi)	Immunolabeling with α -syn antibodies have become the reference standard in the assessment of LBs and Lewy neurites in both the CNS and peripheral nervous system. Hence, α -synucleinopathy affects all levels of the BGA.	
Alzheimer's disease (AD)	(i)	AD is characterized by a deposition of amyloid beta ($A\beta$) followed by the formation of plaques, characterized by a progressive decline in cognitive function.	(Wang et al., 2014,
	(ii)	Gut microbiota produce amyloids which aid bacterial cell binding, and form part of the biofilm protecting these from destruction by host immune factors.	Jouanne et al., 2017).
	(iii)	Bacterial amyloid proteins exposure to the host, from the gut, may be detrimental since they prime of the host's immune system against endogenous production of neuronal amyloids in the brain.	(Friedland and Chapman, 2017). (Kowalski et al., 2019). (Zhao et al., 2017).
	(iv)	Bacterial lipopolysaccharides are increased in the neocortex and the hippocampus in AD.	(Kowalski et al., 2019).
	(v)	Calprotectin is indicative of inflammation and has be detected in elevated amounts in the CSF, brain and fecal matter of AD patients.	
Multiple sclerosis (MS)	(i)	MS is a demyelinating disease, clinically associated with autoimmune disease. Progressive degradation of the integrity of the epithelia that comprise cellular barriers essential to maintaining the integrity of both intestine and CNS, have been associated in MS patients suffering from autoimmunity, resulting in paralysis and other related symptoms of MS.	(Dendrou et al., 2015, Ochoa-Repáraz et al., 2018, Ochoa-Repáraz and Kasper, 2014).
	(ii)	Clinical signs of MS are relapse of sensory, motor and cerebellar complications; while an acute disease stage is a characteristic feature of the relapsing-remitting MS (the latter of which are often diagnosed with neuronal dysfunction).	(D'Amico et al., 2016, Connick et al., 2018, Johnston Jr and Joy, 2001).
	(iii)	Secondary-progressive MS develops and transcends into progressive neurological impairment.	(D'Amico et al., 2016).
	(iv)	Dysbiosis affects the immunological responses of the host to the microbiota, as described in an experiment where germ-free mice with an immune dysfunction, were characterized by an imbalance between pro- and anti-inflammatory immune cells in the gut, where after colonization of the gut with commensal microbes restored immune function.	(Ochoa-Repáraz et al., 2018, Mazmanian et al., 2005).
			(Wang et al., 2011,
	(i)	Dysbiosis in children with ASD has been show to contribute to both gastrointestinal and CNS abnormalities.	Santocchi et al., 2016).

Neuropsychiatric Autism spectrum disorders (ASD)	(ii)	Short-chain fatty acid producing bacteria, and their metabolites, especially propionic acid, has been indicated to adversely affect the CNS and contribute to autism behavior by modulating the BGA.	(De Angelis et al., 2015).
	(iii)	Behavioral abnormalities are accompanied by imaging abnormalities in the sensory and emotion regulation regions of the brain.	(Green et al., 2013)
	(iv)	Abnormally elevated levels of lipopolysaccharides have also been associated with the pathogenesis of autism.	(Fattorusso et al., 2019).
	(v)	40% of ASD patients complain of GI symptoms; abnormalities such as chronic diarrhoea, constipation, vomiting, feeding problems, reflux and abdominal pain, as well as anxiety.	(Mayer et al., 2014, Fattorusso et al., 2019).
	(vi)	Patients with ASD also have high fecal and urinary levels of bacterially derived p-cresol, and further exposure to p-cresol has been shown to contribute to the severity of behavioral symptoms and cognitive impairment in ASD.	(Altieri et al., 2011, Gabriele et al., 2014, Persico and Napolioni, 2013).
	(vii)	Optimized remedies that are practiced include rehabilitation, educational therapy and psycho-pharmacological approaches.	(Fattorusso et al., 2019).
			(Bercik et al., 2011, Jiang et al., 2015, Kelly et al., 2016, Park et al., 2013).
Depression, anxiety, and major depressive disorder (MDD)	(i)	Pre-clinical studies of depression, anxiety and MDD indicate that the altered brain function associated with these, can partly be attributed to disturbances in the gut microbiota composition.	
	(ii)	Studies have shown that the microbiome has the capacity to influence on emotional behavior, and is associated with various parameters relating to depression pathogenesis and severity.	(Cryan and Dinan, 2012, Bercik et al., 2011, Clemente et al., 2012).
	(iii)	Hippurate, dimethylamine and dimethylglycine, all by-products of gut microbiota, have been detected in abnormal concentrations in MDD patients which further substantiates the aforementioned observations.	(Zheng et al., 2013, Zheng et al., 2016).
	(iv)	Increased severity in depression and anxiety have been noted following bacterial infection in patients.	(Naseribafrouei et al., 2014).

the enteric microbiota indirectly via an altered intestinal permeability, or directly via signaling molecules released into the gut lumen from immune and enterochromaffin cells, thereby increasing motor, sensory and secretory modalities of the GIT (Rhee et al., 2009; Grenham et al., 2011; Eisenstein, 2016). Those signaling systems that allow the brain, in this crosstalk communication, to influence gut-microbiome functions in the GIT, are: (1) the endocrine-immune system, (2) the hypothalamus–pituitary–adrenal (HPA) axis, (3) the sympathetic and parasympathetic arms of the autonomic nervous system (ANS), and (4) enteric nervous system (ENS) (Rhee et al., 2009; Grenham et al., 2011; Cong et al., 2015). These signaling systems are interlinked systematically to form a complex reflex network, with afferent and efferent fibers (O’Mahony et al., 2011). Hence, activation of any of these signaling systems, either alone or in combination, might influence the composition and functionality of enteric microbiota (Rhee et al., 2009). For instance, under conditions of chronic stress the brain recruits these same mechanisms, by activation of the HPA axis in the brain, to regulate cortisol secretion. Cortisol in turn affects various immune cells (including cytokine secretion) locally in the gut, subsequently inducing changes to microbiota composition, and increasing the gastrointestinal permeability (de Punder and Pruimboom, 2015; Kelly et al., 2015; Farzi et al., 2018). Hence, an exceedingly complex array of signaling systems, all interlinked, lies between the brain and gut in the “top-down” concept (Aziz and Thompson, 1998; Collins and Bercik,

2009; O'Mahony et al., 2009; Forsythe et al., 2014; Khlevner et al., 2018; Weltens et al., 2018; Zhao et al., 2018).

The CNS is well shielded by the BBB, the major site of blood–CNS exchange. The barrier comprises microvascular endothelial cells, astrocytes and pericytes, and is tasked with the regulated passage of molecules into and out of the brain (Abbott et al., 2010; Sochocka et al., 2017b). Neurotropic bacteria are capable of evading host defenses, gaining access to the CNS (Dando et al., 2014), with >95% of brain abscesses caused by bacterial infection(s) (Sonneville et al., 2017). Furthermore, the brain may become particularly susceptible to bacterial infection(s), if the BBB is chronically compromised by an initial infection (Mendes et al., 1980; Cantiera et al., 2019). Various brain cells – microglia (resident macrophages), endothelial, ependymal, neuronal and glial (astrocytes and oligodendrocytes) – convey innate immune molecules that prompt the recruitment of leukocytes into the infected CNS compartments, in order to combat invading neurotropic bacteria (Klein et al., 2017). This process results in a series of initial neuroinflammatory events within the brain, as well as phagocytosis of the infecting bacteria, in an attempt to control disease progression. Neuroinflammation in the CNS is mediated by the production of cytokines and chemokines, that are pivotal in the coordinated communication between the immune system and the brain (DiSabato et al., 2016). The host's inflammatory reaction in the CNS is initiated by the recognition of the invading pathogens, which in turn leads to the local production of mediators by the glial cells comprising microglia and astrocytes (Grandgirard et al., 2013). Thus, acute inflammatory feedback is triggered by rapid and early activation of mediators released by activated glial cells in the CNS due to the infectious agent. However, when the presence of an infectious agent persists, a chronic state of inflammation within the brain results (Sochocka et al., 2017a) and the activated glial cells are altered beyond “normal” proportions, which results in progressive neurodegeneration (Kempuraj et al., 2017; Sochocka et al., 2017a). Pattern recognition receptor (Newton and Dixit, 2012; Suresh and Mosser, 2013) activation initiates the release of pro-inflammatory cytokines and chemokines, in order to modulate the immune response, leading to pleocytosis of white blood cells (Janowski and Newland, 2017). This in turn triggers an increased BBB permeability and the influx of leukocytes from the blood into the CNS at the site(s) of infection (Waisman et al., 2015; Kempuraj et al., 2017). Although this is the mechanism by which the brain attempts to restore homeostasis and protect itself against the invading pathogen (More et al., 2013), the chronic production of immune cells induces neurodegeneration. Since activated microglia have both neuroprotective and neurotoxic functions (Kim, 2003; Nimmerjahn et al., 2005; Dando et al., 2014; Liechti et al., 2015; Doran et al., 2016), various toxic molecules released by the microglia during the immune response may also inflict neuronal injury.

2.4 Bacterial infections of the CNS And their effect on The Brain–Gut Axis

Most bacterial CNS infections present acutely, including subacute and chronic forms. Common acute bacterial CNS infections involve *Streptococcus agalactiae*, Gram-negative bacilli including *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* (Durand et al., 1993; Gray, 1997; Grandgirard et al., 2013; Zhou, 2019), while subacute and chronic bacterial CNS infections, besides *Mycobacterium tuberculosis*, involve *Borrelia burgdorferi*,

Leptospira interrogans, *Treponema pallidum*, *Mycobacterium leprae*. Microbial pathogens can gain entry into CNS by penetrating the BBB or via the olfactory (Kristensson, 2011). The nasopharynx is the usual portal of entry for major meningeal pathogens. Pathogens penetrate the olfactory epithelium, and could potentially cross epithelial barriers into the subarachnoid space; compromising the epithelial tissue by exposure to bacterial virulence factors, directly infecting the olfactory sensory neurons (Dando et al., 2014; Rey et al., 2018). Meningeal invasion subsequently follows via penetration of the cellular barriers of the CNS. The putative cascade of events caused by bacterial infection(s) of the brain that alter permeability of the gut – discussed in detail below, ultimately leads to dysbiosis.

- (1) Within the cascade, the first step of bacterial invasion involves transitioning across the compromised BBB into the subarachnoid space. Pathogens can cause disruption of the BBB, which enables their passage into the brain. The various host defenses are usually inadequate to control the infection. Leukocytes traverse the BBB and patrol the brain parenchyma under normal conditions.

During inflammation, as result of infection, the BBB junctions (adherens and tight) that regulate the flux of ions, polar molecules, and macromolecules from the systemic circulation can be compromised, thus traffic is greatly increased at these junctions. Bacteria may cross the BBB by transcellular penetration after bacterial adhesion to endothelial cells or via infected leukocytes. Pinocytosis, increased by leukocytes combating bacteria that might have invaded following disruption of tight junctions or via the “Trojan horse” mechanism – phagocytes infected with the pathogen transverse the BBB (Kim, 2003; Pulzova et al., 2009). Leukocytes, activated by inflammatory molecules released during infection, cross the BBB by a multistep process that involves attachment to, and invasion through, the post-capillary venule wall and the surrounding endothelial and parenchymal basement membranes which differ in their laminin composition and permeability (Owens et al., 2008; Kristensson, 2011; Dando et al., 2014). During infection of the CNS various acute pathological events may occur which further compromise the CNS. The brain parenchyma is populated by resident immune cells, the microglia, which are highly specialized tissue macrophages.

- (2) Microglia cells, the primary immune effector cells in the brain, continuously survey the brain parenchyma and respond to very subtle alterations in their microenvironment and in the brain's structural integrity (Nimmerjahn et al., 2005). Microglia are highly motile immune effector cells in the brain that respond to neuronal infection and damage. The role of microglia in a healthy brain, along with immediate reaction to brain damage, is paramount in response to the prevention of any kind of major brain damage. Microglia are considered essential for communication in the intrinsic immune system of the CNS, as well for intercellular crosstalk between astrocytes and neurons (Kreutzberg, 1996; Stollg and Jander, 1999; Streit, 2002; Streit et al., 2004; Akiyoshi et al., 2018). Microglia maintain CNS health via mediators involved in the function of neurogenesis, modeling of synapses, excitotoxicity prevention and regulation of neuroinflammation. Short-chain fatty acids derived from the gut-microbiome play a pivotal role in the function and maturation of microglia. Hence, microglia are crucial mediators in the interaction between the CNS and the gut microbiota (Wang et al., 2018; Abdel-Haq et al., 2019).
- (3) Bacterial cell wall material, enzymes, and toxins cause direct injury to neurons and indirect damage by increasing vascular permeability that causes edema and further injury. Microglial cells respond to

bacterial pathogens and neuronal injury by the production of reactive oxygen species (ROS), nitrous oxide, and peroxynitrite. Immune response also contribute to neurotoxicity via release of proteases and excitatory amino acids. Several signaling molecules, such as catecholamines, serotonin, dynorphin and cytokines, used by the host for neuronal and neuroendocrine signaling, are also likely to be secreted into the gut lumen (Rhee et al., 2009).

- (4) Bacterial pathogens may target neurons and glial cells, inducing inflammation and exerting direct cytopathic effect due to the release of their products. Thereafter, brain cell apoptosis begins to occur. For example, Pneumolysin and hydrogen peroxide (H_2O_2) are direct triggers of *Streptococcus pneumoniae*. H_2O_2 rapidly diffuses through eukaryotic cell membranes to damage intracellular targets thus increasing intracellular Ca^{2+} , damaging mitochondria, and causing the release and translocation of mitochondrial apoptosis-inducing factor. Increased intracellular ROS and Ca^{2+} precedes morphologic changes that lead to brain cell apoptosis (Mitchell and Andrew, 1997; Lipton and Nicotera, 1998; Braun et al., 2002; Janowski and Newland, 2017). Brain cell apoptosis leads to neuronal injury in the form of brain manifestations, such as: basal ganglia and thalami communication that become obstructive, cranial nerve dysfunction, minor focal neurological signs, infiltrates of inflammatory cells, exudation of protein-rich fluid, and edema (Gray, 1997; Hussein and Shafran, 2000; Van de Beek et al., 2004; Østergaard et al., 2005; Al Khorasani and Banajeh, 2006; Hähnel and Bendszus, 2009; Abdulrab et al., 2010).
- (5) Pathogenic bacteria that causes meningitis exhibit antiphagocytic capsular polysaccharide ability which enables survival within the blood. Hence, changes in the gut involves hematogenous dissemination of bacteria, initiating meningitis via mucosal adhesion of the organism and subsequent systemic invasion (Seib et al., 2009; Harvey et al., 2011; Dando et al., 2014). The intestinal immune system is tasked to maintain homeostasis within the gut-microbiome via the processes of minimizing direct contact between intestinal bacteria and the epithelial cell surface (stratification) and confining penetrant bacteria to intestinal sites and limiting their exposure to the systemic immune compartment (compartmentalization) (Hooper et al., 2012; Macpherson and McCoy, 2013). Mucosal surfaces represent the major interface and constitute the point of entry of most infectious pathogens and are in contact with potentially injurious antigens (Janeway et al., 2001; Kaetzel, 2005).
- (6) Stratification of intestinal bacteria on the luminal side of the epithelial barrier also depend on secreted immunoglobulin A (IgA). IgA specific for intestinal bacteria is produced with the help of intestinal dendritic cells that sample the small numbers of bacteria penetrating the overlying epithelium. Some meningeal pathogens produce proteases that cleave to human immunoglobulin subclasses (e.g., IgA1), allowing adherence of bacterial strains to mucosal surfaces and crossing the mucosal barrier (Lorenzen et al., 1999; Hooper et al., 2012; Brooks and Mias, 2018). IgA1 proteases separate the pathogen-recognition (Fab) and host signaling (Fc) components of the antibody, thereby severing communication with host defense cells. This also leaves pathogens coated with cleaved Fab fragments and camouflaged from the immune system. IgA1 proteases disable this important defense immune molecule allowing for direct escape of the invading pathogen from host immunity (Woof and Russell, 2011; Marshall et al., 2017). This communication/crosstalk involving the gut microbiota from the CNS encompasses several channels along various neural, enteric and immune systems. Sensory

and motor fibers from the vagus nerve connect the gut and the brainstem, and serve as a conduit for neural signals involving the microglia. Increased CNS inflammation signals vagal efferent nerves to relay information about the immune status of the brain to the gut and the gut microbes. In the same manner, vagal afferents transduce and relay information from the GIT to the CNS, signaling microglia via increased production of various pro-inflammatory cytokines that modulate neuroinflammation (Goehler et al., 1999, 2005; Borovikova et al., 2000; Forsythe et al., 2014; Abdel-Haq et al., 2019).

2.5 Urine reflects dysbiosis within bacterial CNS infection(s)

The CNS can communicate with the gut via signaling molecules carried by the CSF and blood, which in turn may alter gut composition and physiology. Evidence for this communication between the gut and the brain includes the following: (1) it is well known that toxins or abnormal metabolites that enter the bloodstream are ultimately removed from the blood, in an attempt to maintain a state of cellular homeostasis, and excreted via the urine (Li, 2015; Wu and Gao, 2015); (2) biomarkers for various neurological diseases are detected using body fluids including CSF, blood and urine (An and Gao, 2015). The CSF transfers waste products to the blood, which is filtered by the kidneys, whereby blood-borne waste products accumulate in the urine and are then excreted (Wu and Gao, 2015). It is also well known that various perturbations or other physiological changes in the human body – such as an altered microbiome, for instance – may change what is considered a normal urinary metabolome fingerprint into a new disease specific fingerprint (Want et al., 2010; Emwas et al., 2015; Wu and Gao, 2015). There exists well-described examples in the literature of metabolites found in urine that are associated with microbial metabolism or microbial–host co-metabolism and found to change in response to diseases where gut dysbiosis is the predominant perturbation (Holmes et al., 2011; Vernocchi et al., 2016; Dumas et al., 2017; Malatji et al., 2019). Furthermore, urine is considered the preferred sample matrix for the detection of certain metabolites, which are otherwise difficult to detect from a blood sample due to their low concentrations. Moreover, urine collection is considered relatively non-invasive (Bouatra et al., 2013; Li, 2015). For these reasons, the metabolomics of urine has been successfully exploited for new biomarker discovery in various diseases, including neuropsychiatric disorders, such as schizophrenia, major depressive disorder, bipolar disorder, and autism spectrum disorder (Yap et al., 2010; Cai et al., 2012; Zheng et al., 2013; Chen et al., 2014), and various neurodegenerative diseases, such as PD, AD, and MS (Luan et al., 2014). Based on the premise that the urine contains the accumulation of all end-product metabolites of the body, logic dictates that chronic bacterial infection(s) of the CNS should, in principle, result in persistent feedback on the gut via the BGA, communicated via the CSF and blood, leading to dysbiosis and an altered urinary metabolome.

In research on infectious diseases, urinary profiling has received much attention, in particular regarding pulmonary tuberculosis (TB) – a disease caused by *Mycobacterium tuberculosis* (Mtb) – about which several studies have been conducted using urine for the detection of clinically relevant biomarkers (Banday et al., 2011; Bonkat, 2012; Das et al., 2015; Luies and Loots, 2016; Luies et al., 2017; Preez et al., 2017; Isa et al., 2018). The detection of lipoarabinomannan (LAM), for instance, a *Mycobacterium*-specific liposaccharide from the Mtb cell wall, is an example of the basis of a well-studied commercial ELISA assay that shows promise for its diagnostic use in urine with a reported sensitivity of 74% and

specificity of 86.9% in a study performed on 148 confirmed TB patients (Tessema et al., 2001); a sensitivity of 80.3% and specificity of 99% in a study conducted on 132 confirmed TB patients (Boehme et al., 2005); and a sensitivity of 44% and specificity of 89% in a study conducted on 195 TB-positive patients in a high-HIV prevalence setting (Mutetwa et al., 2009). Within TBM cases (see **Box 1**), the direct LAM-ELISA assay of CSF has similarly shown a sensitivity of 64% and specificity of 86.9% in a study including 50 TBM cases in a high-HIV-prevalence setting (Patel et al., 2009); and a sensitivity of 43% and specificity of 91% for definite TBM cases in a study performed on CSF collected from the 4th ventricle, post-mortem (Cox et al., 2015). However, Bahr et al. (2015) determined that this LAM-based TB antigen test yielded negative results for all the CSF samples (~100) analyzed in their study, of whom 18 had a confirmed diagnosis of TBM. In a short communication the following year, Bahr et al. (2016) voiced their concern about the reliability of the LAM assay for

BOX 1 | Tuberculous meningitis (TBM).

TBM, a severe infectious disease caused by *Mtb*, is a chronic form of bacterial meningitis (BM), resulting in chronic neuroinflammation often associated with irreversible neurological damage/dysfunction. TBM develops in severity in progressive stages (TBM stages I, II and III), and a uniform case definition (definite, probable and possible TBM) for diagnosis has been standardized

(Marais et al., 2010). TBM is the most common form of CNS-tuberculosis (TB) (Van Well et al., 2009) and is considered severe due to its high associated prevalence of mortality and morbidity (Rohlwink et al., 2019). Transmitted via infectious aerosols into the lung, *Mtb* may enter the circulatory system, traverse the BBB and then enter the brain meninges (Rock et al., 2008; Nicholas et al., 2012). Microglia, the resident macrophages of the brain, are the cells preferentially infected by the *Mtb* bacilli (Rock et al., 2005). The Rich foci (Rich and McCordock, 1933), lesions that form in the meninges, eventually rupture, spilling the *Mtb* microbes, cytokines and chemokines into the subarachnoid space, resulting in infection and extensive inflammation of the meninges (Dastur et al., 1995; Donald et al., 2005; Rock et al., 2008). The pathogenesis of TBM is dynamic and *Mtb* bacteria exhibit a resilience that allows them to survive hostile environments, which results in a persistent neuroinflammatory response if not treated correctly and swiftly (de Carvalho et al., 2010; Beste et al., 2011, 2013; Warner, 2015). Despite all efforts toward improved solutions to curbing TB since the discovery of *Mtb* as the causative agent in 1882, there is still a very limited understanding of *Mtb* infection within the host, especially so for TBM, and hence the need for new biomarkers better describing this.

use on CSF for diagnosis of TBM, and also discussed the study by Cox et al. (2015). Ultimately, the LAM-ELISA, like many other TB diagnostic tests, is not sufficient as a stand-alone assay for a definitive diagnosis of TB.

Of particular interest, as it pertains to our review, is that bacterial antigen-specific assays perform particularly poorly when used for diagnosing bacterial CNS infection from urine collected from patients, even in documented septicemia cases (Barnes et al., 1998). Barnes et al. postulated that the reason for this is that these complex polysaccharide antigens break down before excretion in urine. Using the well-tested LAM-ELISA assay, Blok et al. (2014) analyzed urine collected from 21 TBM cases and obtained a sensitivity of only 4.8% and specificity of 93.1%, and hence concluded that urinary LAM detection offers little value for the diagnosis of TBM. Although LAM is detectable in the urine of TB cases and the CSF of TBM patients, it is almost undetectable in urine collected from patients with TBM. A postulated reason for this inconsistency is the inability of LAM to transgress the BBB. This hypothesis can likely be extended to complex bacterial antigens in general, as supported by the results of Barnes et al. (1998). We therefore conclude from these *Mtb*-antigen-specific assay studies that the diagnosis of bacterial infection(s) of the CNS, based on the detection of bacterial antigens in urine, is not a viable option.

For this reason, we believe that the detection of the catabolic components (metabolites) of complex signaling pathways is a better option for the accurate and sensitive differential diagnosis of bacterial CNS infection(s), using urine collected from patients. Mason et al. (2016) provided proof-of-concept by using an untargeted gas chromatography–mass spectrometry (GC-MS) metabolomics approach to analyze the urine of 12 confirmed TBM cases, 19 non-TBM cases (sick controls proven negative for both TB and meningitis) and 29 controls. This explorative study identified urinary metabolite markers that showed two important changes in the TBM cases: (1) a dysfunctional host metabolism, and (2) indicators of an altered host–microbe response in TBM (Mason et al., 2016). The indicators of dysfunctional host metabolism

included: lipolysis and ketosis (elevated 2-hydroxybutyric acid, 3-hydroxybutyric acid, 2methyl-3-hydroxybutyric acid, and acetoacetic acid); perturbed energy metabolism (elevated branched-chain amino acid derivatives, citric acid cycle intermediates and vanillylmandelic acid); liver damage (from the presence of 4-hydroxyphenyllactic acid and 4-hydroxyphenylacetic acid, and highly elevated 4-hydroxyphenylpyruvic acid). Of greater importance to this review was the discovery of those markers serving as indicators of an altered host–microbe response in TBM, as is discussed in greater detail below.

First, Mtb-induced changes to tryptophan metabolism was evident, due to the presence of elevated urinary concentrations of indole-3-acetic acid, 5-hydroxyindole acetic acid, tryptophan, kynurenic acid and quinolinic acid, accompanied by significantly elevated levels of N-acetylanthranilic acid (the N-acetylated product of anthranilic acid; Paul and Ratledge, 1970, 1971, 1973), the latter of which is a novel microbial metabolite indicative of gut microbiota involved in the perturbed host's tryptophan metabolism (Mason et al., 2016). Using a similar but more sensitive metabolomics analytical platform (GC × GC–TOFMS), Luies and Loots (2016) independently compared urine collected from 46 confirmed TB adults to 30 TB-negative healthy controls, and identified similar urinary markers indicative of the same alterations for the host's tryptophan metabolism. They attributed these to the result of an inflammatory response due to releases of cytokines, specifically IFN- γ . Hence, an inflammatory response induced by Mtb-infection, whether in the lungs or brain, results in the release of IFN- γ , which stimulates the upregulation of tryptophan catabolism (Yoshida et al., 1981; Taylor and Feng, 1991; Blumenthal et al., 2012; Hashioka et al., 2017; Lu et al., 2017). The presence of increased urinary tryptophan catabolites therefore contributes to a differential diagnosis of Mtb-based infection, but they do not serve as uniquely distinctive biomarkers.

Second, Mtb–host related metabolites were identified. In particular, significantly elevated concentrations of methylcitric acid were speculated to be likely to have originated from the well-characterized methylcitrate cycle of Mtb (MuñozElías et al., 2006; Savvi et al., 2008). Interestingly, a positive correlation between urinary quinolinic acid and methylcitric acid concentrations was observed by Mason et al. (2016) in all the TBM patients' urine samples collected both before and after Mtb specific treatment commenced. Hence, the roles of quinolinic acid and methylcitric acid in the host are intertwined during Mtb infection, and its treatment.

Lastly, urinary metabolite markers associated with alterations to the gut-microbiome were identified as a major consequence of perturbed metabolism associated with TBM. Of the significant urinary metabolites, those that are linked to gut microbiota were identified as uracil, hippuric acid, 4-hydroxyhippuric acid, phenylacetylglutamine and 4-cresol (Mason et al., 2016). Luies and Loots (2016) also identified elevated urinary concentrations of oxalic acid and rhamnulose, as evidence for an altered gut-microbiome in pulmonary TB. In a follow-up study by Luies et al. (2017), the failure of treatment of TB via standard anti-TB combination therapy was characterized by an imbalanced gut-microbiome, with the two largest predictors for a poor treatment outcome being two altered microbiome urinary markers [3,5 dihydroxybenzoic acid and 3-(4-hydroxy-3-methoxyphenyl)propionic acid]. Additionally, another independent GC-MS metabolomics longitudinal treatment study conducted on TB patient urine (Das et al., 2015) showed a treatment-dependent trend of a deregulated tyrosine–phenylalanine axis, also associated with an abnormal microbiome. Considering these urinary TB metabolomics studies, although not yet fully

understood, strong evidence exists for the association of TB disease and an altered microbiome, detectable via altered metabolite markers present in urine collected from TB patients.

Independent urinary metabolomics studies on pulmonary TB, therefore, although not related to the CNS but still involving an infectious disease distinguished by chronic inflammatory response(s), support the findings of Mason et al. (2016) in characterizing chronic neuroinflammation from TBM through urinary profiling. Herein lies the strength of untargeted metabolomics studies – the complementary evidence of three independent, open-minded analyses of metabolomics data obtained from urine on a similar analytical platform with a common, general hypothesis of the importance of the gut microbiota. For the remainder of this review, we focus on TBM and take a validated 3-marker CSF immunological signature of TBM and discuss it in conjunction with previously identified, altered urinary metabolomics markers of TBM.

2.6 Validated 3-marker CSF immunological signature of TBM

Bacteriological confirmation of TBM from CSF is not always possible, especially in children, so that diagnosis is mostly based on a combination of clinical findings, CSF analysis and radiological results (Marais et al., 2010). Since various biomarker based tests of the host have shown promise in extrapulmonary pleural-TB diagnostics, it has been thought that these same tests could also be used to diagnose TBM (Chegou et al., 2008). Recent technology has allowed for the screening for many such biomarkers, using as little as 3 μ L of CSF via Luminex multiplex cytokine-beaded arrays. With clinical application, host biomarkers could potentially be added to the current TBM diagnostic armamentarium, in order to provide an earlier and more efficient diagnosis.

A preliminary 3-marker CSF biosignature, comprising VEGF, IL-13 and cathelicidin LL-37 (cut-off values 42.92, 37.26, and 3221.01 pg/mL, respectively), correctly diagnosed childhood TBM with a sensitivity and specificity of 52 and 95%, respectively (Visser et al., 2015). The same 3-marker CSF biosignature, tested on a different cohort of 23 children, however, revealed lower sensitivity (30.4%), yet a similar specificity (91.7%), with different cut-off values. In this same cohort of 23 children with TBM and 24 controls, VEGF, IFN- γ , and MPO provided good accuracy with an AUC of 0.97, up to 91.3% sensitivity and up to 100% specificity, with cut-off values of >9.4, >99.5, and >25,823 pg/mL, respectively (Manyelo et al., 2019). Hence, VEGF, IFN γ , and MPO in combination was validated by Manyelo et al. (2019) as a 3-marker CSF immunological signature of TBM. The background behind these three markers is now described, in order to provide insights into how they led to our predictive metabolic model.

2.7 Vascular endothelial growth factor (VEGF)

VEGF, a 46 kDa glycosylated homodimeric cytokine protein, is expressed intracellularly in several cell types, including microglia (Cohen et al., 1996). It is a potent growth factor inducer of vascular endothelial cell proliferation, vascular permeability (Soker et al., 1997) and angiogenesis (Connolly, 1991; Yancopoulos et al., 2000). Endothelial changes associated with VEGF include: (1) separation of intercellular tight junction, (2) increased vesicle transport, and (3) formation of vesico-vacuolar organelles, all of which results in increased macromolecular transport over the endothelial barrier (Feng et al., 1996;

Wang et al., 2001). Classically associated with chronic inflammatory diseases, such as rheumatoid arthritis (Fava et al., 1994), VEGF is also associated with the increased permeability, and subsequent dysfunction, of the BBB (Dobrogowska et al., 1998; Proescholdt et al., 1999; Harrigan et al., 2002) and in the pathogenesis of brain edema related to ischemia, trauma, vasculitis and tumors (Van Bruggen et al., 1999; Viac et al., 1999). VEGF exhibits direct neuroprotective effects during *in vitro* ischemia (Jin et al., 2000). Another study showed that topical application of VEGF on the cerebral cortex induces a reduction of infarct size in a rat model of transient cerebral ischemia (Hayashi et al., 1998).

In 2001, Van der Flier et al. showed no detectable CSF VEGF concentrations in patients with viral meningitis (VM), whereas 30% (11/37) of those patients with bacterial meningitis (BM) displayed detectably elevated concentrations of CSF VEGF (ranging from <25 to 633 pg/mL). Furthermore, elevated VEGF has been associated with an upregulation of MMP-9 (Wang and Keiser, 1998) – see **Box 2** – which additionally contributes to BBB disruption in BM (Paul et al., 1998). Van der Flier et al. (2001) also indicated the VEGF index in BM (calculated as $[\text{VEGF}_{\text{CSF}}/\text{VEGF}_{\text{plasma}}]/[\text{albumin}_{\text{CSF}}/\text{albumin}_{\text{plasma}}]$) to be 6.2 [0.6–42], which indicates that CSF VEGF is a result of intrathecal production. This increase in CSF VEGF could be associated with: (1) a change in mental status, (2) seizures, (3) an elevated CSF WBC count (with neutrophils being the main source of VEGF), (4) elevated CSF protein and higher CSF:serum albumin ratios (marker of BBB breakdown), (5) severe BBB disruption, and, eventually, (6) death.

Within TBM, VEGF is localized in the microvessels and perivascular cells (Matsuyama et al., 2001). Tumor necrosis-alpha (TNF- α), associated with pathogenesis of TBM (Tsenova et al., 1999), is a known inducer of VEGF (Ryuto et al., 1996). In a follow-up investigation conducted by Van der Flier et al. (2004), the prevalence of elevated CSF VEGF concentrations in TBM patients was 58% (15/26) (at 98 ± 31 pg/mL) with a calculated VEGF index of 486 ± 976 , the latter once again indicative of

BOX 2 | Matrix metalloproteinases (Kolb et al., 1998; Leib et al., 2000; Shapiro et al., 2003; Lee et al., 2004).

MMPs are a large family of zinc-dependent proteolytic enzymes. Their main function involves remodeling of the connective tissues by degrading extracellular matrix molecules and are regulated by tissue inhibitors of metalloproteinases. These many compounds are subdivided according to their main substrates:

- Gelatinases: MMP-2, MMP-9.
- Collagenases: MMP-1, MMP-8, MMP-13.
- Stromelysins: MMP-3, MMP-10, MMP-11.

MMP-2 and MMP-9 digest type IV collagen and are subsequently implicated in the breakdown of the BBB via dissolution of the basement membrane underlying the endothelial cells. MMP-2 and MMP-9 production is strongly correlated with the development of neurological sequelae and induced by pro-inflammatory cytokines (IFN- γ) and other mediators (such as MPO). The amount of MMP present in CSF varies, depending on the severity of inflammation. MMP-2 and MMP-9 are detected in elevated amounts in the CSF of meningitis cases (TBM, VM and BM), with MMP-9 correlating strongly with the number of neutrophils in VM.

Table 2.2 | Summary of CSF VEGF concentrations in different types of meningitis.

	TBM	BM	VM
CSF VEGF	142.8 pg/mL [28.1–225.7] ^a 144.4 ± 75.1 pg/mL ^d 106 ± 50 pg/mL [44.9–336] ^e	14.5 pg/mL [8.7–86.5] ^a 47 ± 9 pg/mL [<10–174] ^b 37.5 pg/mL [<20–160] ^c 80.1 ± 49.5 pg/mL ^d	27.9 pg/mL [7.9–48.7] ^a 27.6 ± 26.3 pg/mL ^d

^aVisser et al. (2015). ^bVan der Flier et al. (2005). ^cCoenjaerts et al. (2004). ^dMatsuyama et al. (2001). ^eHusain et al. (2008).

intrathecal production. Van der Flier et al. furthermore associated the elevated concentrations of CSF VEGF in TBM with: (1) significantly greater mononuclear cell counts; (2) elevated CSF protein and higher CSF:serum albumin ratios; (3) not being significantly correlated with the elevated ICP, decreased CSF glucose nor with cerebral infarct on a CT scan; and (4) the inhibition explained the clinical effect of adjuvant corticosteroid therapy. In 2008, Hussain et al. similarly indicated significantly increased CSF VEGF levels (106 ± 50 pg/mL [44.9–336 pg/mL]) in TBM, accompanied by a strongly positive correlation between microvessel density and VEGF expression. Additionally, the investigation revealed that in excised tuberculomas: (1) VEGF expression was highest in regions of the granulomatous reaction; (2) no VEGF was present in the areas of caseous necrosis; (3) areas of caseation were devoid of angiogenesis; and (4) inflammatory mononuclear cells were positive for VEGF antigen (these included epithelioid cells, histiocytes and macrophages). Furthermore, immunohistochemical staining of excised tuberculoma demonstrated an elevated expression of VEGF in the granulomatous areas, with positivity in inflammatory mononuclear cells, Langhan's giant cells, as well as reactive astrocytes and fibrocytes.

Matsuyama et al. (2001) and Visser et al. (2015) both indicated CSF VEGF to be significantly increased in TBM compared with other types of meningitis (**Table 2**). Among the TBM cases, CSF VEGF was additionally significantly higher in those patients with hydrocephalus (196.3 ± 60.2 pg/mL vs. 119.8 ± 69.6 pg/mL) and there was a significant correlation with increased CSF protein and CSF total cell counts (Matsuyama et al., 2001). Visser et al. (2015) associated elevated CSF VEGF with raised hydrocephalus and CSF protein (>1 g/L), along with basal meningeal enhancement and hyperdensity in the basal cisterns on non-contrast CT scans. Lastly, Matsuyama et al. (2001) indicated that CSF VEGF localizes to microvessels and perivascular cells in TBM.

2.8 Myeloperoxidase

Myeloperoxidase (MPO), a heme enzyme (EC 1.11.1.7) and pro-inflammatory mediator present in the primary granules of polymorphonuclear leukocytes (PMNs), participates in oxygendependent microbiocidal activity of PMNs and triggers oxidative stress during acute and chronic inflammatory processes, resulting in the production of ROS. MPO can be measured in CSF as an index of inflammation (Liechti et al., 2014) and leukocyte influx (Grandgirard et al., 2012). In a review by Ray and Katyal (2016), MPO was clearly associated with the etiology of neurodegenerative disorders.

MPO is synthesized in reaction to infection (Pohanka, 2013), resulting in elevated ROS. The occurrence of oxidative stress in meningitis patients is well-described in the literature (Koedel and Pfister, 1999; Ray et al., 2000; Tsukahara et al., 2000; Christen et al., 2001; Kastenbauer et al., 2002; Klein et al., 2006; Hamed et al., 2009; Loro, 2009; Koedel et al., 2010; Miric et al., 2010; Barichello et al., 2011). Furthermore, significant increases in MPO activity have been shown in BM-induced rats (Giridharan et al., 2017), particularly within the hippocampus and frontal cortex (Barichello et al., 2011, 2014). In a study of 59 pediatric BM cases, Miric et al. (2010) showed no significant correlation between MPO and neutrophil count in CSF; however, CSF MPO activity did correlate with various lipid peroxidation products. Additionally, H_2O_2 levels in CSF were associated with elevated BBB permeability, CSF albumin concentrations, and serum H_2O_2 concentrations. Lastly, it is important to note that MPO reacts with cell matrix metalloproteinases (MMPs – see **Box 2**), or their tissue inhibitors, and this is thought to contribute to the BBB dysfunction seen in such cases.

Borelli et al. (1999) proved that purified MPO, in the presence of H_2O_2 , exerts a consistent killing effect on *Mtb*, and that the MPO activity is both time and dose dependent; it also requires chloride ions for efficacy. This $MPO-H_2O_2-Cl_2$ system produces hypochlorous acid (HOCl) via activated leukocytes (Klebanoff, 2005), which in turn serves as a strong, non-radical oxidant of a wide range of biological compounds, although it is more selective than hydroxyl radicals (Hampton et al., 1998), with the following characteristics: (1) it has a preferred substrate selectivity toward thiols and thioethers, (2) an ability to convert amines to chloramines, (3) promotes chlorination of phenols and unsaturated bonds, (4) oxidizes iron centers, (5) crosslinks proteins, and (6) is membrane permeable. HOCl has also been characterized as covalently modifying lipids and/or proteins, resulting in local tissue damage and amplification of the inflammatory cascade. Furthermore, HOCl, in the presence of nitrite (NO_2^-) formed by stimulated PMNs, forms 3-chlorotyrosine (3Cl-Tyr), and to a lesser degree, 3-nitrotyrosine (3NO₂-Tyr) and N-chlorotaurine (Eiserich et al., 1998). The 3Cl-Tyr is considered a specific marker of MPO-catalyzed oxidation (Hazen and Heinecke, 1997), with GC-MS being the preferred method for quantifying it (Hazen et al., 1997; Winterbourn and Kettle, 2000). Other biomarkers of MPO derived HOCl include: chlorohydrins, protein carbonyls, antiHOP (hypochlorous acid-oxidized protein), antibodies, 5-chlorocytosine, and glutathione sulfonamide. Each with their advantages and disadvantages is described by Winterbourn and Kettle (2000). Based on the analyses of CSF collected from 79 confirmed pediatric BM cases, Rugemalira et al. (2019) indicated that elevated ratios of 3Cl-Tyr:para-tyrosine serves as a marker for MPO activation in CSF in pediatric BM cases, and potentially also for grading the severity of neuroinflammation. Furthermore, Rugemalira et al. (2019) also proved that 3NO₂-Tyr can be used as a biomarker for peroxynitrite formation and is associated with an unfavorable outcome of BM. In a study of 59 children

with confirmed BM (Miric et al., 2010), CSF MPO activity, although relatively low, was significantly increased at baseline compared to controls ($n = 23$), increasing even further by day 5 of treatment. It was concluded that MPO may be involved in the oxidative stress associated with BM, as well as potentially contributing to BBB disruption. Marais et al. (2016) indicated a significant increase in neutrophil-dependent inflammatory response biomarkers, including MPO, in adult TBM and HIV co-infection patients with paradoxical immune reconstitution inflammatory syndrome. Lastly, Üllen et al. (2013) indicated that BBB dysfunction associated with neuroinflammation caused by MPO can be partially reversed by using para-aminobenzoic acid (PABA) hydrazide, first shown by Forghani et al. (2012) to effectively treat multiple sclerosis in mice. PABA (or vitamin Bx) is non-essential for humans, but exhibits anti-fibrotic properties. Fibrosis in the brain occurs via the proliferation or hypertrophy of glial cells, such as microglia – microgliosis, during neurotrauma caused by infection. Subsequently, PABA may later be considered for its use as a possible adjunctive therapeutic agent in TBM, since the inhibition of MPO has been posited to be a valuable therapeutic approach to reduce oxidative-stress-mediated damage in neurodegenerative diseases (Green et al., 2004).

2.9 Interferon- γ

Interferon- γ (IFN- γ) is predominantly produced by CD4⁺ T cells and functions by activating microglia, thereby stimulating lymphocyte Th1 differentiation (Farrar and Schreiber, 1993) and antimicrobial activity of the microglia (Mastroianni et al., 1997), after infection. A plethora of literature studies report the performance of IFN- γ release assays (IGRAs) for diagnosing TB under different conditions. These studies are comprehensively covered by systematic reviews and meta-analyses and include applications to diagnosing: (1) latent Mtb infection (53 studies: Diel et al., 2011); (2) latent Mtb infection in rheumatic patients (11 studies: Ruan et al., 2016); (3) latent TB in patients with autoimmune diseases under immunosuppressive therapy (17 studies: Wong et al., 2016); (4) active TB (27 studies: Sester et al., 2011); (5) active TB among HIV-seropositive individuals (11 studies: Huo and Peng, 2016); (6) active TB in immunocompetent children (15 studies: Laurenti et al., 2016), immunodiagnosis of TB (75 studies: Pai et al., 2004); (7) active and latent TB in HIV-positive populations (32 studies; Overton et al., 2018); and (8) extra-pulmonary TB (22 studies: Zhou et al., 2015). Similarly, several studies (**Table 3**) using IGRAs have also been performed using CSF as a possible sample matrix for diagnosing TBM, with the two main commercially used IGRAs tested being T-SPOT.TB and QuantiFERON-TB. IGRAs function by measuring the release of IFN- γ from T cells, after *in vitro* stimulation with Mtb antigens, such as early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP10); they are influenced by (1) the antigenic load, (2) host responsiveness to antigens, and (3) host–pathogen interactions (Lu et al., 2017).

Consolidating from the literature, the CSF studies on IGRAs as a diagnostic tool for TBM (**Table 3**), a weighted average of the diagnostic performance of IGRAs (pooled from 326 TBM cases) was calculated to give an overall average sensitivity and specificity of 65 and 87%, respectively – insufficient for application as a stand-alone diagnostic tool. On similar data, a meta-analysis of 6 studies from the literature, all using IGRAs conducted on CSF, showed a pooled (156 cases) sensitivity of 77% (69–84%) and specificity of 88% (74–95%) for TBM diagnostic applications (Yu et al., 2016). Furthermore, IGRAs

require 3–7 mL of CSF, a volume often unobtainable, especially from children and infants. Moreover, the measure of sensitivity and specificity is dependent upon a pre-defined cut-off point which is currently not yet standardized.

The use of IGRAs for the differential diagnosis of meningitis has, however, yielded a practical outcome. Chonmaitree and Baron (1991) analyzed CSF from 16 VM and 41 BM cases and determined that elevated concentrations of IFN- γ were present in 75 and 24% of these patient groups, respectively. A review of the literature (1964–1991) by Chonmaitree and Baron (1991) revealed a similar trend, showing elevated concentrations of IFN- γ in 68% (133/196) of all VM patients (based on 11 studies), whereas in patients with BM, only 28% (59/189) showed elevated IFN- γ in the pooled population (8 studies used). Hence, patients with VM exhibit higher IFN- γ levels than those with BM. Based upon quantified data in 50 patients with VM, using a radioimmunoassay, Minamishima et al. (1991) determined CSF IFN- γ to be on average 9.8 ± 7.5 UI/mL. Minamishima et al. additionally suggested that IFN- γ produced in the inflamed intrathecal space may be associated with the pathogenesis of the disease, and associated the elevated CSF IFN- γ levels with (1) CSF protein concentrations, (2) total cell counts, and (3) number of febrile episodes. San Juan et al. (2006), also using a radioimmunoassay on CSF collected from patients, calculated a mean IFN- γ for definite ($n = 12$) and probable ($n = 8$) TBM patients to be 28.7 ± 8.2 and 10.6 ± 2.8 UI/L, respectively. However, Ohga et al. (1994) showed only 3 out of the 13 BM patients investigated, and Kornelisse et al. (1997) only 20 of 35 BM patients investigated, to have CSF IFN- γ elevated to concentrations above the detection limit of 10 pg/mL. In an analysis of 30 TBM patients, Lu et al. (2016) determined, via ELISA, a mean CSF IFN- γ value for patients with TBM to be 350.97 ± 372.94 pg/mL. Lu et al. also determined that in 10 of these TBM patients the average CSF IFN- γ levels were 500.48 pg/mL before treatment and 103.62 pg/mL following 4 weeks of treatment, indicating that while IFN- γ decreased significantly (5-fold), it still remained elevated compared to the norm, after 4 weeks of treatment (that is, inflammation in the brain persisted). Mansour et al. (2005) reported a highly elevated mean concentration of CSF IFN γ (794 ± 530 pg/mL) in 39 patients with TBM (all of whom were HIV negative) prior to receiving medication, which was correlated with markers of neuroinflammation in these individuals. Mansour et al. (2005) also showed that the CSF IFN- γ remained elevated for many weeks after treatment was begun in patients with TBM, whereas in those cases diagnosed with VM and BM the CSF IFN- γ returned to undetectable concentrations within a couple of days post-treatment.

Table 2.3 | Performance of IGRAs on CSF from TBM cases as a stand-alone diagnostic tool.

References	IGRA	TBM cases (n)	Sensitivity % (range)	Specificity % (range)
Pan et al. (2017)	T-SPOT.TB	53	61 (40–92)	97 (75–100)
Lu et al. (2017)	T-SPOT.TB	61	62 (49–74)	73 (62–82)
Qin et al. (2015)	T-SPOT.TB	12	92 (62–100)	93 (76–99)
Park et al. (2012)	T-SPOT.TB	25	72 (51–88)	79 (66–89)
Kim et al. (2010)	T-SPOT.TB	31	71 (51–86)	89 (72–98)
Patel et al. (2010)	T-SPOT.TB	38	58 (41–74)	94 (83–99)
Thomas et al. (2008)	T-SPOT.TB	10	90 (56–100)	100 (59–100)
Caliman-Sturdza et al. (2015)	QuantiFERON-TB	63	84	98
Vidhate et al. (2011)	QuantiFERON-TB	36	13 (2–40)	63 (35–85)
Weighted average diagnostic performance of IGRAs		329	65	87

Considering all of the above, patients with VM and TBM exhibit a similar increase in CSF IFN- γ levels, both far greater than in patients with BM. This suggests that CSF IFN- γ could potentially be used as a differential diagnostic marker for the exclusion of BM. Furthermore, CSF IFN- γ levels in TBM cases remain elevated for weeks following treatment, differentiating TBM from VM. However, as described previously, in order to acquire a definitive TBM diagnosis additional measures of CSF parameters are needed.

In summary, the overall trend across all CSF VEGF studies is a significantly higher concentration of VEGF in TBM patients than in other cases of meningitis. Of further note, Van der Flier et al. (2004) reports significantly increased CSF VEGF (178 ± 52 pg/mL) in TBM patients with nausea and vomiting, indicating that elevated CSF VEGF has a potential direct impact on the BGA, leading to a perturbed gut. CSF IFN- γ levels show a similar increase in TBM and VM but less so in BM. Hence, CSF IFN- γ levels could potentially be used for the exclusion of the diagnosis of BM. The HOCl produced by the MPO–H₂O₂–Cl₂ system yields similar oxidative markers in both TBM and BM. The addition of VEGF and MPO with IFN- γ , as part of a 3marker immunological biosignature of TBM in CSF (Manyelo et al., 2019), has yielded a diagnostic measure with an AUC of 0.97, and a sensitivity and specificity of up to 91.3% and up to 100%, respectively. Hence, this 3-marker biosignature yields excellent results for diagnosis of TBM from a CSF sample. But, what of the urinary metabolomics profile? If these three immunological markers are present in the CSF of a TBM patient, then a downstream metabolic effect, based upon the BGA, should be reflected in the urine. This concept is explored in our proposed predictive metabolic model that follows.

2.10 Proposed predictive metabolic model of TBM in the brain based upon IFN- γ , MPO and VEGF

Given the background of IFN- γ , MPO, and VEGF described above, and the associated metabolic pathways of these signaling compounds, we propose a predictive metabolic model for TBM in the brain based upon previously published biochemistry fundamentals. This model, illustrated in **Figure 1**, shows the interaction of the overlapping metabolic cascades initiated by TBM, and its associated 3-marker CSF immunological signature.

Our predictive metabolic model shows how increased levels of VEGF result in a persistent metabolic burst caused by the induction of angiogenesis (Stapor et al., 2014; Treps et al., 2016), whereby glycolysis, and the release of glycogen from astrocyte stores to fuel glycolysis, is increased significantly. Secondary pathways that are subsequently upregulated include: (1) the pentose phosphate pathway, that contributes to an elevated synthesis of glutathione (Ben-Yoseph et al., 1996), elevated xylulose-5-phosphate (also via phosphoenolpyruvate in the glycolysis pathway) to fuel tryptophan catabolism (Stephanopoulos and Simpson, 1997; Simpson et al., 1999; Maria et al., 2018), and elevated purine and pyrimidine synthesis (Zimmer, 1988, 1996); (2) the hexosamine pathway, which contributes to increased O- and N-protein glycosylation, imperative for the host's immune response since glycosylation controls cell migration, host defense, and antigenicity (Varki, 1993); (3) increased β -oxidation providing substrate in the form of diacylglycerol from downstream catabolism of dihydroxyacetone phosphate and activation of protein kinase C from VEGF (Takahashi et al., 1999; Harhaj et al., 2006), ultimately yielding increased acetyl-CoA; and (4) the boosted mitochondrial citric acid (TCA) cycle, due to the increased acetyl-CoA. The elevated TCA intermediate α -ketoglutarate (α -KG), previously indicated to be a urinary marker of TBM (Mason et al., 2016), contributes to glutamate synthesis and downstream glutathione (GSH) production, the latter being a needed antioxidant, synthesized in response to the elevated MPO.

Increased levels of IFN- γ , stimulated by Mtb-induced antigens (Blumenthal et al., 2012; Lu et al., 2017), specifically upregulate indoleamine dioxygenase (Yoshida et al., 1981; Taylor and Feng, 1991; Hashioka et al., 2017), the initial enzyme in the tryptophan catabolic pathway. A massive burst in tryptophan catabolism results in astrocyte-based kynurenic acid and microglia-based quinolinic acid synthesis – also previously identified urinary markers of TBM (Mason et al., 2016). Several enzymes within the tryptophan metabolic pathway require pyridoxal-5phosphate (P-5-P), an active form of vitamin B₆, as a cofactor.

Deficiency of P-5-P diverts tryptophan metabolism from production of NAD to the excessive formation of xanthurenic acid (Oxenkrug, 2013), and subsequently apoptosis. One of the mechanisms of insulin resistance is inflammation-induced upregulation of tryptophan metabolism in combination with P-5-P-deficiency-induced diversion of tryptophan metabolism leading to formation of xanthurenic acid and other kynurenine derivatives that affect insulin activity (Oxenkrug, 2013). It has been shown that reduced insulin levels may lead to uncontrolled glucose metabolism – previously described in individuals with diabetes (American Diabetes Association, 2013), pulmonary TB (Preez et al., 2017) and, recently, in runners after a marathon (Stander et al., 2018), all of which are associated with a severe inflammatory response. As depicted in our model, uncontrolled glucose metabolism can result in glucose being oxidized via glucose oxidase to produce gluconolactone, in addition to hydrogen peroxide, previously reported to occur in both diabetes and pulmonary TB

(Preez et al., 2017). The gluconolactone subsequently becomes siphoned into the pentose phosphate pathway via hydrolysis to form gluconic acid and phosphorylation with ATP to produce 6-phosphogluconate lactone (Dickens and Glock, 1951; Rohatgi et al., 2014). The consequential elevated H_2O_2 , and its interaction with raised MPO, leads to the activation of various oxidative stress pathways (Hampton et al., 1998; Podrez et al., 2000; Klebanoff, 2005), as depicted in **Figure 1**, and described above. The two final urinary markers of elevated MPO and of HOCl, via the $MPO-H_2O_2-Cl_2$ system, are predicted to be glutathione sulfonamide and 3-chlorotyrosine (Winterbourn and Kettle, 2000).

Furthermore, the burst from both the glycolysis (via pyruvate) and tryptophan catabolism (via hydration of kynurenine to form alanine; Kotake and Nakayama, 1941) pathways yields increasingly elevated levels of extracellular lactate. This lactate pool is essential in the lately proposed astrocyte–microglia lactate shuttle (AMLS, Mason et al., 2015), since lactate plays a dual role of being a preferred source of energy in the brain during TBM as well as also being neuroprotective (Mason, 2017), diverting lactate away from neurons (thereby deactivating neurons to protect them) toward activated microglia in an attempt to eradicate the immediate insult/infection. This increase in lactate in TBM is predicted to be localized in the brain for immediate use and is not expected to be present in elevated amounts in the urine of TBM patients.

The predictive metabolic model presented here, although speculative, is based upon three validated immunological markers of TBM. The subsequent activated metabolic pathways in the brain are based upon biochemistry fundamentals and supported by the literature, as discussed. The end-product metabolites that act as metabolic markers of TBM are expected to cross the BBB and travel in the blood circulation and interact with the gut. The principal limitation of our model is that, in its current form, it can predict only the endproduct metabolites from the TBM-infected brain, with the assumption that no other systemic co-infection is present. The complex interactions with the gut microbiota are poorly understood and require further research. However, based upon previous urinary metabolomics studies reported in this review, experimental evidence is emerging that points toward an altered gut metabolism. Hence, changes in gut metabolism became the fourth component of our proposed urinary metabolic profile of a TBM patient. The specifics of the complex relationship between host and gut-microbiome and the details of the altered metabolic profile of the gut under pathophysiological states remain a hot topic.

2.11 Conclusion

The significance of this review is that it takes a newly established paradigm within the neurosciences – the BGA – and critically examines the literature from a relatively unexplored niche perspective – chronic neuroinflammation caused by CNS bacterial infection(s) of the brain, using TBM as an example. We posit that if the BGA exists then chronic neuroinflammation within the brain caused by pathogenic bacteria (*Mtb*) will influence the gut microbiota, and the ideal biofluid to analyze this, reflecting the associated systemic changes, is urine. We support our postulate with data from published studies on urinary metabolomics, as follows.

First, the strength of untargeted urinary metabolomics is clearly demonstrated in the literature. A previous untargeted urinary metabolomics study conducted on TBM cases, by Mason et al. (2016), yielded data that, when analyzed in a non-biased, holistic manner, resulted in a putative urinary metabolic signature characterizing TBM that was interpreted in a hypothesis-generating perspective. Independently, using similar analytical platforms in metabolomics, Das et al. (2015) and Luies and Loots (2016) examined urinary metabolomics profiles of pulmonary TB patients, and came to similar conclusions – the most significant of which, in the context of this review, were that infection by *Mtb* results in an altered gut-microbiome and this is substantiated by altered microbiome markers in the urine of these patients.

Second, we take an independent, and initially unrelated, study that closely examined the immunological profile of TBM, in which three specific immunological markers in the CSF associated with neuroinflammation – VEGF, IFN- γ , and MPO – were validated as diagnostic markers of TBM. We explored the background behind this 3-marker CSF immunological signature of TBM, in the context of its influence on the gut-microbiome and the subsequently altered urinary metabolome, using previously discovered urinary metabolites in TBM patients as proof (such as α -KG, and the tryptophan catabolites 3-hydroxykynurenine acid and quinolinic acid) (Mason et al., 2016). By extension, we also predict other metabolic pathways that would be expected to be changed within our model.

Third, we combined the sciences of immunology and metabolomics to create a novel integrated predictive metabolic model of TBM in the brain. By integrating relevant information from systems biology, our predictive cascading metabolic model should be adjustable to account for other types of bacterial infection(s) of the CNS that cause chronic neuroinflammation, such as neurosyphilis, bacterial brain abscesses and Lyme disease, as well as chronic non-bacterial CNS infections that are common in resource-constrained settings of poor communities, and sometimes difficult to distinguish from TBM, such as cerebral malaria and cryptococcal meningitis. Being so identified, based upon the literature, patients with VM and TBM exhibit a similar increase in CSF IFN- γ levels, both far greater than in patients with BM. Hence, a predictive metabolic model of cerebral malaria and cryptococcal meningitis would likely exclude CSF based IFN- γ and its subsequent downstream cascading metabolic influence – that is, no downstream tryptophan metabolic catabolites. What remains to be done is to identify the unique immunological markers associated with these other bacterial infection(s) of the CNS and predict and confirm their associated downstream metabolic markers that should be reflected in urine, which could be used diagnostically or to characterize these diseases better.

In short, analysis of urinary metabolic profiles offers a wealth of metabolic information that can be traced back to an altered gut microbiome, and to an inherently changed BGA, induced by chronic

neuroinflammation from bacterial infection(s) of the CNS. This metabolic information from urine holds within it the potential to contribute to improved and early differential diagnosis of bacterial infection(s) in the CNS – a quicker and less invasive method of diagnosis than currently available. The review presented here provides support that, by taking existing validated immunological markers of infectious diseases in conjunction with metabolomics data and biochemistry fundamentals, it is possible to predict downstream metabolic products, most likely detectable via urinary metabolic profiling methods.

Author contributions

SM conceptualized the manuscript. SI, SM, and DL planned the outline of the manuscript. SI wrote the manuscript. SM and DL supervised SI in the writing of the manuscript by providing critical feedback. RS provided clinical input and critically read the manuscript. All co-authors read and approved the final draft for submission.

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**PART 2 URINARY METABOLIC CHARACTERIZATION OF ADVANCED
TUBERCULOUS MENINGITIS CASES IN A SOUTH AFRICAN
PAEDIATRIC POPULATION**

CHAPTER 3

Urinary metabolic characterization of advanced tuberculous meningitis cases in a South African paediatric population



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3.1 Abstract

Tuberculous meningitis (TBM) is a severe form of tuberculosis with high Neuro-morbidity and mortality, especially among the paediatric population (aged ≤ 12 years). Little is known of the associated metabolic changes. This study aimed to identify characteristic metabolic markers that differentiate severe cases of paediatric TBM from controls, through non-invasive urine collection. Urine samples selected for this study were from two paediatric groups. Group 1: controls ($n = 44$): children without meningitis, no neurological symptoms and from the same geographical region as group 2. Group 2: TBM cases ($n = 13$): collected from paediatric patients that were admitted to Tygerberg Hospital in South Africa on the suspicion of TBM, mostly severely ill; with a later confirmation of TBM. Untargeted ¹H NMR based metabolomics data of urine were generated, followed by statistical analyses via MetaboAnalyst (v5.0), and the identification of important metabolites. Twenty-nine urinary metabolites were identified as characteristic of advanced TBM and categorized in terms of six dysregulated metabolic pathways: 1) upregulated tryptophan catabolism linked to an altered vitamin B metabolism; 2) perturbation of amino acid metabolism; 3) increased energy production—metabolic burst; 4) disrupted gut microbiota metabolism; 5) ketoacidosis; 6) increased nitrogen excretion. We also provide original biological insights into this biosignature of urinary metabolites that can be used to characterize paediatric TBM patients in a South African cohort.

KEYWORDS

urine, metabolic, paediatric, tuberculous meningitis (TBM), untargeted metabolomics, Proton nuclear magnetic resonance (¹H NMR) spectroscopy

3.2 Introduction

The most lethal form of extra-pulmonary tuberculosis (TB)– tuberculous meningitis (TBM)–affects 1%–5% of TB infected individuals globally (Donovan et al., 2020). The pediatric age group (aged ≤12 years) are the most at risk for contracting the disease, and account for 12% of all TBM cases, with one in five affected children dying and only one in three surviving without long-term neurological sequelae (WHO, 2020; Basu Roy et al., 2021). According to the World Health Organization, South Africa is among the eight countries that account for two-thirds of the global total TB burden (WHO, 2020). In the Western Cape province of South Africa, TBM is the most common form of paediatric meningitis detected (Donald et al., 1996; Wolzak et al., 2012). Early diagnosis and timely introduction of appropriate therapy can potentiate a positive treatment outcome (Donovan et al., 2020). However, the timely and accurate diagnosis of TBM is challenging, since the early symptoms are usually nonspecific (van Toorn and Solomons, 2014). There is a pressing need to improve current TBM diagnostic strategies (Hasbun et al., 2018). Stand-alone methods currently used to diagnose TBM in children are unreliable (Manyelo et al., 2021). Existing diagnostic techniques for TBM are invasive, complex and time-consuming, subsequently delaying treatment, and putting patients at a high risk of mortality. Recently, efforts have been made to better understand the pathophysiology through new research focused on finding novel TBM biomarker signatures (Rohlwink et al., 2017; Manyelo et al., 2019). Yet, there are still no clear biomarker(s), nor biosignature(s), for TBM in the cerebrospinal fluid (CSF), let alone one from patient samples collected non-invasively, such as from urine. Urine is rich in metabolic information that describes the systematic state of an individual and is a relatively unexplored biofluid in TBM research. Urinary metabolomics profiling in pulmonary TB patients has been an efficient means of diagnosing and monitoring an individual's response to treatment (Luies et al., 2017). Mason *et al.* (2016b) holistically illustrated the metabolic complexity of TBM and provided proof-of-concept that a putative biosignature of urinary metabolites (methylcitric, 2-ketoglutaric, quinolinic and 4-hydroxyhippuric acids) can be defined with the potential to be used for the non-invasive metabolomics diagnosis and prognosis of paediatric TBM patients. In another metabolomics study, Chatterji *et al.* (2016) observed reduced malonic acid and elevated 2-hydroxybutyric acid, acetic acid, creatine and glycerophosphocholine, in the urine of TBM adults. These pioneering metabolomics studies have provided proof-of-concept that urine provides a wealth of metabolic information in TBM cases and deserves further investigation.

Metabolomics provides analytical, chemical and physiological insights into metabolite interactions (Mason et al., 2016a). These metabolites are vital constituents of biological systems and are highly informative of their functional state, serving as biomarkers of disease and also reflective of therapeutic response (Goodacre, 2010). van der Greef *et al.* (2004) describe metabolomics as a prospective tool for clinical use in the early detection of a metabolic perturbation in a biological system, before the disease symptoms actually present themselves. Nuclear magnetic resonance (NMR) spectroscopy is one such technique commonly used for the metabolite profiling and analysis of complex biofluids (NaganaGowda et al., 2008). Studying the molecular level shift in equilibrium in patient urine will improve the systematic understanding of TBM and may also help develop transformable solutions related to novel diagnostics

(Blankley et al., 2014). The need for research, such as that reported here, in order to generate knowledge which can be used to develop timely and accurate diagnosis, improve treatment outcomes, and ultimately reduce the dire mortality and neuro-morbidity in paediatric TBM disease in children (and adults), has become a priority (Organization, 2018). Hence, this study aimed to identify metabolites in urine that characterize the metabolic profile of severe TBM in paediatric cases, using ^1H NMR metabolomics. Characterization here is defined as explaining the biochemistry underpinning TBM, in terms of altered metabolic pathways and increased/decreased metabolites.

3.3 Materials and methods

3.3.1 Sample origin and selection criteria

The sample population used in this study were infants and children (aged ≤ 12 years) from the Western Cape Province of South Africa, an area with high prevalence of TB (681 per 100,000), especially among children ≤ 12 years of age (100 per 966) (Donald et al., 1996; KANABUS, 2020). Participants were referred from primary and secondary level healthcare facilities to the paediatric department at Tygerberg Hospital, Stellenbosch University. The participants were divided into two groups (Group 1: control; $n = 44$) and (Group 2: TBM; $n = 13$). Criteria for collection of the controls (Group 1) were paediatric patients without meningitis, no neurological symptoms, and from the same geographical region as the TBM patients. Urine samples from the control group were requested from children undergoing routine follow up at Paediatric Outpatient Clinics, none of whom were acutely ill during sampling. The TBM urine samples (Group 2) used in this study were collected from pediatric patients (van Elsland et al., 2018) under initial treatment that were admitted to the hospital on suspicion of TBM, most of them severely sick; with a later confirmation of “definite TBM” according to the uniform research case definition for TBM (i.e., *M. tuberculosis* (*M. tb*) identified on CSF by microscopy, culture and/or detection by commercial nucleic-acid amplification test (Marais et al., 2010)) at an advanced stage (with or without focal neurological deficit) (Van Toorn et al., 2012). The urine sample used in this study was the first urine sample collected as an out-patient (discharged from hospital once stabilized) as a home-treatment program (van Elsland et al., 2018). Clinical information was recorded for all TBM cases (see Table 1). As per ethics requirements, all urine samples were collected after written informed consent from parent(s) and assent from the child, if older than 7 years and able to do so, was obtained, under the Health Research Ethics Committee (HREC) approval of Stellenbosch University (ethics approval no. N16/11/142 and N11/03/061 for group 1 and 2 respectively), the Western Cape provincial government, as well as by the HREC of the North-West University, Potchefstroom campus (ethics approval no. NWU-00063-18-A1-01). Participants with an unknown or positive HIV status were excluded from this study because HIV co-infection further confounds an already complex metabolic profile.

Table 3.1 | Demographic, clinical, laboratory and imaging findings of 13 paediatric patients diagnosed with advanced tuberculous meningitis.

CRITERIA	n (%)
Gender: male/female	6 (46.2)/7 (53.8) ^a
Age (months) (Median [range])	43 [22–140]
Clinical symptoms	
Fever	11/13 (84.6)
Night sweats	2/13 (15.4)
Poor feeding	5/13 (38.5)
Weight loss	5/13 (38.5)
Vomiting	4/13 (30.8)
Coughing	None
Headache	1/13 (7.7)
Seizures	4/13 (30.8)
Lethargy	2/13 (15.4)
Neurological signs	
GCS: (median [range])	10 [7–15]
Meningism	3/13 (23.1)
Focal motor deficit	4/13 (30.8)
Cranial nerve palsy	3/13 (23.1)
Raised ICP	4/13 (30.8)
Neuroimaging (CT brain)	
Hydrocephalus	11/13 (84.6)
Infarctions	1/13 (7.7)
Tuberculoma	2/13 (15.4)
Meningeal enhancement	7/13 (53.8)
VP shunt	4/13 (30.8)
Convulsions	1/13 (7.7)
Hemiparesis	4/13 (30.8)
CXR (signs of pulmonary TB)	1/13 (7.7)
Laboratory values	
Blood albumin (g/L) (median [range])	41 [40–52]
Blood sodium (mmol/L) (median [range])	131.5 [120–141]
Blood total protein (g/L) (median [range])	77.5 [75–80]
Blood glucose (mmol/L) (median [range])	7.1 [4.9–7.7]
Blood lipids (median)	3.9
CSF protein (g/L) (median [range])	0.84 [0.21–3.0]
CSF glucose (mmol/L) (median [range])	2.2 [0.2–4.8]
CSF pleocytosis (cells/ μ L) (median [range])	40 [3–129]
CSF lymphocytes (cells/ μ L) (median [range])	57 [3–148]
BCG positive status	3/13 (23.1)

^a= no significant difference; ICP = intracranial pressure; VP = ventriculoperitoneal; BCG = *Bacillus Calmette–Guérin* vaccine; GCS = glasgow coma scale; CSF = cerebrospinal fluid; CXR = chest X-ray.

3.3.2 Sample transport, storage and handling

Upon collection, urine samples were stored at -80°C in a dedicated freezer at the division of Molecular Biology and Human Genetics at Stellenbosch University. Once all samples were collected, they were collectively couriered overnight–frozen, on dry ice, to a dedicated freezer (-80°C) in a biosafety level 3 (BSL3) laboratory at the Centre for Human Metabolomics, situated on the Potchefstroom campus of North-West University. All urine samples were thawed in a biological safety cabinet, after which 600 μL of each sample was aliquoted into a separate tube for NMR analysis and an additional 50 μL for the purpose of making a pooled quality control (QC) sample. The pooled QC sample was then vortexed and re- aliquoted into 20 equal-volume quantities. All aliquoted samples (including QC samples) were kept at -80°C until NMR analysis.

3.3.3 Sample preparation and ^1H NMR analysis

All urine samples were thawed to room temperature prior to processing. A volume of 600 μL of urine was centrifuged at 12,000 g for 5 min to sediment any particulates and macromolecules. A volume of 540 μL of the supernatant was collected in a micro- centrifuge tube, with 60 μL of the NMR buffer solution [1.5 M potassium phosphate solution in deuterium oxide with internal standard TSP (trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid); pH 7.4]. The sample was briefly vortexed to ensure homogeneity before being centrifuged at 12,000 g for 5 min. For ^1H NMR analysis, 540 μL of the supernatant was transferred to a 5 mm NMR glass tube and analyzed using a Bruker Avance III HD NMR spectrometer with a triple-resonance inverse (TXI) ^1H (^{15}N , ^{13}C) probe head and x, y, and z gradient coils, at 500 MHz, in a randomized sequence, with QC samples interweaved at regular intervals. With a spectral width of 12,000 Hz, ^1H spectra were recorded as 128 transients in 32 K data points. The sample temperature was kept constant at 300 K, and the H_2O resonance was pre-saturated using single-frequency irradiation with a 4-s relaxation delay and an 8- μs excitation pulse, using the noesygppr1d water presaturation pulse program. Sample shimming was performed automatically based on the deuterium signal. TSP and metabolites had resonance line widths of <1 Hz. Fourier transformation, phase and baseline correction were performed automatically. Bruker Topspin (V3.5) was used to process the NMR data. For metabolite identification and quantification, Bruker AMIX (V3.9.14) was employed (Erasmus et al., 2019).

3.3.4 Data pre-processing and statistical analysis

The ^1H NMR spectral output was binned at 0.02 ppm widths, relative to creatinine, to create a data matrix of spectral intensity with the bins as the columns and samples as the rows. A t-test on the peak integral region of the creatinine peak between the control and TBM group revealed a p -value of 0.065. The noise level was identified as less than the limit of detection (LOD), calculated as: $\text{LOD} = \text{average of blank bins (bins with no discernible peaks)} + 3.3 * \text{standard deviation of blank bins}$ (Westgard, 2008), and bins identified as noise were zeroed. All zeroes were replaced with $1/5$ of the lowest value using MetaboAnalyst (V5.0). Bins around the suppressed water peak ~ 4.72 ppm were also removed. The final data matrix gave a total of 468 bins. Throughout the batch analysis, 20 aliquots of a pooled QC sample were processed at predefined intervals to ensure trustworthy data. Quality assurance was based

on QC observations, with bins with a coefficient of variation (CV) value greater than 30% were removed from the spectral binned data matrix. Because spectral intensity does not always reflect biological significance, data were log transformed and Pareto scaled to account for skewed distributions and to put bins on an even footing when presented for multivariate analysis. Statistical analyses and identification of important metabolites were conducted using MetaboAnalyst (V5.0)—a comprehensive platform dedicated for metabolomics data analysis via a web-based interface that enables high-throughput analysis for both targeted and untargeted metabolomics (Pang et al., 2021); included univariate statistics (fold changes and t-tests, displayed using a volcano plot) and multivariate statistics, specifically principal component analysis (PCA), partial least squares–discriminant analysis (PLS-DA). To project the data onto fewer, more manageable dimensions and to highlight entirely data-driven connections between instances, unsupervised PCA analyses were used with a 95% confidence interval (CI) ellipsis to detect natural separation between group centroid, to remove outliers in the two experimental groups, and to examine QC distribution to assess overall method reliability. Hierarchical cluster analysis was also performed, based on Euclidean distance using Ward’s linkage method. Quantitative statistical data were used to identify variables of importance: PLS-DA VIP of >1.0 for components 1 and 2, a t-test p -value ≤ 0.05 , corrected for multiple testing (FDR, false discovery rate), and a fold change ≥ 2.0 . Discriminatory metabolites were identified using pure compound 1D ^1H NMR spectral libraries and confirmed using 2D correlation spectroscopy (COSY) and J-resolved (JRES) ^1H – ^1H NMR data. Important identified metabolites were quantified relative to the creatinine peak ($\mu\text{mol}/\text{mmol}$ creatinine) and additional univariate measures, including t-test p -values (adjusted using Bonferroni–Holm) and Cohen’s d -values, were calculated. Statistical significance ($p \leq 0.05$) was used to generalize findings, that is, what is the probability that we won’t find a difference if we take another sample? On the other hand, practical significance ($d > 0.6$) indicates magnitude of difference and answers the “so what” question, namely, whether the effect is large enough to care about. A summary of the experimental design for this study is illustrated schematically in Figure 1. The use of online metabolite databases, such as the Human Metabolome Database (HMDB) and the Kyoto Encyclopedia of Genes and Genomes (KEGG), were used to assist in the biological interpretation of the results.

3.4 Results

Prior to statistical analysis, a quantitative quality assurance check was performed on all QC samples. A total of 24 bins across 20 QC samples had a CV greater than 30% and were removed from the spectral binned data matrix. This reduced the number of bins from 468 to 444. Hence, the variation exhibited across bins between the two groups can be attributed towards biological variation. A PCA of all cases, including the QC samples (Figure 2A), shows that the 20 QC samples cluster closely together, indicating overall method reliability (i.e., no batch or machine drift). An unsupervised PCA, excluding QC samples, was performed Supplementary Figure S1 and four outliers were identified in the

control group and removed from further analysis; no outliers identified in the TBM group. Next, an unsupervised PCA analysis of the 40 controls against the 13 TBM cases was performed (Figure 2B), which yielded almost complete natural separation between the groups, with their 95% CI ellipses slightly overlapping as a result of two control samples. The results of the unsupervised PCA confirm that the two groups are indeed differentiated, providing confidence before using the supervised method of PLS-DA.

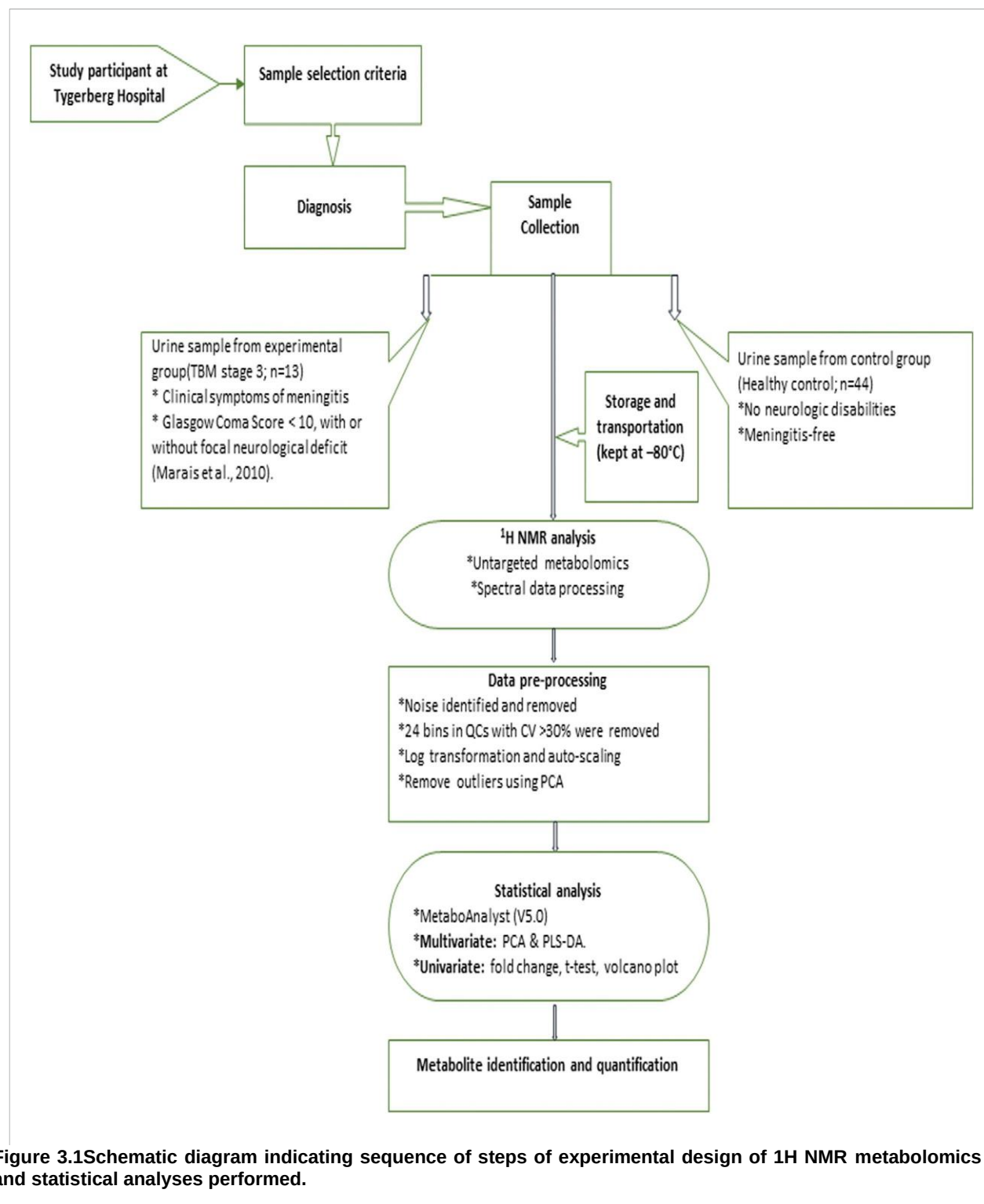


Figure 3.1 Schematic diagram indicating sequence of steps of experimental design of ^1H NMR metabolomics and statistical analyses performed.

Subsequent hierarchical cluster analysis (**Supplementary Figure S2**) identified two clusters: one homogeneous cluster containing control cases (n = 38) at the top, with one TBM case, and the other (bottom) cluster containing 12 TBM cases and two control cases. The hierarchical cluster analysis further illustrates that there was some overlap between the two groups, but overall there was differentiation.

A supervised PLS-DA (**Figure 3.3**) was performed in order to detect differentiating bins and avoid false discoveries. The PLS-DA model generates meaningful information that can be used to identify important variables and assess the significance of class discrimination by performing a permutation test using the optimal number of components determined by cross validation. The supervised PLS-DA model for the data exhibited an R^2 of 96%, and was validated by a Q^2 of 81%, and a permutation p -value of 0.0004 (188/2000). Various bins differentiating the TBM and control groups were observed through loading plots for the PLS-DA and identified through a variable of importance in projection (VIPs) cut-off criteria of greater than 1.0 ($VIP > 1.0$) for components 1 and 2. Additionally, univariate statistics of fold change analysis and Wilcoxon t -tests are illustrated as a volcano plot in (**Figure 3.4**). Thresholds of absolute fold change ≥ 2.0 and an FDR p -value ≤ 0.05 were used to identify statistically significant bins.

Based upon the quantitative statistical data of the ^1H -NMR spectral bins, the important bins were identified using the following

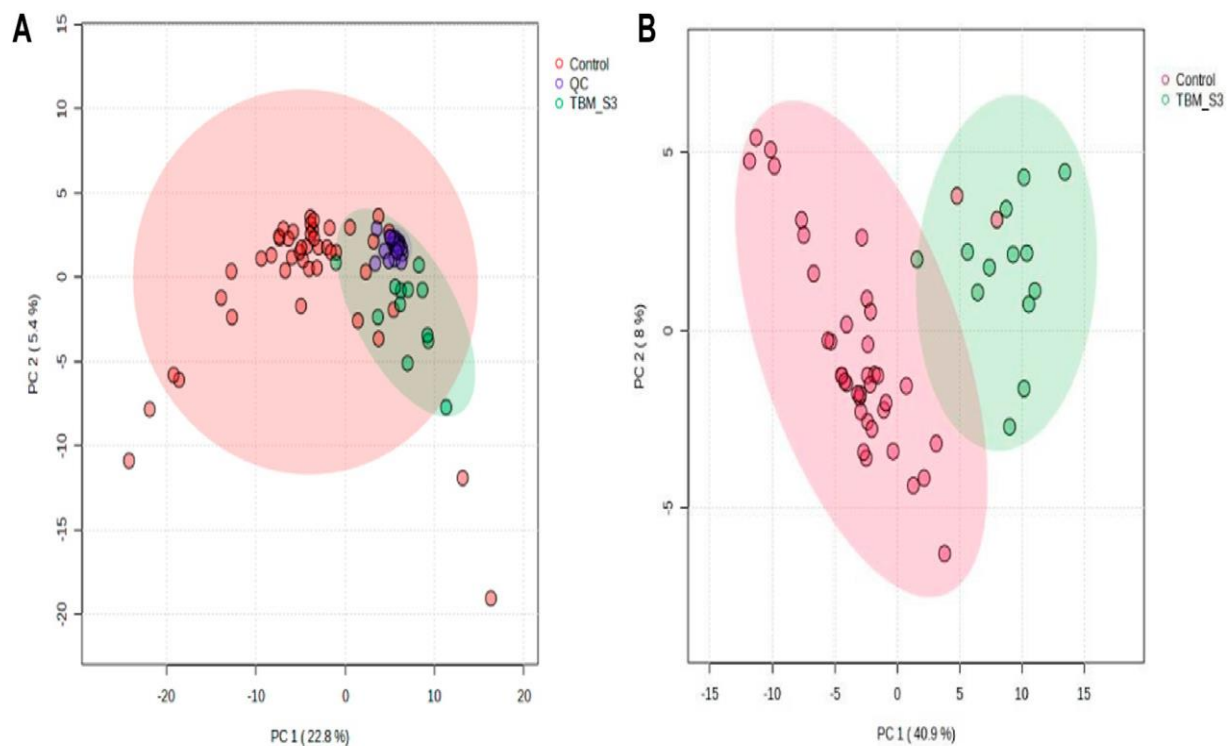


Figure 3.2 PCA scores plots. (A) (Left): Quality controls (QCs) and all cases—20 QCs, 44 controls and 13 TBM cases at initial treatment (TBM), with 95% CI ellipses.

The total explained variance from PC1 and PC2 is 28.2%. (B) (Right): Reduced cases (without QCs)—40 controls and 13 TBM cases at initial treatment (TBM), with 95% CI ellipses. The total explained variance from PC1 and PC2 is 48.9%.

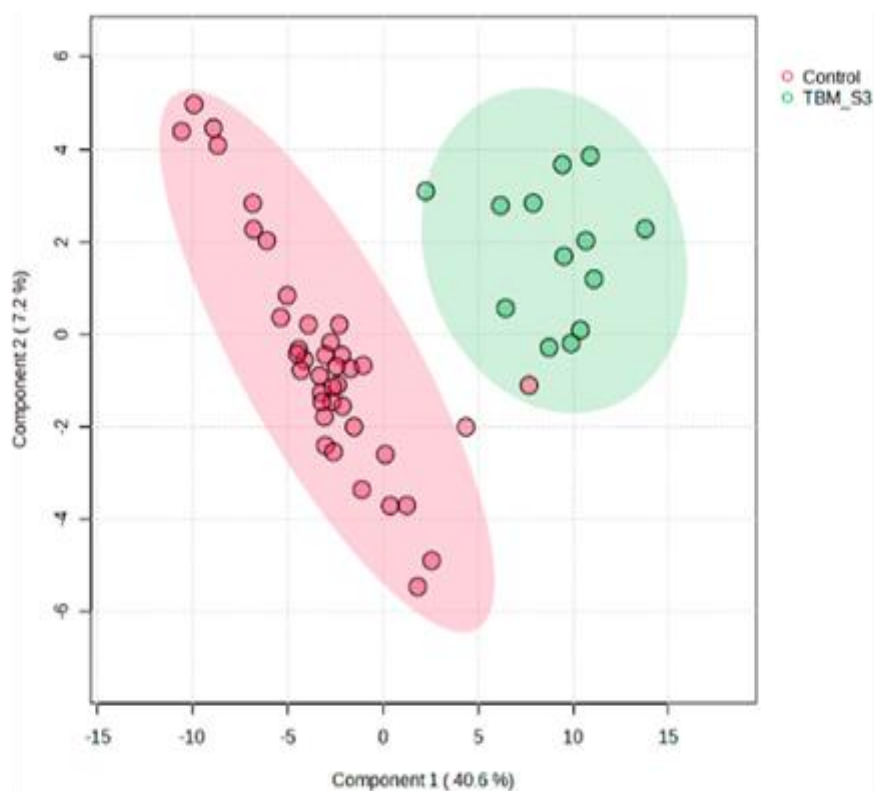


Figure 3.3 Supervised PLS-DA scores plot of 40 controls and 13 TBM cases at initial treatment (TBM), with 95% CI ellipses. The total explained variance from component 1 and component 2 is 47.8%.

rule: (VIP comp one and comp 2 > 1.0) OR (p -value FDR ≤ 0.05 and absolute fold change ≥ 2.0) (see Supplementary Table S1). Pure compound ^1H NMR spectral libraries were used to annotate most of the important bins that discriminated between the 40 controls and the 13 cases of TBM at initial treatment. Several important bins contained either pure forms of medications, or their metabolites: 1,2-propanediol, acetaminophen, isoniazid, isonicotinic

methylnicotinamide, e) trigonelline]; increased nitrogen excretion Figure 3.8: f) N-acetylglutamine, as well as increased urea—not quantified].

3.5 Discussion

The 29 significant urinary metabolites identified in this study can be categorized in terms of six dysregulated metabolic pathways. 1) Upregulated tryptophan catabolism (quinolinic acid) linked to an altered vitamin B3 metabolism (1-methylnicotinamide, trigonelline). 2) Perturbation of amino acid metabolism (leucine, lysine, isoleucine, glycine, tyrosine, valine). 3) Increased energy production—metabolic burst (3-hydroxyisobutyric acid, 3- hydroxyisovaleric acid, glucose, mannose, myo-inositol, pyruvic acid, sucrose). 4) Disrupted gut microbiota metabolism (4- hydroxyphenylacetic acid, arabinose, formic acid, hippuric acid, m-cresol, methylamine, methylguanidine, o-cresol). 5) Ketoacidosis (acetic acid, acetoacetic acid, acetone). 6) Increased nitrogen excretion (urea, N- acetylglutamine). We provide biological context by describing the characterization of the urinary metabolic profile of TBM in the discussion below.

3.5.1 Upregulated tryptophan catabolism

Quinolinic acid (Figure 3.5A) was significantly elevated in the urine of the TBM patients (71.57 ± 49.42 $\mu\text{mol}/\text{mmol}$ creatinine) compared to the controls (0 $\mu\text{mol}/\text{mmol}$ creatinine; $p < 0.001$, $d = 2.9$). Quinolinic acid was completely absent (or below the detection limit of the NMR spectrometer) in all control cases. This suggests that quinolinic acid would be an excellent candidate as a potential urinary diagnostic marker for TBM—to be tested in a future study. Tryptophan is a key amino acid required for protein biosynthesis, and a precursor for the synthesis of a diversity of other metabolites (Davis et al., 2019); the most reported on, in the case of TB (Campbell et al., 2014; Manyelo et al., 2019), is the kynurenine pathway, via the *M. tb* infection-induced pro-inflammatory cytokines IL-6, TNF- α , and IFN- γ , upregulating indoleamine 2,3-dioxygenase (IDO) (Campbell et al., 2014). Quinolinic acid (a downstream metabolite of tryptophan), induced via IDO-1 (Heyes et al., 1992), has been associated with a variety of inflammatory disorders, elevated, and also detected in elevated concentrations in the CSF and brain tissue of patients with a wide range of infectious and other neurological diseases (Heyes et al., 2001), produced in large quantities by activated macrophages and microglia (Heyes et al., 1996). Of note, a study on the CSF collected from adult TBM cases done by van Laarhoven *et al.* (2018) identified tryptophan metabolism as one of the most upregulated metabolic pathways in TBM cases, with downstream upregulated tryptophan metabolism reflected by the kynurenine pathway. Their study examined an adult cohort of 33 TBM cases against 22 controls; with a further validation study of 101 TBM cases versus 17 controls. Of the metabolites identified in the initial study, 250 metabolites were increased and 18 decreased in TBM. Van Laarhoven *et al.* further examined the initial 33 TBM cases and identified 16 as survivors and 17 as non-survivors; 13 metabolites were shown to be increased in TBM survivors compared to controls and even higher in TBM non-survivors compared to TBM survivors. Of the 13 metabolites

identified, three metabolites, including tryptophan, were found to be decreased in non-survivors compared to controls, and also decreased in TBM survivors *versus* TBM non-survivors. Van Laarhoven *et al.* then carried out a validation study on CSF tryptophan with 101 TBM cases *versus* 17 controls; that identified IDO-1 as having greater expression in TBM. However, in all of these investigation studies, there is no report of the metabolites that characterize TBM. In a study characterizing the CSF immunological signature of TBM in a cohort of 23 children (Manyelo *et al.*, 2019), a 3-marker signature associated with

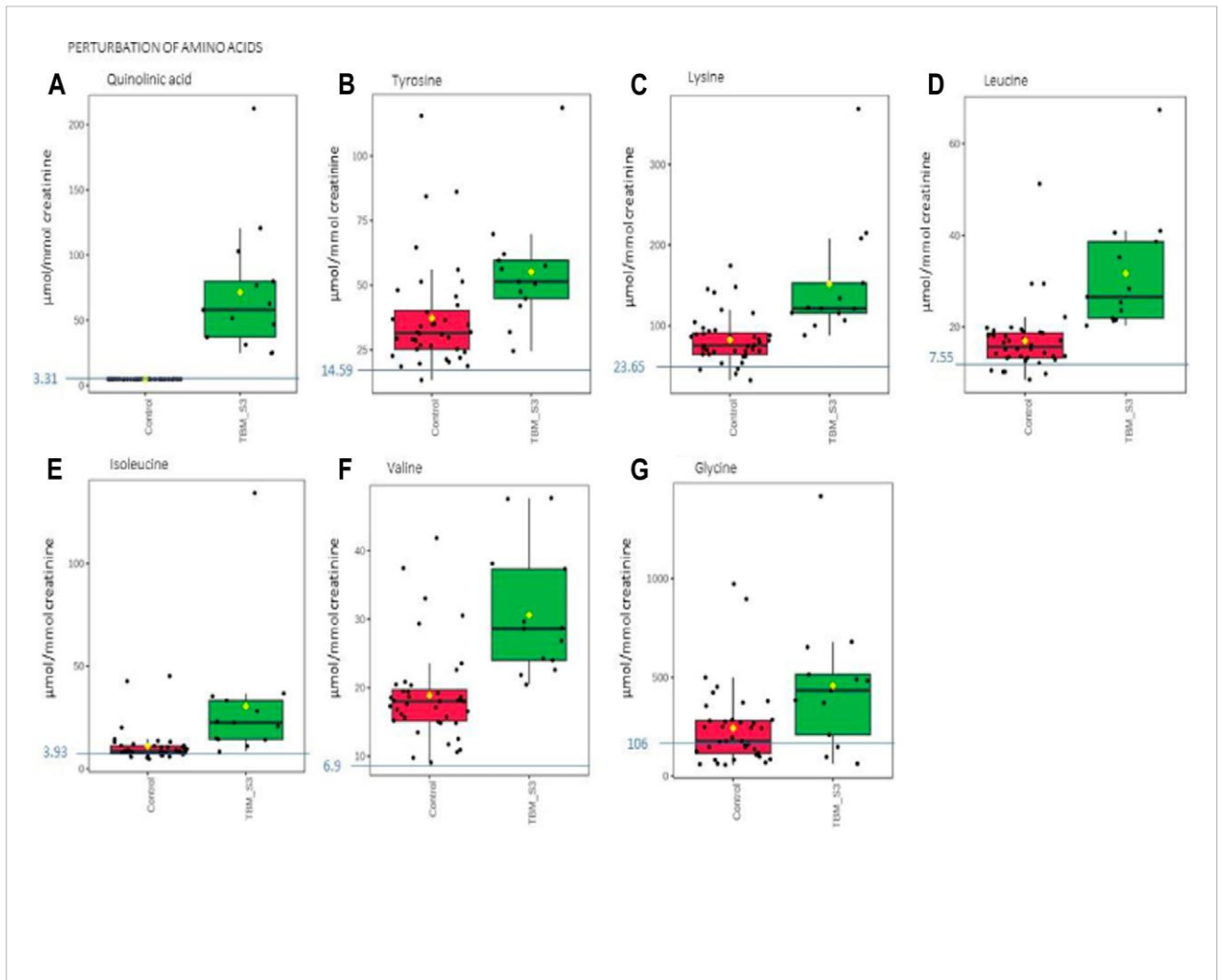


Figure 3.5 Box plots of the relative concentration (μmol/mmol creatinine) of seven urinary metabolites of Perturbation of amino acids.

(A) quinolinic acid, (B) tyrosine, (C) lysine, (D) leucine, (E) isoleucine, (F) valine, (G) glycine] identified as being part of perturbation of amino acid metabolism in TBM. Green = TBM cases; red = controls. Blue line (and number) indicates normal reference value, based upon www.hmdb.ca and Bouatra *et al.* (2013).

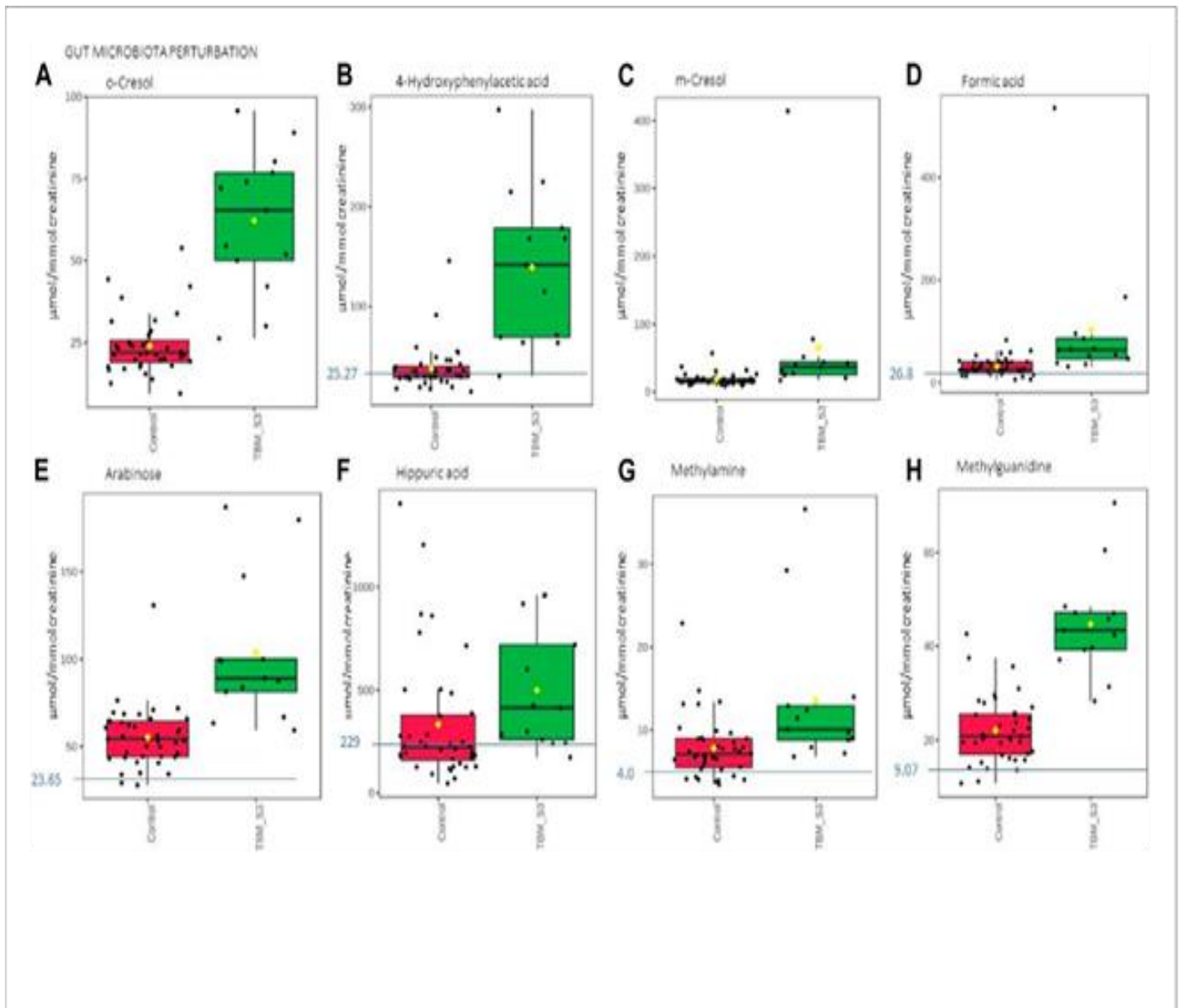


Figure 3.6 Box plots of the relative concentration ($\mu\text{mol}/\text{mmol creatinine}$) of eight urinary metabolites of Gut microbiota perturbation.

(A) o-Cresol (B) 4-hydroxyphenylacetic acid, (C) m-cresol, (D) formic acid, (E) arabinose, (F) hippuric acid, (G) methylamine, (H) methylguanidine] identified as being part of gut microbiota perturbation in TBM. Green = TBM cases; red = controls. Blue line (and number) indicates normal reference value, based upon www.hmdb.ca and Bouatra *et al.* (2013)

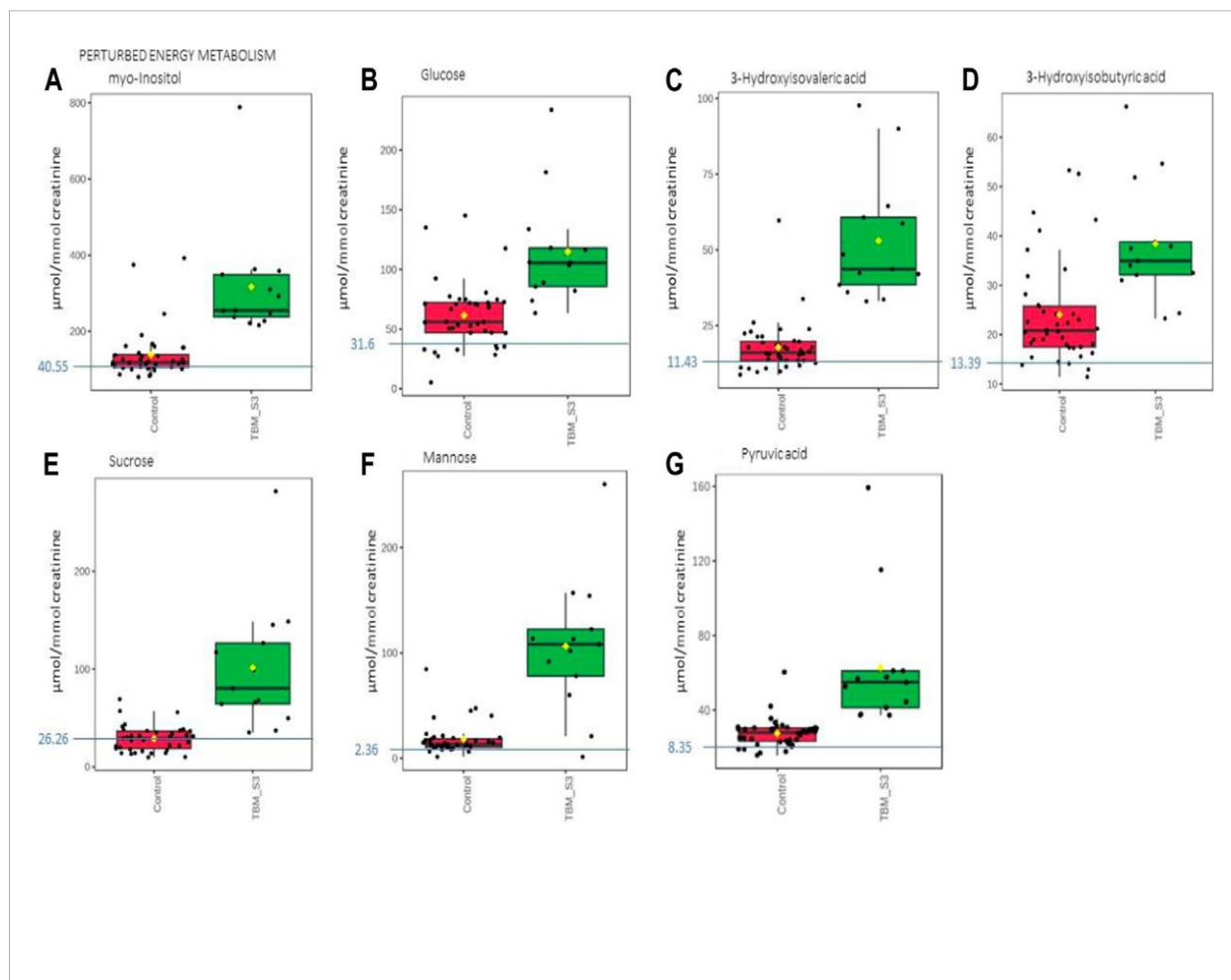


Figure 3.7 Box plots of the relative concentration ($\mu\text{mol}/\text{mmol creatinine}$) of seven urinary metabolites of perturbed energy metabolism.

(A) myo-inositol, (B) glucose, (C) 3-hydroxyisovaleric acid, (D) 3-hydroxyisobutyric acid, (E) sucrose, (F) mannose, (G) pyruvic acid] identified as being part of perturbed energy metabolism in TBM. Green = TBM cases; red = controls. Blue line (and number) indicates normal reference value, based upon www.hmdb.ca and Bouatra *et al.* (2013).

neuroinflammation (VEGF, IFN- γ , and MPO) showed strong potential as a diagnostic tool for TBM in children with promising accuracy. We modelled the downstream metabolic effects expected from VEGF, IFN- γ , and MPO and predicted pivotal altered metabolic pathways that would be reflected in the urinary profiles of TBM subjects (Isaiah *et al.*, 2020). Quinolinic acid was one of the major metabolic end-products that we predicted to come from this, within the *M. tb*-infected brain, induced by an increased IFN- γ . Urinary quinolinic acid was also observed in the TBM patients' urine samples in a similar untargeted urinary ^1H NMR metabolomics study carried out by Mason *et al.* (2016b). Quinolinic acid is not only expected in abundance in the *M. tb*-infected brain, but also produced by the enteric nervous system in the gut caused by dysbiosis, in conjunction with a perturbed gut–brain axis (Isaiah *et al.*, 2020).

3.5.2 Altered vitamin B3 metabolism

Trigonelline (1-methylnicotinic acid) was significantly elevated (Figure 3.8E) in the urine of the TBM patients ($18.65 \pm 12.58 \mu\text{mol}/\text{mmol creatinine}$) when compared to that of the controls ($6.94 \pm 7.96 \mu\text{mol}/\text{mmol creatinine}$; $p = 0.008$, $d = 1.14$). 1-Methylnicotinamide, the methylated amide of nicotinic acid, was also significantly elevated (Figure 3.8D) in the urine of the TBM patients ($45.30 \pm 25.22 \mu\text{mol}/\text{mmol creatinine}$) when compared to that of the controls ($10.61 \pm 12.83 \mu\text{mol}/\text{mmol creatinine}$; $p < 0.001$, $d = 1.82$). Both 1-methylnicotinic acid and 1-methylnicotinamide are products of an upregulated kynurenine metabolism (Cazzullo et al., 1976), and, within

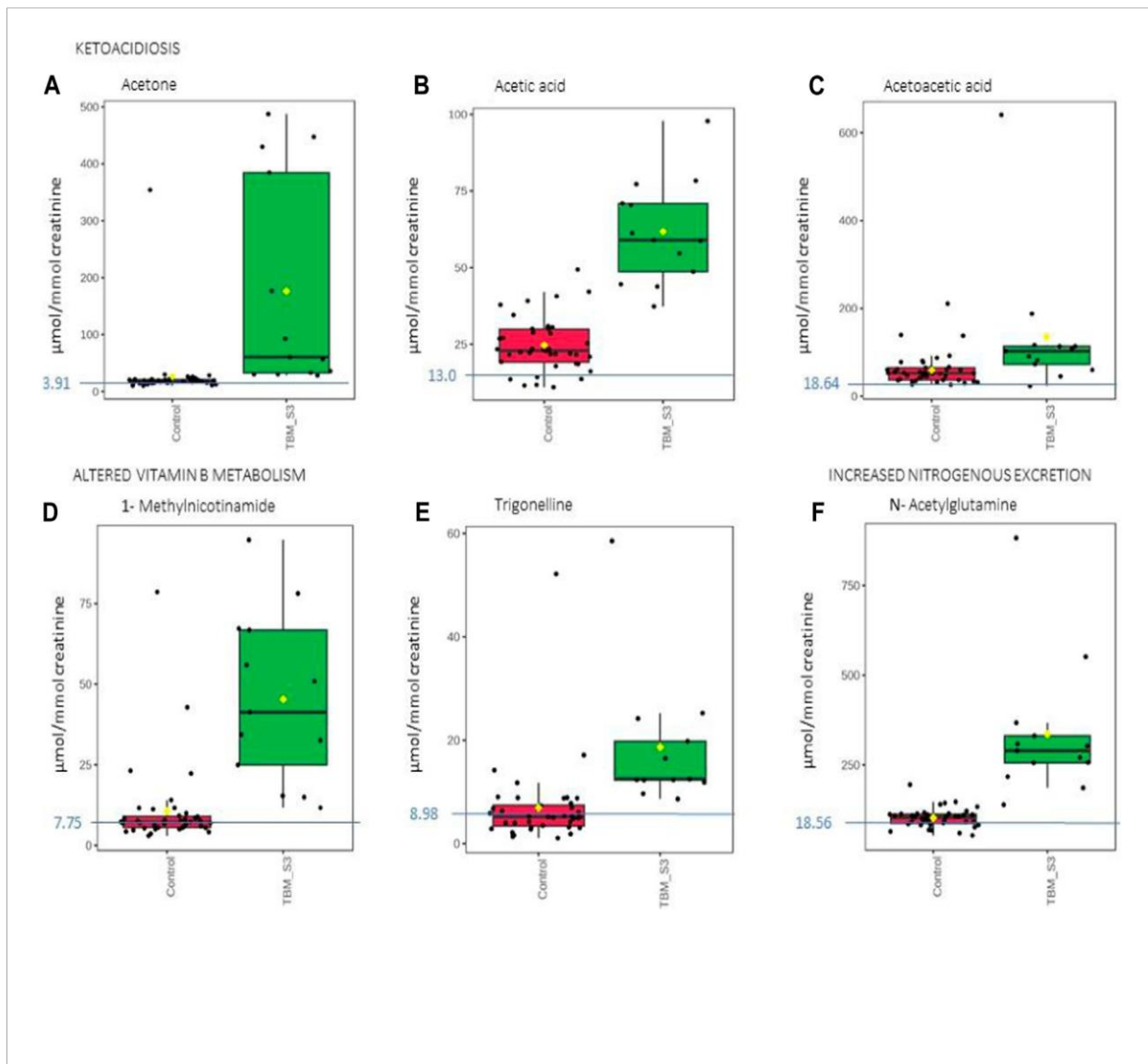


Figure 3.8 Box plots of the relative concentration (μmol/mmol creatinine) of six urinary metabolites identified as being part of ketoacidosis, altered vitamin B metabolism and increase nitrogen excretion.

(A) acetone, (B) acetic acid, (C) acetoacetic acid], altered vitamin B metabolism (D) 1-methylnicotinamide, (E) trigonelline] and increased nitrogen excretion (F) N- acetylglutamine] in TBM. Green = TBM cases; red = controls. Blue line (and number) indicates normal reference value, based upon www.hmdb.ca and Bouatra et al. (2013).

the tryptophan–nicotinic acid metabolism, 1-methylnicotinamide is an end-product of nicotinamide (vitamin B3) metabolism. Nicotinamide is the precursor of the coenzymes β -nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which are involved in a variety of enzyme-mediated oxidation and reduction reactions (Li et al., 2006; Zhou et al., 2009). Excess nicotinamide is methylated, oxidized or hydroxylated to 1-methylnicotinamide, nicotinamide-N-oxide or 6-hydroxynicotinamide, respectively, and then 1-methylnicotinamide is further oxidized to the pyridones 1-methyl-2-pyridone-5-carboxamide (2Py) and 1-methyl-4-pyridone-3-carboxamide by aldehyde oxidase (Zhou et al., 2009; Mayneris-Perxachs et al., 2016). 2Py and 1-methylnicotinamide are the major common nicotinamide metabolites found in human urine (Kitamura et al., 2008). A study by Zhou et al. (2009) showed that 1-methylnicotinamide clearance was delayed in diabetic patients, suggesting that 1-methylnicotinamide could cause oxidative stress and insulin resistance (discussed further below). 1-Methylnicotinamide may also play a role in the progression of Parkinson's disease, as a result of a superoxide anion formed by 1-methylnicotinamide via mitochondria (Fukushima et al., 2002; Williams et al., 2005), associated with elevated oxidative stress (Smeitink et al., 2004), and also with major depressive disorders (Zheng et al., 2013); hence, associating increased urinary 1-methylnicotinamide to several neurological and inflammatory conditions. In this study, the significantly elevated concentrations of 1-methylnicotinamide, is most likely due to the increased flux through the kynurenine pathway induced by INF- γ , and generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS)—the consequences of severe oxidative stress and impaired redox status, associated with TB (Reddy et al., 2009; Miric et al., 2013).

3.5.3 Perturbation of amino acid metabolism

The urinary concentrations of tyrosine (Figure 3.5B) were significantly elevated in the TBM patients (55.09 ± 21.81 $\mu\text{mol}/\text{mmol creatinine}$) compared to the controls (37.2 ± 20.14 $\mu\text{mol}/\text{mmol creatinine}$; $p = 0.021$, $d = 0.85$). Phenylalanine and tyrosine are aromatic amino acids, synthesized from phosphoenolpyruvate and erythrose 4-phosphate—intermediates of glycolysis and the pentose phosphate pathway, respectively (Ferreira and Teixeira, 2003). Phenylalanine is catabolized into acetoacetic acid and fumaric acid via tyrosine (Komoda and Matsunaga, 2015). Tyrosine is formed by the hydroxylation of phenylalanine in the liver when the intake of tyrosine is low (Litwack, 2018). Both these amino acids serve as precursors for the synthesis of many biologically/ neurologically active compounds that are essential for maintaining a variety of biological functions (Han et al., 2019). The neurotransmitters epinephrine and norepinephrine are synthesized from the tyrosine metabolite L-3,4-dihydroxyphenylalanine (L-DOPA). Parkinson's disease and schizophrenia are thought to be caused by, amongst other factors, a lack of these neurotransmitters (Komoda and Matsunaga, 2015). Hyperphenylalaninemia (HPA), a disorder resulting in levels of phenylalanine that are excessive, is caused by a deficiency of the hepatic phenylalanine-4-hydroxylase (PAH) or its cofactor tetrahydrobiopterin (BH4), and clinically presents with a number of neurological signs and symptoms, such as irritability, hyperkinesia, and severe cognitive deficiency, often associated with TBM (Blau et al.,

2014). In TB patients, altered metabolism of phenylalanine and tyrosine was previously observed in a pilot study that compared the urine metabolic profiles of 21 adults (Van laarhoven et al., 2018); this alteration was confirmed in *M. tb*-infected mice (Shin et al., 2011; Weiner et al., 2012). These studies support our findings of significantly elevated tyrosine in advanced TBM when compared to controls.

Glycine concentrations (Figure 3.5G) were elevated in the TBM patients (457.19 ± 336.45 $\mu\text{mol}/\text{mmol}$ creatinine) when compared to that of the controls (242.51 ± 194.75 $\mu\text{mol}/\text{mmol}$ creatinine; $p = 0.053$, $d = 0.81$), at a practically significant level ($d > 0.6$). Glycine functions as a neurotransmitter in the brain, allowing neurons to communicate with one another, and subsequently regulates neuronal activity (Shahsavar et al., 2020). Many of the clinical signs and symptoms of TBM, such as low muscle tone, lethargy, seizures, coma, and apnea requiring ventilator support, are associated with glycine accumulation in the brain and neural tissue (DeArmond et al., 2017). Glycine transporters, both astrocytic GlyT1 and presynaptic neuronal GlyT2, are critical for proper glycine recycling at glutamatergic and glycinergic synapses.

Table 3.2 Data of the quantified (concentration: $\mu\text{mol}/\text{mmol}$ creatinine) important metabolites (n = 28) that characterize TBM. A p-value ≤ 0.05 indicates statistical significance, and a Cohen's d effect size ≥ 0.6 indicates practical significance.

Metabolites (chemical shift)	HMDB ID	p-value	Effect size
1-Methylnicotinamide (9.29)	HMDB0000699	<0.001	1.82
3-Hydroxyisobutyric acid (1.08)	HMDB0000023	0.001	1.3
3-Hydroxyisovaleric acid (1.27)	HMDB0000754	<0.001	2.48
4-Hydroxyphenylacetic acid (6.88)	HMDB0000020	<0.001	2.07
Acetic acid (1.92)	HMDB0000042	<0.001	2.97
Acetoacetic acid (2.29)	HMDB0000060	0.111	0.81
Acetone (2.33)	HMDB0001659	0.014	1.29
Arabinose (4.54)	HMDB0000646	0.001	1.67
Formic acid (8.46)	HMDB0000142	0.08	0.99
Glucose (4.67)	HMDB0000122	0.001	1.48
Glycine (3.57)	HMDB0000123	0.053	0.81
Hippuric acid (7.66)	HMDB0000714	0.096	0.57
Isoleucine (1.03)	HMDB0000172	0.052	0.99
Leucine (0.99)	HMDB0000687	0.002	1.49
Lysine (1.76)	HMDB0000182	0.007	1.34
Mannose (5.20)	HMDB0000169	<0.001	2.3
m-Cresol (2.31)	HMDB0002048	0.135	0.85
Methylamine (2.60)	HMDB0000164	0.043	0.93
Methylguanidine (2.83)	HMDB0001522	<0.001	2.56
myo-Inositol (4.09)	HMDB0000211	0.001	1.71
N-Acetylglutamine (2.08)	HMDB0006029	<0.001	2.21
o-Cresol (2.21)	HMDB0002055	<0.001	2.58
Pyruvic acid (2.39)	HMDB0000243	0.004	1.70
Quinolinic acid (8.03)	HMDB0000232	<0.001	2.9
Sucrose (5.42)	HMDB0000258	0.002	1.89
Trigonelline (9.13)	HMDB0000875	0.008	1.14
Tyrosine (6.91)	HMDB0000158	0.021	0.85
Valine (1.05)	HMDB0000687	<0.001	1.48

Note: chemical shifts (ppm) of metabolites are given in brackets. HMDB ID refers to the identity number assigned to the metabolite in the Human Metabolome Database (www.hmdb.ca).

A number of key studies have shown that microglial activity is modulated by astrocyte-derived glycine and L-serine (Van Den Eynden et al., 2009). Glycine at micromolar concentrations changes the morphology of microglial cells and increases the secretion of nitrogen oxide, superoxide, acid phosphatase, and their metabolic activity when induced by lipopolysaccharides (LPS) (Van Den Eynden et al., 2009). LPS are also well-known for their ability to stimulate the production of proinflammatory cytokines (Jansky et al., 2003; Schildberger et al., 2013), especially IL-6 and IFN- γ , all of which (LPS and the associated cytokines) are also elevated in TB patient blood (Feruglio et al., 2013; Gallucci et al., 2021). Several of the clinical symptoms associated with HPA and an accumulation of glycine in the brain are similar to those clinical symptoms seen in TBM—e.g., altered consciousness and behavior. Thus, the treatment of these amino acid imbalances is a potential basis of therapy to alleviate the symptoms found in TBM cases—something that needs to be tested in a future study.

The branched chain amino acids were all increased in the TBM group—leucine (Figure 3.5D): 31.67 ± 12.64 $\mu\text{mol}/\text{mmol}$ creatinine, isoleucine (Figure 3.5E): 30.5 ± 31.27 $\mu\text{mol}/\text{mmol}$ creatinine and valine (Figure 3.5F): 31.67 ± 12.64 $\mu\text{mol}/\text{mmol}$ creatinine, relative to the controls—leucine: 16.95 ± 7.05 $\mu\text{mol}/\text{mmol}$ creatinine; $p = 0.002$, $d = 1.49$, isoleucine: 10.95 ± 8.08 $\mu\text{mol}/\text{mmol}$ creatinine; $p = 0.052$, $d = 0.99$, valine: 18.88 ± 6.9 $\mu\text{mol}/\text{mmol}$ creatinine; $p < 0.001$, $d = 1.48$. One of the most crucial building blocks for gluconeogenesis are free amino acids, which become elevated when protein synthesis is impaired. These findings are in line with other reports of wasting (cachexia) and malnutrition, also known as “anabolic block,” in TB patients. Here, the term “anabolic block” describes a higher proportion of ingested amino acids being oxidized than being used for protein anabolism (MacAllan et al., 1998; Macallan, 1999; Schwenk and Macallan, 2000). Hence, the significant increase in BCAAs may likely be caused by the increased proteolysis needed to meet the elevated demand for amino acids that are used as fuel sources for energy production during infection (Levin et al., 1983). It is also well recognized that branched chain amino acids (BCAAs) contribute to important metabolic processes in the brain, such as the gluconeogenesis that occurs in activated microglia, as a source of energy during an activated immune response (Sweatt et al., 2004). Furthermore, numerous other pathological conditions are associated with changes to amino acid levels in body fluids (Binici et al., 2023). The level of total amino acids in CSF was measured and examined in a 1981 study on patients who had viral meningitis and TBM. Patients with TBM were found to have significantly higher concentrations of total amino acids, which included leucine, isoleucine and valine (Corston et al., 1981). In a recent investigation of the predictive value of amino acids for bacterial, aseptic, and tuberculous meningitis ($n = 41$, 41 , and 21 , respectively), against healthy controls ($n = 64$), carried out by Binici et al. (2023), all BCAAs were found to be significantly increased in TBM subjects, confirming the results. In a ^1H NMR-based metabolomics study, serum BCAAs were increased in *M. tb*-infected rats, as compared to control rats (Shin et al., 2011) giving support to what we are seeing in this paediatric study population. In conditions of perturbed energy metabolism, as reported by Mason et al. (2016b), as analyzed using urine from a similar pediatric TBM cohort, elevated concentrations of hydroxyl acids derived from BCAAs leucine (3-hydroxyisovaleric acid) isoleucine (2-methyl-3-hydroxybutyric acid) and valine (3-hydroxyisobutyric acid) were shown in a putative urinary biosignature. The present study also indicates a comparable metabolic increase in BCAAs to that observed in CSF of children with TBM by Mason et al. (2015).

Lastly, lysine, an important amino acid known to facilitate protein synthesis (Chang and Gao, 1995) (Figure 3.5C) was also significantly increased in the TBM cases (151.55 ± 72.73 $\mu\text{mol}/\text{mmol}$ creatinine) when compared to that of the controls (82.59 ± 29.84 $\mu\text{mol}/\text{mmol}$ creatinine; $p = 0.007$, $d = 1.34$). Via its already well described neuroprotective and neurotrophic effects, lysine has been shown to enhance neurological function and cerebral blood flow in patients with ischemic stroke (Kondoh et al., 2010). According to reports, intellectual disability and other motor neuron impairment are associated with elevated levels of lysine in the CSF of TBM patients (Shaw et al., 1995a). Additionally, it has been demonstrated to form adducts with various substances, including acrolein-lysine, a marker of lipid peroxidation in pediatric meningitis (Tsukahara et al., 2002). Children with persistent atopic dermatitis have also been shown to exhibit considerably higher urine excretion of acrolein-lysine adducts than do healthy children (unpublished data) (Tsukahara et al., 2002). In this investigation, all TBM patients

showed elevated lysine concentration, which is in support of the aforementioned reports, and that of Mason *et al.* (2017), who investigated the utilization of amino acids in CSF, in order to distinguish TBM from healthy controls.

3.5.4 Metabolic burst energy metabolites

In this study, urinary glucose (Figure 3.7A) was significantly elevated in the TBM patients ($114.82 \pm 44.86 \mu\text{mol}/\text{mmol creatinine}$) compared to the controls ($61.35 \pm 27.63 \mu\text{mol}/\text{mmol creatinine}$; $p = 0.001$, $d = 1.48$). Other significantly increased urinary saccharides in TBM included mannose ($106.26 \pm 62.42 \mu\text{mol}/\text{mmol creatinine}$) and sucrose ($101.48 \pm 63.86 \mu\text{mol}/\text{mmol creatinine}$) when compared to the controls, respectively ($17.83 \pm 14.63 \mu\text{mol}/\text{mmol creatinine}$; $p < 0.001$, $d = 2.3$ and $28.83 \pm 12.95 \mu\text{mol}/\text{mmol creatinine}$; $p = 0.002$, $d = 1.89$). Myo-inositol (Figure 3.7A) and pyruvic acid (Figure 3.7G) were also identified by statistical analysis in this study as being significantly increased in TBM ($317.02 \pm 145.37 \mu\text{mol}/\text{mmol creatinine}$ and $62.79 \pm 34.04 \mu\text{mol}/\text{mmol creatinine}$, respectively) when compared to the controls ($137.99 \pm 64.29 \mu\text{mol}/\text{mmol creatinine}$; $p = 0.001$, $d = 1.71$ and $27.41 \pm 7.49 \mu\text{mol}/\text{mmol creatinine}$; $p = 0.004$, $d = 1.70$, respectively).

These increased metabolites—glucose, mannose, sucrose, myo- inositol, pyruvic acid, 3-hydroxyisobutyric acid and 3- hydroxyisovaleric acid—can be linked to metabolic burst, associated with an increased need for energy production in the *M. tb*-infected brain (Mason *et al.*, 2015; Mason, 2017; van Zyl *et al.*, 2020). Glucose is the primary metabolite that is catabolized for neuroenergetics purposes. However, uncontrolled glucose utilization in the brain may subsequently lead to insulin resistance, and transitory glucose oxidation via glucose oxidase during unregulated glucose metabolism. According to a growing body of evidence, chronic neuroinflammatory diseases, such as TBM, are known to be linked to insulin resistance (Isaiah *et al.*, 2020). The oxidation of glucose produces gluconolactone, an inflammatory marker previously observed in pulmonary TB (Preez *et al.*, 2017), when insulin becomes depleted. Gluconolactone is siphoned into the pentose phosphate pathway and leads to elevated levels of hydrogen peroxide (Preez *et al.*, 2017). Various studies (Hampton *et al.*, 1998; Podrez *et al.*, 2000; Klebanoff, 2005; Manyelo *et al.*, 2019) have shown that myeloperoxidase, identified as an immunological marker of TBM, interacts with hydrogen peroxide and triggers a variety of oxidative stress pathways. In our previously published downstream metabolic model of TBM (Isaiah *et al.*, 2020), we predicted end products of myeloperoxidase activation, namely, glutathione sulfonamide and 3- chlorotyrosine. Although we did not detect these two products in the urine of the TBM patients using ^1H -NMR, it is highly likely that other more sensitive analytical methods (e.g., liquid chromatography coupled to mass spectrometry) may detect these and this could be considered for a later study.

In the brain, lactate has neuroprotective properties, associated with a shuttling neuroenergetic mechanism (Mason, 2017). However, this shuttling role of lactate is compartmentalized within the brain during TBM, and urinary lactate levels do not significantly increase during such times. Systemically, lactate is converted to pyruvate, leading to significantly increased urinary levels of pyruvate in TBM, and

an increased conversion of NAD⁺ to NADH. This signals an increased level of redox and oxidative stress, as previously described in the predictive urinary TBM model by Isaiah *et al.* (2020). This is evidenced when looking at the urinary lactate:pyruvate (Lac:Pyr) ratio, and TBM cases have a significantly lower ($p = 0.004$) Lac-Pyr ratio of 2.43, when compared to that of the controls (6.32). An additional measure of the redox status can be done by determining the ratio of 3-hydroxybutyric acid:acetoacetic acid (3HB:AAA), which is significantly lower ($p < 0.001$) in the TBM cases (1.12), than in the controls (1.93).

3-Hydroxyisobutyric acid, a downstream catabolite of valine metabolism (Ko *et al.*, 1991) (Figure 3.7D), was significantly increased in the TBM group ($38.41 \pm 11.78 \mu\text{mol}/\text{mmol creatinine}$), as well as was the leucine catabolite 3-hydroxyisovaleric acid (Figure 3.7C, $53. \pm 20 \mu\text{mol}/\text{mmol creatinine}$), when compared to the controls, respectively ($24.06 \pm 10.32 \mu\text{mol}/\text{mmol creatinine}$; $p = 0.001$, $d = 1.3$ and $17.85 \pm 8.39 \mu\text{mol}/\text{mmol creatinine}$; $p < 0.001$, $d = 2.48$). Both of these hydroxy acids gave further support for the metabolites associated with a perturbed energy state in TBM (Mason *et al.*, 2016b). The accumulation of 3-hydroxyisobutyric acid and 3-hydroxyisovaleric acid, subsequently feeds into the anaplerotic Krebs cycle, for primary energy production. Coincidentally, the *M. tb* bacilli also utilize the TCA cycle for their own energy production (Savvi *et al.*, 2008). An additional indicator of perturbed energy metabolism is the elevated myo-inositol detected in the paediatric TBM patients. Myo-inositol is mostly made from glucose (Hauser and Finelli, 1963) and is a key signaling molecule required for immunological responses, such as microglia activation. Myo-inositol has previously been linked to microglia and astrocyte activation, as well as a pathogenic response seen in neurodegenerative illness and neuroinflammation (Pears *et al.*, 2005). Increased CSF myo-inositol in TBM patients has also been reported previously (van Zyl *et al.*, 2020), and this study shows that this is a systemic condition in TBM that is likely linked to uncontrolled glucose utilization.

Mannose is interesting because it is described as being ubiquitous (i.e., it occurs everywhere). It is grouped under energy metabolism here since it is a sugar but mannose can also come from lipoarabinomannan (LAM), one of the key components of the *M. tb* cell wall (Van Toorn *et al.*, 2014). In lab grown *M. tb*, LAM was shown to be composed of D-arabinose (55%–60%), D-mannose (36%–40%), and fatty acyls (1%–3%; palmitate C:16; tuberculostearate (TBSA) C:19:1) (Amin *et al.*, 2021). Further examination of the role of mannose is however still needed.

3.5.5 Disrupted gut microbiota metabolism

Eight of the metabolites listed in Table 3.2 are urinary indicators of altered metabolism associated with the gut microbiota. A significant increase of 4-hydroxyphenylacetic acid (Figure 3.6B) was observed in TBM urine ($138.78 \pm 75.85 \mu\text{mol}/\text{mmol creatinine}$) compared to the controls ($37.45 \pm 22 \mu\text{mol}/\text{mmol creatinine}$; $p < 0.001$, $d = 2.07$). Elevated levels of o-cresol (Figure 3.6A) and m-cresol (Figure 3.6C) ($62.15 \pm 20.88 \mu\text{mol}/\text{mmol creatinine}$ and $65.57 \pm 101.77 \mu\text{mol}/\text{mmol creatinine}$, respectively) were found in TBM compared to the controls ($24.02 \pm 8.67 \mu\text{mol}/\text{mmol creatinine}$; $p < 0.001$, $d = 2.58$ and $18.5 \pm 8.7 \mu\text{mol}/\text{mmol creatinine}$; $p = 0.135$, $d = 0.85$, respectively). Other elevated urinary gut microbiota metabolites were

formic acid (Figure 3.6D), methylamine (Figure 3.6G) and methylguanidine (Figure 3.6H) ($102.66 \pm 129.4 \mu\text{mol}/\text{mmol creatinine}$, $13.56 \pm 8.65 \mu\text{mol}/\text{mmol creatinine}$ and $44.7 \pm 10.8 \mu\text{mol}/\text{mmol creatinine}$, respectively), when compared to the controls ($31 \pm 16.06 \mu\text{mol}/\text{mmol creatinine}$; $p = 0.08$, $d = 0.99$, $7.82 \pm 3.64 \mu\text{mol}/\text{mmol creatinine}$; $p = 0.043$, $d = 0.93$ and $22.03 \pm 6.9 \mu\text{mol}/\text{mmol creatinine}$; $p < 0.001$, $d = 2.56$). Hippuric acid was identified by statistical analyses as significant based upon the ^1H NMR spectral data (Table 3.2). However, the concentration data (Figure 3.6F) of hippuric acid did not show statistical significance ($p = 0.096$, $d = 0.57$). Lastly, arabinose (Figure 3.6E) was identified from the untargeted ^1H -NMR data as being statistically significant in TBM cases ($103.45 \pm 40.2 \mu\text{mol}/\text{mmol creatinine}$) compared to the controls ($55.47 \pm 17.21 \mu\text{mol}/\text{mmol creatinine}$; $p = 0.001$, $d = 1.67$). Arabinose is another ubiquitous metabolite that is produced by the host and gut microbiota, but is also a component of LAM (Amin et al., 2021). Previous studies have shown arabinose as a proxy for LAM in active TB (De et al., 2015). Hence, its significantly elevated concentrations in this pediatric cohort of TBM cases, could be classified as a potential biomarker of *M. tb*—this requires further investigation. Arabinose has also been associated with other diseases. In a case of two autistic brothers, without any known metabolic disease, urinary arabinose concentrations were found to be six times greater than that in healthy children (Shaw et al., 1995b). Furthermore, increased levels of arabinose have been reported to have an inhibitory effect on sucrase (Seri et al., 1996), which could account for the increased levels of urinary sucrose in TBM cases found in this study.

Some organic acids are produced, at least in part, by intestinal gut bacterial metabolism. Clinically, these organic acids can be used as an indirect indicator of dysbiosis (Chapman et al., 2020), and the higher the quantities of these bacterial metabolites in the urine, the greater the bacterial quantities and activity in the gastrointestinal tract (Chapman et al., 2020). Elevated urinary concentrations of gut microbiota metabolites. Chalmers *et al.* (1979) indicated that 4- hydroxyphenylacetic acid is synthesized when amino acids, such as phenylalanine and tyrosine—both elevated in TBM—are metabolized by intestinal bacteria. Tyrosine is degraded to tyramine, and then deaminated and oxidized eventually to form 4-hydroxyphenylacetic acid, which is then excreted unchanged and unconjugated in the urine (van Der Heiden et al., 1971; Fellaman et al., 1977). The latter occurs in children with small-bowel disease or in various bacterial overgrowth syndromes; Chalmers *et al.* (1979) subsequently concluded that urinary 4-hydroxyphenylacetic acid serves as a useful marker for the screening of gut disorders in children (Chalmers et al., 1979). It has also been determined that roughly 50% of cystic fibrosis patients, and some patients with confirmed or suspected TB, have elevated urinary concentrations of 4-hydroxyphenylacetic acid in both adults and children with different types of pulmonary disease (Garrettson et al., 1971; van Der Heiden et al., 1971). The occurrence of 4- hydroxyphenylacetic acid has also been linked to the breakdown of amino acids.

Hippuric acid, also known as benzoylglycine or benzoylamino- acetate, is the glycine conjugate of benzoic acid (Lees et al., 2013). Increased levels of urinary hippuric acid have been linked to several diseases, including dysbiosis. This supports its recognition as a biomarker for microbial changes in the gut (Williams et al., 2010; Chapman et al., 2020). In a gas chromatography–mass spectrometry (GC-MS) metabolomics study conducted on a similar paediatric cohort, Mason *et al.* (2016b) observed a

significant increase of hippuric acid in the urine of TBM patients and attributed the observation to a drug-like phase II metabolic response of the host to products generated by the microbiota, indicating a highly active glycine-conjugated GLYAT-biotransformation system in TBM. Hence, the presence of increased urinary hippuric acid in TBM is supported by our previous metabolomics study, in which an alternative analytical platform was used.

Methylamine has been found in numerous tissues and bodily fluids, and it has been hypothesized that methylamine, and other closely similar short-chain aliphatic amines, may be involved in the abnormalities of the central nervous system seen during hepatic and renal disease, particularly when the blood-brain barrier is damaged. When compared to trimethylamine and dimethylamine, there is little information in the literature about the human urinary excretion of methylamine (Mitchell and Zhang, 2001; Pirisino et al., 2005; Guba et al., 2022). Methylguanidine is the immediate precursor to methylamine, thereby linking it to microbial metabolism, however, methylguanidine is also known to be an uremic (neuro)toxin (De Deyn et al., 2001) and an inhibitor of inducible nitric oxide synthase (iNOS), which is known to affect *M. tb* cerebral infection (Poh et al., 2022). Hence, methylguanidine is another metabolite of interest that is potentially produced directly by *M. tb*, and requires further investigation.

Formic acid is a common organic acid used and excreted by bacteria and no specific link has previously been made to TBM, however, formic acid has been associated as a potential biomarker of Alzheimer's disease, as a byproduct of the metabolism of formaldehyde (Wang et al., 2022). To our knowledge, no literature exists on the two cresols (o-cresol and m-cresol) detected as increased in TBM in this study, in relation to neuropathology.

Thus, these eight increased microbial metabolites originate almost exclusively from altered bacterial metabolism in the gut and are attributed to the severe TBM disease in this study. The occurrence of dysbiosis in TBM is supported by several studies and the results support further investigation of the gut-brain axis in TBM (Isaiah et al., 2020). A major limitation of this study is that we cannot account for treatment given to the TBM cases prior to admission to hospital, nor upon admission, and their effects on the gut microbiota.

3.5.6 Ketoacidosis

Ketoacidosis is a condition defined by increased ketone bodies, such as acetone, acetic acid and acetoacetic acid. Acetone (Figure 3.8A) and acetic acid (Figure 3.8B) were significantly elevated in the TBM cases (176.48 ± 179.09 $\mu\text{mol}/\text{mmol}$ creatinine and 61.77 ± 16.25 $\mu\text{mol}/\text{mmol}$ creatinine, respectively)

compared to the controls (26.82 ± 52.59 $\mu\text{mol}/\text{mmol}$ creatinine; $p = 0.014$, $d = 1.29$ and 24.73 ± 8.7 $\mu\text{mol}/\text{mmol}$ creatinine; $p < 0.001$, $d = 2.97$, respectively). However, acetoacetic acid (Figure 3.8C) in the TBM group was not statistically different from the control group ($p = 0.111$), but were practically significant ($d = 0.81$).

Ketones are metabolic end-products of fatty acid metabolism that occurs in the liver (Dhillon and Gupta, 2021; Cruzat et al., 2018). During perturbed energy metabolism, or in the absence/diminished availability of carbohydrates, fats become the primary source of energy, and large levels of ketones are produced as a metabolic by-product. Hence, the occurrence of ketoacidosis also supports evidence for insulin resistance, as elevated glucose levels are found in TBM patients. The increased levels of ketone bodies indicate that this elevated glucose is not being used for energy production; instead, fatty acid metabolism has become the major source of energy production in TBM (Blau et al., 2014; Dhillon and Gupta, 2021).

Acetone, which may give the breath of ketotic patients a typical odor, is formed via the non-enzymatic decarboxylation of acetoacetate (Blau et al., 2014). The TBM group's urinary ketone values in this study are indicative of their poor clinical health. Ketoacidosis also supports the metabolic burst that we described previously. Several ketoacidosis markers were also identified in our previous GC-MS metabolomics study of TBM (Mason et al., 2016b), which included the ketosis markers 2- hydroxybutyric acid, 3-hydroxybutyric acid, 2-methyl-3- hydroxybutyric acid, and acetoacetic acid, in the urine of the TBM patients.

3.5.7 Increased nitrogen excretion

Urea was identified in the statistical analyses of the raw data as being significantly increased in the urine of the TBM cases compared to the controls. Increased urea is irrevocably linked to elevated nitrogen excretion. Urea in a ^1H NMR spectrum presents as a very distinctive broad singlet near to the suppressed water signal. Owing to the width of the urea signal in the ^1H NMR spectra, and the known influence of water suppression on the urea peak, accurate quantification of urea was not possible.

N-Acetylglutamine (Figure 3.8F), another metabolite of increased nitrogen excretion, was significantly elevated in the TBM patients ($335.45 \pm 184.47 \mu\text{mol}/\text{mmol creatinine}$) compared to the controls ($102.43 \pm 25.98 \mu\text{mol}/\text{mmol creatinine}$; $p \leq 0.001$, $d = 2.21$). N-Acetylglutamine is an amino acid derivative, and the downstream metabolite of D-glutamine and D-glutamate metabolism (Zhou et al., 2018). N-acetylglutamine is used as a neurotransmitter in the brain, and perturbed N-acetylglutamine can be linked to altered neuronal stability and/or function. In a study that conducted RNA-sequencing on whole blood and CSF of ventricular and lumbar punctures of paediatric patients treated for TBM, Rohlwick *et al.* (2019) indicated immune responses and neural excitotoxicity which depicts the immunological events that occurs in the brain. Of the 389 differentially expressed genes, 45 genes are mainly associated with glutamate excitotoxicity, key excitatory neurotransmitter in learning and memory also, mediator associated with several neurological diseases (Rohlwick et al., 2019). N-Acetylglutamine is also classified as a uremic toxin and increased urinary levels indicate severe neurological complications (typical in TBM), advanced catabolism and/or possible kidney damage. Glutamine supplementation is a well-known strategy to treat some critically ill patients, such as prematurely born infants (Darmaun et al., 2018), and could be considered in paediatric TBM.

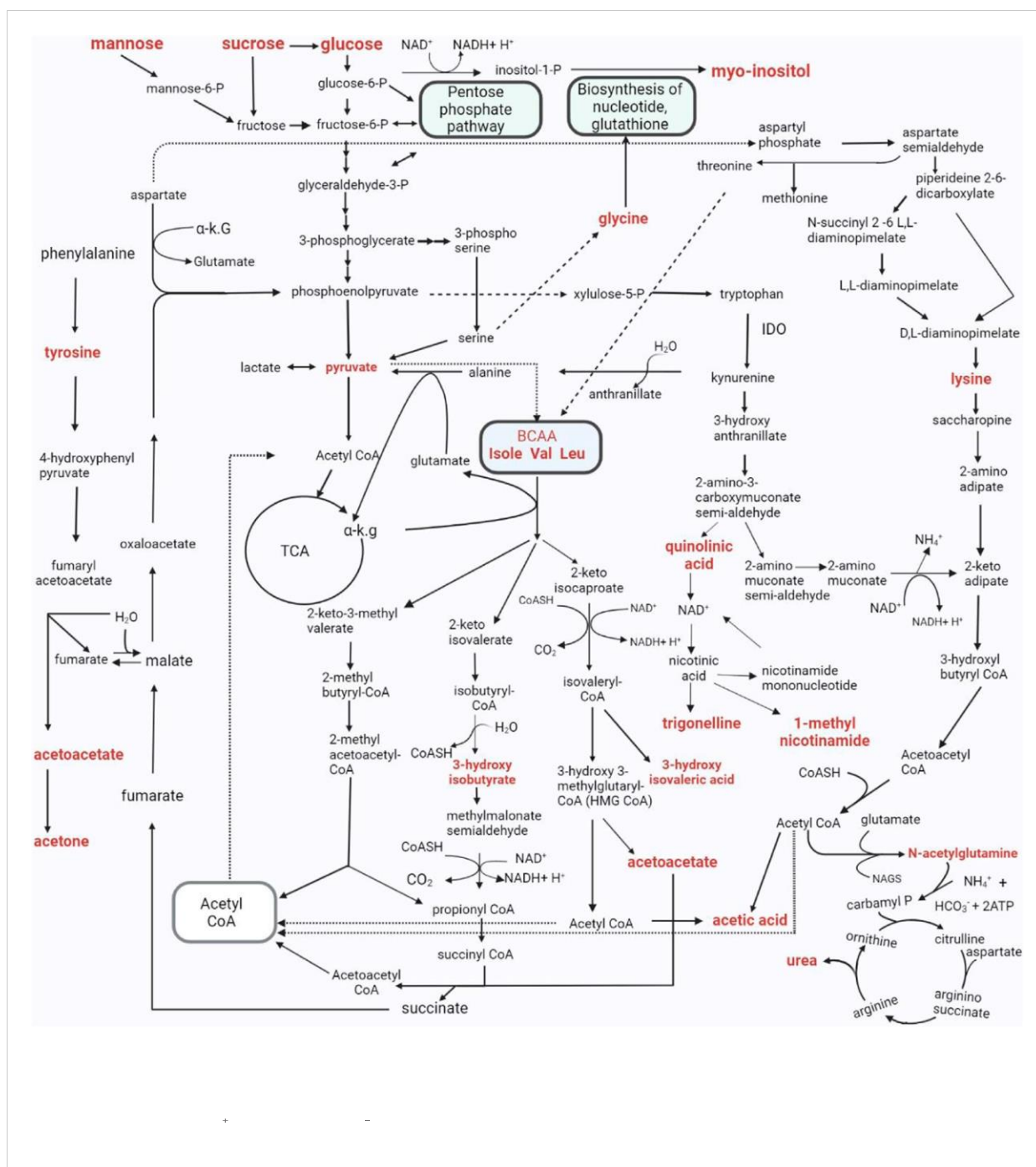


Figure 3.9 The metabolic pathways characterizing the changed urinary metabolic profiles in TBM stage 3 patients.

The metabolites in bold denote those detected in significantly elevated concentrations in the TBM stage 3 patients when compared to the controls. As indicated in the pathway, P=phosphate; α K.G=alpha ketoglutarate; IDO - Indoleamine 2, 3-dioxygenase; Isole=Isoleucine; Val=valine; Leu=leucine; CoA, CoASH=coenzyme A; TCA – tricarboxylic acid cycle; NH_4^+ – ammonium; HCO_3^- – bicarbonate; ATP=adenosine triphosphate; NAD=nicotinamide adenine dinucleotide; BCAA=branched-chain amino acids.; H_2O =water; NAGS=N-acetyl glutamate synthase.

3.6 Urinary metabolic map of TBM

We took all of the above described metabolic pathways characterizing the urinary profiles of advanced hospitalized TBM paediatric cases, and connected them into a metabolic map (Figure 3.9). Figure 3.9 illustrates how all these metabolic pathways connect with each other, providing new connections

(information) of metabolic pathways, and their complexity, in advanced TBM in paediatric patients. With this new information, we offer new insights and approaches into future TBM research.

3.7 Limitations of the study and TB medications

The limitations in this study included: 1) a small sample size (no *a priori* sample size considerations were factored in because the samples were retrospectively selected from a previous study), 2) no validation sample cohort, 3) there was no information available on the timing and type of treatment regime administered to the undiagnosed TBM patients at the referral healthcare centers, before being taken to the hospital, 4) the controls were heterogeneous and, due to ethical restrictions, the only information available for the controls were that they were pediatric patients without meningitis, no neurological symptoms, and from the same geographical region as the TBM patients. Upon inspection of the ^1H -NMR spectra, it was found that pyrazinamide (PZA), isoniazid (IZA) and ethionamide were detected in all the time zero stage three (T0) TBM cases urine samples, except for 2 cases (103 and 114) which had no IZA and no ethionamide. Rifampicin and its two metabolites—3-formylrifampicin and 25-deacetyl-rifampicin, were not detectable in the ^1H -NMR spectra in any of the urine samples. The inability to see rifampicin and its two known urinary metabolites could be that either the rifampicin has been detoxified in the body into a form that we do not know of, or, more likely, the NMR analytical platform lacks the sensitivity (i.e., below the detection limit of the NMR).

3.8 Conclusion

We consider the significant metabolites identified in this study as being the best urinary metabolic characterization of TBM, to date. The precise outcome of this study indicated that TBM under initial treatment could be well differentiated from controls, based upon urinary metabolic profiles analyzed by ^1H NMR spectroscopy. These results support metabolomics data produced in our previous GC-MS metabolomics study conducted using a similar paediatric cohort. Thus, these results support the use of ^1H NMR metabolomics in characterizing TBM, and in the pursuit of non-invasive metabolic markers of TBM to aid in the early differential diagnosis and subsequent treatment of TBM—something that is greatly needed in the paediatric population, the patient group most at risk of mortality.

Moreover, several clinical aspects of the studied cohort of advanced TBM were inferred from the urinary metabolomics data, which need to be confirmed from blood samples collected from TBM cases. The findings related to illness-induced-malnutrition can have therapeutic impact. Malnutrition can be subdivided in deficiency of micronutrients (e.g., vitamins) and macronutrients (e.g., proteins). We found (a) deficient vitamin B3 status expressed by increased urinary 1-methylnicotinamide—upregulated utilization of vitamin B3 from increased tryptophan catabolism, and increased urinary trigonelline; (b) increased nitrogen excretion, which reflects the elevated catabolic state of patients and their related malnutrition. For instance, shortage of the semi essential amino acid glutamine can lead to immune dysfunction (Cruzat et al., 2018). Thus, it is our recommendation that the nutritional status of TBM cases should be assessed with comparison made to pulmonary TB cases or controls, in order to determine whether the findings are specific to TB or TBM. Potentially, additional supplementation of vitamin B and

glutamine may be a consideration in the nutritional component of the future overall management of TBM.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the Health Research Ethics Committee (HREC) of Stellenbosch University (ethics approval no. N16/11/142 and N11/03/061 for group 1 and 2 respectively), as well as by the HREC of the North-West University, Potchefstroom campus (ethics approval no. NWU-00063-18-A1-01). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

SI: Data curation, Formal Analysis, Investigation, Methodology, Software, Writing—original draft. DL: Formal Analysis, Investigation, Methodology, Supervision, Writing—review and editing. MR: Formal Analysis, Methodology, Writing—review and editing. RS: Data curation, Methodology, Resources, Writing—review and editing. SE: Data curation, Methodology, Writing—review and editing. AT: Funding acquisition, Project administration, Resources, Supervision, Writing—review and editing. MK: Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing—review and editing. SM: Conceptualization, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmolb.2024.1253983/full#supplementary-material>

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**PART 3 URINARY MARKERS OF MYCOBACTERIUM TUBERCULOSIS AND
DYSBIOSIS IN PAEDIATRIC TUBERCULOUS MENINGITIS CASES UNDERGOING
TREATMENT**

CHAPTER 4

RESEARCH

Open Access



Urinary markers of *Mycobacterium tuberculosis* and dysbiosis in paediatric tuberculous meningitis cases undergoing treatment

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4.1 Abstract

Background The pathogenesis of tuberculous meningitis (TBM) involves infection by *Mycobacterium tuberculosis* in the meninges and brain. However, recent studies have shown that the immune response and inflammatory processes triggered by TBM can have significant effects on gut microbiota. Disruptions in the gut microbiome have been linked to various systemic consequences, including altered immunity and metabolic dysregulation. Inflammation caused

by TBM, antibiotic treatment, and changes in host immunity can all influence the composition of gut microbes. This complex relationship between TBM and the gut microbiome is of great importance in clinical settings. To gain a deeper understanding of the intricate interactions between TBM and the gut microbiome, we report innovative insights into the development of the disease in response to treatment. Ultimately, this could lead to improved outcomes, management strategies and quality of life for individuals affected by TBM.

Method We used a targeted liquid chromatography–tandem mass spectrometry (LC-MS/MS) approach to investigate metabolites associated with gut metabolism in paediatric participants by analysing the urine samples collected from a control group ($n = 40$), and an experimental group ($n = 35$) with confirmed TBM, which were subdivided into TBM stage 1 ($n = 8$), stage 2 ($n = 11$) and stage 3 ($n = 16$).

Findings Our metabolomics investigation showed that, of the 78 initially selected compounds of microbiome origin, eight unique urinary metabolites were identified: 2-methylbutyrylglycine, 3-hydroxypropionic acid, 3-methylcrotonylglycine, 4-hydroxyhippuric acid, 5-hydroxyindoleacetic acid, 5-hydroxyhexanoic acid, isobutyrylglycine, and phenylacetylglutamine as urinary markers of dysbiosis in TBM.

Conclusion These results – which are supported by previous urinary studies of tuberculosis – highlight the importance of gut metabolism and of identifying corresponding microbial metabolites as novel points for the foundation of improved management of TBM patients.

Keywords Urine, Microbiota, Paediatric, Tuberculous meningitis (TBM), Metabolomics, Liquid chromatography- tandem mass spectrometry (LC-MS/MS)

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4.2 Introduction

Before the coronavirus (COVID-19) pandemic, tuberculosis (TB) was ranked as the leading cause of infectious disease mortalities worldwide [1, 2]. Tuberculous meningitis (TBM) is a severe form of extrapulmonary TB, resulting in high morbidity and mortality, especially in children under the age of 12 years [3]. Compared to that of pulmonary TB, less research has been conducted on TBM, mainly due to difficulty in attaining research sample material, particularly cerebrospinal fluid, comparatively lower prevalence of TBM and difficulty in diagnosis. Hence, much still needs to be done to better understand *Mycobacterium tuberculosis* (*M. tb*), the causative agent, in the context of TBM, the subsequent immunopathological interactions and the resulting systemic metabolic adaptations/response of the host, which in turn typically leads to new therapeutic approaches [2–7]. There is also an urgent need for more effective and earlier diagnostic tools to detect TBM, especially in children, at an early stage of the disease.

The human gut microbiome is a complex dynamic ecosystem. Dysbiosis – disruption of the gut microbiota has been found to be strongly correlated with many infectious diseases, including TB and TBM. Mason et al. [8] previously identified four urinary metabolites – methyl citric, 2-ketoglutaric, quinolinic and 4-hydroxyhippuric acids, ascribed to an altered host microbiome in children with TBM. Zhou et al. [9] observed that the microbiota of both intra and extra-TB lesions are similar, and identified *Mycobacteria* and *Porphyromonas* as co-factors for lesion formation. Krishna et al. [10] reported significant changes to the microbial composition of the lung micro-biome of pulmonary TB patients vs. control groups, and also different opportunistic pathogens characterized the *M. tb*-infected cases. Isaiah et al. [11] additionally identified 15 urinary metabolites characterising advanced stage TBM, resulting from six dysregulated metabolic pathways and a disrupted gut microbiome. Luies et al. [12] identified 50 urinary metabolite markers characterising pulmonary TB, as well as investigated successful and unsuccessful treatment outcomes of standard anti-TB therapy, with one of the main reasons for unsuccessful treatment outcome being an altered gut microbiome.

Metabolomics is a modern scientific approach, based upon using the latest analytical and bioinformatics methods, and capable of accurate quantitative characterization of metabolites [13]. The discipline has resulted in a substantial and rapid advancement of knowledge by identifying new biomarkers associated with various diseases, and metabolomics research into *M. tb* infection is no exception [14–16]. As described, various untargeted metabolomics

studies have identified dysbiosis as an important component of *M. tb* infection. We postulate that a targeted microbiome metabolite metabolomics study, using urine samples collected from patients with confirmed TBM during the 6 month treatment regimen, would show changes in microbiome metabolites associated with: (1) the gut microbiome, (2) host–microbe interaction, and also possibly (3) *M. tb* death – identifiable by metabolites associated with the *M. tb* cell wall. Thus, this study aimed to identify urinary metabolites associated with host and/or microbial changes associated with dysbiosis in TBM cases during the course of treatment.

4.3 Materials and methods

4.3.1 Sampling

The sampling cohort in this study were from a population of infants and children (< 13 years) living in the Western Cape province of South Africa, a region well known for a high prevalence of TB [17–19]. Paediatric patients from primary and secondary healthcare service centres were referred to the paediatric department at the Tygerberg Hospital in Cape Town. Written and informed consent or assent was obtained from all participants.

The urine samples collected from the participants were divided into two groups: Group 1, the controls ($n = 40$) and Group 2, confirmed TBM ($n = 35$), which were sub- divided into TBM stage 1 ($n = 8$), stage 2 ($n = 11$) and stage 3 ($n = 16$), as described by van Toorn et al. [20]. The controls (Group 1) were age-matched and collected from the same geographical region as the TBM paediatric patients. They were seen at the general paediatrics outpatient clinic of Tygerberg Hospital for follow up of an underlying subacute or chronic illness. None of them had meningitis or neurological disease during sampling. The TBM urine samples (Group 2) were collected from paediatric cases admitted to Tygerberg Hospital due to suspected meningitis, most of whom were seriously ill, and later confirmed to be “definite TBM”, identified by the presence of *M. tb* in their cerebrospinal fluid by microscopy, culture, and/or detection by nucleic-acid techniques, with or without a focal neurological deficit [21, 22]. The TBM patients were stabilized before hospital discharge (the median time from admission to discharge in the study setting over the past 38 years was 16 days, inter- quartile range 12–23 days) and a baseline urine sample was collected upon initiation of home treatment, as described by [23]. Ethical approval (numbers N16/11/142 and N11/03/061 for groups 1 and 2, respectively), was obtained by the Health Research Ethics Committee (HREC) at Stellenbosch University in accordance with the Western Cape provincial government policy and the HREC at North-West University, Potchefstroom campus (ethics approval number NWU-00063-18-A1-01). All participants with a confirmed positive HIV status

were excluded from this study, since HIV co-infection would significantly complicate an already complex metabolic profile. Supplementary Table [4.S1](#) shows demographic information of study cohort.

4.3.2 Sample handling and storage

After collection, all urine samples were stored at -80°C in the Molecular Biology and Human Genetics Division of the University of Stellenbosch. All samples were transported overnight on dry ice and stored in a designated freezer (-80°C) in a biosafety level 3 (BSL-3) laboratory at North-West University, Potchefstroom campus. Initially, all urine samples were thawed in a biosafety cabinet and 100 μL of each sample (TBM and controls) was aliquoted into a single tube to create a pooled urine quality control sample (QC). A separate pooled urine sample consisting of 100 μL aliquots from the TBM cases only was also prepared (PS). Each pooled sample (QC and PS) was then vortexed and equal aliquots of each sample were placed in Eppendorf microcentrifuge tubes. These two types of quality control samples were used to monitor the analytical performance during the duration of the experiment. All samples (experimental, QC and PS) were stored again at -80°C .

4.3.3 Chemicals and standards

All mobile phase solutions were prepared with UPLC–MS grade solvents of water and acetonitrile from Anatech (Burdick and Jackson), and formic acid was obtained from Merck (South Africa). Supplementary Table [S3](#) shows the 78 organic acid standards used in this study, and their source. NOTE: these specific 78 organic compounds were selected for this targeted study because they are considered to be part of gut microbial metabolism.

4.3.4 Sample preparation and LC–MS/MS analysis

Urine samples were thawed at room temperature and homogenized by vortex mixing. A 50 μL volume of urine was aliquoted into a microcentrifuge tube and to each sample, 50 μL of internal standard (heptanoylglycine: 576.8 μM) and 200 μL of HPLC water with 0.1% formic acid were added, and the sample vortexed. A 200 μL volume of the prepared sample was then transferred into a 2 mL glass vial containing an insert and capped. The samples were analysed (in 19 batches of 12–14 samples interspaced with QC and PS samples) on an Infinity II 1290 UHPLC–MS/MS system (Agilent Technologies, Chemetrix, South Africa). Prior to sample analysis, Mobile phase (A and B) standard solutions were freshly prepared: (A) HPLC water with 0.1% v/v formic acid, (B) acetonitrile with 0.1% v/v formic acid. The temperature within the multi-sampler was set at 4°C and the column temperature at 22°C . The flow rate was set at 0.4 mL/min, sample injection at 2

μL and the run time was 27 min. LC-separation was accomplished using an Acquity C18 column UPLC HSS T3 1.8μm (2.1 × 100 mm) with an Acquity VanGuard Pre-Column HSS T3, 2.1 × 5 mm (Waters, South Africa). The gradient programme of the 27 min run in negative ionization mode and the MS conditions are described in Supplementary Table 4.S5. The PS were randomized across the batches with QCs placed at the start, middle and end of each batch run. All QC and PS extracts were injected twice. Data acquisition and processing was conducted using Agilent Mass Hunter workstation software, specifically Acquisition V10 and Quantitative Analysis for QQQ V10 Software. The monitored ions and their retention times, which were determined using standards, are listed in Table 4.S4. The peak area was used for quantification and each analyte quantified relative to the internal standard. A data matrix was generated in Excel that consisted of all cases as rows, and the 78 metabolites as columns, with each entry representing a relative concentration.

4.3.5 Statistical analysis

For all multivariate statistical analyses, a log transformation of the data was conducted to correct for data distribution (i.e., Gaussian distribution; parametric data) and heteroscedasticity, and auto scaling was applied to allow different values with differences in magnitudes of order (concentrations) to be more comparable. All univariate statistical analyses were done on the non-parametric (untransformed) data. MetaboAnalyst 5.0 (www.metaboanalyst.ca) was used to provide an initial qualitative visualization of the distribution and variation of the data at a multivariate level by using unsupervised principal component analysis (PCA). MetaboAnalyst was also used for the multivariate statistical analyses of the data using supervised partial least squares discriminant analysis (PLS-DA) – which was tested for overfitting by conducting a permutation test – and univariate assessment of the data by a one-way ANOVA (Kruskal-Wallis test) with a statistical significance set at a p-value cut-off at a false discovery rate (FDR) less than 0.01. The PLS-DA model was used quantitatively to identify significant metabolites as having variables important in projection (VIP) values > 1.0 for components 1 and 2. Additional univariate statistics was conducted using the non-parametric equivalent of a one-way ANOVA, with a significant adjusted p-value < 0.01. Microsoft Excel was used to calculate effect size, in order to determine practical significance. The most important metabolites, best describing the variation between the compared groups, were identified using the following criteria: PLS-DA VIP value (components 1 and 2) > 1.0 and ANOVA Kruskal-Wallis FDR p-value < 0.01 and effect size d-value ≥ 0.8. GraphPad Prism (V 10.0.2) was used to create violin plots of the metabolites identified as important – illustrating distribution of data frequency, medians, and interquartile ranges, and

statistically significant differences (with multiple corrections) noted as ANOVA Kruskal–Wallis FDR p-value < 0.01, as well as linear regression plots.

4.4 Results

4.4.1 Quality assurance

PCA of all the batches ($n = 19$) was conducted to determine if there were any batch effects. Notably, one case (014_T3) stood out as an outlier (see supplementary information (SI) – Fig. 4.S1) and was removed because this was a highly diluted sample (creatinine concentration = 0.026 mM) and normalizing this case to creatinine overinflated all other values. Reassessment of the PCA of all batches after removal of this single outlier case showed no batch effects, therefore batch correction was not necessary. An assessment of the PCA (Fig. 4.S2) showed that all the QC and PS samples clustered closely together, confirming that there were no batch effects in the data, supporting the data for further statistical analyses and metabolite marker selection. An additional quantitative quality check was done on all the QC samples by calculating the coefficient of variation (CV) of each, and across each batch. Of the 78 metabolites analysed, three metabolites had an overall CV greater than 20%, namely, propionylglycine (55.06%), suberylglycine (38.7%), and 3,4-dihydroxybenzoic acid (22.41%), which were subsequently removed from the metabolite list. Hence, quality assurance checks of the data removed one TBM case and three metabolites, yielding 75 metabolites suitable for analysis and biological interpretation.

4.4.2 Initial urinary profile

The first urine sample collected from each TBM participant after discharge from hospital was labelled “T1” to represent the initial urine sample. There were initially 7, 11 and 16 urine samples representing TBM stages 1, 2 and 3, respectively. The PCA indicated that the metabolome of the control group differentiated significantly from the TBM cases, with PCA 1 & 2 explaining 48% of the total variation (Fig. 4.1). The natural separation in the PCA between the control group and TBM cases provided confidence in using PLS–DA modelling. The PLS–DA model generated (Fig. 4.S3) is also supported by a permutation p-value of 0.001 (Fig. 4.S4), thus the data were not over-fitted in the PLS–DA model. Quantitative data indicating 30 metabolites at T1 that are of statistical and practical importance are shown in Table 4.S2. No differentiation between TBM stages at T1 could be seen based upon PCA (Fig. 4.S3).

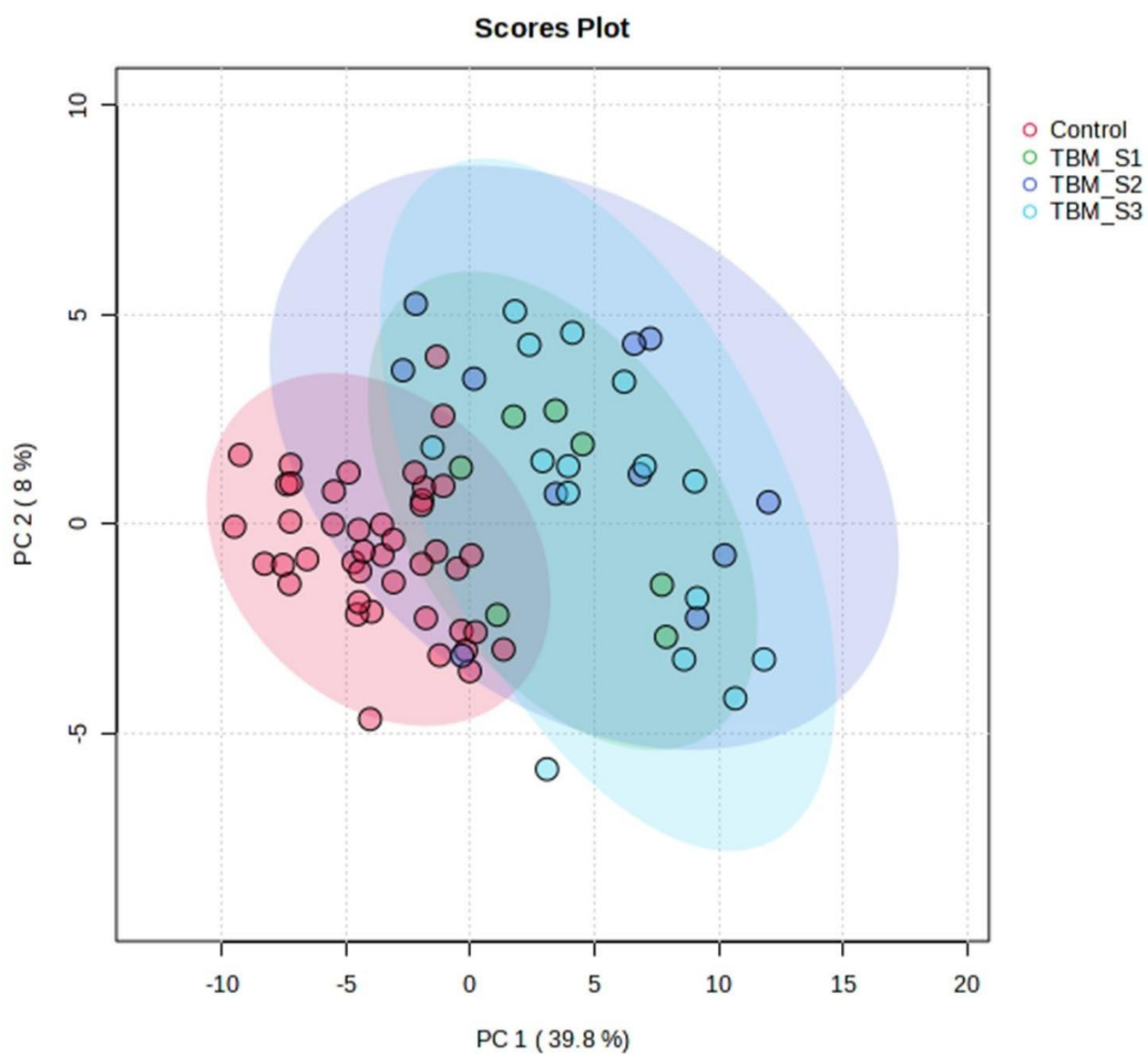


Figure 4.1 PCA scores plot of initial urinary profile for all 75 metabolites analysed at T1. PCA 1 & 2 explained 48% of the total variation.

4.4.3 Longitudinal urinary profile during TBM treatment

The same statistical approach as described for the initial urinary profile comparisons was adopted for time points 2 to 6 (T2–T6), for the samples collected monthly during the 6-month treatment regimen. Hence, T2 was one month after the initial sample was collected and represented the altered metabolome after one month of treatment; T6 was collected five months after the initial sample and represented five months of treatment. It is important to note that not all TBM cases had 100% adherence to IZN, PYR and RIF throughout the entire treatment period. By T4, there was a decrease in adherence for IZN: 100–89.7%; PYR: 100–95.2%; and RIF: 65.5–34.5%; although, RIF adherence declined over time apparently due to its bad taste [23]. For all longitudinal time points (T2–T6), the PCAs similarly reflected (see SI) some TBM cases beginning to cluster closer to controls; however, a clear differentiation between the control group and all three TBM stages remained. Similarly to the results of the initial urinary profile comparison, the PLS–DA model remained reliable (permutation p-value < 0.05). Following the same rule described for the initial urinary profile comparison, five additional data sets were generated (see SI). A Venn graph of the common and unique metabolites for all six time points is shown in Fig. 4.2. Furthermore, similarly to T1 (Fig. S3), TBM stages did not differentiate from each other across the longitudinal comparison of the study.

Figure 4.2 shows that eight significant metabolites (2-methylbutyrylglycine, 3-hydroxypropionic acid, 3-methylcrotonylglycine, 4-hydroxyhippuric acid, 5-hydroxyindoleacetic acid, 5-hydroxyhexanoic acid, isobutyrylglycine, and phenylacetylglutamine – see violin plots in Fig. 4.3 and linear regression plots in Fig. 4.4) uniquely occur only in T1. Since these eight metabolites occur exclusively in the T1 samples (not significant in T2–T6), they represent the metabolites induced by TBM disease and hence will be interpreted in this context. Other significant metabolites that occurred uniquely at the other time points were butyrylglycine at T6, and 2-ketoglutaric acid and 3-hydroxyglutaric acid at T3. The 13 significant metabolites common across all time points included: 2-hydroxyadipic acid, 2-hydroxyglutaric acid, 3-hydroxyisovaleric acid, 3-methylglutaconic acid, 4-hydroxyphenylacetic acid, aconitic acid, ethylmalonic acid, glucaric acid, hexanoylglycine, isocitric acid, N-acetylaspartic acid, N-acetylphenylalanine, and vanillic acid. Since these 13 metabolites were common across all six treatment time points, they are markers of treatment and will be discussed further in this context. Other significant metabolites that occurred commonly at some, but not all, time points were: 2-hydroxyphenylacetic acid at T1, T3 and T4; 2-octenedioic acid at T1, T3,

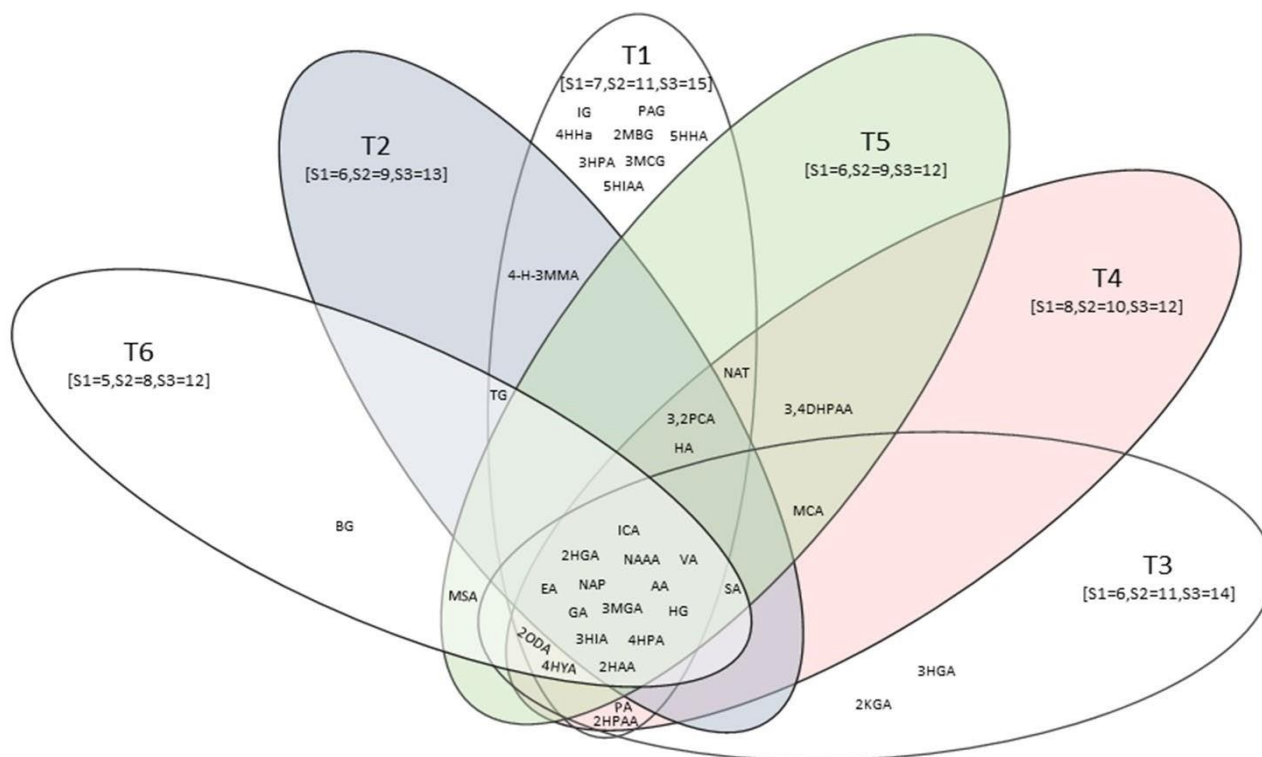


Figure 4.2 Venn diagram of significant metabolites across all six time points of TBM treatment.

Number of cases according to TBM stages per time point are given in square brackets. Abbreviations: 2HGA, 2-hydroxyglutaric acid; 3HIA, 3-hydroxyisovaleric acid; 3MGA, 3-methylglutaconic acid; 4HPAA, 4-hydroxy-phenylacetic acid; AA, aconitic acid; EA, ethylmalonic acid; GA, glucaric acid; HG, hexanoylglycine; ICA, isocitric acid; NAAA, N-acetylaspartic acid; NAP, N-acetylphenylalanine; VA, vanillactic acid; 2HAA, 2-hydroxyadipic acid; 2HPAA, 2-hydroxyphenylacetic acid; 2KGA, 2-ketoglutaric acid; 2MBG, 2-methylbutyrylglycine; 2ODA, 2-octenedioic acid; 2,3PCA, 2,3-pyridinecarboxylic acid; 3HGA, 3-hydroxyglutaric acid; 3HPA, 3-hydroxypropionic acid; 3MCG, 3-methylcrotonylglycine; 3,4DHPAA, 3,4-dihydroxyphenylacetic acid; 4-H-3MMA, 4-hydroxy-3-methoxymandelic acid; 4HPLA, 4-hydroxyphenyllactic acid; 4HHA, 4-hydroxyhippuric acid; 5HIAA, 5-hydroxyindoleacetic acid; 5HHA, 5-hydroxyhexanoic acid; BG, butyrylglycine; HA, homovanillic acid; IG, isobutyrylglycine; MCA, methylcitric acid; MSA, methylsuccinic acid; NAT, N acetyltyrosine; PAG, phenylacetylglutamine; PA, pimelic acid; SA, suberic acid; TG, tiglylglycine

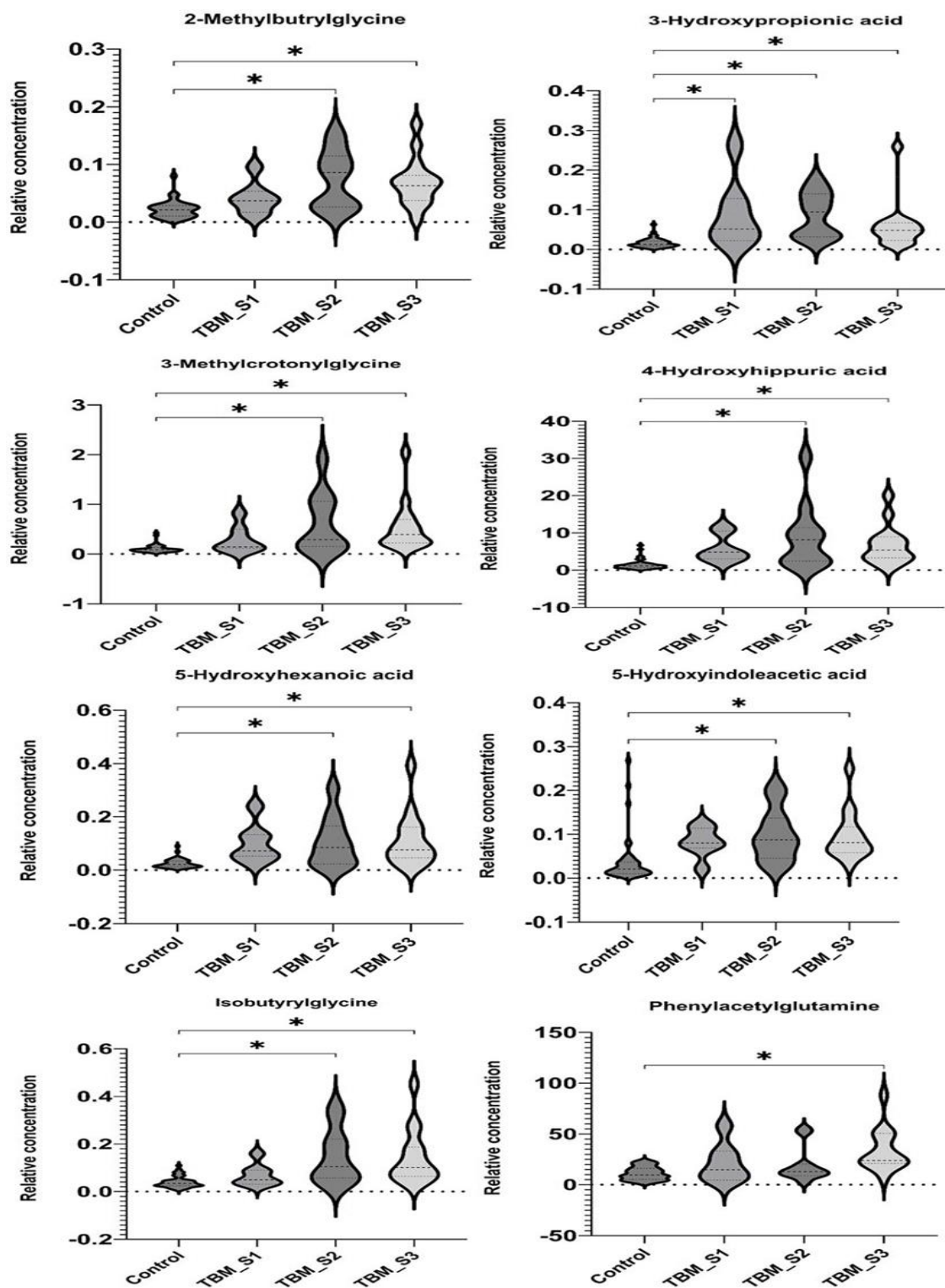


Figure 4.3 Violin plots of eight significant metabolites at T1.

2-methylbutyrylglycine, 3-hydroxypropionic acid, 3-methylcrotonylglycine, 4-hydroxyhippuric acid, 5-hydroxyindoleacetic acid, 5-hydroxyhexanoic acid, isobutyrylglycine, and phenylacetylglutamine. Control, TBM S1, TBM S2 and TBM S3 consist of 40, 7, 11 and 15 number of samples, respectively. Plots show distribution of data frequency, as well as the median and inter-quartile ranges. Statistically significant differences (ANOVA Kruskal-Wallis FDR $p < 0.01$, with multiple corrections) indicated by *

T4, T5 and T6; 2,3-pyridinecarboxylic acid (quinolinic acid) at T1, T2, T4 and T5; 3,4-dihydroxyphenylacetic acid at T4 and T5, 4-hydroxy-3-methoxymandelic acid at T1 and T2; 4-hydroxyphenyllactic acid at T1, T3, T4, T5 and T6; homovanillic acid at T1, T2, T4 and T5; methylcitric acid at T3, T4 and T5; methylsuccinic acid at T5 and T6, N-acetyltyrosine at T1, T4 and T5; pimelic acid at T3 and T4; suberic acid at T2, T3, T4, T5 and T6; and tiglylglycine at T1, T2 and T6.

4.5 Discussion

In this targeted LC–MS/MS metabolomics investigation, of initially 78 selected compounds of microbiome origin, we identified eight unique, statistically significant urinary metabolites at T1: 2-methylbutyrylglycine (2MBG), 3-hydroxypropionic acid (3HPA), 3-methylcrotonylglycine (3MCG), 4-hydroxyhippuric acid (4HHA), 5-hydroxyindoleacetic acid (5HIAA), 5-hydroxyhexanoic acid (5HHA), isobutyrylglycine (IG), and phenylacetyl- glutamine (PAG). Five of these eight significant metabolites (2MBG, 3MCG, 4HHA, 5HIAA, and PAG) showed a constant elevated level for TBM stage 1 (Fig. 4.4) across the entire treatment period and we consider this to be the background effect of the treatment. We consider these eight metabolites to be associated with the disease pathogenesis (TBM), as markers of the breakdown of *M. tb* cell wall caused by anti-TB medication, and/or dysbiosis commonly associated TBM. We discuss the biological significance of these eight metabolites in terms of: (1) associated metabolic pathways and corroboration from the literature; (2) their origin – linking these metabolites to either microbial or host origin, or a combination of both (a co-metabolite); (3) the insight(s) into the biology of the breakdown of *M. tb* cell wall within the host; (4) understanding the gut dysbiosis associated with TBM.

4.5.1 Metabolic map and link to previous TB marker studies

It should be noted that seven of the eight significant metabolites identified in this study have previously been linked to pulmonary TB. IG, 3MCG and 2MBG are glycine conjugates of intermediates (isobutyryl- CoA, 3-methylcrotonyl-CoA and 2-methylbutyryl-CoA, respectively) of branched-chain amino acid catabolism (Fig. 4.5). Based upon a transcriptomics study by Jiang et al. [24], the expression of the enzyme branched-chain amino acid transferase-1 (BCAT1) is decreased, whereas BCAT2 increases, in TB cases. Both BCAT1 and BCAT2 are of human origin, with BCAT1 being localized in the cytoplasm and BCAT2 compartmentalized in the mitochondria. An increased expression of (mitochondrial) BCAT2 is in line with the enhanced energy metabolism associated with TB [8]. However, raised BCAT2 activity is not specific to TB only, but has also been linked to other inflammatory diseases [25]. Furthermore, propionyl-CoA

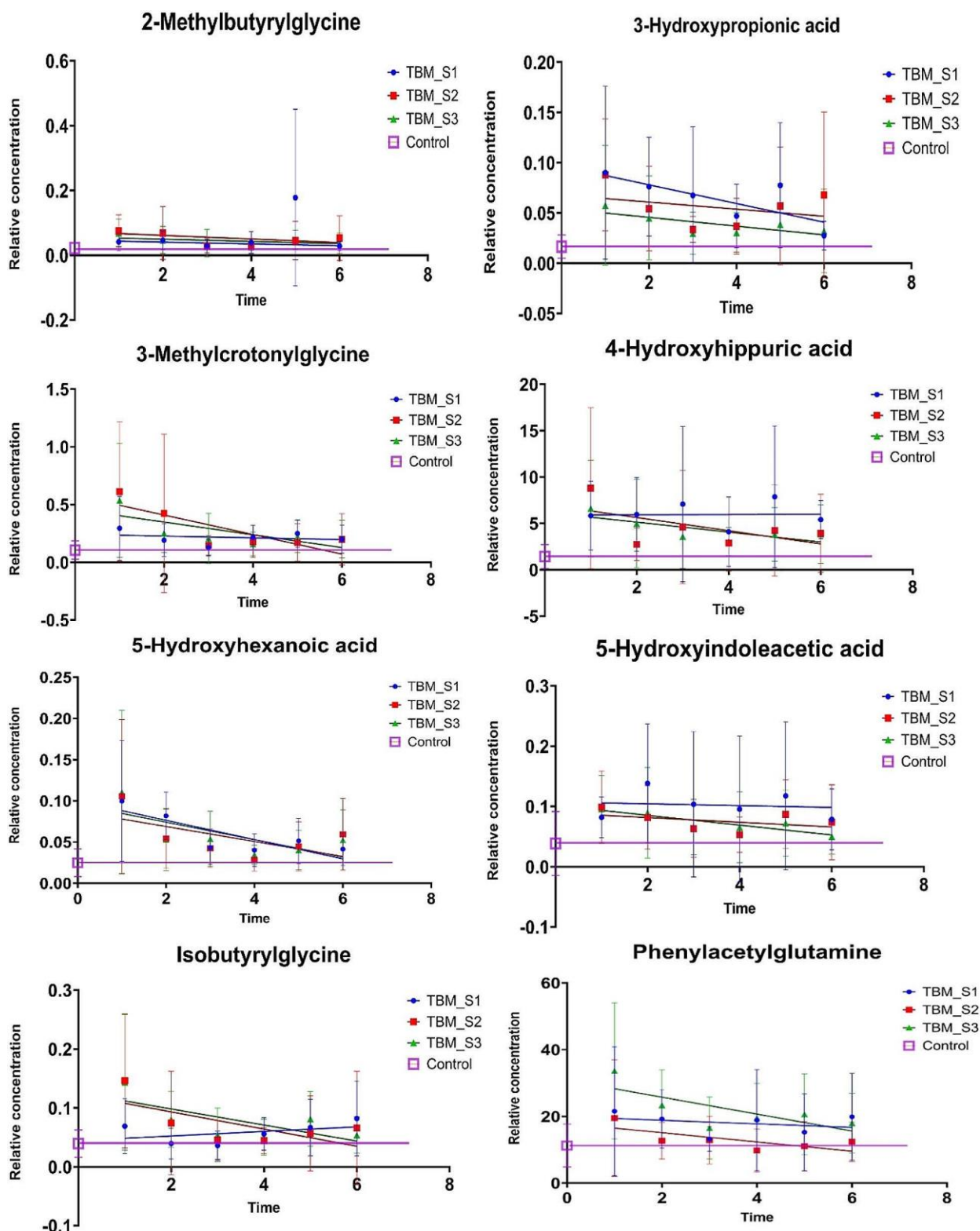


Figure 4.4 Linear regression plots with error plots across all six TBM treatment points (T1–T6), for control, TBM_S1, TBM_S2 and TBM_S3, of eight metabolites found to be significant only at T1.

All eight metabolites show increased levels at T1, returning to normal (control) levels at final treatment point (T6) for all S2 and S3, while some metabolites remain consistently elevated for S1. Metabolites: 2-methylbutyrylglycine, 3-hydroxypropionic acid, 3-methylcrotonylglycine, 4-hydroxyhippuric acid, 5-hydroxyindoleacetic acid, 5-hydroxyhexanoic acid, isobutyrylglycine, and phenylacetylglutamine

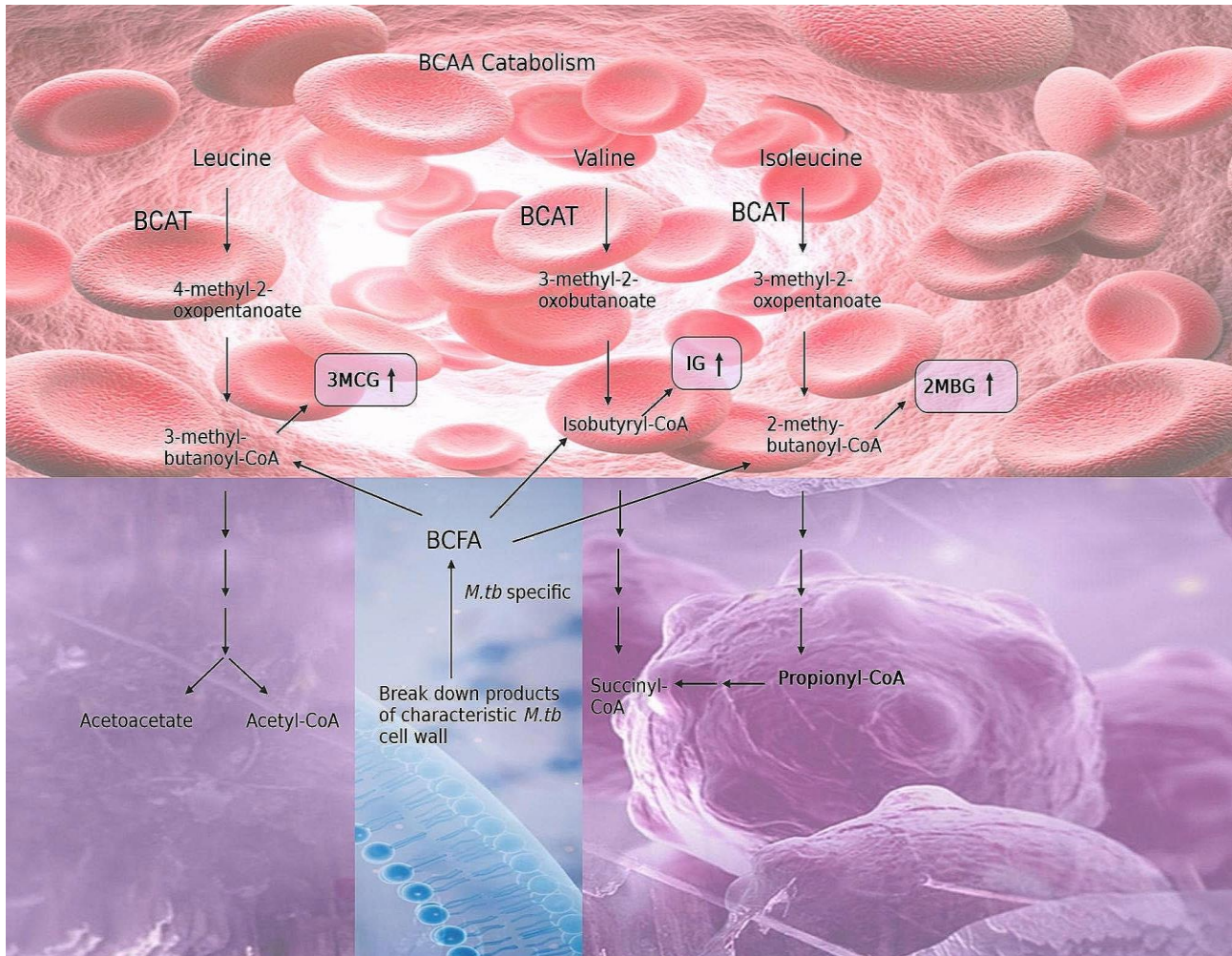


Figure 4.5 Branched-chain amino acid (BCAA) catabolism.

(in the host: red; and as co-metabolism (both host and microbial): purple). Elevated 3-methylcrotonylglycine (3MCG), isobutyrylglycine (IG), and 2-methylbutyrylglycine (2MBG) are urinary markers of dysbiosis at T1 of TBM treatment. It should be noted that the three BCAA intermediates (3-methylbutanoyl-CoA, isobutyryl-CoA and 2-methylbutanoyl-CoA) can also be linked to branched-chain fatty acids that are specific to deterioration of the unique cell wall of *M. tb* (microbial specific: blue). Propionyl-CoA is also an end product that can be linked to Fig. 4.6

is an end product of valine and isoleucine catabolism, and is a microbial metabolite, as discussed later.

Four of the eight significant metabolites – 3HPA, 5HIAA, 5HHA, and PAG – can also be linked to urinary markers of pulmonary TB as part of the altered host metabolome, caused by *M. tb* [26]. 3HPA is a microbial product of glycerol [27], a fatty acid that was identified as increased in TB cases by Luier and Loots [26]. 5HHA (a fatty acid) and 5HIAA (a tryptophan catabolite) were found to be increased in our study and also by Luier and Loots [26]. PAG, increased in this study, is a biotransformation product of phenylacetic acid, and also found to be increased by Luier and Loots [26].

4.5.2 3-Hydroxypropionic acid (3HPA) – host immune response, gut microbiome and *M. tb* metabolism

The metabolite 3HPA was the only metabolite that was significantly (adjusted $p < 0.01$) increased in all three TBM stages at T1 (Fig. 4.3). Treatment of TBM stages 1 and 3 (Fig. 4.4) showed 3HPA returning to that of the control levels by T6. TBM stage 2 showed a similar linear trend as stages 1 and 3, up to T4; thereafter, there was a re-emergence of increased 3HPA. Elevated levels of 3HPA in the host usually can be caused by defect in the enzyme propionyl-CoA carboxylase. Certain metabolic conditions in the host have been linked to appearance of high concentration of 3HPA in urine. These include: propionyl-CoA carboxylase deficiency, combined carboxylase deficiencies resulting from a defect of biotin metabolism, methylmalonic semialdehyde dehydrogenase deficiency, and 4-hydroxybutyric aciduria [28–31]. Significant amounts of 3HPA can bring about functioning as an acidogen (induce metabolic acidosis) and metabotoxin (prompts adverse effect at chronic high levels) [32, 33]. In a study reported by Pollitt et al. [29], three infants died due to a peculiar pattern of a significant urinary excretion of 3HPA, attributed to abnormal bacterial metabolism in the gut. 3HPA is an intermediary in the degradation of gut-produced propionic acid and branched-chain amino acids, as well as an intermediary metabolite in the microbial bi-cycle of propionyl-CoA and acetyl-CoA, and for carbon fixation via the microbial cycle of hydroxypropionic acid and hydroxybutyric acid (Fig. 6). Furthermore, 3HPA is a key intermediate in propionyl-CoA metabolism, which is used by *M. tb* via three mechanisms: (1) the protein ISL1 converts propionyl-CoA to succinic acid and pyruvic acid for energy production via either isocitrate lyase in the glyoxylate shunt or methylisocitrate lyase in the methylcitric acid cycle; (2) propionyl-CoA carboxylase converts propionyl-CoA to methylmalonyl-CoA for the vitamin B12-dependent methylmalonyl pathway, which is used for succinyl-CoA production; (3) propionyl-CoA, in the form of methylmalonyl-CoA, is used to generate building blocks for the *M. tb* cell wall [34]. Hence, 3HPA is a metabolite of the host, but

also has strong association with altered gut microbiota metabolism; our study and previous reports indicate a clear link to *M. tb* metabolism.

4.5.3 Prolonged immune response-related protein catabolism to treatment and induced *M. tb* death

IG, 3MCG and 2MBG were significantly elevated at T1 in TBM stages 2 and 3 (Fig. 4.3). In Fig. 4.4 it can be seen that for TBM stage 1, both 3MCG and 2MBG remained above that of the controls at a constant level (i.e., no trend), whereas IG for TBM stage 1 inexplicably increased during the course of treatment. For all three of these metabolites (IG, 3MCG and 2MBG), there was a clear linear trend of being significantly elevated at T1 and returning to control concentration levels by T6, for TBM stage 2 and 3. Loots et al. [35] also reported elevated urinary concentrations of 2MBG, and other organic acids associated with a multiple acyl-CoA dehydrogenase defect (MADD) metabolic profile in the urine of TB patients, supporting the result in this study. Chronic infection has been shown to cause vulnerability of the host, thus creating a cycle for survival, thereby activating specific metabolic pathways resulting in hypermetabolism, increased nitrogen loss, and enhanced gluconeogenesis. *M. tb* largely depends on human-derived nutrients for survival and replication including a source of nitrogen. Amino acids, including the BCAA amino acids isoleucine, leucine, and valine, are the most important organic nitrogen suppliers required by *M. tb* for intracellular growth, and

main signalling molecule involved in several physiological processes and controls gut motility [43–45]. Serotonin, biosynthesized from tryptophan, functions as a neuromodulator and maintains homeostasis as a neuroendocrine and neurotransmitter [46]. Urinary excretion of 5HIAA serves as a marker for determining serotonin in neurological conditions, including Friedreich's ataxia, schizophrenia, and olivoponto-cerebral atrophy [46–48]. The presence of 5HIAA in urine has been reported in a patient with carcinoid heart disease (CHD), with a high significant level as compared to patients without CHD. Kaltsas et al. [49] further confirmed that higher urine 5-HIAA levels are linked to an increased risk of developing CHD. Cheetham et al. [50] reported low levels of 5HIAA in cerebrospinal fluid of patients with depression, indicating increased serotonin breakdown. The biosynthesis of serotonin occurs in serotonergic neurons within the central nervous system in minor quantity, as well as in the enterochromaffin cells that lie along the gastrointestinal tract, where a majority is synthesized. 5HIAA is also a derivative of microbial indole-3-acetic acid and has been found to be a product of human gut microbiota, mostly synthesized in the kidney and liver, and passed in the urine [51, 52]. The microbe-produced indole derivatives of tryptophan have been shown to have a clear link to immune regulation in the host [53]. 5-Hydroxyindole (5HI), a microbial product and possible precursor of 5HIAA, is known to be involved in CNS regulation and antioxidant activity. 5HI protects cells by attenuating oxidative stress and consequently protects against mitochondrial dysfunction [54]. Liu et al. [55] shows that the microbial tryptophan metabolite 5HIAA can be used to assess intestinal homeostasis. Enrichment of microbial 5HIAA activates the Epac/Rap1 signalling pathway, which plays an important role in intestinal homeostasis Zhang et al. [56]. Furthermore, multi-omic profiling has shown that 5HIAA is a sensitive marker for intra-abdominal hyper-tension and sepsis in the host, and also that tryptophan-related metabolites (especially 5HIAA) are associated with microbiota changes, with several microbiota species correlating (positively and negatively) with 5HIAA [57]. Thus, 5HIAA is clearly a host metabolite, but is also a microbial metabolite, making it a co-metabolite. However, what is not clear from the literature is how 5HIAA is produced by gut microbiota. Moreover, a key question we ask is this: if increased flux of tryptophan into the kynurenine pathway is known to occur due to upregulated IDO-1 activity during TB, and other diseases [53, 58], then where/how is there sufficient bioavailability of tryptophan in the host for the increased levels of urinary 5HIAA that have been observed in this study?

4.5.5 Disturbances in fatty acid metabolism

In our study, (5HHA) was significantly increased in TBM stages 2 and 3 at T1 and showed a decreasing linear trend that approached control levels for all three TBM stages by T6. 5HHA is a fatty acid dicarboxylic acid breakdown product that has been identified as a urinary

microbial metabolite, under situations of fasting, aberrant fatty acid oxidation (FAO), and medium-chain triglyceride (MCT) feeding [59–61]. With the aim of elucidating mechanisms of TB, Luier and Loots [26] found anomalies in the host's fatty acid and amino acid metabolism brought on by pulmonary TB. These fatty acids and degradation products comprised 5HHA, glycerol monostearate and 2-octenoic acid. Note that 2-octenedioic acid was significantly elevated in this study at T1, T3, T4, T5 and T6. Other fatty acids that arose at later treatment times as significant in this study were pimelic acid and suberic acid. It is known that *M. tb* uses fatty acids as primary sources of energy results; hence increased urinary fatty acids and their catabolic products are common in TB cases. Additionally, other factors that might influence the host's response are different bacterial processes, which could help to elucidate the associated loss of weight in TB cases [26, 62, 63]. Hence, disturbances in fatty acid metabolism are another landmark of gut dysbiosis induced by TB. However, to our knowledge, 5HAA has not been directly linked to changes in gut microbiota.

4.5.6 Perturbed tyrosine–phenylalanine metabolism

Lastly, 4HHA was significantly increased in TBM stages 2 and 3 at T1, and PAG was significantly increased only in TBM stage 1 at T1. Both metabolites showed similar linear trends – TBM stage 1 stayed elevated through-out treatment, whereas TBM stages 2 and 3 showed a decreasing trend, approaching control levels by T6. 4HHA and PA are components of host tyrosine–phenylalanine (aromatic amino acids) metabolism, and metabolites of microbial origin. 4HHA and PA have been identified as a major consequence of perturbed metabolism associated with diseases linked with alterations to the gut-microbiome [8, 40, 64–66]. A study by Mason et al. [8] examined the urinary profiles of (untreated) infants and children (≤ 13 years) from cohorts of 12 confirmed TBM cases, 12 suspected TBM cases that were later confirmed negative for TBM, and 29 healthy controls. Mason et al. [8] identified four metabolites, including 4HHA, as having potential for a non-invasive diagnostic biosignature, as well as an increase in PAG. In another study, Das et al. [67] compared the urinary metabolic profiles of patients with active pulmonary TB ($n = 11$) under treatment to those of healthy controls ($n = 11$). The urine metabolome of follow-up samples revealed a treatment-dependent trend for numerous metabolites, notably 4HHA; those who were deemed clinically healed displayed a metabolic profile comparable to that of asymptomatic healthy individuals, suggesting that a possible target for TB diagnosis and treatment is revealed by disrupted tyrosine–phenylalanine metabolism [67]. Children with autism have also been found to produce abnormal PA excretion through the urine [68]. PA has also been linked to liver dysfunction and this aligns with the pathogenesis of TBM; namely, by TBM stage 3 the patient has had the infection for two or more weeks and the accumulated hepatotoxins have

been responsible for some damage to the liver. This could explain why only TBM stage 3 cases showed significantly increased PA in our study.

4.5.7 Urinary biomarkers for *M. tb*

Of note, methylcitric acid (MCA) was identified as significant at T3, T4 and T5, and methylsuccinic acid (MSA) was observed as significant at T5 and T6. MCA and MSA have been linked to *M. tb* metabolism by several studies [8, 69–72]. Therefore, we postulate that some of the TBM patients retained a reservoir of *M. tb* somewhere in their body – that is, *M. tb* was not entirely eradicated by treatment. This is an untested postulate, but one that we would like to test in future treatment studies.

4.6 Limitations of this study

Previous work has shown that tuberculostatic antibiotics cause changes in the gut microbiota community, and associated metabolism [73, 74]. These findings have not escaped our attention. It should be stated that we cannot definitively demarcate which metabolites are strictly the response of treatment or disease. Treatment invariably has some effect(s). For our study, however, we focused on eight altered metabolites that we consider to be most closely associated with the disease – TBM, and its associated dysbiosis. Other limitations of this study are that follow-up *M. tb* tests were not done and there are no data on the status of the TBM patients after treatment. Finally, the time of baseline sample (T1), collected upon release from hospital, was variably different for each TBM patient (an uncontrolled confounder). Each TBM patient therefore required a unique amount of time to stabilize in hospital before being discharged.

4.7 Conclusions

We identified 2-methylbutyrylglycine, 3-hydroxypropionic acid, 3-methylcrotonylglycine, 4-hydroxyhippuric acid, 5-hydroxyindoleacetic acid, 5-hydroxyhexanoic acid, isobutyrylglycine, and phenylacetylglutamine as urinary markers of dysbiosis in TBM. These urinary metabolites can be associated with host and/or microbial metabolism and appear to be fundamental to TB. This study lays the foundation for identifying products of microbial metabolites as novel points for management of TBM treatment. It is recommended that further studies be conducted to identify the altered gut microbiota linked to TBM pathogenesis and treatment efficacy. We recommend also that future studies employ both metabolomics and metagenomics on fecal samples collected from TBM cases to properly assess the gut microbiome.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13099-024-00609-9>.

Supplementary Material 1

Author contributions

Conceptualization: Shayne Mason; Data curation: Simon Isaiah, Emarie Davoren, and Shayne Mason; Formal analysis: Simon Isaiah, Du Toit Loots, Elmarie Davoren, and Shayne Mason; Funding acquisition: Martijn van der Kuip and A. Marceline Tutu van Furth; Investigation: Simon Isaiah; Methodology: Simon Isaiah, Du Toit Loots, Elmarie Davoren, Sabine van Elsland, and Shayne Mason; Project administration: Martijn van der Kuip and A. Marceline Tutu van Furth; Resources: Regan Solomons; Supervision: Du Toit Loots, Elmarie Davoren, and Shayne Mason; Writing – original draft: Simon Isaiah; Writing – review & editing: Simon Isaiah, Du Toit Loots, Martijn van der Kuip, Elmarie Davoren, Regan Solomons, A. Marceline Tutu van Furth, Sabine van Elsland, and Shayne Mason.

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Data availability

The entire set of LC/MS data generated during this metabolomics research will be made available to the public in 2024, once the project is completed and

all the data are published. On special request, immediate subsets of data from the study published here can be made accessible. (Email: nmr.nwu@gmail.com)

Declarations

Ethics approval and consent to participate

For every participant in the study, informed consent was obtained.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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**PART 4 THE DIAGNOSTIC POTENTIAL OF URINE IN PAEDIATRIC PATIENTS
UNDERGOING INITIAL TREATMENT FOR TUBERCULOUS MENINGITIS**

Chapter 5



OPEN The diagnostic potential of urine in paediatric patients undergoing initial treatment for tuberculous meningitis

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Tuberculous meningitis (TBM)—the extrapulmonary form of tuberculosis, is the most severe complication associated with tuberculosis, particularly in infants and children. The gold standard for the diagnosis of TBM requires cerebrospinal fluid (CSF) through lumbar puncture—an invasive sample collection method, and currently available CSF assays are often not sufficient for a definitive TBM diagnosis. Urine is metabolite-rich and relatively unexplored in terms of its potential to diagnose neuroinfectious diseases. We used an untargeted proton magnetic resonance (¹H-NMR) metabolomics approach to compare the urine from 32 patients with TBM (stratified into stages 1, 2 and 3) against that from 39 controls in a South African paediatric cohort. Significant spectral bins had to satisfy three of our four strict cut-off quantitative statistical criteria. Five significant biological metabolites were identified—1-methylnicotinamide, 3-hydroxyisovaleric acid, 5-aminolevulinic acid, N-acetylglutamine and methanol—which had no correlation with medication metabolites. ROC analysis revealed that methanol lacked diagnostic sensitivity, but the other four metabolites showed good diagnostic potential. Furthermore, we compared mild (stage 1) TBM and severe (stages 2 and 3) TBM, and our multivariate metabolic model could successfully classify severe but not mild TBM. Our results show that urine can potentially be used to diagnose severe TBM.

Keywords Diagnostic, Tuberculous meningitis (TBM), Proton magnetic resonance (¹H-NMR), Urine, Pediatric, Metabolomics

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5.1 Introduction

Tuberculous meningitis (TBM) is a devastating and severe form of extrapulmonary tuberculosis (TB) that affects the central nervous system, especially the meninges that surround the brain^{1,2}. TBM causes substantial mortality and morbidity worldwide, with sub-Saharan Africa being by far the most severely affected region globally. Infants and children are the most vulnerable group to TBM, resulting in death or neurological deficits in those who are fortunate enough to survive^{3–7}. In South Africa, the Western Cape Province has a high incidence of tuberculosis (TB) (681 per 100,000) in infants and children under the age of 14 years^{8–10}. The diagnosis of TBM is a major clinical challenge since this insidious infection often masks nonspecific symptoms that overlap with a spectrum of neurological complications that mimic other forms of meningitis¹¹. Despite the availability of effective treatments, TBM remains a severe disease in progressive stages with slow onset, particularly in resource-constrained settings. The vague clinical manifestations of TBM make the initial distinction of this inflammatory disease a formidable task, especially in the early stages⁷. A study showed an outcome difference between stages (mortality stage I: 1%, stage II: 10% and stage III: 34% with morbidity of approximately 27% stage I, 41% stage II and 71% stage III)¹², and the lack of a definitive diagnostic gold standard in children makes the diagnosis even more complex¹³. One of the mainstays of TBM diagnosis is the detection of *Mycobacterium tuberculosis* (*M. tb*) in cerebrospinal fluid (CSF), which requires a lumbar puncture—an invasive procedure. Traditional diagnostic methods for TBM, such as microscopy with acid-fast staining and culture, are characterized by their limited sensitivity and the long time required to obtain results^{7,11,14}. Polymerase chain reaction (PCR) is a valuable diagnostic tool; however, a meta-analysis of the commonly used Xpert MTB/RIF in TBM showed high accuracy but was unable to rule out TBM¹⁵. Similarly, Xpert MTB/RIF Ultra improves the sensitivity of Xpert MTB/RIF but is also unable to exclude TBM¹⁶. Currently, no single diagnostic test is sufficient for accurate differential diagnosis of TBM; hence, a combination of multiple diagnostic tests has been proposed.

Biomarkers can be used to diagnose, monitor, or predict the progression of a disease¹⁷. However, currently, there are no highly specific diagnostic biomarkers for TBM. Hence, it remains a priority to identify markers that allow timely and accurate diagnosis for optimal treatment outcomes. Efforts to overcome these diagnostic challenges in TBM continue to progress, and researchers are working to improve and refine diagnostic approaches to allow for the earlier identification and subsequent treatment of TBM patients. Urine-based diagnostic tests are becoming increasingly

useful for diagnosing TB because they are non-invasive and easy to perform. Urinary TB lipoarabinomannan (TB-LAM) assay detects the presence of LAM, a cell wall protein of *M. tb*, especially in HIV-infected people with low CD4 counts, and Xpert MTB/RIF Ultra also detects the presence of *M. tb* DNA in urine^{18–20}; however, the results of TB-LAM detection have been suboptimal, and there is a paucity of *M. tb* DNA in urine. Metabolomics is the study of the complete set of small molecule metabolites in a biological system²¹. Metabolites are the end products of metabolic pathways, and their levels can be affected by a variety of factors, including diseases²². From our urinary metabolomics studies on TBM, we described the biochemical characterization of the urinary profile of TBM²³ and identified markers of dysbiosis²⁴, paving the way for the use of urinary metabolomics for diagnostic purposes. In this investigation, we used ¹H-NMR spectroscopy because it is a rapid, highly repeatable, and robust metabolomics analytical platform. By highlighting the diagnostic difficulties associated with TBM, this study aimed to identify biomarkers of TBM with high precision and specificity using noninvasively collected urine.

5.2 Materials and methods

5.2.1 Sampling

This study included infants and children (13 years or younger) from the Western Cape province of South Africa, a region with a high burden of TB, particularly in children^{8,9,25,26}. Children with clinical symptoms and signs suggestive of meningitis who initially arrived at basic-level and regional health centres were referred to the Department of Paediatrics and Child Health at Tygerberg Hospital in the Western Cape Province of South Africa. According to the universal research case definition criteria for TBM⁷, all participants in our study (n = 32) were diagnosed with definite TBM and staged according to the revised British Medical Research Council (BMRC) criteria, which were used to classify TBM severity as follows: stage 1, Glasgow Coma Scale (GCS) of 15, without focal neurological deficit; stage 2, GCS of 15 with focal neurological deficit or GCS of 11–14 with or without focal neurological deficit; and stage 3, GCS < 11 with or without focal neurological deficit²⁷. TBM diagnosis was based on the identification of *M. tb* in CSF by microscopy, culture, and/or commercial nucleic acid amplification tests. All patients with TBM were stabilized in the Tygerberg Hospital Paediatric Neurology ward and treated with a short-intensified anti-*M. tb* drug regimen that included high-dose rifampicin, isoniazid, pyrazinamide, and ethionamide given for 6 months if HIV uninfected and 9 months if HIV co-infected^{28,29}. Urine samples from TBM patients were collected upon discharge from the hospital; the median time

from admission to discharge in the study setting over the past 38 years was 16 days, the interquartile range was 12–23 days²⁴. HIV co-infection considerably complicates an already complex metabolic profile; hence, participants with a positive or unknown HIV status were excluded from this investigation.

5.2.2 Sample collection

This study compared the urine of a control group (n = 39) with that of an experimental group of paediatric patients (n = 32). The controls, anonymously collected with written and informed consent, were classified as 'normal' paediatric patients who were admitted to Tygerberg Hospital. Here, 'normal' is based on the following benchmark: paediatric patients without meningitis, without neurological symptoms, and from the same geographic location as the patients with TBM. No further clinical data on the controls were available due to ethical constraints. The group of patients with TBM was subdivided into stage 1 TBM (n = 8), stage 2 TBM (n = 10) and stage 3 TBM (n = 14). Clinical symptoms included headache, fever, nausea/vomiting, photophobia, meningeal irritation, and/or neck stiffness⁹. The first urine sample after hospital discharge was obtained and classified as time point 1 (T1). Table 1 provides a more detailed description of the demographics and clinical information of the TBM patients.

5.2.3 Sample transport, storage, and handling

The urine samples were stored at – 80 °C in a dedicated freezer at Stellenbosch University's Division of Molecular Biology and Human Genetics. These samples were then collectively transferred overnight—frozen on dry ice—to the BSL-3 Laboratory at Human Metabolomics North-West University, Potchefstroom campus and stored at – 80 °C. On the day that the metabolomics analyses were performed, the samples were allowed to thaw in a biological safety cabinet. A pooled quality control (QC) urine sample was created by aliquoting 50 µL of each sample and pooling them into a single tube.

5.2.4 Sample preparation and ¹H NMR analysis

Prior to analysis, all urine samples were thawed to room temperature. To remove any particulates and macromolecules, 600 µL of urine was centrifuged at 12,000 g for 5 min. A 540 µL volume of supernatant was added to

Table 5.1 | Demographic and clinical characteristics of the TBM patients in this study stratified into stages 1, 2 and 3.

Criteria	Stage 1 TBM n (%)	Stage 2 TBM n (%)	Stage 3 TBM n (%)
Male gender	4 (50)	6 (60)	7 (50)
Age (months)*	65 [30–117]	45.5 [10–135]	42 [22–140]
Clinical symptoms			
Fever	4/8 (50)	4/10 (40)	12/14 (86)
Night sweats	0	1/10 (10)	2/14 (14)
Poor feeding	2/8 (25)	2/10 (20)	5/14 (36)
Weight loss	3/8 (36)	2/10 (20)	5/14 (36)
Vomiting without diarrhoea	3/8 (36)	1/10 (10)	4/14 (29)
Persistent (> 14 days) coughing	1/8 (13)	0	0
Headache	2/8 (25)	2/10 (20)	1/14 (7)
Seizures	3/8 (36)	1/10 (10)	5/14 (36)
Lethargy	2/8 (25)	1/10 (10)	2/14 (14)
Neurological signs			
GCS*	15 [15]	13 [12–15]	10 [7–15]
Meningism	0	4/10 (40)	3/14 (21)
Focal motor deficit	0	2/10 (20)	4/14 (29)
Cranial nerve palsy	0	1/10 (10)	3/14 (21)
Raised ICP	0	2/10 (20)	4/14 (29)
Neuroimaging (CT brain)			
Hydrocephalus	2/8 (25)	5/10 (50)	12/14 (86)
Infarctions	0	2/10 (20)	1/14 (7)
Tuberculoma	2/8 (25)	1/10 (10)	2/14 (14)
Meningeal enhancement	2/8 (25)	2/10 (20)	6/14 (43)
VP shunt present	0	1/10 (10)	4/14 (29)
Other radiology			
CXR (signs of pulmonary TB)	1/8 (13)	3/10 (30)	2/14 (14)
Laboratory values			
Blood sodium (mmol/L)*	139 [136.7–142.2]	136 [134.7–137.7]	131.5 [124.5–138]
Blood total protein (g/L)*	34 [17–51]	74 [73–75]	78.5 [76.7– 80.2]
Blood glucose (mmol/L)*	0	2.95 [1.47–4.42]	7.1 [6 –7.4]
Blood lipids*	0	0	1.95 [0.97–2.92]
CSF protein (g/L)*	0.53 [0.12–6.20]	0.93 [0.83–1.27]	0.84 [0.76–1.93]
CSF glucose (mmol/L)*	2.6 [1.27–3.6]	2.05 [1.2–2.71]	1.8 [0.8–2.85]
CSF lymphocytes (cells/μL)*	40 [20–60]	157 [103–410]	57 [40–83]

CSF lymphocytes (%)*	43 [21.7–65.2]	92 [88–94.8]	96 [94.5–100]
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CXR chest radiography, GCS Glasgow coma scale, Sex (M = male, F = female), CSF cerebrospinal fluid, ICP intracranial pressure, VP Ventriculoperitoneal. *Median with interquartile range.

60 μ L of NMR buffer solution [1.5 M potassium phosphate solution in deuterium oxide with internal standard trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid (TSP); pH 7.4]. The sample was vortexed and centrifuged at 12,000 g for 5 min, and 540 μ L of the supernatant was transferred to a 5 mm NMR glass tube and capped. All the samples were loaded in random order onto the NMR autosampler, with the QC samples periodically interspaced. The QC samples were used as a quality assurance method to monitor the analytical variation. The instrument used was a Bruker Avance III HD 500 MHz NMR spectrometer with a triple-resonance inverse (TXI) ^1H (^{15}N , ^{13}C) probe head and x, y, and z gradient coils. The ^1H spectra were captured as 128 transients at 32 K data points with a spectral width of 12,000 Hz. The sample temperature was kept constant at 300 K, and the H_2O resonance was pre-saturated using single-frequency irradiation with a 4 s relaxation delay and an 8 μ s excitation pulse. The deuterium signal was used to automatically shim the samples. The resonance line widths of half of the TSP peak were less than 1 Hz, indicating good resolution. Automatic Fourier transformation and phase and baseline correction were performed. The NMR data were processed using Bruker Topspin (V3.5). Bruker AMIX (V3.9.14) was used for identification and quantification³⁰.

5.2.5 Data pre-processing

The ^1H NMR spectral output was binned at set widths of 0.02 ppm, and probabilistic quotient normalization³¹ was applied to account for dilution differences in the urine samples. A data matrix was constructed, with bins in columns and samples in rows and data entries as the spectral data intensity. The bins affected by the suppressed water peak at ~ 4.72 ppm were removed. The coefficient of variation (CV) was checked in all bins for the QC samples, and any bin with a QC CV > 10% was removed. Therefore, all remaining bins were considered reliable (< 10% analytical variation) for biological interpretation.

5.2.6 Statistical analysis

All the statistical analyses were performed with Microsoft Excel, MetaboAnalyst 5.0, an online metabolomics tool (www.metaboAnalyst.ca/), and GraphPad Prism 10. The data were log transformed, and Pareto scaled for all multivariate analyses [principal component analysis (PCA),

orthogonal partial least squares discriminant analysis (OPLSDA), and random forest (RF) alongside OPLSDA to support the results (discriminatory variables were compared)], while all univariate statistics were analysed using nonparametric tests. Unsupervised PCA was used to obtain a visual overview of the data—qualitative assessment of group differentiation and/or clustering. Subsequently, four quantitative statistical tests were performed with defined and strict critical cut-off criteria. A spectral bin was considered important for further investigation of its diagnostic potential if it met at least three of the following four criteria: 1) OPLSDA with a VIP > 1.5 (supported by an RF out-of-the-bag (OOB) value < 0.05), 2) a Wilcoxon t test with an FDR p value < 0.01, 3) a fold change > 5.0, or 4) Hedge's effect size g value > 1.0. The metabolites within the important bins were identified based on 1D and 2D ¹H-NMR pure compound spectral libraries, and the metabolite concentrations were calculated as mmol/mol creatinine. Subsequently, violin plots were used to illustrate the distribution of the frequency of the concentrations of these important metabolites, as well as their medians, interquartile ranges, and statistical significance (p < 0.01, with multiple test corrections). Receiver operating characteristic (ROC) curves of the most important biological variables were generated to assess their diagnostic potential, and correlation analysis was performed to assess whether the biological diagnostic markers and unknowns had any strong correlations (r > 0.6), with statistical significance (p < 0.05) with the metabolites of the medication. Figure 5.1 illustrates the overall experimental design.

5.2.7 Ethics approval and consent to participate

All research was performed in accordance with relevant guidelines/regulations, and informed consent was obtained from all participants and/or their legal guardians. All research was performed in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Stellenbosch University Health Research Ethics Committee (HREC) (ethics approval number: N16/11/142 and N11/03/061 for TBM cases), the Western Cape Department of Health and Wellness, and the HREC of North–West University, Potchefstroom campus (ethics approval number: NWU-00063-18-A1-01).

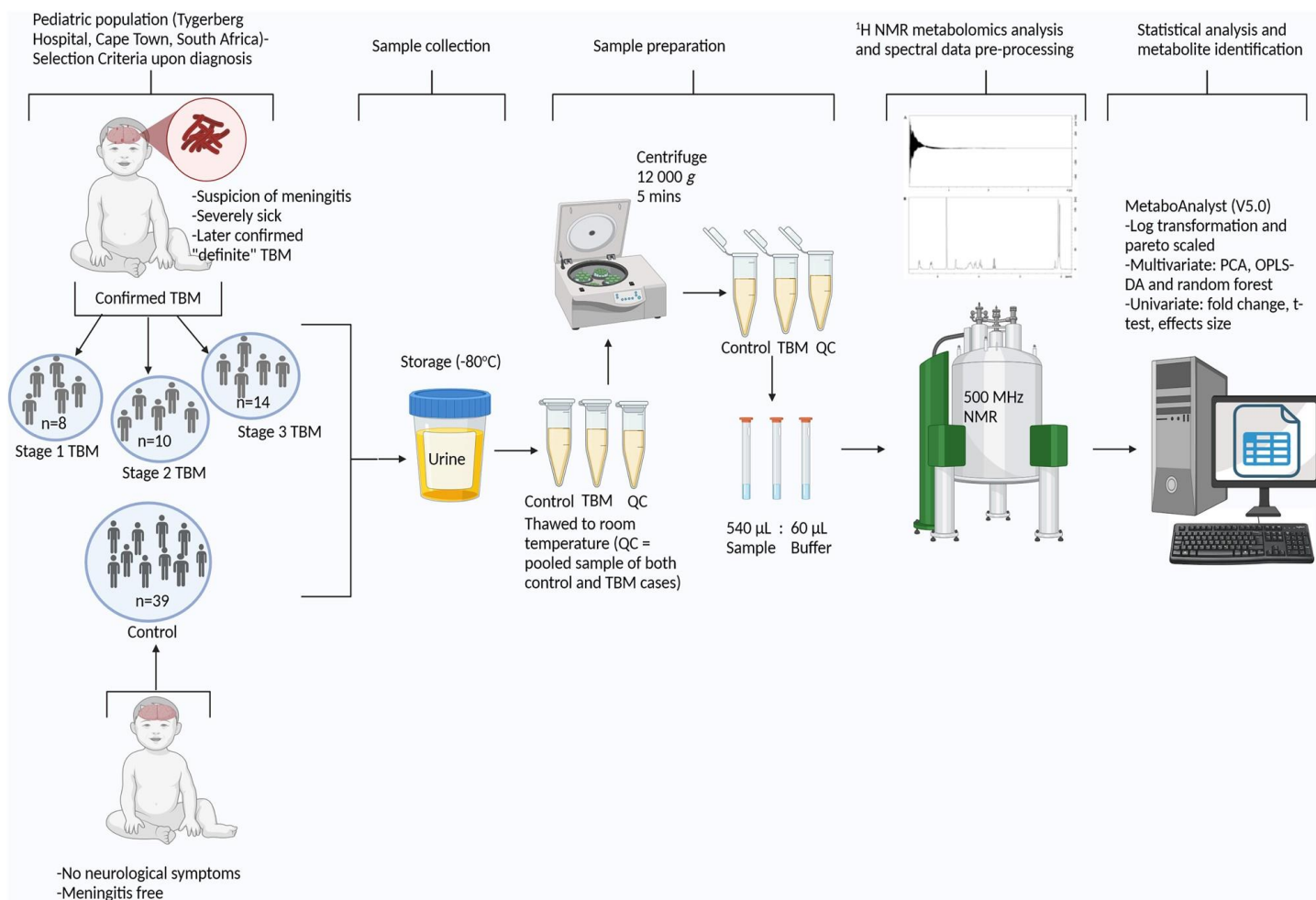


Figure 5.1 Schematic study design illustrating the experimental workflow – from (left to right): sampling, sample collection, sample preparation, ¹H-NMR analysis, and statistical analysis.

5.3 Results and discussion

5.3.1 Quality assurance

A total of 21 QC samples were run at regular intervals throughout the ^1H -NMR analysis. Quantitatively, the coefficient of variation (CV) was calculated in Excel for all spectral bins, and the bins with a QC CV greater than 10% (i.e., more than 10% analytical variation, likely due to horizontal shifting in the ^1H -NMR spectra in regions that contain peaks that are sensitive to slight pH differences) were removed from the entire data matrix. Hence, we can assure the quality of the remaining data and that we will assess biological variances with negligible analytical variation present in the data. Furthermore, qualitatively, an evaluation of the PCA scores plot (Figure S5.1) showed that the QCs clustered closely together; therefore, no experimental drift of the analytical pipeline was observed in this study.

5.3.2 Qualitative overview: multivariate statistics

The unsupervised PCA scores plots show natural differentiation between controls and all stages of TBM (Fig. 5.2A), as well as between individual comparisons (Figs. 5.2B-D). These qualitative results provide confidence in proceeding with additional quantitative statistical analyses—OPLSDA with RF and univariate assessment.

5.3.3 Quantitative statistical evaluation

Four quantitative statistical tests, with strict critical cut-off criteria, were evaluated in our binned ^1H -NMR data set: 1) OPLSDA with a VIP > 1.5 (supported by RF with similar discriminatory bins and OOB values < 0.05—Fig. 5.3), 2) Wilcoxon t test with FDR p value < 0.01, 3) fold change > 5.0, and 4) Hedge's effect size g value > 1.0. Comparisons were made between the controls and each stage of the TBM individually. If a bin achieved at least three of the four critical cut-off criteria, then that bin was considered important for the investigation of diagnostic markers. Table 5.2 shows a summary of these statistically significant quantitative results. As expected, several anti-TB drugs and their metabolites were identified as significant in differentiating TBM patients on treatment from controls. Of interest to our study were the biological elements identified as significant: N-acetylglutamine, succinic acid, citric acid, 5-aminolevulinic acid, 1-methylnicotinamide, and quinolinic acid. Furthermore, 14 unknown compounds that were not found in our spectral library databases were identified as important.

5.3.4 Correlations

We also performed a correlation analysis of all the significant variables identified in Table 5.2 for all patients with TBM (Fig. 5.4). Of the five biological diagnostic metabolic markers, only 3-hydroxyisovaleric acid showed any strong correlations ($r > 0.6$), with statistical significance (p -value < 0.05), with other significant variables, namely, propylene glycol and unknowns 4, 6 and 9. Hence, only 3-hydroxyisovaleric acid showed some correlation with a known medication compound (propylene glycol). The other four diagnostic markers, 1-methylnicotinamide, 5-aminolevulinic acid, N-acetylglutamine and methanol, did not show strong and/or statistically significant correlations with other significant variables. These data support our argument that 1-methylnicotinamide, 5-aminolevulinic acid, and N-acetylglutamine are diagnostic markers of TBM and are not directly associated with medication.

5.3.5 Evaluation of diagnostic markers for TBM

Since most of the significant variables annotated in Table 5.2 were medications and their known metabolites, we reran the statistical pipeline on a reduced data set in which all the bins related to known medications were removed. Furthermore, Fig. 5.2 and Table 5.2 show that TBM stages 2 and 3 exhibit similar characteristics. Therefore, to improve the power of the analysis, TBM stages 2 and 3 were combined and labelled 'severe TBM', and the remaining cases of stage 1 TBM were labelled 'mild TBM'. Figure 5.5 shows the PCA score plots for this revised data set—controls vs mild TBM show some overlap, and controls vs severe TBM show complete natural differentiation. Random Forest showed that mild TBM could not be successfully classified using the current multivariate metabolic model (71.4% misclassified and OOB = 0.109). However, the random forest misclassified only one severe TBM case (4% misclassification) and had an OOB value < 0.05 . Therefore, our multivariate metabolic model can successfully classify severe but not mild TBM. Furthermore, compared with the OPLSDA results, the random forest results showed similar discriminating bins.

Based on univariate statistics, using the Wilcoxon t test with an FDR p value < 0.01 and Hedge's effect size g value > 2.0 , several bins were identified as statistically and practically significant in differentiating controls from mild and severe TBM patients. Table 5.3 shows these quantitative statistical values, along with the annotations of the metabolites.

Five biological metabolites from Table 5.3 (1-methylnicotinamide, 3-hydroxyisovaleric acid, 5-aminolevulinic acid, N-acetylglutamine and methanol) were quantified (mmol/mol creatinine); the

violin plots are presented in Fig. 5.6. ROC curve analysis of the diagnostic potential of these five metabolites was carried out based on the area under the curve (AUC), sensitivity, and specificity (Fig. 5.7 and Table 5.4).

As shown in Fig. 5.7, all five metabolites have diagnostic potential. 1-Methylnicotinamide had AUCs of 0.99 and 0.95 for mild TBM and severe TBM, respectively; 3-hydroxyisovaleric acid had AUCs of 0.89 and 0.99 for mild TBM and severe TBM, respectively; 5-aminolevulinic acid had AUCs of 0.94 and 0.99 for mild TBM and severe TBM, respectively; N-acetylglutamine had AUCs of 0.99 and 0.99 for mild TBM and severe TBM, respectively; and methanol had AUCs of 0.92 and 0.98 for mild TBM and severe TBM, respectively. Table 5.4 shows the sensitivity of the specific cut-off concentrations, all with a specificity of 97.5% and a 95% confidence interval of 87%-100%.

1-Methylnicotinamide and N-acetylglutamine have 100% sensitivity as early diagnostic markers of mild TBM and > 90% sensitivity for severe TBM, while 3-hydroxyisovaleric acid has greater sensitivity for severe TBM, and 5-aminolevulinic acid has equivalent sensitivity as a diagnostic marker for both mild and severe TBM. Therefore, 1-methylnicotinamide, 3-hydroxyisovaleric acid, 5-aminolevulinic acid and N-acetylglutamine show potential as diagnostic markers of TBM but do not have sufficient power to differentiate mild-stage from severe-stage TBM. Methanol showed statistical significance (Fig. 5.6) but lacked diagnostic sensitivity for use as a diagnostic marker for TBM.

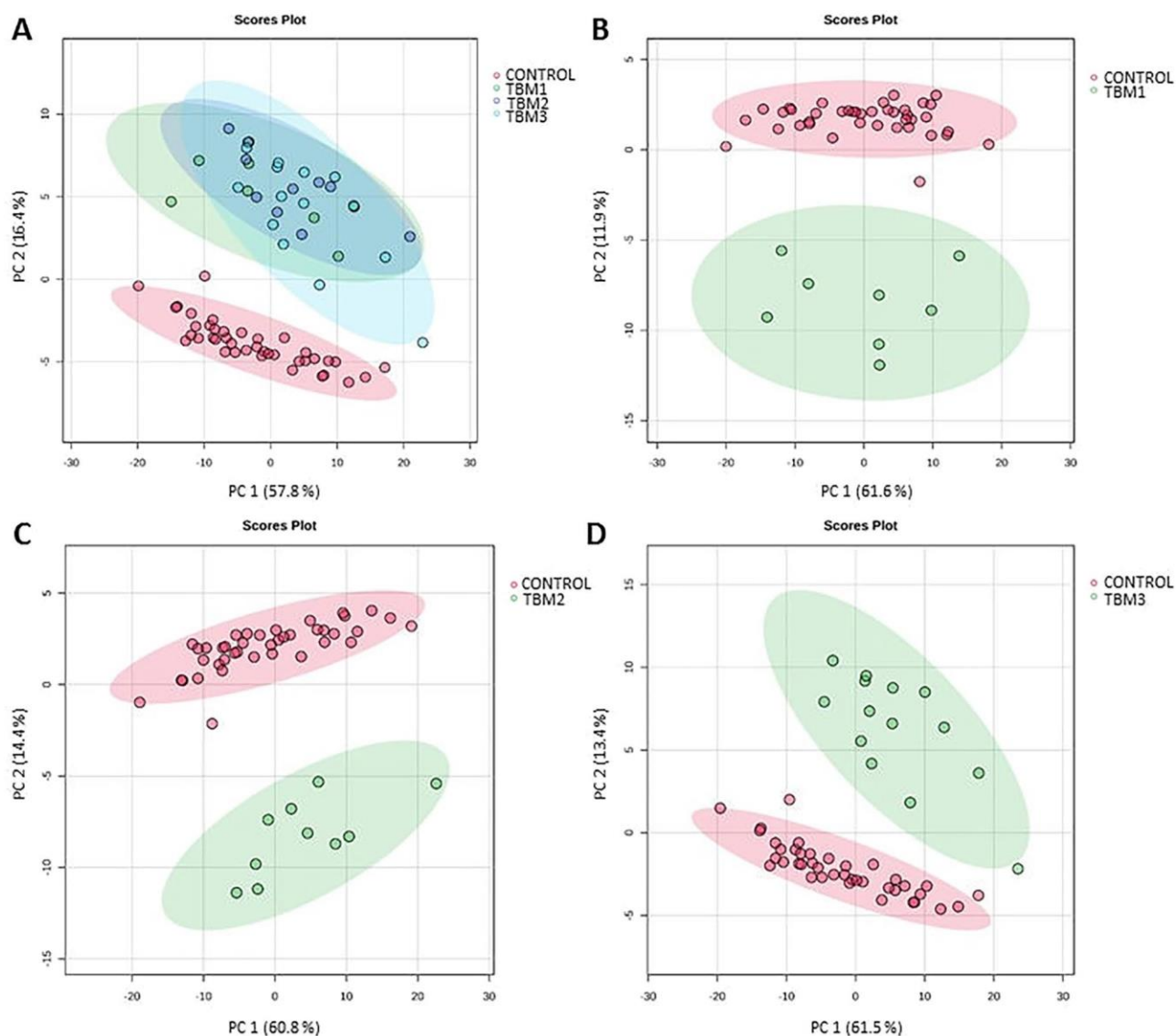


Figure 5.2 PCA score plots of (A) Controls vs. all three TBM stages, (B) Controls vs. Stage 1 TBM, (C) Controls vs. Stage 2 TBM, (D) Controls vs. Stage 3 TBM.

These PCA scores plots give qualitative confirmation that natural differentiation exists between the control group and each TBM stage, supporting the use of quantitative multivariate statistical analysis.

5.3.6 Biological context for the four TBM diagnostic metabolites

Three (1-methylnicotinamide, 3-hydroxyisovaleric acid, and N-acetylglutamine) of the four urinary metabolic markers of TBM, as described above, have been discussed in terms of their biological context in our previous study-see²³ for more details. Here, we report 5-aminolevulinic acid as

being a significant urinary metabolite in TBM for the first time. 5-Aminolevulinic acid has been defined as a non-proteinogenic amino acid, meaning it is not used in the synthesis of proteins. This endogenous metabolite is biosynthesized in the body by the condensation of glycine and succinyl-CoA, catalysed by the enzyme 5-aminolevulinate synthase. 5-Aminolevulinic acid is required in the metabolism of heme in the body and it is involved in maintaining normal mitochondrial function.

To the best of our knowledge, this is the first time that 5-aminolevulinic acid has been identified in any type of sample matrix collected from TBM patients. However, it is important to state that 5-aminolevulinic acid is not a specific marker of TBM. 5-Aminolevulinic acid has been identified in the urine of patients following an acute attack of p orphyria³², as well as in patients diagnosed with the tyrosinemia type I³³. Moreover, due to its anti-inflammatory and immunoregulatory properties³⁴, 5-aminolevulinic acid has been identified as a novel therapeutic for inflammatory bowel disease³⁵, and has potential in treating type II diabetes mellitus³⁶, and COVID-19³⁷.

Hence, 5-aminolevulinic acid is likely an endogenous metabolic marker of the immune response to severe inflammation (severe and chronic neuroinflammation is a classic symptom of TBM). But it is also important to note that some studies³⁸ have identified 5-aminolevulinic acid as being neurotoxic. Therefore, future studies should examine the levels of this metabolite in the brain of TBM patients.

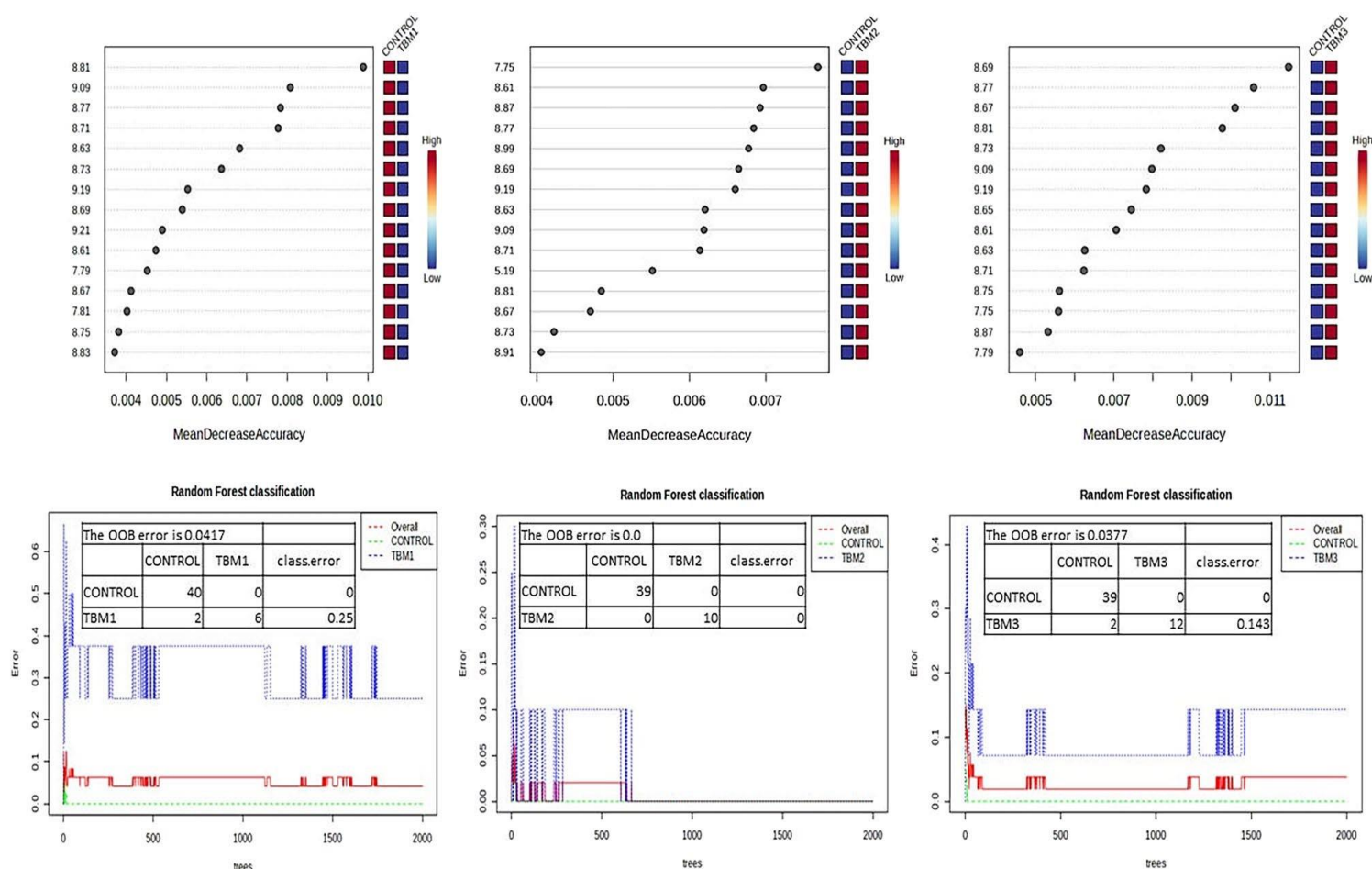


Figure 5.3 Random Forest outputs supporting the OPLSDA results.

The results are shown in the left, centre, and right panels for the Stage 1 TBM, Stage 2 TBM, and Stage 3 TBM, respectively. The top part of each panel shows that similar discriminating bins were found in comparison to the OPLSDA results, and the out-of-bag (OOB) values for each panel are < 0.05 , supporting the OPLSDA model. The bottom part of each panel shows the Random Forest classification: stage 1 TBM cases were misclassified 25% of the time as controls, and no misclassifications occurred for stage 2 TBM, while stage 3 TBM cases were misclassified 14.3% of the time as controls.

5.3.7 TBM stage differentiation

In addition to identifying potential diagnostic markers in each TBM stage (comparing TBM patients with controls), the TBM stages were also compared to each other to determine whether any urinary metabolic marker could differentiate the TBM stages. Based on the PCA score plot (Figure S2), no qualitative differentiation could be detected between the stages of TBM (i.e., the metabolic urinary ^1H -NMR profiles could not differentiate the stage of TBM). Quantitative statistical data

were checked and did not show statistically significant differences. Therefore, no urinary metabolites could differentiate the stages of TBM.

5.3.8 Unknown compounds—Biological or medication related?

Ten significant unknown compounds were identified, as shown in Table 5.3, and quantified. Based on the correlation data (Fig. 5.4), none of the unknowns were correlated with the identified anti-TB medications. However, unknowns 6, 9 and 10 were significantly correlated with propylene glycol and may be related to other forms of medication. The other unknowns (1, 2, 3, 4, 5, 7 and 8) had no correlation with medication and may be of biological origin.

5.3.9 Speculation of the identities of unknowns

Based on the 2D JRES and COSY NMR spectral data, a doublet at 1.55 ppm (Unknown 2) was correlated with a quartet at 5.19 ppm (Unknown 8) (see Figure S3 in the supplementary information for more details). This ^1H -NMR chemical shift information implies that a terminal methyl group is present next to a double bond CH component. This chemical information suggests that one of the unknowns is likely to have a chemical structure similar to that of a short-chain unsaturated carboxylic acid. The ^1H -NMR spectra of several suspected short-chain unsaturated carboxylic acids were investigated, including propionic acid (C3), butyric acid (C4), and isobutyric acid (C4). However, while the patterns were similar, none of these pure compound spectra matched the unknown spectra sufficiently. We suspect that the unknown compound that contains a doublet at 1.55 ppm and a quartet at 5.19 ppm is a short branched-chain organic acid that is methylated. Short branched-chain organic acids originate from micro-organisms, and these unknowns unique to the TBM cases could be degradation products of the unique cell wall of *M. tb*. It is our recommendation that additional cohorts of urine samples collected from treated TBM patients should undergo targeted gas chromatography–mass spectrometry (GC–MS) metabolomics analyses, with a focus on short and branched-chain organic acids that are methylated.

Table 5. 2 | Quantitative statistically significant results (achieved 3 of 4 demarcated critical cut-off criteria of controls compared to TBM stages 1, 2 and 3).

The annotations given to the bins were based upon 1D and 2D ¹H-NMR spectral library matches. *VIP* variables important in projection (OPLSDA). *FDR* Wilcoxon p value adjusted for multiple tests. *FC* fold change. *ES* Hedge's g value effect size. X indicates an unknown compound.

Annotations	Bins	Stage 1 TBM				Stage 2 TBM				Stage 3 TBM			
		VIP	FDR	FC	ES	VIP	FDR	FC	ES	VIP	FDR	FC	ES
Rifampicin/3-formylrifampicin	0.59	2.21	< 0.01	7.65	2.27								
Rifampicin/3-formylrifampicin	0.61	2.04	< 0.01				< 0.01	8.02	2.84		< 0.01	5.50	2.06
Propylene glycol	1.13				2.26	1.56	< 0.01	35.30	3.00		< 0.01	22.53	2.11
Propylene glycol	1.15				2.84								
X1	1.55				2.70	1.52	< 0.01		3.32				
X2	1.57				4.13	1.61	< 0.01		4.79	1.52	< 0.01		3.59
N-Acetylglutamine	2.07	1.50	< 0.01			1.54	< 0.01		4.38		< 0.01	5.87	2.66
Acetylisoniazid	2.15	1.88	< 0.01		3.49	1.61	< 0.01		3.79				
Succinic acid	2.41	1.56	< 0.01		3.11	1.50	< 0.01	8.16	3.50				
5-Aminolevulinic acid	2.53	1.87	< 0.01		2.94								
Citric acid	2.67	1.51	< 0.01								< 0.01	8.01	1.62
5-Aminolevulinic acid	2.81				3.85	1.58	< 0.01		3.53	1.58	< 0.01		3.73
X3	3.17	1.73	< 0.01		3.39	1.60	< 0.01		4.26				
X4	3.39						< 0.01	7.46	1.22				
Propylene glycol	3.45				1.01								
Propylene glycol	3.47				2.53	1.56	< 0.01	5.52	2.31				
Propylene glycol	3.55	1.62	< 0.01			1.56	< 0.01		3.14	1.52	< 0.01		3.14
Propylene glycol	3.89	1.65	< 0.01				< 0.01	5.32	2.15		< 0.01	5.11	1.80
N-Acetylglutamine	4.15	1.58	< 0.01		2.16	1.63	< 0.01	8.21	2.31	1.52	< 0.01	7.59	2.35

X5	4.29				2.04	1.60	< 0.01	14.04	2.77	1.54	< 0.01	11.79	2.02
X6	4.41	1.52	< 0.01				< 0.01	5.78	1.60				
X7	4.61	1.56	< 0.01		1.96		< 0.01	5.43	1.22		< 0.01	5.87	1.30
X8	5.17				1.41		< 0.01	5.55	1.77				
X9	5.19	1.86	< 0.01				< 0.01	7.55	1.16		< 0.01	8.66	1.26
Rifampicin/3- formylrifampicin	6.33	1.62	< 0.01		3.15		< 0.01	10.10	2.08		< 0.01	9.96	2.02
Rifampicin/3- formylrifampicin	6.35	1.95	< 0.01				< 0.01	5.81	3.08		< 0.01	6.66	2.00
X10	6.81				4.40	1.57	< 0.01	23.83	4.51		< 0.01	19.22	2.81
X11	7.21				3.68		< 0.01	8.15	2.52		< 0.01	6.31	2.18
Isoniazid	7.71	1.89	< 0.01		2.19	1.50	< 0.01	28.72	1.64		< 0.01	22.71	1.22
X12	7.73	1.80	< 0.01		3.36		< 0.01	43.01	3.12		< 0.01	31.00	2.34
Isonicotinic acid	7.75	2.24	< 0.01	8.39			< 0.01	6.19	1.26		< 0.01	5.89	1.64
Isonicotinic acid	7.77	1.86	< 0.01		2.75								
2-Pyridin-4- formidoacetic acid	7.79	2.27	< 0.01	12.13	1.48						< 0.01	5.67	1.31
Acetylisoniazid	7.81	2.18	< 0.01	12.57	1.27		< 0.01	10.95	2.33		< 0.01	6.94	1.29
Quinolinic acid	8.05						< 0.01	12.68	1.10				
X13	8.59	1.78	< 0.01		1.55		< 0.01	7.40	2.21		< 0.01	7.94	1.92
Isonicotinic acid	8.61	2.34	< 0.01	21.60			< 0.01	16.45	1.00		< 0.01	8.17	1.05
Isonicotinic acid	8.63	2.38	< 0.01	44.73		1.56	< 0.01	9.51	1.22				
X14	8.65	2.02	< 0.01		1.20	1.68	< 0.01	16.13	1.65	1.62	< 0.01	16.73	1.25

5.4 Conclusions

The identification of urinary biomarkers for TBM is a promising area of research. Our multivariate metabolic model can successfully classify severe but not mild TBM. The four metabolites (1-methylnicotinamide, 3-hydroxyisovaleric acid, 5-aminolevulinic acid and N-acetylglutamine) identified in this study show good diagnostic potential for severe TBM (stage 2 and 3 TBM patients combined), but they have not yet been established as definitive diagnostic tools. Future studies are needed to validate these biomarkers, including the significant unknowns identified in this study, to determine their clinical utility and explore their specificity and sensitivity for the diagnosis of TBM. Further research is needed, but we believe that urine from TBM patients will potentially aid in the earlier diagnosis and treatment of this disease.

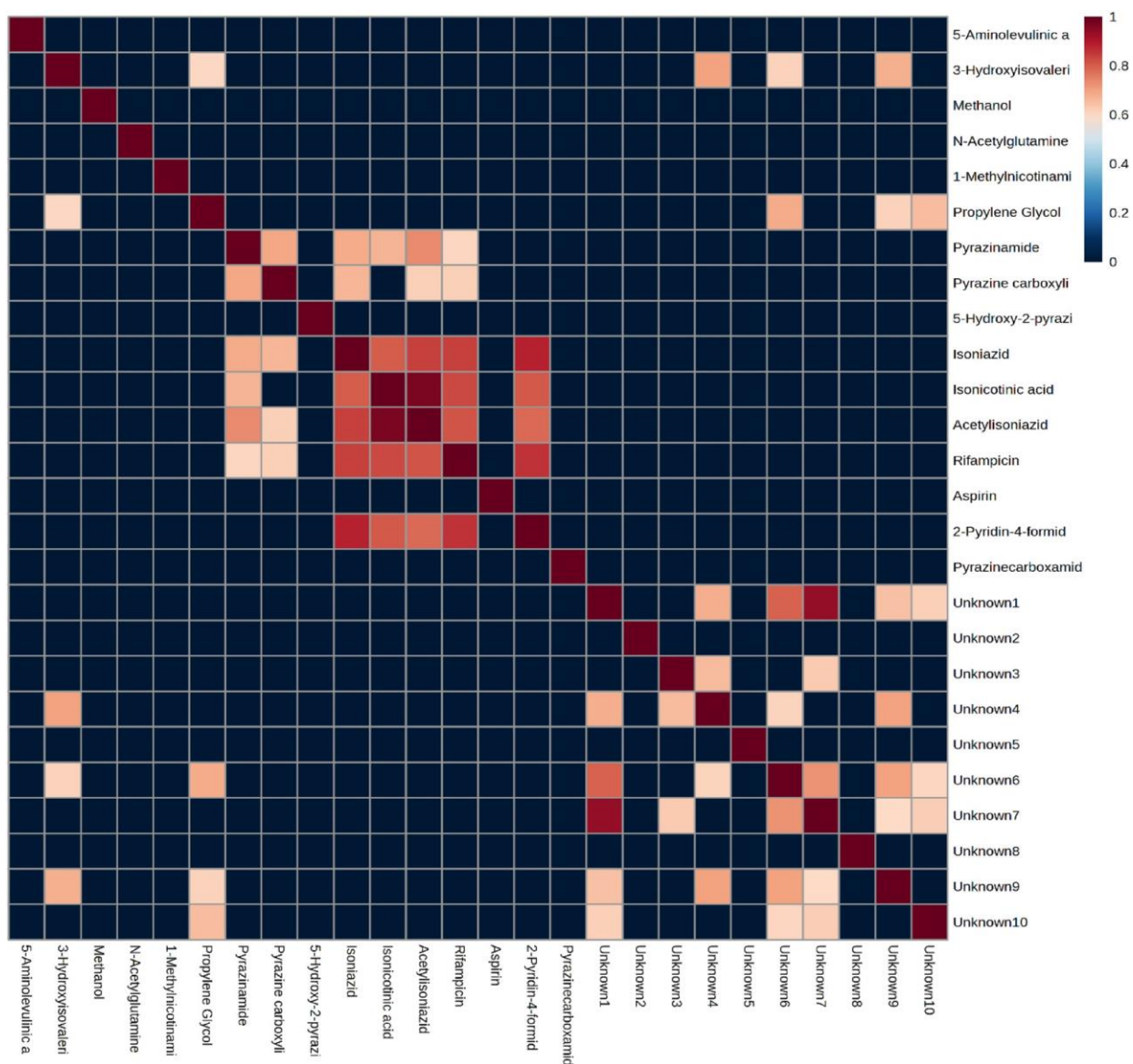


Figure 5. 4 Plot showing correlations with an r value greater than 0.6 and a p value < 0.05 for all important quantified variables in Table 5.2 for all the TBM cases.

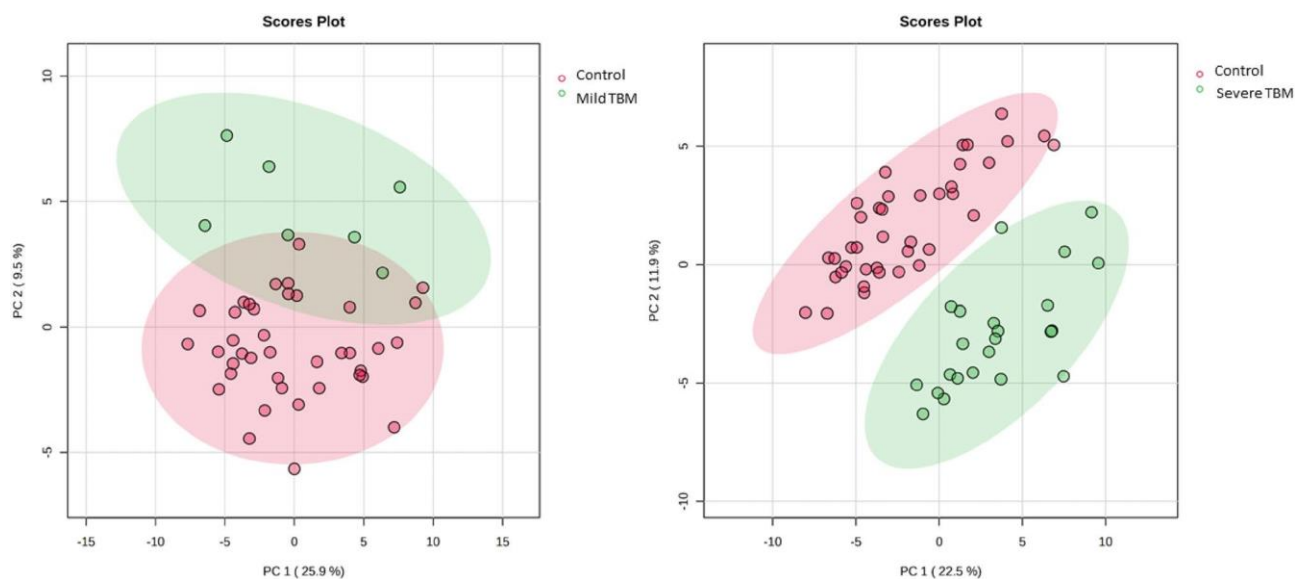


Figure 5.5 PCA score plots of revised data sets of (LEFT) controls vs mild TBM, showing some overlap, and (RIGHT) controls vs severe TBM, showing complete natural differentiation between the groups.

Table 5. 3 | Statistically and practically significant bins of the reduced data set based on the Wilcoxon t test with an FDR p value < 0.01 and Hedge's effect size g value > 2.0.

Annotations given to bins, based upon 1D and 2D ^1H -NMR spectral library matches. The Wilcoxon p values, adjusted for multiple tests, was < 0.01 for all comparisons. ES Hedge's g value effect size. X indicates an unknown compound.

Controls vs Mild TBM			Controls vs Severe TBM		
Annotations	Bins	ES	Annotations	Bins	ES
X1	1.43	2.85	3-Hydroxyisovaleric acid	1.27	2.37
N-Acetylglutamine	2.07	2.96	X2	1.55	2.59
X5	2.21	2.65	X3	1.57	2.16
X6	2.23	2.64	5-Aminolevulinic acid	2.81	2.76
5-Aminolevulinic acid	2.81	3.61	X4	2.85	2.43
X4	2.85	2.57	Methanol	3.37	2.18
X7	3.39	3.33	X7	3.39	2.55
X8	5.19	3.70	X8	5.19	2.44
X9	7.97	2.53	1-Methylnicotinamide	8.91	2.03
X10	8.33	2.09	1-Methylnicotinamide	8.99	2.47
1-Methylnicotinamide	8.99	2.59			

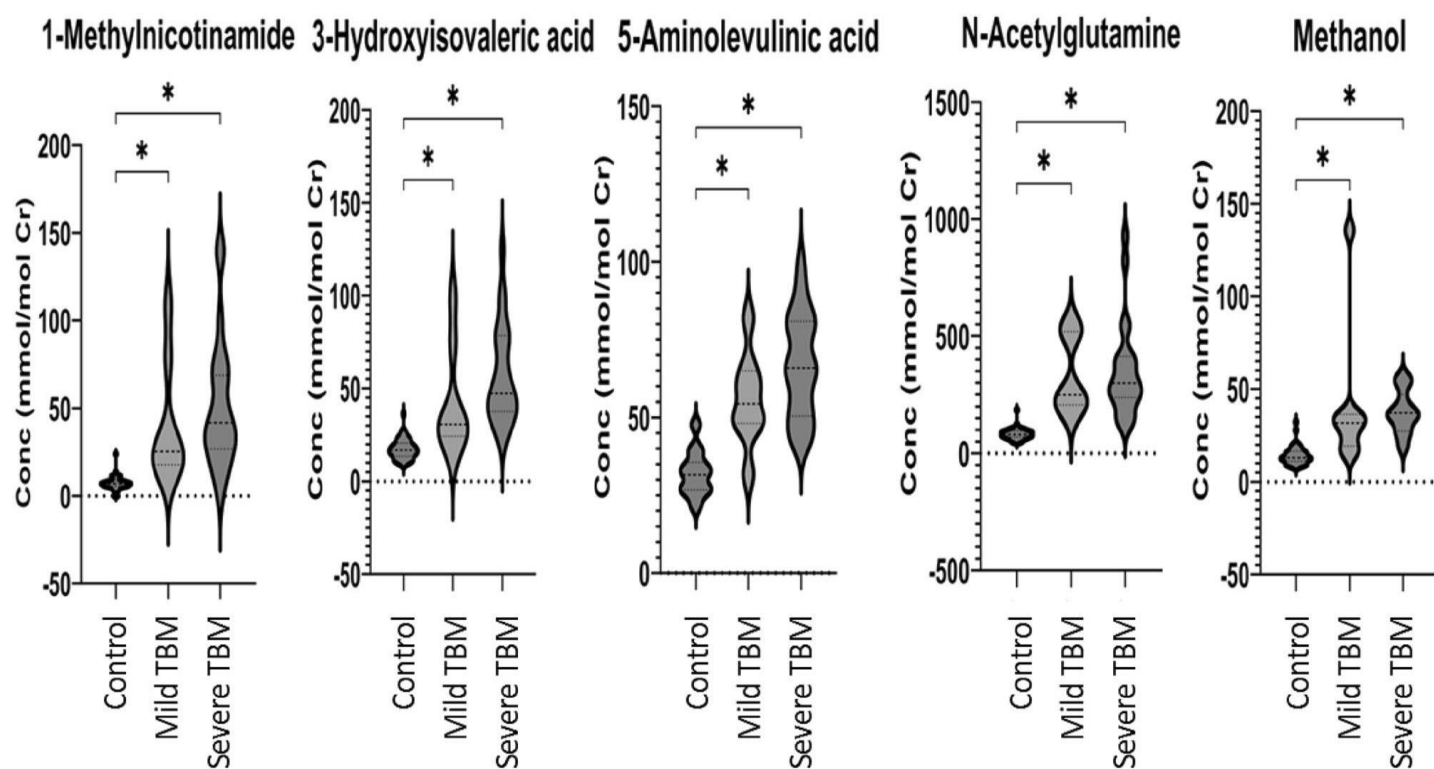


Figure 5.6 Violin plots depicting the five significant metabolites (1-methylnicotinamide, 3-hydroxyisovaleric acid, 5-aminolevulinic acid, N-acetylglutamine, and methanol) in mild and severe TBM.

The concentration is given as mmol/mol creatinine in the y-axis. Statistical significance (FDR $p < 0.01$) is indicated by *.

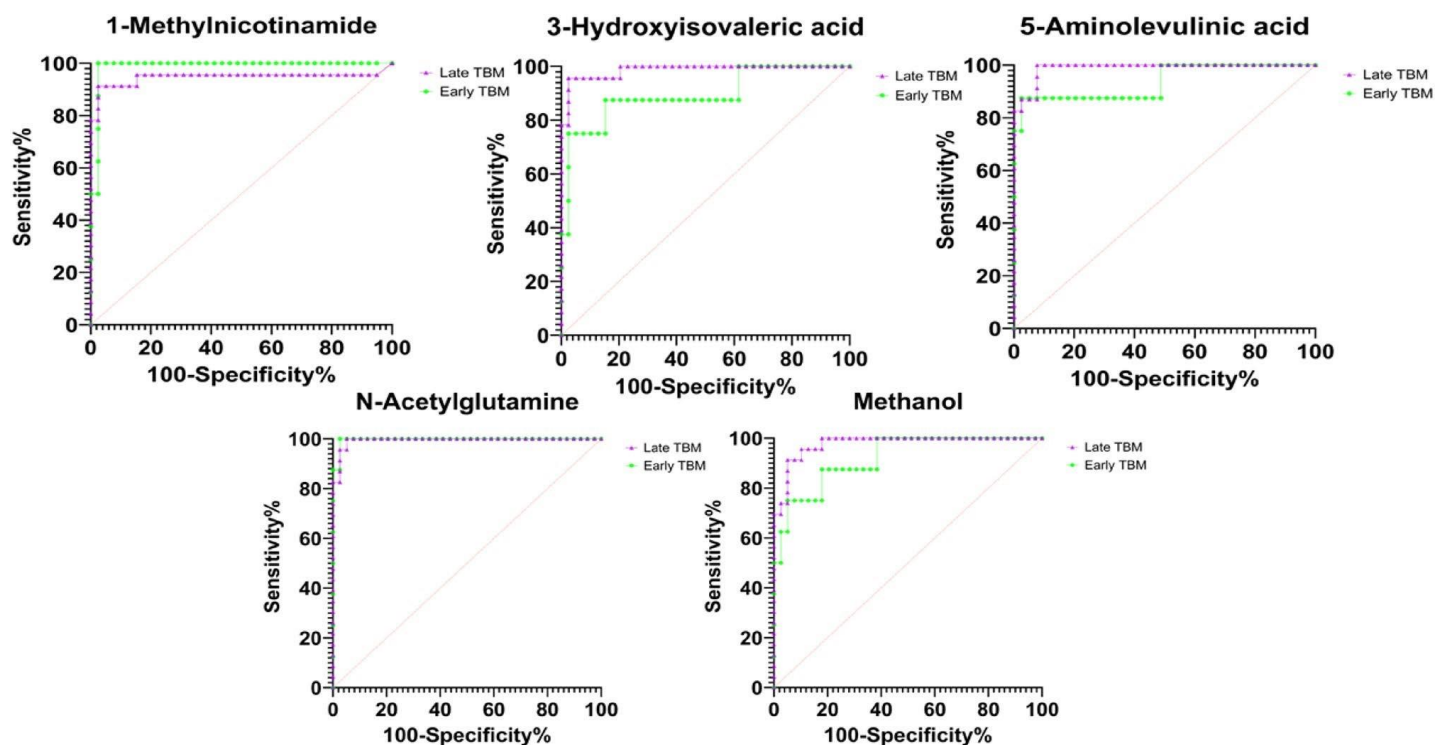


Figure 5.7 ROC curves of 1-methylnicotinamide, 3-hydroxyisovaleric acid, 5-aminolevulinic acid, N-acetylglutamine and methanol. These five ROC curves indicate that each metabolite has diagnostic potential.

Table 5.4 | Diagnostic sensitivity of the five urinary metabolic markers of TBM at specified cut-off concentrations. The specificity for all was 97.5%, with a 95% confidence interval of 87%-100%.

Metabolite	Diagnostic criteria	Mild TBM	Severe TBM
1-Methylnicotinamide	Conc (mmol/mol cr)	> 14	> 14.45
	Sensitivity %	100 (67.6–100)	91.3 (73–98.5)
3-Hydroxyisovaleric acid	Conc (mmol/mol cr)	> 27.33	> 27.41
	Sensitivity %	75 (41–95.6)	95.7 (79–100)
5-Aminolevulinic acid	Conc (mmol/mol cr)	> 47.89	> 47.84
	Sensitivity %	87.5 (53–99)	87 (68–95.5)
N-Acetylglutamine	Conc (mmol/mol cr)	> 117.3	> 127.5
	Sensitivity %	100 (67.6–100)	95.7 (79–100)
Methanol	Conc (mmol/mol cr)	> 29.46	> 29.86
	Sensitivity %	62.5 (30.5–95.6)	73.9 (53.5–87.5)

Data availability

All metabolomics research data associated with this project will be made available online in a data repository once the project is completed and all the data are published. On special request, immediate subsets of data from the study published here can be made accessible via email: nmr.nwu@gmail.com.

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Competing interests

The authors declare no competing interests.

Additional information

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PART 5 Exploratory investigation of urinary alkanes and other volatile organic compounds in paediatric patients with tuberculous meningitis

CHAPTER 6

Exploratory investigation of urinary alkanes and other volatile organic compounds in paediatric patients with tuberculous meningitis

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Abstract

Background: Alkanes are a class of biologically important metabolites that are used for growth and survival strategies in bacteria. Various species of infectious mycobacteria use alkanes specifically to survive challenging conditions. Here, we investigated the altered alkanes and other volatile organic compounds (VOCs) in the urine from paediatric cases with tuberculous meningitis (TBM) – a disease caused by *Mycobacterium tuberculosis* (*M. tb*) infection of the brain.

Method: We used untargeted gas chromatography coupled with time-of-flight mass spectrometry (GC-TOFMS) to analyse and compare all volatile underivatised compounds present in the urine from 27 confirmed cases of paediatric TBM over a treatment period of six months, as well as a control group (n = 13).

Result: Four alkanes (pentadecane, 5,7-dimethyl-undecane, 4,7-dimethyl-undecane, and 2,6-dimethyl-undecane), three alkenes (2,5-dimethyl-2-hexene, 4,4-dimethyl-1-pentene, and 3-methoxy-1-pentene), and three other VOCs of biological interest (2-butenic acid methyl ester, 3-heptanone, and 2-pyrrolidinone) were identified as statistically significant. These volatile compounds remained perturbed during the TBM treatment.

Conclusion: This study discovered new systemic metabolic information about *M. tb* in the host and the role of alkanes and VOCs in the potential persistence of *M. tb*. We demonstrate the value of targeting alkanes and other VOCs for future metabolomics studies of *M. tb*.

Keywords: urine; alkanes; volatile organic compounds (VOCs); paediatric; tuberculous meningitis (TBM); metabolomics; gas chromatography coupled with time-of-flight mass spectrometry (GC-TOFMS)

6.1 Introduction

Alkanes are saturated hydrocarbons, meaning that they consist of a series of single-bonded carbon and hydrogen atoms. It is known from the literature that many species of bacteria use alkanes as a carbon source (Rojo, 2009; Wentzel *et al.*, 2007). Although sparse in the literature, some studies have shown that species of *Mycobacterium* use alkanes as a carbon source. Dunlap and Perry showed that C13-C17 alkanes were incorporated into the fatty acid metabolism of an OFS lab strain of a *Mycobacterium* species (Dunlap and Perry, 1967); however, alkanes shorter than C13 or longer than C17 were not incorporated into the cellular fatty acids of mycobacteria without some form of degradation (Dunlap and Perry, 1968). Churchill *et al.* (1999) isolated a CH1 lab strain of a *Mycobacterium* species and showed that mycobacteria could use a wide range of alkanes [linear (dodecane and hexadecane); branched-chain (pristane); long-chain (octadecane, docosane, and octacosane)] as the sources of carbon and energy. Another study by Van Beilen *et al.* (2002) showed that various species of mycobacteria could use C6-C24 alkanes as carbon sources. To use alkanes, bacteria need the alkane hydroxylase system, which consists of three protein components: alkane hydroxylase (AlkB), rubredoxin (AlkG), and rubredoxin reductase (AlkT) (Smits *et al.*, 2002). Stokas *et al.* (2022) showed that *Mycobacterium tuberculosis* (*M. tb*), the pathogen responsible for tuberculosis (TB), regulates a highly conserved alkane hydroxylase/rubredoxin system that contributes to the success of these specific mycobacteria in human macrophages. Hence, *M. tb* uses alkanes for growth and survival.

The breath of active cases of pulmonary TB has been investigated (Phillips *et al.*, 2010; Phillips *et al.*, 2007) and it was found that the alkanes tridecane, 4-methyldodecane, and 3,7-dimethyldecane were among the volatile organic compounds (VOCs) of the breath that were diagnostic markers of TB. However, alkanes and VOCs have never been targeted in any TB study nor investigated in other biofluids (urine, plasma, serum, etc.) from TB cases. VOCs in biofluids are low molecular weight compounds that are easily vaporised when heated. Here, we conducted an exploratory investigation of alkanes and other VOCs in urine collected from paediatric cases undergoing treatment for confirmed tuberculous meningitis (TBM; TB of the brain). TBM is the most severe extrapulmonary form of TB and if not diagnosed early can result in a high probability of death or long-term neurological complications. Hence, there is a desperate need to discover new potential diagnostic markers to aid in quicker and more efficient diagnosis of TBM. For this study, we used gas chromatography coupled with time-of-flight mass spectrometry (GC-TOFMS)

as the analytical approach, with the aim of the study to evaluate the urinary profile of alkanes and other VOCs in cases of TBM.

6.2 Materials and methods

6.2.1 Sampling and ethics

This investigation involved retrospectively collected urine samples (van Elsland *et al.*, 2018) from infants and children (aged < 13 years) residing in the Western Cape Province of South Africa, a geographical location known for its high prevalence of TB (681 per 100,000), especially among children (Donald *et al.*, 1996; Mandalakas *et al.*, 2021; van Toorn and Solomons, 2014). Children exhibiting clinical symptoms and signs indicative of meningitis, initially seen at the basic level and regional health facilities, were referred to the Department of Paediatrics and Child Health at Tygerberg Hospital in the Western Cape Province of South Africa. Following the application of the universal research case definition criteria for TBM (Marais *et al.*, 2010), all TBM participants in our cohort (n = 27) were diagnosed with definite TBM. The diagnosis of definite TBM was based on the detection of *M. tb* in CSF by microscopy, culture, and/or a commercial nucleic acid amplification test. All TBM patients were stabilised in the Tygerberg Hospital Paediatric Neurology ward and treated with an intensified anti-tuberculous drug regimen comprising high-dose rifampicin, isoniazid, pyrazinamide, and ethionamide (Van Toorn *et al.*, 2012). Urine samples were collected with the consent of the parents or guardians and the assent of the child if older than 7 years and can comprehend. All experimental samples were collected at baseline (time 0; T0) and at one-month intervals, up to six months (T1-T6), until the completion of the TBM treatment (van Elsland *et al.*, 2018). The control group (n = 13) were anonymous paediatric patients with written and informed parental or guardian consent and child's assent if older than 7 years, negative for meningitis, without neurological symptoms, and from the same geographic region as the TBM patients; classified as 'normal and healthy cases'. Ethical approval was granted by the Stellenbosch University Health Research Ethics Committee (HREC) (ethics approval number: N16/11/142), the Western Cape Department of Health and Wellness, and the HREC of North-West University, Potchefstroom campus (ethics approval number: NWU-00063-18-A1-01). The presence of HIV co-infection further complicates an already intricate metabolic profile; hence, individuals with positive or unknown HIV status were excluded from this study.

6.2.2 Sample handling, storage, and transportation

The Division of Molecular Biology and Human Genetics at Stellenbosch University stored the urine samples in a dedicated freezer at -80°C. Subsequently, all urine samples designated for metabolomics analysis were sent overnight by courier, frozen and on dry ice, to the Human Metabolomics BSL-3 Laboratory at the North-West University, Potchefstroom campus, where they were aliquoted and stored at -80°C. A volume of 50 µL was collected from each urine sample and combined into a single tube to form a pooled quality control (QC) urine sample.

6.2.3 Sample preparation and GC-TOFMS analysis

Before analysis, the samples were defrosted to room temperature in a biological safety cabinet. A 140 µL volume of extraction solvent, containing 70 µL ethyl acetate and 70 µL acetonitrile with an internal standard (IS; 50 ppm concentration of 2E-pentenoic acid), was mixed with 140 µL urine in a ratio of 1:1:2. The material was thoroughly mixed by vortex for 1 min and allowed for proper mixing in a rotator for a further 20 mins. Centrifugation at 12,000 x g at 4°C was performed for 5 mins on the 280µL of mixture to facilitate phase separation and sediment any particles and macromolecules. After centrifugation, 120 µL of the top organic phase was transferred to a glass GC vial and sealed. Samples were analysed in a random order, with QC samples inserted at regular intervals, on a GC-TOFMS (Leco Pegasus BT with Agilent 7890 GC).

Prior to analysis, a new liner and septum were installed into the GC-TOFMS to prevent undesired reactions and surface adsorption occurrences. Furthermore, routine maintenance procedures were conducted prior to analysis, encompassing leak detection, tuning, and mass calibration. The samples were analysed in 12 batches, following a randomised approach. A 1 µL volume was injected into the GC-TOFMS using a 1:10 split ratio, with purified helium as a carrier gas, maintained at a constant flow of 1 mL/min. Chromatographic separation was carried out using a Restek Rxi-5MS capillary column (30 m length; 250 µm diameter; 0.25µm film thickness). Throughout the complete chromatographic procedure, the initial inlet temperature was maintained at 270°C, the transfer line at 250°C, and the ion source was set at 200°C. The initial GC oven protocol began at 50°C and sustained for 2 mins. Subsequently, the temperature was increased in the following manner: 20°C/min to 120°C, 16°C/min to 180°C, 12°C/min to 250°C, and ultimately 8°C/min to 300°C, maintained for 1 min. The total duration of each sample analysis was approximately 23 mins. Prior to the acquisition of MS data, a 180 s solvent delay was

implemented, during which no mass spectra were recorded. Mass spectra were collected across a spectrum of 50–800 m/z at a rate of 20 spectra per second.

6.2.4 Data pre-processing

Prior to the execution of statistical data analyses, a standardised procedure for the pre-processing of GC-TOFMS metabolomics data was applied. All compounds underwent normalisation utilizing the mass spectral total useful signal (MSTUS) by computing the total useful signal for each sample (Chetwynd *et al.*, 2016). Subsequently, variables that exhibited no variation between the groups were excluded and a data filtering process was executed on each variable to remove those that exhibit more than 50% zero values within each group (Smuts *et al.*, 2013). A quantitative mass merge was then carried out. Thereafter, the coefficient of variation (CV) was evaluated across all quality control (QC) samples, with any variable with a CV > 70% being discarded.

6.2.5 Statistical analysis

All statistical analyses were done using MetaboAnalyst 6.0 (Pang *et al.*, 2024) and Microsoft Excel. Univariate statistical analysis was done when comparing two groups and a one-way ANOVA was done when comparing more than two groups. A Wilcoxon rank-sum test, with multiple testing, was calculated to determine statistical significance (FDR p-value < 0.05). Since our sample size was small, a Hedge's effect size (instead of a Cohen's effect size) was calculated to determine practical significance. A small effect (low practical significance) has a $g \leq 0.2$, a medium effect (moderate practical significance) has a $0.2 < g \leq 0.8$, and a large effect (high practical significance) has a $g > 0.8$. All data were log-transformed to allow for visual comparisons between groups (box plots).

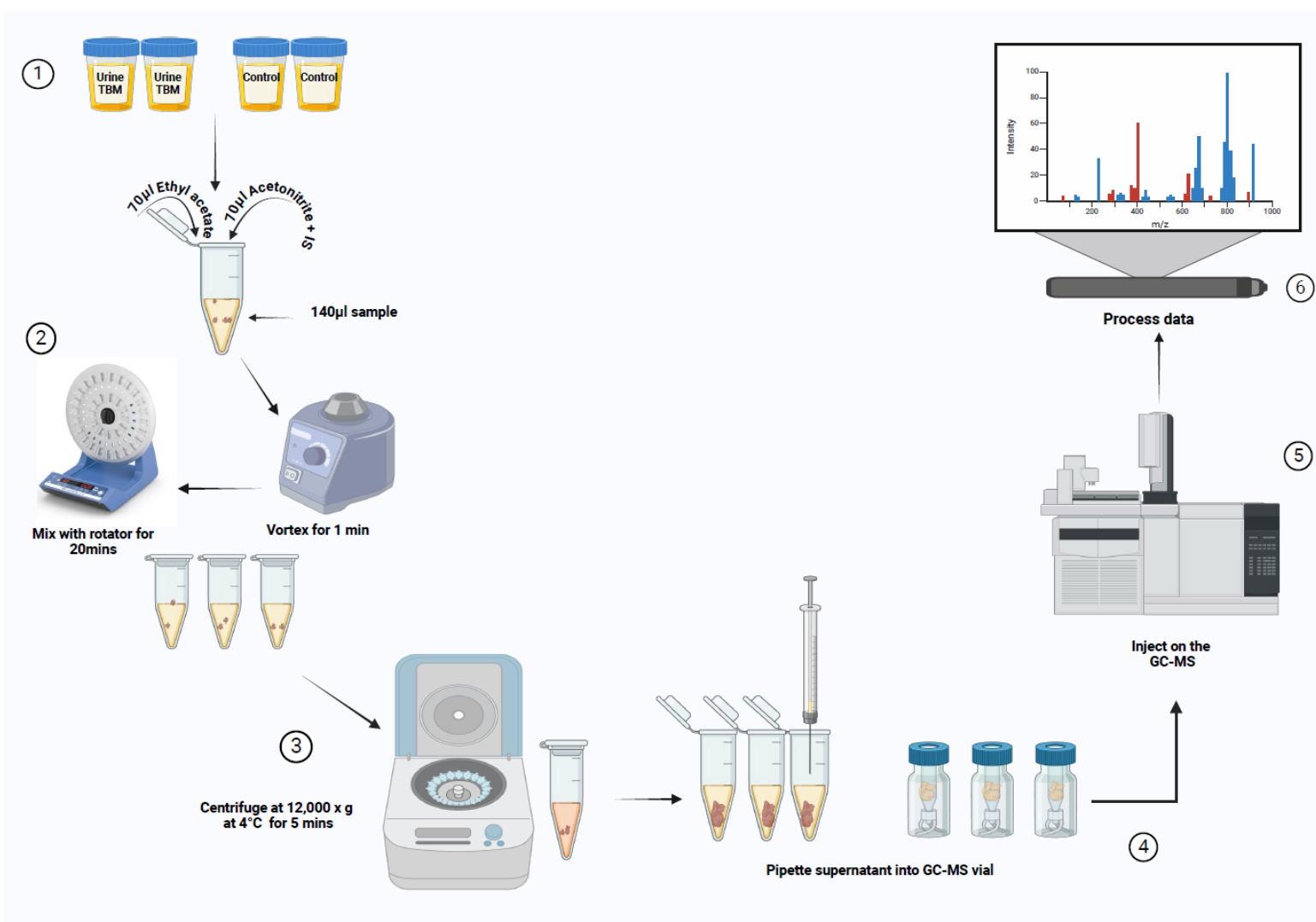


Figure 6.1 Schematic study design depicting the experimental workflow.

6.3 Results

Univariate statistical results identified 13 urinary metabolites to be statistically significantly different (FDR p-value < 0.05) when comparing the urine collected from paediatric TBM patients at baseline (T0) and the control group. Six of these 13 compounds had a medium effect size ($0.2 < g \leq 0.8$) and seven compounds had a large effect size ($g > 0.8$). The quantitative measures of these 13 statistically significant compounds are given in Table 1.

Table 6.1 | Statistically significant (FDR p-value < 0.05) compounds in the baseline paediatric TBM patients when compared to the control group. Hedge's effect sizes are also given as g-values, with a medium effect size identified as $0.2 < g \leq 0.8$, and a large effect size identified as $g > 0.8$.

Compound	FDR p-value	Hedge's effect size (g-value)
3-Methoxy-1-pentene	0.014	1.52
4,7-Dimethyl-undecane	0.042	1.39
Pentadecane	0.003	1.30
3-Heptanone	<0.001	1.27
2-Pyrrolidinone	0.018	1.14
2,6-Dimethyl-undecane	0.042	1.07
Dodecane	0.042	1.05
5,7-Dimethyl-undecane	0.021	0.62
Dichloro-acetic acid, ethyl ester	0.021	0.56
2,5-dimethyl-2-hexene	0.042	0.46
2-Ethyl-trans-2-butenal	0.049	0.44
4,4-Dimethyl-1-pentene	0.042	0.41
2-Butenoic acid, methyl ester	0.018	0.36

6.3.1 Alkanes

Pentadecane (a 15-carbon alkane) and three 11-carbon alkanes with two attached methyl groups (5,7-dimethyl-undecane, 4,7-dimethyl-undecane, and 2,6-dimethyl-undecane) were found to be statistically significantly different in the paediatric TBM urine at T0 when compared to the controls. Pentadecane remained significantly increased (FDR $p < 0.05$) throughout the entire treatment duration (6 months of TBM treatment), except at T2 (2 months of TBM treatment) (FDR $p = 0.083$). The Hedge's effect size g-value of pentadecane remained > 0.8 (large effect) for all TBM treatment points, except at T2, ($g = 0.61$ -medium effect).

4,7-Dimethyl-undecane showed a similar trend to pentadecane – large effect size ($g > 0.8$) at all TBM treatment points and statistical significance at T3 and T5 when compared to the controls. All four alkanes remained increased and did not return to approximate control levels during TBM treatment. These results suggest that the TBM treatment was insufficient to lower these *M. tb*-related alkanes to control levels. Unfortunately, we cannot comment on the possible persistent presence of *M. tb* because the samples used in this study were collected from a retrospective study and follow-up metadata (e.g. *M. tb* presence/load) at various treatment points and treatment outcomes were not collected (van Elsland *et al.*, 2018).

The other two remaining alkanes (5,7-dimethyl-undecane and 2,6-dimethyl-undecane) also differed statistically significantly in the TBM group at T0 when compared to the controls. However, no statistically significant differences occurred during the TBM treatment; although, they continued to show practical significant differences (medium effect size [$0.2 < g \leq 0.8$]) for T1-T6

when compared to the controls. See Figure 2 for box plots and Table 2 for univariate measures of pentadecane, 5,7-dimethyl-undecane, 4,7-dimethyl-undecane, and 2,6-dimethyl-undecane over the TBM treatment points.

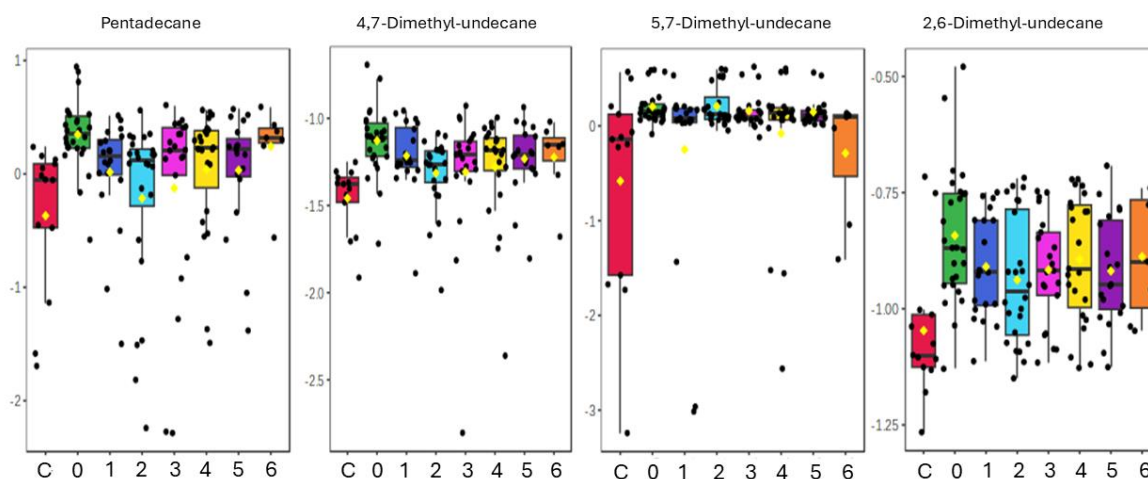


Figure 6.2 Box plots of the four urinary alkanes identified as showing significant changes across the longitudinal TBM data set.

The groups are shown as controls (C; red), baseline TBM (0; green), one month of TB treatment (1; blue), two months of TB treatment (2; light blue), three months of TB treatment (3; pink), four months of TB treatment (4; yellow), five months of TB treatment (5; purple), and six months of TB treatment (6; orange). All data are log-transformed.

Table 6.2 | Four urinary alkanes over TBM treatment points compared to controls. Statistical significance indicated by FDR p-value < 0.05. Hedge's effect sizes indicate a medium effect size at $0.2 < g \leq 0.8$ and a large effect size at $g > 0.8$. The groups are shown as controls (C), one month of TB treatment (1), two months of TB treatment (2), three months of TB treatment (3), four months of TB treatment (4), five months of TB treatment (5), and six months of TB treatment (6).

Alkane	Univariate measure	Control vs TBM treatment points					
		C vs 1	C vs 2	C vs 3	C vs 4	C vs 5	C vs 6
Pentadecane	FDR p-value	0.01	0.083	0.012	0.009	0.007	0.011
	g-value	1.07	0.62	1.04	1.05	1.14	1.48
4,7-Dimethyl-undecane	FDR p-value	0.161	0.083	0.038	0.122	0.031	0.141
	g-value	1.36	0.92	1.01	1.09	1.28	1.57
5,7-Dimethyl-undecane	FDR p-value	0.306	0.083	0.232	0.113	0.299	0.403
	g-value	0.37	0.63	0.43	0.57	0.38	0.40
2,6-Dimethyl-undecane	FDR p-value	0.214	0.39	0.149	0.121	0.614	0.562
	g-value	0.87	0.63	0.81	0.94	0.77	0.95

6.3.2 Other volatile organic compounds (VOCs)

Three alkenes (3-methoxy-1-pentene; 4,4-dimethyl-1-pentene; 2,5-dimethyl-2-hexene) and an aliphatic ketone (3-heptanone) were also found to be statistically significant. 2,5-Dimethyl-2-hexene and 4,4-dimethyl-1-pentene showed a statistically significant decrease in the TBM baseline samples compared to the controls and remained significantly decreased through the six-month TBM treatment period, with 4,4-dimethyl-1-pentene showing a large effect size ($g > 0.8$) and 2,5-dimethyl-2-hexene showing a medium effect size ($0.2 < g \leq 0.8$). Methoxy-1-pentene was significantly increased at T0, T2-T4, and T6, but decreased (returning close to control levels) at T1 and T5 of TBM treatment. 3-Heptanone remained significantly decreased in the TBM cases throughout treatment, with a large effect size (high practical significance).

2-Butenoic acid methyl ester was significantly decreased and 2-pyrrolidinone was significantly increased at baseline TBM, and 2-butenoic acid methyl ester remained statistically significant at T1. However, for all other time points these two compounds were not statistically significant but retained a medium effect size. Hence, 2-butenoic acid methyl ester and 2-pyrrolidinone are likely compounds that can be linked to the disease (TBM) because they were statistically significant at TBM baseline and as the disease (but not necessarily the *M. tb* infection) resolved, these compounds also resolved. In particular, 2-pyrrolidinone returned to control levels by T1 of TBM treatment. See Figure 3 for box plots and Table 3 for the quantitative measures of these six VOCs over the TBM treatment period.

Dichloro-acetic acid, ethyl ester and 2-ethyl-trans-2-butenal are also VOCs but no link to biological function could be found in the literature and were thus not considered further in this study.

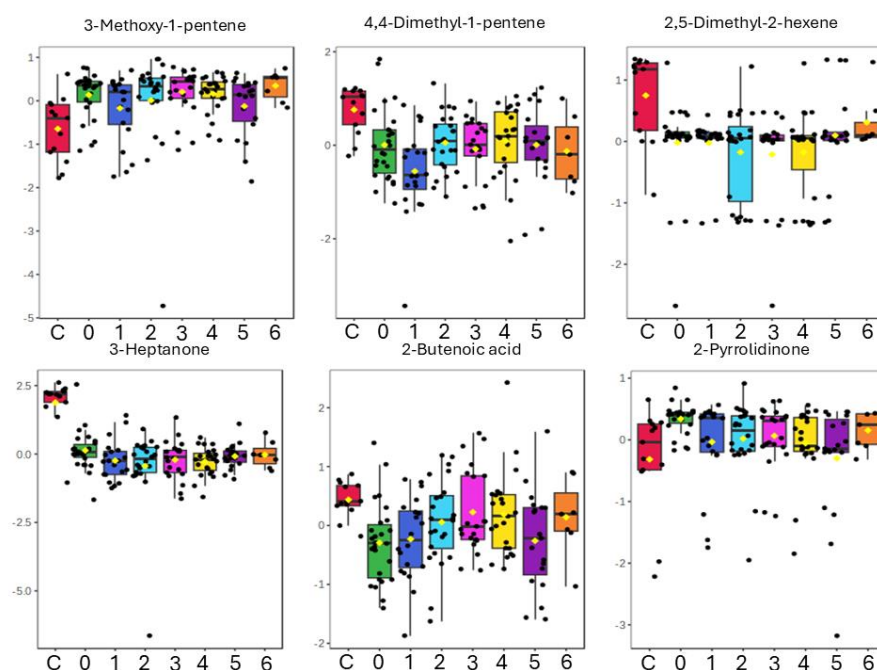


Figure 6.3 Box plots of six urinary compounds identified as showing significant changes across the longitudinal TBM data set.

The groups are shown as controls (C; red), baseline TBM (0; green), one month of TB treatment (1; blue), two months of TB treatment (2; light blue), three months of TB treatment (3; pink), four months of TB treatment (4; yellow), five months of TB treatment (5; purple), and six months of TB treatment (6; orange). All data are log-transformed.

Table 6.3 | Quantitative measures for the other statistically significant VOCs over TBM treatment points compared to controls. Statistical significance indicated by FDR p-value < 0.05.

Hedge's effect sizes indicate a medium effect size at $0.2 < g \leq 0.8$ and a large effect size at $g > 0.8$. The groups are shown as controls (C), one month of TB treatment (1), two months of TB treatment (2), three months of TB treatment (3), four months of TB treatment (4), five months of TB treatment (5), and six months of TB treatment (6).

Alkane	Univariate measure	Control vs TBM treatment points					
		C vs 1	C vs 2	C vs 3	C vs 4	C vs 5	C vs 6
3-Methoxy-1-pentene	FDR p-value	0.058	0.006	0.001	0.002	0.064	0.007
	g-value	0.7	1.14	1.59	1.42	0.95	1.64
4,4-Dimethyl-pentene	FDR p-value	<0,001	0.002	<0,001	0.002	0.02	0.032
	g-value	2.17	1.51	2.04	1.46	1.29	1.47
2,5-Dimethyl-hexene	FDR p-value	<0,001	<0,001	<0,001	<0,001	0.028	0.106
	g-value	0.32	0.59	0.35	0.39	0.27	0.32
3-Heptanone	FDR p-value	<0,001	<0,001	<0,001	<0,001	<0,001	0.007
	g-value	2.02	2.14	2.05	2.11	1.98	1.54
2-Butenoic acid	FDR p-value	0.009	0.63	0.54	0.581	0.604	0.566
	g-value	0.04	0.14	0.22	0.21	0.22	0.27
2-Pyrrolidinone	FDR p-value	0.241	0.282	0.204	0.581	0.935	0.311
	g-value	0.68	0.49	0.71	0.3	0.38	0.49

6.4 Discussion

6.4.1 Alkanes

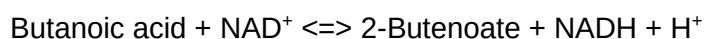
Several species of mycobacteria produce distinctive patterns of VOCs that act as chemical 'fingerprints' (Phillips *et al.*, 2007) of the organism and the associated diseases they cause. Alkanes and methylalkanes have been progressively employed by physicians as an innovative approach to the diagnosis of various pathological conditions, circumventing the discomforts associated with invasive methodologies (Cheepsattayakorn and Cheepsattayakorn, 2014). To facilitate the uptake of alkanes, bacteria secrete glycolipid surfactants (Ron and Rosenberg, 2002). The process starts with the oxidation of the terminal methyl group by alkane hydroxylases, leading to the formation of a primary alcohol, which is subsequently oxidised to an aldehyde and finally converted to fatty acids for energy or cell wall biosynthesis (Rojo, 2009). Understanding the adaptation and replication of *M. tb* in the intracellular environment of the host will aid in our understanding of the pathogenesis of TBM. A study by Loots (2014), in an isoniazid-resistant *M. tb* strain, characterised the TetR transcriptional regulator Rv3249c, which regulates a conserved alkane hydroxylase. Rv3249c represses the operon for AlkB, a predicted alkane hydroxylase that enables the utilization of medium- and long-chain (C5-C16) alkanes as carbon sources. It also shows similarity to membrane-bound fatty acid desaturases. Fatty acid desaturation entails the enzymatic removal of hydrogen from a methylene group within an acyl chain (Shanklin and Cahoon, 1998). The findings by Schnappinger *et al.* (2003) indicate that *M. tb* experiences heightened oxidative stress in the intra-phagosomal environment, leading to increased reliance on fatty acid oxidation for carbon and energy. Loots (2014) also noted that isoniazid-resistant strains with *katG* mutations likely face greater oxidative stress, prompting enhanced alkane uptake for fatty acid synthesis and energy utilisation. Additionally, alkanes and their derivatives have been detected in human breath as VOCs resulting from oxidative stress (Kneepkens *et al.*, 1994; Phillips *et al.*, 2000). Patients with TBM are known to exhibit increased oxidative stress (Davis *et al.*, 2019). In this current study, we also identified urinary alkenes (2,5-dimethyl-2-hexene; 4,4-dimethyl-1-pentene; 3-methoxy-1-pentene) associated with *M. tb*. Other studies have also reported the presence alkene derivatives in patients with TB (Kolk *et al.*, 2012; Makhubela *et al.*, 2023). Research endeavours should continue to explore the role of VOCs in the diagnosis of TBM. For instance, Phillips *et al.* (2007) identified a spectrum of VOCs correlated with TB, and 3-heptanone, one of the markers identified in this investigation, has previously been reported as a significant biomarker for breath analysis in the context of TB.

6.4.2 2-Pyrrolidinone

2-Pyrrolidinone is a lactam cyclization product of the neurotransmitter gamma-aminobutyric acid (GABA) (Hyder *et al.*, 1999). A study (Petroff *et al.*, 1999) also found that antiepileptic drugs increase GABA and its related metabolites homocarnosine and 2-pyrrolidinone. Moreover, 2-pyrrolidinone is seen to induce long-lasting facilitation of hippocampal synaptic transmission by enhancing nicotinic acetylcholine (ACh) receptors in brain responses via a protein kinase C pathway (Miyamoto *et al.*, 2003). AChs contribute to the control of the resting membrane potential, modulation of synaptic transmission, and mediation of fast excitatory transmission (Hogg *et al.*, 2003; Miyamoto *et al.*, 2003). GABA serves as the primary inhibitory neurotransmitter in the human nervous system, and the metabolism of GABA to succinic semialdehyde helps regulate its levels and neurotransmitter activity (Kennedy *et al.*, 2019). The accumulation of GABA, either by enzymatic inactivity 4-aminobutyrate aminotransferase (ABAT) or by medical intervention, can result in elevated levels of 2-pyrrolidinone, due to GABA cyclization (Callery *et al.*, 1978). 2-Pyrrolidinone can also be converted to succinimide through a two-step reaction, and the opening of the hydrolytic ring of cyclic imides such as succinimide can occur through enzymatic and non-enzymatic routes (Kurono *et al.*, 2008; Lerner *et al.*, 2013; Maguire and Dudley, 1978). Inhibition of ABAT activity results in the accumulation of GABA, β -alanine, homocarnosine, and 2-pyrrolidinone (Jaeken *et al.*, 1990; Parviz *et al.*, 2014). Increased levels of GABA leads to symptoms including hypotonia, hyperreflexia, lethargy, refractory seizures, abnormal brain magnetic resonance imaging, and electroencephalogram abnormalities (Dracopoulos *et al.*, 2010; Hussain *et al.*, 2017; Pearl *et al.*, 2009; Schonstedt *et al.*, 2015). Kennedy *et al.*, (2019), compared four patients with GABA-transaminase deficiency, using an untargeted metabolomics approach that compared the biochemical profile in different matrices (plasma, urine, and CSF) for clinical screening of 2-pyrrolidinone and succinimide. Three patients showed elevated levels of 2-pyrrolidinone, succinimide, or its opened ring form (succinamic acid) in plasma, urine, and CSF, and/or homocarnosine in urine and CSF. To elucidate these mechanisms, Kennedy *et al.* (2019) further indicated that the identification of medications alongside the levels of 2-pyrrolidinone gave insight into the mechanism of the elevation of 2-pyrrolidinone as a clinical biomarker. Hence, 2-pyrrolidinone has several links to neurological functions, indicating impairment (shunting) of GABA metabolism in TBM.

6.4.2 2-Butenoic acid

2-Butenoic acid, also known as crotonic acid, is a straight, aliphatic, unsaturated carboxylic acid with a strong apolar nature. 2-Butenoic acid is produced by the oxidation of 2-butanoic acid, coupled with the reduction of NAD⁺ to NADH:



Crotonic acid and its derivatives permeate cell membranes through passive diffusion and active transport mechanisms utilising sodium-coupled monocarboxylate transporter-1 or monocarboxylate transporter-1, subsequently undergoing conversion to crotonyl-CoA, which serves as a principal substrate for the crotonylation of histone lysine residues. However, the comprehensive biological pathways that govern the metabolism of crotonic acid are not yet adequately elucidated (Yang *et al.*, 2023). A segment of the scholarly community posits that crotonic acid, analogous to butyric acid, may function as a metabolic byproduct or an intermediary generated by specific microbial activity in the body; however, empirical substantiation for this hypothesis is currently absent (Fischer *et al.*, 2010). Crotonyl-CoA, an intermediate metabolite synthesised from fatty acid catabolism, is derived from crotonic acid through the enzymatic action of acyl-CoA synthetase short-chain family (ACSS2) or the oxidative breakdown of amino acids such as lysine, tryptophan, and butyrate (Fang *et al.*, 2021). It has been demonstrated to play a crucial role in a variety of neurological disorders, including neuropathic pain, Alzheimer's disease, neonatal hypoxic-ischemic encephalopathy, and various neurodevelopmental diseases (Yang *et al.*, 2023). Crotonic acid was also identified as a marker of the presence of poly-β-hydroxybutyrate (PHB), microbial-origin (Watt *et al.*, 1991). In the context of aerobic facultative physiology, bacteria exhibit a propensity to amass PHB as a reservoir for carbon and energy. During microbial proliferation and energy production, the tricarboxylic acid (TCA) cycle and the PHB metabolic pathway engage in competition for the substrate acetyl-CoA (Korotkova and Lidstrom, 2001). A study in search of drug targets for *M. tb* (Purohit *et al.* (2007) showed a truncated citric acid cycle with the glyoxylate shunt, suggesting an option for survival by the pathogen and pathogenesis. Purohit *et al.* (2007) proposed that precursors to support this pathway could also be generated via enzymatic conversion involving PHB. They supported this claim by using available genome sequence data, which were analysed for possible enzymatic conversions that can generate glyoxylate, acetyl CoA, and other enolases that can also be useful for various fatty acid transformations. Therefore, crotonic acid is another *M. tb*-related metabolite that needs further investigation.

6.4.3 3-Heptanone

3-Heptanone, also referred to as ethyl n-butyl ketone (EBK), is an aliphatic ketone. The principal metabolite of EBK is identified as 2,5-heptanedione, which serves as a neurotoxic analogue of 2,5-hexanedione (O'Donoghue *et al.*, 1984). Notably, 2,5-hexanedione is recognized as the most neurotoxic metabolite arising from the metabolism of n-hexane and methyl n-butyl ketone (MnBK), a neurotoxin that was reported in the literature (Mendell *et al.*, 1974; Spencer *et al.*, 1975). The increased metabolic conversion of EBK to 2,5-heptanedione may lead to neurotoxic effects (O'Donoghue *et al.*, 1984). 3-Heptanone is also a metabolite of valproic acid (VPA) or 2-propylpentanoic acid, which is classified as branched-chain fatty acid. VPA can exert its effects by inhibiting voltage-gated sodium channels, thereby enhancing the inhibitory actions of GABA (Erhart *et al.*, 2009). The metabolic pathway of VPA includes the conversion to unsaturated compounds via hydrogenation, the generation of hydroxylated metabolites through oxidation, and the formation of carbonyl metabolites through further oxidation. The β -oxidation of VPA produces 3-oxo-VPA, which subsequently undergoes spontaneous decarboxylation to produce 3-heptanone (Erhart *et al.*, 2009; Feriduni *et al.*, 2019). The fact that this particular VOC remained significantly increased (with a very large effect size – high practical significance) in the TBM cases, despite 6 months of TBM treatment, suggests that this VOC is linked to a possible persistent perturbation caused by the *M. tb* infection. Future *M. tb* studies should target this VOC and evaluate its significance.

6.5 Conclusions

To the best of our knowledge, this is the first GC-TOFMS analysis of urinary alkanes and other VOCs in individuals infected with *M. tb*. This study identified eight compounds related to *M. tb* metabolism: four alkanes (pentadecane, 5,7-dimethyl-undecane, 4,7-dimethyl-undecane, and 2,6-dimethyl-undecane), three alkenes (2,5-dimethyl-2-hexene, 4,4-dimethyl-1-pentene, and 3-methoxy-1-pentene), an aliphatic ketone (3-heptanone), and two additional biological compounds (2-butenic acid methyl ester and 2-pyrrolidinone). By understanding the complexity of TBM and targeting the metabolism of *M. tb*, this study contributes to our understanding of the disease (TBM) and the role of VOCs as a carbon source for the persistent survival of *M. tb*. This study shows that alkanes and other VOCs in urine are an important consideration in *M. tb*-related diseases and should be further investigated with larger cohorts of *M. tb*-infected individuals and across

various biofluids. Therefore, this study improves our understanding of *M. tb* infection but also takes us one step closer towards using urine as a potential non-invasive diagnostic measure for TBM.

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**PART 6 ANALYSIS OF THE CENTRAL CONCLUSIONS OF THIS THESIS AND
POTENTIAL FUTURE DIRECTIONS FOR THIS RESEARCH.**

CHAPTER 7

This thesis is a study of TBM in children with a focus on characterising the urinary metabolic profile of the disease, to better understand treatment response, the interactions between the pathogen (*M. tb*) and the host, and how this relates to the severity of the infection disease. To understand these pathogen-host interactions, it is important to recognise that it is currently difficult to accurately characterise TBM, especially in the early disease stages due to the non-specific symptoms of general ill health. Nonetheless, the research presented in this thesis describes the urinary metabolic profile of the infected host, attempting to demarcate specific metabolites to either host or pathogen metabolic pathways. A detailed examination of the overarching conclusions and implications rendered by this thesis, together with the potential future directions in which this research could progress, shows promise that the urinary metabolic profile of TBM can be further explored to enhance our understanding of this devastating disease.

According to the WHO, the African region witnessed the occurrence of the second highest count globally (23%) of new cases of TB (WHO, 2023a; WHO, 2023b). South Africa is one of the countries with the highest prevalence of TB in the world. TBM is the most severe manifestation of TB and accounts for an estimated 20% of TB-related deaths in South Africa (**Chapter 1**) (Solomons, 2023, Rohlwink et al., 2016). However, there is currently a lack of knowledge on the global effects of paediatric TBM. Age is considered the most important risk factor in the development of TBM, most commonly occurring in children aged 0 to 4 years, with children younger than 2 years of age with TB, if left untreated, typically developing TBM (Wolzak et al., 2012). The reason why children are more vulnerable to developing TBM is due to their still underdeveloped immune systems. Children can present with various general symptoms, partly dependent on the stages of the disease, differences in immune responses, and other different host factors. Hence, TBM is a serious health issue within South Africa and is particularly dangerous in infants and children. It is known that TBM in children is associated with neurological sequelae (Chiang et al., 2014). However, there can be a high survival rate if diagnosed early and, subsequently, early treatment.

The gold standard for the definite diagnosis of TBM (definite TBM) requires a positive culture of *M. tb* from collected CSF. A lumbar puncture is performed to collect CSF, but this procedure has numerous potential health and safety risks, particularly in children. The lack of bacillary presence and limited CSF volume collected from paediatric patients presenting with symptoms complicates the confirmation of the presence of *M. tb*. Hence, diagnosing TBM remains a significant challenge, with many TBM cases primarily diagnosed through clinical assessments, such as identifying

abnormalities in the CSF and brain (probable TBM). Considering this, a non-invasive diagnostic approach is still needed to accurately diagnose TBM, especially in children. To the best of our knowledge, this thesis is the first comprehensive report (incorporating various analytical platforms) on the urinary metabolic profile of TBM in paediatric cases.

In this thesis, untargeted proton nuclear magnetic resonance (^1H -NMR) spectroscopy (**Chapter 3 and 5**), semi-targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) (**Chapter 4**), and semi-targeted gas chromatography coupled with time-of-flight mass spectrometry (GC-TOFMS) (**Chapter 6**) was used. These three analytical platforms were used to identify the urinary metabolic markers characteristic of TBM in its various stages to gain a better understanding of the disease and identify potential urinary diagnostic markers of TBM in paediatric patients. Metabolomics is a promising technique that is used to advance our understanding of the mechanism of infection/disease and the identification of metabolites for new diagnostic targets (de Carvalho et al., 2010, Zhang et al., 2018). Thus, new analytical approaches, such as metabolomics, are needed to shift paradigms in understanding the metabolism of TBM, and ultimately develop new diagnostic approaches. The discussion that follows describes the insights gained from the urinary metabolomics evaluation of TBM.

7.1 The first goal of the thesis: gain biological insight into the pathogenesis of TBM

Objective 1: to acquire an understanding of the comprehensive metabolite landscape at the locus of infection (the brain) in TBM through a holistic view of the examination of the urinary profile. An overview of the pathogenesis of TBM, as described in the literature, is given in **Chapters 1 and 2**. It is well established in the literature that infection in the central nervous system (CNS) influences the gut microbiome through the gut-brain axis, and the resulting metabolic alterations in both the CNS and the gut, which in turn are released into the bloodstream, and then filtered by the kidneys and excreted via urine (**Chapter 2**). After the onset of pulmonary TB, *M. tb* may also disrupt the BBB, which enables their passage into the brain. The various defences of the host are usually inadequate to control the infection. Inflammation follows from infection and may, in turn, compromise the BBB junctions that regulate the flux of ions, polar molecules, and macromolecules. The result is a BBB with increased permeability. This then results in more infectious bacteria crossing the BBB by transcellular penetration after bacterial adhesion to endothelial cells or via infected leukocytes, as well as via the phagocytes infected with the pathogen. When bacteria penetrate the BBB and establish an infection, there is a cascade of

events within the brain, modulating a feedback effect on the host's gut microbiome. Thus, the perturbed metabolism in both the CNS and the gut will release metabolites into the blood. The gastrointestinal tract (GIT) is a highly complex organ involved in multiple dynamic physiological processes while interacting with the gut microbiome. Additionally, the brain-to-intestinal signalling pathway affects host–bacteria interactions in the GIT by influencing the enteric microbiota indirectly via altered intestinal permeability, or directly through signalling molecules released into the gut. This crosstalk changes the gut-microbiome functionality and the activation of any of these signalling systems, alone or in combination, affects the composition and functionality of the enteric microbiota. Short-chain fatty acids derived from the gut microbiome play a pivotal role in the functionality and maturation of the microglia in the brain. Microglia are considered crucial mediators in the brain and intestinal microbiota interaction. The CNS communicates with the gut through signalling molecules carried by the CSF and blood, which are also known to alter the gut composition and physiology. This communication encompasses several channels along various neural, enteric and immune systems [i.e., gut-brain axis (**Chapter 2**)].

An in-depth understanding of TBM can be significantly enhanced through the application of metabolomics, which systematically explores and characterises the intricate biochemical profiles and metabolic alterations that occur within biological systems, thus illuminating the complex pathophysiological mechanisms underpinning this serious infectious disease. To do this, an untargeted ¹H-NMR metabolomics approach (**Chapter 3**) was initially used in this thesis, since NMR is not biased to any metabolite class and provides a robust and comprehensive perspective on the altered metabolic profile of the infected host. Characterisation of the metabolic profile involves an in-depth biochemical description of its characteristics, including its aetiology, pathogenesis, clinical manifestations, epidemiology, and prognosis. After meticulous examination and biological elucidation of the altered metabolites observed in urine samples collected from paediatric subjects with TBM, we successfully identified several promising candidates that may serve as urinary biomarkers indicative of the pathological condition. A biosignature of TBM was established, comprising 29 distinct urinary metabolites identified as characteristic of advanced TBM (stage 3) and were categorized into six dysregulated metabolic pathways. Upregulated tryptophan catabolism indicated a significant alteration in amino acid metabolism. Quinolinic acid, the downstream metabolite of tryptophan metabolism, was also identified as a potential marker, reflecting immune dysregulation in TBM; produced by the enteric nervous system in the intestinal tract caused by dysbiosis. Of further mention, was that the majority of the significantly altered urinary metabolites (VIPs) identified through our untargeted metabolomics investigation in TBM-

confirmed paediatric cases, were also previously observed in an independent research endeavour that also used NMR metabolomics as an exploratory approach (Mason et al., 2016) to examine TBM. We additionally observed a suboptimal vitamin B3 status in these individuals, indicated by increased urinary concentration of 1-methylnicotinamide, which reflects an enhanced utilisation of vitamin B3, resulting from the aforementioned increased tryptophan catabolism. Increased energy production and a metabolic burst associated with increased energy production were also observed, suggesting a heightened metabolic state in patients with TBM. Mannose, another indicator of this metabolic burst, as was previously investigated in the serum of children infected with TB (Conde et al., 2022), and one of the key components of *M. tb* cell wall lipoarabinomannan (LAM) (van Toorn et al., 2014), was also identified as a significantly altered metabolic marker in the current study. Ketoacidosis and increased nitrogen excretion were also identified as characterising paediatric TBM. Lastly, eight elevated microbial metabolites, derived primarily from modified microbiome metabolism, were characteristic of the severe manifestation of TBM, as delineated in this study. The presence of dysbiosis in TBM is supported by previous findings, which warrants further examination of the gut-brain axis in the context of TBM (**Chapter 2**). These results show that TBM, after initial treatment, could still be well differentiated from controls, according to the altered urinary metabolic profiles of the urine samples of these TBM patients, elucidated via ¹H-NMR metabolomics.

7.2 Fulfilling the second goal of the thesis: Identify metabolite profiles that characterize the three stages of TBM and potential biosignatures for an earlier differential diagnosis.

The pursuit of the second objective was greatly aided by two fundamental pieces of contextual knowledge, as described below.

Objective 2 was to gain more insight into the altered metabolic pathways associated with TBM. To do this we used a semi-targeted LC-MS/MS metabolomics approach (**Chapter 4**). Identifying early (stage 1) TBM currently presents significant challenges and remains problematic. This emphasises the importance of identifying metabolite markers that may, in turn, lead to a novel approach in accurately identifying children recently exposed to *M. tb*, in the CNS, via alterations to their urinary metabolite profiles. To achieve this objective, a novel method was first developed to eventually study the underlying perturbation of the altered gut microbiome in stages 1, 2, and 3 of TBM. Using this LC-MS/MS approach, 78 targeted compounds of interest of microbiome

origin were selected, using previously purchased standards/isotopes (**Annexure B**). In TBM, the immune response and inflammation can affect the gut microbiota, leading to disruptions that affect immunity and metabolism, and highlighting the importance of understanding this relationship for better treatment results. This LC-MS/MS investigation discovered eight unique urinary metabolites that correlated with TBM, including 5-hydroxyindoleacetic acid (5HIAA), 5-hydroxyhexanoic acid (5HHA), 4-hydroxyhippuric acid (4HHA), and methylcitric acid. These metabolites are associated with the metabolic pathways of tryptophan, fatty acids, and *M. tb*, respectively. In addition, these metabolites are associated with urinary biomarkers indicative of pulmonary TB, a consequence of the modified host metabolome induced by *M. tb*. Urinary excretion of 5HIAA also serves as a marker for determining serotonin in neurological conditions. Derivatives of microbial indole-3-acetic acid are a product of gut dysbiosis. Microbe-produced indole derivatives of tryptophan have been shown to have a clear link to immune regulation in the host, while the 5-hydroxyindole precursor of 5HIAA is known to be involved in CNS regulation (O'Mahony et al., 2015; Roth et al., 2021; Fiore and Murray, 2021). An important question we posed was this: 'If the increased flow of tryptophan into the kynurenine pathway is known to occur due to upregulated IDO-1 activity during TB and other diseases, then how is there sufficient bioavailability of tryptophan in the host for the increased levels of urinary 5HIAA that have been observed in this study?' In fasting situations, 5HHA has been identified as an aberrant fatty acid oxidation (FAO) urinary microbial metabolite. Intending to elucidate the mechanisms of TB, Luies and Loots (2016) found anomalies in the metabolism of fatty acids and amino acids in the host caused by pulmonary TB, including 5HHA. Hence, disturbances in fatty acid metabolism are another landmark of TB-induced gut dysbiosis. However, to our knowledge, 5HAA has not been directly linked to changes in gut microbiota. Investigating urinary TBM profiles of infants and children (untreated), Mason et al. (2016) identified four metabolites, including 4HHA, as having the potential for a non-invasive diagnostic biosignature. These significant metabolites showed a constant elevated level for stage 1 TBM throughout the treatment period. Hence, this discovery indicates that the gut microbiome represents a significant factor in the intricate interactions between TBM infection and the host's immune response. Further investigations are essential to clarify the mechanisms that govern this association and to formulate targeted therapeutic strategies that take advantage of the potential of the gut microbiome as a potential therapeutic target in the fight against TBM.

In addition to the semi-targeted LC-MS/MS investigation, we also conducted a novel semi-targeted GC-TOFMS investigation (**Chapter 6**) to evaluate volatile organic compounds (VOCs).

It has been noted in microbiological studies on lab strains of mycobacteria that these adaptive bacteria can grow and survive on alkanes alone. However, there has never been an investigation into alkanes and other VOCs in TBM. For the first time, we showed that alkanes and other VOCs are important to consider. This may emphasize the possible role of alkanes and VOCs in the potential persistence of *M. tb*. Hence, we provided proof of concept of a novel class of compound to investigate in future diagnostic and treatment studies of TBM.

Objective 2 was also to use the innovative insights gained from the holistic view of the characterisation of urinary metabolomics in the pursuit of establishing new diagnostic biomarkers for the non-invasive identification of TBM in children within the cohort under investigation. We delved deep into the heart of the main goal through an in-depth ¹H-NMR metabolomics study on urine collected from a TBM paediatric cohort. The objective was to identify biomarkers of TBM with high precision and specificity and the outcome of the study (**Chapter 5**) indicated that five significant biological metabolites were identified: 1-methylnicotinamide, 3-hydroxyisovaleric acid, 5-aminolevulinic acid, N-acetylglutamine, and methanol which did not correlate with medication metabolites. Four of these metabolites (1-methylnicotinamide, 3-hydroxyisovaleric acid, 5-aminolevulinic acid, and N-acetylglutamine) were identified as showing promising diagnostic potential for severe TBM. Additionally, 14 unknown compounds were identified as important for differentiating TBM patients from controls. Three of the four urinary metabolic markers of TBM (1-methylnicotinamide, 3-hydroxyisovaleric acid, and N-acetylglutamine) have already been comprehensively discussed in terms of their biological context (**Chapter 3**). In the context of tryptophan–nicotinic acid metabolism, 1-methylnicotinamide is an important recurring metabolite that represents a terminal product of nicotinamide (vitamin B3) metabolism, and the observed increase in urinary levels of 1-methylnicotinamide has been previously associated with various neurological and inflammatory disorders (Smeitink et al., 2004; Zheng et al., 2013). Remarkably, elevated levels of 1-methylnicotinamide are predominantly attributed to an increased metabolic flux through the kynurenine pathway (**Chapter 3**), which is stimulated by INF- γ , alongside the production of ROS and RNS. These results are indicative of severe oxidative stress and compromised redox balance, which are particularly relevant and related to TB (Reddy et al., 2009). A disturbed energy status in TBM, in addition to a metabolic burst and an increased requirement for energy in the brain infected with *M. tb*, have been associated with 3-hydroxyisovaleric acid, a leucine catabolite. Functioning as a neurotransmitter in the brain, N-acetylglutamine is a downstream product of D-glutamine and D-glutamate metabolism. Its accumulation has been previously shown to result in neuronal instability (**Chapter 3**). Ultimately,

our metabolic model could accurately classify severe TBM but could not classify early (stage 1) TBM.

In conclusion, the metabolomics data generated via the methodologies employed in our investigation can be deemed successful in providing new insights into TBM. The identification of urinary biomarkers for TBM can be asserted with a notable degree of confidence; however, it remains a tentative proposition as it is exclusively relevant to the specific cohort examined. At this point, it is imperative to highlight that a distinct urinary biosignature for TBM has been identified, but it has not yet been established as a definitive diagnostic tool, and further validation studies for this purpose are needed. This underscores the principal significance of the entire study... based on the results from the TBM cohort analysed in our research, it is feasible to say that TBM could be diagnosed using urinary markers (i.e., utilising a non-invasive sample collection technique instead of a lumbar puncture). Therefore, this study paves the way for subsequent research and validation efforts, which could culminate in the formulation of a medical algorithm for the diagnosis of TBM based on the urinary markers determined from this metabolomics study.

7.3 Limitations

This thesis has given a deeper understanding of the metabolic characterisation, diagnostic criteria, and prognostic implications of TBM in children, but many questions remain unanswered. The constraints inherent in this study necessitate careful consideration. Future inquiries should aim to address these limitations comprehensively. The limited sample size presents a significant constraint, as well as the absence of meta-data regarding the timing and nature of the treatment protocols administered to the undiagnosed TBM patients at the referral healthcare facilities. Upon meticulous examination of the ^1H -NMR spectra, it was determined that a subset of patients had previously received pharmacological interventions. These interventions were likely administered at regional/local health centres to stabilise the patient before their transfer to Tygerberg Hospital, which boasts a highly proficient paediatric cohort specializing in TBM; however, the biochemical alterations of these medications in the body remain unidentified. Furthermore, given the retrospective nature of the urine sample collection, the precise timing of sample acquisition was not documented; rather, a baseline urine specimen was obtained at the commencement of home treatment. Considering that the median time duration from admission to discharge in this study context over the past 38 years was recorded as 16 days, with an interquartile range of 12–23 days, means that the time of baseline sample collection was variably different for each TBM patient (an uncontrolled confounder). Therefore, each TBM patient had a varying duration of time

to stabilise in the hospital before being discharged and commencing home treatment. Another major limitation was the inability to definitively demarcate the demographic data of the control group, due to ethical restrictions. The only information available for the controls was that they were paediatric patients without meningitis, no neurological symptoms, and from the same geographical region as the TBM patients. Another limitation was the lack of follow-up *M. tb* testing during the period of treatment and no meta-data on the status of the TBM patients during treatment, especially regarding follow-up studies on the potential diagnostic biosignature and verification of the candidate. Due to these limitations of this study, **Objective 3** – evaluation of the treatment response based on the metabolic profiles over the 6-month treatment period, could not be accurately determined.

7.4 Future perspectives

Nonetheless, the implications of the findings in this thesis warrant further exploration within diverse contexts. It is imperative to recognise the potential for broader application of these results in subsequent research endeavours. As is typically observed in metabolomics research, particularly concerning diseases that exhibit low prevalence, the stated limitations of this study present avenues for subsequent investigations. Future investigations should corroborate the findings of quinolinic acid as a prospective urinary diagnostic marker for TBM while exploring amino acid imbalance treatments as a therapeutic approach to mitigate TBM symptoms. This thesis also proposed therapeutic implications (**Chapter 3**), such as additional vitamin B and glutamine supplementation, in the management of TBM symptoms.

As a follow-up study to this thesis, with the aim to validate the results given here, the ideal prospective study design should incorporate the following: a larger cohort of children with TBM ($n > 100$ for stages 1, 2, and 3), various sample types (urine, stool, and blood) with a baseline date at initial point of admission, ideally before stabilising the patient with treatment, a sizeable number of case-matched control subjects with clinical data and selection criteria; excluding comorbidity (with HIV) from the large cohort based on patients clinical information at point of care.

Lastly, the ultimate goal of the larger project, eventually, would be to develop a non-invasive point-of-care (POC) diagnostic device for quick and reliable detection of early TBM in the field (outside the hospital) using the urinary markers detected in this investigation. To design such a POC device we would first need to designate validated urinary metabolic biomarkers to TBM, approach a biotechnology company to design nanoparticles that would attach specifically to these

biomarkers, and for that the nanoparticle-metabolite complex to create a colour change on a dipstick to indicate the positive presence of the biomarkers. Thus, the high-tech, expensive instruments used in metabolomics would not be necessary in the field; instead, a cheap and quick POC device is all that would be needed to save the many lives that would have been lost to TBM through insufficient diagnostics. The impact of TBM on public health, especially children, has put societies and underdeveloped African countries in pain. Every child deserves a healthy life. Unfortunately, most African regions, including South Africa, still suffer from this epidemic. Urinary metabolic profile screening can provide a way for early detection for quicker treatment and give public health a second chance.

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Annexure A:

Supplementary Information:

Urinary metabolic characterization of advanced tuberculous meningitis cases in a South African paediatric population

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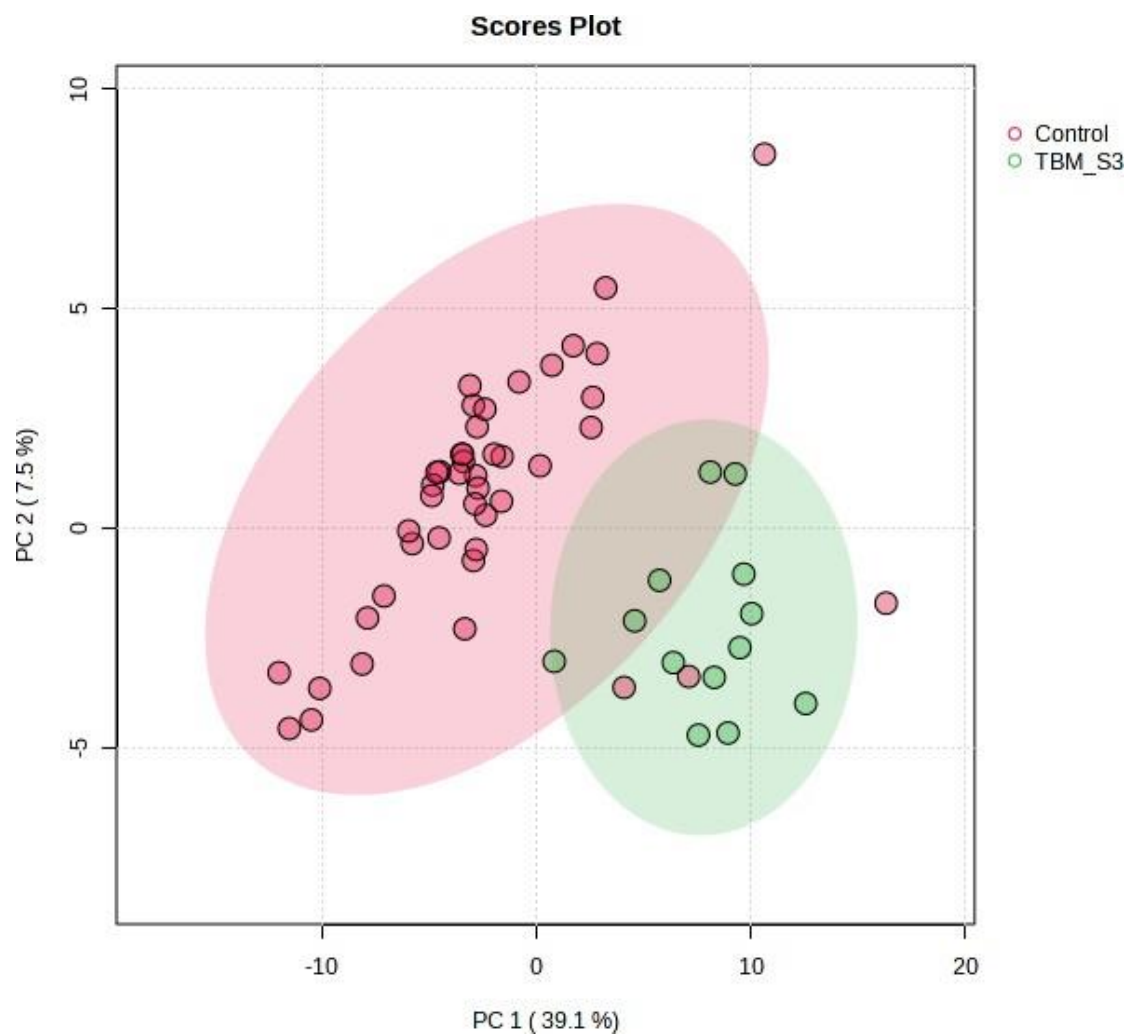


Figure S3.1 Four outliers identified in control group using PCA with 95% CI.

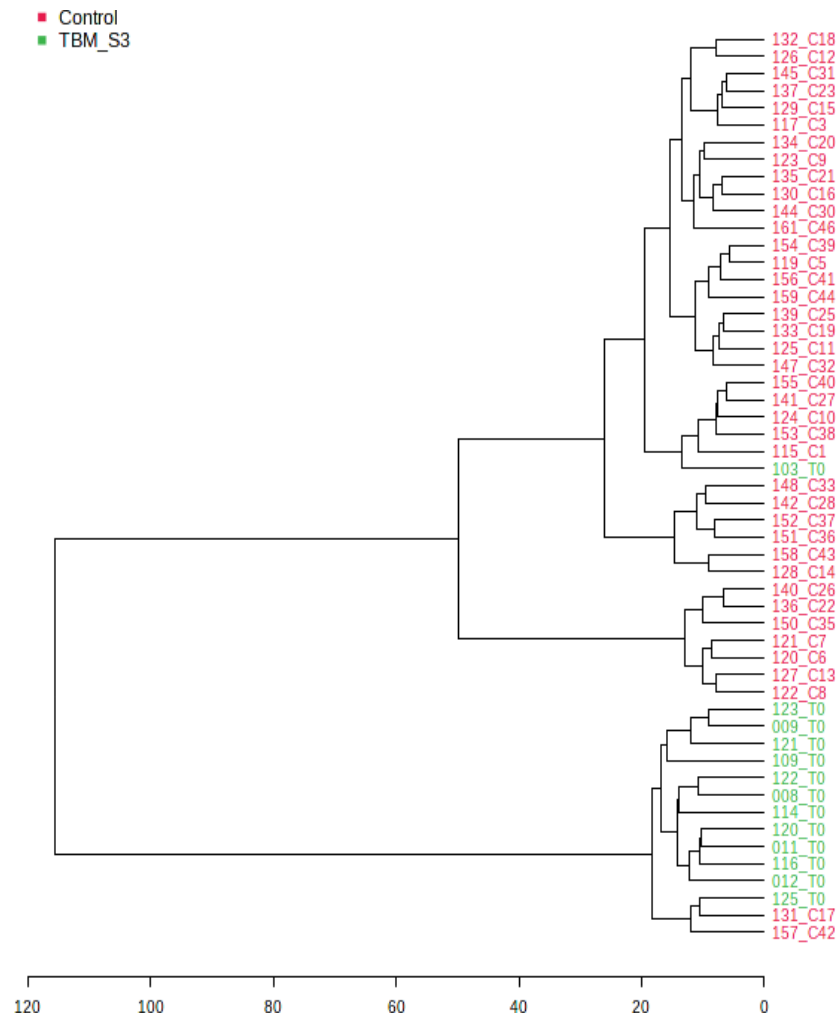


Figure S3.2 Hierarchical cluster analysis results shown as dendrograms (Euclidean distance measure, and clustering algorithm using Ward's method) All cases of controls (n = 40, in red) and TBM cases (n = 13, in green).

Table S3.1 Quantitative statistical data of the 137 ¹H-NMR spectral bins, with metabolite annotations, that were identified as important based upon the rule: VIP comp 1 and comp 2 > 1.0 OR p-value FDR ≤ 0.05 and absolute fold change ≥ 2.0.

Metabolite annotation	Bin	VIP Comp. 1	VIP Comp. 2	p-value FDR	Fold Change
Isoleucine	0.93	0.99	1.05	<0.00001	2.23
Isoleucine/Leucine	0.95	1.22	1.23	<0.00001	2.65
Leucine	0.97	1.28	1.24	<0.00001	2.67
Valine	0.99	1.13	1.01	0.0006	3.30
Isoleucine/Valine	1.01	1.21	1.07	0.0001	3.91
Valine	1.03	1.08	1.06	0.0001	3.62
Valine	1.05	1.00	0.91	0.0006	2.71
3-Hydroxyisobutyric acid	1.07	1.30	1.17	0.0001	2.97
	1.09	1.49	1.44	<0.00001	3.78
1,2-Propanediol	1.13	1.86	1.65	<0.00001	7.51
	1.15	2.24	1.99	<0.00001	18.81
	1.17	0.91	0.94	0.0005	2.13
3-Hydroxyisovaleric acid	1.27	1.38	1.24	<0.00001	2.92
1.29	1.29	1.20	1.09	<0.00001	2.63
1.41	1.41	1.09	1.18	<0.00001	2.34
1.43	1.43	1.26	1.20	<0.00001	2.96
1.45	1.45	1.22	1.29	<0.00001	2.74
1.51	1.51	1.38	1.31	<0.00001	3.21
1.55	1.55	1.50	1.34	<0.00001	3.83
1.57	1.57	1.48	1.32	<0.00001	3.75
1.61	1.61	1.16	1.14	<0.00001	2.52
Lysine	1.75	1.06	0.98	<0.00001	2.39
	1.77	0.97	0.89	<0.00001	2.13
Acetic acid	1.93	1.09	0.97	<0.00001	2.05
N-Acetylglutamine	2.07	1.42	1.33	<0.00001	3.24
Acetaminophen	2.15	1.76	1.57	<0.00001	5.61
2.19	2.19	1.02	1.12	0.0001	2.46
o-Cresol	2.21	1.32	1.19	<0.00001	3.08
Acetone	2.23	1.12	1.00	<0.00001	2.98
Acetoacetic acid	2.29	0.81	0.78	0.0005	2.13
m-Cresol	2.31	0.94	0.94	<0.00001	2.08
Aspirin	2.33	1.53	1.41	<0.00001	6.17
Pyruvic acid	2.39	1.10	0.98	<0.00001	2.13
2.59	2.59	1.07	0.97	0.0004	2.68
Methylamine	2.61	1.30	1.17	<0.00001	3.10
2.75	2.75	1.26	1.17	<0.00001	2.61
2.77	2.77	1.40	1.28	<0.00001	3.07

2.79	2.79	1.15	1.14	<0.00001	2.37
2.81	2.81	2.01	1.84	<0.00001	6.58
Methylguanodine	2.83	1.05	1.13	<0.00001	2.22
2.85	2.85	1.89	1.73	<0.00001	6.38
2.87	2.87	1.34	1.21	<0.00001	2.94
2.89	2.89	1.48	1.34	<0.00001	3.19
2.95	2.95	1.11	1.00	<0.00001	3.08
2.97	2.97	1.57	1.46	<0.00001	3.47
myo-Inositol	3.31	1.20	1.10	<0.00001	4.00
3.35	3.35	0.93	1.06	<0.00001	2.05
3.37	3.37	1.16	1.12	<0.00001	2.48
Glucose	3.41	1.21	1.17	<0.00001	2.75
Glucose/1,2-Propanediol	3.45	1.68	1.55	<0.00001	4.77
Glucose	3.47	1.47	1.31	<0.00001	4.13
	3.49	1.14	1.08	<0.00001	2.46
	3.51	1.18	1.07	<0.00001	2.34
myo-Inositol/Glucose	3.53	1.37	1.22	<0.00001	2.98
1,2-Propanediol	3.55	1.70	1.52	<0.00001	5.77
Glycine	3.57	1.12	1.00	<0.00001	2.47
3.59	3.59	1.31	1.17	<0.00001	2.83
myo-Inositol	3.61	1.17	1.06	<0.00001	2.27
Arabinose	3.69	1.12	1.00	<0.00001	2.23
Glucose	3.71	1.26	1.12	<0.00001	2.69
	3.73	1.11	0.99	<0.00001	2.27
	3.75	1.10	0.98	<0.00001	2.12
	3.83	1.20	1.08	<0.00001	2.47
	3.87	1.21	1.08	<0.00001	2.52
	3.89	1.44	1.28	<0.00001	3.69
Arabinose/Glucose	3.91	1.28	1.14	<0.00001	2.84
Sucrose	4.23	1.53	1.36	<0.00001	3.58
4.25	4.25	1.51	1.35	<0.00001	3.19
4.27	4.27	1.10	1.10	<0.00001	2.32
4.29	4.29	1.32	1.23	<0.00001	3.33
4.31	4.31	1.17	1.13	<0.00001	2.47
4.33	4.33	1.66	1.47	<0.00001	3.97
4.35	4.35	1.20	1.26	<0.00001	2.28
4.37	4.37	1.47	1.35	<0.00001	3.11
4.43	4.43	1.18	1.05	<0.00001	2.23
Trigonelline	4.45	1.18	1.06	<0.00001	2.41
4.47	4.47	1.72	1.63	<0.00001	4.45

1-Methylnicotinamide	4.49	1.98	1.94	<0.00001	6.43
Arabinose	4.51	1.13	1.24	<0.00001	3.41
	4.53	1.63	1.72	<0.00001	5.67
4.57	4.57	1.61	1.59	<0.00001	5.47
4.59	4.59	1.41	1.49	<0.00001	4.73
4.65	4.65	0.83	0.75	0.0031	2.54
4.67	4.67	0.70	0.69	0.0038	2.44
Mannose	5.19	1.61	1.65	<0.00001	5.28
5.21	5.21	1.12	1.04	0.0002	2.02
Arabinose/Glucose	5.25	1.81	1.77	<0.00001	5.06
Sucrose	5.41	1.10	1.09	<0.00001	3.90
	5.43	0.63	0.66	0.0036	2.25
Urea	5.51	0.48	0.49	0.0202	2.13
	5.55	0.78	0.75	0.0006	2.61
	5.61	0.74	0.66	0.0056	2.37
	5.63	0.77	0.69	0.0074	2.24
	5.65	0.79	0.71	0.0141	2.01
	5.97	0.82	0.73	0.0063	2.28
6.67	6.67	0.60	0.68	0.0036	2.01
6.71	6.71	0.78	0.72	0.0006	2.58
6.85	6.85	1.11	1.10	<0.00001	3.86
4-Hydroxyphenylacetic acid	6.87	1.77	1.63	<0.00001	6.04
Tyrosine	6.89	0.87	0.82	0.0106	2.57
	6.91	1.04	0.99	0.0006	2.80
7.01	7.01	0.35	0.77	0.0436	0.43
7.09	7.09	0.73	0.93	0.0287	2.32
4-Hydroxyphenylacetic acid	7.17	1.46	1.32	<0.00001	4.06
7.51	7.51	0.72	0.69	0.0327	2.93
7.59	7.59	1.22	1.11	<0.00001	4.24
7.67	7.67	0.74	0.66	0.0238	2.29
7.69	7.69	1.52	1.37	<0.00001	3.37
Isoniazid	7.73	1.65	1.48	<0.00001	5.82
Isonicotinic acid	7.75	2.42	2.29	<0.00001	15.61
2-Pyridin-4-formidoacetic acid	7.77	1.97	1.87	<0.00001	8.43
Acetylisoniazid	7.81	2.64	2.47	<0.00001	20.51
Hippuric acid	7.87	1.29	1.21	0.0004	5.67
7.89	7.89	1.65	1.63	<0.00001	5.53
7.91	7.91	1.43	1.27	<0.00001	4.80
7.93	7.93	1.06	0.94	0.0036	2.25
7.95	7.95	1.51	1.49	<0.00001	5.85

Quinolinic acid	8.01	1.22	1.08	0.0003	4.46
	8.03	1.51	1.37	0.0001	5.31
	8.05	1.04	0.93	0.0031	5.00
5-Hydroxy-2-pyrazine carboxylic acid	8.07	0.51	0.51	0.0202	2.90
1-Methylnicotinamide	8.17	1.30	1.17	0.0003	6.04
8.33	8.33	1.72	1.78	<0.00001	9.95
8.35	8.35	0.86	0.78	0.0090	4.26
Quinolinic acid/Formic acid	8.47	1.21	1.11	<0.00001	3.74
8.49	8.49	0.61	0.57	0.0036	2.04
Isonicotinic acid	8.61	1.60	1.55	<0.00001	7.17
	8.63	2.30	2.22	<0.00001	14.18
8.67	8.67	0.63	0.58	0.0036	2.27
Pyrazine carboxylic acid	8.69	2.45	2.40	<0.00001	13.68
	8.71	2.10	2.02	<0.00001	8.16
Acetylisoniazid	8.73	2.57	2.47	<0.00001	21.19
8.75	8.75	1.94	1.74	<0.00001	9.57
Pyrazine carboxamide	8.77	1.53	1.37	<0.00001	5.69
	8.81	0.81	0.72	0.0011	2.50
	8.83	1.29	1.16	<0.00001	4.25
Pyrazine carboxylic acid	9.09	2.22	2.19	<0.00001	10.55
Pyrazine carboxamide	9.19	1.20	1.08	<0.00001	3.56

Annexure B:

Supplementary Information

Urinary markers of *Mycobacterium tuberculosis* and dysbiosis in paediatric tuberculous meningitis cases undergoing treatment

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#Shared last authorship

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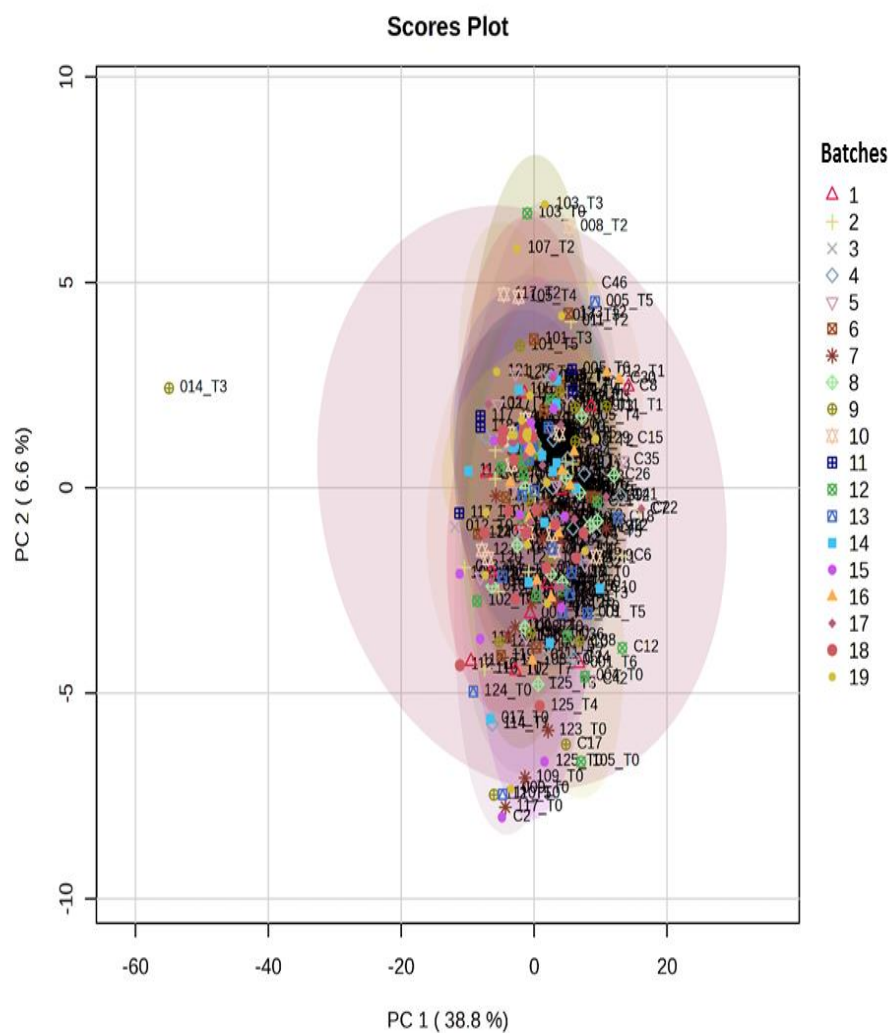


Figure S4.1 A PCA of all the batches(n=19). Notably, one case (014_T3) stood out as an outlier, and was removed from analysis.

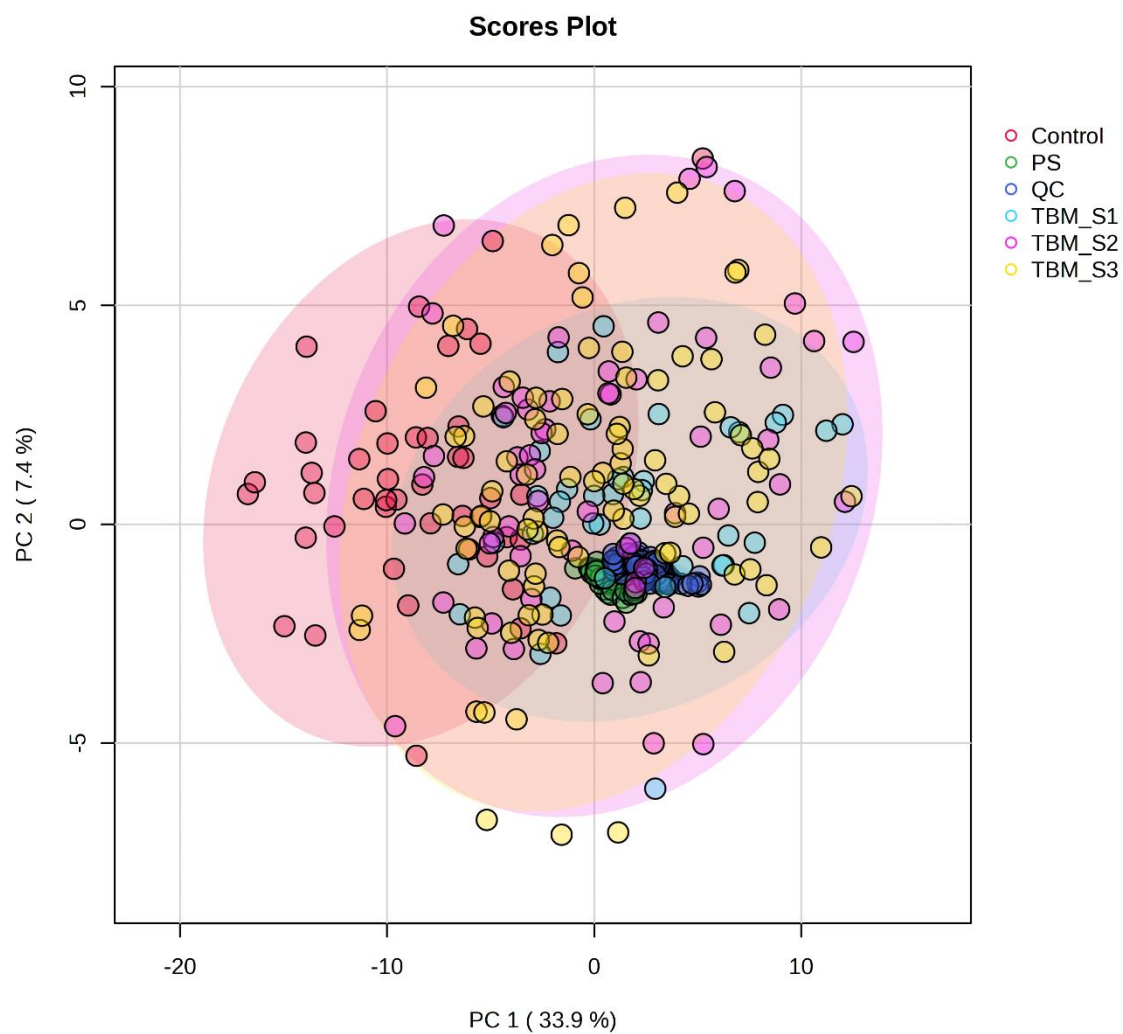


Figure S4.2 PCA showing that the samples of the QC.

(dark blue; pooled quality control urine sample) and PS (green; pooled patient urine sample) clustered closely together; supporting the conclusion that there was no batch effect from the analytical instrument.

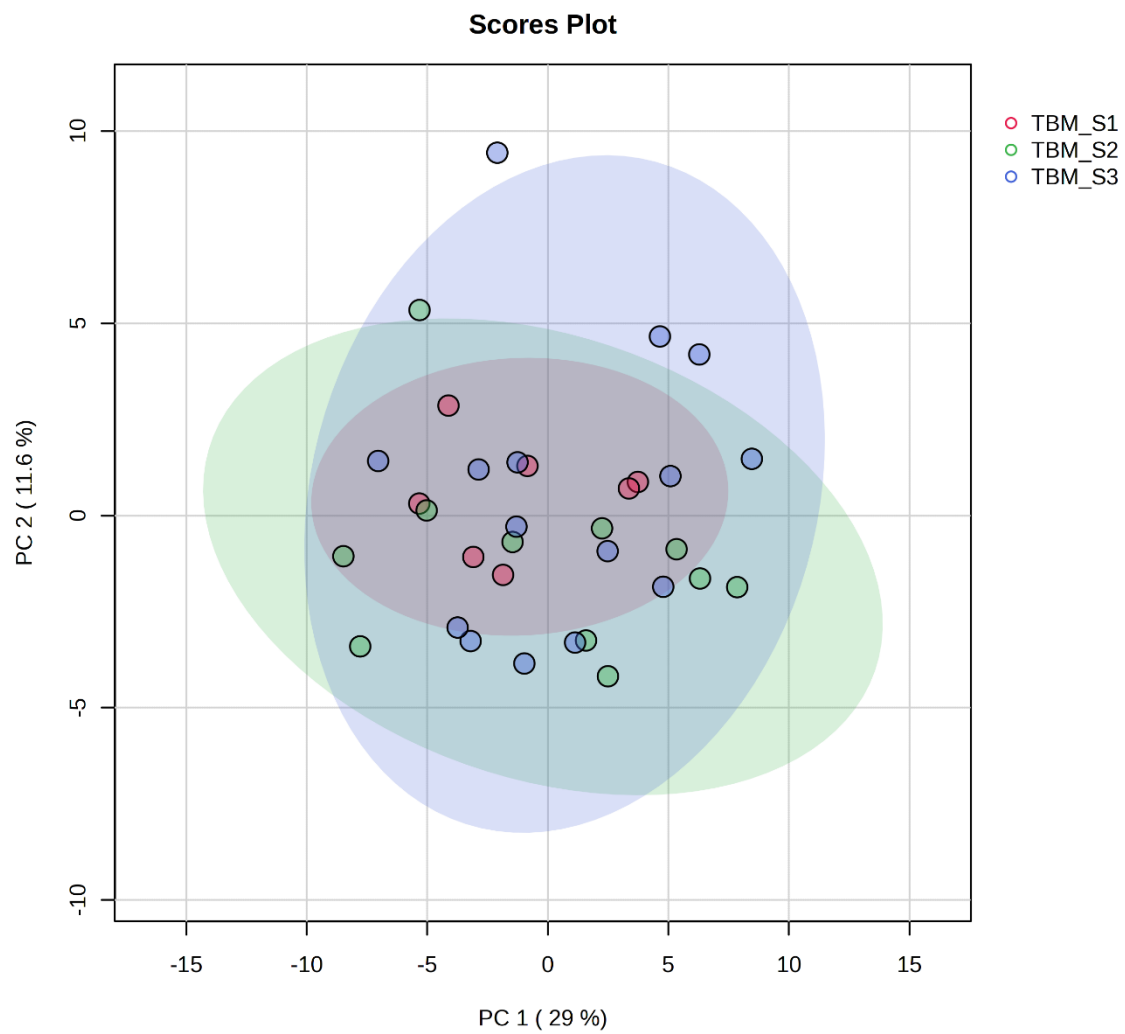


Figure S4.3 PCA of the three TBM stages at T1 shows no characteristic grouping nor differentiation between the stages.

Table S4.1 | Demographics of experimental study cohort affirmed as definite TBM, organized into stages (1, 2 and 3).

CRITERIA	TBM 1 n/N (%)	TBM 2 n/N (%)	TBM 3 n /N (%)
Male gender	4/8 (50)	7/11 (63.6)	9/16 (56.3)
Age (months) (Median [IQR])	65 [30 – 117]	46 [10 – 135]	43 [22 – 158]
Clinical symptoms			
Fever	4/8 (50)	5/11 (45.5)	14/16 (87.5)
Night sweats	0	2/11 (18.2)	2/16 (12.5)
Poor feeding	2/8 (25)	2/11 (18.2)	5/16 (31.3)
Weight loss	3/8 (35.7)	2/11 (18.2)	5/16 (31.3)
Vomiting without diarrhoea	3/8 (35.7)	2/11 (18.2)	4/16 (25)
Persistent coughing >14d	1/8 (12.5)	0	1/16 (6.3)
Headache	2/8 (25)	3/11 (27.3)	1/16 (6.3)
Seizures	3/8 (35.7)	1/11 (9.1)	5/16 (31.3)
Lethargy	2/8 (25)	2/11 (18.2)	2/16 (12.5)
Neurological signs			
GCS: (median [IQR])	15 [15]	14.5 [12 – 15]	10.5 [7 – 15]
Meningism	0	4/11 (36.4)	3/16 (18.7)
Focal motor deficit	0	2/11 (18.2)	4/16 (25)
Cranial nerve palsy	0	1/11 (9.1)	3/16 (18.7)
Raised ICP	0	2/11 (18.2)	5/16 (31.3)
Neuroimaging (CT brain)			
Hydrocephalus	2/8 (25)	6/11 (54.5)	11/16 (68.7)
Infarctions	0	2/11 (18.2)	1/16 (6.3)
Tuberculoma	2/8 (25)	1/11 (9.1)	3/16 (18.7)
Meningeal enhancement	2/8 (25)	2/11 (18.2)	8/16 (50)
VP shunt present	0	1/11 (9.1)	4/16 (25)
Other radiology			
CXR (signs of pulmonary TB)	1/8 (12.5)	3/11 (27.3)	2/16 (12.5)
Laboratory values			
Blood sodium (mmol/L) (median [IQR])	139 [130 – 139]	136 [135 – 139]	131.5 [126 – 141]
Blood total protein (g/L) (median [IQR])	68 [68]	74 [73 – 75]	78.5 [75 – 82]
Blood glucose (mmol/L) (median [IQR])	0	5.9 [0 – 5.9]	7.1 [4.9 – 7.7]
Blood lipids (median [IQR])	0	0	3.9 [0 – 3.9]
CSF protein (g/L) (median [IQR])	0.12 [0.12 – 0.53]	0.94 [0.87 – 2.1]	0.84 [0.78 – 2.73]
CSF glucose (mmol/L) (median [IQR])	2.6 [2.6 – 3.6]	2.05 [1.4 – 2.7]	1.8 [0.67 – 2.82]
CSF lymphocytes (cells/ μ L) (median [IQR])	0 [0 – 40]	157 [103.5 – 346.7]	57 [40 – 80]
CSF lymphocytes (%) (median [IQR])	0 [0 – 43.5]	92.2 [78.9 – 93.18]	95.6 [94.5 – 100]
HIV	0	0	0
Key: IQR = Interquartile range, CXR = Chest X-ray, GCS= Glasgow coma scale, CSF = Cerebrospinal fluid, ICP = Intracranial pressure, VP = Ventriculoperitoneal, HIV = human immunodeficiency virus.			

Table S4.2 | Quantitative data indicating 30 metabolites at T1 that are of statistical and practical importance [PLS-DA VIP value (component 1 AND 2) >1.0 AND ANOVA Kruskal-Wallis adjusted p-value < 0.01 AND effect size d-value > 0.8]. For Effect size conditional highlighting: d < 0.8 = red dot; 0.8 ≤ d ≤ 1.0 = yellow dot; d > 1.0 = green dot.

PLS-DA	Comp. 1	Comp. 2	ANOVA (KW)	chi.square	FDR	Effect size	TBM_S1	TBM_S2	TBM_S3
2 Hydroxyadipic acid	1.4591	1.3033	2 Hydroxyadipic acid	46.976	6.59E-09	2 Hydroxyadipic acid	● 3.09	● 0.88	● 1.53
2 Hydroxyglutaric acid	1.0574	1.0325	2 Hydroxyglutaric acid	23.408	7.78E-05	2 Hydroxyglutaric acid	● 1.13	● 0.81	● 0.94
2 Hydroxyphenylacetic acid	1.2093	1.074	2 Hydroxyphenylacetic acid	30.397	4.27E-06	2 Hydroxyphenylacetic acid	● 1.01	● 1.04	● 1.15
2 Methylbutyrylglycine	1.1739	1.0692	2 Methylbutyrylglycine	29.199	6.10E-06	2 Methylbutyrylglycine	● 0.7	● 1.05	● 1.13
2 Octenedioic acid	1.2087	1.1182	2 Octenedioic acid	29.879	4.99E-06	2 Octenedioic acid	● 0.93	● 0.7	● 0.59
2,3-Pyridinecarboxylic acid	1.4065	1.314	2,3-Pyridinecarboxylic acid	43.023	3.04E-08	2,3-Pyridinecarboxylic acid	● 0.61	● 0.87	● 0.76
3 Hydroxyisovaleric acid	1.4407	1.2804	3 Hydroxyisovaleric acid	44.406	1.86E-08	3 Hydroxyisovaleric acid	● 0.97	● 1.28	● 1.11
3 Hydroxypropionic acid	1.1864	1.0753	3 Hydroxypropionic acid	37.892	2.56E-07	3 Hydroxypropionic acid	● 0.85	● 1.28	● 0.69
3 Methylcrotonylglycine	1.3612	1.2392	3 Methylcrotonylglycine	34.806	9.13E-07	3 Methylcrotonylglycine	● 0.69	● 0.84	● 0.87
3 Methylglutaconic acid	1.3092	1.2094	3 Methylglutaconic acid	31.973	2.49E-06	3 Methylglutaconic acid	● 0.51	● 1.13	● 1.03
4 Hydroxy 3 Methoxymandelic acid	1.2094	1.0842	4 Hydroxy 3 Methoxymandelic acid	27.066	1.47E-05	4 Hydroxy 3 Methoxymandelic acid	● 1.11	● 1.03	● 1.12
4 Hydroxyphenylacetic acid	1.6068	1.5431	4 Hydroxyphenylacetic acid	47.449	6.59E-09	4 Hydroxyphenylacetic acid	● 1.02	● 0.82	● 1.33
4 Hydroxyphenyllactic acid	1.1519	1.0836	4 Hydroxyphenyllactic acid	30.615	4.27E-06	4 Hydroxyphenyllactic acid	● 1.09	● 0.41	● 0.54
4-Hydroxyhippuric acid	1.3049	1.2654	4-Hydroxyhippuric acid	38.283	2.56E-07	4-Hydroxyhippuric acid	● 1.18	● 0.85	● 1
5 Hydroxyindoleacetic acid	1.1961	1.1034	5 Hydroxyindoleacetic acid	30.497	4.27E-06	5 Hydroxyindoleacetic acid	● 0.82	● 1.01	● 1.04
5-Hydroxyhexanoic acid	1.2127	1.1351	5-Hydroxyhexanoic acid	29.346	6.07E-06	5-Hydroxyhexanoic acid	● 1.02	● 0.86	● 0.86
Aconitic acid	1.182	1.0597	Aconitic acid	31.034	3.69E-06	Aconitic acid	● 1.65	● 0.81	● 1.06
Ethylmalonic acid	1.1867	1.0811	Ethylmalonic acid	32.231	2.34E-06	Ethylmalonic acid	● 1.44	● 0.87	● 0.98
Glucaric acid	1.5705	1.5324	Glucaric acid	49.399	4.02E-09	Glucaric acid	● 1.03	● 1.59	● 1.25
Hexanoylglycine	1.4562	1.4627	Hexanoylglycine	37.827	2.56E-07	Hexanoylglycine	● 0.85	● 1.02	● 0.98
Homovanillic acid	1.3241	1.2578	Homovanillic acid	33.347	1.57E-06	Homovanillic acid	● 0.92	● 0.98	● 1.24
Isobutyrylglycine	1.2794	1.1598	Isobutyrylglycine	28.263	9.23E-06	Isobutyrylglycine	● 0.63	● 0.94	● 0.9
Isocitric acid	1.2421	1.1096	Isocitric acid	29.295	6.07E-06	Isocitric acid	● 1.99	● 0.81	● 1.06
N Acetylasparitic acid	1.1264	1.0706	N Acetylasparitic acid	27.716	1.16E-05	N Acetylasparitic acid	● 1	● 0.64	● 1.04
N Acetylphenylalanine	1.3323	1.1879	N Acetylphenylalanine	35.165	8.43E-07	N Acetylphenylalanine	● 0.85	● 0.79	● 0.78
N Acetyltyrosine	1.1676	1.0827	N Acetyltyrosine	27.573	1.20E-05	N Acetyltyrosine	● 0.84	● 0.57	● 0.68
Phenylacetylglutamine	1.0603	1.0319	Phenylacetylglutamine	22.603	0.000108	Phenylacetylglutamine	● 0.53	● 0.47	● 1.1
Pimelic acid	1.2319	1.096	Pimelic acid	34.4	1.02E-06	Pimelic acid	● 1.81	● 1.14	● 0.95
Tiglylglycine	1.3397	1.2088	Tiglylglycine	32.3	2.34E-06	Tiglylglycine	● 0.74	● 1	● 1.33
Vanillactic acid	1.494	1.3889	Vanillactic acid	50.427	4.02E-09	Vanillactic acid	● 2.67	● 0.66	● 1.82

Table S4.3 | Seventy-eight (78) targeted compounds of interest used in this study, including standards/isotopes.

Compounds of interest including internal standards/isotopes	Type	Distributor	Purity	CAS no	Product code
1,3-Dimethyluric acid	Standard	ChemCruz			sc-
2,3-Pyridinedicarboxylic acid	Standard	Aldrich	99%	89-00-9	P63204-25G
2,5-Furandicarboxylic acid	Standard	Sigma	97%	3238-40-2	722081-5G
2-Furoylglycine	Standard	Sigma	98%	5657-19-2	SMB00966
2-Hydroxy-3-Methylbutyric acid	Standard	Aldrich	99%	4026-18-0	219835-1G
2-Hydroxyadipic acid	Standard	Sigma	95%		92826-10MG
2-Hydroxydecanedioic acid	Standard	Industrial Analytical	98%	103963-71-9	H939450
2-Hydroxyglutaric acid disodium salt	Standard	Sigma	98%	63512-50-5	61313-10MG
2-Hydroxymethylbutyric acid	Standard	Sigma			96045-10MG
2-Hydroxyphenylacetic acid	Standard	Aldrich	99%	614-75-5	H49804-10G
2-Ketoglutaric acid disodium salt hydrate	Standard	Sigma	95%	305-72-6	K3752-5G
2-Methylbutyrylglycine	Standard	VUMC - Organic Synthesis Laboratory	95%		
2-Octenedioic acid	Standard	Sigma	95%	124791-62-4	92675-10MG
3-(3,5-Dihydroxyphenyl) propanoic acid	Standard	Industrial Analytical			TRCD267960
3-(3-Hydroxyphenyl)-3-Hydroxypropionic acid (HPHPA)	Standard	Sigma	95%	3247-75-4	06704-10MG
3-(3-Hydroxyphenyl)-propionic acid	Standard	Sigma		621-54-5	91779-50MG
3,4-Dihydroxybenzoic acid	Standard	Sigma	97%	99-50-3	08992-50MG
3,4-Dihydroxyphenylacetic acid (L-DOPAC)	Standard	Industrial Analytical	98%	102-32-9	D454250
3-Hydroxy-2-methylbutyric acid (HMBA)	Standard	Industrial Analytical	95%		H946485
3-Hydroxybutyric acid (DL-) sodium salt	Standard	Sigma	98%	150-83-4	H6501-5G

3-Hydroxydodecanedioic acid	Standard	Industrial Analytical	93%	34574-69-1	H939615
3-Hydroxyglutaric acid	Standard	Sigma	95%	638-18-6	04725-10MG
3-Hydroxyisobutyrate sodium	Standard	Sigma	96%	1228078-57-6	11161-100MG
3-Hydroxyisovaleric acid/3-Hydroxy-3-methylbutyric acid	Standard	Sigma	95%	625-08-1	55453-1G
3-Hydroxypropionic acid	Standard	Sigma		503-66-2	792659-1G
3-Hydroxysebacic acid	Standard	Industrial Analytical	97%	73141-46-5	H953770
3-Indoleacetic acid	Standard	Aldrich	98%	87-51-1	I3750-5G-A
3-Methylcrotonylglycine	Standard	VUMC - Organic Synthesis Laboratory	95%		
3-Methylglutaconic acid	Standard	Fluka	97%	372-42-9	44108-10MG
3-Phenyllactic acid	Standard	Aldrich	98%	828-01-3	P7251-1G
4-Hydroxyhippuric acid	Standard	Industrial Analytical	95%	2482-25-9	H827810
4-Hydroxyphenylacetic acid	Standard	Sigma	98%	156-38-7	55206-10MG
4-Hydroxyphenyllactic acid (DL-)	Standard	Aldrich	97%	6482-98-0	H3253-500MG
4-Pyridoxic acid	Standard	Aldrich	98%	82-82-6	P9630-25MG
5-Hydroxyhexanoic acid	Standard	ChemCruz			sc-352672
5-Hydroxyindoleacetic acid (5-HIAA)	Standard	Sigma	98%	54-16-0	H8876-100MG
5-Hydroxymethyl-2-furancarboxylic acid	Standard	Sigma	95%	6338-41-6	902845-50MG
Acetylaspartic acid	Standard	Sigma	99%	997-55-7	00920-5G
Aconitic acid	Standard	Aldrich	98%	4023-65-8	122750-25G
Azelic acid	Standard	Sigma	99%	123-99-9	95054-100MG
Benzoic acid	Standard	Sigma	99%	65-85-0	242381-100G
Butyrylglycine	Standard	VUMC - Organic Synthesis Laboratory	95%		
Citric acid	Standard	Sigma	99%	77-92-9	C0759-500G

Ethylmalonic acid	Standard	Aldrich	97%	601-75-2	102687-5G
Glucaric acid (D-Saccharic acid potassium salt)	Standard	Sigma	98%	576-42-1	S4140-10G
Glutaric acid	Standard	Aldrich	99%	110-94-1	G3407-25G
Heptanoylglycine	Internal Standard	Sigma	Analytical std	23783-23-5	93539-50MG
Hexanoylglycine	Standard	VUMC - Organic Synthesis Laboratory	95%		
Hippuric acid	Standard	Sigma	98%	495-69-2	112003-100G
Homogentisic acid	Standard	Sigma	98%	451-13-8	H0751-100MG
Homovanillic acid (HVA)	Standard	Sigma	98%	306-08-1	H1252-250MG
Hydantoin-5-propionic acid/3-(2,5-Dioxoimidazolidin-4-yl) propionic acid	Standard	Industrial Analytical	95%	5624-26-0	D486445
Isobutyrylglycine	Standard	VUMC - Organic Synthesis Laboratory	95%		
Isocitric acid (trisodium salt hydrate, DL-)	Standard	Sigma	93%	1637-73-6	I1252-1G
Isovalerylglycine	Standard	VUMC - Organic Synthesis Laboratory	95%		
Kynurenic acid	Standard	Sigma	98%	492-27-3	K3375-1G
Malic acid	Standard	Aldrich	98%	6915-15-7	M0875-100G
Methylcitric acid/2-Methylcitric acid	Standard	Industrial Analytical	97%	6061-96-7	TRCM265080
Methylmalonic acid	Standard	Aldrich	99%	516-05-2	M54058-25G
Methylsuccinic acid	Standard	Sigma	99%	3641-51-8	415596-5G
Methyluric acid	Standard	ChemCruz			sc-208662
Methylxanthine	Standard	Sigma	97%	6136-37-4	69720-250MG
N-Acetylphenylalanine	Standard	Aldrich	99%	2018-61-3	857459-1G
N-acetyltyrosine	Standard	Sigma	100%	537-55-3	PHR1173-1G
Phenylacetyl-L-glutamine	Standard	Sigma	95%	28047-15-6	SMB00962-25MG

Pimelic acid	Standard	Aldrich	98%	111-16-0	P45001-25G
Propionylglycine	Standard	VUMC - Organic Synthesis Laboratory	95%		
Pyroglutamic acid (5-oxoproline)	Standard	Aldrich	99%	98-79-3	83160-25G
Sebacic acid	Standard	Aldrich	99%	111-20-6	283258-5G
Suberic acid	Standard	Aldrich	98%	505-48-6	S5200-5G
Suberylglycine	Standard	VUMC - Organic Synthesis Laboratory	95%		
Succinic acid	Standard	Sigma	99%	110-15-6	S3674-100G
Tiglylglycine	Standard	VUMC - Organic Synthesis Laboratory	95%		
Tricarballic acid	Standard	Aldrich	99%	99-14-9	T53503-25G
Uracil	Standard	Sigma	99%	66-22-8	U0750-5G
Vanillic acid (DL-Vanillic acid lithium salt hydrate)	Standard	Sigma	97%	2475-56-1 (free acid)	89127-50MG
Vanillic acid	Standard	Sigma	97%	121-34-6	94770-10G
Vanillylmandelic acid (VMA)/DL-4-Hydroxy-3-methoxymandelic acid	Standard	Sigma	98%	55-10-7	H0131-100MG
Xanthurenic acid	Standard	Aldrich	96%	59-00-7	D120804-1G

Table S4.4 | Multiple reaction monitoring (MRM's) values and expected retention times of compounds of interest.

Name of compound	MRM	Expected RT*
1,3-Dimethyluric acid	195.1 -> 180.1	5.86
2 5 Furandicarboxylic acid	155 -> 111.1	5.03
2 Furoylglycine	168 -> 67.1	5.54
2 Hydroxy 3 Methylbutyric acid	117 -> 71.2	5.73
2 Hydroxyadipic acid	161 -> 71	4.14
2 Hydroxydecanedioic acid	217.1 -> 155.2	10.19
2 Hydroxyglutaric acid	147 -> 85.2	1.82
2 Hydroxymethylbutyric acid	117 -> 87.2	5.41
2 Hydroxyphenylacetic acid	151 -> 107	9.00
2 Ketoglutaric acid	145 -> 101	1.37
2 Methylbutyrylglycine	158 -> 74	6.30
2 Octenedioic acid	171.1 -> 127.2	9.16
2,3-Pyridinecarboxylic acid	166 -> 122	1.81
3 4 Dihydroxybenzoic acid	153 -> 109.1	6.20
3 4 Dihydroxyphenylacetic acid	167 -> 123.1	6.71
3 Hydroxy 2 Methylbutyric acid	117 -> 73.1	4.82
3 Hydroxybutyric acid	103 -> 59.2	2.83
3 Hydroxydodecanedioic acid	245.1 -> 59.2	12.28
3 Hydroxyglutaric acid	147 -> 85.1	2.16
3 Hydroxyisobutyric acid	103 -> 73.2	3.17
3 Hydroxyisovaleric acid	117 -> 59.2	4.74
3 Hydroxypropionic acid	89 -> 59	1.29
3 Hydroxysebacic acid	217.1 -> 59.1	9.51
3 Indoleacetic acid	174.1 -> 130.1	11.61
3 Methylcrotonylglycine	156.1 -> 74.1	6.62
3 Methylglutaconic acid	143 -> 99.1	6.66

3 Phenyllactic acid	165.1 -> 119.1	6.48
3-(3,5-Dihydroxyphenyl)propionic acid	181.1 -> 137.1	7.40
3-(3-Hydroxyphenyl)-3-Hydroxypropionic acid	181.1 -> 121.1	7.00
3-(3-Hydroxyphenyl)-propionic acid	165.1 -> 119.1	9.89
4 Hydroxy 3 Methoxymandelic acid	197 -> 137	4.65
4 Hydroxyphenylacetic acid	151 -> 107.1	7.91
4 Hydroxyphenyllactic acid	181.1 -> 135.1	6.83
4 Pyridoxic acid	182 -> 108	4.73
4-Hydroxyhippuricacid	194 -> 100.1	6.28
5 Hydroxyindoleacetic acid	190.1 -> 146.1	7.80
5-Hydroxyhexanoic acid	131 -> 85.2	6.18
5-Hydroxymethyl-2-Furancarboxylic acid	141 -> 97	5.30
Aconitic acid	173 -> 129.2	2.90
Azelic acid	187.1 -> 125.1	11.23
Benzoic acid	121 -> 77.2	10.80
Butyrylglycine	144 -> 74.2	4.88
Citric acid	191 -> 111	2.28
Ethylmalonic acid	131 -> 87.1	5.43
Glucaric acid	209 -> 191	0.79
Glutaric acid	131 -> 87.2	4.75
Heptanoylglycine (IS)	186 -> 74.2	11.70
Hexanoylglycine	172.1 -> 74.1	9.42
Hippuric acid	178.1 -> 134.1	7.80
Homogentisic acid	167 -> 122.1	5.49
Homovanillic acid	181.1 -> 137.1	8.49
Hydantoin 5 propionic acid	171 -> 100.1	3.48
Isobutyrylglycine	144.1 -> 74.2	4.67
Isocitric acid	191 -> 111	1.18

Isovalerylglycine	158.1 -> 74.1	6.58
Kynurenic acid	188 -> 144.1	7.29
Malic acid	133 -> 115	1.13
Methylcitric acid	205 -> 125.1	4.62
Methylmalonic acid	117 -> 73.2	3.08
Methylsuccinic acid	131 -> 87.1	5.42
Methyluric acid	181 -> 138.1	5.01
Methylxanthine	165 -> 80.2	5.34
N Acetylaspartic acid	174 -> 88.1	1.67
N Acetylphenylalanine	206.1 -> 91.2	9.73
N Acetyltyrosine	222.1 -> 178.1	7.07
Phenylacetylglutamine	263.1 -> 127.1	7.90
Pimelic acid	159.1 -> 97.2	7.96
Propionylglycine	130 -> 74.2	2.58
Pyroglutamic acid (Positive mode)	130 -> 84.1	2.27
Sebacic acid	201.1 -> 183.2	12.75
Suberic acid	173.1 -> 111.2	9.63
Suberylglycine	230.1 -> 212.3	7.92
Succinic acid	117 -> 73	2.80
Tiglylglycine	156.1 -> 112.1	6.46
Tricarballic acid	175 -> 69.2	3.40
Uracil	111 -> 42.3	1.65
Vanillic acid	211.1 -> 193.3	7.52
Vanillic acid	167 -> 152.1	8.16
Xanthurenic acid	204 -> 160.2	6.92

Table S4. 5 | Binary gradient conditions and experimental parameters of LC-MS/MS.

Time	Mobile Phase 1 (H2O with 0.1% Formic acid)	Mobile Phase 2 (Acetonitrile with 0.1% Formic acid)
0.00 min	100%	0
1.50 min	100%	0
2 min	98%	2%
2.5 min	95%	5%
3.5 min	93%	7%
6 min	85%	15%
7.5 min	80%	20%
9 min	75%	25%
10.5 min	70%	30%
12 min	60%	40%
15 min	25%	75%
17 min	5%	95%
22 min	100%	0
25 min	100%	0

Agilent Jetstream ESI Source Parameters

Gas Temp	170°C
Gas Flow	10 l/min
Nebulizer	25 psi
Sheath Gas Temp	350°C
Sheath Gas Flow	12 l/min
Capillary	3000 V Negative
Nozzle Voltage	500 V

Annexure C:

Supplementary information:

The diagnostic potential of urine in paediatric patients undergoing initial treatment for tuberculous meningitis

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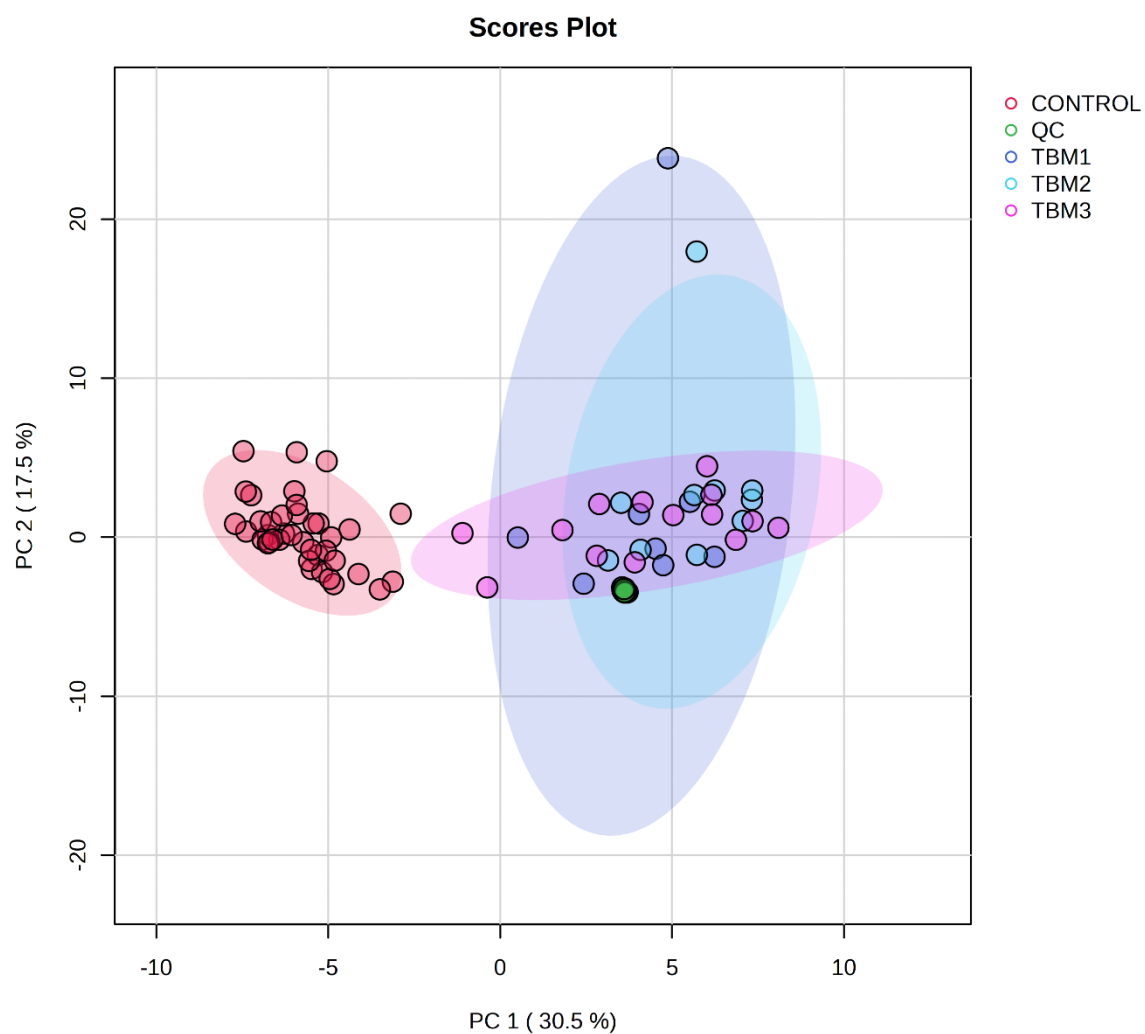


Figure S5.1 PCA scores plot of all patients and QC samples.

The QC samples (green dots) clustered closely together, indicating that there was no discernible analytical drift during the course of all the ^1H -NMR analyses.

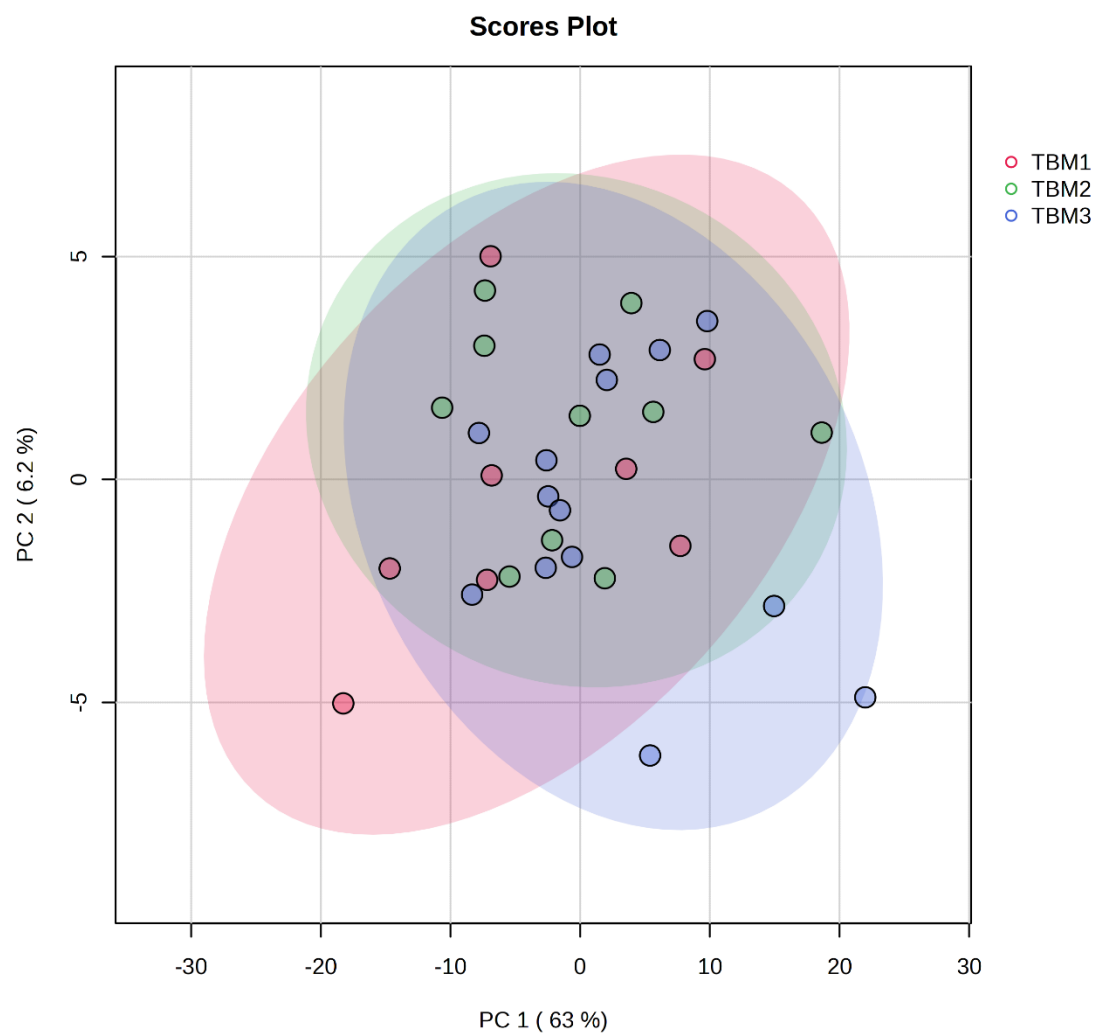


Figure S5.2 PCA scores plot of all the TBM stages showing no differentiation.

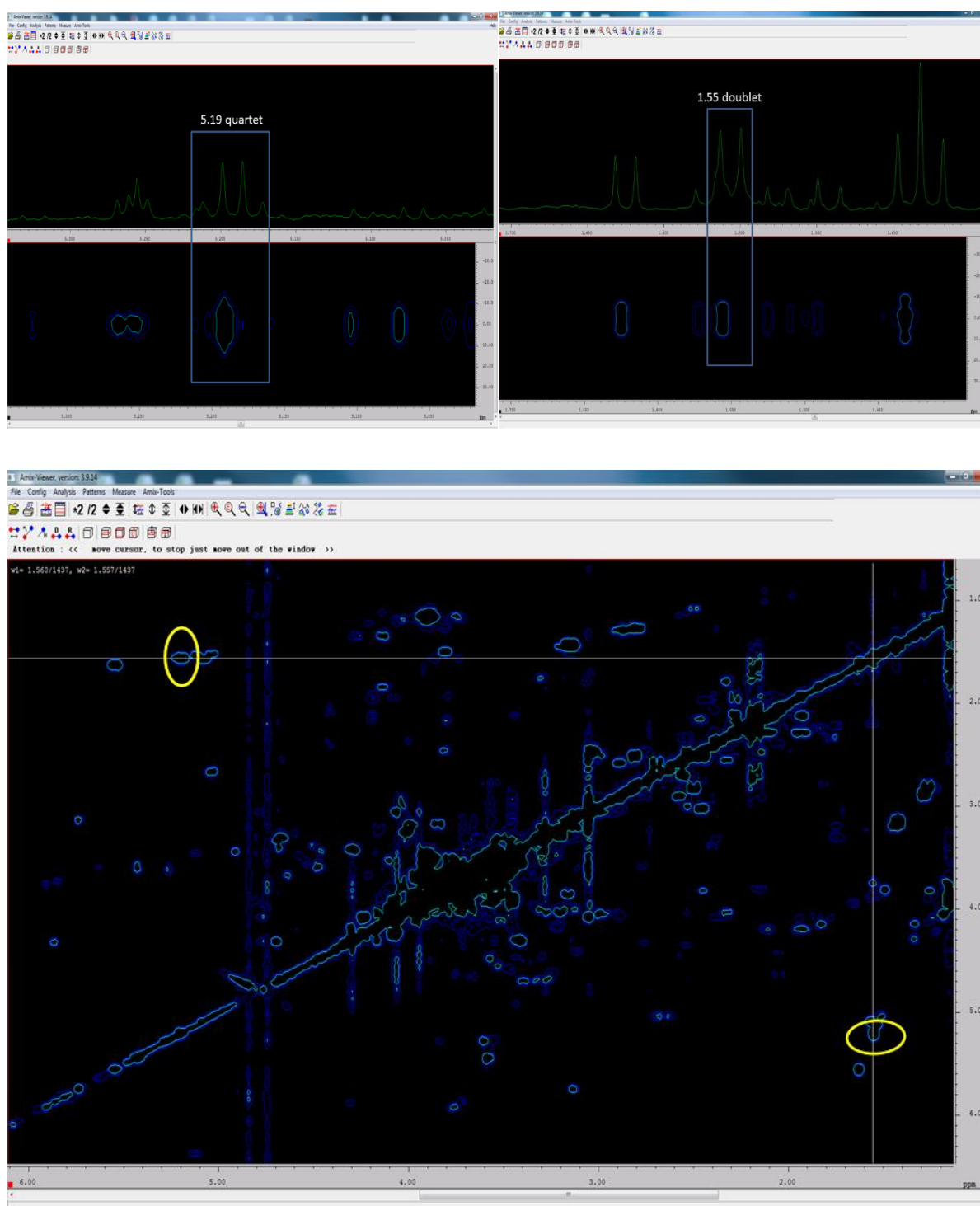


Figure S5.3 Above: 1D and 2D JRES NMR spectra showing a doublet at 1.55 ppm (above right) and quartet at 5.19 ppm (above left).

Bottom 2D COSY NMR spectrum showing (yellow circles) that the doublet at 1.55 ppm and the quartet at 5.19 ppm are correlated (i.e., they are part of the same chemical compound).

Annexure D:

Material and methods in summary

Patient cohort and urine sampling: 45 TBM patients, 40 non-neurological ('healthy') controls and 40 non-meningitis ('sick') controls. A diagnosis of TBM is based on the uniform research case definition of Marais et al. (2010). Sick controls are defined as suspected meningitis cases that later are proven to be negative for meningitis (i.e. alternative diagnosis). In the patient group, five urine samples are collected from each patient – upon diagnosis (T=0), and monthly following treatment, up to 4 months. Total of 225 patient and 80 control samples. TBM grading distribution at diagnosis (T=0): stage 1: 33.3%; stage 2: 28.9%, stage 3: 37.8%. Illustration of sampling design:

T=0	T= 1 Mo	T= 2 Mo	T= 3 Mo	T= 4 Mo
TBM Stage 1 N=15				
TBM Stage 2 N=13				
TBM Stage 3 N=17				
Non TBM N=40				
Health Con N=40				

Clinical data collection: caregiver (gender, language, relation to child, marital status, education, date of birth) and child (gender, education, date of birth). clinical course and out-patient follow up (diagnosis, TBM grading, clinical symptoms, treatment date commenced, discharge from hospital, date follow up visit, date treatment to be completed, weight, height and medication). Exclusion criteria: HIV co-infection

Metabolomics

This study was a two-step metabolomics approach. The first step used an untargeted approach. Urine samples were analyzed, in complement, with proton nuclear magnetic resonance (^1H -NMR) spectroscopy, this was done as an explorative approach. The results was global metabolic profiles. The second step was semi-targeted approach. Liquid chromatography–tandem mass spectrometry (LC-MS) and gas chromatography time-of-flight mass spectrometry (GC-TOF-MS).

The analysis of a small set of compounds related by pathway, function or compound class. This approach is normally seen as hypothesis-driven.