Pathogenesis of Tuberculous Meningitis

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Declaration

I Angharad Grace Davis confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Tuberculous Meningitis (TBM) is the most severe form of tuberculosis, affecting >100,000 people/year (Wilkinson et al., 2017, Marais et al., 2011). TBM arises when *Mycobacterium tuberculosis* crosses the blood-brain barrier, causing severe inflammation and tissue damage (Davis et al., 2019b). Inflammation also initiates metabolic derangement, mediating neuronal injury despite treatment (Davis et al., 2019b, Rohlwink et al., 2019, Rohlwink et al., 2017). In HIV-1 coinfection, TBM mortality is reported to be as high as 50% (Marais et al., 2011). Half of TBM survivors are affected by neurological disability (Thwaites et al., 2003). Thus, improved therapeutic strategies are needed, including targeted and nuanced modification of the injurious host inflammatory response. To develop these, we must understand the immune induced tissue damaging responses and metabolic disturbances contributing to brain damage which drive acute and long-term neurological complications.

The goals of this PhD thesis were to investigate the safety of a novel drug regimen in HIV-associated TBM and through a series of nested sub-studies, understand pathogenic mechanisms of acute and long term neurological sequalae in TBM. Specifically, this thesis presents:

i) A study protocol and results from a phase 2A randomised controlled trial of high dose rifampicin and adjunctive linezolid with and without aspirin in HIV-associated TBM
ii) Results from a case-control study of cognitive and functional outcomes in HIV-associated TBM
iiI) Results from a study investigating *in vivo* markers of brain injury in HIV-associated TBM via magnetic resonance spectroscopy

iv) Results from a study investigating *ex vivo* markers of poor outcome via Luminex multiplex analysis of blood and cerebrospinal fluid.

Given the timing of this PhD, this thesis also contains results of an observational case-control study to understand neurological complications of COVID-19 via systematic analysis of cerebrospinal fluid of patients presenting with neurological symptoms during the first wave of the COVID-19 pandemic in South Africa. These results are included within the appendix of the thesis.

Impact Statement

Tuberculous meningitis (TBM) arises when *Mycobacterium tuberculosis* crosses the blood-brain barrier and is the most lethal and disabling form of tuberculosis. In some contexts, including HIV co-infection, TBM mortality can be as high as 50% despite therapy, and long-term disability is common amongst survivors. These injuries are induced by tissue damaging immune responses and metabolic disturbances contributing to neurotoxic and degenerative neurological damage. Poor outcomes are thought in part due to i) inadequate antibiotic penetration into the central nervous system, ii) poor understanding of the host inflammatory response in the acute and longer term phases of illness and iii) a lack of adequate therapies which dampen a counterproductive host inflammatory response. This PhD contributes knowledge to these areas.

A phase 2 randomised controlled trial (RCT) demonstrated that i) high-dose rifampicin and adjunctive linezolid can safely be added to standard of care and ii) high-dose aspirin is safe when used in combination with intensified antibiotics in HIV-associated TBM. Results of this trial, now published in Clinical Infectious Diseases (Davis et al., 2022b), will inform the design of phase 3 RCT in TBM.

A case-control study in TBM shows that cognitive impairment occurs in half of patients at 6 months post diagnosis and that impairment is above and beyond that attributable to HIV or the systemic effects of TB alone. These results, now published in Clinical Infectious Diseases (Davis et al., 2022a), provide new knowledge on neurological complications in TBM. They also provide rationale for new studies to evaluate outcomes at longer-term timepoints and explore the relationship between cognitive performance, functional status and treatment adherence. The results also provide new

methods for clinical correlation of emerging knowledge in TBM pathogenesis which suggests that key pathways share similar mechanistic characteristics to neurodegenerative conditions.

Ex vivo analysis of blood and CSF in TBM provides insight into potential inflammatory mediators driving the immune response. The work highlights mediators which may serve as biomarkers of poor outcome or druggable targets in the development of host directed therapies; findings around IL-1β and inflammasome driven innate immune responses provide further rationale for the consideration of drugs which target these pathways. Studies using larger cohorts and including longer term timepoints are now needed to further investigate and validate these findings.

A pilot study evaluated a novel method for *in vivo* measurement of brain injury markers in TBM. It showed that single voxel magnetic resonance spectroscopy can feasibly measure glutamate and GABA within the brains of patients with TBM, providing methodology for future studies. Although results need validation in a larger cohort, they demonstrate that glutamate is significantly raised in the acute phase of TBM compared to healthy controls. This work provides a platform for further development of this technique which is important given the emerging importance of glutamate driven excitotoxicity in TBM.

Alongside this work, a case-control study evaluating the spectrum of neurological manifestations of COVID-19 provides knowledge on the neurotropism of SARS-CoV2, demonstrating that penetration of virus to the CNS is uncommon even in those with COVID-19 and neurological symptoms.

Acknowledgement

I would firstly like to thank my primary supervisor Professor Robert J Wilkinson from whom I have received unwavering support and guidance over the last 5 years. Thank you trusting me to lead a clinical trial, for your diligence in your role as my supervisor, and for your honest and invaluable mentorship. These have been the most rewarding years of my professional life and I will always be deeply grateful for the opportunities I have been given. Thanks also to Professor Anne O'Garra for your teaching and guidance; it has been a privilege to learn from you. Likewise to Professor Kaila Srai for your guidance in navigating the administration of a PhD at University College London and for your always enthusiastic support.

I would like to express my upmost respect and gratitude for the clinical trial teams at CIDRI Africa who work tirelessly to recruit and care for study participants despite challenging settings. In particular thank you to my close colleagues Professor Sean Wasserman, Mpumi Maxebengula and Dr Cari Stek; your support, friendship and guidance has been invaluable. I am also grateful for the opportunity to have worked with Anna Dreyer, A/Professor Sam Nightingale, Dr Frances Robertson and Professor John Joska at the University of Cape Town. I have learnt a huge amount through this work with you and developed a new research interest which I endeavour to pursue far beyond this PhD. Thank you for sharing your expertise and passion with me. And of course thank you to the participants who agreed to be part of numerous clinical studies within this PhD; without your time and trust none of this would be possible.

Finally I would like to thank my family. My parents for teaching me to believe in myself when things don't go to plan. My husband Dave whose commitment to helping me

realise my career goals is limitless; thank you for always sharing the load and for your constant love. And lastly to my two children, Rowan and Cerys, both born during the course of this PhD. Thank you for your sacrifice, patience and for the joy you bring me every single day.

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Thesis outline

Chapter 1 provides an introduction to the burden of Tuberculous Meningitis globally and sets out the goals of this PhD. Thereafter it provides a more detailed overview on i) current treatment options for TBM and its neurological sequalae, ii) potential novel targets for host directed therapies and iii) pathogenic mechanisms in TBM. These insights are largely taken from three review papers published throughout the course of my PhD (Davis et al., 2018, Davis et al., 2019b, Davis et al., 2020). The scientific rationale for the research conducted during the PhD is explained.

Chapter 2 is a published research paper outlining the study protocol for the phase 2 randomised controlled trial evaluating the safety of high dose rifampicin, adjunctive linezolid with and without high dose aspirin in HIV-associated TBM ('The LASER-TBM Trial') (Davis et al., 2021).

Chapter 3 is a research paper describing the results from the LASER-TBM Trial (published in Clinical Infectious Diseases at time of thesis submission (Davis et al., 2022b)).

Chapter 4 is a case-control study describing cognitive and functional outcomes in HIV associated TBM (published in Clinical Infectious Disease at time of thesis submission (Davis et al., 2022a)).

Chapter 5 describes results from *ex vivo* immunological analysis of blood and CSF arising from the LASER-TBM trial using Luminex platform technology.

Chapter 6 describes results from a pilot study using magnetic resonance spectroscopy to measure *in vivo* concentrations of GABA and glutamate in patients with HIV-associated TBM recruited to the LASER-TBM trial.

Chapter 7 provides a brief discussion of key findings from the thesis and describes next steps in relation to this work.

The appendix includes a case-control study describing the spectrum of neurological manifestations in SARS-CoV2 infection (available in medarchive at time of thesis submission).

Author Contributions

The work undertaken within this PhD included several collaborative projects. Outlined below are details on the contributions to each chapter made by myself (AGD) and research collaborators.

Chapter 1: This chapter brings together three review papers published at the start of my PhD, which have been updated in line with the current literature. For the original publications, AGD was involved in paper conceptualisation, and produced the first written draft. She was the first and corresponding author on each submission. Radiological images included within this chapter were kindly provided by colleagues at University of Cape Town, Professors Graeme Meintjes and Tony Figaji.

Chapter 2: Within this study protocol AGD was involved in study conceptualisation and design. She produced the first written draft of the study protocol and undertook subsequent edits following comments from listed co-authors. She also developed or oversaw the development of all study documentation (informed consent forms, standard operating procedures, case report forms, ethical and regulatory applications) relating to the trial. Professors Sean Wasserman and Graeme Meintjes at The University of Cape Town and my primary supervisor Robert J Wilkinson oversaw study conceptualisation and design and provided regular review of drafts of the protocol.

Chapter 3: AGD wrote the statistical analysis plan, with supervision from the trial statistician related to this analysis (published as supplementary material in (Davis et al., 2021). AGD cleaned the data, undertook the analysis, interpreted the results and produced the first draft of the manuscript. Professors Sean Wasserman, Graeme

Meintjes and Robert J Wilkinson, as well as the trial statistician (Jason C Liang) provided close supervision via data presentations at interim points throughout the analysis.

Chapter 4: AGD conceived the idea for this study and designed the sub-study written into the LASER-TBM protocol. Colleagues at The University of Cape Town Anna Dreyer, A/Professor Sam Nightingale and Professor John Joska contributed to the study design. Data within this chapter is presented from cognitive test battery data arising from the LASER-TBM sub-study, but also includes;

i) cognitive test battery data arising from an unpublished data set submitted as part of an MSc thesis project by Dr Christine Albertyn at the University of Cape Town (additional TBM cases, and a population of patients with non-CNS TB) – included to increase statistical power and as a comparator group;

ii) a subset of cognitive test battery data arising from an unpublished prospective study of cognitive impairment in HIV (the CONNECT study) – included as a comparator group of people living with HIV.

With permission, AGD combined the raw data from these additional studies to that arising from LASER-TBM. AGD cleaned the data, undertook the analysis, interpreted the results and produced the first draft of the manuscript. This work was supervised by Anna Dreyer and A/Professor Sam Nightingale.

Chapter 5: AGD conceived the idea for the study, cleaned the data, analysed the results and wrote the chapter. The laboratory work (processing of CSF by Luminex Multiplex commercial kit) was performed by a University of Cape Town collaborator (Dr

Muki Shey) as travel to South Africa at the time of this work was prohibited due to the COVID-19 pandemic. Supervision and guidance in interpretation of the results and chapter draft review was provided by Professors Anne O'Garra and Robert J Wilkinson.

Chapter 6: AGD designed the study and wrote documentation related to the study (consent forms, standard operating procedures, ethical and regulatory approvals). The imaging protocol was developed in collaboration with colleagues at University of Cape Town (Dr Frances Robertson and Professor Ernesta Meinjtes). AGD cleaned the data, analysed the results, interpreted the findings, and wrote the chapter. Supervision and guidance in interpretation of the results and chapter draft review was provided by Dr Frances Robertson and Professor Robert J Wilkinson.

In the study of neurological manifestations of COVID-19 included within the appendix of this thesis AGD conceived the idea for the study and wrote the study protocol as well as documentation related to the study (standard operating procedures, case report forms, ethical approvals). AGD cleaned the data, analysed the results and produced the first draft of the manuscript. The laboratory work related to i) Luminex analysis of CSF and ii) detection of SARS-CoV2 in CSF were performed by University of Cape Town collaborators (Dr Muki Shey and Dr Georgia Schafer) as travel to South African at the time of this work was prohibited due to the COVID-19 pandemic. Oversight and supervision was provided by Professor Robert J Wilkinson.

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Chapter 1. Introduction

1.1 Background and PhD goals

Tuberculosis remains a major global health problem. In 2015, an estimated 10.4 million new cases of TB occurred worldwide. The World Health Organization's 'End TB Strategy' calls for a 90% reduction in TB related deaths and 80% reduction in TB incidence rate by 2030, 15 years on from its declaration. In 2015 the rate of reduction in yearly incidence was 1.5%, which falls below the required target of 4-5%. These figures reflect the ongoing evolving challenges faced in the prevention and treatment of tuberculosis[1].

Tuberculous meningitis (TBM) is the most lethal and disabling form of tuberculosis (TB), affecting >100,000 people per year (Wilkinson et al., 2017). TBM arises when *Mycobacterium tuberculosis (Mtb)* crosses the blood-brain barrier, causing severe inflammation and tissue damage. Inflammation also initiates metabolic derangement, contributing to ongoing neuronal injury despite effective antibiotics and anti-inflammatory therapy (Davis et al., 2019b). In HIV co-infection, TBM mortality approaches 50% while on therapy, and long-term disability is very common amongst survivors (Marais et al., 2011). The central hypothesis driving work undertaken within my PhD is that poor outcome in TBM and HIV-TBM is due to treatment which fails to provide adequate antibiotic penetration into central nervous system whilst simultaneously dampening counterproductive host responses.

The goal of my PhD was to address these failures by:

i) evaluating the safety of a novel drug regimen which aims to increase bactericidal activity within the CNS in a phase 2A randomised controlled trial
ii) embedding pathogenesis studies within the clinical trial to enable *in vivo* and *ex vivo* analysis of pathogenic mechanisms at play in HIV-associated TBM
iii) developing robust quantitative clinical endpoints for TBM studies to better understand neurological outcomes, in particular cognitive and functional outcomes, and in turn inform studies of pathogenesis

This work was extended to include a case-control study of neurological complications related to SARS-CoV2 infection, which contributed knowledge to this field whilst serving as a control group for *ex vivo* analysis.

This introduction aims to cover two key areas, namely;

i) current treatment options in TBM
ii) current understanding of pathogenic mechanisms in TBM

1.2 Current treatment options in Tuberculous Meningitis

In 1948 the modern era of tuberculosis treatment saw the first evidence of therapeutic response to streptomycin in pulmonary TB (Streptomycin in Tuberculosis Trials Committee, 1948). Isoniazid followed in 1952 with a key trial demonstrating improved efficacy when added to streptomycin (Anonymous, 1952) and in 1971 the addition of rifampicin and pyrazinamide led to reduction in treatment duration from 2 years to 6 months (Nitti, 1971). However, unlike in pulmonary TB where decades of clinical trials have instructed and refined treatment regimens in drug-sensitive and more recently drug-resistant TB, comparatively little evidence exists to guide optimal treatment in tuberculous meningitis. Here I evaluate evidence guiding the treatment of TBM in adults considering three aspects to successful management; i) effective antimicrobial treatments ii) controlling the host inflammatory response and iii) supportive

interventions to reduce raised intracranial pressure. I discuss TBM complicated by HIV co-infection, in particular timing of antiretroviral therapy and review the evidence for emerging therapies.

1.2.1 Antibiotics

Current WHO guidelines for TBM are based on those developed to treat PTB and suggest treatment with two months of rifampicin, isoniazid, pyrazinamide and ethambutol followed by up to ten months of rifampicin and isoniazid for all patients (Anonymous, 2010). Although initiation of this regimen before the onset of coma is the strongest predictor of survival from TBM (Prasad et al., 2016), this regimen does not take into account the differential ability of antituberculosis drugs to penetrate the brain (Donald, 2010).

Introduced in 1952, isoniazid made immediate impact on mortality in all forms of tuberculosis. This drug penetrates the CNS freely (Ellard et al., 1993), and is a key chemotherapeutic agent in TBM with proven potent bactericidal activity within the first 2 days of treatment (Mitchison, 2000). Isoniazid metabolism occurs via the genetically polymorphic NAT2, with both fast and slow metabolisers described (Weber and Hein, 1979). In pulmonary TB studies have demonstrated an association between phenotype, exposure and clinical outcome (Weiner et al., 2003). One recent pharmacokinetic study has evaluated this association in TBM and found, using pharmacodynamic modelling that isoniazid exposure was associated with outcome, with a higher mortality rate in in fast metabolizers with lower isoniazid exposures (Ding et al., 2020). These findings provide rationale to investigate higher doses of isoniazid in TBM, particularly in fast acetylators; a NAT2 stratified, randomized controlled trial in TBM is currently underway (NCT03787940).

Rifampicin does not penetrate the blood brain barrier as well with concentrations in CSF only 10-20% of that reached in plasma (Donald, 2010). Several studies have now investigated the efficacy of higher dose rifampicin, with varying results. In 2013 an open-labelled randomized phase 2 study in 60 Indonesian adults with TBM showed a 50% reduction in mortality with high dose rifampicin (600 mg, about 13mg/kg intravenously) compared to standard dose rifampicin (450mg, about 10mg/kg orally) (Ruslami et al., 2013b). A larger randomized placebo-controlled trial in Vietnam recruiting between 2011 and 2014 tested a higher dose of oral rifampicin (15mg/kg orally) alongside levofloxacin against standard therapy and did not show a mortality benefit (Heemskerk et al., 2016). A recent pharmacokinetic (PK) study in Uganda suggested that doses higher than 15mg/kg may improve outcomes by demonstrating ~8 and ~6 fold higher CSF exposures with 35mg/kg (oral) and 20mg/kg (IV) doses respectively compared to standard oral dose (10mg/kg) (Cresswell et al., 2021). Alongside this work, studies in PTB have shown that doses up to 35mg/kg are safe and well tolerated in the first two weeks of therapy (Boeree et al., 2015). Within this study, the highest doses of 30 and 35mg/kg showed highest early bacteriocidal activity measured by fall in colony forming units (CFU) and time to positivity. At two weeks 8 out of 14 patients taking 35mg/kg of rifampicin were culture negative compared 5 of 14 taking 20mg/kg, 0 of 15 taking 25mg/kg, 2 of 15 taking 30/kg and 3 of 8 controls (Boeree et al., 2015). This data may suggest that 15mg/kg within the aforementioned phase 3 RCT in TBM (Heemskerk et al., 2016) was not high enough and provides rationale for evaluating doses up to 35mg/kg in TBM. Several ongoing studies are now evaluating either the pharmacokinetics and/or efficacy of high dose rifampicin in adult and paediatric TBM (SIMPLE; NCT03537495; HARVEST; ISRCTN 15668391; SURE; ISRCTN40829906; ALTER; NCT04021121; INTENSE-TBM; NCT04145258). The safety of high dose rifampicin, in the context of HIV-associated TBM and when used in conjunction with

other adjunctive antibiotics and/or host directed therapies however is not well described.

Pyrazinamide was first introduced as an adjunctive agent to rifampicin to shorten treatment regimens in pulmonary TB (East African/British Medical Research Councils, 1972), and remains a pivotal drug in TB. Pyrazinamide has good CSF penetration with measured concentrations in CSF close to that of serum (Donald, 2010); however its dose in TBM has not been optimised. Though pyrazinamide has poor bactericidal activity in the first 2-4 days of treatment, studies in pulmonary TB have shown that thereafter (i.e. days 4-14) its activity matches that of isoniazid and rifampicin (Jindani et al., 1980, Botha et al., 1996). A recent pharmacokinetic study demonstrated good CSF penetration as well as high correlation between dose, plasma exposure and CSF concentration (Stemkens et al., 2019). The effect of higher CSF exposure on clinical outcome however is not known; studies to evaluate this whilst considering possible increased risk of hepatotoxicity and neurotoxicity (Pasipanodya and Gumbo, 2010, Torok et al., 2018) are needed. This is interesting given that pyrazinamide may also possess anti-inflammatory properties, via reduction of pro-inflammatory cytokine production (Manca et al., 2013).

Of the current recommended first line drugs, penetration of ethambutol is the poorest (Donald, 2010); highlighting whether ethambutol has a role within the standard regimen in TBM. This is also important in considering drug resistance in TBM, for which mortality rate approaches 100% (Tho et al., 2012, Patel et al., 2004, Vinnard et al., 2011). WHO guidelines for the treatment of rifampicin resistant- and multi-drug resistant TBM state that at least five effective drugs should be used initially including a fluoroquinolone and an injectable second line agent, and treatment should last 18-24 months (Falzon et al., 2017). Recommended core second line agents and their CSF penetrance are listed in Table 1.1.

Of the fluoroquinolones, ofloxacin was the first to be recognized as a potential effective treatment for tuberculosis (Tsukamura et al., 1985). In 2011 a randomized study in Vietnam investigated the pharmacokinetics and exposure-response relationships of three fluoroquinolones (ciprofloxacin, levofloxacin or gatifloxacin) in TBM (Thwaites et al., 2011). Population pharmacokinetic models describing the disposition of fluoroquinolones in CSF and plasma were used to determine exposure-response relationships through univariable analysis of clinical outcomes. Significant higher proportions of death and disability were observed for patients with lower or higher plasma and CSF fluoroquinolone exposures than for patients with intermediate exposures; a finding which may be explained by the increased permeability of the BBB in severe meningeal disease and/or reduced creatinine clearance in those with more severe systemic involvement and therefore those more likely to die. Nonetheless the study demonstrated improved clinical outcomes measured by survival, burden of disability and incidence disease relapse of fluoroquinolones when used prior to the onset of coma and informed dose finding for future studies (Thwaites et al., 2011). Subsequently two randomized controlled trials have evaluated the safety and efficacy of adjunctive fluoroquinolones in adult TBM with or without high dose rifampicin. In a phase 3 RCT 817 adults with TBM received either standard therapy or an intensified regimen including a higher dose of rifampicin (15mg/kg) as well as levofloxacin (20mg/kg); in this study levofloxacin did not improve outcomes (Heemskerk et al., 2016). The second, phase 2 RCT in Indonesia randomized 60 adults with TBM to receive standard dose or high dose rifampicin with either moxifloxacin (400mg) or ethambutol (750mg) and demonstrated no relationship between exposure to moxifloxacin in plasma and CSF and survival (Ruslami et al., 2013b). In a recently published RCT, children with TBM received either high dose rifampicin with or without levofloxacin (Paradkar et al., 2022). Although high dose rifampicin improved neurocognitive outcomes, this was not

statistically significant in the arm that included levofloxacin. It is possible that in this study levofloxacin negated in part the benefit of high dose rifampicin, or that the small sample size precluded a thorough assessment of potential benefit of adjunctive levofloxacin (van der Laan et al., 2016). This remains an area for consideration in future research trials, with no current RCT planned to evaluate adjunctive fluoroquinolones in either adult or paediatric TBM.

Linezolid a synthetic antimicrobial and the first agent of the oxazolidinone class, was licensed in 2000 for treatment of nosocomial pneumonia and skin infections caused by select gram positive bacteria (Ford et al., 2001, Brickner et al., 2008). The role of linezolid in tuberculosis was first investigated in the context of MDR tuberculosis. Early studies reported rapid sterilization of *M. tuberculosis* cultures following the administration of linezolid 600mg BD in addition to standard treatment (Fortun et al., 2005, von der Lippe et al., 2006) with subsequent studies demonstrating a role for linezolid as an effective treatment in drug resistant tuberculosis (Sotgiu et al., 2012). Broad tissue penetration of linezolid, including into the CNS (Nau et al., 2010) makes linezolid a favourable drug in TBM; however evidence to support the use in this context is limited. An observational study by Li et al demonstrated favourable clinical outcomes and a non-significant difference in adverse events in children with drug sensitive TBM treated with linezolid compared to control (Li et al., 2016). However, the study was a retrospective observational analysis with unblinded assessment of clinical outcomes. In adult TBM a retrospective cohort study of 33 adults with severe TBM demonstrated more rapid improvement in CSF parameters, recovery of consciousness and reduction of fever with adjunctive linezolid (Sun et al., 2014).

There has however been concern regarding safety of linezolid. The most common adverse events associated with LZD use in TB treatment are haematological toxicity

(mainly dose-dependent, occurring in up to 25%) and peripheral neuropathy (mainly duration-dependent, occurring in up to 31%) (Zhang et al., 2015). In one systematic review, haematological toxicity appeared to be dose related, with a significantly higher prevalence of anaemia (31.3% vs 13.6%, *p=0.007*) and a lower prevalence of peripheral neuropathy (23.5% vs 37.2%, *p=0.018*) in those taking a dose > 600 mg/day, compared to those taking < 600 mg/day (Zhang et al., 2015). Since LZD toxicity is thought to be related to trough concentrations once daily dosing is likely to result in less toxicity than twice daily dosing. This was demonstrated in a PK-toxicity analysis of a randomized controlled trial of LZD for TB, which demonstrated an inverse correlation between LZD trough concentrations and mitochondrial function. Furthermore, higher LZD trough concentrations correlated with the development of mitochondrial toxicity-related AE (Song et al., 2015). These findings were replicated in a hollow-fibre infection model demonstrating a favourable toxicity profile with once daily LZD dosing (Brown et al., 2015). The onset of symptoms related to linezolid toxicity are well described. Typically, anaemia usually occurs within 2 months, whereas peripheral neuropathy occurs between 2-4 months of therapy (Tang et al., 2015). Two recent studies however have shown that toxicity associated with linezolid in MDR-TB is rarely severe (Wasserman et al., 2022) and largely reversible (Conradie et al., 2020) and therefore may be acceptable in a disease where mortality is so high. Several ongoing and planned studies, besides LASER-TBM are evaluating either the pharmacokinetics or efficacy of linezolid in adult and paediatric TBM (SIMPLE; NCT03537495; ALTER; NCT04021121 and INTENSE-TBM NCT04145258).

1.2.2 Host directed therapies

In TBM adjunctive host-directed immune interventions which either enhance protective immunity or regulate pathological tissue-damaging immunity are needed (Davis et al., 2020).

Corticosteroids are the most widely used host directed therapy. The first randomised controlled trial of corticosteroids in TBM was conducted in 1969 and demonstrated a non-significant reduction in mortality (RR 0.53, 95% CI 0.39 to 1.37; n = 23)(O'Toole et al., 1969). Since then, a further 6 published RCT have investigated efficacy of corticosteroids in reducing mortality associated with TBM (Girgis et al., 1991, Kumarvelu et al., 1994, Chotmongkol et al., 1996, Malhotra et al., 2009, Schoeman et al., 1997, Thwaites et al., 2004). A recent Cochrane review found that in a pooled analysis of these 7 trials alongside data from two unpublished trials there were 25% fewer deaths with corticosteroids (RR 0.75, 95% CI 0.65 to 0.87; 1337 participants) (Prasad et al., 2016). The largest of these was a randomised, double-blind placebo-controlled trial conducted in Vietnam in 545 patients with TBM (Thwaites et al., 2004). In this trial treatment with dexamethasone was associated with a reduction in risk of death (RR 0.69, 95% CI 0.52 to 0.92; *p = 0.01*). Of the 9 trials included within the Cochrane review, 8 reported on neurological disability at 2 to 24 months' post diagnosis (Prasad et al., 2016). Pooled analysis of data from 1314 participants demonstrated no effect of corticosteroids between the two groups (RR 0.92, 95% CI 0.71 to 1.20). In the aforementioned Vietnamese study, there was also no significant reduction in the proportion of severely disabled patients (34 of 187 (18.2%) among survivors in the dexamethasone group vs 22 of 159 patients (13.8%) in the placebo group, p=0.27) or on the combined outcome of death and severe disability at 9 months (OR 0.81, 95% CI 0.58 to 1.13; *p = 0.22*). At 5 years there was no significant

association between dexamethasone treatment and disability status (p=0.32) (Thwaites et al., 2004). This trial, as well as the analysis from this recent Cochrane review highlight the lack of evidence to support the efficacy of corticosteroids to reduce disability in TBM. Further work is required to investigate this.

There is a lack of efficacy data to inform the use of corticosteroids in HIV-associated TBM. Of the nine RCT taking place since 1969, only the 2004 Vietnamese study enrolled patients with HIV-1 co-infection (Thwaites et al., 2004). In this study, 98 of the 545 patients were co-infected with HIV. Although not powered to address the question of the efficacy of dexamethasone in HIV-associated TBM, there was no significant effect of dexamethasone on the combined endpoint of death and disability or on death alone (stratified relative risk of death 0.78; 95% CI 0.59 to 1.04; *p=0.08*). A larger study to evaluate the role of dexamethasone in HIV-associated TBM has been completed and results are awaited (NCT03092817). The role of corticosteroids in the treatment of TBM-associated immune reconstitution inflammatory syndrome (IRIS) is unclear. Although in other forms of TB-IRIS use of prednisolone improves outcomes (Meintjes et al., 2010), there is a lack of evidence to support their use in TBM-IRIS. In a study to describe pathogenesis of TBM-IRIS, the use of corticosteroids often failed to prevent the onset of IRIS in HIV-1 infected patients with TBM (Marais et al., 2017).

A focus of recent research is the identification of genetic polymorphisms in immune response genes, in particular a single polymorphism in the leukotriene A4 hydrolase (LTA4H) promotor which plays a role in the balance of proinflammatory and anti-inflammatory eicosanoids thereby influencing expression of TNF alpha (Tobin et al., 2010). Studies in zebrafish and subsequently in humans have shown that expression of LTA4H can determine susceptibility to disease as well as response to corticosteroids (Tobin et al., 2012). In a retrospective analysis of patients enrolled to a trial of

adjunctive dexamethasone in TBM, survival benefit was restricted to homozygotes with a TT genotype of the LTA4H (hyperinflammatory) in contrast to CC (hypoinflammatory) genotypes where dexamethasone was associated with harm (Tobin et al., 2012). More recently in an analysis of patients enrolled to a study of intensified antituberculous regimens and adjunctive dexamethasone, LTA4H genotype predicted survival in HIV-1-uninfected patients with the TT genotype patients significantly more likely to survive than those with the CC genotype. In this study patients with the LTA4H TT genotype had high pro-inflammatory cytokine concentrations (IL-1β, IL-2, and IL-6). However, those with CT and CC genotypes had intermediate or lower concentrations respectively (Thuong et al., 2017). This may suggest that the suppression of inflammation by dexamethasone leads to survival benefit in patients with the TT genotype, however, it may be non-beneficial or even harmful in those with CT or CC genotypes. This highlights the potential role for individualized immunotherapy where adjunctive corticosteroids are given on the basis of pre-treatment genotyping and provides rationale for the LTA4H genotype stratified, randomised placebo-controlled phase III noninferiority trial evaluating adjunctive dexamethasone currently ongoing in Vietnam (NCT03100786).

Improved understanding of immunopathogenesis in TBM has led to discovery of target sites for immunotherapies. The cytokine TNF alpha has been a target in both animal and human studies. Thalidomide has a complex mechanism of action, including but not exclusively via inhibition of TNF-alpha. In a rabbit model of TBM, thalidomide was associated with survival benefit (Tsenova et al., 1999). In a safety and tolerability study using thalidomide at escalating doses, thalidomide was safe and well tolerated as an adjunctive therapy to treat children with stage 2 TBM (Schoeman et al., 2000). Clinical and radiological data also suggested improved outcome. The results of this study

supported a phase 3 randomised controlled trial in paediatric TBM to test thalidomide against placebo in stage 2 and 3 disease (Schoeman et al., 2004). Thalidomide was given at a dose of 28mg/kg/day for the first 28 days of treatment. Forty-seven children were enrolled, of which 30 received thalidomide. This study was terminated early as all adverse events and deaths occurred in the thalidomide arm. Debate around the influence of the high dose and late stage of disease on the adverse outcomes remains. Subsequent studies have suggested that thalidomide may still have a role in tuberculous mass lesions where treatment with corticosteroids has failed (van Toorn et al., 2015) and in children with CNS TB-related complications (van Toorn et al., 2021).

Early (within 2-4 weeks of commencing antitubercular therapy) antiretroviral (ART) therapy of HIV-associated tuberculosis is associated with survival benefit in patients with low CD4 counts (Abdool Karim et al., 2010, Blanc et al., 2011, Havlir et al., 2011). A metanalysis including 8 randomised controlled trials in pulmonary TB compared survival in patients in whom antiretroviral therapy was started within 1-4 weeks vs 8-12 weeks. Results demonstrated a survival benefit in patients newly diagnosed with tuberculosis and a CD4 count of less than 50/mm^3 where antiretroviral therapy was commenced within 1-4 weeks of diagnosis (Uthman et al., 2015). However, initiating such otherwise life-preserving therapy early during TB treatment may be complicated by more frequent immune reconstitution inflammatory syndrome (IRIS). In the CNS TBM-IRIS itself is associated with increased mortality (Marais et al., 2013). Early initiation of ART in TBM does not improve outcomes but rather increases the chance of grade 4 adverse events (Torok et al., 2011a); this data therefore supports the current recommendations stating that initiation of ART should be deferred until 4-6 weeks of anti-TB therapy has been completed.

Aspirin acts by irreversibly inhibiting the cyclooxygenase pathway of arachidonic acid metabolism, and thus reducing the downstream production of prostanoids (Vane, 1971). At low doses aspirin prevents ischaemic infarction through inhibitory effect on platelet and thrombus formation (Richman and Owens, 2017); which at high doses may be further augmented by production of 15-epi-lipoxins, 17R-resolvins and protectins known to contribute to resolution of inflammation (Spite and Serhan, 2010, Tobin et al., 2012). This potential to prevent infarction, as well as hasten resolution of cerebral inflammation, has led to its evaluation for use as an HDT in TBM. Three RCT have thus far investigated its potential. The first demonstrated a trend towards lower 3-month mortality and incidence of stroke in adults with TBM treated with 150mg OD of aspirin (Misra et al., 2010). The second showed a significant reduction in the incidence of new hemiplegia in children receiving high dose (1000mg OD) vs low dose (75mg OD) aspirin (Schoeman et al., 2011). The third demonstrated a potential reduction in new infarcts and deaths by day 60 in the aspirin-treated adults with microbiologically confirmed TBM (11/32 (34.4%) events in placebo *vs*. 4/27 (14.8%) in aspirin 81 mg *vs*. 3/28 (10.7%) in aspirin 1000 mg; *p=0.06*) (Mai et al., 2018). In the latter, planned CSF analysis demonstrated aspirin dose-dependent inhibition of thromboxane A2 and upregulation of pro-resolving CSF protectins. Although no increase in adverse events with high doses have been observed in these referenced studies, the safety of aspirin in combination with intensified antibiotics and in the context of HIV co-infection is not known.

Infliximab is a TNF-alpha inhibitor which is FDA approved for use in inflammatory bowel disease, rheumatoid arthritis and some seronegative arthropathies. Although most reports of infliximab in TB relate to the reactivation of latent tuberculosis, there are several case reports where corticosteroids have failed to control inflammation yet subsequent reintroduction of infliximab has led to a near complete resolution of

symptoms(Marais et al., 2021, Molton et al., 2015, Lee et al., 2012a). Other therapies to consider include interleukin receptor 1 inhibitors anakinra (IL-1α and β)(Keeley et al., 2020) and canakinumab (IL-1β only).

1.2.3 Potential Pathways for Future Host Directed Therapies for Tuberculous Meningitis

Although host directed therapies are in use, they are limited in either efficacy or availability; in particularly in HIV associated TBM. Therefore, the quest for more effective therapeutics is ongoing. Here I discuss potential therapies which target pathways highlighted in recent pathogenesis studies or draw on insights from other forms of TB or inflammatory conditions with shared mechanisms of pathogenesis.

Statin Therapy Pathways

HMG-CoA reductase inhibitors ('statins') are ubiquitously used in prevention and treatment of cardiovascular disease, but are also known to have immunomodulatory, anti-inflammatory and anti-oxidative properties. Several *in vitro* studies have demonstrated that statins enhance anti-inflammatory and inhibit pro-inflammatory functions in microglial cells and inhibit mechanisms involved in neurodegeneration (Kata et al., 2016, Churchward and Todd, 2014, Cordle and Landreth, 2005, McFarland et al., 2018). Anti-inflammatory properties may be due to modulation of isoprenylation (Waiczies et al., 2008) with downstream effects on inhibitory and stimulatory transcription pathways, or via allosteric inhibition of leucocyte function antigen (LFA)-1 integrin (Weitz-Schmidt et al., 2001) which is involved in the transmigration of activated T cells through the blood brain barrier. Neuroprotective effects may be due to

modulation of excitotoxicity, vascular function, angiogenesis, and/or reduced oxidative damage through nitric oxide stimulas (Bosel et al., 2005, Ponce et al., 2008). Importantly, some studies have shown increased neuronal death with higher concentrations of statins (Michikawa and Yanagisawa, 1999, Tanaka et al., 2000, Schulz et al., 2004).

The potential of statins to effect CNS inflammation and neurodegeneration in other conditions are of interest given the shared mechanistic pathways in TBM. For example, animal models of multiple sclerosis (MS) show that statins skew immune responses towards an anti-inflammatory T-helper cell 2 response, inhibiting pro-inflammatory cytokines IL-2, IL-12 and IFN-γ (Youssef et al., 2002). Patients with secondary progressive MS benefited from statin therapy (Chataway et al., 2014) with a phase 3 trial underway (NCT03387670). In a mouse model of traumatic brain injury, atorvastatin led to profound attenuation of T cell, neutrophil and natural killer cell invasion into the CNS, and reduction in production of pro-inflammatory cytokines (IFN-y and IL-6) and chemokines (CCL5 and CXCL10) (Xu et al., 2017). In a double-blind randomised trial involving 36 patients with traumatic brain injury, rosuvastatin given for 10 days in the acute phase of injury significantly reduced TNF-α which correlated with a reduction in disability scores (Sanchez-Aguilar et al., 2013). Other conditions where the role of statins has been explored include Alzheimer's disease (Jick et al., 2000), and Parkinson's disease (Yan et al., 2019). Further, statins may be associated with reduced risk of tuberculosis (Lai et al., 2016). In a TB murine model, adjunctive simvastatin shortened time to culture clearance by 1 month, enhanced bacterial killing, and decreased culture-positive relapse and enhance bacterial killing (Dutta et al., 2016, Skerry et al., 2014, Parihar et al., 2014). Clinical trials (NCT03456102, NCT04147286) will investigate the efficacy of statins in pulmonary tuberculosis. Given their potential use as an adjunctive TB therapy, their lipophilic properties allowing good penetration to

the CNS, as well as their potential as an anti-inflammatory and neuroprotective agent, statins may have a role as a HDT in TBM; trials to explore this hypothesis are needed.

Glutamate 'grabbing' drugs

Excessive glutamate and neuro-excitotoxicity are thought to contribute to brain injury and cell death in TBM. In one study, RNA sequencing of whole blood and CSF from children with TBM demonstrated significant enrichment of transcripts associated with neural excitotoxicity predominantly driven by glutamate release, NMDA receptor binding and uptake (Rohlwink et al., 2019). This mechanism is thought to contribute to brain injury and cell death in other neurological conditions such as stroke, epilepsy, traumatic brain injury, Alzheimer's and Huntington's disease (Meldrum, 2000, Wang and Reddy, 2017). Therapeutics which aim to reduce glutamate excitotoxicity either by i) modulating the downstream effects of glutamate via NMDA receptor binding or ii) reducing extracellular glutamate (e.g. glutamate 'grabbing') may have a role in the treatment of TBM. In acute stroke, a similar approach was taken however although animal studies were promising, randomised trials in humans assessing efficacy of NMDA antagonists largely failed (Jia et al., 2015, Kalia et al., 2008, Grupke et al., 2015). Therapeutics have been designed to reduce glutamate induced excitotoxicity by lowering blood glutamate concentration thus leading to a larger natural glutamate gradient between the brain and blood thereby facilitating the efflux of extracellular brain glutamate into the blood (Castillo et al., 2016). In an animal study riboflavin (vitamin B_2), selected for its ability to interact with Glutamate-Oxaloacetate transaminase (GOT) to significantly reduced blood glutamate levels compared to placebo (da Silva-Candal et al., 2018). In a randomised trial, riboflavin was correlated with improvement of disability when given intravenously in adults with acute stroke (da Silva-Candal et al., 2018). A number of studies have explored the neuroprotective properties of riboflavin including in conditions such as migraine and Parkinson's disease (Marashly and

Bohlega, 2017). It is unclear whether drugs such as riboflavin, or others which reduce glutamate neuro-excitotoxicity, have a role as an adjunctive therapy to promote neuroprotection in TBM; however, given the emerging body of evidence which suggest involvement of the glutamate-glutamine pathway, this is a potential area of interest for future studies.

Tryptophan Pathway Drug Targets

Tryptophan is an essential amino acid which can either be converted to serotonin or oxidized kynurenines via indoleamine 2,3-dioxygenase (IDO1). Further oxidization occurs to convert kynurenine to kynurenic acid, which has neuroprotective properties. Prior studies have shown that *M.tb* induces marked upregulation of IDO-1 expression in both human and murine macrophages *in vitro* (Blumenthal et al., 2012); and that blockade of IDO activity reduces both clinical manifestations of TB as well as microbial and pathological correlates of the human TB syndrome in macaques.(Gautam et al., 2018). In an observational cohort study of TBM, *low* CSF tryptophan levels were found in those who survived, compared to non-survivors or controls (van Laarhoven et al., 2018). It is therefore unclear in TBM whether drugs which block IDO-1 such as indoximod, an immunometabolic adjuvant that is current under investigation in cancer therapy (Fox et al., 2018), would cause benefit or harm. It is plausible that improved survival seen in those with low CSF tryptophan is due to increased availability of kynurenic acid which has neuroprotective action via glutamate receptors and reactive oxygen species. Further investigation into the influence of tryptophan and its downstream metabolites on pathogenesis in TBM is required in order to establish suitable targets along this pathway for HDT.

Eicosanoid Modulating Drugs

Eicosanoids are arachidonic acid derived lipid mediators that trigger pro-and anti-inflammatory responses and include prostaglandins, resolvins, lipoxins, and leukotrienes which serve as signalling molecules, modulating inflammation and cell death in TB (Ricciotti and FitzGerald, 2011). A delicate balance in eicosanoid levels is crucial for *M.tb* control and regulating the production of pro-inflammatory cytokines (Young et al., 2020).

Non-steroidal inflammatory drugs (NSAID), which exert their effects by inhibiting cyclooxygenase (COX) activity may lead to reduction of excessive inflammation in TBM. As discussed, aspirin, a non-selective COX inhibitor has been investigated in three trials in TBM with variable outcomes (Mai et al., 2018, Schoeman et al., 2011, Misra et al., 2010). New generation NSAID with more selective inhibition of COX2 may have more favourable safety profiles. Phase 1 trials to assess the safety and bactericidal activity of celecoxib and etoricoxib in healthy volunteers with a view to developing these agents as HDTs for drug sensitive TB are currently underway (NCT02602509; NCT02503839). Although trials to further investigate the role of aspirin in TBM are underway, future research should consider the potential contribution of newer more selective COX2 inhibitors in TBM.

Phosphodiesterase Inhibitors

Phosphodiesterase inhibitors (PDE-i) are small-molecule inhibitors that reduce inflammation by increasing intracellular cyclic adenosine monophosphate and cyclic guanine monophosphate (Page and Spina, 2011). Phosphodiesterase 4 (PDE-4) inhibitors such as roflumilast have shown to be effective in the treatment of numerous inflammatory conditions including chronic obstructive inflammatory disease (Calverley et al., 2009). PDE-4 is expressed within the cortex and hippocampus and animal models suggest that inhibition of PDE-4 may have a beneficial role in CNS conditions

where inflammation plays a role in pathogenesis (Schaal et al., 2012, Atkins et al., 2007, Gong et al., 2004, Gonzalez-Garcia et al., 2013, Wu et al., 2017). In animal models of pulmonary TB, inhibition of PDE-3 (cilostazol), PDE-4 (roflumilast) and PDE-5 (sildenafil) have all increased bacterial clearance and reduced pro-inflammatory cytokines which contributed to a reduction in neutrophil infiltration and lung pathology (Subbian et al., 2011, Maiga et al., 2015, Maiga et al., 2012, Konrad et al., 2015). The role of phosphodiesterase inhibitors has not been studied in TBM but the properties above make them intriguing candidates for adjunctive therapy in TBM.

1.2.4 Supportive therapies

Rich and McCordock were the first to describe the pathogenic mechanisms which lead to central nervous system tuberculosis (RicH, 1933). Research since then has enabled better understanding of the natural history including the neurological sequelae such as hydrocephalus, vasculitis leading to cerebral infarction and metabolic abnormalities especially hyponatremia. Early recognition and management of these phenomena remains integral to the treatment of patients with TBM.

- Hydrocephalus and raised intracranial pressure

The inflammatory infiltrate within the subarachnoid space or the ventricular pathways may lead to disruption of CSF flow resulting in hydrocephalus. Hydrocephalus can be communicating (caused by abnormal flow through the basal cisterns), or non-communicating (usually a later complication due to obstruction at the level of the fourth ventricle). Communicating hydrocephalus is more common and can be managed medically however may require intervention if progressing. Non-communicating hydrocephalus requires rapid intervention. CSF diversion techniques such as

ventriculoperitoneal shunts (VPS) and endoscopic third ventriculostomy are the mainstay of surgical treatment for hydrocephalus (reviewed in (Rajshekhar, 2015). Evidence as to which technique is most effective is lacking.

A systematic review of 1038 adults and children with TBM and hydrocephalus demonstrated good outcome, defined as Glasgow Outcome Scale 4 or 5 (Table 1.2) in 58.2% of patients. Good outcomes were observed in more patients with less severe disease specifically those found to be Grade I (78.57%) and II (65%) compared to those with more severe (Grade IV disease) where only 31.5% survived (Table 1.2). Subgroup analysis demonstrated that good outcomes occurred in significantly fewer patients with HIV-1 associated TBM with only 25% patients of patients achieving a good outcome compared to 61% of HIV negative patients (Rizvi et al., 2017). In a study of 30 participants with HIV-associated TBM and hydrocephalus, participants underwent VP shunt placement and outcomes were compared to age and gender matched HIV negative controls. Patients were followed up at two time points; discharge (short term) and three months after VP shunt insertion (long term). Although short term outcomes were only marginally better in the HIV negative group, long term outcomes differed significantly with 66.7% mortality and 76.2% poor outcome in HIV positive patients compared to 30.8% mortality and 34.6% poor outcome in the HIV negative controls. Their study demonstrated that HIV seropositivity is an independent predictor of poor outcome, although they did identify that in patients with less severe disease at presentation, 80% had good outcomes. By contrast to previous studies these results suggest a role of VP shunting in HIV-associated TBM in patients with less severe disease (Sharma et al., 2015). In paediatric TBM, a recent study showed that there is an association between the severity of hydrocephalus and CSF immune biomarkers GFAP and S100B (Rohlwink et al., 2017). It remains unclear as to whether this is due to the secondary compressive effect on brain parenchyma or whether these inflammatory mediators are involved in the

pathogenesis of hydrocephalus. Further research is required to establish best evidence-based practice for the treatment of this common complication in TBM in particular for HIV associated disease.

Although hydrocephalus is the most common cause of raised ICP, elevated ICP can also be caused by other pathological processes within the CNS. In TBM, meningeal pathology may extend into the parenchyma and lead to encephalitis, whilst obliterative vasculitis within the vessels leads to infarction. These processes may result in cytotoxic and vasogenic oedema. The presence of parenchymal pathology may lead to failure of cerebral vascular autoregulation. Metabolic abnormalities such as hyponatraemia, hyperthermia and hypercapnia can cause further dysregulation. Thus, clinical management should be directed at the frequent monitoring and correction of abnormalities in gas exchange and tissue oxygenation, through mechanical ventilation (if necessary), meticulous fluid and electrolyte management, monitoring and intervention to treat raised intracranial pressure where appropriate as well as adequate temperature control. When there is no surgical intervention indicated, yet ICP remains high hyperosmolar agents, most commonly mannitol, may be effective yet a randomised control trial to test this hypothesis is required (Oddo et al., 2009, Francony et al., 2008).

- Hyponatraemia

Hyponatraemia defined as a plasma sodium level <135mmol/L occurs in 40-50% of patients with TBM (Misra et al., 2016). Several mechanisms exist. Cerebral salt wasting (CSW) is characterised by natriuresis, hyponatremia and volume contraction in response to brain injury (Sterns and Silver, 2008). The syndrome of inappropriate anti-diuretic hormone (SIADH) is also associated with brain injury and occurs due to excessive release of antidiuretic hormone from the posterior pituitary gland resulting in

inappropriate, continued secretion or action of the antidiuretic hormone arginine vasopressin (AVP) despite normal or increased plasma volume leading to hyponatraemia (Moller et al., 2001). In a prospective hospital-based study conducted in India, of 76 patients with TBM, 34 (44.7%) had hyponatremia due to CSW in 17, SIADH in 3 and miscellaneous causes in 14 (Misra et al., 2016). Distinguishing between CSW and SIADH is critical: their presentations are similar, but management is different. By convention, SIADH is managed by fluid restriction and cerebral salt wasting by fluid administration. Some suggest that both conditions can be treated with hypertonic saline (Sterns and Silver, 2008), whereas others state that fluid restriction, the traditional treatment for SIADH, has had little benefit in meningitis and might result in worsening hypovolaemia and harm (Moller et al., 2001). This complex and often overlooked complication in TBM should be further investigated to define optimal investigation and management.

- Tuberculomas

Tuberculomas can occur together with or independently of TBM. Clinical presentation depends on site and includes seizures, focal neurological weakness or symptoms of raised intracranial pressure due to hydrocephalus or mass effect. Tuberculomas commonly present as a feature of paradoxical worsening in patients treated for TB or in HIV-1 infected patients starting ART. In a randomized study to assess effect of dexamethasone on TBM related cerebral MRI changes in Vietnam, 43 patients receiving either dexamethasone (n=24) or placebo (n=19) underwent serial MRI scans. The number of patients with one or more tuberculomas rose from 64% (14 of 22) before treatment to 74% (20 of 27) after 60 days. There was no effect of dexamethasone on incidence of tuberculoma formation or on resolution of tuberculomas (Thwaites et al., 2007). The mainstay of treatment remains antituberculous therapy, the duration of which

is debated due to a lack of evidence in this area. It is unknown as to whether persistent radiological enhancement of intracranial tuberculomas following completion of 9-12 months of anti-TB therapy represents ongoing active TB, an inflammatory response in a lesion without active TB, or revascularisation. A recent consensus report suggested that ongoing enhancement does not represent treatment failure, however studies to evaluate optimal duration of treatment for CNS tuberculomas are required (Marais et al., 2019).

In some cases, where there is diagnostic doubt or where the size and anatomical location of the tuberculoma is causing clinical worsening, surgical excision may be required. Stereotactic craniotomy and excision of superficial small tuberculomas and microsurgery are procedures now used. In cases where there is no response to dexamethasone alternative anti-inflammatory agents have been tried, particularly when the tuberculoma involves the optic chiasm and threatens vision including thalidomide or infliximab (Schoeman et al., 2006, de la Riva et al., 2013, Roberts et al., 2003, Molton et al., 2015, Lee et al., 2012a).

- Vasculitis and stroke

Stroke in TBM occurs in 15-57% of patients with TBM depending on the imaging modality used: CT reveals stroke in 13-35% and MRI in ~60% (Thomas et al., 1977, Shukla et al., 2008, Kalita et al., 2009). They are usually multiple, bilateral and occur most commonly in deep grey matter structures including the caudate, anterior thalamus, anterior and genu of the internal capsule, namely the ‘tubercular zone (Misra et al., 2011) (Figure 1.1). The macroscopic pathological appearance in the brain vasculature is that of gelatinous fibrocellular leptomeningeal infiltrates initially enveloping the vessels including the carotid arteries, middle cerebral arteries and their branches. Vasculitis within the affected vessels may occur with intimal proliferation.

These process with or without superadded thrombosis likely leads to cerebral infarction(Thomas et al., 1977). There is no established prevention or treatment for stroke in TBM. There is no evidence to support corticosteroids in the prevention of stroke (Thwaites et al., 2004). Aspirin discussed earlier within this chapter has antiplatelet, anti-aggregant, anti-inflammatory and antioxidant properties, and in some cases has been shown to prevent stroke(Schoeman et al., 2011, Mai et al., 2018). It is unknown however whether other antiplatelet therapies such as clopidogrel may have a role, or whether treatment of acute stroke occurring within a 4 hour window should consider thrombolysis. Further research in the area of stroke prevention and treatment in TBM is required.

1.2.5 Conclusions and research priorities

- The neurological presentations of tuberculosis are the most lethal and under-researched manifestations of TB which remains a major global health problem.
- Current anti-tuberculous therapy regimens for TBM are based on those which are efficacious in PTB but do not consider the differing efficacy of drugs across the BBB.
- Further research is required to investigate the safety and efficacy of intensified therapy regimens and newer anti-tuberculous agents to treat CNS tuberculosis.
- Corticosteroids have proven mortality benefit except in HIV-associated TBM where, as yet, no sufficiently powered study has been able to prove benefit or lack thereof. More research is required to develop and evaluate novel host directed therapies. Immune response phenotypes and genetic polymorphisms may direct individualized immune therapies and mediators of the innate immune response may provide targets for the development novel therapies.
- Stroke is a major cause of morbidity and mortality in TBM. Recent studies have shown a potential benefit of aspirin in the prevention of stroke as well as in the modulation of the host immune response in TBM.
- There is currently no significant evidence base to guide management of hydrocephalus in HIV-1- infected TBM. A large randomized clinical trial is required to investigate outcomes comparing available CSF diversion techniques in this particularly vulnerable subgroup of patients.
- A treatment algorithm (Figure 1.2) gives a practical holistic approach to the management of patients with tuberculous meningitis

1.3 Pathogenesis of Tuberculous Meningitis

1.3.1 From primary infection to the central nervous system

The systemic immune response to tuberculous infection

Tuberculosis (TB) infection occurs through the inhalation of infectious droplets of aerosolised *Mycobacterium tuberculosis (M.tb)*, which cross the lung epithelium and infect lung alveolar macrophages, neutrophils and dendritic cells (DC). This leads to the secretion of antimicrobial peptides, cytokines (including interleukin-1α and β, tumour necrosis factor alpha (TNF-α), interleukin (IL)-6 and -12, chemokines, lipoxins that may stimulate necrosis and contribute to immune protection, and prostaglandins that may induce apoptosis (O'Garra et al., 2013). Under the influence of IL-12 and chemokines CCL-19 and -21, infected DC migrate to the local draining lymph nodes to stimulate the differentiation of T-helper I (Th1) cells. Th1 cell release of interferon gamma (IFN-γ) and TNF-α at the site of infection activates macrophages and DC to produce cytokines and antimicrobial factors that contribute to containment of the TB bacillus (O'Garra et al., 2013). This inflammatory process results in the formation of a granuloma, which encapsulates the infected cells and retards the replication of TB bacilli and can lead to latent infection. However, in the elderly, immunocompromised or very young in particular, the ongoing immune response may progress to active primary TB disease associated with tissue destruction in the lung and dissemination of the TB bacillus to other organ systems (Kumar and Kumar, 2010, Coico and Sunshine, 2009).

Dissemination to the brain

Dissemination of TB involves seeding of *M.tb* to other sites including the central nervous system (CNS). Various mechanisms by which the bacilli migrate into the lymphatic system or blood stream have been suggested (Krishnan et al., 2010, Jain et

al., 2006, Nguyen and Pieters, 2005). Bacterial proteins- early secretory antigenic target 6kDa (ESAT-6) and culture filtrate protein 10kDA (CFP-10) are involved in cell lysis, while heparin binding haemagglutinin adhesion (HBHA) aids *M.tb* translocation across the epithelium without lysis (Krishnan et al., 2010). *M.tb* may also invade and traverse vascular endothelial cells (Jain et al., 2006), replicate in lymphatic endothelial cells (Lerner et al., 2016), and be trafficked to distant locations in phagocytes (Krishnan et al., 2010). Furthermore, mycobacteria are able to survive and replicate in infected macrophages and lymphatic endothelial cells (LEC) surrounding granulomas in the lymph nodes. Research on LEC demonstrates that although *M.tb* bacilli are initially phagocytosed upon infecting the cell, through their genetic locus termed the region of difference 1 (RD1), the bacilli are able to escape from the phagosomes into the cytosol, where they more readily replicate. For those bacteria remaining in the phagosome they are able to prevent fusion of lysosomes with the phagosome, also in an RD1 dependent manner, thereby allowing bacterial replication in the phagosome and contributing to lymphatic tuberculosis (Lerner et al., 2016). The activation of LEC by IFN-γ is key to restricting these RD1 mechanisms of replication (Lerner et al., 2016). Additionally, host immunity and *M.tb* strain variation may play a role; polymorphisms in the genes encoding for antigen recognition and macrophage activation (Hawn et al., 2006) or impaired pro-inflammatory cytokine release may influence the ability of the initial innate response to control infection (Krishnan et al., 2010). TB strains assigned greater virulence, like the lineage 2 strain, which is postulated to subvert the innate immune response, promoting its survival and replication and thereby more severe disease (Caws et al., 2008, Fernando et al., 2007, Caws et al., 2006).

The CNS is protected from influx of potentially harmful blood-borne bacteria by 2 vascular barriers; the blood-brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB) (Pulzova et al., 2009) (Figure 1.3). The BBB is mainly formed by brain

microvascular endothelial cells that are characterised by intercellular tight junctions and a paucity of endocytic vesicles and fenestrae, and exhibit dedicated transport mechanisms to regulate influx and efflux across the CNS and blood compartments (Be et al., 2009, Pulzova et al., 2009). Pericytes, embedded within a basement membrane, and astrocytes' end-feet support the endothelial cells and also make an indispensable contribution to BBB integrity. In contrast, the BCSFB is composed of choroid plexus epithelial cells joined together by tight junctions and the arachnoid membrane. However, despite these protective mechanisms, *M.tb* bacilli migrate across these barriers. *In vitro* and animal models demonstrate that *M.tb* invades and traverses brain endothelial cells in the microvasculature through rearrangement of their actin (Jain et al., 2006, Be et al., 2009). Further, the *M.tb* gene *Rv0931c* (pknD) has been identified as a potential virulence factor promoting CNS infection in certain TB strains, as it enables the bacilli to interact with extracellular factors on the brain endothelium facilitating bacillary endothelial adhesion (Be et al., 2009). Another potential route of entry is the 'Trojan horse' mechanism by which *M.tb* are trafficked in infected macrophages and neutrophils across the BBB (Nguyen and Pieters, 2005).

Once the TB bacilli gain access to the brain, limited local innate immunity allows their survival and replication and the development of silent tuberculous lesions. Based on postmortem studies, Rich and McCordock suggested that TBM is initiated by the rupture of one of these lesions, the Rich focus, located under the cortical pia or adjacent to the meninges or ventricles, which releases *M.tb* bacilli into the sub-arachnoid space causing a granulomatous infection of the meninges (Rock et al., 2008, Donald et al., 2005, Rich A, 1933, Dastur et al., 1995) and the subsequent induction of inflammation. Recently the relationship between the Rich focus and the onset of miliary tuberculosis has been reviewed. Rich and McCordock did not acknowledge a role of miliary tuberculosis in the pathogenesis of TBM, however, several studies since have

suggested that the bacteraemia seen in these cases increased the likelihood that a meningeal or sub-cortical focus is established with subsequent rupture giving rise to TBM (reviewed in (Donald et al., 2005)).

1.3.2 Pathogenic and pathophysiological mechanisms within the brain

The host immune response to tuberculosis in the brain

Microglia within the cerebral parenchyma are the principal CNS cells infected by *M.tb* (Peterson et al., 1995, Rock et al., 2005) and are involved in immune regulation. Other CNS cells that have potential roles in this process are astrocytes and neurons (Rock et al., 2005, Randall et al., 2014) (Figure 1.3). Randall *et al* have demonstrated direct infection of neurons with *M.tb*, however, the effect on neuronal function and implications for intercellular interactions is not clear (Randall et al., 2014). Although not as prominent as microglia in their role, astrocytes have also been implicated with a study by Rock and colleagues demonstrating 15% of astrocytes having cell associated bacilli (average of 1.3 bacilli per cell) compared to 76% of microglia (average of 4.2 bacilli/cell) under the same conditions (Rock et al., 2005).

M.tb is recognized by microglial cells via innate immune and neuro-specific receptors, including pattern recognition receptors. The toll-like receptors (TLR), a family of ten pattern recognition molecules, play a crucial role in innate immunity. Internalisation of *M.tb* by human microglia is dependent on CD14, a monocyte differentiation antigen, which binds to lipopolysaccharide with TLR-4 (Wright et al., 1990). This was demonstrated in a study in which uptake of non-opsonized tubercle bacilli by microglia was reduced by 64% and 62% in the presence of anti-CD14 monoclonal antibodies and soluble CD14 ligand respectively. This is in contrast to peripheral mononuclear phagocytes where CD14 neutralising anti-CD14 antibodies did not affect bacillary

uptake (Shams et al., 2003), but interestingly the presence of CD14 led to upregulation of CD14 expression on these cells perhaps facilitating pathogenic immune responses. This receptor, along with the β2-integrin CD18 and TNF-α, is also involved in the formation of histologically characteristic multinucleated giant cells seen at autopsy and experimentally identified in porcine microglia infected with *Mycobacterium bovis* (Peterson et al., 1996).

Activation of microglia leads to secretion of a number of cytokines (Figure 1.3). In murine microglia the intracellular signalling pathways leading to cytokine release are *M.tb* inducible leading to a pro-inflammatory response through NAPDH oxidase-dependant reactive oxygen species (ROS) generation (Yang et al., 2007). Although cytokines play a critical role in the host defence to infection with *M.tb*, they can also mediate inflammation. Tumour Necrosis Factor (TNF) is central to the pathogenesis of central nervous system tuberculosis (Mastroianni et al., 1997, Tobin et al., 2012, Tsenova et al., 1999). It has a protective role in the immune response to mycobacteria (Kaplan and Freedman, 1996); but is also associated with pathology *in vivo* via induction of fever, activation of the hypothalamo-adrenal axis and by triggering the release of other cytokines (Hashimoto et al., 1991, de Vries et al., 1997, Ramilo et al., 1990). Local TNF-α production in the CNS also increases permeability of the BBB and thus influx of other immune mediators to the CNS (de Vries et al., 1997). In a murine CNS TB model, Tsenova *et al* demonstrated a correlation between levels of TNF-α and the extent of cerebral pathology as measured by CSF leukocytosis, protein accumulation, meningeal inflammation, persistence of bacillary load and clinical deterioration (Tsenova et al., 1999). TNF-α antagonists such as thalidomide (Tsenova et al., 1998) and analogues thereof (Tsenova et al., 2002), used in rabbit models of TBM, downregulated the production of TNF-α and subsequently improved survival. This finding was not replicated in human studies where a clinical trial of thalidomide

used in conjunction with standard antituberculous therapy and corticosteroids in children with TBM was stopped early due to adverse events associated with thalidomide use (Schoeman et al., 2004). Since then, there has been some suggestion of benefit with the use of thalidomide in the context of tuberculous mass lesions (van Toorn et al., 2015).

CSF concentrations of IL-6 independently associate with more severe presentation of TBM (Simmons et al., 2006). In this context it is unclear whether this is due to its pro-inflammatory or anti-inflammatory effects. In murine models of TB, IL-6 has been implicated in the stimulation of IFN-γ production but not necessarily essential for the protective immunity to *M.tb* (Saunders et al., 2000). It may also have an anti-inflammatory role by suppressing gene expression of pro-inflammatory cytokines (Xing et al., 1998). In a study by Rock *et al*, pro-inflammatory cytokines other than TNF-α, IFN-γ and IL-6 found to be secreted by microglia in response to TBM included IL-1β, CCL2, CCL5 and CXCL-10. In contrast to microglia, astrocytes produced only moderate amounts of CXCL10 (Rock et al., 2005). Other cytokines, confirmed experimentally, to be secreted by microglia following *M.tb* stimulation include: IL-1α, IL-10, IL-12p40, G-CSF, and GM-CSF (Curto et al., 2004, Yang et al., 2007).

Recent focus has turned to the pathogenic role of inflammatory mediators such as DAMPs (Damage Associated Molecular Patterns) and PAMPs (Pathogen Associated Molecular Patterns) (Chen et al., 2016), their validity as biomarkers of cerebral injury (Rohlwink et al., 2017), and as potential targets for novel host directed therapies in TBM (Berger et al., 2018). PAMPs are by-products released from pathogens that are recognised by host cell receptors subsequently leading to activation of the innate immune response. DAMPs, which are released by damaged host immune cells, interact with PAMPs leading to an accelerated cycle of cell death and injury. Host

poly(ADP-ribose) polymerase 1 (PARP1; also known as ARTD1) is an ADP-ribosylating enzyme essential for initiating various forms of DNA repair (Ko and Ren, 2012). Recent studies have suggested a role for PARP1 in the pathogenesis of TBM via its potential to modulate the release and activation of DAMPs. This includes high mobility group box-1 (HMGB1), a non-histone nuclear DNA binding protein expressed in all mammalian cells, and S100B, a protein synthesised by astrocytes, oligodendrocytes and Schwann cells known to be involved in cell-to-cell communication, cell growth, and intracellular signal transduction, as well as the development and maintenance of the CNS (Heizmann et al., 2002). These biomarkers of cerebral injury are known to increase in TBM (Rohlwink et al., 2017), and therefore suggest that PARP1 may be a potential new target in the development of host directed therapies (Mahon and Hafner, 2015). S100A8/9, also from the S100 family has a role in neutrophil chemoattraction and stimulation (Ryckman et al., 2003) and is implicated in the pathogenesis of tuberculosis in pulmonary disease. In HIV-1 uninfected patients raised S100A8/9 in serum correlated with increased radiographic disease severity (Pechkovsky et al., 2000, Gopal et al., 2013). In TBM Marais *et al* demonstrated that in patients with TBM and HIV infection, levels of S100A8/9 were significantly elevated two weeks after the initiation of antiretroviral therapy in those who developed Immune Reconstitution Inflammatory Syndrome (IRIS) defined as a paradoxical worsening of infection despite adequate treatment following the initiation of antiretroviral drugs, compared to those who did not. This observation may explain in part the ongoing paradoxical inflammation observed in IRIS (Marais et al., 2014).

In addition to the inflammatory milieu described above, there are several other factors implicated in the pathogenesis of TBM, in particular the subsequent increasing permeability of the BBB and influx of inflammatory mediators and cells (Figure 1.3). Vascular Endothelial Growth factor (VEGF) is a potent endothelial growth factor playing

diverse roles in vasculogenesis and angiogenesis. In tuberculosis it is now considered a useful biomarker of disease, where it may be used as an indicator of active *vs* latent disease activity or a marker of extrapulmonary *vs* primary lung disease. In several types of cancer, VEGF inhibitors such as Bevacizumab are well established as an effective therapeutic approach. In ischaemic conditions of the brain, VEGF has a pathological and protective role depending on pathogenic stage due to either effect on permeability of the microvasculature or subsequent reparative angiogenesis. In age related neurological conditions such as Alzheimer's disease, Parkinson's disease and motor neuron disease however, VEGF is thought to be pathogenic due to its effect on BBB dysfunction (Lange et al., 2016). In these conditions the effect of VEGF on increasing endothelial permeability is clear, however the mechanism by which this happens remains less understood, with possibilities including its effect on cell-cell junctions including tight junctions and adherens junctions, as well as on transcytosis (Ayata and Ropper, 2002). In TBM, VEGF disrupts the permeability of the BBB (van der Flier et al., 2004, Kim et al., 2008), which has been proposed as a mechanism by which dexamethasone exerts efficacy as a host directed therapy in TBM. Also interesting are its neuroprotective effects which have been more thoroughly explored in the context of amyotrophic lateral sclerosis where low VEGF has been reported with BBB dysfunction and the therapeutic use of a VEGF analogue (VEGF-A_{165}) is currently under investigation in a clinical trial (NCT02269436). The release of intercellular and vascular adhesion molecules (ICAM and VCAM) as well as matrix metalloproteinases (MMP) from inflammatory cells within the CNS have also been shown to increase the permeability of the BBB (Rai et al., 2014).

1.3.3 Metabolic factors in the host

Neuroendocrine-associated metabolic abnormalities are common in TBM. In an observational study of patients newly diagnosed with TBM, neuroendocrine dysfunction occurred in half (More et al., 2017). This is likely due to the tendency for TBM to affect the basal structures such as the interpeduncular fossa, cisterna ambiens and cisterna pontis, which surround the pituitary gland, pituitary stalk and hypothalamus (Figure 1.6). Exudates here lead to oedema, perivascular infiltration and subsequent microglial reactions known collectively as 'borderzone encephalitis'. Subsequent metabolic abnormalities include gonadotropin deficiency, hyperprolactinemia, thyrotropin deficiency, corticotropin deficiency and somatotropic hormone deficiency (More et al., 2017). Hyponatraemia is also common, discussed earlier within this chapter. Corticotropin deficiency may be modulated by treatment with dexamethasone; pituitary dysfunction and its role in the pathogenesis of TBM remains an area of interest, particularly given the observations of worsening outcomes and possible benefit of cortisol replacement.

Glucose and its metabolic effects are also of interest. In a pivotal study where adjunctive dexamethasone was found to decrease short term mortality in HIV-1 uninfected patients with TBM, low glucose at presentation predicted poor outcome (Thwaites et al., 2004). In more recent studies, including those in children, CSF glucose, lactate and protein levels have been linked to poor outcome (Bang et al., 2016, Simmons et al., 2006). If these metabolic markers are simply markers of disease activity as in most forms of neurological infection, then this finding is unsurprising. Mason *et al* demonstrated that the increase in lactate levels commonly observed in the CSF of patients who go on to develop poor outcomes is of the L-form and therefore solely a response of the host to infection, rather than being of microbial origin (Mason

et al., 2016). This finding contributes to our research group's hypothesis that in the context of neuroinflammatory-inducing infection, energy flow in brain metabolism is shifted away from the neurons and shunted towards the microglia. They theorise that this leads to lactic acid production by glycolysis in astrocytes, which subsequently participate in the activated immune response by contributing to oxidative phosphorylation and hence production of high levels of adenosine triphosphate (ATP) and forms of ROS required for degradation of the invading pathogen.

The metabolism of tryptophan is also of interest in TBM. Tryptophan is a key amino acid required for protein biosynthesis, and a precursor for various metabolites, including serotonin and melatonin (serotonin pathway) and kynurenine and quinolinic acid (kynurenine pathway) (Lesniak et al., 2013) (Figure 1.4). Pro-inflammatory cytokines such as IL-6, TNF-α and IFN-γ are known to trigger the kynurenine pathway by stimulating indoleamine 2, 3-dioxygenase (IDO) (Campbell et al., 2014, O'Connor et al., 2009). Once IDO is activated, the kynurenine pathway is promoted at the expense of the serotonin pathway. Microglia and astrocytes then secrete quinolinic acid and kynurenic acid, respectively. Both products have opposite roles, while kynurenic acid acts as an antagonist of the glutamate N-methyl-D-aspartate receptor (NMDAr), quinolinic acid acts as its agonist and leads to neurotoxicity (Campbell et al., 2014). Activation of the kynurenine pathway is also known to exacerbate progression of neurodegenerative diseases and has been described in HIV-associated dementia (Majlath et al., 2013).

Recently, Van Laarhoven *et al* conducted a metabolomic study examining serum and CSF of TBM patients. Their results showed that while most metabolites demonstrated elevated concentrations in TBM patients compared to controls, concentrations of tryptophan were low in TBM and further reduced in patients who survived. They further

demonstrated upregulation of the gene *IDO1* and identified 11 trait loci that correlated with tryptophan concentrations and were prognostic of survival when combined with sex and age. The prognostic potential of these genetic correlates was demonstrated in a validation cohort. These data suggest that tryptophan metabolism may play an important role in TBM outcome, and that further investigation into this metabolic pathway is warranted (van Laarhoven et al., 2018).

While *M.tb* may directly stimulate of IDO at the site of infection (Weiner et al., 2012), vitamin D on the other hand is an essential cofactor of the tryptophan hydrolase (TPH) and promotes serotonin production and thus neurotransmission. Furthermore, Vitamin D alone or bound to the vitamin D receptor (VDR) expressed on various cell types, including astrocytes and microglia, leads to a decrease in inflammatory response (Wobke et al., 2014). Thus tryptophan metabolism could be a modality by which the vitamin D status of an individual modulates both susceptibility to *M.tb* infection and TBM pathogenesis. By restoring a non-inflammatory environment and restricting *M.tb* replication, vitamin D could promote the serotonin pathway at the expense of the kynurenine one and protect the brain from neurotoxicity.

1.3.4 Host genetic factors

A number of immune response genes encoding the pathways described are under genetic influence. For instance, mutations and polymorphisms within the genes involved in the TLR pathway have been associated with susceptibility to infection in tuberculosis. A recent study of patients with TBM in Vietnam was the first to demonstrate an association between single nucleotide polymorphisms (SNP) in the TLR9 gene region and TBM (Graustein et al., 2015). Further, a Vietnamese study found an association between TLR2 SNP T597C and the development of TBM and millary TB, suggesting that TLR2 influences the dissemination of *M.tb* (Thuong et al.,

2007). Toll Interleukin 1 receptor domain containing adaptor protein (TIRAP) mediates signals from TLR1, -2, -4 and -6 to activate macrophages and dendritic cells. Hawn *et al* investigated the association of the TIRAP SNP C558T with i) susceptibility to TB (odds ratio, 2.25, $p < 0.001$) and ii) susceptibility to meningeal TB (OR, 3.02; $p < 0.001$) vs pulmonary TB (OR, 1.55; $p = 0.22$). They also demonstrated that compared to the to the 558CC genotype, the 558TT genotype was associated with decreased whole-blood interleukin-6 production, suggesting that TIRAP influences disease susceptibility by modulating the inflammatory response (Hawn et al., 2006).

Another gene of interest is leukotriene A4 hydrolase (LTA4H). LTA4H influences the balance of pro- and anti- inflammatory eicosanoids and subsequent TNF-α regulation through either reduced inflammation due to excess lipoxins or augmented inflammation due to excess leukotriene B4. In a study of zebrafish and humans, mutations in the gene encoding LTA4H led to immunosuppressive Lipoxin A4 (LXA4) accumulation and increased susceptibility to mycobacteria (Tobin et al., 2012). In this study, heterozygosity at several LTA4H SNPs was associated with protection against meningeal tuberculosis (TBM) (Tobin et al., 2012). These findings supported a hypothesis that excess LTA4H activity leads to a 'hyperinflammatory state', whereas lack of LTA4H activity leads to an inadequate host response (Tobin et al., 2010). In a later study by the same group the LTA4H promoter region SNP rs17525495, defining 3 genotypes—TT, CT, and CC— was identified as a likely molecular determinant of genetic susceptibility. In this study lymphoblastoid cell lines with SNP genotype CC conferred a hypoinflammatory and TT a hyperinflammatory phenotype (Tobin et al., 2012). It was also demonstrated that i) genotype correlated with pre-treatment CSF leukocytosis and survival and ii) those benefiting from adjunctive dexamethasone were carriers of the 'hyperinflammatory' TT genotype (Tobin et al., 2012). Several follow-up studies to further investigate the relevance of this finding have tended to reproduce an

association with susceptibility to disease, but not necessarily outcome (Yang et al., 2014a, Dunstan et al., 2015, van Laarhoven et al., 2017, Thuong et al., 2017). The two largest of these both published in 2017 investigated the association in Vietnamese (Thuong et al., 2017) and Indonesian (van Laarhoven et al., 2017) populations. The first showed that individuals with the LTA4H CC genotype had a higher risk of early death, whereas the second did not find an association between genotype and mortality. Possible explanations may include differences in linkage disequilibrium as well as the observed overall differences in patient characteristics such as mortality (40.7% in Indonesia vs 18.9% in Vietnam), frequency of culture confirmed diagnosis (55.3% vs 42.8% in Vietnam), severity of disease (BMC grade I severity in Vietnam 37% vs 11% in Indonesia) and median age of patients (41 years vs 29 years in Vietnam) (reviewed in (Fava and Schurr, 2017)). Nonetheless this remains an area of great interest and a clinical trial is under way to determine whether LTA4H genotype, defined at randomisation, influences dexamethasone's clinical efficacy when added to the first 6-8 weeks of anti-tuberculosis treatment of TBM (NCT03100786).

There are a number of other polymorphisms documented in the literature that relate to the pathogenesis of TBM. Polymorphisms in CD43 encoding a surface glycoprotein involved in *M.tb* adhesion and pro-inflammatory cytokine induction has been associated with decreased survival from TBM (Campo et al., 2015). The mannose binding protein (MBP) binds mycobacteria and acts as an opsonin *in vitro*. Although MBP plays a role in the first line of defence against *M.tb*, it may also facilitate the spread of the intracellular pathogen (Hoppe et al., 1997). A hypothesis was therefore advanced that phenotypes in which levels of MBP are low may result in protection from TBM. In a study to test this, a mutation in the MBP B allele (G54D), which leads to disruption of the collagen region of the MBP protein, was found to be associated with reduced dissemination to the brain and suggested protection against TBM (Hoal-Van

Helden et al., 1999). Polymorphism in the PKP3-SIGIRR-TMEM16J gene region encoding a negative regulator of toll-like receptor/IL-1R signaling has been associated with reduced survival in both pulmonary TB (PTB) and TBM (Horne et al., 2012). Lastly, Vitamin D Receptor gene polymorphisms with heterozygous (TC) and mutant (CC) genotypes of Taq1 VDR SNP associate with TBM (Rizvi et al., 2016), although further research is required to investigate whether this is universal across all populations (Areeshi et al., 2017).

1.3.5 Pathogen virulence factors and their effect on pathogenesis

M.tb was formerly regarded as an organism exhibiting little genetic variation. More recent studies using genetic typing techniques to analyse *M.tb* isolates from diverse geographic populations have revealed a cladal phylogeographic distribution with variation between different lineages (Gagneux and Small, 2007, Gagneux et al., 2006). Seven lineages are now identified classified as “ancient” (lineages 1, 5, 6 and 7) or “modern” (2, 3 and 4) (Comas et al., 2013). One lineage of particular interest has been lineage 2, which is highly prevalent in East Asia (and therefore known as ‘Beijing’) and has been ascribed hypervirulence in a rabbit model (Tsenova et al., 2005) as well as some human studies demonstrating increased risk of disseminated disease (Kong et al., 2007). This has been attributed to an intact polyketide synthase (*pks 15/1*) gene and the production of a phenolic glycoprotein (PGL), which is thought to attenuate the early host immune response leading to a reduced production of pro-inflammatory cytokines (Dormans et al., 2004). A later study by Caws *et al* comparing bacterial and host genotype across two groups of Vietnamese adults with pulmonary or meningeal TB found that disease caused by the Euro-American lineage 4 was significantly more likely in pulmonary disease, however, by contrast found no association between the lineage 2 and disease phenotype (Caws et al., 2008).

Epidemiological studies have reported several differences in disease phenotype between *M.tb* lineages in terms of pathogen virulence (Guerra-Assuncao et al., 2015b, Reed et al., 2004), transmission of disease (Coscolla and Gagneux, 2014, Gagneux et al., 2006, Guerra-Assuncao et al., 2015a), progression from latent to active disease (de Jong et al., 2008) and in response to treatment (van Crevel et al., 2001, Parwati et al., 2010). *In vitro* studies have explored whether the host immune response is specific to genotype. A study in South Africa found differential mycobacterial growth and levels of early pro-inflammatory cytokines including TNF and IL-12p40 between three modern lineages (Sarkar et al., 2012). Others have found differences in human macrophage responses between lineages and have hypothesised that the lack of early pro-inflammatory response observed with modern lineages may contribute to more rapid disease progression and transmission and therefore confers survival advantage for these strains of *M.tb* (Portevin et al., 2011). In Madagascar differences between ancient and modern lineages were characterised by contrasting IFN-γ responses (Rakotosamimanana et al., 2010). Specifically, comparison of the IFN-γ responses with the spoligotype of the infecting clinical strains showed that modern *M.tb* strains tended to induce lower IFN-gamma responses than ancient strains in index cases and their household contacts (Rakotosamimanana et al., 2010). The aforementioned study by Caws *et al* was the first to demonstrate an interaction between pathogen and host genetic factors as a predictor of disease phenotype by showing that individuals with the C allele of the TLR02-T597C genotype were more likely to have tuberculosis caused by the Beijing genotype (OR 1.57; 95% CI 1.15 – 2.15) (Caws et al., 2008). The most recent study in this field performed whole genome sequencing of *M.tb* strains from 322 HIV-1 uninfected patients with TBM (n=106) and PTB (n=216). Unlike the previous studies (Caws et al., 2008) (Kong et al., 2007) there was no association with disease phenotype and lineage, however using a homoplasy based association analysis they

identified three *M.tb* genes associated with disease phenotype. This included Rv0218, a secretome gene encoding a protein that influences pathogen recognition and host-pathogen interaction. They hypothesise that a SNP in the region of Rv0218 would alter the appearance of the surface of *M.tb* therefore allowing it to evade recognition and the immune response, enabling dissemination to extrapulmonary sites.

1.3.6 Differences in the intracerebral immune response in HIV

There is a relative paucity of data directly comparing the intracerebral immune response to TBM in HIV-1 infected and uninfected hosts. The impact of HIV-1 infection on the clinical presentation of TBM is debated, some studies report that HIV-1 infection does not alter the cerebral features of TBM (Katrak et al., 2000, Karande et al., 2005), while others suggest a higher rate of extra-meningeal and extra-pulmonary disease associated with HIV-1 infection (Thwaites et al., 2005, Karstaedt et al., 1998, Azuaje et al., 2006). These studies consistently report that HIV-1 infection associates with death (Katrak et al., 2000) and decreased efficacy of treatment against *M.tb* (Karstaedt et al., 1998). A better characterisation of the effect of HIV-1 in the pathogenesis of tuberculous meningitis is important.

A study conducted in Vietnam demonstrated a small reduction in IFN-γ yet more significant reduction in the anti-inflammatory cytokine IL-10 in HIV-1 infected patients, the latter being 6-fold decreased in the context of HIV. This skewed the CSF IFN-γ: IL-10 ratio towards excess IFN-γ with subsequent analysis that was independently associated with HIV co-infection (odds ratio (95% CI), 2.47 (1.25 – 4.88), *p=0.009*).

Torok *et al* (Torok et al., 2008) described a higher percentage of CSF neutrophils and suggested this may be due to prior HIV-1 infection. Indeed, this elevated percentage of

CSF neutrophils in HIV-1 infected patients may be caused by the microglial response to HIV-1 infection and HIV-1 proteins gp120, Tat (Trans-activator of transcription), Nef (Negative regulatory factor) and Vpr (Viral protein R). Atluri *et al* demonstrated that these four proteins disrupt the BBB integrity by triggering the decline of tight proteins expression on brain microvascular endothelial cells, MMP expression and apoptosis, and leading to infiltration of leukocytes (Atluri et al., 2015). Moreover, it has been reported that microglia *in vitro* secrete numerous pro-inflammatory cytokines and chemokines when infected by HIV-1, including the pro-inflammatory neutrophil chemotactic factor IL-8 (Rock et al., 2005). Those cells also secrete TNF, a cytokine implicated in BBB permeability and whose role in pathogenesis of *M.tb* is critical, indicating the influence of HIV-1 in the promotion of TBM and *M.tb* spread in the CNS.

Further insight into the mechanisms underlying immunopathogenesis of TBM have been observed through the study of paradoxical immune reactions. A paradoxical reaction in tuberculosis is characterised by worsening of pre-existing tuberculous lesions, or the appearance of new tuberculous lesions despite effective anti-tuberculosis treatment in patients who have demonstrated initial improvement to therapy. In HIV-1 co-infection, paradoxical worsening often occurs following the introduction of antiretroviral therapy (ART): a phenomenon known as immune reconstitution inflammatory syndrome (IRIS). Although initiation of ART during treatment of TBM is associated with a reduced six month mortality (HR 0.3, 95% CI=0.08–0.82) (Marais et al., 2011) IRIS in this context carries a poor prognosis (Marais et al., 2013). The role of inflammasomes, immune complexes of receptors and sensors that mediate innate immune responses and lead to inflammation, have been described in this setting (Lai et al., 2015, Tan et al., 2015, Tan et al., 2016).

In TBM specifically, IRIS is characterised by high CSF neutrophil counts and *M.tb* positivity at presentation (Marais et al., 2013). In those who develop IRIS, CSF TNF was found to be high whereas CNF IFNγ was low (Marais et al., 2013). Further analysis of predictive factors has demonstrated the role of neutrophil mediators, in particular S100A8/9, which two weeks after the initiation of ART was found to be significantly higher in the CSF of those who develop TBM-IRIS compared to those who do not(Marais et al., 2014). Using longitudinal microarray analysis of blood from patients with HIV and TBM, Marais *et al* have also demonstrated that neutrophil abundant transcripts were significantly more copious in those who develop IRIS from before ART initiation until the onset of TBM-IRIS (Marais et al., 2017). After initiation of ART a significantly higher number of transcripts associated with canonical and non-canonical inflammasome activation were found in patients who went on to develop IRIS than in those who did not (Marais et al., 2017). These observations together with the finding that inflammasome activation can contribute to pyroptosis (i.e. cell death triggered by proinflammatory signals and associated with inflammation_(Bergsbaken et al., 2009) suggest that tissue injury observed in TBM may be partly induced by inflammasome-mediated pyroptosis. It is unclear as to whether similar mechanisms exist in the absence of HIV.

HIV-1 infiltrates the CNS soon after systemic infection and around half of HIV-1 infected people develop HIV-associated neurocognitive disorders (Tozzi et al., 2005). It has been reported that IRIS may worsen established HIV related cognitive impairment (Miller et al., 2004). Little is known of the pathological mechanisms which lead to worsening cognition in the context of IRIS but some have postulated a role of the HIV-1 Trans-Activator of Transcription (Tat) protein (Johnson et al., 2013). They described the presence of Tat in the CSF of the virologically controlled ART-treated population

and proposed that Tat would contribute to neuroinflammation and IL-17 production by infiltrating Th17 cells. Given that an increase of IL-17 secretion predisposes to IRIS in HIV-1 infected individuals in the context of cryptococcal meningitis (Boulware et al., 2010), it is possible that Tat is a key player in the pathogenesis of HIV related cognitive impairment in the context of IRIS.

1.3.7 Macroscopic manifestations of the disease in relation to the immune response

The characteristic feature of TBM in post-mortem studies is the presence of a thick, gelatinous inflammatory exudate in the basal cisterns and subarachnoid spaces of the brain (Figure 1.5), which may extend into the spinal canal (Dastur et al., 1995, Daniel, 1949, Shinoyama et al., 2012). The predominantly basal location has important implications; firstly, the major cerebral vessels originating from the base of the brain become encased with exudate; secondly, exudate blocks the circulation of CSF; and thirdly, it envelopes and compresses the local cranial nerves resulting in cranial nerve palsies (Dastur et al., 1995).

Vasculitis

As the exudate spreads along the cisternal extensions from the base of the brain it coats the large arteries and their small perforators, although it has a predilection for extension into the Sylvian fissures. Therefore, the middle cerebral artery (MCA) and its perforators around the floor of the 3rd ventricle are most commonly involved, resulting in ischemic damage to the sub-thalamic nuclei and lower internal capsule (Dastur et al., 1995) (Figure 1.7). Involvement of small vessels supplying the mid-brain may lead to infarction (Daniel, 1949) and exudate in the interpeduncular cistern can compromise the vessels supplying the hypothalamic region and precipitate hypothalamic symptoms

in these patients (Daniel, 1949). Vascular pathology includes infiltration of inflammation from the adventitia of arteries and veins inwards, resulting in peri- or pan-arteritis that involves segments or the full thickness of the vascular wall tissue (Lammie et al., 2009). Evolution of vascular inflammation may involve thickening of the vessel intima resulting in vessel stenosis or occlusion (Lammie et al., 2009). Both cerebral and spinal vessels may be affected by any or all of these processes and this may reflect the duration of illness (Lammie et al., 2009). Infarcts (Figure 1.5) and changes on angiography are reported in the majority of TBM patients (Rohlwink et al., 2016) and are associated with post-mortem cerebral and vascular pathology (Dastur et al., 1995).

Hydrocephalus

Extension of exudative material into the basal cisterns results in a collar of exudate around the midbrain and a blockage in CSF flow around the upper brain stem. Exudate may also collect around the cerebral aqueduct of the 3rd and 4th ventricles hindering the flow of CSF through the ventricular system (Figure 1.5). Consequently, hydrocephalus is common, occurring in 75-80% of TBM patients (Rohlwink et al., 2016, van Well et al., 2009, Thwaites et al., 2007). The pressure of expanding ventricles and the opposing pressure of brain oedema due to pathological processes can negatively impact the grey and white matter, leading to pallor and diffuse loss of myelin (Dastur et al., 1995), and the raised intracranial pressure can severely compromise cerebral blood flow.

Tuberculomas and Tuberculous brain abscess

Tuberculomas may coexist with TBM or occur independently and are thought to originate from expanding tubercles in the parenchyma (Figure 1.5). These lesions are typically granulomatous consisting of a necrotic centre surrounded by epithelioid cells (which may merge to form Langhans giant cells), lymphocytes and a border of astrocytes, and are associated with peripheral oedema and vascular proliferation (Rock

et al., 2008, Dastur et al., 1995). With time the tubercles become discretely organised and bound by a rim of connective tissue of reticulin fibres, which enhances on contrasted brain imaging. They may occur throughout the brain in the cerebrum, cerebellum and brainstem (Dastur et al., 1995, Rohlwink et al., 2016).

TB brain abscesses may follow TBM or develop independently (Schoeman et al., 2006, Kumar R, 2004), however, they occur infrequently (Kumar et al., 2002). Abscesses manifest as encapsulated collections of pus that contain tubercular bacilli in the absence of typical TB granuloma features including epithelioid and giant cells and mononuclear cell infiltrates (Kumar et al., 2002). These abscesses have been reported in the cerebrum(Schoeman et al., 2006, Kumar et al., 2002) and posterior fossa (Schoeman et al., 2006, Schoeman et al., 1998, Saini et al., 2011), occur infrequently in the brainstem (Kumar R, 2004), and may be singular or multiple (Rock et al., 2008).

Spinal TB

Spinal TB may develop as a primary tuberculous lesion, from the downward extension of intracranial TBM, or secondary to vertebral TB (Dastur et al., 1995, Moghtaderi and Alavi Naini, 2003, Hernandez Pando et al., 2010). It involves the cord, meninges and nerve roots and manifests as spinal arachnoiditis, intradural (extramedullary) tuberculomas or rarely as intramedullary tuberculomas (Rohlwink et al., 2016, Srivastava and Kochar, 2003, Skendros et al., 2003, Lim et al., 2013). Spinal nerve roots may become entrapped and matted in exudate filling the subarachnoid space, and complete obliteration of the thecal space can occur in severe cases (Rohlwink et al., 2016). Exudate involvement of the lower lumbar segments is associated with difficulty in performing lumbar punctures and a high CSF protein (Dastur et al., 1995, Rohlwink et al., 2016). The microscopic appearance of spinal exudate is identical to intracranial exudate, consisting of giant cells and caseous areas, and causing vasculitis

of the spinal veins and arteries, which may lead to cord ischemia and infarction (Dastur et al., 1995, Kato et al., 1997).

1.3.8 Research gaps and the path forward

Intracerebral and spinal pathology in TBM is mediated by a dysregulated inflammatory response that contributes to meningitis, tuberculoma formation, arteritis, obstruction of cerebrospinal fluid (CSF) flow, and vascular complications including stroke. The development of animal or *in vitro* cellular models could aid understanding of the underlying immune mechanisms and point the way to adjunctive anti-inflammatory and improved antibiotic therapies. In human studies high-resolution MRI or CT imaging to assess TBM disease activity could provide a more detailed clinical phenotype for TBM than is currently available. Unbiased application of 'omics' technologies (principally transcriptomics); in particular, analysis of cells and fluid arising from the site of disease is likely to be particularly valuable. Genes encoding proteins involved in metabolism, cell growth, transport, immune response, cell communication and signal transduction are particularly of interest. Longitudinal sampling of blood or CSF during observational studies or randomized controlled trials is highly informative regarding the dynamics of the disease, such as disease progression and drug response.

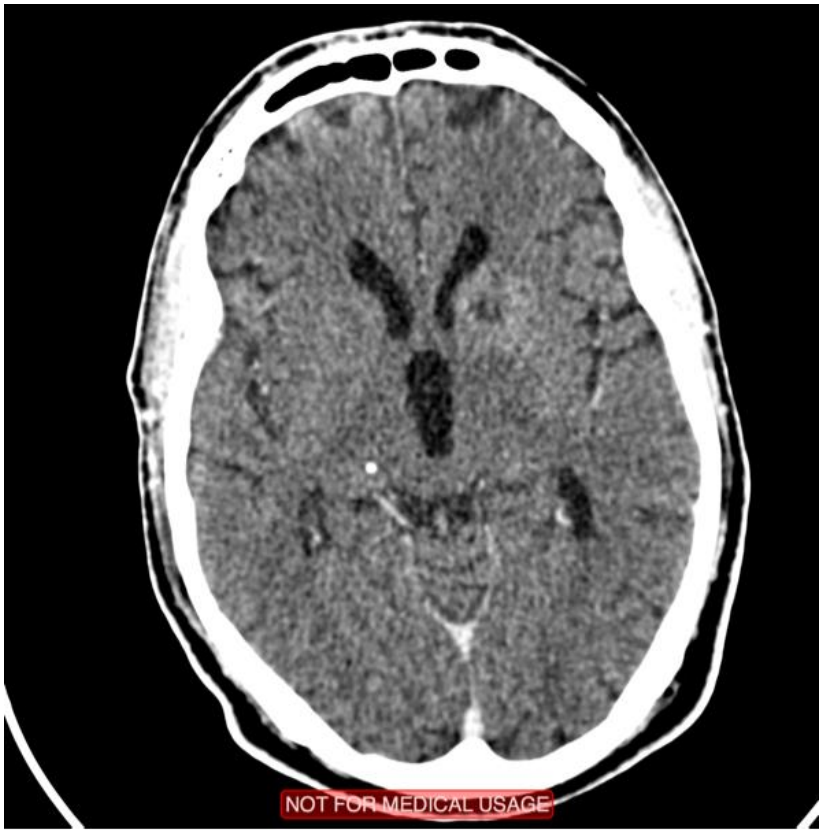
Table 1.1 Currently available antibiotics for treatment of TBM

Drug	WHO recommended daily dose	WHO recommended duration	CSF penetrance (CSF:plasma concentration)
First-line drugs for treatment of drug sensitive TBM in adults			
Rifampicin	10 mg/kg (range 8–12 mg/kg); max 600 mg	12 months	10–20%
Isoniazid	5 mg/kg (range 4–6 mg/kg); max 300 mg	12 months	80–90%
Pyrazinamide	25 mg/kg (range 20–30 mg/kg)	2 months	90–100%
Ethambutol	15 mg/kg (range 15–20 mg/kg)	2 months	20–30%
Second-line drugs for treatment of TBM in adults			
Levofloxacin	10–15 mg/kg	Throughout treatment	70–80%
Moxifloxacin	400 mg	Throughout treatment	70–80%
Amikacin	15 mg/kg; max 1g. IV or IM.	Intensive phase only	10–20%
Kanamycin	15 mg/kg; max 1g. IV or IM.	Intensive phase only	10–20%
Capreomycin	15 mg/kg; max 1g. IV or IM.	Intensive phase only	No data (probably very low)
Ethionamide or Prothionamide	15–20 mg/kg; max 1 g.	Throughout treatment	80–90%
Cycloserine	10–15 mg/kg; max 1 g	Throughout treatment	80–90%
Linezolid	600 mg	Throughout treatment	30–70%
Other drugs used in treatment of multi-drug resistant TB but of uncertain benefit in TBM			
Clofazimine	1 mg/kg	No recommended duration	Limited data (probably low)
p-Aminosalicylic acid	200–300 mg/kg	No recommended duration	No data (probably very low)
Bedaquiline	Not determined	New drug. Limited availability.	More recent study demonstrating equivalent concentrations to plasma
Delamanid	Not determined	New drug. Limited availability.	Animal data only, however this suggests adequate penetration to the CSF

Table 1.2 Clinical rating scores used within surgical management of TBM

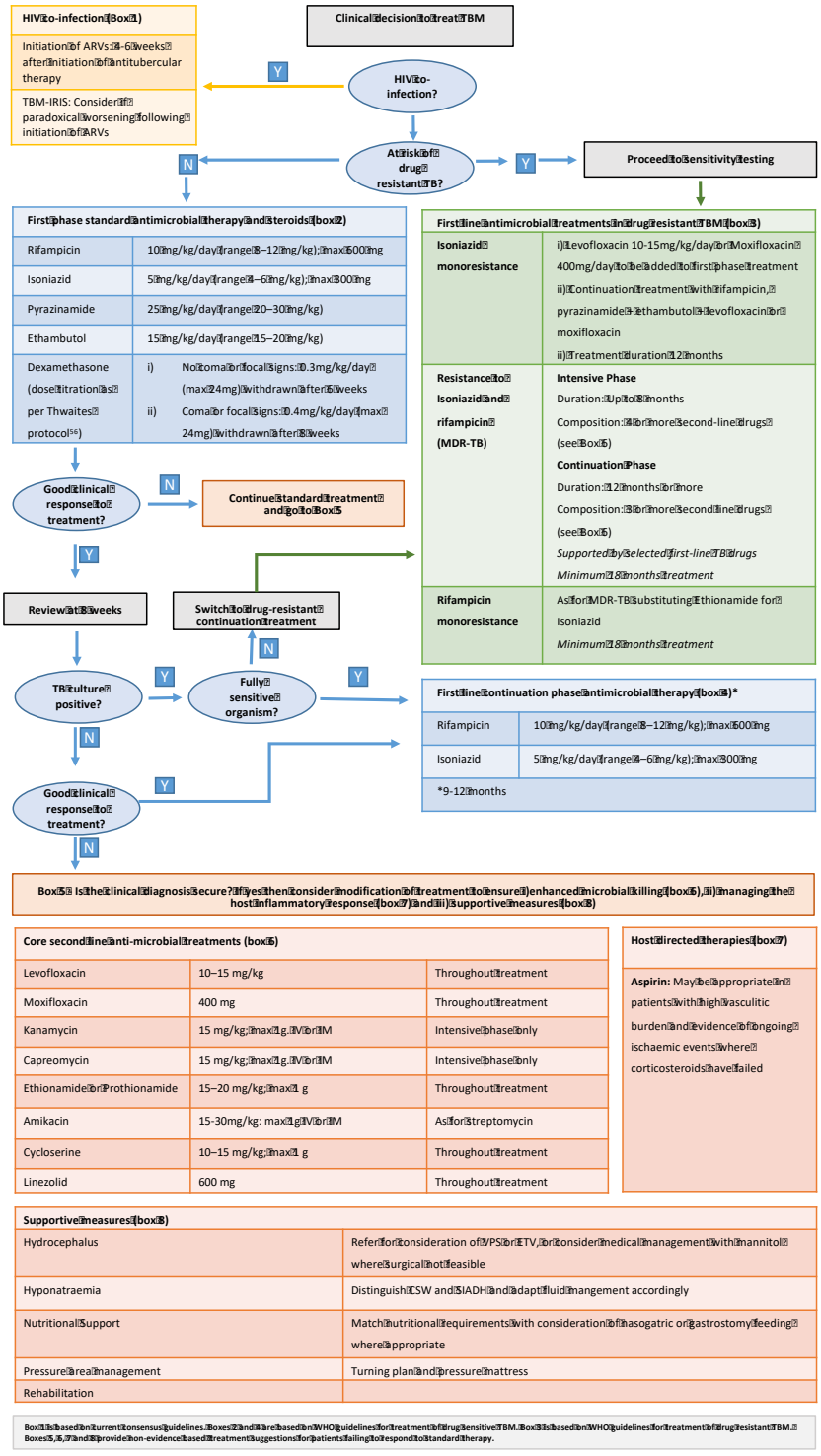
Glasgow outcome scale[97]		
1	Death	Severe injury or death without recovery of consciousness
2	Persistent vegetative state	Severe damage with prolonged state of unresponsiveness and a lack of higher mental functions
3	Severe disability	Severe injury with permanent need for help with daily living
4	Moderate disability	No need for assistance in everyday life, employment is possible but may require special equipment
5	Low disability	Light damage with minor neurological and psychological deficits
Modified vellore grading scale for TBM-induced hydrocephalus[98]		
Grade	Glasgow Coma Scale	Clinical features
I	15	Headache, vomiting +/- neck stiffness No neurological deficit
II	15	Neurological deficit present
III	9-14	Neurological deficit may or may not be present
IV	3-8	Neurological deficit may or may not be present

Figure 1.1 Computerised tomography of the head in a patient with TBM



Evidence of left MCA territory infarct within the ‘tubercular zone’ likely due to compromise of lenticulostriate perforator arteries

Figure 1.2 Treatment algorithm for the management of TBM



Abbreviations: ART; antiretroviral therapy: VPS; ventriculoperitoneal shunt: ETV; endoscopic third ventriculostomy: CSW; cerebral salt wasting: SIADH; syndrome of inappropriate antidiuretic hormone release

Figure 1.3 Summary of the pathogenesis of TBM

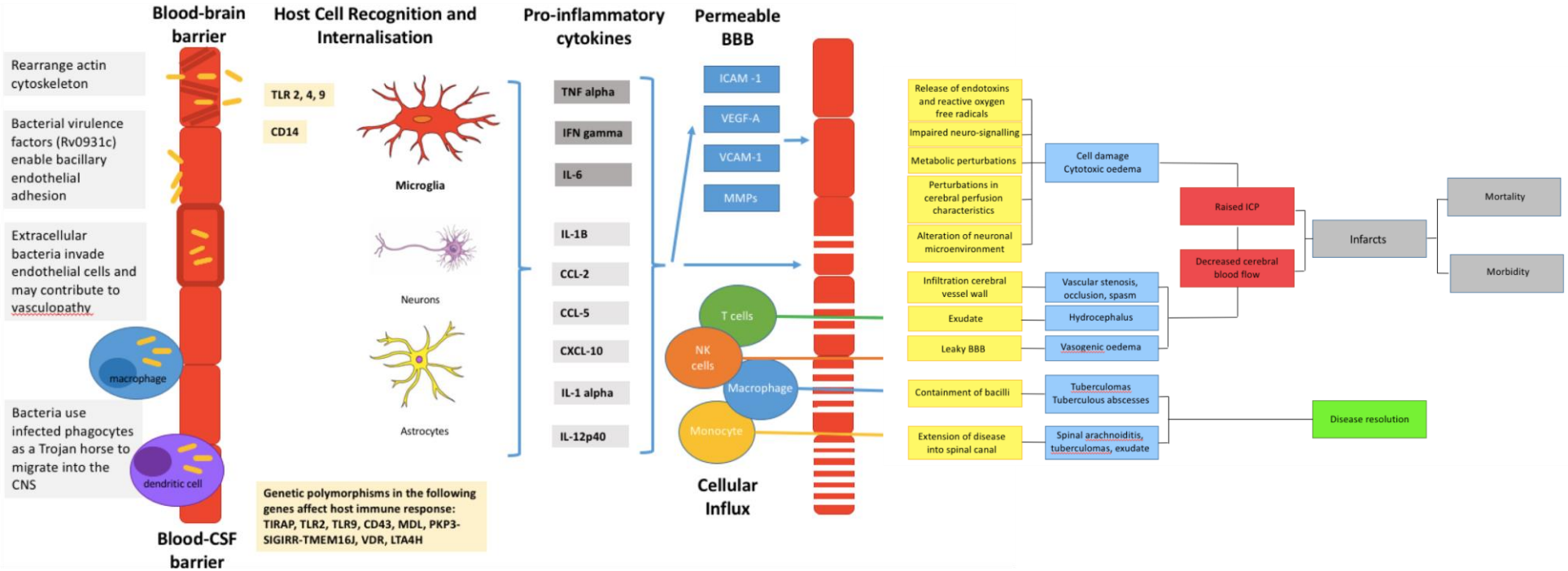
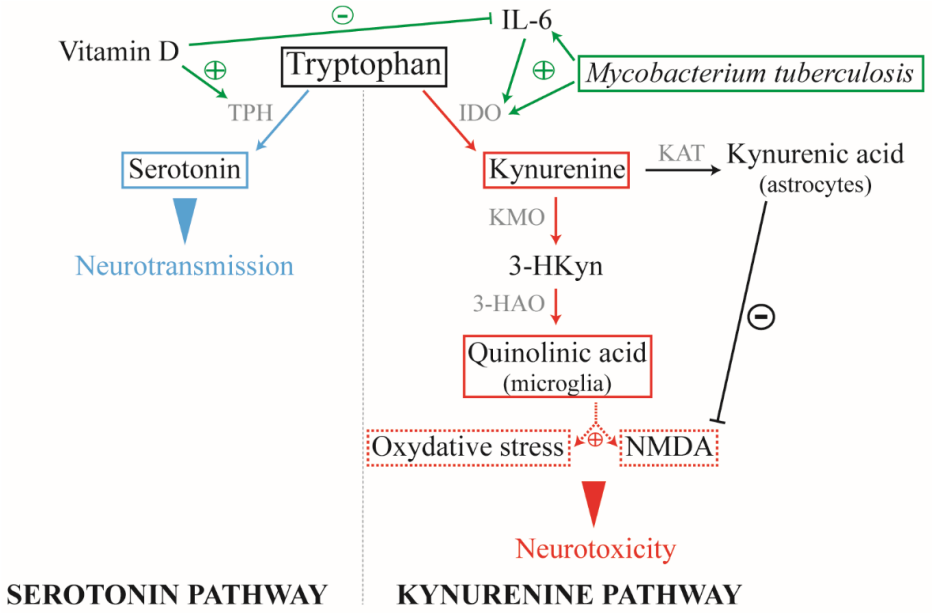


Figure 1.3 description: A: Mycobacterium tuberculosis bacilli (M.tb) disseminate from the primary site of infection in the lung to seed the brain. The bacilli are able to traverse the blood brain barrier (BBB) and blood cerebrospinal fluid barrier (BCSFB) through various virulence factors that enable the invasion of and migration through cerebral vascular endothelial cells, or are carried into the central nervous system (CNS) by infected peripheral innate immune cells. B: In the CNS antigen recognition and internalisation by microglia, neurons and astrocytes occurs, mediated by numerous host genetic factors. C: The resulting immune response stimulates the release of pro-inflammatory cytokines and chemokines and other immune mediators that contribute to the breakdown of the BBB and the influx of

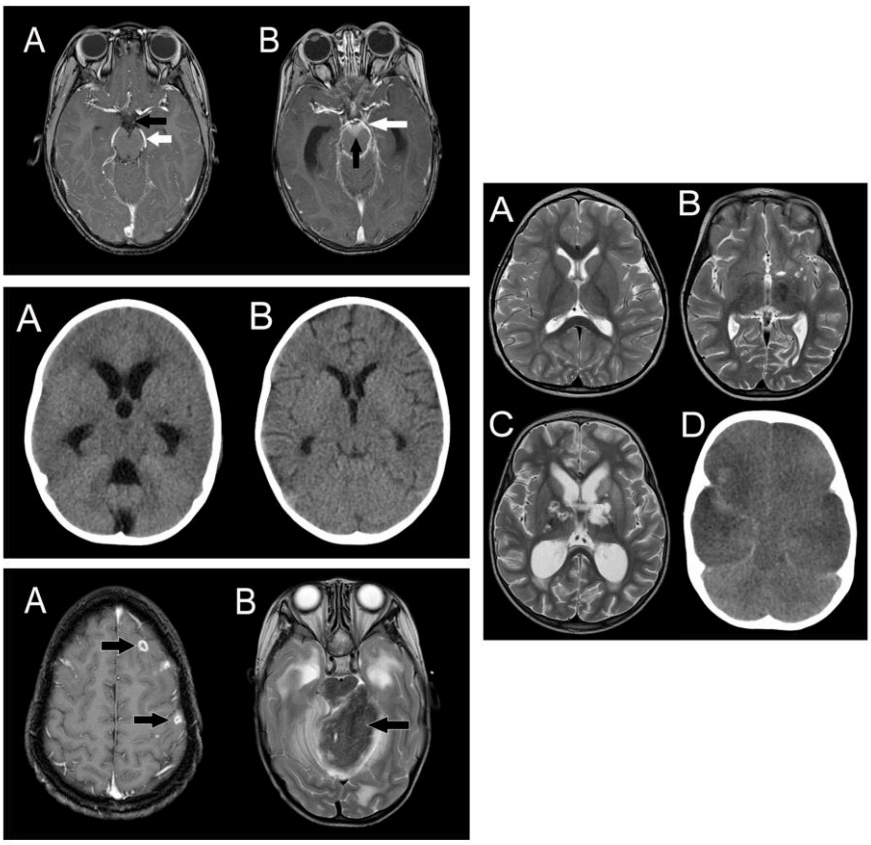
innate and adaptive immune cells from the periphery. D: A prolific inflammatory response ensues. The inflammatory exudate in the basal cisterns contributes to cerebral vascular pathology and the development of hydrocephalus and raised intracranial pressure. Vasogenic oedema due to an influx of proteins through the leaky BBB, and cytotoxic oedema as a result of cellular damage contribute to the raised pressure. The overall decrease in cerebral blood flow puts the brain at risk of ischaemia, infarction and poor patient outcomes. In some cases the infection is controlled in discrete tuberculomas or abscesses, which resolve with treatment and time. Extension of the disease into the spinal canal manifests as spinal arachnoiditis, tuberculomas, or collection of exudate.

Figure 1.4 *M.tb* and vitamin D impact on tryptophan metabolism



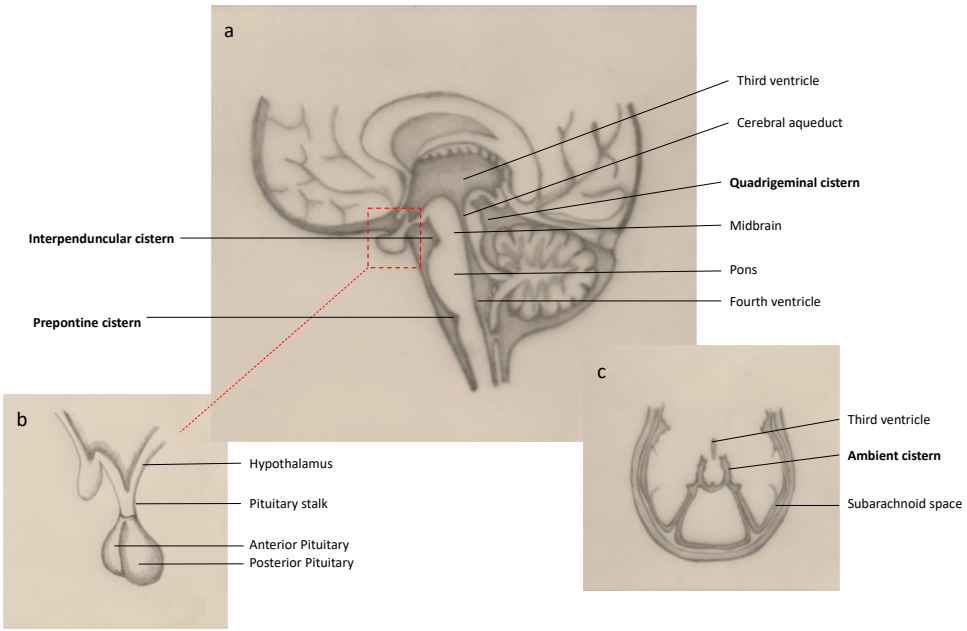
M.tb infection activates the inflammatory response resulting in pro-inflammatory cytokine secretion. Along with *M.tb*, these cytokines can trigger the kynurenine pathway by stimulating IDO. Once the pathway is activated, astrocytes and microglia respectively produce kynurenic acid and quinolinic acid, the latter is neurotoxic. Thus, an imbalance in the synthesis of these two products may result in neurotoxicity. Vitamin D, cofactor of TPH, promotes serotonin production and neurotransmission. Alone or when bound with one of its receptor, VDR, vitamin D attenuates the inflammatory response. *M.tb*: *Mycobacterium tuberculosis*; IDO: indoleamine 2, 3-dioxygenase; TPH: tryptophan hydrolase; KAT: kynurenine aminotransferase; KMO: kynurenine-3-monooxygenase; 3-HAO: 3-hydroxyanthranilic acid dioxygenase; NMDA: *N*-methyl-d-aspartate; VDR: vitamin D receptor. Blue: neuroprotection; red: neurotoxicity; green: influential factors.

Figure 1.5 Radiological features of TBM



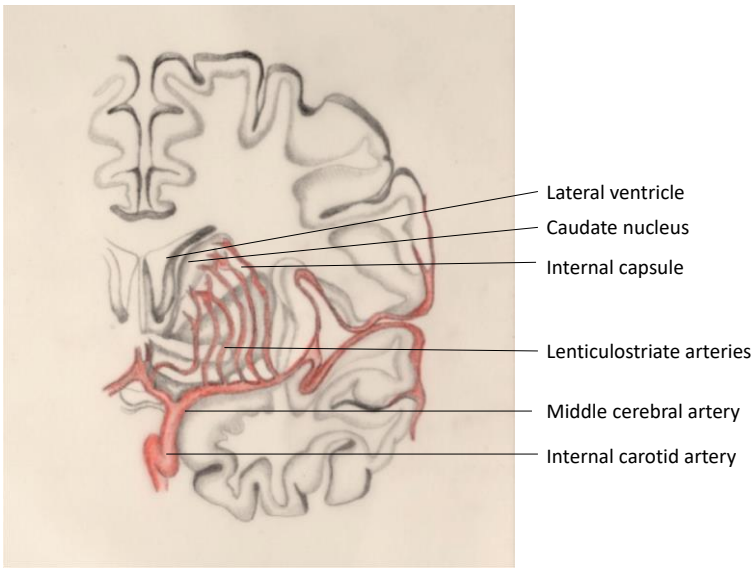
Top left: Exudate Contrast-enhanced T1-weighted MRI scan images: A: Normal scan showing cerebrospinal fluid in the cisterns (interpeduncular cistern in front of the midbrain, black arrow) and vessels at the base of the brain in normal cisterns (posterior cerebral artery, white arrow); B: Scan of a patient with TBM showing exudate in the basal cisterns of the brain (interpeduncular cistern, anterior to the brainstem and beneath the hypothalamus, black arrow) and vessels coursing through the exudate in the cisterns (posterior cerebral artery, white arrow).
Middle left: Hydrocephalus A: Initial head CT scan images of a patient with TBM showing acute hydrocephalus with dilated ventricles and a compressed brain; B: Head CT scan of the same patient after 3 weeks of medical therapy showing resolution of the hydrocephalus.
Lower left: Tuberculomas MRI scans demonstrating different patterns and imaging characteristics of brain tuberculomas: A: Contrast-enhanced T1-weighted MRI scan showing multiple ring-enhancing small tuberculomas (arrowed); B: T2-weighted MRI scan showing a large tuberculoma in the cerebellum compressing the brainstem, surrounding oedema, and hydrocephalus from obstruction of the cerebral aqueduct.
Lower Right: Infarcts Patterns of infarction in in TBM, A: T2-weighted MRI scan showing normal ventricular size and no infarcts; B: T2-weighted axial MRI scan of a patient with TBM showing discrete unilateral small perforator vessel infarcts; C: T2-weighted MRI scan showing more extensive infarcts in the thalami and basal ganglia; D: Head CT scan of a patient with TBM showing global infarction with hypodense hemispheres bilaterally and a swollen brain.

Figure 1.6 Basal cisterns and pituitary anatomy



A: Basal cisterns affected in TBM are represented here in a sagittal view of the brain. Note the quadrigeminal cistern which extends laterally to become a thin sheet like cistern surrounding the midbrain and posterior thalamus, named the ambient cistern shown in **C**. **B**: Anatomy of the pituitary gland and surrounding structures.

Figure 1.7 Lenticulostriate arteries



Lenticulostriate arteries branching from the M1 segment of the middle cerebral artery supply the basal ganglia and internal capsule.

Chapter 2. Study protocol for A phase 2A trial of the safety and tolerability of increased dose rifampicin and adjunctive linezolid, with or without aspirin, for HIV-associated tuberculous meningitis (The LASER-TBM trial)

2.1 Background

In Africa, HIV-1 associated tuberculous meningitis (TBM) has a 2-month mortality approaching 50% (Marais et al., 2011). Although early antiretroviral therapy (ART) is of proven benefit in other forms of tuberculosis (TB) (Blanc et al., 2011) this has not been demonstrated for TBM (Torok et al., 2011b), a finding potentially contributed to by exacerbated immunopathology in the confined space of the central nervous system (CNS) (Marais et al., 2013). Furthermore, only a few clinical trials have addressed the recognized poor penetration of several antibiotics into cerebrospinal fluid (CSF) and adjunctive corticosteroids have not shown unequivocal benefit for HIV-1 co-infected patients in clinical trials (Thwaites et al., 2004). The recommended management of patients with TBM has remained unchanged for decades. The need to develop an effective drug treatment regimen combining agents to ensure effective bacterial killing, as well as therapies to control the host immune response is urgent.

Linezolid

Linezolid (LZD) is currently used as an effective rescue therapy in extensively drug resistant TB (Sotgiu et al., 2012, Schecter et al., 2010, Migliori et al., 2009, Anger et

al., 2010, Lee et al., 2012b, Singla et al., 2012). Its use is also established for the treatment of gram positive infections including pyogenic brain abscesses where patients receive 1200mg for four weeks (Nau et al., 2010). LZD is an attractive agent for the treatment of TBM due to its potent efficacy against *Mycobacterium tuberculosis (M.tb)* as well as its excellent CNS penetration (Nau et al., 2010). Two published studies have investigated its potential role in TBM; the first, an observational study demonstrated favorable clinical outcomes and a non-significant difference in adverse events in children with drug sensitive TBM treated with LZD compared to control (Li et al., 2016); the second, a retrospective cohort study of 33 adults with TBM the addition of LZD to a standard regimen led to more rapid improvement in CSF parameters, recovery of consciousness and reduction of fever (Sun et al., 2014).

LZD toxicity has however limited its use. The most common adverse events (AE) associated with LZD use in TB treatment are haematological toxicity (mainly dose-related) and peripheral neuropathy (mainly duration-dependent) (Zhang et al., 2015). These are usually mild and are reversible with dose reduction or treatment interruption if identified early. In a systematic review, AE related to LZD use at doses > 600 mg/day occurred at a median of 252 days (IQR 120 – 540) of LZD exposure (Ahuja et al.). In the *NiX-TB* trial where LZD was given at a total dose of 1200mg per day for 6 months, peripheral neuropathy occurred in 81% of cases with the majority of these occurring after 3 months of treatment (Conradie et al., 2020). Median time to return to a normal or mild neuropathy score was 3 months. Myelosuppression was the second most common AE occurring in 48% of cases. Although these AE led to frequent treatment interruption (66% of cases had treatment interruption) all 109 participants completed 26 weeks of treatment. In the context of TBM, where morbidity and mortality is high, the risk-benefit of this potent antituberculous agent with good CNS penetration requires further evaluation in the context of a phase II safety trial.

Aspirin

Aspirin (ASA) is a safe, widely available and inexpensive drug with effects on the pathogenic processes recognised as integral to the pathogenesis of TBM and its complications (Bousser, 2009, Hovens et al., 2008). In a placebo-controlled trial of ASA in 118 adult patients with TBM in India, 150mg daily ASA was associated with a significantly lower 3-month mortality and a lower incidence of stroke albeit not significant (Misra et al., 2010). Following this a similar study in South Africa randomized 146 children with TBM to receive low dose (75mg/24hours) (n=47) or high dose (1000mg/24 hours). In this trial there was no significant effect of ASA on mortality however there was a significant reduction on incidence of new hemiplegia in those receiving high dose ASA (Schoeman et al., 2011). In a recent study in Vietnam, HIV-1 uninfected individuals with TBM received ASA in addition to standard care. Patients were randomized to receive either placebo, ASA 81mg OD or ASA 1000mg OD for 56 days. The pre-specified sub-analysis of results demonstrated a potential reduction in new infarcts and deaths by day 60 in patients with microbiologically confirmed TBM receiving 1000mg OD of ASA (Mai et al., 2018). Its safety for use in HIV-1 infected individuals with TBM, particularly when used in combination with an intensified antituberculous regimen, has yet to be investigated.

High dose rifampicin

Rifampicin (RIF), one of the four first line treatments for TBM demonstrates poor penetration of the blood brain barrier (BBB) with total concentrations in CSF only 10-20% of that reached in plasma (Donald, 2010). *In vitro*, animal and early bactericidal activity studies suggest that the standard 600mg once daily dose is at the lower end of the dose response curve (van Ingen et al., 2011). This has prompted a series of studies in both pulmonary and extrapulmonary tuberculosis investigating the safety and

efficacy treatment regimens containing higher doses of RIF (Heemskerk et al., 2016, van Toorn et al., 2014, Ruslami et al., 2013b, Boeree et al., 2015, Boeree et al., 2017, Aarnoutse et al., 2017, de Steenwinkel et al., 2013, Steingart et al., 2011). None of these studies have detected a significant safety signal thereby supporting the safety of RIF up to doses of 35mg/kg. Similarly, they provide evidence to suggest superior efficacy when used at a dose of 35 mg/kg compared to 20 mg/kg (Boeree et al., 2015, Boeree et al., 2017, de Steenwinkel et al., 2013, Steingart et al., 2011).

In TBM, the use of higher RIF doses is appealing given its incomplete penetration into the central nervous system. In 2013 an open-labelled randomized phase 2 study in 60 Indonesian adults with TBM showed a 50% reduction in mortality with higher dose intravenous RIF (13 mg/kg, which equates to an approximate oral dose of 20mg/kg) compared with standard dose oral therapy (Ruslami et al., 2013a). This intensified treatment did not result in increased toxicity and was associated with a substantially lower 6-month mortality. A subsequent large randomized placebo-controlled trial in Vietnam evaluated a combined regimen of oral RIF 15 mg/kg plus levofloxacin. Unlike the previous trial using intravenous therapy (at higher equivalent oral doses of 20 mg/kg) there was no effect of mortality (Heemskerk et al., 2016). These results, plus evidence from pre-clinical studies and pulmonary TB, provide adequate justification to systematically assess the effect on outcomes in TBM with RIF doses > 20 mg/kg.

The proposed trial combines three drugs for which there is sufficient evidence to suggest adequate safety profiles and potential benefit in a condition in which there is high mortality and inadequate treatment. Their safety in combination and in the context of HIV-1 co-infection requires careful evaluation in a controlled Phase II trial, before this regimen can be tested in the context of a phase III clinical trial.

Our primary hypothesis is that increased dose RIF plus LZD and ASA can be safely added to standard therapy for HIV-1-associated TBM.

2.2 Methods and Study Design

Study aims

The primary aim is to investigate the safety of enhanced antimicrobial therapy including increased dose RIF and LZD with or without adjunctive ASA added to standard therapy for TBM in HIV-1 infected adults.

The **secondary aims** are:

a. To determine CSF *M.tb* culture positivity and Gene Xpert® Ultra positivity at baseline and at 3 and 28 days post treatment by allocation.
b. To evaluate the effect of ASA and enhanced TB treatment on the incidence of Immune Reconstitution Inflammatory Syndrome (IRIS) in participants starting ART
c. To evaluate the effect of high dose RIF and LZD with and without ASA on CNS imaging (CT, MRI and MR Spectroscopy) in conjunction with clinical, immunological and transcriptional profiling.
d. To determine i) whether host genotype, including leukotriene A4 hydrolase (LTA4H) genotype, influences therapeutic effect of ASA in HIV-TBM and ii) the pharmacogenetic influence on RIF and LZD exposures and toxicity.

Three sub studies will recruit all consenting participants with the following aims:

Sub study 1 (Pharmacokinetic-Pharmacodynamic):

1. To describe the plasma and CSF PK of LZD and high dose RIF.
2. To evaluate the relationship between drug exposures, toxicity and efficacy.
3. To compare exposures between intravenous and oral RIF administration.
4. To investigate the impact of high dose RIF on LZD availability.

Sub study 2 (Pathogenesis):

1. To evaluate the effect of high dose RIF and LZD, with and without ASA on the transcriptional signature derived from whole blood and CSF RNA sequencing, as well as the metabolomic and proteomic profiles, in TBM.

Sub study 3 (Cognitive and functional outcomes):

1. To describe the frequency and characteristics of cognitive impairment following HIV-associated TBM
2. To compare cognitive outcomes with: i) presence and location of structural abnormalities on magnetic resonance imaging, ii) radiological makers of metabolic dysfunction on magnetic resonance spectroscopy, iii) *in vivo* markers of neurodegeneration and brain injury within the central nervous system
3. To quantify the functional impairment (including effect on quality of life) of TBM associated cognitive impairment

A strategic aim of LASER-TBM is to serve as a planning study to generate data which will inform a planned phase 3 RCT of intensified treatment in TBM (INTENSE-TBM). Data from LASER in particular i) pharmacokinetic data on exposure in intravenous versus high oral dose rifampicin and ii) safety data to exclude any signal which would preclude commencement of INTENSE-TBM will in part dictate the resulting sample size. If no safety signal is detected, and PK endpoints meet with adequate power then

LASER-TBM recruitment may cease prior to the maximum sample size of 100 participants to allow timely commencement of INTENSE-TBM.

Study design, recruitment and duration

LASER-TBM is a parallel group, randomized, multi-arm Phase 2A trial evaluating the safety of increased dose RIF plus LZD, with or without ASA, for the treatment of HIV-infected adults with TBM (Figure 2.1). HIV-1 infected adults with newly-diagnosed TBM (up to n = 100) will be recruited from five public-sector hospitals across South Africa. Participants will be randomised in a 1.4:1:1 ratio across two experimental (n = 30 each) and one standard of care (n = 40) arms (Figure 2.1). Relatively more participants will be randomised to the control group to account for the higher mortality anticipated in the standard of care arm. Treatment will be provided in all arms for 56 days, after which participants will be referred back to public sector facilities to complete standard therapy for HIV-associated TBM. All participants will receive antitubercular chemotherapy as well as corticosteroids as per standard practice. Participants allocated to experimental arms 2 and 3 will receive additional RIF (total oral dose 35 mg/kg/day) for 56 days plus oral LZD 1200mg daily for the first 28 days, reduced to 600 mg daily for the next 28 days. Those randomized to experimental arm 3 will also receive oral aspirin 1000 mg daily for 56 days. A second randomization will take place before receipt of study drug for participants in the experimental arms (n = 60) to receive either oral (35 mg/kg) or intravenous (20 mg/kg) RIF. This will be continued for 3 days, after which all participants will receive oral RIF for the remainder of the intervention period (53 days). There are six scheduled study visits, which will occur at study sites or affiliated stepdown facilities. Visits will involve interviews, clinical examination, phlebotomy, lumbar puncture and brain imaging at the timepoints shown in Figure 2.1. In those who consent, intensive PK sampling will take place at day 3. Trial participation will be for 180 days post-randomization: primary safety endpoints and secondary

efficacy endpoints will be evaluated at day 56; additional secondary endpoints will be evaluated at day 180 through record review.

Endpoints

The primary endpoint of the study is: The incidence of solicited treatment-related AE (see Table 2.1) and death at 56 days associated with increased dose RIF plus LZD with or without adjunctive ASA, when administered alongside standard antitubercular therapy.

Secondary study endpoints are:

- Death and severe disability (Modified Rankin Scale Grade 5) at 56 days (Box 2.3).
- Death at 56 and 180 days.
- Disability at 56 and 180 days, stratified by baseline MRC grade.
- Grade 3 or 4 AE.
- Permanent discontinuation of experimental drugs.
- Severity and frequency of haematologic and neurologic AE related to LZD use.
- Severity and frequency of major bleeding (gastrointestinal and intracerebral) related to ASA use.
- *M. tb* culture status and time to positivity in automated liquid culture (MGIT) and Gene Xpert® Ultra cycle threshold (C_t) values at days 28 and 56.
- The occurrence of TBM-IRIS assessed by the modified International Network for the Study of HIV-associated IRIS (INSHI) criteria (Meintjes et al., 2008).
- MRI and CT changes at day 56.

Study participants for LASER TBM must be adults (aged 18 or over), with proven HIV-1 seropositivity, and a diagnosis of TBM meeting criteria for ‘possible’, ‘probable’ or ‘definite’ as per the published consensus definition(Marais et al., 2010).

Potential participants will be excluded if they meet any of the exclusion criteria outlined in Box 2.1. The published uniform case definition criteria used to define 'definite', 'probable' and 'possible' TBM in described in Box 2.2.

2.3 Recruitment, randomisation, retention and withdrawal

Recruitment will be from inpatients at the participating hospital sites in South Africa (Groote Schuur Hospital, Mitchells Plain Hospital, New Somerset Hospital and Livingstone Hospital). Suitable patients will be identified by attending ward doctors and co-investigators at each site and referred to the study staff for screening.

Participant identification numbers (PID), assigned at the screening visit, will be used throughout the study. After signing the informed consent document; eligible participants will be randomized to one of the treatment arms using an electronic randomization tool. The randomisation list will be generated and updated by the trial pharmacist who will have no direct contact with trial participants or involvement with the assessment for eligibility in the trial. The second randomisation to IV or oral RIF will take place immediately, prior to receipt of study drug, for all participants allocated to experimental arms. The trial is open-label, and regimens will not be masked.

All trial procedures will take place in hospital during the admission period. The decision to discharge trial participants will be made by clinical, and not trial, staff. Site-specific standard operation procedures (SOP) will be developed for trial follow up visits following discharge or referral to a stepdown facility.

A participant will be withdrawn from the study if:

- The initial *M.tb* strain is found to be RIF-resistant on confirmatory testing;
- HIV-1 result is found to be negative on confirmatory testing;
- An alternative diagnosis is established within 5 days of antitubercular treatment initiation which leads the treating physician to discontinue antitubercular therapy;
- Withdrawal of informed consent.

Participants who withdraw consent prior to completion of the study will not undergo any further study procedures or data collection. In such cases, consent for the use of data collected prior to withdrawal of consent will be sought from the withdrawing participants. There will be no replacement for participants withdrawn from the trial.

2.4 Interventions

Study drug regimens

Participants enrolled to the study will receive study drug regimens as outlined in Table 2.2. Dosing of the RHZE fixed dose combination (FDC) will be according to WHO weight bands. Study drugs will be given orally, either as tablets/capsules or crushed, depending on the clinical circumstances. Half of participants in experimental arms will be randomised to receive IV RIF for the first 3 days of therapy and switched to the oral formulation thereafter. Study drugs will be prescribed by trial doctors, packaged and distributed by trial pharmacists.

Oral RIF Dosing Bands

Simulations were performed to determine the dose of RIF required to achieve the most equitable drug exposures across the weight range, 30 to 100 kg. Demographic data of a reference cohort of TB patients (n = 1225), with or without HIV-1 coinfection, recruited in clinical trials conducted in West Africa and South Africa were used for the simulations (Chigutsa et al., Lawn et al., Diacon et al., McIlleron et al.). An additional 12 250 virtual patients were generated using the weight and height distributions of the 1225 patients to increase the number of patients with a weight close to the boundaries of the weight range. Parameter estimates of the population PK model for RIF were used to simulate (100 replicates) RIF exposures (Chirehwa et al.). Four dosing scenarios were evaluated using the weight-band based dosing with 4-drug FDC tablets and extra RIF tablets with each tablet containing 150 mg or 600 mg RIF. The FDC tablets were assumed to have 20% reduced bioavailability based on data from a clinical trial where the same formulation was used (Court et al.).

Intravenous RIF

Participants allocated to experimental arms will be randomised (1:1) to receive either oral RIF 35 mg/kg or IV RIF 20 mg/kg for the first 3 days of therapy (in addition to HZE and LZD with or without ASA, according to the experimental arm). Those randomised to IV RIF will receive the full RIF dose intravenously, plus additional antitubercular drugs as individual tablets (at standard doses). IV RIF will be administered as an infusion as per the package insert and according to a detailed SOP.

Concomitant medications

Corticosteroids

All participants will receive corticosteroids for the first 8 weeks of TBM treatment as used in a randomized controlled trial demonstrating mortality benefit in patients TBM (Thwaites et al., 2004).

Antiretroviral Therapy (ART)

ART will be commenced by treating clinicians after 4-6 weeks of antitubercular therapy in all participants based on the single randomized controlled trial of ART timing in TBM, which showed no benefit for earlier ART (Torok et al., 2011a). If available, a dolutegravir-based regimen will be used in accordance with international (Gunthard et al.) and local guidelines (Meintjes G).

Gastric protection

Participants can be prescribed omeprazole 40mg daily. A higher starting dose of 40mg OD was selected to account for the interaction between proton pump inhibitors (PPI) with rifampicin via CYP2C19 and CYP3A4 leading to reduction in levels of the PPI. In participants with persistent symptoms the dose will be titrated to 80mg daily and gastroscopy considered. Although the study initially planned to use ranitidine for this indication, this was withdrawn as a concomitant medication due to concerns over a potential contamination with the probable carcinogen N-nitrosodimethylamine (NDMA)(Mahase, 2019), making the medication unavailable for use in South Africa.

Pyridoxine

Participants will receive pyridoxine supplementation as per the South African guidelines for prevention of anti-tuberculosis drug-related peripheral neuropathy(South Africa Department of Health, 2014).

Disallowed medications

The medicines listed in Box 2.4 have been shown to interact with study drugs and are therefore contraindicated for concomitant use during the study

2.5 Study procedures, schedule and clinical assesments

Participants will undergo 6 scheduled study visits after screening, plus ascertainment of vital status and disability assessment at 6 months. Table 2.3 describes planned investigations at each study visit

Clinical assessment

Clinical assessment will include clinical history, conscious level by GCS, presence of new or ongoing focal neurological deficit, all adverse events, new medications started and adherence to drugs. The neurological examination at D180 is extended to assess for such as language, visuospatial deficit, visual agnosia and praxis: focal cognitive deficits which may be present in people with TBM.

Modified Brief Peripheral Neuropathy Score

This purely clinical early screening tool was adapted from the subjective peripheral neuropathy score (SPNS) validated for the assessment of HIV associated distal sensory polyneuropathy (DSP) (McArthur, 1998) and used previously in trials to assess LZD toxicity.

Modified Total Neuropathy Score

This screening tool, initially developed for the assessment of chemotherapy induced neuropathy (Cornblath et al., 1999), has been modified for use in the HIV research setting where it has shown acceptable sensitivity and specificity (85% and 80% respectively) (Evans et al., 2008). Prior studies have used a simplified 16-point (Robinson-Papp et al., 2009) or 20-point clinical scoring system (Maritz et al., 2010) as markers of HIV-associated DSP severity.

Insomnia Severity Index

All participants will complete the Insomnia Severity Index (ISI) at the Day 28 and 56 visits. The ISI is a brief screening measure of insomnia which has been validated for use in in insomnia research (Bastien et al., 2001). Dolutegravir has been associated with neurotoxicity presenting with neuropsychiatric symptoms such as insomnia (Hoffmann et al., 2017), and this will be assessed as part of the PK/PD assessment.

Measures of cognitive function

These measures where possible will be done in the participants preferred language.

i) Montreal Cognitive Assessment

The Montreal Cognitive Assessment (MoCA) assesses six broad domains of ability and cognitive function(Nasreddine et al., 2005) and has been used to screen for cognitive impairment in previous studies within South Africa (Robbins et al., 2013). This will be carried out at day 56 and day 180.

ii) Cognitive Assessment Tool-Rapid Version (CAT-Rapid). This instrument includes four questions about subjective cognitive complaints, as well as tasks assessing learning and memory and cognitive sequencing. CAT-rapid was developed in South Africa (Joska et al., 2016), in response to the need to develop a brief screening tool that includes functional symptom questions and a measure of executive function. The CAT-rapid incorporates aspects of the International HIV Dementia Scale and includes four symptom questions, as well as tasks assessing learning and memory and cognitive sequencing.

iii) Brief neuropsychological battery

Neuropsychological testing will be carried out by a trained neuropsychometric tester and clinical research worker at day 180 and will include a cognitive test battery and assessment of contributing mental health symptoms. The cognitive

battery comprised 12 standardized tests, each of which assessed performance in one of six cognitive domains commonly affected by TBM. The domains, tests, and outcome variables were: (1) Executive Functioning: Color Trails Test 2 (CTT2) - completion time; Wisconsin Card Sorting Test (WCST) - perseverative errors; (2) Learning and Memory: Hopkins Verbal Learning Test-Revised (HVLT-R) - total learning total and delayed recall total; Brief Visuospatial Memory Test-Revised (BVMT-R) - total learning total and delayed recall total; (3) Generativity/fluency: category fluency - total number of animals / total number of fruits and vegetables named in 1 minute; (4) Attention/Working Memory: Wechsler Adult Intelligence Scale-Third Edition (WAIS-III) Digit Span - total score; (5) Processing Speed: CTT1 - completion time; Wechsler Adult Intelligence Scale-Third Edition (WAIS-III) Digit Symbol Coding - total score; WAIS-III Symbol Search- total score; (6) Motor Function: Grooved Pegboard Test (GPT) dominant (DH) and nondominant hand (NDH) - completion time; Finger Tapping Test (DH and NDH) - completion time. Tests were administered in either English or Xhosa depending on the participant's preference. Mental Health measures were the Centre for Epidemiological Studies-Depression (CES-D), State Trait Anxiety Inventory-trait (STAI-trait), Alcohol Use Disorders Identification Test (AUDIT) and Drug Use Disorders Identification Test (DUDIT).

Measures of functional status

i) Modified Rankin Score

The MRS a commonly used clinical outcome measure for patients suffering from stroke (Rankin, 1957), has demonstrated good inter-rater reliability (van Swieten et al., 1988) and is the most commonly used outcome measure to assess neurological disability in TBM (Saver et al., 2010).

ii) Modified Patients Assessment of Own Functioning Inventory (PAOFI)

This patient reported outcome measure is designed to evaluate a patient's sense of his or her functional capacity in everyday activities concerning memory, language and communication, use of hands, sensor perception, higher level cognitive and intellectual functions, and work/recreation(Chelune).

iii) Lawton-SA is designed to assess independent living skills, considered more complex than basic activities of daily living. This was developed in 1969(Lawton and Brody, 1969), but since modified for use in the South African context(Joska et al., 2010).

Blood

Testing will be done at specified timepoints (as per table 2.3), and may be repeated to follow-up on abnormal results, for example after occurrence of an AE. Samples for haematology and biochemistry and HIV testing will be processed in National Health Laboratory Service (NHLS) laboratory according to local SOPs. Samples for non-clinical assays (immune markers, RNA, metabolomics, proteomics) and future use will be collected and transported to the Institute of Infectious Disease and Molecular Medicine at the University of Cape Town (UCT) for processing and storage. PK samples will be centrifuged *within an hour* of being taken at 1500 x g at room temperature for 10 minutes, the plasma aliquoted into cryovial tubes, stored at -80^{o}C and transported to UCT Clinical Pharmacology laboratory for storage and processing.

Urine

Bedside pregnancy testing will be done on urine at screening. Urine will be sent to chemistry laboratories for osmolality and electrolyte testing in the context of

hyponatraemia at the discretion of the investigator. Further urine will be collected for biobank storage.

Cerebrospinal fluid analysis

CSF will be obtained via lumbar puncture at Days 3 and 28, in accordance with a detailed SOP. CSF collected for diagnosis in routine care (baseline) will be retrieved where possible. Routine microbiology, cell count, and biochemistry will be done in NHLS laboratory according to local SOPs. Samples for non-clinical testing (RNA sequencing, metabolomic and proteomic analysis and immunological assays) will be collected and transported to the IDM at UCT for processing and storage. CSF to determine drug concentrations for the PK study will be frozen at -80 degrees immediately following collection and transported to UCT Clinical Pharmacology laboratory for storage and processing.

Magnetic Resonance Imaging (MRI)

MRI scans will be performed in all participants who can tolerate or access the investigation at baseline and day 56. A 3-Tesla (3T) MRI scanner located at Groote Schuur Hospital will be used for all imaging in the Cape Town area, whilst a 1.5T scanner located at Livingstone Hospital will be used for participants recruited in the Port Elizabeth Area. Gadolinium enhanced imaging will be performed on participants with eGFR > 30mL/min/1.73m^2.

Image sequences will include the following:

- T1 weighted sequences with or without gadolinium
- T2 weighted sequences
- Diffusion weighted images (DWI)

- Susceptibility weighted images (SWI)
- T2 Fluid-attenuated inversion recovery (FLAIR)
- Point resolved spectroscopy (PRESS/MEGA-PRESS) to estimate brain metabolite changes

Computed Tomography (CT)

If participants are unable to tolerate or access MRI, CT will be used as an alternative imaging method at the same time points as stated for MRI. Participants with eGFR > 30 mL/min/1.73m^2 will have contrast enhanced imaging. Pre- and post- contrast sequences will be available for analysis. A standardised reporting form including positive and negative radiological findings will be used.

2.6 Statistical considerations

Sample size

Arm 1 - control - (standard-of-care): 40

Arm 2 - experimental - (standard-of care + high dose rifampicin + linezolid): 30

Arm 3 - experimental - (standard-of care + high dose rifampicin + linezolid + aspirin): 30

Total participants required for primary safety analysis = 100

Sample size justification

This Phase IIA trial will focus on evaluating adverse events in the experimental arm relative to the standard of care arm. Solicited treatment related AE (Table 2.1) plus

deaths will be evaluated, and the Data Safety Monitoring Board (DSMB) will provide recommendations accordingly. The DSMB will review all safety events and approve the ongoing conduct of the trial. Analyses that will aid their decision-making will be based on several sources:

First, a test of proportions will compare the AE rates between the standard-of-care arm and the experimental arms. Concerns about a worse safety profile will be flagged using a two-sided type I error rate of 0.1. Consider a scenario in which there are 10 out of 20 AE in the standard-of-care arm and 14 out of 20 in the experimental arms. This corresponds to a two- sided p-value of 0.053 using Boschloo's test and would be reason for the DSMB to consider stopping the trial.

Similarly, a Bayesian posterior probability (with an uninformative prior) of the probability that the AE rate in the experimental arm is worse than that in the control arm. This will provide an additional means of interpreting the relative results. If this probability is high, the DSMB may recommend stopping the study. For example, in the 10/20 and 14/20 scenario the posterior probability that the experimental arm has a worse rate of AEs is 94%. If the split was 10/20 versus 14/20, this probability would be 89%. The DSMB will be unblinded to safety data after every 15 patients recruited (5 per arm). At each point, absence of a significant safety signal (as outlined in the DSMB charter) will permit ongoing recruitment.

Statistical analysis plan

A detailed statistical analysis plan for LASER-TBM detailing the planned analysis for the primary and PK endpoints, co-authored and authorised by the trial statistician (JL) is included within the supplementary material of this manuscript.

2.7 Adverse events

Assessment of AE

Study participants will be monitored and assessed for new AE at all scheduled study visits (as shown in Table 2.3). At each visit, trial staff will also assess the evolution and outcome of previously recorded AE. Safety monitoring of the study will be performed by a DSMB as described below.

Severity of AE

All AE will be assessed for severity by study clinical investigators and graded as per the Division of AIDS (DAIDS) criteria(U.S. Department of Health and Human Services). Each AE will be assigned a grade 1 to 5. For events not included in the protocol-defined grading systems, the following general definitions from grades 1 to 4 will be applied to classify event severity:

Changes in the severity of an AE should be documented to allow an assessment of the duration of the event at each level of intensity to be performed. AE characterised as intermittent require documentation of onset and duration of each episode.

Solicited treatment related AE

Table 2.1 lists AE of special interest which are considered ‘solicited treatment related AE’ and therefore comprise primary safety endpoints of this study. These AE are reported regardless of causal relationship to study drugs. For each AE there is a specific objective measure incorporating the DAIDS grading criteria and other parameters of clinical significance. The management of each AE is summarised.

Management of adverse events

Treatment must be discontinued for safety reasons if any clinical AE, laboratory abnormality, intercurrent illness, or other medical condition or situation occurs such that continued exposure to treatment would not be in the best interest of the participant. Detailed guidance for management of AE is provided in the manual of operating procedures.

2.8 Safety monitoring

Safety oversight

Safety oversight will be under the direction of an independent DSMB. Comprised of independent internationally-recognized HIV-TB researchers and an independent statistician, the DSMB will meet after each 15 participants enrolled. The DSMB may also decide to convene an unscheduled review if warranted by safety or data quality concerns. The data for review will be prepared by an independent statistician.

The task of the DSMB will thus be to review study recruitment, data quality and trial drug safety and advise the sponsor of major safety issues and data quality issues. The DSMB may advise that trial enrolment should be paused or stopped entirely based on the decisions regarding the frequency and severity of solicited treatment related AE as outlined in Table 2.1 (‘Solicited Treatment Related Adverse Events’).

Pausing and stopping rules

Halting rules for safety reasons will be detailed in the DSMB charter. In the event of serious safety concerns, the DSMB chair will consult the full DSMB by email or

teleconference. Pending the DSMB response, the chair may use his/her discretion to recommend one or more if the following: Halt in study (arm) enrolment; halt in study (arm) dosing; provision of additional intervention; no action. After review, the DSMB will issue a recommendation to the trial steering committee to continue, modify (one or more arms) or terminate the trial.

2.9 Data access and handling

Source documents

Source data are original records of clinical findings, observations, or other activities necessary for the evaluation of the trial. Examples of these original documents and data records include, but are not limited to: hospital records, laboratory reports, and radiological images. CRF may also be acceptable source documents. A complete list of source documents will be created prior to trial initiation.

The following individuals and groups will have access to study records:

- Members of the study team
- Relevant institutional review board (IRB)
- Regulatory agencies (South African Health Products Regulatory Authority - SAHPRA)
- Study Monitor
-

All site staff, the sponsor, and any sponsor representatives will preserve the confidentiality of all participants taking part in the study in accordance with ICH GCP, applicable South African national and local regulations and (to the extent applicable) the U.S. Health Insurance Portability and Accountability Act of 1996 (HIPAA). Subject

to the requirement for source data verification by the study personnel by reference to the participant's notes confidentiality of all participant identities will be maintained. Only participant study number and initials will be used on the CRF and in all study correspondence, as permitted. No material bearing a participant's name will be kept on file by the Sponsor. The written informed consent will contain a clause granting permission for review of the participants' source data.

2.10 Data collection, management and storage

Procedures to ensure data quality will be detailed in a Data Management plan. Data will be collected and captured onto hardcopy CRF on site and then entered into an electronic database. Clinical data will be entered onto paper CRF directly from the source documents on site. Other study data, such as laboratory reports and telephonic interviews will either be recorded on a specific study form before data entry or entered directly into the electronic database. CRF will be cross-checked for accuracy, authenticity and completeness at the site by study staff; checks for consistency will be implemented at the data entry level on site and centrally after data entry.

The data will be managed and stored using a GCP-compliant web-based REDCap® database custom-designed for the study. The REDCap® data entry and user permission structures provide auditing trails in line with international requirements. Access to the database is password controlled and will be limited to delegated trial staff with data entry and data management responsibilities.

Publication of research findings

The definition of publication for this purpose is any public presentation of the data emerging from this study. All unpublished information given to the investigator by the

Sponsor shall not be published or disclosed to a third party other than to the responsible IRB, within the understanding of the confidentiality of their nature, without the prior written consent of the Sponsor. Results of this research will be submitted for publication as soon as feasible upon completion of the study in the form of a joint publication(s) between the Sponsor and investigator(s), including site clinical and laboratory investigators, as appropriate.

2.11 Trial committees, ethical procedures and sponsorship

A Trial Management Group (TMG) responsible for the day-to-day management of the trial at the UCT CRC includes; National Trial Coordinator (Ms Mpumi Maxebengula), Lead Clinician (Dr Angharad Davis), Research Medical Officers (Dr Cari Stek, Dr Remy Daroowala, Dr Marise Bremer, Dr Stephani Botha, Dr Saalika Aziz), Project Manager (Ms Rene Goliath), Pharmacists (Ms Sonya Koekemoer, Mr Yakub Kadernani). The group will communicate weekly to discuss trial progress.

The Trial Steering Committee (TSC) is composed of Professor Guy Thwaites (chair, Infectious Disease Physician, University of Oxford), Professor Graeme Meintjes (site principal investigator), Dr Sean Wasserman (site principal investigator), Dr John Black (site principal investigator), Professor Robert J Wilkinson (National Principal Investigator) and Dr Angharad Davis (lead investigator). The role of the TSC is to provide overall supervision for the trial and provide advice through its independent chair. The ultimate decision for the continuation of the trial lies with the TSC.

The Data Safety and Management Board (DSMB) is composed of Professor David Lalloo (chair, Director of the Liverpool School for Tropical Medicine and a Professor of Tropical Medicine), Dr David Meya (Infectious Diseases clinician, Senior Lecturer at the College of Health Sciences at Makerere University and Adjunct Associate Professor in the Division of Infectious Diseases and International Medicine at the University of Minnesota), Dr Evelyne Kestelyn (Head of the Clinical Trials Unit at the Centre for Tropical Medicine and Global Health, University of Oxford), Dr Maryline Bonnet (Medical Epidemiologist Institute of Research for Development and Epicentre), Dr Angela Crook, (Trial Statistician). The role of the DSMB is to protect and serve LASER-TBM trial patients and to assist and advise the Principal Investigators, so as to protect the validity and credibility of the trial.

ETHICS

The trial has ethics approval from the University of Cape Town Human Research Ethics Committee (293/2018), Walter Sisulu University Human Research Committee (Ref 012/2019) and the South African Health Products Regulatory Authority (reference number 20180622). The trial is registered on the South African National Clinical Trials Register (DOH-27-0319-6230) and Pan African National Clinical Trials Register (PACTR201902921101705).

TRIAL SPONSOR

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PORT ELIZABETH

Livingstone Hospital, Standford Road, Korsten, Port Elizabeth, 6020, Republic of South Africa

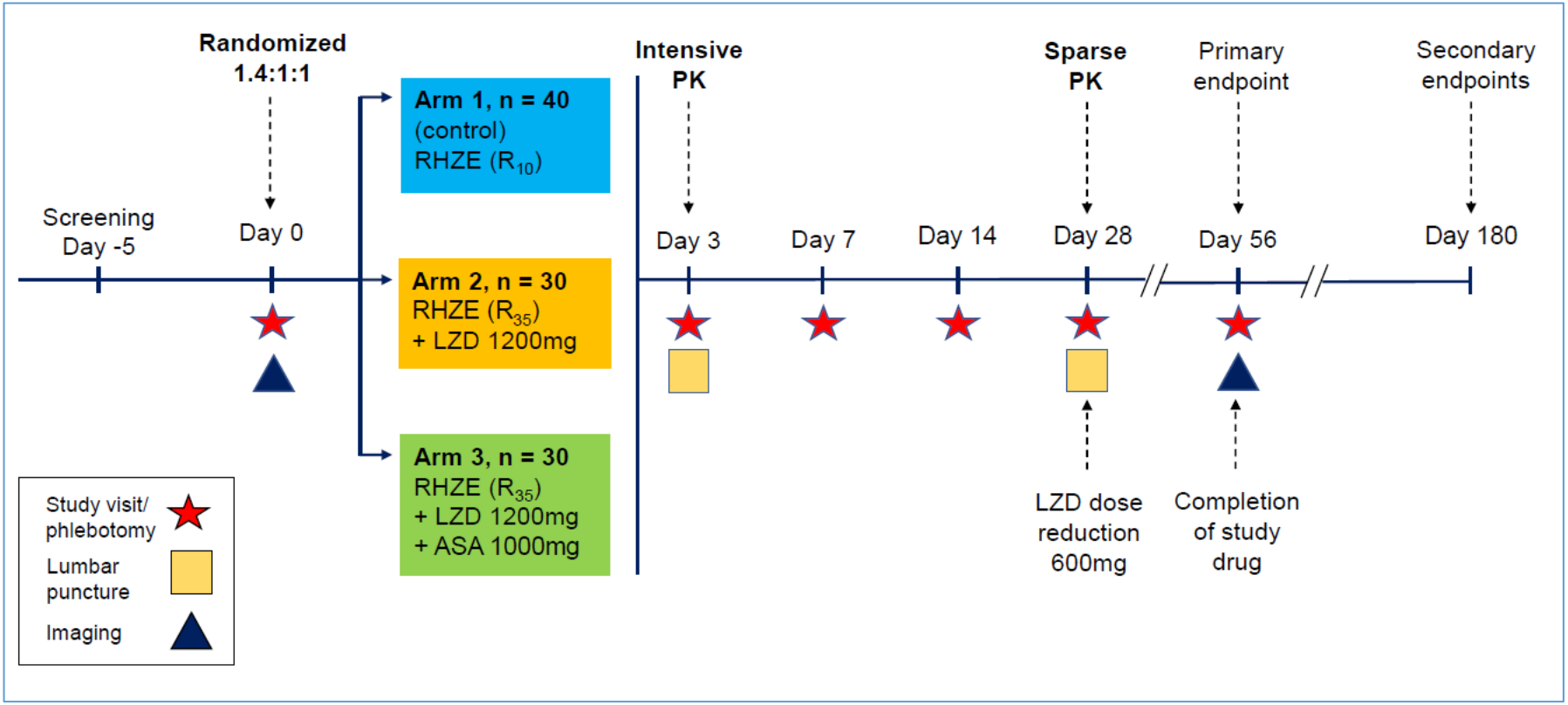
2.12 Version control and protocol amendment policy

Submitted version of the protocol: V6 (dated 11 May 2020)

Any change to the protocol will be affected by means of a protocol amendment. The PI, HREC, and Sponsor must agree on all amendments. No amendment will be

implemented until approved by the relevant authorities and signed by all required parties. Exceptions to this are when the PI considers that the participant's safety is compromised. No deviations from or changes to the protocol should be initiated without prior written approval from the IRB and regulatory authority. The PI, or designated site staff, is responsible for documenting and explaining any deviations from the protocol. Protocol deviations must be sent to the Sponsor and IRB in accordance with standard procedures.

Figure 2.1 LASER-TBM Study Design



Study design schematic describing randomisation to study arms, treatment intervention per am, visit schedule, overview of clinical procedures and timepoints relating to primary and secondary endpoint data collection. Abbreviations: RHZE: Rifampicin, Isoniazid, Pyrazinamide, Ethambutol; R10: Rifampicin 10mg/kg/day; R35: Rifampicin 35mg/kg/day; LZD: Linezolid; ASA; Aspirin;

Box 2.1 LASER-TBM eligibility criteria

Inclusion criteria

- Age >18 years
- proven HIV-1 seropositivity
- Diagnosis of 'possible', 'probable' or 'definite' TBM

Exclusion criteria

- Rifampicin-resistant *M.tb* detected on any clinical specimen;
- History of allergy or hypersensitivity to RIF, isoniazid, ethambutol, pyrazinamide, LZD or ASA;
- Received more than 5 days of antitubercular therapy in the 30 days prior to screening;
- Receipt of regular daily ASA or NSAID prior to TBM diagnosis
- CSF unobtainable by lumbar puncture or another procedure;
- Evidence of bacterial or cryptococcal meningitis;
- Severe concurrent uncontrolled opportunistic infection including, but not limited to, active cytomegalovirus-associated disease, Kaposi sarcoma, *Pneumocystis jirovecii* pneumonia, HIV related or unrelated malignancy, or gastrointestinal bleeding;
- Any other form of immunosuppressive therapy, including antineoplastic and biologic agents, apart from corticosteroids;
- More than 17 weeks pregnant at baseline;
- Peripheral neuropathy scoring Grade 3 or above on the BPNS;
- Any disease or condition in which the use of the standard anti-TB drugs (or any of their components) are contraindicated. This includes, but is not limited to, allergy to any TB drug or their components;
- The presence of one or more of the following:

- Estimated glomerular filtration rate (eGFR) < 20ml/min/1.73 m2*
- INR > 1.4 and/or clinical evidence of liver failure or decompensated cirrhosis;
- Haemoglobin < 8.0 g/dL;
- Platelets < 50 x109 /L;
- Neutrophils < 0.5 x 109 cells/L;

- Any disease or condition in which any of the medicinal products listed in the section pertaining to prohibited medication (See Box 2.4) is used and cannot be safely stopped;
- Known or suspected history of drug abuse or any other reason that is, in the opinion of investigators, sufficient to compromise the safety or cooperation of the participant.

*Calculated using the Cockcroft-Gault equation; INR: International normalised ration; BPNS: Brief Peripheral Neuropathy Score; NSAID: Non Steroidal Anti Inflammatory Drug;

Box 2.2 Summary of uniform case definition criteria for tuberculous meningitis used to define 'definite', 'probable' and 'possible TBM in LASER-TBM (Marais et al., 2010)

All patients require symptoms and signs suggestive of meningitis including one of more of the following; headache, irritability, vomiting, fever, next stiffness, convulsions, focal neurological deficits, altered consciousness or lethargy. An alternative diagnosis must be excluded. In this context they should then be subsequently assessed on the following criteria.	
Criteria	**Score**
Clinical (maximum category score = 6)	
Symptom duration of > 5 days	4
Weight loss, night sweats or persistent cough for > 2 weeks)	2
Focal neurological deficit (excluding cranial nerve palsy)	1
Cranial nerve palsy	1
Altered consciousness	1
CSF (maximum category score = 4)	
Clear appearance	1
Cells 10-500/μL	1
Lymphocytic predominance > 50%	1
Protein concentration greater than 1g/L	1
CSF to plasma glucose concentration of less than 50% or an absolute CSF glucose concentration less than 2.2mmol/L	1
Cerebral Imaging criteria (max score 6)	
Hydrocephalus	1
Basal meningeal enhancement	2
Tuberculoma	2
Infarcts	1
Pre-conntrast basal hyperdensity	2
Evidence of TB elsewhere (max score 4)	

CXR suggestive of active TB	4 (miliary) 2 (other active TB)
CT/MRI/Ultrasound showing evidence for TB outside the CNS	2
AFB identified or M.tb cultured from another source or positive Urine LAM	4
Scoring – scores should be added up and the one of the following diagnostic categories assigned	
Definite Acid fast bacilli seen in CSF *and/or* M.tb culture from CSF	
Probable Total diagnostic score of 12 (cerebral imaging available) or 10 (cerebral imaging unavailable). At least 2 points should come from CSF or cerebral imaging criteria.	
Possible Total diagnostic score of 6-11 (cerebral imaging available) or 6-9 (cerebral imaging unavailable).	

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Box 2.3 Modified Rankin Score

SCORE	DESCRIPTION
0	No symptoms
1	No significant disability. Able to carry out usual activities, despite some symptoms.
2	Slight disability. Able to look after own affairs without assistance, but unable to carry out all previous activities.
3	Moderate disability. Requires some help, but able to walk unassisted.
4	Moderately severe disability. Unable to attend to own bodily needs without assistance, and unable to walk unassisted.
5	Severe disability. Requires constant nursing care and attention, bedridden, incontinent.
6	Dead

Box 2.4 LASER-TBM contraindicated medications

Tricyclic antidepressants: Amitriptyline, Amoxapine, Clomipramine, Desipramine, Doxepin, Imipramine, Nortriptyline, Protriptyline, Trimipramine. **Selective Serotonin Re-uptake Inhibitors (SSRI's):** Citalopram, Escitalopram, Fluoxetine, Fluvoxamine, Paroxetine, Sertraline. Serotonin-Noradrenaline Re-uptake Inhibitors (SNRI's) Venlafaxine, Duloxetine, Levomilnacipran, Milnacipran. **Serotonin Receptor Agonist**, Buspirone, **Mono-amine Oxidase Inhibitors (MOAIs**), Isocarboxazid, Nialamide, Phenelzine, Tranylcypromine, Selegiline, Rasagaline, Toloxatone. **Reversible MOA Inhibitors (RIMAs):** Moclobemide, Pirlindole. **Migraine medications**: Triptans. **Macrolide antibiotics**: Clarithromycin, Erythromycin, Troleandomycin. **Opiate analgesics**: Methadone, Tramadol, Pentazocine. **Stimulants**: MDMA (ecstacy), Cocaine, Methamphetamine **Hormonal treatment**: Gestodene, Testosterone. **Other medications**: Antiretrovirals – Atazanavir, Anti-arrhytmic – Quinidine, Anti-malarial – Quinine, Chemotherapy – Doxorubicin, Asthma –Furafylline, Hypertension – Hydracarbazine, Antifungal – Ketokonazole, Amino-acid - Tyramine

Bold represents class of drug. Underlined medications represent commonly used medications in South Africa.

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791 **Table 2.1 Solicited treatment related adverse events, objective measures for assessment and management plan in each setting**

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Adverse Event (responsible study drug)	Objective measure	Parameter	Management
Gastrointestinal haemorrhage (ASA)	Clinical and laboratory measures suggesting GI haemorrhage.	i) Vomiting fresh or changed blood of any volume, ii) Melena, iii) Unexplained drop in Hb concentration of >2g/L or iv) > 5mls of fresh or changed blood aspirated from nasogastric tube.	Discontinue study drug permanently.
Intracerebral haemorrhage (ASA)	Radiological evidence of haemorrhage.	CT or MRI finding	Discontinue study drug permanently.
Transaminitis (RIF)	alanine transferase (ALT), bilirubin	New Grade 3 or above	Discontinue study drug (and other potentially hepatotoxic agents). Place on alternative treatment for TBM if background regimen affected. Re-test every 2 days. Resume study drug with an escalating dose rechallenge once ALT < 100 IU and total bilirubin within normal range.
Anaemia (LZD)	Hemoglobin (Hb)	New Grade 3	Discontinue study drug (plus any other myelosuppressive drugs as appropriate). Consider transfusion with packed cells or erythropoietin therapy. Monitor Hb twice weekly. Restart at 50% dose once Hb ≥ 8 mg/dL
		New Grade 4	Discontinue study drug permanently. Consider hospital admission and/or transfusion with packed cells or erythropoietin therapy. Re-test every 2 days.
Neutropenia (LZD)	Neutrophils	New Grade 3	Discontinue study drug. Monitor white cell count (WCC) every week. Restart at 50% dose once neutrophil count 0.5 x 10^9 cells/L.
		New Grade 4	Discontinue study drug permanently. Consider therapy with GM-CSF. Monitor WCC every 1 – 2 days.
Thrombocytopenia (LZD)	Platelet (Plt) count	New Grade 3	Discontinue study drug (plus any other myelosuppressive drugs as appropriate). Monitor Plt count twice weekly. Restart at 50% dose once Plt count > 50 x10^9 cells/L.
		New Grade 4	Discontinue study drug permanently. Consider hospital admission and/or transfusion pooled Plts. Re-test every 1 - 2 days.

Peripheral Neuropathy (LZD)	Full neurological history and examination, Brief Peripheral Neuropathy Score (BPNS) and Modified Total Neuropathy Score (mTNS)	Change in clinical history of examination resulting in: I) 1 grade increase in BPNS ii) 2 grade change in any modality on mTNS	Review with a view to discontinuing study drug (plus any other neuropathic drugs like isoniazid). Consider restarting at 50% dose once completely resolved.
Optic Neuropathy (LZD)	14-plate Ishihara Test, visual acuity measured by logMAR chart	Change in score of 2 on 14-plate Ishihara Colour Test or new or worse logMAR score of 0.2	Stop study drug and EMB and refer for formal ophthalmological assessment. If assessment consistent with optic neuritis do not restart drug.

Grade relates to DAIDS criteria(Services, Corrected Version 2.1 July 2017).

Table 2.2 Details and dosing of study drug regimen provided for 56 days post randomisation

	Drug					
Arm	**Rifampicin**	**Isoniazid**	**Ethambutol**	**Pyrazinamide**	**Linezolid**	**ASA**
1	10 mg/kg O.D.	5 mg/kg O.D.	15 mg/kg O.D.	25 mg/kg O.D.		
2	35 mg/kg O.D.	5 mg/kg O.D.	15 mg/kg O.D.	25 mg/kg O.D.	1200 mg O.D. (28 days) then 600 mg O.D. (28 days)	
3	35 mg/kg O.D.	5 mg/kg O.D.	15 mg/kg O.D.	25 mg/kg O.D.	1200 mg O.D. (28 days) then 600 mg O.D. (28 days)	1000 mg O.D.

806 **Table 2.3 Planned study assessments and procedure per study date**

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Visit (window in days)	SCR	ENR	Day 3 (+/- 1)	Day 7 (+/- 2)	Day 14 (+/- 2)	Day 28 (+/- 3)	Day 56 (+/- 4)	Day 180
Bedside								
Study Informed Consent	x	x						
Vital Signs	x	x	x	x	x	x	x	
Medical History	x	x	x	x	x	x	x	x
Physical Examination	x	x	x	x	x	x	x	
BPNS and mTNS	x							
Modified Rankin Score		x		x	x	x	x	x
Insomnia Questionnaire						x	x	
MOCA, IHDS, EQ5d5L							x	
Neurocognitive mini-battery								x
AE/SAE, Adherence Monitoring		x	x	x	x	x	x	
Randomisation and treatment assignment		x						
Blood								
Weight		x	x	x	x	x	x	
Haematology: FBC and white cell differential								
Biochemistry: Creatinine, eGFR, electrolytes, LFTs	x		x	x	x	x	x	
INR	x		x			x		
HIV-1 ELISA +/- HIV Rapid Test (x2) if required	x							
CD4+ count, HIV Viral Load		x						
Plasma for PK sub-study (sparse sampling)						x	x	
Plasma for PK sub-study (intensive sampling – if consented)			x					
Stored plasma for immunological, proteomic and metabolomic profiling		x	x	x	x	x	x	
PBMC for storage		x	x			x		
Whole blood for RNA extraction		x	x	x	x	x	X	
Whole blood for DNA extraction (if consented)		x						
Urine								
Urine for pregnancy test	x							
Urine for storage		x			x		x	
Cerebrospinal Fluid Analysis								
Cell count, MC+S, TB culture, GeneXpert Ultra (inc Rif resistance)			x			x		
Biochemistry: protein and glucose			x			x		

Stored CSF for immunological, cellular, proteomic and metabolomic profiling;			x			x		
CSF for RNA extraction								
CSF for PK sub-study			x			x		
Imaging								
MRI Head, or CT Head if MRI not tolerated (+/- 5 days)		x					x	

Table Abbreviations: SCR: Screening; ENR: Enrolment; AE: Adverse Event; BPNS: Brief Peripheral Neuropathy Score; CSF: Cerebrospinal Fluid; CT: Computerised Tomography; FBC: Full Blood Count; LFT: Liver Function Tests; IHDS: International HIV Dementia Score; MC+S: microscopy, culture and sensitivity; MOCA: Montreal Cognitive Assessment; mTNS: modified Total Neuropathy Score; MRI: Magnetic Resonance Imaging; PAOFI: Patients Assessment of Own Functioning Inventory; PBMC: Peripheral Blood Mononuclear Cells; PK: Pharmacokinetic;

Chapter 3. Results from a phase 2A trial of the safety and tolerability of increased dose rifampicin and adjunctive linezolid, with or without aspirin, for HIV-associated tuberculous meningitis (The LASER-TBM Trial)

3.1 Introduction

The background to the clinical trial is described within chapter 2 of the thesis.

3.2 Methods

The study methods are described in detail in chapter 2 of the thesis. The following information adds further detail, not included within chapter 2 on the final sample size and statistical analysis.

Sample size

No formal statistical power calculation was performed. Even as single adjunctive therapies, there was limited available data on the use of these drugs in TBM to predict likely rate of AESI (table 3.1) and/or death. Given this would be further complicated when considering likely event rate when the drugs were combined, it was felt a more pragmatic approach was to create a recruitment target of 100 participants with frequent blinded review of cumulative safety events by an

independent DSMB. A secondary aim of LASER-TBM was also to serve as a planning study to generate PK and safety data to inform a phase 3 RCT of intensified treatment in TBM (NCT04145258), which in part would influence resulting sample size of that study. The decision to stop recruitment prior to 100 participants enrolled was made as a result of the following:

- The rate of recruitment was slower than anticipated due to the COVID-19 pandemic
- Funding for the trial was due to cease in March 2021 and therefore recruitment could only take place up until January 2021
- DSMB review of safety data (both during recruitment to the trial and following enrolment of last recruit) had revealed no reason for the planned RCT not to go ahead.

Statistical analysis

Analysis was performed in GraphPad Prism v.9.0 and R v.3.6.0. The primary analysis was performed in the modified intent-to-treat population (those who receive any dose of the study drug). A sensitivity analysis was planned for the per-protocol population (those who completed treatment as specified in the protocol). However given the small sample size and since these populations were similar, here we report the most conservative analysis (modified intention to treat).

The primary endpoint, frequency of AESI or death (where data is censored at the first event prior to day 56) was summarized and compared across arms using a chi-squared test. A time to event analysis was performed for worst grade (in each individual participant) AESI or death; comparisons between study groups were made using the log-rank test. Neurological disability (measured by Modified Rankin Score), as well as radiological outcomes at day 56 were compared across treatment arms using chi-squared test. We used spaghetti plots to visually represent longitudinal CSF parameters (lymphocytes,

polymorphonuclear cells, protein and glucose) over time and used t tests to compare longitudinal summaries (mean and SD) of each individual trajectory across treatment arms.

Details of further analysis can be found in the full statistical analysis plan published alongside the study protocol (Davis et al., 2021).

3.3 Results

98 patients were screened and 52 were randomised between June 2019 and January 2021 (Figure 3.1). Reasons for screening exclusion are summarised in Table 3.6. One participant was randomised but excluded prior to any study IP being dispensed due to emergence of an exclusion criterion (eGFR <20) on a hospital blood test performed prior to randomisation. Another participant was excluded from the modified intention to treat analysis as they died prior to receiving any dose of study drug. Six participants discontinued the study prior to day 56, and a further four participants discontinued between day 56 and day 180 (Table 3.7).

The baseline characteristics of the participants stratified by treatment arm are described in Table 3.2. Most participants were male (71%) and the median age was 39 (34-46). Most participants had mild disease (MRC Grade 1 59%; Grade 2 39%; Grade 3 2%). A third (33%) of participants had microbiologically confirmed TBM at baseline, with the remaining participants defined as possible (41%) or probable TBM (25%) as per the uniform TBM case definition(Marais et al., 2010).

The primary endpoint analysis was performed in the modified intention to treat population (n=50; arm 1, 20; arm 2, 14; arm 3, 16). The composite primary endpoint of AESI or death occurred in 6/20 in arm 1, 4/14 in arm 2, and 10/16 participants in arm 3, (p=0.083). The occurrence of each category of AESI stratified by treatment arm are summarised in table 3.3 with further detail on timing and outcome of each of these events listed in table 3.4. Frequency of death prior to day 56 was similar across arms (n=7; arm 1, 3; arm 2, 1; arm 3, 3; *p=0.649*) and in no case was cause of death related to study investigational product (table 3.5). Grade 3 or 4 AE (grade 3: arm 1, 7 vs arm 2, 7 vs ar, 3, 9, *p=0.44*; grade 4: arm 1, 2 vs arm 2, 4 vs arm 3, 4, *p=0.38*) or serious adverse events for reasons other than death (arm 1, 6 vs arm 2, 8 vs arm 3, 7, *p=0.37*) were similar across treatment arms.

The cumulative incidence of the composite endpoint of worst grade AESI or death at day 56 demonstrated worse outcomes when comparing arm 3 vs arm 1 (p=0.043), with similar proportions observed in other pre-specified analysis (arm 2 *vs* arm 1 (*p=0.3*), arm 2+3 combined *vs* arm 1 (*p=0.5*)) (Figure 3.2, log rank test). Similarly, analysis for death alone demonstrated no difference between arms (Figure 3.3). The cumulative incidence of AESI events was greater in arm 3 vs arm 1 (p=0.02), however, when arms 2 and 3 were combined and compared to arm 1 this difference was less marked (p=0.18) (Figure 3.4).

The frequency of grade 5 MRS (severe disability) *or* death was 4 (arm 1) vs 3 (arm 2) vs 5 (arm 3), p=0.774. The frequency of good (defined as MRS grade 0-3), and bad outcomes (MRS Grade 4-6) were similar across arms (p=0.616) (Figure 3.5). *Post hoc* analysis of change in neurological function (as measured by MRS) found similar changes of MRS from baseline to day 56 between the

three arms (Figure 3.4a). Few IRIS events occurred (arm 1, 2; arm 2, 2; arm 3, 3), of which 4/7 were defined as neurological IRIS. Within the first 56 days of treatment, four participants developed new onset lower limb weakness (TB myelopathy 2; TB radiculomyelopathy/arachnoiditis 1; other (no cause found prior to death) 1); three participants developed a new onset hemiplegia; one patient developed a new onset isolated cranial nerve palsy (lower motor neuron VII). Thirteen participants presented with new onset seizures at TBM diagnosis. A further nine participants had new onset seizures within the first 2 months of follow up (arm 1, 5; arm 2; 2; arm 3, 2; *p=0.54*). Baseline and follow up imaging was performed in only 9 patients at the timepoints pre-specified within the protocol. Follow up imaging demonstrated new or worsening leptomeningeal enhancement in 2/9 participants (arm 1 and arm 2), new evidence of infarction in 2/9 participants (arm 1 and arm 2), new or worsening tuberculomas in 2/9 participants (arm 1 and arm 2) which was associated with worsening sulcal effacement in 1/9 participant (arm 1).

Spaghetti plot analysis of longitudinal CSF parameters (lymphocyte and polymorphonuclear cell count, protein and glucose) over time demonstrated downward trend of parameters across all three treatment arms (figure 3.6). Individual values are plotted and the superimposed line represents the mean values at each timepoint in each treatment arm. T tests comparing mean and variance at each time point demonstrated no difference between arms.

3.4 Discussion

The LASER-TBM study was a phase 2a RCT which evaluated the safety of high-dose rifampicin (35mg/kg daily), adjunctive linezolid (1200mg reducing to 600mg after 28 days) and adjunctive aspirin (1000mg daily) for the first 56 days of treatment in HIV-associated TBM. Primary endpoint analysis showed no significant difference in the incidence of AESI or death between treatment arms, although there was a trend towards an increase in events in arm 3. There was no difference in death or disability at day 56 across arms; and a similar frequency of clinical or radiological events occurred in each arm. Exploratory analysis found no difference in change in CSF parameters over time by arm.

Although secondary analysis revealed a significantly higher number of events (AESI or death) in arm 3 vs arm 1 ($p=0.04$), it is reassuring that no deaths were attributed to aspirin. Only one bleeding event occurred, after minimal exposure to high-dose aspirin (1 dose), resolved immediately following discontinuation of the drug, and was not associated with any laboratory markers to suggest significant gastrointestinal bleeding. Toxicity attributable to linezolid was similarly mild; of seven events potentially attributable to linezolid occurring in participants randomised to experimental arms, 3/7 were due to an alternative cause and 2/7 had recovered prior to the subsequent study visit. No patient was formally diagnosed with peripheral neuropathy, which is expected given the most recent studies showing a median time to onset of neuropathy occurs after 10 weeks of treatment (Wasserman et al., 2022, Conradie et al., 2020). Only one participant developed a change in visual acuity, which may have been due to linezolid, although on review by an ophthalmologist was assessed as more likely due to ethambutol. The number of participants in whom potential abnormalities were detected using the LogMAR and tumbling E assessments, compared to the confirmed number of cases of optic neuropathy calls into question the specificity of these outcome measures. Given that linezolid has potential to treat TBM as

well as drug resistant TB, we must consider whether better outcome measures can be developed to reliably detect abnormalities attributable to these drugs in both the clinical and research setting in order to prevent overestimation of toxicity, particularly when used for a short duration. Toxicity due to rifampicin was similarly infrequent with only two participants developing clinically significant transaminitis. In both cases the transaminitis recovered with treatment interruption. These results suggest that toxicity associated with the enhanced antitubercular regimen (rifampicin 35mg/kg and adjunctive linezolid) is not common when used in combination for two months to treat HIV-associated TBM. This is encouraging in the context of a disease where no specific evidenced-based antitubercular regimen exists and provides rationale for the ongoing phase 3 RCT (NCT04145258) where participants are randomised to receive both high-dose rifampicin and linezolid at doses identical to that used in this study.

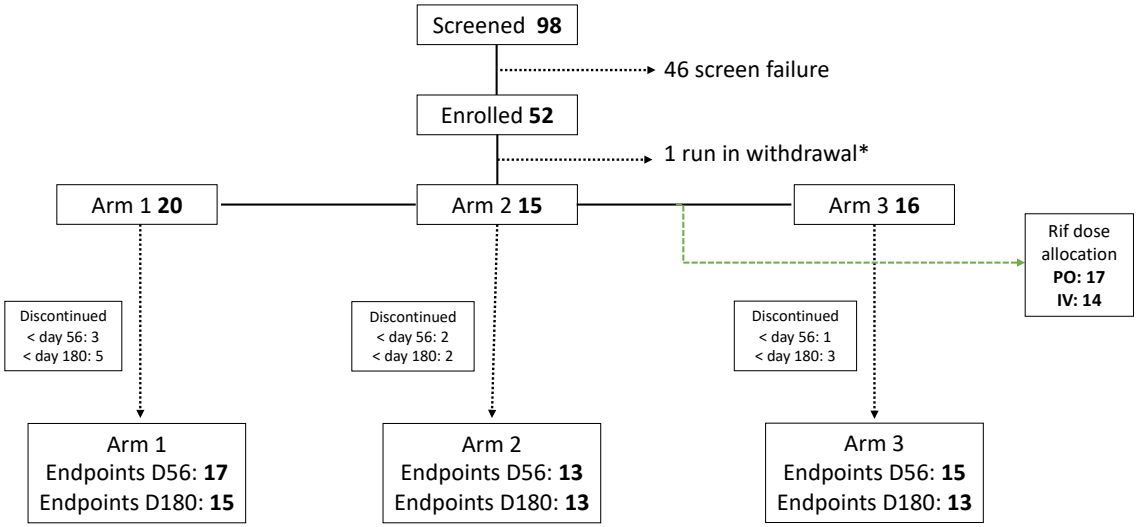
There are several limitations to this study. Although no formal power calculation took place, final sample size was substantially smaller than the target of 100 participants. The COVID-19 pandemic adversely affected recruitment to the study, and in January 2021 a decision was made to stop recruitment to enable commencement of a similar phase 3 study which was ready to start resulting in a lower than proposed sample size. It is unknown whether the significantly higher number of AESI or death in arm 3 vs arm 1 demonstrated within the secondary analysis reflects a true safety risk of the regimen containing aspirin, or is due to chance given the lower than anticipated numbers of participants recruited. Secondly, the majority participants recruited had mild TBM. The reasons for this is likely multifactorial including i) patients dying prior to screening given that up to 5 days of TB treatment was allowed prior to enrolment and ii) patients with decreased levels of consciousness arriving at hospital alone and therefore not having available next of kin available for proxy consent. In the latter case a

protocol amendment was approved to allow deferred consent in these patients, however, is likely to explain in part the higher rate of mild disease in our cohort. The mild level of disease within our patient cohort likely explains the low level of mortality; 16% 2-month mortality contrasted the oft quoted 50% mortality within the literature (Marais et al., 2011). The primary endpoint of AESI *or* death was designed with the assumption that observed mortality would be near or approaching 50%. The relatively few numbers of deaths led to a greater proportion of AESI in the composite endpoint of AESI *or* death. Given that all of the listed AESI were proportionally more likely to occur in the experimental arm 3, it is unsurprising that the number of events within the composite endpoint of AESI or death occurred in arm 3 where the greatest number of interventions was given. This is supported by the observation that when considering AESI alone, the cumulative incidence of events was significantly greater in arm 3, suggesting that the composite endpoint of AESI or death was driven by the higher rate of AESI in arm 3. The bias towards milder disease may also have affected the efficacy analysis. Given the nature of TBM and clinical trial research, it is challenging to ensure inclusion of those with severe TBM, in particular around gaining proxy or deferred consent in unconscious patients and ensuring early referrals to include those who are most likely to die early within the disease course. Future trials especially phase 3 RCT must endeavour to overcome these hurdles and include such patients to ensure generalisability of results.

Our study is the first RCT to evaluate linezolid in TBM and demonstrates that this important drug can be safely added to standard of care to treat HIV-associated TBM. It is also the first study to date to systematically evaluate the safety of a novel drug regimen containing enhanced antitubercular treatments alongside a host directed therapy in TBM, demonstrating that this approach can be safe. Our results reassure that high-dose rifampicin and linezolid may be safely combined

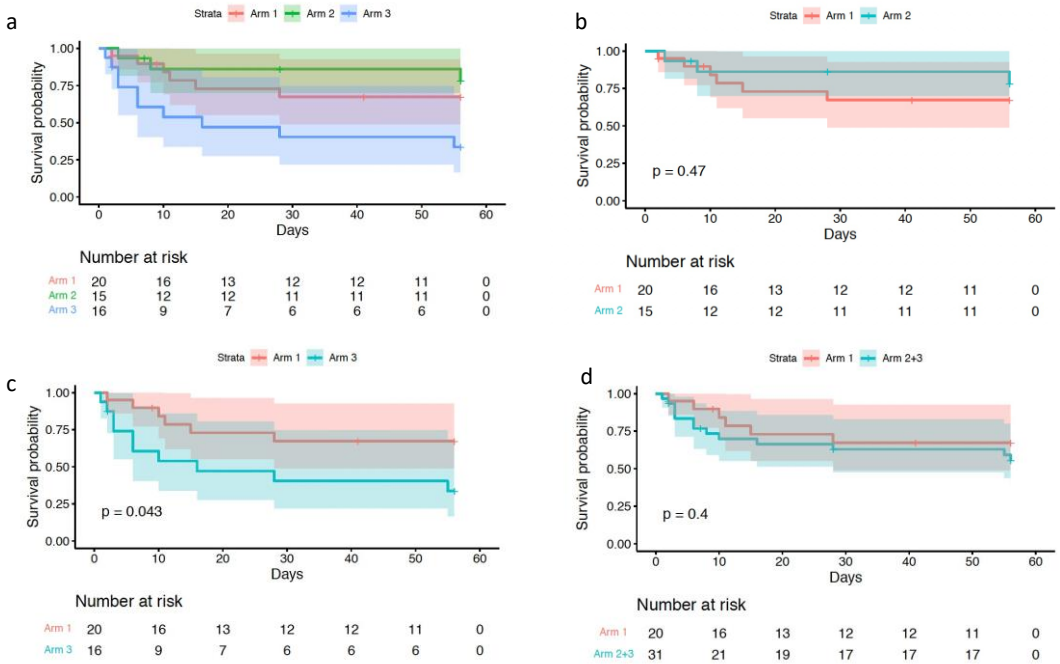
in HIV-associated TBM and supports evaluation of the efficacy of these drugs either alone or in combination in phase 3 trials. Within our study we did not see any significant bleeding events with the use of high-dose aspirin: a larger study is now required to see if potential harm is offset by a morbidity and mortality benefit.

Figure 3.1 CONSORT diagram for LASER-TBM



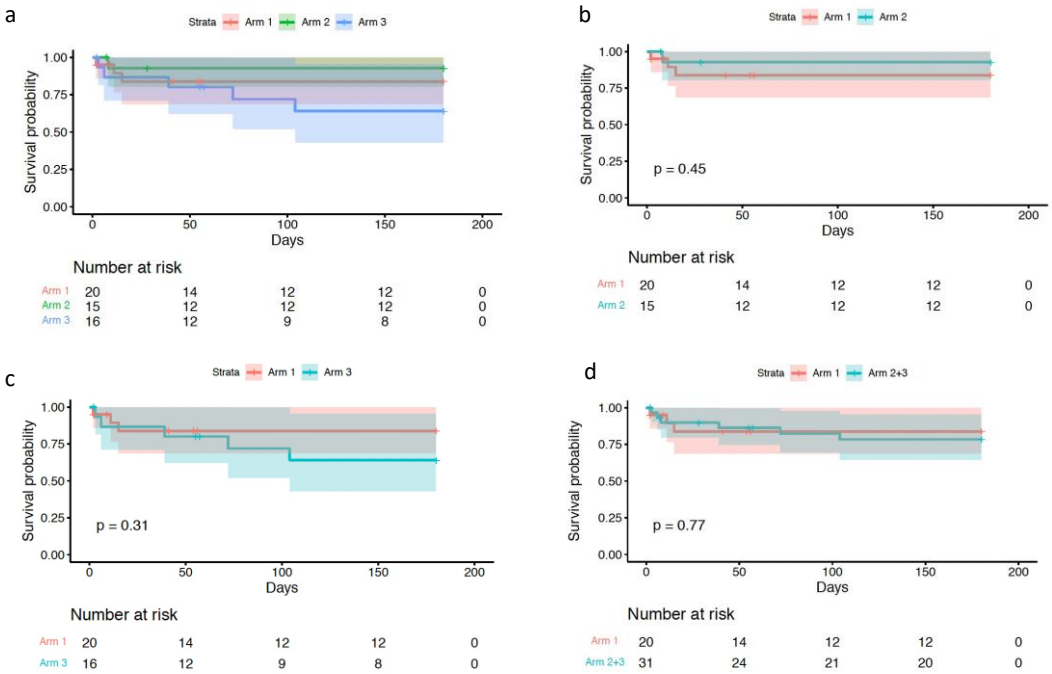
CONSORT diagram to describe recruitment and arm allocation. Reasons for screening exclusions and early study withdrawals are listed in Tables 3.6 and 3.7; Rif, rifampicin; *patient randomized but withdrawn prior to receiving study IP due to emergence of exclusion criteria

Figure 3.2 Kaplan-Meier analysis of time to worst grade adverse events of special interest or death



Kaplan-Meier analysis of time to worse grade AESI or death, comparing arm 1, 2 and 3 (a), arm 2 vs arm 1 (b), arm 3 vs arm 1 (c) and arm 2 and 3 combined vs arm 1 (d).

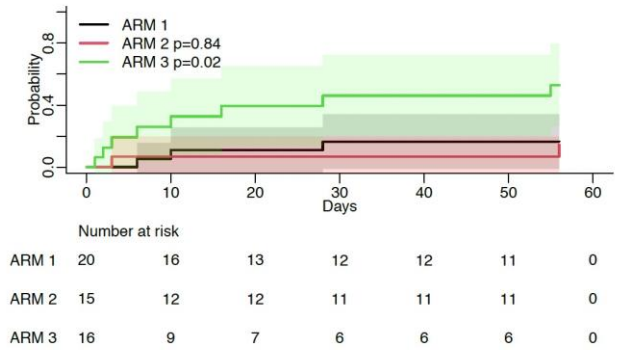
Figure 3.3 Kaplan-Meier analysis time to death



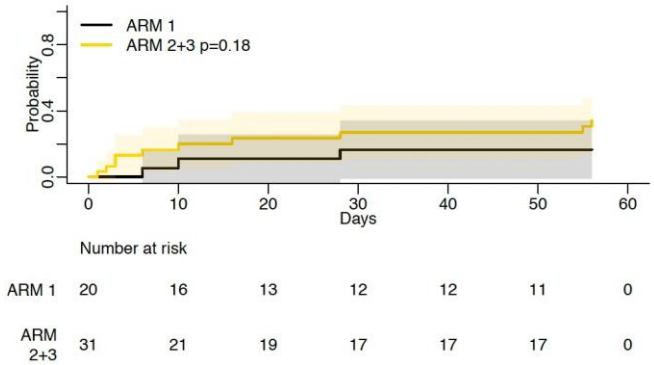
Kaplan-Meier analysis of time to death, comparing arm 1, 2 and 3 (a), arm 2 vs arm 1 (b), arm 3 vs arm 1 (c) and arm 2 and 3 combined vs arm 1 (d).

Figure 3.4 Kaplan-Meier analysis time to worst grade adverse event of special interest

a



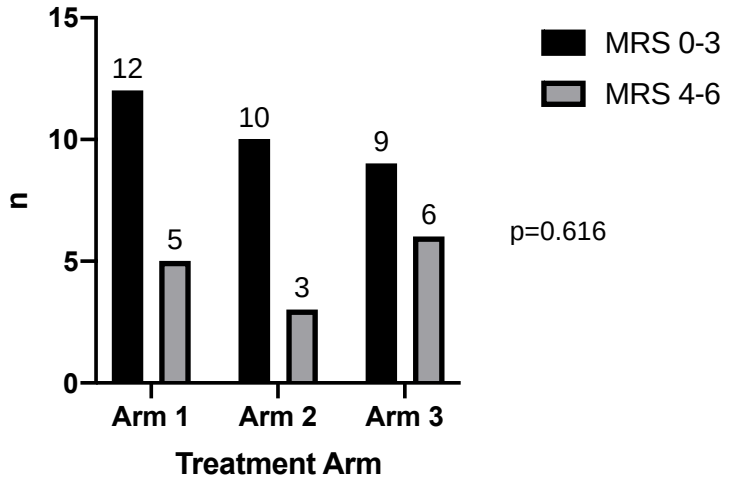
b



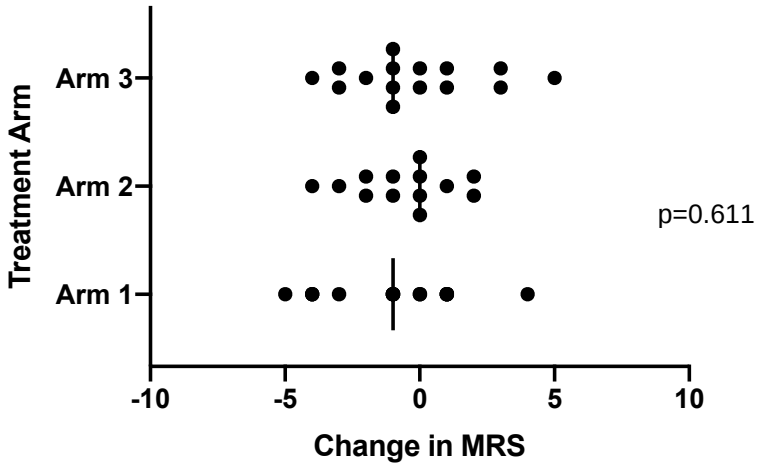
Kaplan-Meier analysis of time to adverse event of special interest (AESI), comparing arm 1, 2 and 3 (a), and arm 2 and 3 combined vs arm 1 (b).

Figure 3.5 Functional neurological outcome at day 56 as defined by modified Rankin scale

a

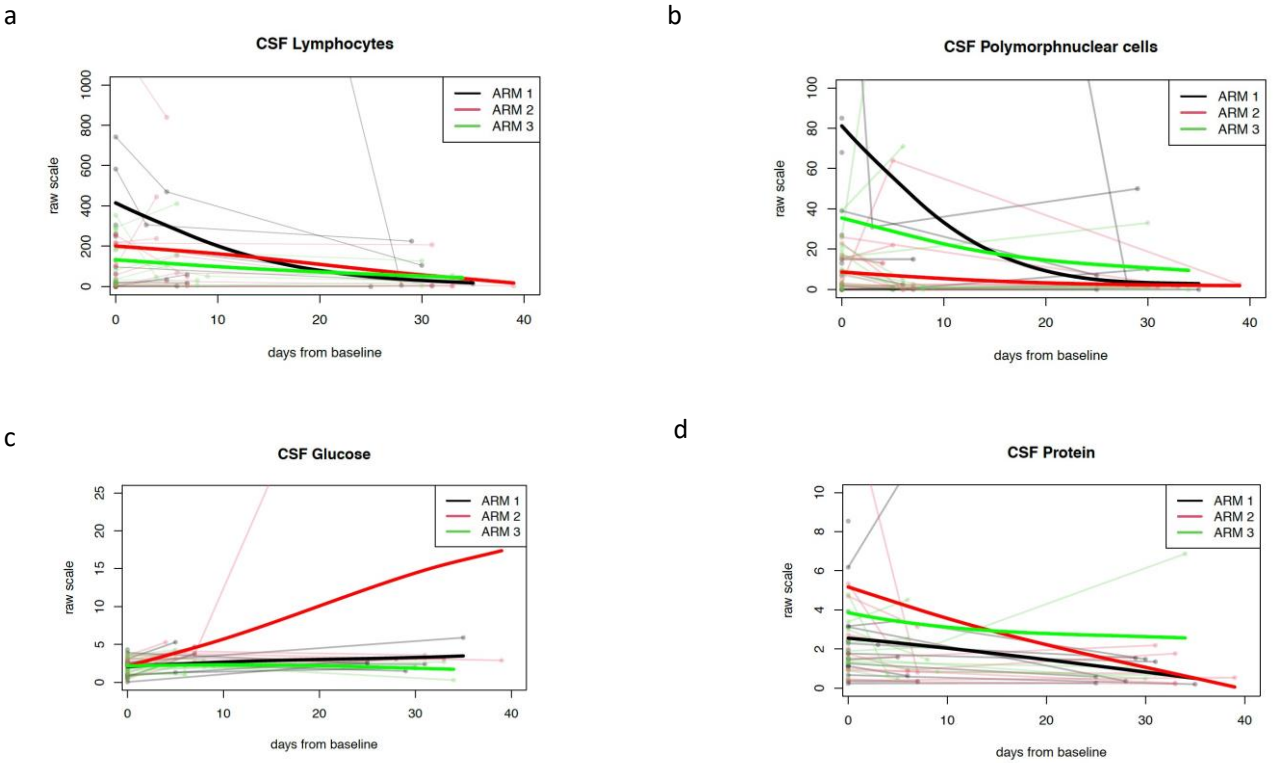


b



A comparison between good outcome (MRS 0-3) vs bad outcome stratified by arm at day 56; 'b' compares change in MRS between enrolment and day 56, across treatment arms.

Figure 3.6 Change in CSF parameters over time



Spaghetti plots for CSF parameters (lymphocyte count (a), polymorphonuclear cells (b), glucose (c), protein (d)) plotted as individual values over time (faint lines), with mean values for each treatment arm represented by superimposed line (bold lines).

Table 3.1 Adverse events of special interest (AESI) assessed in LASER-TBM

AESI	Investigational product	Objective measure
Gastrointestinal haemorrhage	Aspirin	Clinical and laboratory measures to suggest haemorrhage
Intracerebral haemorrhage	Aspirin	Radiological evidence of haemorrhage
Transaminitis	Rifampicin	ALT, bilirubin (DAIDS criteria, Grade 3 and 4)
Anaemia	Linezolid	Hb (DAIDS criteria, Grade 3 and 4)
Neutropenia	Linezolid	Neutrophils (DAIDS criteria, Grade 3 and 4)
Thrombocytopenia	Linezolid	Plt count (DAIDS criteria, Grade 3 and 4)
Peripheral Neuropathy	Linezolid	1 grade increase on the BPNS and/or a 2 grade change in any modality on the mTNS
Change in LogMAR score (visual acuity)	Linezolid	Change of 0.2 on the LogMAR/Tumbling E Chart

ALT, alanine transaminase; DAIDS, Division of AIDS; BPNS, Brief Peripheral Neuropathy Score; mTNS, modified Total Neuropathy Score.

Table 3.2 Baseline demographics and clinical characteristics in LASER-TBM

	Arm 1 (n=20)	**Arm 2 (n=15)**	**Arm 3 (n=16)**
Age, median (IQR) years	39.5 (34-48.5)	37 (34.5–42.5)	41.5 (31.8-46)
Gender, male n (%)	10 (50)	10 (66.7)	16 (62.5)
Uniform case definition, n (%) - Definite - Probable - Possible	 8 (40) 5 (25) 7 (35)	 3 (20) 4 (26.7) 8 (53.3)	 6 (37.5) 4 (25.0) 6 (37.5)
BMRC TBM Grade, n (%) - Grade 1 - Grade 2 - Grade 3	 11 (55) 8 (40) 1 (5)	 11 (73.3) 4 (26.7) 0 (0)	 8 (50) 8 (50) 0 (0)
CD4 T-cell count, median (IQR), cells/μL	116.5 (58.6-283)	131 (82.5-186)	158.5 (85.5-331.5)
HIV viral load, median (IQR), copies/mL	89,150 (1000-203,711)	37,960 (2428 – 394,839)	2686 (1361-777,620)
ART status, n (%) - On ART - Previous ART - ART naive	 6 (30) 3 (15) 11 (55)	 5 (33) 6 (40) 4 (27)	 5 (31) 5 (31) 6 (38)
Of those on ART, duration in weeks, median (range)	288.9 (22.4-459.3)	23.7 (0.4-83.6)	355 (2.9-879.1)
CSF cell count/biochemical data available (n)	17	14	13
Polymorphonuclear cells, median (IQR), cells/ μL	13 (0-85)	4 (2-16)	16 (3-22)
Lymphocytes, median (IQR), cells/ μL	63 (10-259)	79 (11-218)	82 (28-278)
Protein, mg/dL	1.78 (1.13-3.13)	1.89 (0.95-4.2)	1.9 (1.32-2.99)
CSF Glucose, mg/dL	2.2 (0.9-2.5)	2.4 (1.9-2.9)	1.7 (1.2-3.3)
Baseline radiology available (n)	16	12	11

Hydrocephalus n (%)	1 (6.3)	1 (8.3)	1 (9.1)
Meningeal enhancement n, (%)	4 (25)	2 (16.7)	6 (54.5)
Tuberculoma(s) n, (%)	1 (6.3)	2 (16.7)	2 (18.2)
Infarct(s) n. (%)	4 (25)	1 (8.3)	3 (27.3)

Abbreviations: IQR, interquartile range; ART, antiretroviral therapy; BMRC, British Medical Research

Table 3.3 AESI stratified by treatment arm

	Arm 1 (n=20)	**Arm 2 (n=14)**	**Arm 3 (n=16)**	***p value* ****
Bleeding, n* (%)	0 (0)	0 (0)	1 (6)	*0.338*
Transaminitis, n (%)	0 (0)	0 (0)	2 (13)	*0.109*
Hematological, n (%)	2 (10)	0 (0)	1 (6)	*0.481*
Peripheral Neuropathy, n (%)	2 (10)	2 (14)	4 (25)	*0.46*
Change in LogMAR score, n (%)	0 (0)	2 (14)	2 (13)	*0.231*

*individuals with an event
** arm 3 *vs* arm 1

Table 3.4 Details of AESI by event

AE name	Treatment arm	Days of treatment	DAIDS grade	Pre-existing	Outcome
Melaena	3	1	1	No	Two episodes of black stool. No associated change in Hb or urea. Aspirin stopped and not restarted as per protocol. No further events.
Transaminitis	3	16	3	No	Improved to grade 2 but not restarted on high-dose rifampicin at discretion of site PI.
Transaminitis	3	6	4	No	Improved, successfully rechallenged with rifafour FDC. High-dose rifampicin not restarted per protocol.
Neutropenia	1	13	3	Yes	No change in study medication (arm 1)
Neutropenia	3	28	3	No	Linezolid stopped, resolved. Not restarted.
Anemia	1	28	3	No	No change in study medication (arm 1)
Neurosensory symptoms	1	10	2	No	No change in study medication (arm 1). Normal at subsequent visit.
Neurosensory symptoms	1	6	2	No	No change in study medication (arm 1). Normal at subsequent visit.
Neurosensory symptoms	2	3	1	No	Linezolid stopped. MRI show anterior cord changes (possible ischaemic or inflammatory aetiology). Not restarted on linezolid although felt clinically not to be consistent with peripheral neuropathy.
Neurosensory symptoms	2	7	1	No	All study medication stopped due to relocation of participant and therefore withdrawal from study. No follow up BPNS performed.
Bilateral lower limb weakness	3	18	2	No	Linezolid stopped. MRI showed changes consistent with TB radiculopathy//arachnoiditis. Linezolid not restarted at discretion of site PI , although clinically unlikely peripheral neuropathy.
Paresthesia left leg	3	18	1	No	
Neurosensory symptoms	3	3	1	No	Linezolid stopped. Normal at subsequent visit although linezolid not restarted at discretion of site PI.
Neurosensory symptoms	3	10	1	No	Linezolid stopped. Participant subsequently died, cause of death not related to linezolid.

Asymptomatic increase in BPNS	3	13	1	No	Linezolid stopped. Normal at subsequent visit. Linezolid restarted at 600mg as per protocol.
Increase in LogMAR	2	56	1	No	Noted on day 56 visit, therefore no change in study medication. No follow up notes.
Increase in LogMAR	2	55	1	No	Optic neuropathy ruled out by ophthalmology. Linezolid restarted.
Change in visual acuity with related change in LogMAR	3	42	4	No	Seen by ophthalmology. Diagnosis: Parietal stroke +/- ethambutol related optic neuropathy.
Increase in LogMAR	3	55	1	No	Noted on day 56 visit, therefore no change in study medication. No follow up notes.

Abbreviations: Hb; haemoglobin; FDC, fixed dose combination; BPNS, Brief Peripheral Neuropathy Score; LogMAR, Logarithm of the Minimum Angle of Resolution.; MRI, Magnetic Resonance Imaging; PI, principal investigator.

Table 3.5 Timing and cause of death prior to day 56

Cause of death	Treatment Arm	Days of IP
Renal Failure	1	11
TB Meningitis	1	15
TB Meningitis	1	2
TB Meningitis	2	8
TB Meningitis	2	0*
Pulmonary embolism	3	39
TB Meningitis	3	6
TB Meningitis	3	3

*death prior to receiving study IP and therefore excluded from modified intention to treat population analysis

Table 3.6 Reasons for screening exclusion

Reason*	n	%
Chronic aspirin or NSAID use	9	13.2
HIV uninfected	8	11.8
Investigator discretion	8	11.8
Received more than 5 days of anti-TB treatment in 30 days prior to enrolment	7	10.3
Not ‘possible’, ‘probable’ or ‘definite’ TBM	6	8.8
Haemoglobin < 8 g/dL	5	7.4
INR > 1.4	4	5.9
No consent for enrolment given by the patient	3	4.4
Peripheral neuropathy scoring Grade 3 or above on modified BPNS	3	4.4
Standard TB treatment contraindicated	2	2.9
Known rifampicin resistance during this episode	2	2.9
Previous drug resistant TB	2	2.9
Uses a 'disallowed medication' that cannot safely be stopped	2	2.9
Pregnant (>17 weeks at baseline)	1	1.5
Allergy to RHZE, LZD, aspirin	1	1.5
Died before enrolment	1	1.5
Evidence of bacterial or cryptococcal meningitis	1	1.5
eGFR < 20	1	1.5
Platelet count < 50 109/L	1	1.5
Relocation prior to enrolment	1	1.5

Abbreviations: NSAID, non-steroidal anti-inflammatory drug; BPNS; brief peripheral neuropathy score; Rif, rifampicin; RHZE; rifafour; LZD, linezolid; ASA, aspirin

*more than one reason can be assign to each participant

Table 3.7 Reasons for study withdrawal prior to day 56

Withdrawal	Reason
1	Participant relocated to Malawi and therefore unable to attend study follow up visits
2	Participant relocated to Kwazulu-Natal and therefore unable to attend study follow up visits
3	Participant withdrew consent
4	Participant withdrew consent
5	Participant developed acute psychosis and was unsafe to follow up
6	Participant lost to follow up

Chapter 4. Cognitive Impairment in Tuberculous Meningitis

4.1 Introduction

Tuberculous meningitis (TBM) affects approximately 100,000 people per year (Wilkinson et al., 2017). Cognitive impairment is often referred to as a common complication in TBM, however only four studies have reported its frequency. Two of these used brief screening tests rather than comprehensive cognitive batteries (Ranjan et al., 2003, Kalita et al., 2007), and another gathered information on cognition from the clinical history alone (Anderson et al., 2010). The only study to undertake more comprehensive cognitive testing assessed a small group of 17 TBM participants (Chen et al., 2015). No studies have systematically evaluated the frequency of cognitive impairment following TBM including affected cognitive domains or described the effect of cognitive performance on functional outcome. Moreover, no published studies have compared findings to an appropriate control group, considered the contribution of HIV co-infection or non-central nervous system (CNS) TB disease, or been undertaken in an African setting where TBM is endemic.

Better understanding of cognitive impairment in TBM is crucial for several reasons. Firstly, objective measures of cognition are a quantitative measure of clinical outcome which are infrequently used in clinical trials in adult TBM. By contrast, paediatric studies routinely consider these as part of a neurodevelopmental outcome assessment (Davis et al., 2019a). Developing an accessible battery of cognitive tests which taps into

cognitive domains known to be impaired in TBM, for use in TBM endemic settings, would improve the precision of measurable outcomes for TBM studies. Secondly, recent TBM pathogenesis studies have unveiled mechanisms of brain injury such as the upregulation of neuroexcitatory pathways (Rohlwink et al., 2019) and release of damage associated proteins also seen in neurodegenerative conditions (Herbst et al., 2019). These findings must encourage us to better understand whether cognitive impairment leads to longer-term disability in TBM. In particular, whether cognitive impairment is focal, attributable to discrete structural abnormalities in the brain e.g. stroke or tuberculomas and/or whether there is a clinical presentation in keeping with a diffuse cortical or subcortical process at play. Most importantly however, understanding cognitive and functional impairment in TBM, particularly its effect on treatment adherence, will improve the long-term care of patients with this condition; including the provision of appropriate resources to aid recovery of these individuals following TBM.

In a case-control study of HIV-associated TBM we aimed to:

i) Evaluate the frequency and nature of cognitive impairment in HIV-associated TBM using formal cognitive testing alongside physician assessment;
ii) Assess the pattern of impairment, and correlate with radiological and neurological measures, to understand whether cognitive impairment relates to focal brain injury, diffuse inflammation, or a combination of these;
iii) Assess the suitability of currently available screening tools to identify cognitive impairment;

iv) Measure the impact of impaired cognitive performance on functional outcomes, including treatment adherence.

4.2 Methods

Participants

We drew participants from three parent studies that took place in Cape Town between 2015 and 2020 (Table 4.1). All studies were performed in similar adult populations in a low-income, peri-urban area of Cape Town, South Africa, with high HIV and TB prevalence.

Participants formed three groups:

i) HIV-associated TBM cases (from the LASER-TBM and Albertyn studies).

ii) Comparator group 1: PLWH, no history of TB (from the CONNECT study).

iii) Comparator group 2: PLWH, non-CNS TB (from the Albertyn study).

Normative cognitive data was drawn from HIV-negative individuals selected to be demographically similar to PLWH in the CONNECT study as described in the statistical methods below.

The studies were each approved by the University of Cape Town's Faculty of Health Sciences Research Ethical Committee (Table 4.1).

Sample size was pragmatic bringing together two unpublished data sets of HIV-associated TBM patients (cases), and non-CNS TB patients

(comparator group 2). The decision to include PLWH (comparator group 1) at a 1:2 ratio was made to increase statistical power for this analysis.

Outcome measures and procedure

Assessment took place at 6 months following diagnosis of TBM or non-CNS TB, or at the time of enrolment in comparator group 1. Timing of follow up was dictated by the umbrella studies from which these participants were recruited.

Baseline assessments

We graded severity of TBM at baseline using the modified British Medical Research Council (BMRC) scale (Thwaites and Tran, 2005), and classified TBM as 'definite', 'probable' or 'possible' as per the uniform case definition for research(Marais et al., 2010). We collected data on educational history, drug and alcohol use; in the LASER and CONNECT cohorts the Alcohol Use Disorders Identification Test (AUDIT) (Barbor, 2001) (cut-off ≥20, 'high risk') and Drug Use Disorders Identification Test (DUDIT) (Berman and https://doi.org/10.1037/t02890-000) (cut-off ≥6 (men), ≥2 (women)) questionnaires were used to ascertain alcohol and drug use respectively. Within the Albertyn cohort, active alcohol or illicit drug use was an exclusion criterion.

Cognitive testing

Cognitive testing was performed in the participants' first language in all cases. An identical cognitive test battery was administered across the three studies to assess 10 measures in 7 cognitive domains: motor skills, processing speed, attention and working memory, fluency, audioverbal and visuospatial learning and memory and executive function (see

Supplementary Material Box 1 published alongside (Davis et al., 2022a)). This is based on a battery with established psychometric validity in South Africa (Nyamayaro et al., 2019). Participants from LASER-TBM also underwent a comprehensive neurological examination, performed by a trained physician, to identify focal cortical syndromes. This included assessment for motor, sensory and cranial nerve abnormalities, language deficits, apraxia, visual agnosia, and right hemisphere dysfunction (visuospatial deficit, anosognosia, sensory neglect). The physician assessment was performed in English with where necessary an isiXhosa translator.

Imaging

Within the LASER cohort we correlated results from cognitive assessment with computed tomography (CT) brain scans (≤2 months since diagnosis) reported by an independent blinded neuroradiologist.

Mental health assessment

All participants completed a Beck Depression Inventory ((BDI) (Upton, 2013) (cut-off 'depression' >17) or CES-D (Radloff, 1977) (cut-off 'depression' ≥16), to assess mood.

Functional measures

We administered the Patient's Assessment of Own Functioning Inventory (PAOFI) (Chelune et al., 1986) in all participants recruited to LASER-TBM. We calculated total score, and total number of responses with 'affirmative responses' as previously described (Woods et al., 2004, Bell et al., 2013). Lower total PAOFI scores indicate higher levels of functioning; ≥3 'affirmative' responses have been previously described as a cut-off for functional impairment. Within LASER-TBM we collected data on treatment adherence for the first 56 days of treatment. Self-reported adherence was assessed by asking participants at each visit if they had missed any doses

since their last visit. Observed adherence was assessed by totalling number of missed doses noted on pill count at each study visit.

Cognitive screening measures

Two screening measures i) Montreal Cognitive Assessment (MoCA) (Nasreddine et al., 2005) (cut-off indicating impairment ≤26) and ii) CatRAPID v2.0 (Joska et al., 2016) (cut-off indicating impairment ≤16) were performed in participants recruited to LASER-TBM.

Statistical analysis

We used R v.4.1.2(2021-11-01), RStudio v.2021.09.0 and GraphPad Prism v.9.3.1, to complete all analyses, with the threshold for statistical significance set at $\alpha=0.05$.

First, we processed and standardized the cognitive test battery data. Normative scores for the cognitive tests were based on healthy control data collected by the CONNECT study. These data were collected between 2018 and 2020 from healthy HIV-negative community-dwelling individuals who presented to the same community health clinics in Gugulethu from which the PLWH comparator group was recruited, within the same area of Cape Town where TBM and TB participant recruitment took place. They therefore shared key demographics (age, ethnicity, language, education), as well as psychosocial and socioeconomic characteristics. We used the control data to calculate demographically corrected z-scores (mean [M] = 0, standard deviation [SD] = 1), using standard regression-based norming processes. The z-scores were then converted to demographically corrected T-scores (M=50, SD=10). If participants had z-scores >5 SD below the mean, the conversion to a T-score resulted in a negative T-score. In these

cases, we assigned a score of 0, the lowest possible T-score to maintain the clinical significance of the low performance. Cognitive performance data were summarised into domain and global T-scores by taking the average of T-scores within each domain and then across domain T-scores. T-scores were converted to deficit scores (See Supplementary Material Box 2 published alongside (Davis et al., 2022a)). The overall global deficit score (GDS) was calculated by averaging deficit scores. A cut-off GDS of ≥0.5 has been considered consistent with 'cognitive impairment' on cognitive test performance (Carey et al., 2004); for the purposes of this study we termed this group as having 'low performance on cognitive testing' whilst the clinical significance and functional impact in TBM is further explored, aligned with recent trends in the HIV literature (Nightingale et al., 2021)

Second, we used the Matchit package in R (https://cran.r-project.org/web/packages/MatchIt/vignettes/MatchIt.htmlR) to select comparator group 1 (PLWH, no history of TB) from the parent study CONNECT with the ratio 1:2 with matched co-variants (age, gender, education) to TBM cases so distributions of covariates in the two groups were approximately equal. We subsequently processed the cognitive test data for this group as described above.

We checked normality of distribution of data with a Shapiro-Wilk test. We determined between group differences in baseline and clinical variables using either parametric T tests or Mann-Whitney (for continuous variables where data was normally or not normally distributed, respectively), or chi-squared tests (for dichotomous variables). We performed a primary comparison of cognitive test performance between HIV-associated TBM cases and comparator group 1, and subsequently HIV-associated TBM

cases and comparator group 2; we did not perform an ANOVA given that our primary aim was to evaluate differences between TBM cases and each comparator group individually, rather than differences between the comparator groups or across the three groups. We compared total PAOFI scores using a Mann-Whitney test of significance in HIV-associated TBM participants with low cognitive performance vs those without. We compared adherence between those with and without low cognitive performance by i) self-report of missing ≥1 dose with Fishers Exact, and ii) observed adherence from number of missed doses on pill count within the first 56 days of treatment with a Mann-Whitney test. We used a 2x2 table of agreement to create a Cohen's Kappa value for agreement between the screening tests (cut-offs: CatRAPID ≤16; MoCA ≥26) and GDS ≥0.5 on the cognitive test battery and assigned levels of agreement as per a published scale (Landis and Koch, 1977).

4.3 Results

We included 34 participants with HIV-associated TBM (cases), 66 participants with HIV and no history of TB (comparator group 1), and 26 participants with HIV and non-CNS TB (comparator group 2) (Figure 4.1). Age, gender and years of education were similar across groups (Table 4.2). IsiXhosa was the first spoken language in 119/126 (94%); in 2/126 (1.6%) it was English and in 3/126 (2.4%) it was Shona. This information was missing in 2 cases. All HIV-associated TBM cases were either mild (BMRC 1) (18/34, 53%) or moderate (BMRC 2) (16/34, 47%) at presentation. Within LASER-TBM, 2/19 participants reported head injury resulting in loss

of consciousness, 2 participants had ‘high risk’ alcohol use, and 0 participants had ‘high risk’ drug use. These were exclusions in the CONNECT and Albertyn studies.

In those with HIV-associated TBM, 16/34 (47%) of participants had low performance on cognitive testing consistent with cognitive impairment (GDS≥0.5). When compared to comparator group 1 (PLWH, no history of TB), those with TBM had worse global T scores (mean score 41 vs 48, *p<0.0001*), with a greater proportion of those within the TBM group meeting the GDS cut-off ≥0.5) (16/34 (47%) vs 17/66 (26%), *p=0.032*). Domain specific T scores for all cognitive domains were significantly worse in the TBM group, with the exception of attention and working memory (Table 4.3A). When compared to comparator group 2 (PLWH, non-CNS TB), global T scores were also worse in those with TBM (mean score 41 vs 46, *p=0.016*). Although a greater proportion of those with TBM met the GDS cut-off for cognitive impairment (≥ 0.5) (16/34 (47%) *vs* 8/26 (31%)), this was not statistically significant (*p=0.201*) (Table 4.3B). Global T scores were worst in those with HIV-associated TBM (cases), better in those with HIV and non-CNS TB (comparator group 2), and best in those with HIV alone (comparator group 1) (Figure 4.2). Radiological assessment (either CT, MRI, or both) was performed in 16/19 participants included in LASER-TBM who had imaging performed either as part of the study or for a clinical indication at presentation. 7/16 (43%) of participants had abnormal imaging; 7/16 (43%) had meningeal enhancement and 4/16 had stroke (33%). 1 participant had multiple calcified granulomas, and another hydrocephalus. In the 4 participants with stroke, 3 had a GDS consistent with low cognitive performance. In these participants the individual domain T scores were reviewed by a clinical neuropsychologist (ADr) and

neurologist (AD) who concluded that the profile of abnormality across the T scores could not be explained by the anatomical location alone. Although we believe that in these cases the infarcts are likely to be contributing to the burden of cognitive impairment, it was felt that the scores represented a more global picture in each of the cases. There was no statistically significant difference in the number of those found to have abnormal imaging in those with low cognitive performance versus those without (6/10 vs 2/6, p0.6084). Physician assessment did not reveal any clear cases where focal motor and sensory deficits correlated to impairment in a discrete corresponding cognitive domain e.g. due to focal stroke or tuberculomas.

The mean score for CATRAPID v2.0 and MoCA screens were performed in 19 TBM participants (the LASER-TBM cohort) and the means (SD) were 16 (3.15) and 21 (3.70) respectively. Using a cut-off of ≤16 for CATRAPID and ≤26 for MoCA, 7 (34%) and 17 (89%) participants respectively would have been flagged as having mild cognitive impairment using these screening tests. A Kappa value of 0.242 (95% CI -0.179-0.661) for CatRAPID and 0.137 (95% CI -0.115-0.389) for MoCA equated to ‘fair’ and ‘slight’ agreement respectively when comparing these measures to a GDS≥0.5 on the cognitive test battery.

Proportionally more participants had depression in the TBM group than the comparator group 1 (8/34 vs 6/66, *p=0.049*); whereas no difference was found between the TBM cases and comparator group 2 (8/34 vs 5/26, *p=0.76*). In the sub-group of participants with TBM where PAOFI was completed (n=19) we found that lower cognitive performance in TBM cases was associated with better functional status (mean (SD) of PAOFI score in

patients with low cognitive performance vs those without, 23.5 (14.8) vs 40.6 (19.1), *p=0.042*). When comparing a cut-off of 3 or more affirmative responses corresponding to 'functional impairment', the difference was not statistically significant (5/11 (45%) 'functionally impaired' in group with low cognitive performance, vs 6/8 (75%) 'functionally impaired' in group without low cognitive performance, *p=0.352*). Given the unexpected finding of worse functional outcomes in those normal cognitive performance, we further explored the cases individually. Although PAOFI is a measure of functional status, the questionnaire centres around self -reporting of cognitive symptoms; it is therefore plausible that low mood, lack of insight and premorbid status may affect reporting of cognitive functioning. We identified one case where performance on the cognitive test battery was severely impaired (GDS 2.85), yet PAOFI scores were low (affirmative score 0, total score 18) suggesting lack of insight and underreporting of impairment. We identified two cases where the BDI suggested clinical depression and PAOFI scores were high (i.e. high burden of cognitive symptoms), yet performance on the cognitive test battery was within normal limits suggesting over-reporting of symptoms associated with low mood. In another case, PAOFI scores were high (affirmative 12, total 71), yet GDS suggested cognitive performance within the low-normal range (GDS 0.428). This individual however had high pre-morbid functioning (completed 12 grades education) and therefore a drop in cognitive functioning may not have been identified using a GDS cut-off of ≥ 0.5.

Within the LASER-TBM cohort, 6/11 (55%) of those with low cognitive performance reported missing medication doses compared to 3/8 (38%) of those with normal cognitive performance. Mean number of missed doses was 2.72 in those with low cognitive performance compared to 0.37 in

those normal cognitive performance. Neither difference was statistically significant.

4.4 Discussion

In this case-control study of HIV-associated TBM we evaluated the frequency and nature of cognitive impairment and functional outcomes, including treatment adherence. Within our study, almost half of participants with HIV-associated TBM demonstrated low performance on cognitive testing. This was a significantly greater proportion of individuals when compared to an age, gender and education matched comparator group of participants with HIV alone; demonstrating that impairment seen in HIV-associated TBM is in addition to that attributable to HIV alone. We also showed that participants with HIV-associated TBM perform worse than those with HIV and non-CNS TB suggesting that low cognitive performance in TBM is due to CNS-specific pathology in addition to any other mechanisms at play in non-CNS TB disease such as systemic inflammation and polypharmacy (Kass and Shandera, 2010, Annane and Sharshar, 2015).

Low cognitive performance was seen across all cognitive domains in participants with TBM when compared to those with HIV only, with the exception of attention and working memory. Within our cohort we did not see cases where motor/sensory deficits on neurological examination and radiological findings correlated with a single focal cognitive deficit. These findings begin to characterise low cognitive performance in TBM as generalised, affecting multiple cognitive domains which are, at least in our

cohort, beyond what is attributable alone to structural deficits e.g. stroke. The predominant motor impairment suggests subcortical damage, and the relative sparing of attention and working memory suggests that ongoing delirium is unlikely to explain our findings. These observations also highlight the limitations of CT as an imaging modality in identifying changes such as cortical and subcortical inflammation and microvascular damage which may present clinically with generalised cognitive deficits. These are important findings when considering i) pathogenic mechanisms in TBM and ii) suitable imaging techniques for identifying those at risk of impairment.

In the subgroup where PAOFI was administered, the finding of better self-reported functional status in those with low cognitive performance compared to those with normal cognition was unexpected. Assessment of individual cases suggested examples where functioning may have been under-reported due to lack of insight, over-reported due to low mood, or not reflected in the GDS cut-off due to higher pre-morbid functioning. These examples illustrate the complexity of measuring cognitive and functional performance in diverse populations, and the potential limitations of dichotomised cut-offs for impairment based on cognitive performance alone. More work is needed to explore the association of cognitive performance with clinical indicators of cognitive impairment, functional outcomes and measures of brain injury; an area currently being explored in the field of cognitive impairment in PLWH (Nightingale et al., 2021). Future studies may consider including i) observer accounts of functional status (eg DECO (Ritchie and Fuhrer, 1996)) to add clarity where other factors e.g. depression may bias self-reporting of functional status and ii) more familiar functional outcome measures in this setting (e.g. Modified Rankin Score) in

order to contextualise the results of more comprehensive measures for the treating physician.

We found no statistically significant difference in self-report or objective measures of treatment adherence between those with and without low cognitive performance, which may be due to the small number of participants where this data was available (n=19). Medication non-adherence is the major cause of poor outcome in TBM, hence this potential association should be further explored in larger cohorts as it may provide new avenues to address adherence and improve outcomes in this group.

There are limitations to this study. Firstly, although all cognitive test batteries were administered by trained individuals, these were different across the three studies. Although training and alignment was overseen by the same neuropsychologist (ADr) to minimise interrater variability we acknowledge the possibility that subtle differences in administration may have influenced outcomes. Along these lines, in 4 of the 7 domains only one measure was used across all three studies and therefore included within the analysis, when ideally >1 measure should be used for each domain. This may explain the unexpected finding of preserved attention and working memory despite this being an early indicator of pathology in many sub-cortical dementias. Secondly, there were differences in the timing of investigations related to HIV status across the three studies; specifically, CD4 counts were collected at different time points (within a range of 12 months) in relation to the cognitive testing making them incomparable between groups. Given the nature of recruitment (inpatient recruitment for unwell TBM subjects, vs outpatient recruitment for well PLWH comparator group) it is highly likely that HIV disease was better

controlled within comparator group 1 than in those with TBM. Nonetheless this does not explain the finding of ~50% low cognitive performance within the TBM group (with no difference in HIV control between those with low cognitive performance and those without) and it is unlikely to entirely explain the large differences we demonstrated between the TBM and PLWH groups. Also, given that CD4 count was on average higher in those with TBM, compared to those with non-CNS TB who performed better on cognitive testing, it is unlikely that between group differences in cognitive performance is likely explained by HIV control alone. Such differences, together with differences in cognitive impairment prior to the development of TBM could be addressed within a prospective study design which should be considered in future research within this field. Thirdly, the lack of TBM cases with severe (BMRC Grade 3) disease may suggest that the frequency of low cognitive performance is underestimated. In LASER-TBM recruitment of stage 3 disease was infrequent, and none of the participants followed up with full cognitive testing had stage 3 disease at baseline. Similarly, no participant included within the Albertyn study had stage 3 disease. Inclusion of participants fortunate enough to survive stage 3 disease should be included within future studies to ensure generalisability of results. Finally, our findings of low cognitive performance at 6 months following TBM diagnosis should not be interpreted as a finding of long-term disability in this population. Longer-term follow up studies are needed to understand whether cognitive performance improves or worsens in the years following active illness. These studies must incorporate detailed assessment of neurobehavioural functioning to understand whether low cognitive performance translates to clinically apparent cognitive impairment following TBM. This is particularly timely given emerging data from *ex-vivo* studies implicating pathogenic mechanisms such as neuroexcitotoxicity

leading to neuronal injury (Rohlwink et al., 2019); mechanisms also described in neurodegenerative conditions (Wang and Reddy, 2017, Barkhoudarian et al., 2016).

Although this study provides important characterisation of neurological sequalae in TBM, it may not be feasible to adopt full cognitive testing in TBM endemic settings. We found only ‘fair’ or ‘slight’ agreement between CatRAPID and MoCA screens and the cognitive test battery when using standard cut-offs. The MoCA characterised most participants as cognitively impaired and may not be appropriate for this setting; this tool was developed and normed for a North American population, whereas in a low-income peri-urban South African population one study found the mean score in cognitively unimpaired, healthy controls was 21.7/30 (Robbins et al., 2013). A Receiver Operating Characteristic analysis is the best method to establish disease-specific cut-offs however we were underpowered to perform this. Given the high frequency of low cognitive performance within our TBM cohort, a larger study to validate and/or adapt cut-offs for impairment in existing screening tools within the TBM context is required, so that potential impairment can be identified where resources are limited. Where resources are available, our results highlight the value of including detailed cognitive and functional outcome assessments as quantitative measures of clinical outcomes within clinical trials in adult TBM.

In summary, our study demonstrates for the first time in adults that low cognitive performance in TBM occurs in approximately half of participants and is characterised by generalised impairment, affecting multiple cognitive domains. We also demonstrate that low cognitive performance in TBM is independent of and additional to the effects of HIV and non-CNS TB

disease. TBM occurring in people without HIV co-infection may have different cognitive sequalae. TBM has different clinical and neuropathological characteristics in PLWH compared to those without HIV (Marais et al., 2011, Cecchini et al., 2009). In addition, PLWH may have underlying HIV-associated brain injury which could decrease cognitive reserve and increase vulnerability to cognitive impairment from TBM neuropathology. A study of TBM cognitive outcomes in those without HIV co-infection would provide further insight into the burden of cognitive impairment in this disease in a different context and would be an important comparison for future studies. Future work is now needed to evaluate outcomes at longer-term timepoints, describe the relationship between cognitive performance, functional status and treatment adherence and validate sensitive context-specific screening tools to identify individuals at risk, in order to improve outcomes for patients with TBM.

Table 4.1 Parent Studies included within the study of cognitive impairment in TBM

	LASER-TBM	Albertyn Study	CONNECT
Study design	Phase 2a randomized, open label clinical trial of intensified antibiotics and high dose aspirin in HIV-associated TBM(Davis et al., 2021)	Prospective case control study evaluating cognitive and functional impairment in HIV-TBM (control group of other forms [non-CNS] TB)	Prospective case control study evaluating cognition, neuropsychiatric symptoms and neuroinflammation in PLWH switching from efavirenz to dolutegravir
Ethical approval	UCT HREC 293/2018	UCT HREC 565/2014	UCT HREC 017/2019
N recruited	52 enrolled	27 TBM 25 controls (non-CNS TB)	180 HIV +ve participants 60 HIV –ve controls
Setting	Inpatient	Inpatient	Outpatient
Inclusion criteria	Adults with TBM (definite, probable, possible) Confirmed diagnosis of HIV	Adults with TBM (definite, probable) Confirmed diagnosis of HIV	180 PLWH studied before and after switch from efavirenz to dolutegravir
Exclusion criteria	Many exclusions related to RCT (see (Davis et al., 2021)) None related to prior head injury, neurological disease or drug/alcohol dependance	Significant prior CNS disease (stroke, opportunistic CNS infection, significant head injury, dementia) Active alcohol or substance abuse/dependance Poor socio-economic support	Excessive drug or alcohol misuse History of CNS infection (inc meningitis) Previous stroke Major head injury (loss of consciousness >30 minutes)
Follow up timepoint	6 months	6 months	Baseline and after switch assessments (6-12 months)

Table 4.2 Baseline demographics and clinical characteristics of participants included within the study of cognitive impairment in TBM

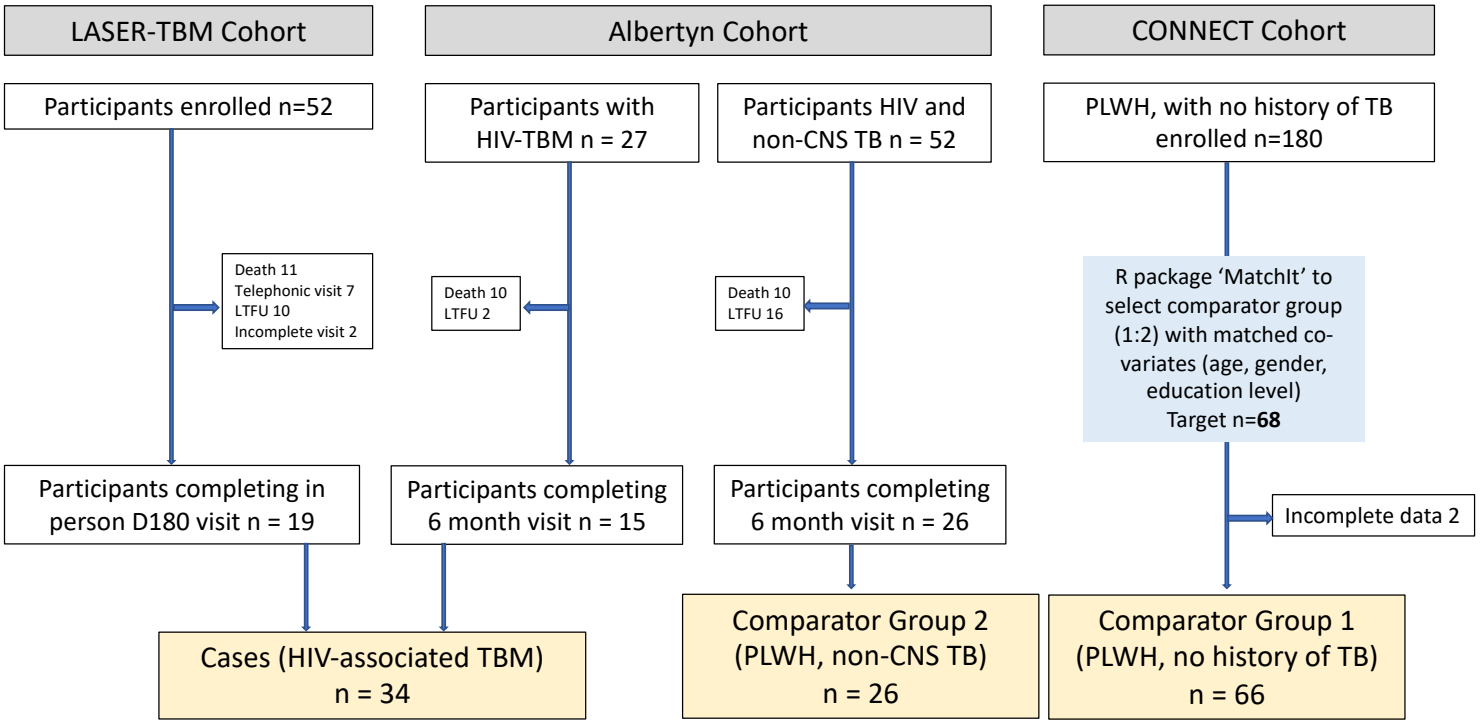
	HIV-associated TBM (n= 34)	PLWH (n=66)	Non-CNS TB (n=26)
Age in years (mean, SD)	36.4 (8.9)	37.4 (7.5)	35.3 (8.0)
Gender (% male)	47	41	58
Years of Education (mean, SD)	9.6 (3.1)	10.4 (1.5)	10.2 (1.6)
First language spoken: n (%) isiXhosa English Shona Missing data	 34 (100) 0 0 0	 59 (89) 2 (3) 3 (5) 2 (3)	 26 (100) 0 0 0
Baseline BMRC Grade Grade 1 Grade 2 Grade 3	 18 (53) 16 (47) 0	 n/a	 n/a
Uniform Case Definition TBM Category Possible Probable Definite	 7 (21) 13 (38) 14 (41)	 n/a	 n/a
HIV details (n) available in CD4 count (cells/mm^3) (mean, SD)	 34* 177 (183)	 41** 533 (293)	 26* 88.6 (90.4)

Table 4.3 (A) Comparison of Domain specific T scores in TBM cases vs comparator group 1 (PLWH, no history of TB) and (B) Comparison of Domain specific T scores in TBM cases vs comparator group 2 (PLWH non-CNS TB)

A	HIV-associated TBM cases (n=34)	Comparator group 1 (PLWH) (n=66)	p value
Domain *T* Score (Mean, SD)			
Motor skills	38 (14)	46 (11)	*<0.05**
Processing speed	36 (15)	47 (7)	*<0.0001****
Attention and working memory	47 (10)	49 (9)	*0.243*
Fluency	45 (10)	49 (7)	*0.029**
Audioverbal learning and memory	39 (15)	49 (9)	*0.0004****
Visuospatial learning and memory	40 (8)	48 (10)	*0.0004****
Executive function	41 (14)	47 (10)	*0.036**
Global *T* score (Mean, SD)	41 (9)	48 (6)	*<0.0001****
GDS suggesting CI (frequency, %)	16 (47)	17 (26)	*0.032*

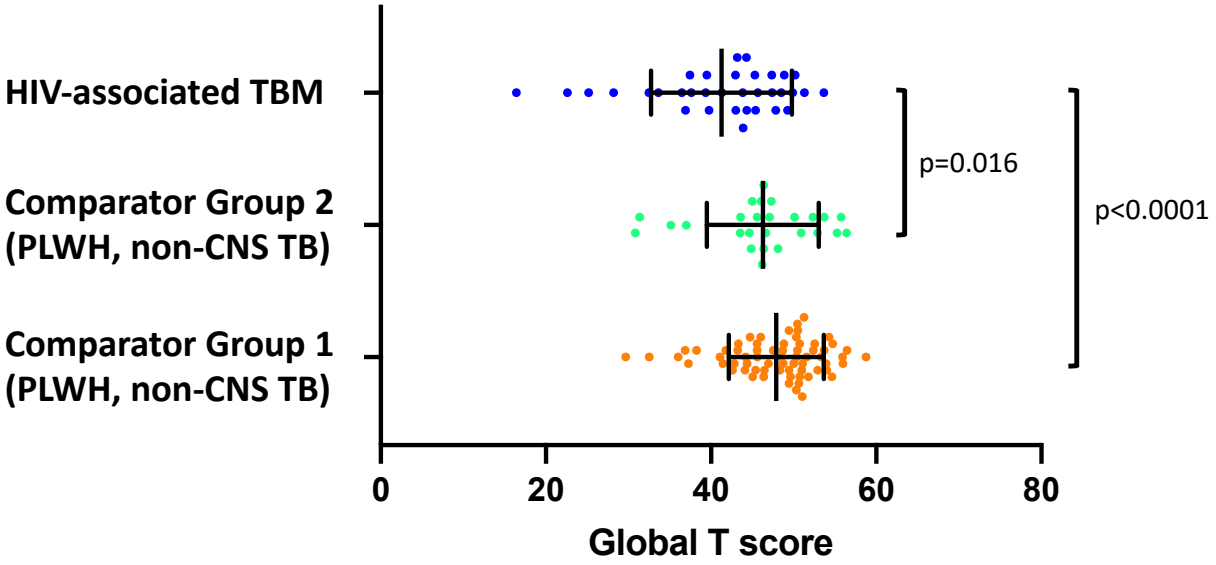
B	HIV-associated TBM cases (n=34)	Comparator group 2 (PLWH, non-CNS TB) (n=26)	P value
Domain *T* Score (Mean, SD)			
Motor skills	38 (14)	44 (8)	*0.108*
Processing speed	36 (15)	43 (9)	*0.096*
Attention and working memory	47 (10)	52 (9)	*0.067*
Fluency	45 (10)	48 (9)	*0.205*
Audioverbal learning and memory	39 (15)	43 (15)	*0.273*
Visuospatial learning and memory	40 (8)	46 (12)	*0.039**
Executive function	41 (14)	48 (10)	*0.156*
Global T score (Mean, SD)	41 (9)	46 (7)	*0.016**
GDS suggesting CI (frequency, %)	16 (47)	8 (31)	*0.201*

Figure 4.1 CONSORT diagram for study of cognitive Impairment in TBM



Consort diagram to describe enrolments across three parent studies

Figure 4.2 Global T Scores across groups



Scatter plot graph with mean and SD as well as individual values plotted in i) participants with HIV-associated TBM, ii) participants with HIV and non-CNS TB (comparator group 2), vs iii) participants with HIV only (comparator group 1).

Chapter 5. Luminex Multiplex analysis in blood and CSF of patients with HIV-associated TBM

5.1 Introduction

The use of host biomarker immunoassays, including the fluorochrome (Luminex) technique are well established in the field of tuberculous meningitis (TBM). Luminex uses a bead-based immunoassay which combines enzyme-linked immunosorbent assay (ELISA) with flow cytometry to simultaneously detect and quantify multiple proteins including cytokines, chemokines and growth factors within a single assay (Faresjo, 2014). The beads (microspheres), which have monoclonal antibodies directed against the proteins of interest, can detect up to 100 analytes simultaneously using a dual laser flow analyser (Faresjo, 2014). Given that the technology measures multiple analytes within a single well only a small sample volume (<25uL) is required. Together with time and cost saving, these features make Luminex preferable to running simple analyte ELISA to detect a large range of inflammatory mediators.

Several studies have harnessed Luminex multiplex platform technology to describe the profile of cytokine responses in the context of neurological sequalae or discover potential diagnostic biomarkers in TBM. In a study of TBM related immune reconstitution inflammatory syndrome (IRIS) all analysed mediators were upregulated in patients going on to develop TBM-IRIS compared to those who did not; implicating both innate and adaptive

immune cellular responses in TBM-IRIS (Marais et al., 2014). Further analysis of selected neutrophil-associated mediators including IL17-A and S100A8/A9 suggested that neutrophil associated inflammation may be a key driver of pathology in TBM-IRIS (Marais et al., 2014). In paediatric TBM, multiplex platform analysis identified a 3 biomarker signature (VEGF, IFNγ and MPO) which showed a sensitivity and specificity of up to 91.3% and up to 100% in the diagnosis of TBM (Manyelo et al., 2019a, Visser et al., 2015); which has since been reproduced in a larger validation cohort (Manyelo et al., 2022). Further sub-group analysis of this cohort identified significantly higher levels of sVCAM-1, MMP-1, sRAGE, and IP-10/ CXCL10 in the CSF of children with stroke compared to those without, providing potential insight into the biology of stroke in TBM, as well as identifying potential biomarkers who may require preventative management (Manyelo et al., 2021). Although a highly compartmentalised immune response is a feature in studies using matched CSF and plasma samples (Marais et al., 2014, Yang et al., 2014b), obtaining cerebrospinal fluid is not without complication. One study has demonstrated that a host serum 3 biomarker signature (adipsin (complement factor D), Aβ42, and IL-10) showed potential in the diagnosis of childhood TBM, with a sensitivity and specificity of 82.6% and 75.0% however larger studies are required to validate this finding (Manyelo et al., 2019b).

These studies support the rationale to use Luminex multiplex technologies to uncover pathogenic mechanisms in TBM, including HIV associated TBM. They also demonstrate the value of matched timepoint CSF and blood analysis with the goal to develop blood-based biomarkers given the complexity of obtaining samples from the site of disease in neurological conditions. Although these studies may have considered mechanistic

drivers of neurological sequalae such as HIV associated TBM-IRIS or stroke, no studies to date have applied this technology to consider mechanistic drivers of all cause poor outcome in adult HIV-associated TBM.

Using a Luminex multiplex assay we:

1. Described immune profiles in the blood and CSF of patients with HIV associated TBM and compare this to a group of patients with neurological presentations of a non-infectious aetiology
2. Identified markers associated with microbiologically confirmed disease and with poor outcome in the blood and CSF of patients with HIV associated TBM
3. Described longitudinal changes in blood and CSF over time and in relation to outcome in HIV associated TBM

5.2 Methods

Setting and Participants

We included within this analysis

i) participants with HIV-associated TBM recruited to LASER-TBM (see chapter 2, LASER TBM study protocol) as our 'cases' group
ii) participants enrolled to a study of neurological complications of COVID-19 (HIATUS-3) (see appendix HIATUS-3 results). We selected from this cohort participants where the discharge documentation diagnosis was due to a non-infectious cause (confirmed with a negative CSF cryptococcal latex agglutination test (CLAT), Gene Xpert Ultra, Gram Stain and bacterial culture) and included these participants as a 'non-infectious comparator' group.

Both studies (LASER-TBM and HIATUS-3) were approved by the University of Cape Town Human Research Ethical Committee (293/2018

and 207/2020) and the Walter Sisulu University Faculty of Health Sciences Research Ethical Committee (012/2019 and 031/2020) respectively.

Procedure

In LASER-TBM lumbar puncture was performed at study visits 3 and 28 days following enrolment (Figure 5.1). A total of 3ml of CSF was collected for immunological, cellular, proteomic and metabolomic analysis where feasible. Samples were spun at 200 x g for 10 mins at 4°C, and CSF supernatant stored separately in aliquots at -80C. By contrast, venepuncture was performed at each study visit. Where feasible up to 6ml of blood was collected for immunological, proteomic and metabolomic analysis within an EDTA tube. Within 4 hours of collection samples were spun at 500 *g* for 10 mins at room temperature, and the plasma aliquoted for storage at -80°C. Within the stored CSF supernatant, samples were prioritised for proteomic and metabolomic analysis, with Luminex Multiplex panel analysis undertaken where at least 200uL of CSF was remaining. In participants with available CSF, plasma from matched timepoints (day 3 and/or day 28), as well as baseline (enrolment) and latest timepoint (day 56) were selected for Luminex Multiplex panel analysis. Clinical, laboratory and radiological data was collected at study visits as described in chapter 2. Clinical outcome was assessed via the Modified Rankin Scale at the day 56 visit. We pre-specified a good and poor outcome (see statistical analysis plan, supplementary appendix) as an MRS between 0 and 3, and 4 and 6 respectively.

In HIATUS-3 lumbar puncture was performed between 3 and 7 days of hospital admission. A total of 2ml of CSF was collected for immunological, cellular, proteomic and metabolomic analysis where feasible. Samples

were frozen within 4 hours of the procedure at -80°C. At the time of this analysis, samples were thawed, spun at 200 g for 10 mins at 4°C and 200uL supernatant used for Luminex Multiplex panel analysis. Clinical, laboratory and radiological data was collected at study visits as described within the HIATUS study (see appendix). No matched timepoint plasma samples were available for analysis from this study.

Luminex Multiplex panel assays

A total of 200uL of CSF supernatant collected as part of LASER-TBM was thawed, filtered, and subsequently analysed using a 65 Plex Human ProcartaPlex™ Panel (catalogue number EPX650-100650-901) as per the manufacturers guidance. Concentrations of cytokines IL-1α, IL-1β, IL-2, IL-2R, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-18, IL-20, IL-21, IL-22, IL-23, IL-27, IL-31, IFNα, IFNγ, TNFβ, MIF, TSLP, LIF, NGFFβ, GM-CSF, G-CSF and M-CSF; chemokines GRO α (CXCL1), CXCL5, IL-8 (CXCL8), MIG (CXCL9), IP-10 (CXCL10), I-TAC (CXCL11), BLC (CXCL13), MCP-1 (CCL2), MIP-A α (CCL3), MIP-1 β (CCL4), MCP-3 (CCL7), MCP-2 (CCL8), EOTAXIN (CCL11), MIP-3 α (CCCL20), MDC, EOTAXIN-2 (CCL24), EOTAXIN-3 (CCL26), FRACTALKINE (CX3CL1); mediators of the TNF superfamily TNF α, TNF RII, TWEAK, CD30, APRIL, TRAIL, BAFF; and other factors VEGF-A, HGF, MMP-1, SCF, SDF-1 α, FGF-2, CD40L were obtained.

Samples obtained from HIATUS-3 were processed in the same way using an 18-plex Human ProcartaPlex™ Panel (catalogue number EPX180-15837-901). Only the concentrations of the common mediators obtained for both LASER and HIATUS panels were selected for this analysis, namely:

G-CSF, IL12p70, IL-10, IL-1 β, IL-6, IP10 (CXCL10), IL-18, IL-17A, MCP1 (CCL2).

Statistical analysis

We compared:

i) Baseline (day 3) mediator concentrations in CSF of patients with HIV associated TBM and compared these with non-infectious comparators;
ii) Baseline (day 3) mediators concentrations in Blood of patients with HIV associated TBM
iii) Baseline mediator concentrations in CSF to blood in a matched timepoint (day 3) and calculated median CSF:blood ratios for each mediator in all patients with HIV associated TBM;
iv) Baseline (day 3) mediator concentrations in blood and CSF in i) those microbiologically confirmed disease (either positive CSF Gene Xpert Ultra, positive CSF TB culture or both) to ii) those without microbiologically confirmed disease
v) Baseline (day 3) mediator concentrations in blood and CSF in i) those with good outcome (MRS 0-3) versus those with poor outcome (MRS 4-6) and ii) those who survived versus those who died (within 2 months of diagnosis).
vi) Baseline (day 3) CSF:Blood ratios in those with good versus those with poor outcome;
vii) Longitudinal change in CSF (day 3 and day 28) and blood (days 1, 3, 28 and 56) mediators in all TBM patients and those with good versus those with poor outcome;

Statistical analysis was performed using GraphPad Prism (version 9.3.1) software. We compared variables between groups using unpaired T Tests (parametric data) or Mann Whitney Tests of significance (non-parametric data). Throughout the analysis we used an unadjusted p value of <0.05 as a nominal threshold for statistical significance. Given the explorative nature of the analysis, and the small sample size we did not account for multiple testing.

5.3 Results

Baseline demographic and clinical results

A total of 18 of the 52 participants recruited to LASER-TBM had sufficient stored CSF to be included within this analysis. Of these, 14 participants had CSF at day 3 timepoint only, 4 at day 3 and day 28 timepoints, and 4 at day 28 timepoint only. All 18 participants had plasma collected at the enrolment visit, and 12 of 18 participants had plasma available from the day 56 timepoint. We analysed 14 and 8 plasma samples at respective day 3 and day 28 timepoints (Figure 5.2). 39 participants provided consent for enrolment to HIATUS-3. Of these 39 participants, CSF from 38 participants underwent Luminex analysis. Of 38 participants, data on 16 participants was not included as final diagnosis was due to an infectious aetiology, or not confirmed as non-infectious aetiology. 22 participants were included in the 'non-infectious' comparator group (Figure 5.2). Baseline demographic and clinical characteristics are outline in Table 5.1.

A summary of mediators found to have statistically significant differences in each of the comparisons listed below are highlighted in figure 5.3. Of note, only IL1beta was highlighted in more than one comparison other than in the comparison of those with microbiologically confirmed disease versus those without. Specifically, IL1β was i) significantly raised in the CSF of those with TBM compared to non-infectious comparators and ii) significantly raised in the blood of those with poor outcome. These are discussed in greater detail in the paragraphs below.

CSF mediator concentrations in patients with TBM and non-infectious comparators

Concentrations of mediators IL-1β, IL10, IL12p70, IL-17A, IL-18, IP-10 (CXCL10), and MCP1 (CCL2) were significantly raised in the CSF of patients with TBM at baseline (day 3) compared to non-infectious comparators (Table 5.2, Figures 5.3, 5.4 and 5.5). IL-6, an interleukin which acts as both a pro-inflammatory cytokine was non-significantly higher in non-infectious comparators compared to patients with TBM (median concentration 20.3 vs 5.79, *p=0.14*). Similarly, concentrations G-CSF, a haemopoietic growth factor were not significantly different between the two groups (median concentration in TBM vs non-infectious comparators, 14.8 vs 18.7, *p=0.61*).

CSF mediator concentrations in patients with microbiologically confirmed TBM vs those without

Of 14 patients with HIV associated TBM included in baseline analysis, 5 had microbiologically confirmed TBM (TB culture positive and/or Gene Xpert Ultra (GXP Ultra) positive CSF). Concentrations of 38/65 mediators measured were found to be significantly different in concentration in those with microbiologically confirmed TBM compared to those without (Table 5.3, Figure 5.6).

Highly compartmentalised inflammatory responses in TBM despite clinical outcome

Within the TBM group, at the day 3 timepoint concentrations of 55 of the 65 mediators measured were significantly higher in CSF when compared to matched timepoint plasma samples (Table 5.4). Figure 5.7 plots median

fold increase in concentration calculated by CSF:Blood ratio demonstrating mediators which highest and least degree of compartmentalisation.

Biomarkers of poor outcome in CSF and blood and effect of outcome on CSF:blood concentration ratio and degree of compartmentalisation.

In the CSF we found 7 mediators (IL-8 (CXCL1), MIP Aα (CCL3), APRIL, TRAIL, MIF, SCF and SDF-1α where concentrations were significantly raised at baseline in the CSF in those with poor versus those with good outcome (Table 5.5, Figures 5.8 and 5.9). A statistically significant difference in the concentration of G-CSF was also noted with outcome although given this was not significantly raised in CSF of patients with TBM compared to non-infectious comparators this was not felt to be a clinically significant finding. In the blood 8 mediators (IL-1 β, IL-27, LIF, IFN γ, BAFF) were significantly raised in those with poor compared to those with good outcome (Table 5.5, Figure 5.10). On comparing CSF:blood ratios in those with differing outcomes, we found 5 mediators where CSF:blood ratios were significantly higher in those with poor outcome (IP-10, CXCL-5, MCP 2, BLC, MDC) (Table 5.5, Figure 5.11). Conversely median CSF:blood ratios of IL-27 were significantly lower in those with poor versus those with good outcome (2.39 vs 20.94, *p=0.0008*). Table 5.5 also shows that in those with poor outcome a fewer number of mediators demonstrated significantly higher concentrations in the CSF compared to blood, than in those with good outcomes (11/65 vs 49/65, *p<0.0001*).

Change in CSF and Blood mediators over time dependant on outcome

We selected mediators for longitudinal comparison that had demonstrated a significant relationship to outcome (i.e. IL-1 β, IL-27, LIF, IFNγ, BAFF in

blood, and IL-8 (CXCL8), MIP-1α (CCL3), APRIL, TRAIL, MIF, SCF and SDF-1α in CSF). Only 4 participants (3 with good outcome, 1 with poor outcome) had CSF at both baseline and follow up timepoints and therefore it was difficult to draw conclusions from longitudinal CSF markers (Figure 5.12). Within the blood IL-1β, IFNγ, IL-4, IL-15, IL-27 and BAFF appeared to increase in participants who died, however in the one participant who survived despite severe disability (MRS 5) mediator concentrations remained at similar levels to those with 'good' outcome (Figure 5.13). When comparing means (and standard error) of mediator concentrations in those who survived vs those who didn't, concentrations appeared to increase prior to death in the two cases who died (Figure 5.14).

5.4 Discussion

We described immune profiles in blood and CSF of patients with HIV associated TBM and compared these to a group of patients with non-infectious aetiology, using a comprehensive immunoassay panel. We have shown, in keeping with the literature, that the immune response in HIV associated TBM is highly compartmentalised and that inflammatory mediators appear to increase prior to death in those with HIV associated TBM. Although small, our study identifies mediators which are associated with microbiologically confirmed disease, and with poor clinical outcome including death. To our knowledge this is the first study to consider how immune profiles change in those with poor outcome in HIV associated TBM (death or disability due to any cause) and how the degree of compartmentalisation differs with clinical outcome, an important

consideration in the search for suitable blood-based biomarkers of poor outcome.

Heterogeneity and microbiologically confirmed disease

We identified 7 mediators in which a significant difference in concentration was found in those with HIV associated TBM compared to a comparator group of patients where neurological infections (bacterial, tuberculous and cryptococcal) had been ruled out. Mediators known to play a role in the innate immune response such as IL-1β, IL-10, IL-17α, as well as those thought to bridge a link between innate and adaptive response such as IL-18, IL-12p70 and IP-10 were raised; in keeping with published data where upregulation of a diverse set of mediators suggests that dissemination of TB to the central nervous system leads to both innate and adaptive immune responses. In those with microbiologically confirmed disease IL-6 and IFNγ previously described in immunopathological pathways in TBM were also upregulated (Saghazadeh and Rezaei, 2022). Also present in higher concentrations in those with microbiologically confirmed disease was IL-16 a pleiotropic cytokine that functions as a chemoattractant, modulator or T cell activation and inhibitor of HIV replication which to our knowledge has not previously been described in TBM (Cruikshank et al., 2000). IP-10 was also present in higher concentrations within this group. This finding is in keeping with the literature where IP-10 concentration is widely quoted as a marker of TB disease activity(Hong et al., 2014, Azzurri et al., 2005). It has also been found in higher concentrations in the CSF of patients with TBM, alongside MIG(Yang et al., 2014b), which was also raised within our microbiologically confirmed TBM participants. These findings need validation in a larger cohort but may provide rationale to investigate IP-10

and MIG as dynamic biomarkers to monitor the efficacy of anti-TB drugs in TBM.

Overall however, although all participants included within the TBM case group were defined as having either possible, probable of definite TBM (Marais et al., 2010), there was significant heterogeneity in the immune mediator profiles between cases, including where TBM was microbiologically confirmed versus those where it was not. This finding echoes the heterogeneity in the clinical presentation of TBM, where a wide spectrum of clinical presentations and neurological sequalae exist; leading to the recognised difficulties when diagnosing TBM, and in part subsequent delays in management. However it also highlights the limitations of the now widely used case definition criteria, in particular when including those with 'possible' TBM where an alternative diagnosis may be mimic TBM thus leading to the subsequent complexities of clinical diagnosis and the need for improved diagnostic tools. Given the size of this study, it would not have been possible to exclude those with possible TBM, however in larger studies the inclusion of only those with microbiologically confirmed or 'probable' TBM may lead to less heterogeneity in cases, and thus more obvious insights into key pathogenic pathways. Comparing definite and probable cases with possible cases within this cohort may also shed light on this issue.

Variation in the compartmentalisation of the host inflammatory response in TBM

Our results align with prior studies which demonstrate a highly compartmentalised inflammatory response within the CSF. We found a

significant ($p<0.05$) difference in the concentration of 55 of the 65 mediators we measured when comparing matched CSF and blood timepoints with median fold increase in concentration up to 250.83. In general, cytokines appeared more likely to be present in high concentrations in CSF compared to chemokines, where often greater concentrations were found in blood compared to CSF. This is an important observation when considering both pathogenic mechanisms but also potential blood biomarkers of disease. Moreover, the number of mediators with significantly different concentrations in CSF compared to blood changed depending on the clinical outcome. Specifically, in those with poor outcome the extent of compartmentalisation appeared to reduce, with only 11/65 mediators found at significantly higher concentrations in CSF compared to blood; compared to 49/65 mediators in those with good outcome. This finding is most likely to be due to the small number of participants included who suffered poor outcome (n=3), but could also reflect blood brain barrier integrity and degree of systemic inflammation in those with severe disease. This observation however provides rationale that blood based biomarkers in TBM may still hold promise as potential diagnostic and management tools, particularly in those with severe disease.

Innate vs adaptive immune responses in TBM

The innate immune response, particularly mechanisms driving neutrophil activation is an important contributor to paradoxical inflammation occurring in HIV-associated TB including TBM (Lai et al., 2013, Lowe et al., 2012, Marais et al., 2017, Marais et al., 2014). The results of our study highlight the role of the innate immune response in TBM, with higher concentrations of IL-8, MIP-1 α and MIF in the CSF and IL-1 β in the blood, in those with

poor clinical outcomes. However, we also demonstrate that in those with poor outcomes, concentrations of mediators known to be involved in T cell regulation and adaptive immune responses, such as TRAIL in CSF as well as BAFF and IL-27 in blood are also upregulated, suggestion that the T cell compartment may also play an important role in severe cases of TBM. This may have important implications for considering biomarkers but also treatment approaches in those with severe disease.

IL-1 β, a potential biomarker of poor outcome and target for host directed therapies?

In IRIS occurring as a complication of HIV-associated TBM, inflammasome activation leads to cleavage and secretion of IL-1β a pro-inflammatory cytokine (Marais et al., 2017). In herpes simplex encephalitis, CSF IL-1β is associated with clinical severity, blood brain barrier permeability and disease outcome (Michael et al., 2016). A recent study in TBM however showed that although CSF IL-1β levels were raised in patients with TBM, this mediator did not associate with mortality (Koeken et al., 2021). Similar to this, our study demonstrated a raised level of IL-1β in the CSF of patients with TBM compared to non-infectious comparator group, although this did not predict poor outcome. However, we did show that IL-1β was raised in the blood of those who went on to have poor clinical outcomes. Moreover, our longitudinal analysis demonstrated that blood IL-1β levels, increased following initiation of treatment reaching highest concentrations prior to death in the two participants who died. These results provide further rationale for the validation of these findings in a larger cohort, particularly given anakinra, a drug which targets the IL-1β pathway has been reported

to be effective in treating paradoxical inflammation in TBM (Keeley et al., 2020).

Our findings around IL-1β may also suggest that existing blood-based biomarkers of inflammation may be effectively utilised; C-reactive protein an acute phase protein is released by hepatocytes in response to IL-6 with it's synthesis enhanced by IL-1β (Mackiewicz et al., 1991). One study showed that a raised serum C-reactive protein was helpful in discriminating TBM from bacterial meningitis at disease onset (Ersoy et al., 2012). In another study, high CSF C-reactive protein predicted mortality with a relative risk of 2.92 ($p=0.027$) however authors discuss how methods for measurement of serum C-reactive protein would need optimisation given that CSF concentrations are significantly lower than in serum (Ratinam et al., 2020). Nonetheless, in a condition where no blood biomarkers or poor outcome exist, the possibility that longitudinal measurements of IL-1β or C-reactive protein as a proxy for IL-1β may predict clinical worsening is an interesting area for future research.

Novel sites for biomarker measurement in TBM

Our study only investigated for potential biomarkers in the blood and cerebrospinal fluid of patients with TBM. In other forms of TB urine biomarkers have been considered. Lipoarabinomannan (LAM) a mycobacterial antigen is commercially available for TB diagnostics, however its use is largely limited to patients with HIV where CD4 counts are low (Songkhla et al., 2019). Recent studies have also highlighted host urine immunological biomarkers for the diagnosis of pulmonary TB (Petrone et al., 2016, Eribo et al., 2020). In other neurological disease, such as multiple

sclerosis urinary biomarkers have been evaluated (Dobson et al., 2013); to our knowledge this has not been explored in TBM. Along these lines, detection of host based biomarkers in saliva have shown promise for the diagnosis of tuberculosis in children (Khambati et al., 2021). It remains unknown whether these assays have a role in extra-pulmonary TB such as TBM. Given that the WHO has prioritised the development of non-sputum-based point-of-care tests on easily obtainable samples like blood, urine, stool, and saliva for tuberculosis (Denkinger et al., 2015), future studies within the TBM field should also consider these novel sites for biomarker development.

Study limitations

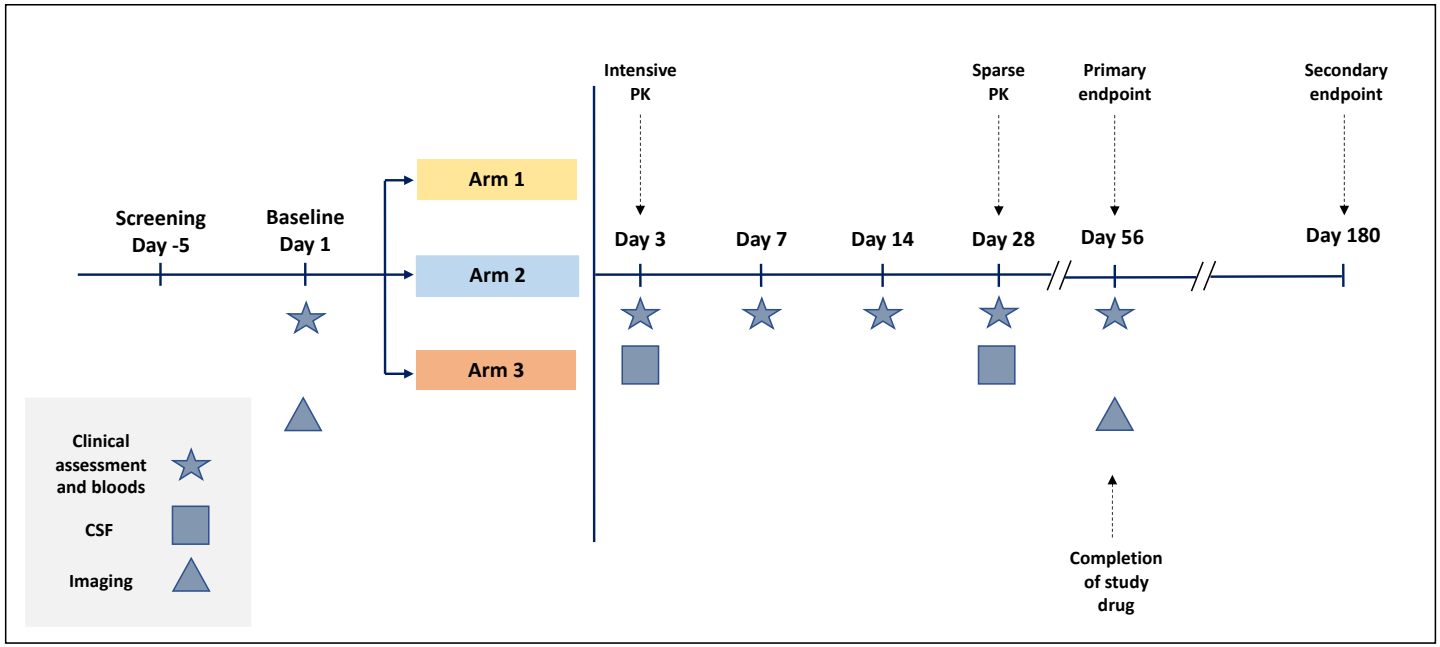
There are several limitations to this study. Firstly, the number of participants included was small and therefore the study was underpowered, particularly in the longitudinal analysis and the comparison of good to poor outcomes where in the latter only 3 participants with poor outcome were included. Due to the small numbers it was also not feasible to account for multiple comparisons within our analysis, and therefore it is possible that a proportion of our significant findings may be due to chance. Larger studies are now required to validate these findings, with a continued effort to acquire matched longitudinal timepoint samples particularly in the CSF. Secondly the heterogeneity within the group may in part be due to the inclusion of patients in whom an alternative diagnosis is more likely. For example, one of the two participants who died repeatedly appeared as an outlier within the analysis. Delving further into this case revealed that this participant, although clearly fulfilling criteria for ‘possible TBM’ using the case definition criteria had a dual diagnosis of neurosyphilis. In cases such

as this the contribution of co-infection, and/or the possibility that TBM was after all an incorrect diagnosis highlights the complexity of pathogenesis research in a condition where no highly sensitive, highly specific diagnostic test exists. Ultimately the design of studies on a scale which allows such cases to be excluded from the analysis will lead to a less heterogenous clinical group and therefore more robust insights into pathogenic mechanisms of disease. A principle component analysis in future analysis would also help to identify outliers. Along these lines, the inclusion of a non-infectious comparator group was pragmatic and therefore, although bacterial, mycobacterial and cryptococcal infectious aetiology had been ruled out in this group the spectrum of final diagnosis was highly heterogeneous. Although acquiring CSF from healthy controls is difficult, only a comparison with such a group can allow us to reliably conclude the full spectrum of raised inflammatory mediators in TBM. Finally, it was interesting to note that in the limited longitudinal CSF data available despite 4 weeks of treatment, multiple inflammatory mediators remain raised. Longer term follow up timepoints would allow us to glean important information as to which inflammatory markers remain raised. In particular, future studies should consider the inclusion of mediators known to be markers of brain injury (eg NSE, S100B, tau) to understand whether the inflammatory mediators we have seen raised in those with poor outcomes continue to rise and/or initiate a cascade of brain injury processes previously described in paediatric TBM, as well as in conditions where there is a recognised interplay between neuroinflammation and neurodegeneration.

5.5 Conclusions and future research

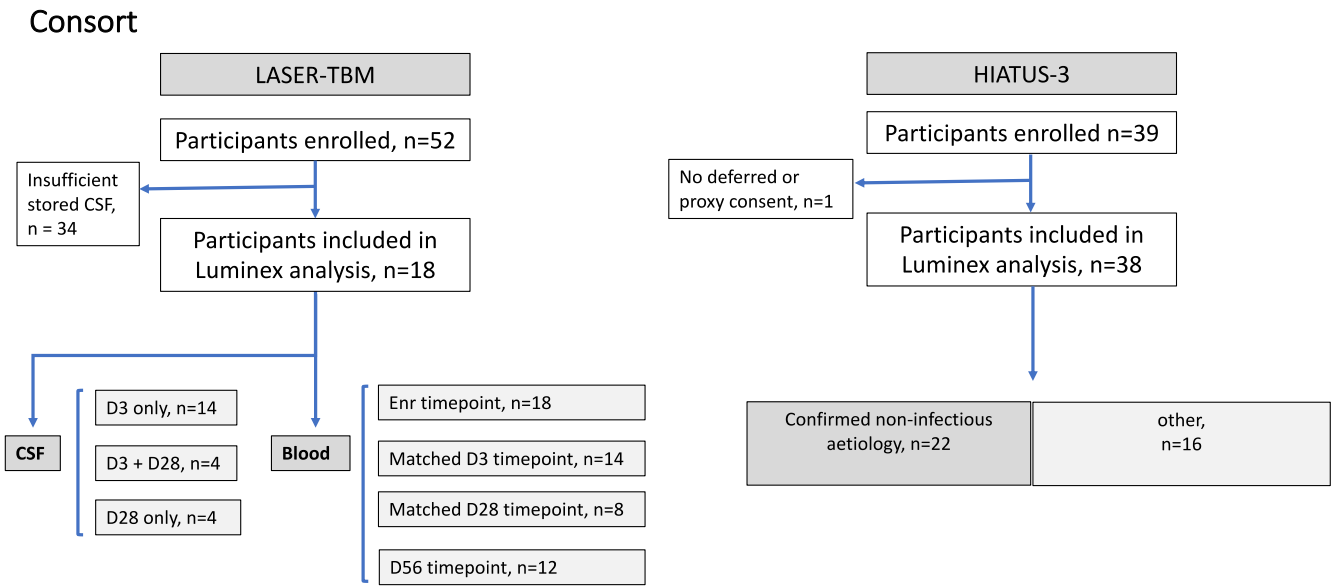
Despite these limitations, these analyses allow us to glean insight into pathogenic mechanisms in TBM, including those who have microbiologically confirmed disease. They highlight potential mediators for further investigation which may serve as useful biomarkers of poor outcome including within the blood and/or serve as useful druggable targets in the development of host directed therapies. In particular, the findings around IL-1β and inflammasome driven innate immune responses provide further rationale for the consideration of drugs which target these pathways. Further studies using larger cohorts and including longer term timepoints, ideally with a healthy control group are now needed to further investigate and validate these findings. Moreover, integration of these results with transcriptomic, metabolomic and proteomic analysis within the same cohort will add depth of knowledge and clarity to pathways of interest. This analysis is planned, funded and will be undertaken by myself as part of the Wilkinson Group at Crick beyond the course of my PhD.

Figure 5.1 Study sampling schedule for LASER-TBM



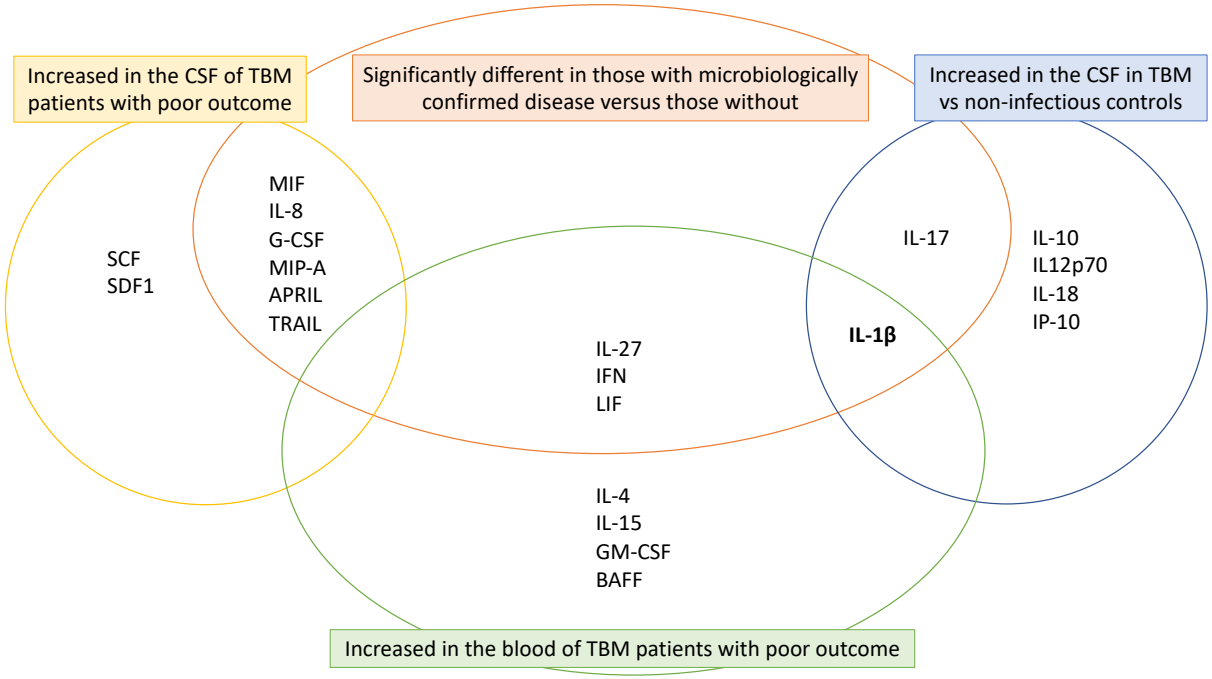
Study participants enrolled to LASER-TBM were seen at 6 timepoints between day 1 (enrolment) and day 56. Blood for immunological analysis was collected at each of these timepoints. Where possible lumbar puncture was performed at day 3 and day 56

Figure 5.2 CONSORT diagram for Luminex analysis study



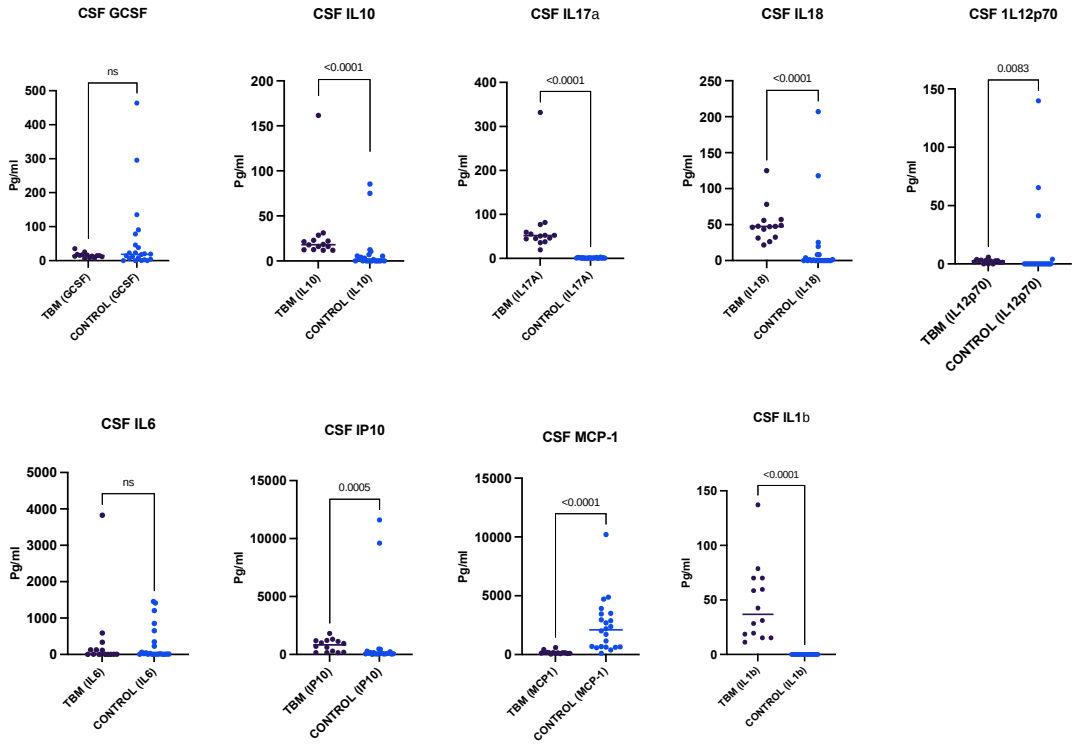
Participants were included as per the consort diagram from the parent studies LASER-TBM and HIATUS-3. Enr; enrolment

Figure 5.3 Summary Venn diagram to describe mediator findings across all analysis groups



This Venn diagram highlights mediators in which a significant difference ($p<0.05$) was found in each of the three comparisons i) comparison of CSF mediators in those HIV associated TBM with good versus poor outcome (yellow), ii) blood mediators in those with HIV associated TBM in good versus poor outcome (green) and iii) CSF mediators in those with HIV associated TBM versus non-infectious comparators (blue). Those overlapping with the red circle are those which were found to be significantly different ($p<0.05$) in those with microbiologically confirmed disease versus those without

Figure 5.4 Scatter graphs plotting individual mediator values in cases with HIV associated TBM compared to non-infectious comparator group

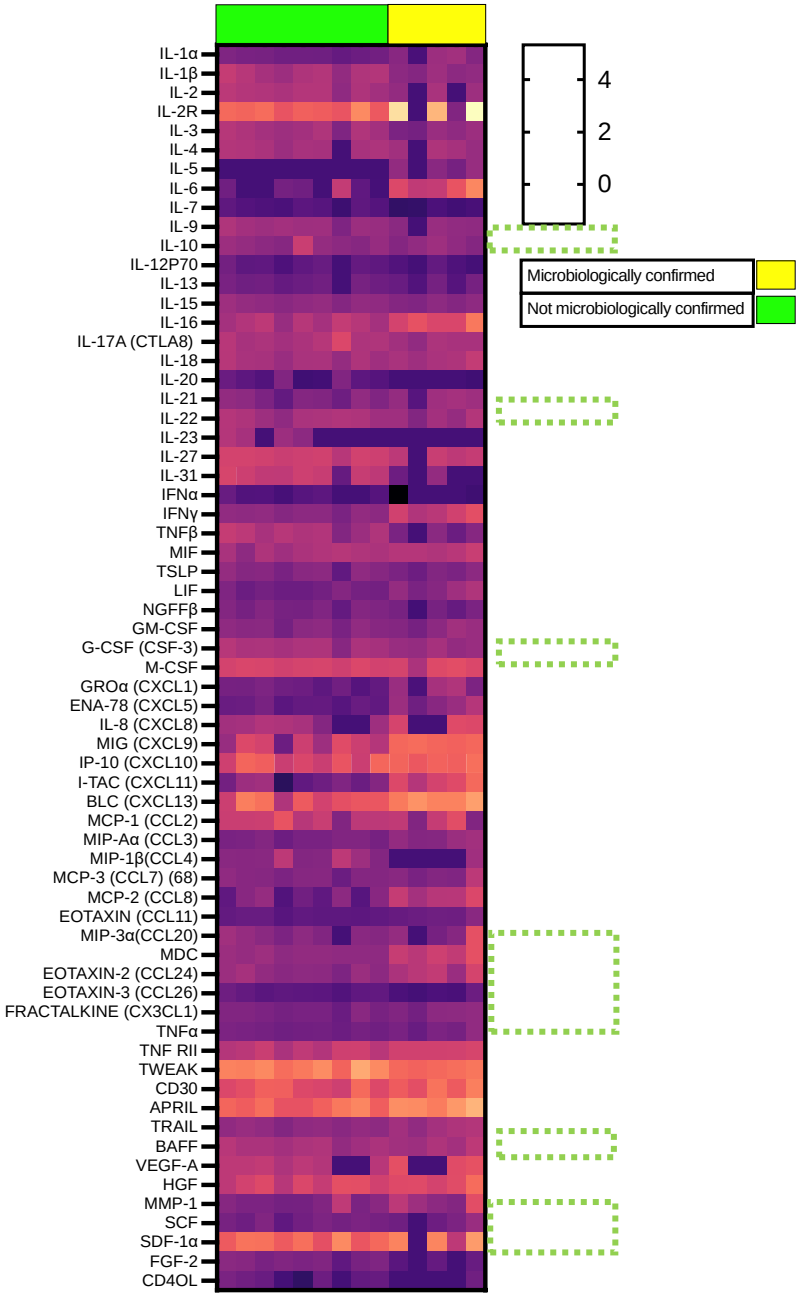


Scatter graphs plot individual mediator concentrations in cases (HIV associated TBM) and comparators (non-infectious aetiology)

Figure 5.5 Day 3 CSF immune mediators in patients with HIV associated TBM compared to non-infectious comparator group

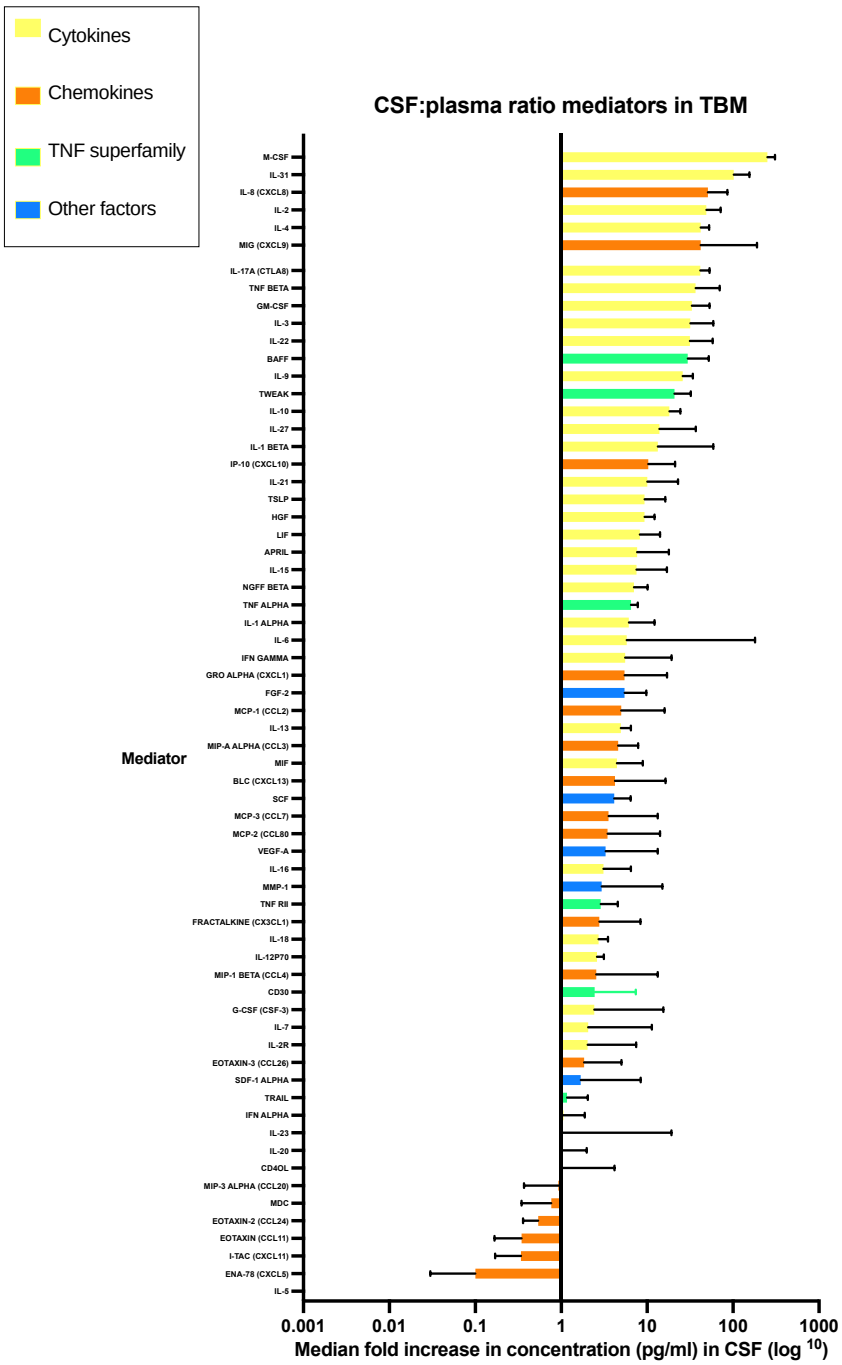
Heatmap plotting individual participants as columns with HIV associated TBM cases on the left (green) and non-infectious comparators on right (yellow). HIV associated TBM cases are ordered from most severe 2 month outcome (MRS 6) on left to best outcome (MRS 0) on right.

Figure 5.6 Day 3 CSF mediators in those with microbiologically confirmed HIV associated TBM versus those without



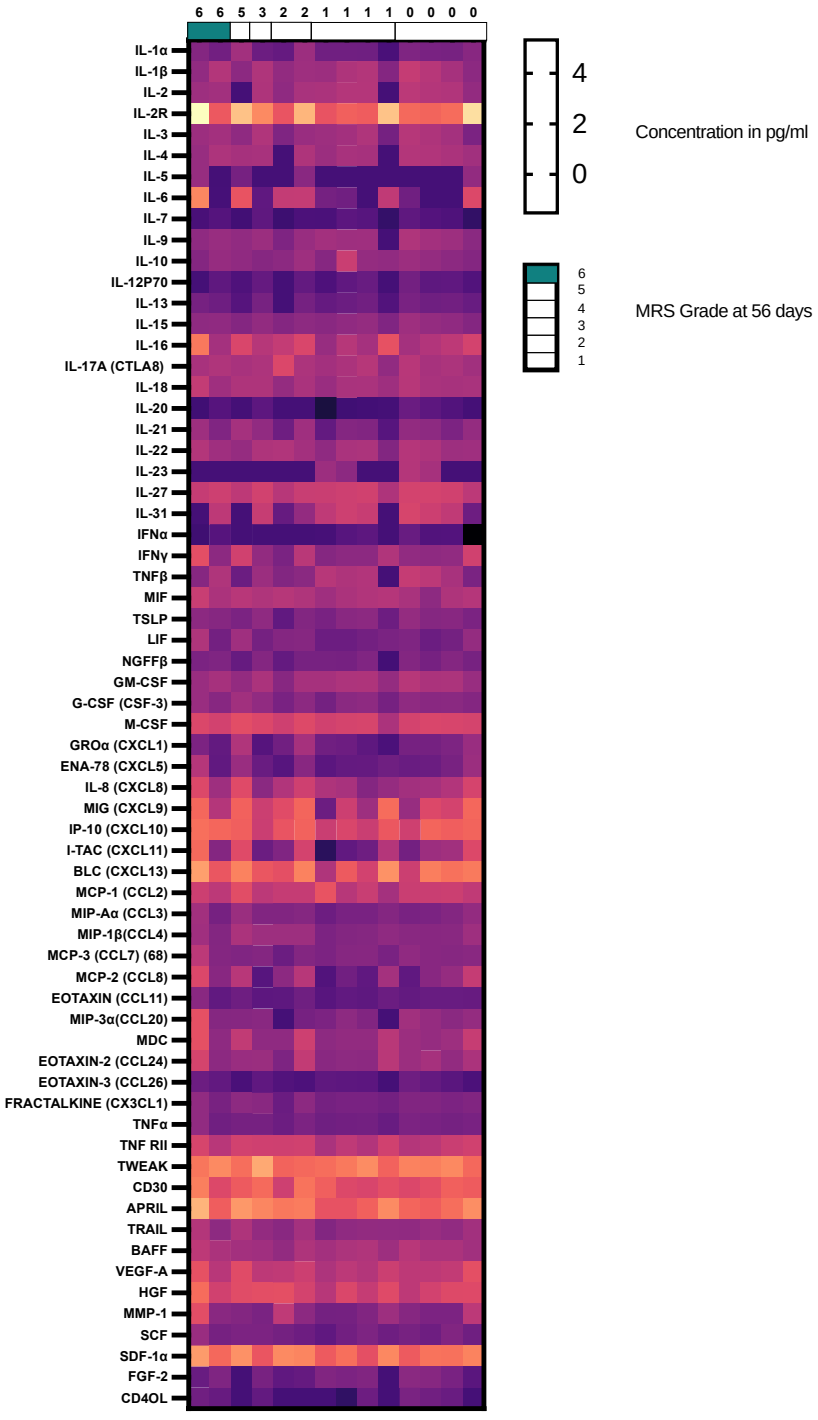
Heatmap plotting individual participants with HIV associated TBM as columns with participants with microbiologically confirmed disease on the right (yellow) and those without microbiologically confirmed disease on the left (green). Highlighted in green dashed boxes are mediators in which obvious differences in concentrations are apparent between the groups.

Figure 5.7 Comparison of baseline mediators in matched CSF and blood timepoint (day 3), CSF to blood ratio ranked by log fold increase



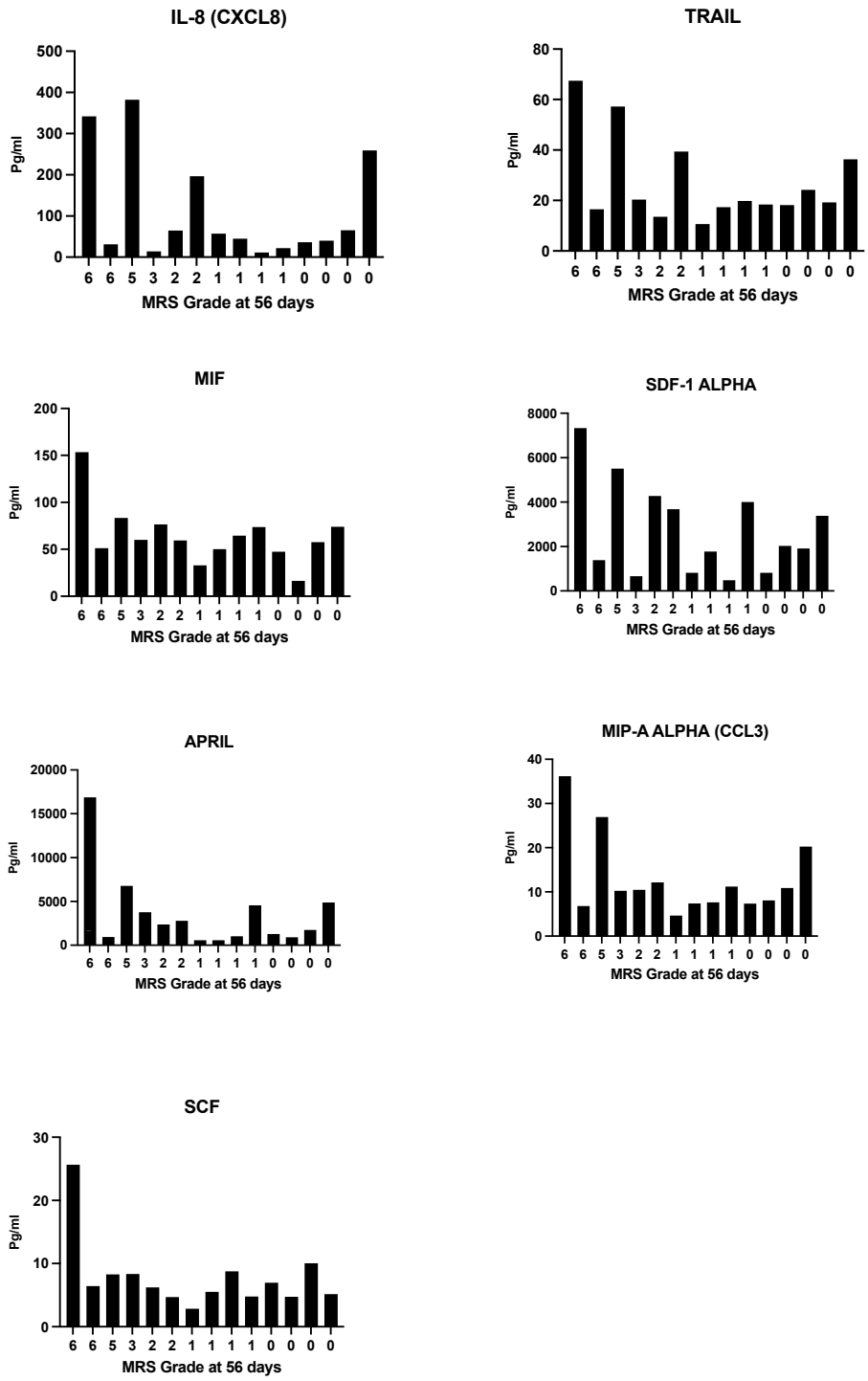
Median fold change in CSF:Blood ratio (pg/ml) (CSF concentration/Blood concentration), converted to a log10 scale, are plotted here for each of the CSF mediators measured in those with HIV associated TBM cases. Mediators are colour coded as per their type (see key).

Figure 5.8 Day 3 CSF mediator concentrations with MRS outcome at D56



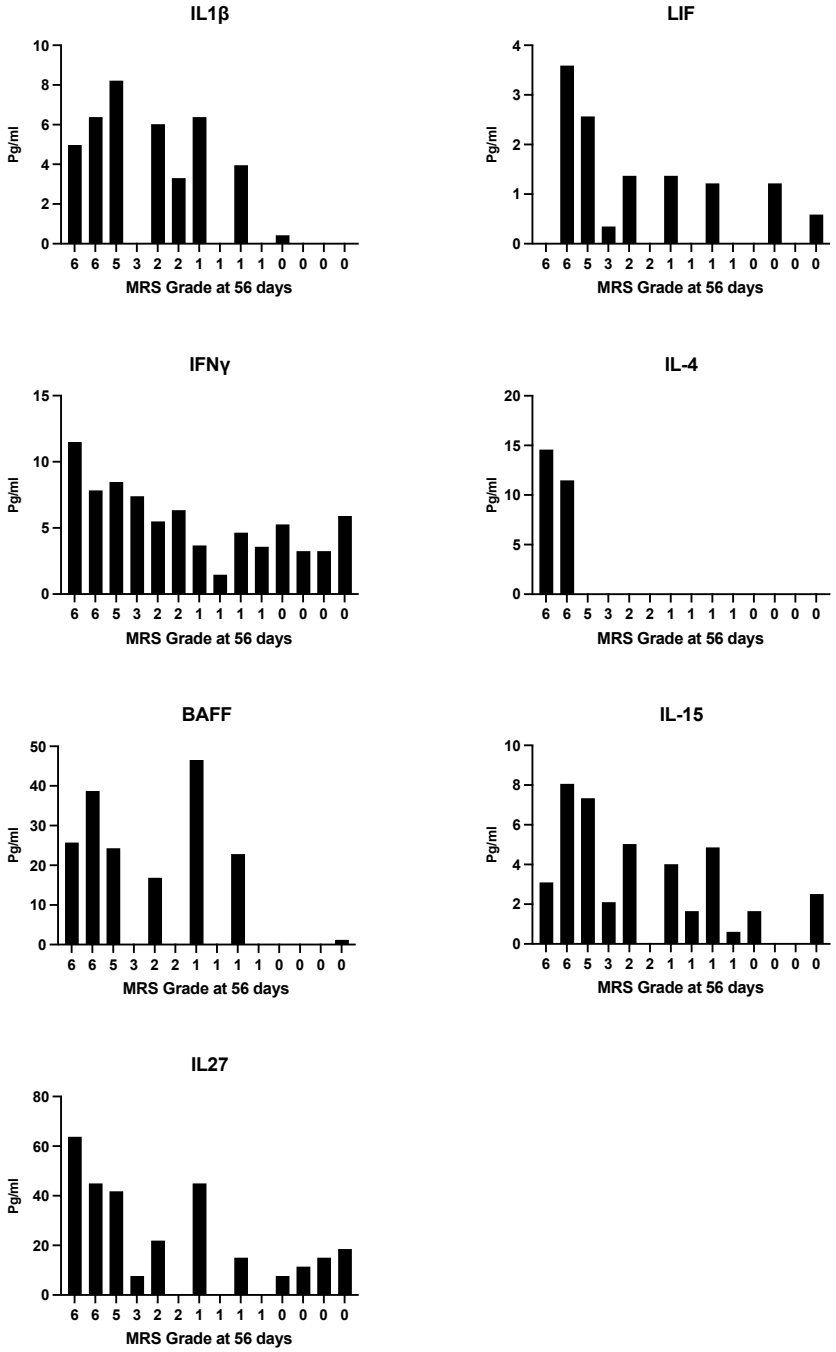
Heatmap in which individual participants with HIV associated TBM are represented as columns with those with most severe outcomes (MRS 6) on left, and those with best outcomes (MRS 0) at day 56 on right.

Figure 5.9 Day 3 CSF markers demonstrating difference (p<0.05) with good (MRS 0-3) versus poor (MRS 4-6) outcome



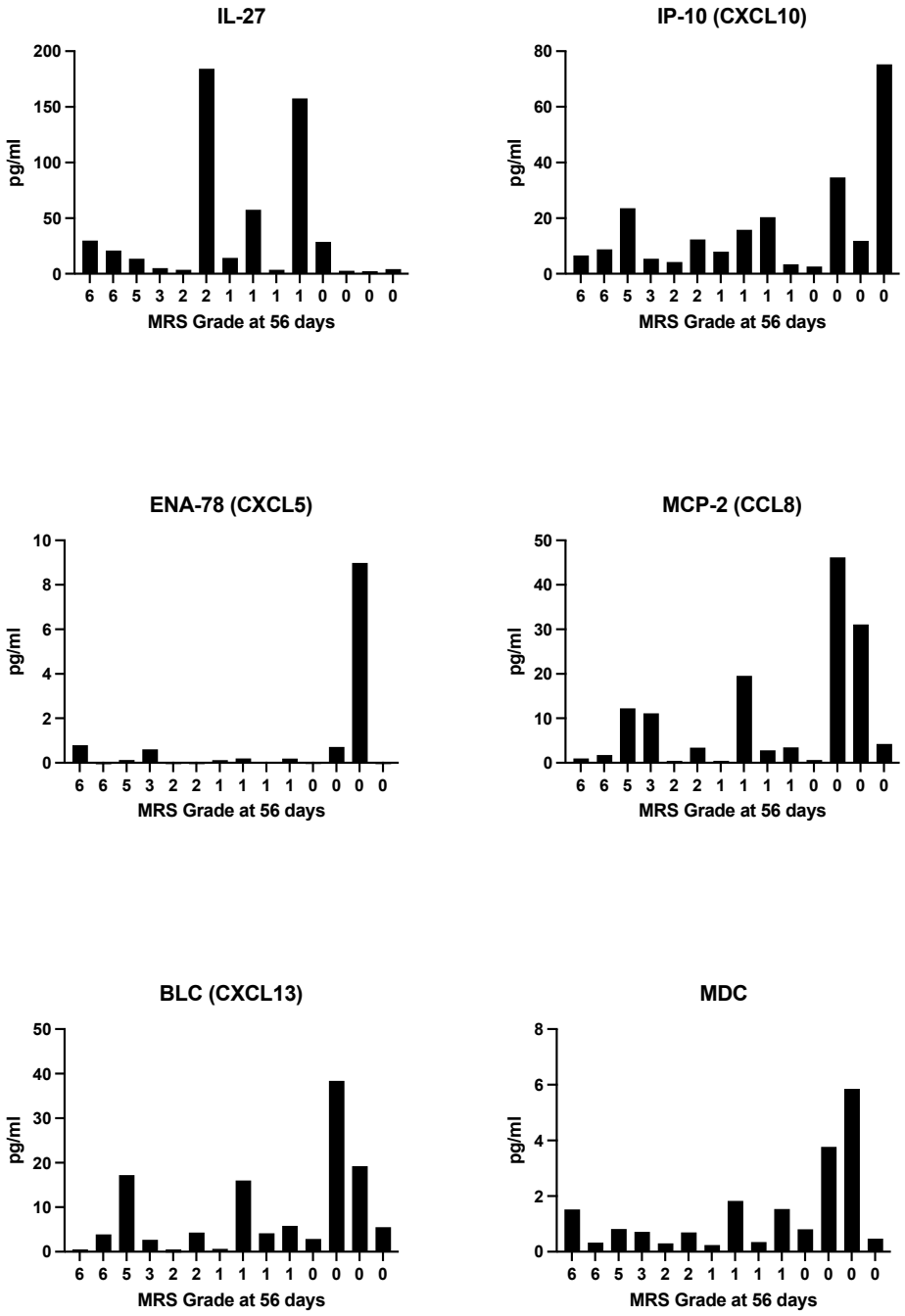
CSF mediators in which a significant difference (p<0.05) in concentration between those with poor versus those with good outcome were selected. Individual participants are represented by individual columns with those with most severe outcomes (MRS 6) on left, and those with best outcomes MRS 0 on right.

Figure 5.10 Day 3 Blood markers demonstrating difference (p<0.05) with good (MRS 0-3) versus poor (MRS 4-6) outcome



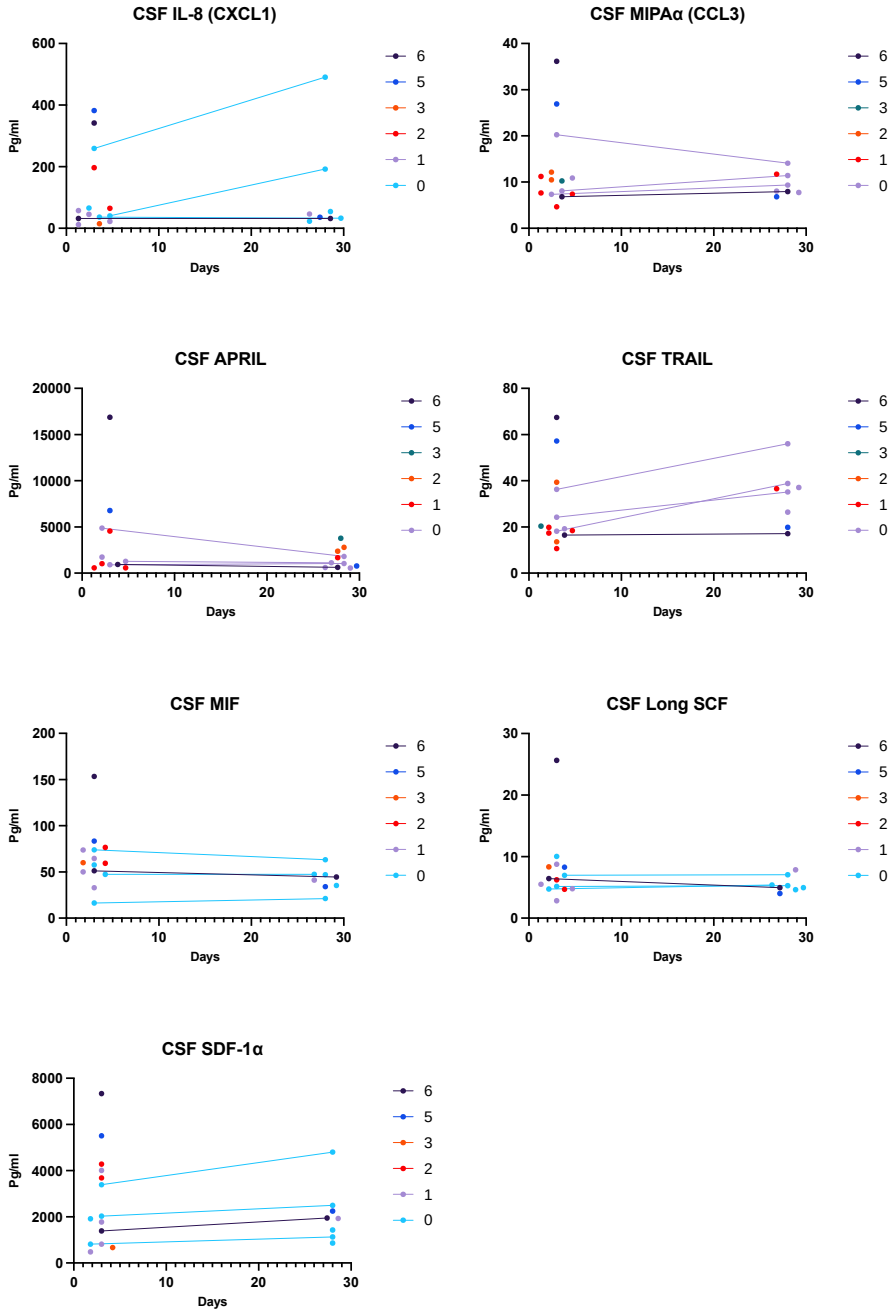
Blood mediators in which a significant difference (p<0.05) in concentration between those with poor versus those with good outcome were selected. Individual participants are represented by individual columns with those with most severe outcomes (MRS 6) on left, and those with best outcomes MRS 0 on right.

Figure 5.11 Day 3 CSF:Blood ratios in mediators demonstrating difference (p<0.05) with good (MRS 0-3) versus poor (MRS 4-6) outcome



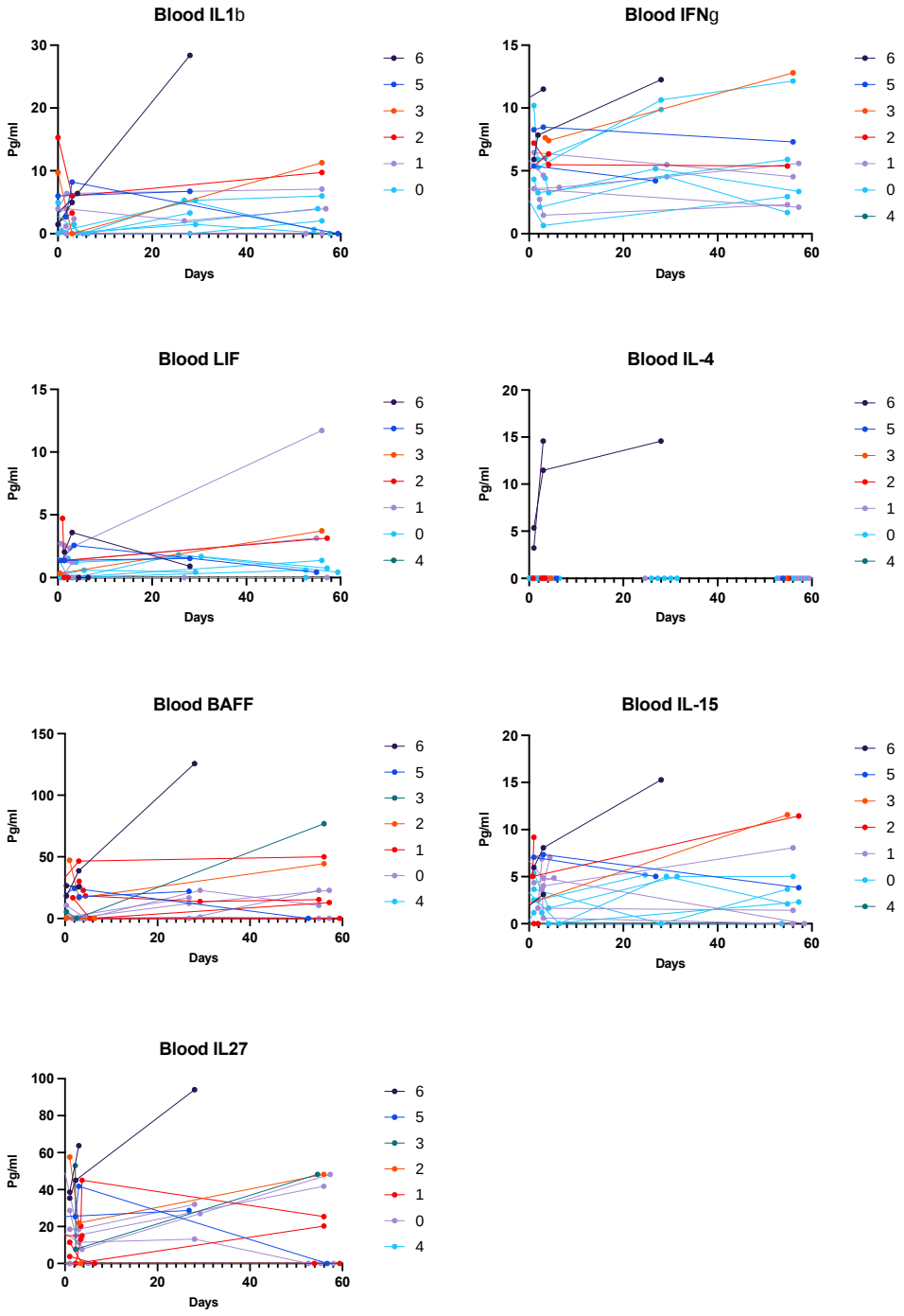
CSF:Blood ratios in mediators where a significant difference in the ratio was found in those with good versus those with poor outcome. In all mediators except IL-27 the trend is towards greater CSF:Blood ratio (ie higher degree of CNS compartmentalisation) in those with milder disease (MRS 0) compared to those with severe disease (MRS 6).

Figure 5.12 Longitudinal change in selected CSF parameters



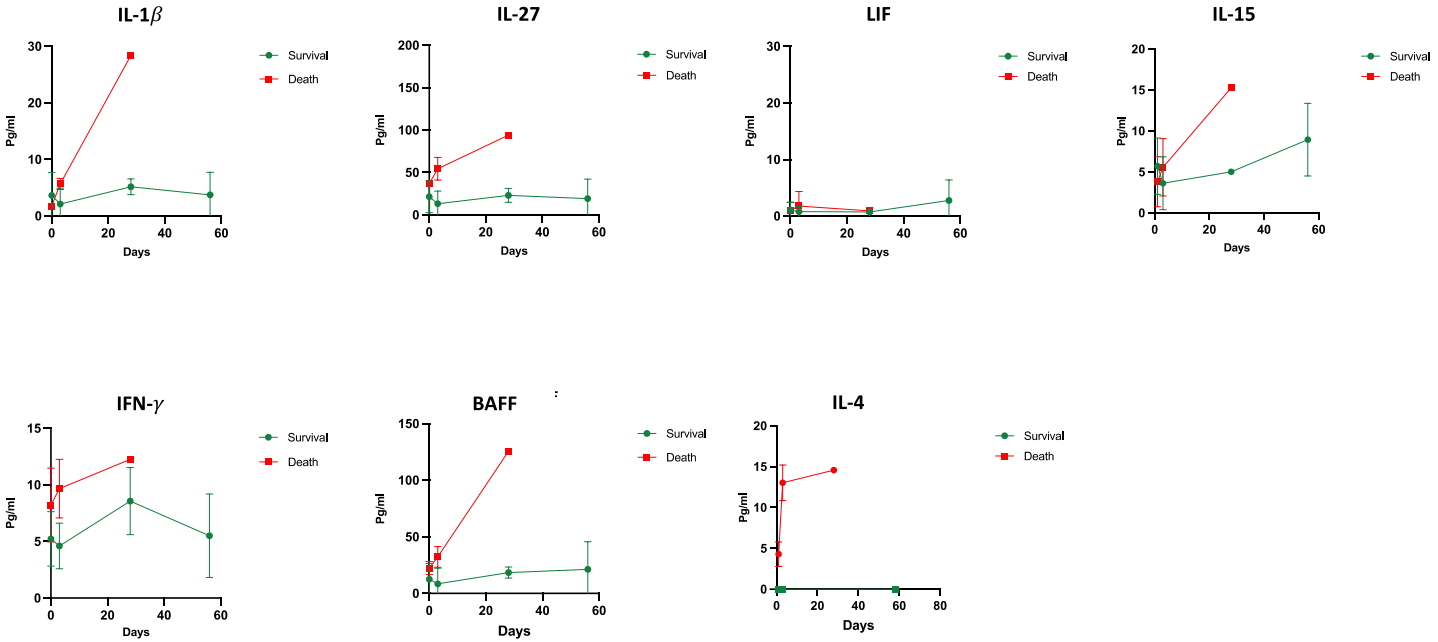
CSF mediators shown to be significantly different in concentration ($p<0.05$) in those with good versus those with poor outcome were selected. Baseline (day 3) and follow up (day 28) were plotted. Participants were colour coded as per the day 56 MRS outcome (0-6). Those with paired samples (ie both a day 3 and day 28 samples) are linked by a single line.

Figure 5.13 Longitudinal change in selected blood parameters



Blood mediators shown to be significantly different in concentration ($p<0.05$) in those with good versus those with poor outcome were selected. The following timepoints were plotted: day 1, day 3, day 28 and day 56 where available, and when from the same participant linked with a single lineParticipants were colour coded as per the day 56 MRS outcome (0-6)

Figure 5.14 Longitudinal change in selected blood markers grouped by survival



Blood mediators shown to be significantly different in concentration ($p<0.05$) in those with good versus those with poor outcome were selected. Participants were grouped into those who survived (green) and those who died (n=2) by day 56 and the median (IQR) of mediator concentrations at the following timepoints were plotted: day 1, day 3, day 28 and day 56 where available.

Table 5.1 Baseline demographics and clinical characteristics of participants included within the Luminex study analysis

	HIV associated TBM (all)	HIV associated TBM Good Outcome (MRS 0-3)	HIV associated TBM Poor Outcome (MRS 4-6)	Non-infectious comparison group
n	18	14	4	22
Age (years) (median, IQR)	38.5	38.5 (28.0-38.5)	40.5 (32.5-44.8)	47.5 (36.8-66.0)
Gender (% female)	39	50	0 (0)	13 (59)
Blood				
WCC	6.07 (3.84-8.68)	5.80 (3.84-9.02)	6.07 (3.72 – 8.01)	8.25 (6.0-15.3)
CD4 count, cells/uL	168 (86.8-383)	168 (122-383)	193 (17.8-749)	n/a
HIV viral load, log_{10}	60,807 (1,812-219,620)	68,497 (5,608 – 378,736)	60,807 (1,812-151,261)	n/a
Cerebrospinal fluid				
Lymphocyte cell count $x10^6$/L	5.5 (1.0-23.0)	5.5 (1.75-23.0)	8.0 (0.25-33.0)	0.0 (0.0-1.25)
Polymorphonuclear cell count $x10^6$/L	19.0 (3.75-308)	27.5 (7.75-411)	5.0 (0.0-64.0)	0.0 (0.0-0.25)
Protein, g/L	2.31 (1.1-3.72)	2.03 (0.865-3.2)	4.25 (1.74-12.8)	0.36 (0.21-0.55)
Glucose, mmol/L	2.2 (0.9-2.95)	2.30 (1.2-3.1)	0.95 (0.625-2.03)	3.70 (2.6-5.73)
GXPU positive, n (%)	5 (27.7)	3 (21.4)	2 (50)	0

Table 5.2 Baseline (Day 3) CSF mediator concentrations in patients with TBM (n=14) and non-infectious comparators (n=22)

	HIV associated TBM (n=14)		Non-Infectious comparator (n=22)		HIV associated TBM vs Non-Infectious Comparators	Direction in HIV associated TBM group compared to non-infectious comparators
Mediator	CSF		CSF			
pg/ml	median	(IQR)	median	(IQR)	p-value	
Mediators measured across both groups (HIV associated TBM and non-infectious comparators)						
Cytokines						
G-CSF	14.8	10.4-17.6	18.7	3.97-54.2	0.6135	↓
IL-6	5.79	0.77-181	20.2	6.8-422	0.1391	↓
IL-12P70	2.61	1.01-3.13	<	0.0-0.0	0.0083	↑
IL-10	18.2	12.6-24.4	0.935	0.0-6.03	<0.0001	↑
IL-1 β	36.9	17.8-70.1	<	0-0	<0.0001	↑
IL-18	47.4	32.3-56.3	<	0-8.21	<0.0001	↑
IL-17A	51.8	42.8-63.9	0.82	0.5-1.66	<0.0001	↑
Chemokines						
IP-10 (CXCL10)	834	186-1198	91.8	50.6-259	0.0005	↑
MCP1 (CCL2)	146	95.3-175	2104	651-3466	<0.0001	↓

Table 5.3 CSF mediator concentrations in those with microbiologically confirmed HIV associated TBM vs those without

	Microbiologically confirmed		Not microbiologically confirmed				Direction (micro confirmed disease vs not)
Mediator	CSF		CSF				
pg/ml *	median	IQR	median	IQR	P value	P value summary	
Cytokines – raised in microbiologically confirmed disease							
IL-16	310	271- 1462	66.8	39.4- 89.4	0.0010	***	↑
IL-5	13.6	3.35- 21.9	0	0-0	0.0050	**	↑
IL-6	337	122- 2208	2.77	0- 5.79	0.0015	**	↑
IFN γ	207	75.4- 341	15.8	13.8- 19.1	0.0029	**	↑
LIF	21.3	10.1- 46.5	5.99	4.44- 7.91	0.0035	**	↑
IL-1 α	13.1	6.35- 33.1	5.26	4.86- 7.16	0.0207	*	↑
IL-21	31.4	12- 36.2	10.2	6.68- 17.6	0.0233	*	↑
MIF	74	66.5- 118	51.3	40.1- 62.3	0.0217	*	↑
G-CSF	15.8	9.07- 30.4	14.8	10.2- 17.1	0.0186	*	↑
IL-2R	18353	5- 137668	1051	715- 1525	0.6064	ns	↑
M-CSF	322	150- 404	251	217- 293	0.5712	ns	↑
IL-18	49	39.4- 90.6	46.4	29.5- 52.5	0.3636	ns	↑
Cytokines – lower in microbiologically confirmed disease							
IL-1 β	15.3	13.3- 24.9	59.6	35.5- 74.4	0.0035	**	↓
IL-2	20.4	0- 38.9	63.8	43.6- 72.5	0.0062	**	↓
IL-7	0.87	0.51- 1.2	1.81	1.35- 2.49	0.0087	**	↓
IL-20	0	0- 0.415	2.43	1.28- 7.04	0.0015	**	↓
IL-31	0	0- 12.6	143	102- 183	0.0018	**	↓
TNF β	7.92	2.16- 13.6	60	36.7- 86.4	0.0062	**	↓
IL-3	17.3	7.2- 27.9	39.6	34.3- 60.6	0.0138	*	↓
IL-9	17.3	7.32- 21.8	31.9	25.9- 37.5	0.0271	*	↓
IL-17A	45.3	27.6- 49.4	55.4	47.7- 79.5	0.0460	*	↓
IL-27	100	47- 148	204	173- 226	0.0120	*	↓
IFN α	0.03	0- 0.865	1.7	0.53- 2.16	0.0476	*	↓
IL-4	36	11.8- 50.4	46.2	34.6- 59.1	0.3169	ns	↓
IL-10	17.9	11.9- 24.8	21.4	14.3- 25.8	0.4166	ns	↓
IL-12P70	1.46	0- 2.36	2.83	1.97- 3.54	0.0867	ns	↓

IL-13	3.7	1.73- 6.57	5.09	3.81- 6.27	0.5025	ns	↓
IL-15	11.8	10.6- 16.9	18.1	15.7- 21.4	0.0565	ns	↓
IL-22	33	16.5- 53.6	53.5	31.6- 61.5	0.2246	ns	↓
IL-23	0	0-0	0	0-36.2	0.1983	ns	↓
TSLP	8.16	6.43- 13.2	13.4	9.85- 16.7	0.2370	ns	↓
NGFF β	7.04	2.22- 7.57	9.49	6.67- 11.1	0.0684	ns	↓
GM-CSF	24.9	21.1- 33.2	51.9	42.2- 55.6	0.2204	ns	↓
Chemokines – raised in microbiologically confirmed disease							
MIG (CXCL9)	1350	1178- 1471	173	27- 291	<0.0001	****	↑
I-TAC (CXCL11)	352	154- 910	5.81	3.62- 20.6	0.0010	***	↑
MCP2 (CCL8)	81.3	60- 232	5.4	2.33- 14.7	0.0010	***	↑
MDC	144	103- 357	19.8	17.3- 25.2	0.0005	***	↑
ENA-78 (CXCL5)	26.4	9.21- 50.9	3.28	2.62- 4.19	0.0015	**	↑
BLC (CXCL13)	3299	2917- 7045	681	199- 1354	0.0012	**	↑
MIP A α (CCL3)	20.3	11.7- 31.5	7.66	7.08- 10.4	0.0027	**	↑
EOTAXIN (CCL11)	4.9	3.97- 9.54	2.96	2.53- 3.43	0.0035	**	↑
EOTAXIN-2 (CCL24)	88.1	37.5- 186	16.6	15- 27.4	0.0070	**	↑
IL-8 (CXCL8)	259	0- 362	36.2	5.76- 51.1	0.0186	*	↑
FRACTALKINE (CX3CL1)	15.9	8.04- 17.7	7.55	6.79- 10.1	0.0370	*	↑
GRO α (CXCL1)	26	4.78- 52.3	5.34	2.87- 6.4	0.1119	ns	↑
IP-10 (CXCL10)	1136	857- 1503	298	165- 1087	0.0829	ns	↑
Chemokines – lower in microbiologically confirmed disease							
MIP 1 β (CCL4)	0	0- 16.7	14.1	11.6- 65.2	0.0340	*	↓
MCP1 (CCL2)	110	10- 284	155	86.4- 168	0.5160	ns	↓
MCP3 (CCL7)	11.1	8.53- 54.1	12.4	10- 12.9	0.9640	ns	↓
MIP 3 α (CCL20)	12.6	3.23- 274	13.8	9.27- 19.3	0.9276	ns	↓
EOTAXIN-3 (CCL26)	1.26	0.58- 2.89	2.68	2.25- 3.11	0.0599	ns	↓
TNF superfamily - raised in microbiologically confirmed disease							
TNF RII	214	208- 249	89	68.9- 178	0.0016	**	↑
APRIL	4879	3671- 11825	1023	741- 2059	0.0020	**	↑
TRAIL	39.4	27.4- 62.3	18.2	15- 20.1	0.0016	**	↑
TNF α	7.53	5.28- 13.7	6.01	5.21- 7.05	0.1898	ns	↑
CD30	883	638- 2516	348	307- 1008	0.0583	ns	↑

TNF superfamily - lower in microbiologically confirmed disease							
Tweak/TNFSF12	1419	1267- 1971	3297	2106- 4426	0.1058	ns	↓
BAFF	37.3	33- 79.2	50.1	35.2- 58.2	0.7840	ns	↓
Other factors – raised in microbiologically confirmed disease							
VEGF-A	401	0- 523	82.9	27.5- 102	0.0299	*	↑
MMP1	30.6	13.2- 274	8.74	7.05- 13	0.0290	*	↑
HGF	385	308- 1004	224	119- 417	0.1119	ns	↑
SDF-1 α	3389	50- 5509	1389	742- 1974	0.2561	ns	↑
Other factors – lower in microbiologically confirmed disease							
FGF-2	2.25	0- 3.61	9.57	7.4- 11.8	0.0008	***	↓
SCF	5.15	2.34- 17	6.44	5.13- 8.54	0.6064	ns	↓
CD40L	0	0- 2.65	3.64	0.23- 5	0.1559	ns	↓

Table 5.4 Baseline (day 3) mediators in HIV associated TBM, a comparison of blood and CSF concentrations

	TBM (n=14)						
	Blood		CSF		CSF:Blood ratio		CSF:Blood
Mediator	median	IQR	median	IQR	median	IQR	p value
Cytokines – higher in CSF compared to blood							
IL-1 α	0.0	0.0-1.2	6.34	5.01-11.9	6.14	2.98-12.20	<0.0001
IL-1 β	1.86	0.0-6.11	36.9	17.8-70.1	13.3	4.28-58.86	<0.0001
IL-2	0.0	0.0-0.0	48.9	19.7-71.6	48.88	19.68-71.61	<0.0001
IL-3	0.0	0.0-0.0	34.3	15.4-58.7	31.86	15.38-58.69	<0.0001
IL-4	0.0	0..0-0.0	44.3	26.5-55.8	42.02	3.99-52.58	<0.0001
IL-5	0.0	0.0-0.0	0.0	0.0-8.42	0.00	0.0-8.42	0.0978
IL-6	0.0	0.0-0.0	5.79	0.77-181	5.79	1.0-180.87	<0.0001
IL-7	0.28	0.07-1.17	1.37	0.84-2.05	2.06	1.38-11.34	0.0022
IL-9	0.0	0.0-0.16	25.9	16.6-34.0	25.94	11.93-34.04	<0.0001
IL-10	0.0	0.0-0.0	18.2	12.6-24.4	18.16	12.59-24.38	<0.0001
IL-12P70	0.0	0.0-0.0	2.61	1.01-3.13	2.61	1.26-3.13	<0.0001
IL-13	0.0	0.0-0.0	4.96	2.99-6.48	4.96	2.99-6.48	<0.0001
IL-15	2.31	0.46-4.90	17.5	11.8-20.2	7.52	3.11-16.97	<0.0001
IL-16	49.9	26.4-101	89.4	40.0-308	3.11	0.88-6.47	0.0571
IL-17A	0.0	0.0-4.58	51.8	42.8-63.9	41.58	12.51-53.32	<0.0001
IL-18	18.7	13.2-22.1	47.4	32.3-56.3	2.72	1.63-3.50	<0.0001
IL-20	0.0	0.0-0.0	0.83	0.0-1.97	1.00	0.67-1.97	0.0103
IL-21	0.0	0.0-4.29	14.1	7.57-24.4	10.06	1.59-22.87	0.0022
IL-22	0.0	0.0-0.85	42.8	28.1-60.0	31.55	14.53-57.76	<0.0001
IL-23	0.0	0.0-0.0	0.0	0.0-19.2	1.00	1.0-19.18	0.0978
IL-27	15.0	5.78-42.6	173	98.5-215	13.88	3.6-36.88	<0.0001
IL-31	0.0	0.0-0.0	102	2.65-155	102.16	2.9-155.40	<0.0001
IFN α	0.0	0.0-0.0	1.01	0.0-1.88	1.03	0.91-1.88	0.0009
IFN γ	5.38	3.5-7.52	19.1	15.4-118	5.56	3.14-19.21	<0.0001
TNF β	0.0	0.0-0.0	36.7	9.99-69.8	36.68	9.99-69.78	<0.0001

MIF	12.2	7.94-42.4	59.7	49.4-74.6	4.46	1.98-8.91	0.0025
TSLP	0.385	0.0-4.18	12.3	8.0-15.9	9.35	1.83-16.27	<0.0001
LIF	0.47	0.0-1.37	7.1	5.6-14.6	8.22	4.21-14.11	<0.0001
NGFF β	0.0	0.0-0.0	7.04	5.78-10.1	7.04	5.78-10.08	<0.0001
GM-CSF	0.0	0.0-9.23	42.2	24.1-53.1	33.23	3.91-53.08	<0.0001
G-CSF	5.86	0.0-14.9	14.8	10.4-17.6	2.43	0.80-15.4	0.0165
M-CSF	0.0	0.0-0.0	258	221-323	250.83	204.05-307.81	<0.0001
Cytokines – lower in CSF compared to blood							
IL-2R	1605	449-4096	1525	885-28,338	2.02	0.67-7.45	0.2649
Chemokines – higher in CSF compared to blood							
GRO α (CXCL1)	0.0	0.0-4.32	5.89	2.99-13.1	5.48	0.63-17.05	0.0026
IL-8 (CXCL8)	0.0	0.0-0.39	51.1	29.2-212	51.15	21.01-85.70	<0.0001
MIG (CXCL9)	6.53	0.0-123	291	62.2-1278	42.0	2.51-189.95	0.0003
IP-10 (CXCL10)	39.5	27.7-143	834	186-1198	10.31	5.14-21.19	<0.0001
BLC (CXCL13)	291	125-484	1354	431-3281	4.22	2.13-16.34	0.0067
MCP1 (CCL2)	23.8	15.2-38.5	146	95.3-175	5.00	3.29-15.90	<0.0001
MIP A α (CCL3)	3.05	0.0-6.20	10.4	7.37-14.2	4.59	2.03-7.85	0.0006
MIP 1 β (CCL4)	6.6	0.0-16.0	15.7	11.8-30.9	2.56	1.41-13.29	0.0117
MCP3 (CCL7)	3.12	0.0-10.8	11.9	9.43-13.2	3.56	0.90-13.23	0.0010
MCP2 (CCL8)	4.32	1.97-8.24	14.7	2.78-80.7	3.47	0.88-14.09	0.0357
EOTAXIN-3 (CCL26)	0.41	0.0-2.50	2.42	1.29-3.07	1.84	0.77-5.02	0.0451
FRACTALKINE (CX3CL1)	3.17	0.0-10.7	9.21	6.99-14.7	2.79	0.75-8.35	0.0278
Chemokines – lower in CSF compared to blood							
ENA-78 (CXCL5)	69.6	31.3-112	4.2	3.13-16.6	0.10	0.03-0.64	<0.0001
EOTAXIN (CCL11)	11.0	6.32-25.5	3.37	2.55-4.47	0.34	0.17-0.47	0.0009
I-TAC (CXCL11)	40.6	24.4-78.1	20.6	4.76-265	0.34	0.17-3.22	0.3346
MIP 3 α (CCL20)	14.2	1.94-28.9	13.2	8.06-21.5	0.93	0.37-7.58	0.9729
MDC (CCL22)	48.8	31.0-83.5	25.2	17.7-126	0.76	0.35-1.61	0.2408
EOTAXIN-2 (CCL24)	27.7	14.1-199	26.2	15.8-59.4	0.54	0.36-1.23	0.7097
TNF superfamily – higher in CSF compared to blood							
TNF α	0.0	0.0-0.0	6.44	5.28-7.75	6.52	5.50-7.75	<0.0001

TNF RII	44.5	27.7-88.6	178	80.9-211	2.88	1.51-4.56	0.0013
Tweak/TNFSF12	155	73.4-358	2379	1413-4128	20.78	7.11-32.23	<0.0001
CD30	249	111-405	645	339-1146	2.45	1.60-7.38	0.0067
APRIL	197	91.8-436	2059	932-4635	7.66	5.79-18.79	<0.0001
BAFF	0.615	0.0-24.7	50.1	34.6-57.5	29.73	1.47-52.04	<0.0001
TNF superfamily – lower in CSF compared to blood							
TRAIL	23.7	11.5-38.2	19.5	17.1-37.1	1.17	0.51-2.04	0.9100
Other factors – higher in CSF compared to blood							
VEGF-A	45.5	23.1-119	109	93.4-235	3.29	0.78-13.26	0.0067
HGF	35.4	20.4-59.8	338	196-431	9.32	4.32-12.18	<0.0001
MMP1	5.32	3.03-11.4	11.6	8.15-46.5	2.96	1.27-15.01	0.0091
SCF	1.72	1.08-2.34	6.34	4.78-8.44	4.15	2.61-6.45	<0.0001
SDF-1 α	709	344-1743	1974	819-4076	1.69	1.16-8.42	0.0141
FGF-2	0.0	0.0-0.0	7.40	2.68-10.4	5.47	2.68-9.78	<0.0001
CD40L	0.0	0.0-2.16	1.81	0.0-4.81	1.00	0.48-4.15	0.1789

Table 5.5 Baseline cerebrospinal fluid (CSF) and blood (Bl) mediator concentrations, and CSF:Blood ratios in patients with HIV associated TBM in those with good (MRS 0-3) vs poor (MRS 4-6) outcome

	HIV associated TBM Good Outcome (MRS 0-3), n=11							HIV associated TBM Poor Outcome (MRS 4-6), n=3							Good outcome vs Poor Outcome*		
Mediator	**Blood**		**CSF**		**CSF vs Blood**	**CSF: Blood ratio**		**Blood**		**CSF**		**CSF vs Blood**	**CSF:Blood ratio**		**Blood**	**CSF**	**CSF:Blood ratio**
pg/ml *	**median**	**(IQR)**	**median**	**(IQR)**	**p value**	**median**	**IQR**	**median**	**(IQR)**	**median**	**(IQR)**	**p value**	**median**	**IQR**	**Direction and p-value**	**Direction and p-value***	**p-value**
Cytokines																	
IL-1 β	0	0-3.95	**42.6**	**19.5-70.1**	<0.0001	17.74	9.44-17.74	6.38	4.97-8.21	**18.7**	**15.3-70.1**	0.1876	3.76	1.87-3.76	↑0.0137	0.5181	0.0604
IL-4	0	0-0	**46.2**	**27.5-58.6**	0.0002	46.21	27.46-46.21	11.5	0-14.6	**42.3**	**23.6-54.9**	0.0354	4.78	1.62-4.78	↑0.0330	0.9771	0.1389
IL-15	1.65	0-3.01	**17.8**	**11.6-21.1**	<0.0001	10.81	4.33-10.81	7.34	3.1-8.06	**17.1**	**11.8-18.1**	0.0186	2.24	1.51-2.24	↑0.0092	0.6288	0.0733
IL-27	11.4	0-18.5	**184**	**94.1-221**	<0.0001	20.94	5.07-20.94	45.0	41.8-63.8	**138**	**100-190**	0.0266	2.39	2.16-2.39	↑0.0008	0.5429	↓0.0220
IFN γ	4.64	3.26-5.91	**19.1**	**15.4-62.6**	<0.0001	5.26	3.30-5.26	8.48	7.84-11.5	**207**	**15.8-468**	0.1671	24.38	2.02-24.38	↑0.0014	0.1978	0.1324
LIF	0.35	0-1.22	**6.40**	**4.44-11.9**	<0.0001	7.79	4.44-7.79	2.57	0-3.59	**32.7**	**5.99-60.3**	0.1203	12.72	1.67-12.72	↑0.0311	0.1786	0.6593
GM-CSF	0	0-0	**51.4**	**24.9-54.1**	<0.0001	51.43	21.67-51.43	20	0-20	**25.4**	**20.6-42.9**	0.1625	2.14	1.03-2.14	↑0.0385	0.2261	0.0606
MIF	13.8	9.15-37.8	**59.4**	**47.4-73.7**	0.0233	3.59	0.93-3.59	4.46	1.98-56.1	**83.4**	**51.3-153**	0.0975	11.51	2.74-11.51	0.2912	↑0.0413	0.1621
G-CSF	5.66	0-11.4	**14.8**	**7.51-16.9**	0.0240	2.44	0.67-2.44	14.2	0-21.7	**25.5**	**12.8-35.3**	0.2396	1.63	0.90-1.63	0.3926	↑0.0137	0.7473
IL-1 α	0	0-0.37	**5.26**	**4.55-9.46**	0.0001	5.26	3.55-5.26	0	0.711	**11.53**	**0.80-11.53**	0.1854	0.95	0.78-0.95	0.6291	0.1648	0.5549
IL-2	0	0-0	**58.4**	**20.4-71.9**	<0.0001	58.38	20.44-58.38	0	0-5.6	**30.4**	**0-36.8**	0.1488	30.42	0.18-30.42	0.2143	0.1231	0.1205

IL-2R	2758	445-7598	**1437**	**919-18353**	0.6063	1.90	0.50-1.90	1258	450-1943	**28125**	**780-203599**	0.2962	22.37	1.73-22.37	0.5549	**0.4560**	0.1703
IL-3	0	0-0	**38.3**	**9.54-59.4**	<0.0001	39.32	9.54-38.42	0	0-1.15	**29.6**	**17.3-37.7**	0.0094	29.61	17.32-29.61	0.3956	**0.5238**	0.5009
IL-5	0	0-0	**0**	**0-0**	0.4762	0.0	0.0-8.42	0	0-0	**6.69**	**0-23.9**	0.2255	0.0	0.0-0.0	n/a	**0.1209**	0.1209
IL-6	0	0-0	**5.19**	**0-127**	0.0010	5.19	1.00-5.19	0	0-2.77	**591**	**1.03-3825**	0.2834	591.31	0.37-591.31	0.2143	**0.2170**	0.4341
IL-7	0.22	0.09-1.18	**1.46**	**0.74-2.34**	0.0077	5.44	1.46-5.44	0.53	0-1.16	**1.12**	**0.87-1.81**	0.1832	1.56	1.12-1.56	0.9038	**0.5657**	0.2253
IL-9	0	0-0	**30.3**	**14.7-37.4**	0.0003	30.25	14.65-30.25	0	0-6.43	**19.3**	**17.3-24.3**	0.0037	17.28	3.78-17.28	0.9167	**0.4498**	0.2119
IL-10	0	0-0	**18.4**	**12.7-28.7**	<0.0001	18.42	12.65-18.42	0	0-0	**17.9**	**11.9-23.0**	0.0054	17.89	11.88-17.89	n/a	**0.6868**	0.6868
IL-12P70	0	0-0	**2.93**	**1.34-3.31**	0.0002	2.83	1.34-2.83	0	0-0	**1.46**	**0-2.63**	0.1476	1.46	0.0-1.46	n/a	**0.3161**	0.1817
IL-13	0	0-0	**5.09**	**3.36-6.38**	<0.0001	5.09	3.36-5.09	0	0-0	**4.83**	**1.87-6.76**	0.0343	4.83	1.87-4.83	n/a	**0.9322**	0.9322
IL-16	55.9	36.7-102	**77.1**	**40.1-234**	0.2169	2.99	0.54-2.99	27.4	23.6-85.7	**308**	**39.2-2371**	0.3078	11.22	1.66-11.22	0.4287	**0.5549**	0.1264
IL-17A	0.00	0-3.19	**52.6**	**37.9-77.2**	<0.0001	37.86	13.94-37.86	0.00	0-19.8	**46.1**	**45.3-59.4**	0.100	45.29	3.0-45.29	0.7527	**0.9780**	0.6273
IL-18	17.4	13.2-23.7	**47.3**	**31.4-49.0**	<0.0001	2.66	1.65-2.66	20.1	17.4-20.6	**56.0**	**32.6-125**	0.1356	2.79	1.58-2.79	0.8887	**0.1232**	0.3294
IL-20	0	0-0	**0.83**	**0-2.43**	0.0039	1.0	0.83-1.0	0	0-7.39	**0.83**	**0-1.82**	0.5645	1.0	0.11-1.0	0.2143	**0.9121**	0.5619
IL-21	0	0-0	**11.7**	**4.92-20.0**	0.0080	8.45	1.93-8.45	0	0-17.1	**31.4**	**9.62-39.9**	0.1172	31.42	0.56-32.52	>0.999	**0.0780**	0.1341
IL-22	0	0-3.39	**48.1**	**30.2-58.4**	0.0006	30.15	10.80-30.15	0	0-0	**33.0**	**22.1-69.6**	0.0444	32.95	22.11-32.95	0.5467	**0.8500**	0.5241
IL-23	0	0-0	**0**	**0-30**	0.0902	1.0	1.0-1.0	0	0-0	**0**	**0-0**	n/a	1.0	1.0-1.0	n/a	**0.5055**	0.5055
IL-31	0	0-0	**110**	**4.71-178**	<0.0001	110.19	4.71-110.19	0	0-0	**0**	**0-97.4**	0.3739	1.0	1.0-1.0	n/a	**0.2073**	0.2098
IFN α	0	0-0	**1.06**	**0-1.91**	0.0039	1.06	1.0-1.06	0	0-0	**0.95**	**0.78-1.87**	0.0239	1.0	1.0-1.0	n/a	**0.9401**	0.7556

TNF β	0	0-0	**45.6**	**10.7-78.3**	<0.0001	45.57	10.68-45.57	0	0-0	**12.4**	4.31-12.4	0.2150	12.41	4.31-12.41	n/a	0.3923	0.3923
TSLP	0	0-2.71	**12.2**	**7.51-16.0**	<0.0001	10.53	4.70-10.53	4.32	0-8.49	**12.5**	8.16-15.9	0.0758	1.89	1.47-1.89	0.0669	0.8576	0.4074
NGFF β	0	0-0	**7.04**	**6.54-10.9**	<0.0001	7.04	6.54-7.04	0	0-0	**8.10**	3.49-9.49	0.0179	8.10	3.49-8.10	n/a	0.8330	>0.9999
M-CSF	0	0-0	**251**	**207-303**	<0.0001	250.83	196.50-250.83	0	0-0	**322**	226-448	0.0066	321.78	226.43-321.78	>0.999	0.1679	0.1252
Chemokines																	
IL-8 (CXCL8)	0	0-0	**45.1**	**22.2-65.7**	<0.0001	45.06	17.43-45.06	0	0-5.3	**342**	**31.6-382**	0.0869	64.50	31.58-64.50	0.69	**↑0.0251**	0.4560
IP-10 (CXCL10)	40.6	29.4-139	**617**	**167-1136**	0.0010	7.96	4.24-7.96	28.9	17.5-153	**1318**	**1002-1814**	0.0055	34.65	11.85-34.65	0.7266	**↑0.0385**	↑0.0076
MIP A α (CCL3)	1.35	0-4.9	**10.2**	**7.38-11.2**	0.0029	7.38	2.00-7.38	5.39	2.42-8.63	**26.9**	**6.82-36.2**	0.1142	4.19	2.82-4.19	0.2720	**↑0.0147**	0.5290
ENA-78 (CXCL5)	71.5	33.6-120	**4.0**	**3.18-7.19**	<0.0001	0.1	0.03-0.64	36.5	8.21-110	**26.4**	**2.96-73.8**	0.6662	0.72	0.03-0.72	0.5549	**0.3544**	↑0.0413
BLC (CXCL13)	347	180-568	**830**	**226-2926**	0.0759	3.88	0.51-3.88	127	85.3-444	**3275**	**698-8532**	0.1622	19.24	5.51-19.24	0.3196	**0.1213**	↑0.0159
MCP2 (CCL8)	3.16	1.76-10.5	**13.3**	**2.73-39.5**	0.2228	2.81	0.51-2.81	2.93	0-6.44	**81.3**	**13.5-327**	0.2286	31.11	4.26-31.11	0.7692	**0.1264**	↑0.0070
MDC	50.4	28.4-81.3	22.7	17.7-86.5	0.1461	0.72	0.33-0.72	13.8	9.15-37.8	120	16.9-528	0.3429	3.77	0.47-3.77	0.8846	**0.5220**	↑0.0078
GRO α (CXCL1)	0	0-2.86	**5.41**	**2.62-8.75**	0.0059	4.59	0.64-4.59	0.59	0-12	**8.31**	**3.11-62.4**	0.3508	14.08	0.26-14.08	0.4286	**0.4560**	0.6593
MIG (CXCL9)	2.23	0-130	**236**	**30.3-1253**	0.0023	10.43	2.04-10.43	10.8	0-121	**1103**	**330-1289**	0.1122	72.87	11.12-72.87	>0.999	**0.2642**	0.8846
I-TAC (CXCL11)	58.4	16.4-87.9	**9.79**	**4.3-72.8**	0.1330	0.34	0.17-3.22	35.9	27.1-41.1	**385**	**11.9-1435**	0.2477	14.20	0.33-14.2	0.3655	**0.0879**	0.0879
MCP1 (CCL2)	23.4	16.3-37.7	**138**	**94.2-163**	<0.0001	5.28	2.69-5.28	4.88	2.02-7.48	**178**	**95.7-434**	0.1086	4.73	4.09-4.73	0.9301	**0.3681**	0.7692
MIP 1 β (CCL4)	5.52	0-19.4	**14.2**	**12-30.5**	0.0876	2.50	1.27-2.50	7.67	0-12.7	**33.4**	**11.1-51.9**	0.1094	2.62	1.45-2.62	0.6003	**0.1229**	0.2026
MCP3 (CCL7)	2.93	0-6.44	**12.4**	**8.58-31.1**	0.0003	3.73	1.18-3.73	10.8	0-12.7	**11.4**	**9.71-94.6**	0.3379	0.90	0.90-0.90	0.2361	**0.8077**	0.8626

EOTAXIN (CCL11)	11.3	7.14-25.5	**3.13**	**2.55-3.78**	0.0008	0.33	0.16-0.33	8.44	3.67-35.9	**4.90**	**2.96-14**	0.4582	0.39	0.35-0.39	0.8110	**0.1813**	0.1703
MIP 3 α (CCL20)	7.9	0-19.4	**13.9**	**6.47-21.2**	0.6622	1.0	0.34-1.0	31.4	14.5-36.9	**12.6**	**11.9-527**	0.4144	0.87	0.38-0.87	0.1236	**0.5330**	>0.9999
EOTAXIN-2 (CCL24)	32.6	14.3-184	**27.1**	**15.8-49.8**	0.4887	0.44	0.30-0.44	19.9	0.68-416	**25.3**	**15.6-250**	0.7714	0.79	0.60-0.79	0.8846	**0.8846**	0.2253
EOTAXIN-3 (CCL26)	0.5	0-2.35	**2.35**	**1.3-2.78**	0.1115	2.16	0.61-2.16	0.28	0-2.94	**3.03**	**1.16-4.49**	0.2471	1.53	1.16-1.53	0.8049	**0.4508**	0.6593
FRACTALKINE (CX3CL1)	1.95	0-9.65	**8.39**	**6.4-10.4**	0.0327	3.28	0.76-3.28	9.87	1.95-14.3	**16.3**	**7.18-19.2**	0.3434	1.34	0.73-1.34	0.2198	**0.0821**	0.6593
TNF superfamily related																	
BAFF	0.00	0-16.8	**50.1**	**33-55**	<0.0001	33.02	2.75-33.02	25.8	24.3-38.7	**50.1**	**37.3-103**	0.1751	1.54	1.29-1.54	↑0.0192	**0.2453**	0.0544
APRIL	267	96.9-411	**1749**	**908-3772**	<0.0001	7.19	5.46-7.19	120	32.5-563	**6779**	**940-16871**	0.1626	29.99	7.81-29.99	0.8564	**↑0.0253**	0.0879
TRAIL	20.6	10.9-39.7	**19.3**	**17.4-24.2**	0.6023	0.99	0.52-0.99	35.4	16-37.7	**57.2**	**16.5-67.4**	0.3649	1.90	0.44-1.90	0.6262	**↑0.0140**	0.3963
TNF α	0	0-0	**6.27**	**5.08-7.53**	<0.0001	6.27	5.08-6.27	0	0-0.83	**6.68**	**5.34-18.8**	0.0806	6.68	6.43-6.68	0.2143	**0.0969**	0.0635
TNF RII	49.1	30.7-72.4	**164**	**72.2-206**	0.0065	1.66	1.34-1.66	19.8	19.6-156	**221**	**83.8-276**	0.1535	4.27	1.77-4.27	0.3681	**0.3019**	0.1398
Tweak/TNFSF12	162	74.7-328	**2539**	**1394-4107**	<0.0001	16.43	7.83-16.43	149	55.2-448	**220**	**1722-4193**	0.0309	28.12	4.96-28.12	>0.999	**0.7692**	0.8846
CD30	279	112-558	**473**	**325-1029**	0.0473	2.09	1.17-2.09	142	50.7-345	**818**	**344-2995**	0.2155	6.80	2.37-6.80	0.3681	**0.2546**	0.1703
Other factors																	
SCF	1.55	1.03-2.22	**5.51**	**4.74-8.33**	<0.0001	3.77	2.32-3.77	2.38	1.83-3.12	**8.28**	**6.44-25.6**	0.1467	4.52	2.71-4.52	0.2745	**↑0.0365**	0.7692
SDF-1 α	738	490-1405	**1919**	**817-3685**	0.0652	1.33	0.96-1.33	182	0-3784	**5511**	**1389-7334**	0.1861	7.64	1.94-7.64	0.5549	**↑0.0492**	0.1264
VEGF-A	47.3	21.1-165	**103**	**96.9-172**	0.0879	2.36	0.69-2.36	42.3	30.1-66.2	**401**	**82.9-560**	0.0984	13.23	1.25-13.23	0.4397	**0.3681**	0.4560

HGF	36.9	26.9-59.3	**314**	**139-385**	<0.0001	8.48	3.33-8.48	21.6	16.7-140	**417**	**214-1590**	0.1889	11.32	9.94-11.32	0.7692	**0.0586**	0.2253
MMP1	4.86	2.84-11.9	**10.2**	**7.74-30.6**	0.0192	3.29	1.19-3.29	8.88	4.16-11.3	**13.6**	**10.9-453**	0.3632	2.62	1.53-2.62	0.5549	**0.2912**	0.3395
FGF-2	0	0-0	**7.69**	**2.82-10.5**	<0.0001	7.10	2.82-7.10	0	0-0	**3.83**	**0-9.57**	0.1835	3.83	1.00-3.83	>0.999	**0.3785**	0.5169
CD40L	0	0-2.05	**0.46**	**0-4.64**	0.2192	1.0	0.46-1.0	0	0-7.51	**3.64**	**0-5.30**	0.8796	1.0	0.71-1.0	0.7527	**0.8242**	0.6257

*If parametric, unpaired T Test; if non-parametric, Mann-Whitney

** Arrow represents direction in those with poor versus goof outcome i.e. if higher in those with poor outcome ↑; if lower in those with poor outcome ↓. Only indicated for those with statistically significant differences ($p<0.05$)

Chapter 6. Magnetic resonance spectroscopy to detect GABA and Glutamate in HIV-associated Tuberculous Meningitis

6.1 Introduction

Nuclear magnetic resonance (NMR) refers to the behaviour of atoms when subjected to a magnetic field and was first described in 1946 by Bloch and Purcell(Packard et al., 1946, Purcell et al., 1946). In biomedical research this principle can be applied via a range of techniques including structural magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS). The latter encompasses a diverse set of methods, including via *in vivo* clinical systems to measure metabolism of well-defined regions of the human body, including the brain. When combined with structural MRI in this context, the tool obtains functional metabolic information in addition to anatomical information within the area of interest.

The basic physical principles of MRS are complex, but the central underlying concept relates to 'nuclear spin' which can be understood as the nucleus of an atom spinning around its own axis. In a large magnetic field, nuclei with spin can undergo resonance and emit a characteristic electromagnetic signal. In ^{1}H MRS, radiofrequency signals arising from the hydrogen atom within tissue metabolites are detected. These signals possess chemically specific frequencies which is determined by the environment (chemical shift)(Tognarelli et al., 2015). These signals are expressed as an MR spectrum i.e. peaks occurring at different frequencies; a plot of signal intensity, proportional to metabolite concentration, against

chemical shift (Figure 6.1). The most important visible peaks on the *in vivo* cerebral proton MR spectra using field strengths of 1.5 to 3.0 Tesla (T) are N-acetyl aspartate (NAA), choline (cho), creatine (Cr) myo-inositol (mI), the combined glutamine and glutamate peak (Glx) and lactate (Lac). More complex to acquire is information regarding concentration of GABA, however validated methods exist to isolate GABA signals from the spectra(Puts and Edden, 2012).

Glutamate is a neuroexcitatory neurotransmitter which acts on receptors classed as either ionotropic (i.e. ligand gated ion channels) or metabotropic (i.e. G protein coupled) on post-synaptic cells, astrocytes, oligodendrocytes and glial cells(Dingledine et al., 1999). Extracellular glutamate levels are regulated by the glutamate-glutamine cycle to prevent excessive glutamate and its associated toxicity(Lipton and Rosenberg, 1994). The balance of glutamate/glutamine and GABA, a neuroinhibitory neurotransmitter, is crucial in maintaining normal neurological function. Prolonged glutamate exposure results in neuronal injury and damage via influx of Ca2+ and Na+ ions across the membrane. This is worsened by the activation of GTP-binding protein-coupled glutamate receptors which stimulate Ca2+ release from the endoplasmic reticulum. When neurons are under oxidative or metabolic stress e.g. due to ischaemia, these glutamate-driven excitotoxic processes can occur even in the context of normal concentrations of glutamate(Gamir-Morralla et al., 2017).

Glutamate driven neuro-excitotoxicity is a key driver of pathogenesis in many neurological conditions including Alzheimer's Disease and traumatic brain injury(Butterfield and Pocernich, 2003, Chamoun et al., 2010). In TBM, a study of ventricular CSF in children with TBM found that transcripts

associated with glutamate release, post synaptic NMDA receptor binding and Ca^{2+} influx were enriched, suggesting that in acute TBM neuro-excitotoxicity may be driven by glutamate release(Rohlwink et al., 2019). A more recent study, also in paediatric TBM supported this hypothesis using ^{1}H metabolomics demonstrating that glutamine and glutamate are elevated in the CSF at disease presentation(van Zyl et al., 2020). Although glutamate and GABA have been evaluated *in vivo* using MRS in many central nervous system diseases including neuro-oncology(Hazany et al., 2007), Alzheimer's disease(JM Scott et al., 2011), psychiatric diseases(Mason and Krystal, 2006), and multiple sclerosis(Cianfoni et al., 2007b), no studies have evaluated its potential in TBM. If feasible, this non-invasive technique would allow correlation of *ex vivo* findings and add depth of understanding relating to the glutamate-glutamine cycle and neuro-excitotoxicity in TBM. It could also, albeit in well-resourced settings, serve as a potentially useful biomarker of clinical outcome, as has been explored in traumatic brain injury(Shutter et al., 2004).

In a pilot study using ^{1}H-MRS in adults with HIV-associated TBM we aimed to examine *in vivo* concentrations of glutamate/glutamine (hitherto referred to as Glx) and GABA within the brain and compare these to a group of healthy control subjects. Our hypotheses were:

i. Magnetic Resonance Spectroscopy is a feasible mechanism to measure *in vivo* concentrations *of* Glx and GABA in HIV-associated TBM
ii. Glx would be found in higher concentrations *whereas* GABA would be found in lower concentrations, in the acute phase of HIV-associated TBM compared to healthy controls

6.2 Methods

This study was approved by the University of Cape Town Human Ethical Research Committee (HREC 293/2018), including the enrolment of up to 10 healthy controls for comparison analysis.

Participants

Participants enrolled to the LASER-TBM study (see chapter 2) were consented for imaging including MRI and MRS at two timepoints; enrolment and day 56. This consent was part of the main study consent and therefore although all enrolled participants were eligible for inclusion, imaging only took place when participants were well enough to travel from the referring hospital site to the University of Cape Town's imaging facility. Participants were required to complete an MRI questionnaire prior to imaging being performed those with contra-indications for MRI were not imaged.

A total of 9 healthy controls provided written informed consent to be included in this study. The study was advertised on the University of Cape Town Health Sciences campus, and participants enrolled were staff and students at the university. Prior to inclusion in the study, any volunteers were required to complete the MRI safety questionnaires; those with contraindications were not included within the study. HC underwent a single timepoint scan.

Neuroimaging

Participants were scanned on a 3T Siemens Skyra MRI scanner at CUBIC using a 32- channel head coil. No sedation was given. Two healthy control volunteers were scanned to evaluate feasibility prior to imaging patients with TBM. These data were analysed and the protocol refined prior to scanning of patients with TBM. Remaining healthy volunteer scans (n=7) were performed following completion of the LASER-TBM participant scans.

Voxel placement

Voxel placement was considered in the context of the common anatomical location of vascular injury in TBM. Specifically, infarcts within the tubercular zone account for around 60% of strokes in TBM(Misra et al., 2011). The tubercular zone comprises the caudate, anterior thalamus, anterior limb and genu of the internal capsule. Its vulnerability to microvascular damage is likely due to the exudate affecting the vessel wall architecture in the medial striate, thalamotuberal and thalamostriate arteries which supply this area. It is also an area vulnerable to further ischaemic injury in the hydrocephalic patients. Therefore, placement of the voxel within this location was thought most likely to yield interesting results (Figure 6.2).

Imaging protocol

An imaging protocol was developed in collaboration with an MRI physicist (Dr Frances Robertson), a physicist (Professor Ernesta Meintjes) and senior radiographer (Petronella Samuels). A structural high resolution T1-weighted magnetisation prepared rapid gradient echo acquisition was acquired for voxel placement with the following variables: Field of View (FOV) 230 mm × 230 mm, Repetition Time (TR) 1720 ms, Inversion Time (TI) 900 ms, Echo Time (TE) = 2.47 ms, flip angle 8 degrees, bandwidth

250 Hz/px, 176 slices, resolution 1 mm × 1.0 mm × 1.0 mm (duration 3 minutes 30s). This was followed by 2 single voxel point-resolved spectroscopy (PRESS) acquisitions from the same voxel with Chemical Shift Selective (CHESS) water suppression: TR=2000 ms, TE =30s, spectral bandwidth 2000Hz, vector size 1024, 64 averages, voxel size 3 x 3 x 3 cm^3. A water reference (2 averages) was acquired in the same voxel without water suppression for subsequent analysis (total duration 2 and a half minutes). Single Voxel Spectroscopy (SVS) acquisitions were then obtained from a 3 x 3 x 3 cm voxel in the same region using Mescher-Garwood point-resolved spectroscopy (MEGAPRESS)(Mikkelsen et al., 2017): TR=2000ms, TE=80ms, 320 averages, bandwidth 2000 Hz, vector size 2048 with ON editing pulse at 1.9 ppm targeting GABA (duration 30 minutes)

Statistical Analysis

We used MEGAPRESS (for GABA) and PRESS (for Glx) acquisitions and subsequently GANNET software(Edden et al., 2014) to estimate metabolite concentrations within the tubercular zone. Metabolite concentrations were measured in institutional units (IU). A combined Glutamine/Glutamate (Glx) concentration was used as a proxy for glutamate concentration, given that these are challenging to differentiate, consistent with previous studies in the literature. Concentrations were corrected to account for the proportion of grey and white matter, as well as CSF within the voxel using published methods(Harris et al., 2015). These 'tissue-alpha corrected' concentrations for both Glx and GABA were subsequently used for analysis. We compared:

- i) Glx and ii) GABA concentrations in patients with TBM at baseline (within 5 days of enrolment) with healthy controls
- i) Glx and ii) GABA concentrations in patients with TBM at baseline and follow up timepoints (day 56 +/- 5 days).
- Glx: GABA ratios in patients with TBM at baseline compared to healthy controls.

Statistical analysis was performed using GraphPad Prism (version 9.3.1) software. We compared variables between groups using unpaired t Tests (parametric data) or Mann Whitney Tests of significance (non-parametric data). Throughout the analysis we used an unadjusted p value of <0.05 as a nominal threshold for statistical significance.

6.3 Results

A total of 11 participants with HIV-associated TBM enrolled to LASER-TBM completed the baseline MRI/MRS imaging protocol (Figure 6.3). 7 of these 11 participants were available for follow up imaging. 4 baseline scans were excluded from the analysis; 2 due to a file formatting error, 2 due to lack of water reference data, which made the data uninterpretable. Of the follow up scans performed, 3 were excluded for similar reasons, leaving 4 for analysis. Baseline characteristics are outlined in table 6.1. Age was not similar across groups, however gender was. The majority of TBM participants had mild disease.

Glx concentrations

We found significantly higher concentrations in those with HIV-associated TBM at baseline compared to healthy controls (median (IQR) concentration

(IU) 15.9 (13.4-16.2) vs 12.2 (11.7-15.5), *p=0.0428* (Figure 6.4(A)). When we compared concentrations at baseline and follow up, we saw a reduction in concentration over time, however this did not reach statistical significance (median (IQR) concentration (IU) 15.9 (13.4-16.2) vs 14.6 (13.8-15.6), p=*0.4136*). Although concentrations of Glx reduced within the first 2 months of treatment, there remained a trend towards raised levels when compared to healthy controls (median (IQR) concentration (IU) 14.65 vs 12.79, *p=0.2190*) (Figure 6.4(B)).

GABA concentrations

There was no statistically significant difference between concentrations of GABA in patients with TBM at baseline compared to healthy controls (median (IQR) concentration (IU) 3.79 (3.34-4.5) vs 4.02 (3.09-4.69), *p=0.69*) (Figure 6.5(A)). Similarly, there was no significant change in concentration from baseline to day 56 those with TBM (median (IQR) concentration (IU) 3.79 (3.34-4.5) vs 3.789 (2.72-4.27), *p=0.65*) (Figure 6.5(B)).

Glx:GABA concentration ratios

We compared ratios of Glx and GABA in those with TBM (at baseline) and healthy controls as a proxy for degree of neuroexcitotoxicity and found a non-significantly higher ratio in those with TBM compared to healthy controls (median (IQR) concentration 3.98 (3.56-4.74) vs 3.31 (2.53-4.19), *p=0.17*) (Figure 6.6).

Glx concentrations and ratios in relation to cognitive outcomes

In the 7 participants in whom baseline data was available, only 3 participants had completed a full cognitive and functional outcome assessment at day 180 as detailed in chapter 4. We compared global T score as a continuous variable indicating cognitive performance and i) glutamate/glutamine concentrations at baseline and ii) the ratio of Glx:GABA concentrations at baselines using simple linear regression. Contrary to our central hypothesis that glutamate driven excitotoxity may be a driver of poor outcome, we found higher glutamate/glutamine: GABA ratios were associated with better neuropsychological performance (*p=0.032*) (Figure 6.7). None of these three participants in whom cognitive data was available had paired timepoint MRS data for comparison of glutamate levels over time.

6.4 Discussion

Within this proof-of-concept study, we demonstrated that *in vivo* measurement of Glx and GABA using ^{1}H MRS is feasible via placement of a single voxel within the deep grey matter of the brain. Within our small cohort, our results suggest that i) Glx concentrations and ii) Glx:GABA ratios are significantly elevated in those with HIV-associated TBM around the time of their diagnosis, when compared to a group of healthy controls. They also show that over a 2 month period, concentrations of glutamate/glutamine decrease, but remain raised compared to healthy controls. As glutamate/glutamine are neuroexcitatory neurotransmitters, and Glx:GABA ratios a proxy for neuroexcitation, this study begins to support findings within the literature(van Zyl et al., 2020, Rohlwink et al.,

2019) which suggest that glutamate driven neuroexcitotoxicity may be a pathogenic mechanism in the acute phase of HIV-associated TBM. Larger studies are now required to validate these findings.

Although our findings of increased glutamate are in keeping with the TBM literature on this topic, it is interesting to consider them within the context of other diseases in which MRS is widely used and in which glutaminergic dysfunction is thought to be a key pathogenic mechanism. For example, in Alzheimer's disease glutaminergic dysfunction is thought to occur via Aβ and tau mediated disruption of glutamate receptor function leading to glutamate accumulation within the synaptic and extra-synaptic spaces(Yeung et al., 2021). Studies using MRS, however, have shown that glutamate is *reduced* in the brains of patients with Alzheimer's, a finding which is thought to represent neuronal loss and injury(Rupsingh et al., 2011). In contrast, a study of four patients with tumefactive demyelinating lesions, a highly active inflammatory form of multiple sclerosis, MRS demonstrated marked elevation of glutamate and glutamine peaks within focal lesions(Cianfoni et al., 2007a); however, in primary and secondary progressive forms of MS longitudinal studies using MRS demonstrate that glutamate and glutamine reduces over time(MacMillan et al., 2016). Our findings in the context of these studies support the hypothesis that in acute TBM, extracellular glutamate is present in excessive levels, potentially driving neuro-excitotoxicity. However, they also raise some important questions, namely what happens to glutamate levels following the acute phase of illness? Although we saw a reduction at 2 months within our study, would longer term imaging timepoints reveal lower levels of glutamate when compared to our healthy controls demonstrating neuronal loss and injury, as seen in neurodegenerative diseases? Given the role of

glutamate in learning and memory(Riedel et al., 2003), and its association with cognitive outcomes in conditions such as multiple sclerosis(Muhlert et al., 2014) and HIV(Mohamed et al., 2010), how would this correlate with cognitive function at longer term timepoints? Further exploration of these questions would allow us to consider whether MRS has a role as a predictive biomarker in TBM, as is the case in traumatic brain injury(Babikian et al., 2006, Ashwal et al., 2000, Eisele et al., 2020).

The number of participants in whom both MRS and detailed cognitive testing at 6 months was performed were too few to draw conclusions; nonetheless in these three participants our results were surprising. Namely we saw that baseline glutamate levels were lower in those with worse cognitive outcomes. Although this is most likely due to the small number of participants included within this analysis, it is also possible that in these three participants baseline cognitive function (which was not assessed) was worse due to poorer HIV control. To address this question, we looked at baseline CD4 count in these three participants. Although the participant with the lowest Glutamate:GABA ratio and the poorest global T score (participant G-2) had the lowest CD4 count (50); the other two participants did not follow this correlation (M-13, CD4 count 468; M-16, CD4 count 373) (see figure 6.7). CD4 count at time of TBM diagnosis is a poor proxy marker for overall HIV control, therefore this explanation may still be plausible. The usefulness of further analysis into only three participants to find explanations for this trend is questionable; however, the result highlights the complexity of interpretation of MRS results in this context given the broad spectrum of variables which may influence metabolite concentration besides TBM itself; namely HIV co-infection, previous traumatic brain injury, aging and co-morbidities such as other forms of

dementia. Further studies considering the utility of MRS must consider these variables, and control for them within the populations assessed.

Within this small study, our aim was to measure glutamate/glutamine and GABA concentrations within our participants. However, data on other metabolites measured within the spectrum may be interesting within TBM. In traumatic brain injury glutamate/choline ratios as well as glutamate levels have been used as a predictive marker of neurocognitive outcome(Shutter et al., 2004). Studies in HIV have shown creatine, choline, myo-inositol and N-acetyl-aspartate have roles in measurement of brain injury in HIV (reviewed in (Chaganti and Brew, 2021)). In multiple sclerosis, N-acetyl-aspartate is a marker of neuronal loss whilst elevated choline peaks represent increased cell membrane turnover and therefore interpreted as a marker of demyelination, inflammation and gliosis; in fact the complete spectrum of metabolite concentrations can be interpreted to establish prognosis, following disease evolution, understand pathogenesis and as a marker of therapeutic efficacy in MS (reviewed in (Narayana, 2005)). Further development of this tool within TBM, learning from other neuroinflammatory and neurodegenerative diseases, in settings where MRS is available, would allow us to harness the full capability of this tool to improve outcomes in TBM.

There are several limitations to this study. Firstly, only a small number of participants were included. This is in part due to the complexity of transporting participants from referring sites across Cape Town to the imaging facility, but also due to COVID-19 related restrictions making the ongoing inclusion of TBM participants impossible after March 2020. Moreover, given the novel nature of our imaging protocol, a number of

imaging sequences could not be included e.g. due to lack of water reference data. A larger study, following further refinement of the protocol to ensure all data collected is interpretable, is required to validate these findings. Secondly, the majority of participants enrolled had mild disease. All participants enrolled to LASER-TBM were eligible for inclusion; however, i) in general most LASER participants had mild or moderate disease and ii) those who were more unwell were in many cases not stable enough to be transported and/or imaged. It is plausible that in more severe disease, our findings may be more striking? However, future studies including patients with severe disease are required to test this hypothesis and to ensure generalisability of results. Thirdly, our healthy controls were younger than our TBM participants, and did not have a diagnosis of HIV. One study has demonstrated that glutamate concentrations within a similar region imaged within our study (the striatum) reduces with age(Zahr et al., 2008), therefore it is possible that differences in glutamate concentrations between our groups are unlikely to have been due to age and may well have been more marked in a better matched group. Other studies however have demonstrated that aging can be associated with different patterns of glutamate changes depending on whether white or grey matter is captured within the single voxel(Kaiser et al., 2005). Along these lines, our study results reflect changes within a single location within the brain where we know microvascular pathology is likely to occur. In TBM however, neurological sequalae can occur in many regions of the brain. Our results in chapter 4 suggest that cognitive impairment is diffuse, affecting multiple domains corresponding to multiple anatomical areas. Therefore, placement of the voxel within other areas, including cortex and subcortical white matter tracts, as well as within lesions such as infarcts or tuberculomas would provide a greater breadth of understanding of metabolic changes

occurring in TBM. Finally, it is unclear whether the changes seen are specific to TBM; comparison to a control group of other forms of meningitis would allow us to differentiate changes specific to TBM.

In conclusion, our study provides *in vivo* correlation of findings within the literature, which suggest that glutamate-driven excitotoxicity may have a role in the pathogenesis of TBM. A recent international survey found that although 76% of TBM settings surveyed did have access to MRI, this wasn't routinely used, likely due to higher burden on available resources(Tucker et al., 2020). This suggests that the framework for developing MRS exists however that in the short term at least its potential may be limited to understanding pathogenic mechanisms via studies undertaken in well-resourced research settings; this study proves that MRS can be developed as a useful tool in this context. Larger studies are now required to validate these findings, measure changes at longer term timepoints and correlate findings with neurological outcomes including cognitive impairment to improve our understanding of pathogenic mechanisms. In time, with greater evidence for its utility and greater resource and expertise in TBM endemic areas, MRS may have a wider role including as a predictive measure and monitoring tool in order to improve outcomes for patients with TBM.

Figure 6.1 Magnetic resonance spectrum of normal brain

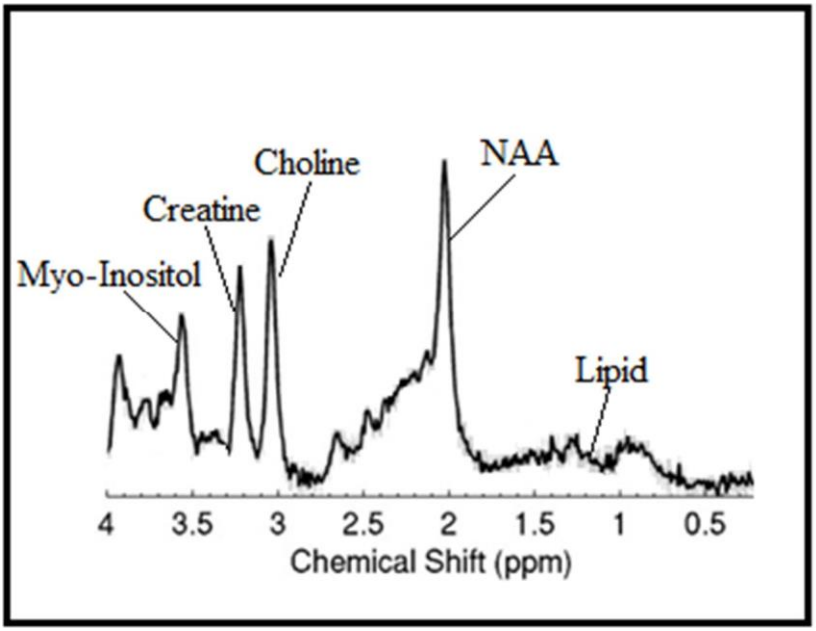
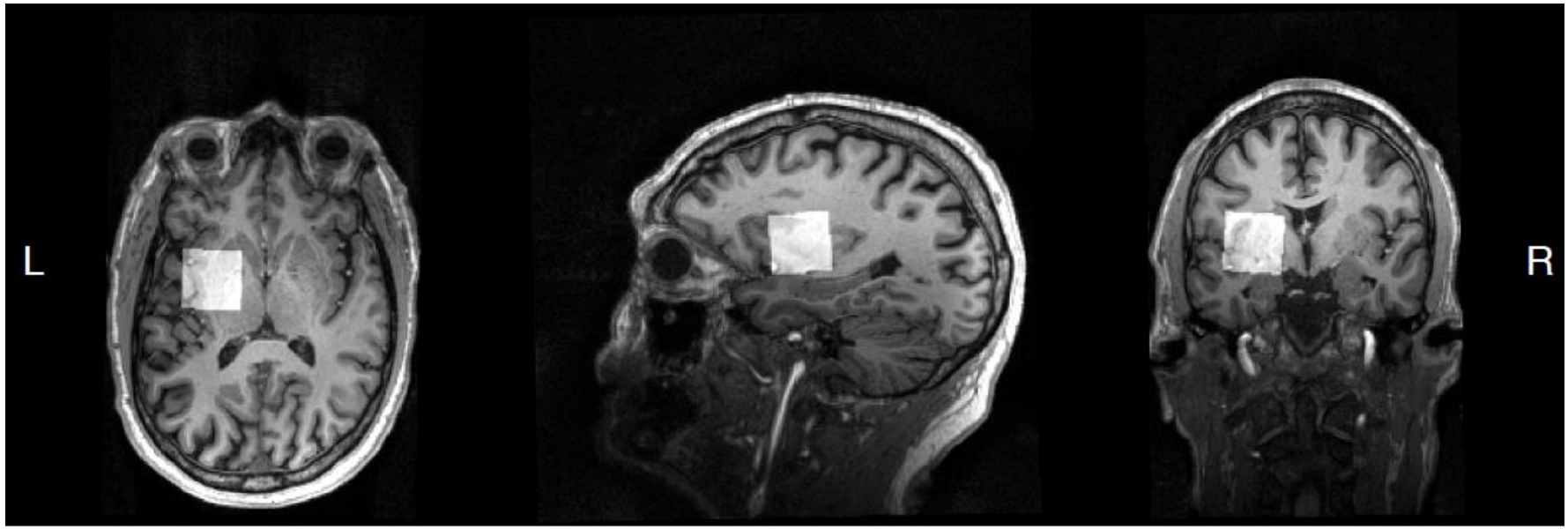


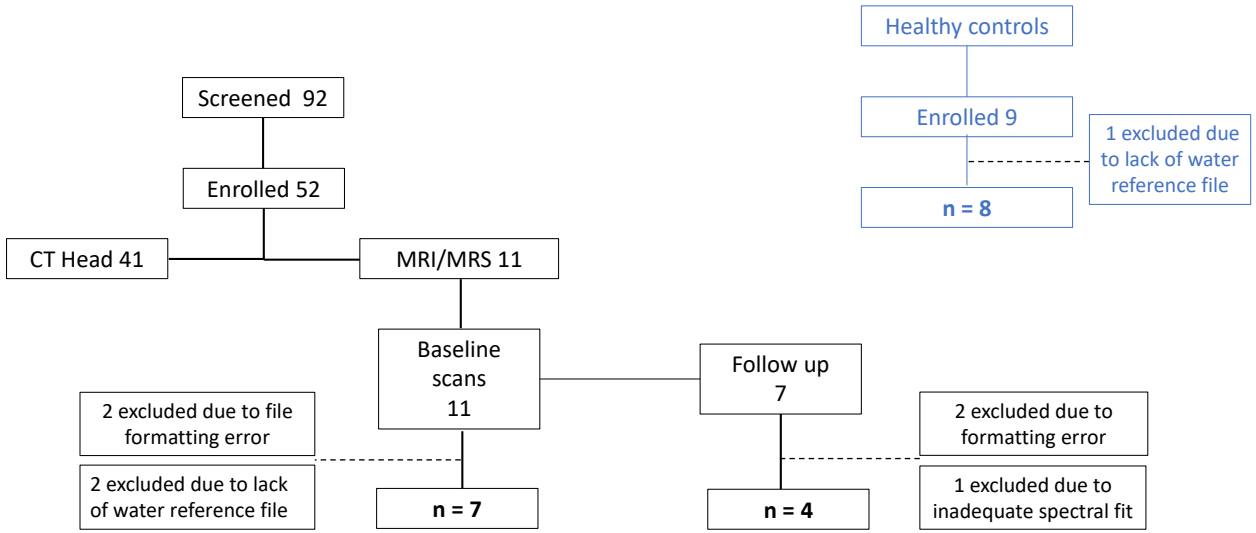
Figure taken from (Manias and Peet, 2018) showing normal MRS spectra. NAA, N-acetyl-aspartate.

Figure 6.2 Voxel placement



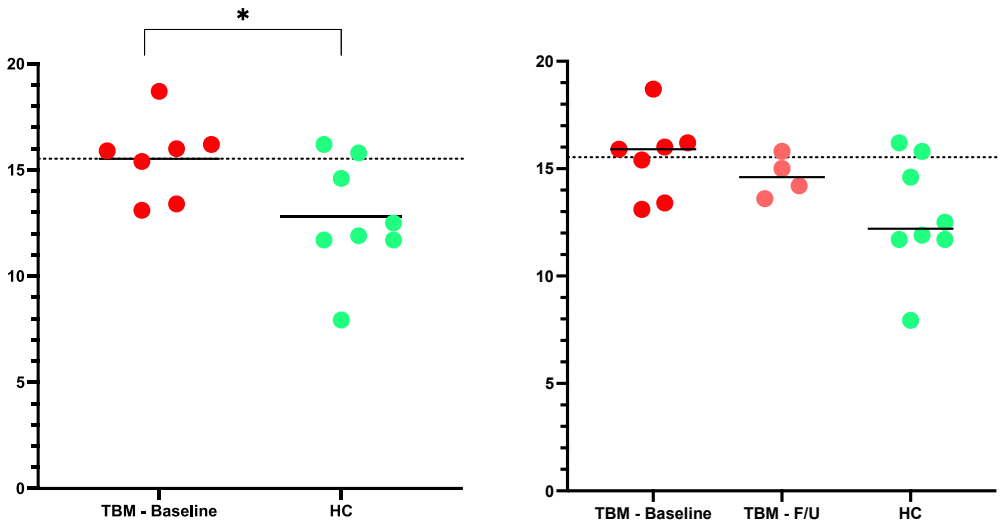
From left to right, axial, sagittal and coronal images demonstrating placement of the single voxel within the ‘tubercular zone’

Figure 6.3 Study CONSORT for magnetic resonance spectroscopy study



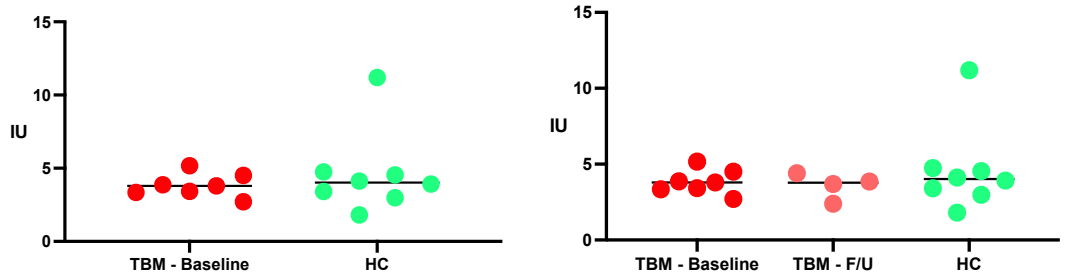
CONSORT diagram demonstrating i) imaging modalities used in LASER-TBM and inclusion of participants for magnetic resonance spectroscopy imaging and ii) recruitment of healthy controls for comparison

Figure 6.4 Glutamate/glutamine (Glx) concentrations in participants with TBM versus healthy controls



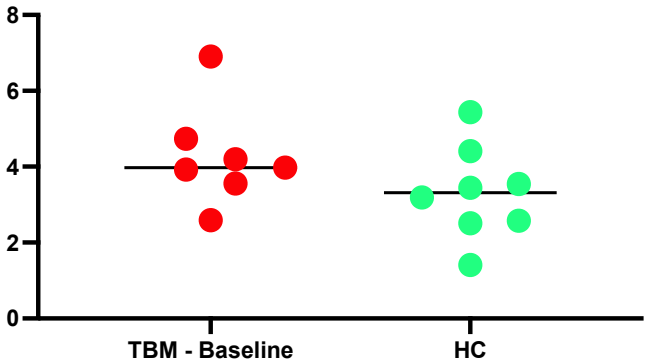
The graph on the left demonstrates that there is significant reduction of Glx concentrations at baseline in TBM participants compared to healthy controls (*p=0.0428)*. The graph on the right demonstrates that these concentrations reduce within the first 2 months of treatment but not to the level of healthy controls. X axis units IU.

Figure 6.5 GABA concentrations in participants with TBM versus healthy controls



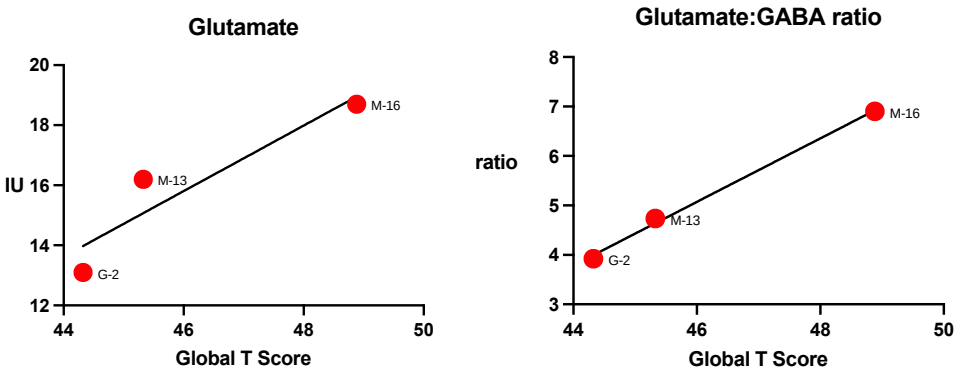
The graph on the left demonstrates that there is no difference in GABA concentrations at baseline in TBM participants compared to healthy controls. The graph on the right demonstrates that these concentrations do not change within the first 2 months of treatment.

Figure 6.6 Glutamate:GABA ratios in participants with TBM versus healthy controls



There is a non-significantly raised Glx:GABA ratio in TBM participants compared to healthy controls (*p=0.173)*

Figure 6.7 Baseline glutamate concentrations and glutamate:GABA concentrations in relation to cognitive outcomes



The graph on the left plots glutamate concentration at baseline scan to global T score on cognitive test performance at 6 months and shows a non-significant correlation by simple linear regression analysis (*p=0.2382*). The graph on the right plots Glutamate:GABA ratio at baseline scan to global T score on cognitive test performance at 6 months and shows a significant correlation by simple linear regression analysis (*p=0.0362*).

Table 6.1 Baseline demographics and clinical characteristics of participants included with the study of magnetic resonance spectroscopy in TBM

	TBM	Healthy controls	*p* value
n	7	8	
Age (median, IQR)	38 (34-43)	23 (22-28)	*p=0.006*
Gender Male Female	 2 4	 4 4	
Median days from enrolment to baseline scan	5		
MRC Grade Grade 1 Grade 2 Grade 3	 7 0 0		
TBM case definition criteria Definite Probable Possible	 1 5 1		

Chapter 7. Discussion

My PhD set the ambitious goal of improving understanding of pathogenesis in TBM. To achieve this goal, under the supervision of my primary supervisor, I set out to design and complete a clinical trial, within which nested sub-studies would enable distinct yet interlinked studies of disease complications and pathogenic mechanisms in TBM. Within the chapters of this thesis, I have:

1. Given a comprehensive summary of current treatment strategies, potential novel drug targets and current understanding of pathogenic mechanisms in TBM
2. Described the design and results of a phase 2A randomised controlled trial evaluating a novel drug regimen in HIV-associated TB
3. Described cognitive and functional impairment in HIV associated TBM
4. Described immune profiles in HIV-associated TBM via ex vivo analysis of blood and CSF using Luminex platform technology
5. Developed a novel protocol to measure GABA and glutamate levels in the brain and compared levels to healthy controls via a pilot study using Magnetic Resonance Spectroscopy in TBM

The COVID-19 pandemic occurred during the course of this PhD, and as such a hiatus to this work enabled me to design a study of neurological manifestations of SARS-CoV2 infection within a wider platform of studies, namely: ‘Health facility-based observational studies to investigate interaction and overlap between SARS- CoV2, HIV-1 and *M. tuberculosis* infections’, the ‘HIATUS’ study. As well as describing neurological presentations to a tertiary COVID-19 referral centre during the first wave of

the pandemic in South Africa, the *ex vivo* analysis provided insights on neurotropism of SARS-CoV2. The cohort also provided a comparison group for the ex vivo analysis of TBM samples.

In each of the results chapters (chapters 3-6) I have discussed the main findings and limitations of each of these studies in turn. Within this chapter, I aim to summarise these and comment upon the implications of these results within the context of advancing our understanding of pathogenesis in TBM.

In chapter 3, we demonstrated that linezolid and high dose rifampicin can be safely combined in the treatment of HIV-associated TBM. We also showed that high-dose aspirin was safe when combined with intensified antibiotics. As well as providing timely safety data to support further evaluation of these important drugs in a phase 3 setting, the trial is the first to evaluate a treatment approach which simultaneously aims to ensure i) effective killing of Mycobacteria within the CNS and ii) control of the dysregulated immune response in TBM. Given the poor treatment outcomes in TBM and the lack of any proven effective host directed therapy in HIV co-infected patients, we believe that this approach is rational to ensure that drug development in this field moves forward at a pace which is timely and appropriate. The nested sub-studies within LASER-TBM have, in the context of my PhD, advanced our understanding of pathogenesis in TBM, which I expand upon in summaries below. However, beyond the scope of this PhD, samples arising from this trial will contribute to our understanding of pathogenic processes via ongoing and planned integrated multi-omic analysis of blood and CSF in patients with HIV-associated TBM.

In chapter 4, we demonstrated that cognitive impairment occurs in 50% of people with HIV-associated TBM at six months following diagnosis. Inclusion of comparator groups of people living with HIV and patients with TB occurring outside of the CNS allowed us to conclude that impairment was above that attributable to HIV, or systemic inflammation and polypharmacy in TB alone. This was, by far, the largest study to date within this field and describes for the first time, using validated and comprehensive neuropsychological assessments, the frequency and nature of this disabling complication in TBM. It also however highlights the value of using objective and quantitative measures of neurological outcome. As we endeavour to uncover drugs which improve survival in TBM, we must also think hard about better ways to evaluate morbidity in this disease. The Modified Rankin Scale has been adopted by the TBM community however is a crude marker of outcome in a condition where neurological complications are diverse and complex. Identifying outcome measures which have the potential to be adapted for both well and poorly resourced settings will not only provide more granular information on the clinical phenotype for pathogenesis studies of TBM, but also allow us to better understand the true impact of this disease for our patients with TBM.

In chapter 5, via a comprehensive immunoassay analysis of CSF and blood, we show that immune responses in TBM are highly compartmentalised, remain persistently raised at four weeks following diagnosis, despite treatment, with highest concentrations evident prior to death. In keeping with the literature, we highlight the role of the innate immune response, but also identify mediators known to be involved in adaptive immune responses in those with poor outcome. We identify IL-1β as a potential blood-based biomarker of poor outcome. These results

require validation in a larger cohort, however, they do contribute insight to pathogenic mechanisms and biomarkers of poor outcome in TBM.

In chapter 6, we demonstrate that MRS is a feasible mechanism to measure concentrations of glutamate and GABA in the brains of patients with TBM. Within this small pilot study, we show that concentrations of glutamate are significantly raised in the acute phase of TBM compared to healthy controls, and there is a trend towards persistently raised levels at 2 months despite treatment. In light of recent publications, which describe the potential contribution of glutamate driven excitotoxicity following TBM, this novel mechanism for validating these findings *in vivo* is exciting. Further work following this PhD now plans to use multi-omic analysis to understand whether pathways associated with glutamate and GABA are associated with poor outcome within the same cohort. Beyond this, MRS may have a broader role in understanding pathogenic mechanisms in TBM, particularly in relation to longer term sequalae such as cognitive impairment as it has done in other neurological diseases.

Within the study included within the appendix of this thesis, during the first wave of COVID-19 at a time when SARS-CoV2 was a new and poorly understood virus, we set out to evaluate the spectrum of neurological manifestations and the potential for this virus to penetrate the CNS. In this study, we described neurological presentations in a setting in where COVID-19 infections were high and, through systematic evaluation of the CSF, demonstrate that penetration of SARS-CoV2 to the CNS is uncommon, even in suspected cases of meningoencephalitis.

Conclusions

Whilst I recognise limitations in my work, I believe that the research contained within this thesis contributes important information for the development of new and effective treatment regimens, the knowledge of neurological sequalae and understanding of pathogenic mechanisms in TBM. TBM is a disease which affects some of the world's most vulnerable people, is a disease of poverty and one with devastating outcomes. Fortunately, senior academics within the field are collectively driving forward a diverse and energetic programme of research which address many of the unanswered questions within this field. The work undertaken within my PhD has provided me the opportunity to work with, learn from and be inspired by some of the most effective researchers across the world who share with me an ambition to improve outcomes for those with TBM. In particular, the work has presented to me many new questions around longer term neurological complications in TBM and the pathogenic mechanisms underlying these. I intend to remain active in the TBM field, building upon the skills I have acquired within this PhD to address these questions and others (whilst asking many more) throughout my research career.

Appendix: Spectrum of neurological manifestations and systematic evaluation of cerebrospinal fluid for SARS-CoV2 in patients admitted to hospital during the COVID-19 epidemic in South Africa (The HIATUS-3 Study)

Introduction

Although COVID-19, the disease caused by SARS-CoV2, is primarily a disease of the respiratory tract, coronaviruses (CoV) are known to be neurotropic with some strains leading to meningitis, encephalitis and cerebral vasculitis (Puelles et al., 2020). In the first case series of SARS-CoV2 conducted in Wuhan, China, it was found that in 62 patients, 21 (34%) reported headache (Xu et al., 2020). In a subsequent retrospective case series to investigate neurological presentations in 214 patients with COVID-19, 36% of patients were found to have neurological signs and symptoms, which were more frequent in severe compared to non-severe COVID-19 (45% vs 30.2%). Since then, a wealth of literature suggests that SARS-CoV2 has the potential to present with neurological manifestations, with or without pulmonary involvement. Reports include cases of meningitis/encephalitis, with (Moriguchi et al., 2020, Khodamoradi et al., 2020b) and without (Espinosa et al., 2020) detection of SARS-CoV2 within the cerebrospinal fluid (CSF), stroke (Beyrouti et al., 2020), transverse myelitis (Kang Zhao, Sarma and Bilello, 2020, Valiuddin et al., 2020), acute disseminated encephalomyelitis (de Miranda Henriques-Souza et al., 2021) and inflammatory polyradiculopathies such as Guillain-Barre Syndrome

(Sedaghat and Karimi, 2020, El Otmani et al., 2020, Alberti et al., 2020, Toscano et al., 2020a). Whether the mechanism by which these phenomena occur are due to, either individually or in combination, direct neurotropic invasion, para- or post-infectious inflammation, a systemic coagulopathic processes or indeed due to separate and co-incidental pathogenic processes is not well understood.

The distinction between direct CNS invasion and other potential mechanisms is however important to make. Clinicians who assess patients presenting with neurological symptoms in the context of a COVID-19 epidemic must know when and how to investigate for neurological complications of SARS-CoV2 infection. Moreover, should SARS-CoV2 commonly lead to neurological symptoms via direct CNS invasion, particularly in the absence of pulmonary symptoms, then patients should be screened routinely for neurological involvement, and physicians must consider this unique potential of the virus. If direct SARS-CoV2 invasion to the CNS does not explain neurological presentations in this context, then greater focus must be towards understanding the nature and contribution of para- and post-infectious inflammatory phenomena and coagulopathy in order to develop effective therapeutics to treat these often severe complications of disease. This is particularly important in resource limited settings where testing for SARS-CoV2 is not always available and as yet, there are no bespoke methods to test for SARS-CoV2 in cerebrospinal fluid (CSF). In these settings, the precise incidence of SARS-CoV2 infection may not be known or be underestimated: in the Eastern Cape of South Africa for instance where this study took place, seroprevalence following the first wave of the epidemic has been found to be as high as 62.5% in adults under the age of 65 years which is 8-fold higher than the official case

count (Sykes et al., 2021), and has led to death in an estimated 1 in 300 people in the region (SAMRC data) (https://www.samrc.ac.za/reports/report-weekly-deaths-south-africa?bc=254). Such high rates of infection and subsequent mortality not only calls for better resource to manage the disease in these settings, but should also alert the clinician to the increased possibility of encountering less typical presentations of SARS-CoV2 infection.

To date no published studies have systematically investigated the presence of SARS-CoV2 within the CNS in patients presenting with neurological symptoms with and without pulmonary manifestations of COVID-19 in a context where COVID-19 infection is the most frequent reason for hospital admission. In a prospective cohort study in the Eastern Cape of South Africa, we described clinical and radiological features, and assessed for the presence of SARS-CoV2 within the CNS in those presenting to hospital with neurological symptoms during the first peak of the COVID-19 epidemic.

Methods

Patient recruitment

We undertook a prospective cohort study at Livingstone Hospital, Eastern Cape. The study was approved by the Faculty of Health Sciences Human Research Ethical Committee of the University of Cape Town (HREC 207/2020) and by the ethical review board at Livingstone Hospital. We sequentially enrolled adults (>18 years) presenting with neurological symptoms who at the discretion of the treating physician required inpatient investigation by lumbar puncture and cerebrospinal fluid analysis between

12th July and 20th October 2020. During this time Livingstone Hospital served as a COVID-19 referral centre in the Eastern Cape with an average admission rate of 40 confirmed COVID-19 cases per day. Written informed consent was taken from the patients where possible in those with capacity to consent. In those with decreased consciousness, patient relatives were approached for proxy consent. In those where no relative was contactable, permission was sought on an individual case basis by the Faculty of Health Sciences Human Research Ethical Committee of the University of Cape Town.

Clinical and radiological data collection

Clinical data was collected on symptoms and signs at presentation as well as relevant past medical history at two timepoints (baseline, and again between 3 and 7 days of enrolment). Computerised Tomography (CT) head images performed as part of standard of care included as routine sagittal and axial views. Two dimensional images were retrieved and independently viewed using a picture archive and communication system (PACS) by a blinded neuroradiologist using a standardised case report form. No specific study procedures took place, however at the time of diagnostic lumbar puncture a total of 6 ml of additional CSF was retrieved for study-specific analysis and biobanking. Similarly, venipuncture was not performed as a study procedure, however 24 ml of additional whole blood samples were collected for study specific blood work up and biobanking. Data on routine blood and CSF investigations performed as part of the diagnostic work up were collected from the National Health Laboratory Database and patient medical record. The outcome of admission (including death and final diagnosis) were recorded retrospectively.

Laboratory methods to detect SARS-CoV2 in CSF

An in-house RT-PCR that compared well with the routine nationally employed test, the Multiplex TaqMan™2019-nCoV kit (Applied Biosystems, Waltham, Massachusetts, USA) was established and used to detect SARS-CoV2 in CSF targeting the E gene and subgenomic RNA (sgRNA). Viral subgenomic mRNA is transcribed only in infected cells and not packaged into virions and therefore a positive sample may indicate evidence of actively infected cells within the CSF(Wolfel et al., 2020). RNA was extracted from patient samples using the E.Z.N.A. Viral RNA kit (Omega Bio-tek), followed by reverse transcription and PCR-amplification of the SARS-CoV2-specific targets E and sgRNA (as well as a human RNA control, RNAseP (RP)) using the TaqPath™ 1-Step Master Mix kit (Thermo Fisher) on a QuantStudio 7 Real-Time PCR machine (Thermo Fisher). Primers and probes for SARS-CoV2 E gene and sgRNA readouts have been published elsewhere (Corman et al., 2020) and were synthesized by Inqaba Biotec (South Africa), while primers and probes for the RP control target were provided by the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel. Each run included a positive control (PC), which for sgRNA runs included a sample from a previously positive patient, a human specimen extraction control (HSC; e.g. HeLa cell RNA) as well as a 'no template' control (NTC, i.e. water). All samples were amplified under the same conditions using 400nM concentrations of each of the primers, as well as 200nM of probe. Thermal cycling involved 10min at 53˚C for reverse transcription, followed by 3min at 95˚C to deactivate Reverse Transcriptase and Taq activation, and 45 cycles of 3s at 95˚C and 30s at 57˚C. A run was considered valid if the control samples yielded the following results with a Ct value < 40 being considered a positive signal: NTC negative for E, sgRNA and RP; HSC negative for E and sgRNA,

positive for RP; PC: positive for E, sgRNA and RP. When all controls exhibited the expected performance, an unknown patient sample was considered negative if the Ct values for E and sgRNA were > 37 AND the Ct value for RP < 37. A specimen was considered positive if the Ct values for E and sgRNA were < 37. Runs with a CT between 37 and 40 were repeated.

Statistical analysis

Data was analysed as an entire cohort with continuous characteristics described in terms of median values and interquartile ranges, and dichotomous variables as counts and percentages. Comparison between patients who tested positive for SARS-CoV2 on nasopharyngeal (NP) swab PCR, and those who did not was assessed using Wilcoxon rank-sum tests to compare continuous variables, and chi squared test for dichotomous variables. All analysis was performed within GraphPad Prism (version 9, Prism for MacOS) software.

Results

At total of 40 participants (24 female, 16 male) were screened for inclusion in the study. One participant was not enrolled as neither deferred and proxy consent were available. Therefore, 39 participants were included within the analysis. The median age at enrollment was 44 years. Baseline characteristics are outlined in table A.1. At the time, routine testing for SARS-CoV2 by NP swab was not available for all inpatients due to limited resource; however, 31/39 participants included within this study underwent testing due to symptomatic presentation, or recent COVID-19 contact. 7/31 participants tested positive for SARS-CoV2 via NP swab. In tables A.1 and 5.2, demographics and clinical characteristics of the cohort are described,

as well as for those who tested positive and negative for SARS-CoV2 via NP swab.

Neurological complaints at baseline are described in table A.1. The most prevalent complaint was confusion (27/39). Less common complaints, occurring with or without confusion included: neck stiffness (14/39), headache (12/39), new onset or increasing frequency of seizures (7/39), acute psychotic symptoms (10/39). On neurological assessment, 5/39 had new onset hemiplegia and 1/39 had new onset bilateral lower limb weakness. In the absence of other motor or sensory disturbance, 1/39 had new onset lower motor neuron facial nerve (VII cranial nerve) weakness, and 1/39 had abducens (VI cranial nerve) weakness. Neurological complaints at presentation in those testing positive for SARS-CoV2 on NP swab included: confusion (4/7) of which 1/7 demonstrated acute psychotic symptoms, new onset seizures (1/7), headache and neck stiffness (1/7), acute onset right sided weakness (1/7) and bilateral lower limb weakness (1/7). Of these 7 participants, 3 had no classical symptoms of COVID-19 (cough, shortness of breath, fever, anosmia or dysgeusia). Between those with and without a positive NP swab for SARS-CoV2, there was no significant difference in the neurological complaints at baseline. In 7 patients with a confirmed diagnosis of COVID-19 (SARS-CoV2 positive PCR on NP swab); 2/7 were thought to have COVID-19 pneumonia with stroke (see table A.4), 1/7 presented with a clinical diagnosis of myelitis treated as possible viral or *M. tb* in aetiology, with the remaining 4/7 patients, all of which presented with confusion, thought to be due to delirium secondary to COVID-19 pneumonia. No further investigation was performed to formally assess for encephalopathy.

Baseline blood and CSF analysis are described in table A.2. This analysis was performed as part of routine care, where there was a clinical indication and therefore not all tests were performed on every participant. There was no difference in CSF markers to suggest an acute infective or inflammatory process in those who tested positive for SARS-CoV2 via NP swab, versus those who did not, this includes: lymphocyte count (*p=0.62*), polymorphonuclear cells (*p=0.39*) and protein (*p=0.59*). CSF glucose was significantly higher in those with a diagnosis of COVID-19 (5.0 vs 3.0, *p=0.01*); however, of note HbA1C (%) was significantly higher in patients with COVID-19 than in those without (13.0 vs 7.25, *p=0.02*), reflecting the non-significant higher proportion of patients with pre-existing diabetes mellitus in those with a diagnosis of COVID-19 compared to those without (2/7 vs 4/32, *p=0.29*).

Computerised Tomography (CT) scans of the brain were performed at baseline in 26/39 participants, of which 2 were performed in patients with a confirmed diagnosis of COVID-19. 3/26 scans were performed with contrast enhancement. Radiological findings at baseline are summarized in table A.3. In the two scans performed in patients with COVID-19, one demonstrated multi-focal subacute infarcts within the left middle cerebral artery (MCA) and anterior deep borderzone territories. In the second, imaging demonstrated multi-territory mature infarcts in in both cerebral hemispheres and in the cerebellum (figure A.1). In both patients, CSF findings were unremarkable (see table A.4), and a diagnosis of COVID-19 pneumonia with presumed diagnosis of stroke (clinical in the former, radiological in the latter) was made.

Multiplex PCR (targeting nucleocapsid (N), spike protein (S) and Orf1ab) runs revealed no evidence of SARS-CoV2 in any of the 39 samples. Using

the primer combinations for E gene readout and subgenomic readout repeatedly gave no or weak (Ct>37) signals that were interpreted as negative. Therefore, none of the 39 samples demonstrated SARS-CoV2 via any of the PCR primer combinations applied. Raw data is presented in table A.5.

Discussion

We describe a cohort of patients presenting with clinical symptoms suggestive of possible neuroinfective or neuroinflammatory aetiology, with and without symptoms and a confirmatory diagnosis of COVID-19 during the first peak of the pandemic in a resource poor setting in South Africa. Examination of the CSF using PCR for multiple targets was negative in all cases therefore suggesting little evidence of direct neurotropic invasion of SARS-CoV2 in CNS.

Published reports provide rationale for direct neurotropic invasion of SARS-CoV2 including cases of meningitis and encephalitis where PCR for SARS-CoV2 was positive in CSF with and without classical symptoms of COVID-19 (Moriguchi et al., 2020) [(Khodamoradi et al., 2020a). At autopsy, SARS-CoV2 RNA has been detected in the brains of patients who have died due to COVID-19 albeit at titers lower than in other affected organs (Wichmann et al., 2020, Puelles et al., 2020). Evidence for direct neurotropic invasion is supported by findings from the 2002 SARS-CoV outbreaks where studies demonstrated the presence of coronavirus particles in the brain (Ding et al., 2004, Gu et al., 2005, Xu et al., 2005), with subsequent studies describing penetration of the CNS via the olfactory nerve (Netland et al., 2008). In SARS-CoV2, a case of olfactory gyrus intracerebral hemorrhage, an

uncommon location for spontaneous hemorrhage, as well as the high rates of anosmia, has highlighted whether SARS-CoV2 can invade neurological structures such as the olfactory bulb via nasal mucosa (Thu et al., 2020). Entry into the CNS via synaptic connections may also provide rationale to consider a centrally-driven contribution to cardiorespiratory dysfunction in coronaviruses, where acute onset respiratory failure leads to significant morbidity and mortality [36]. These observations have, during the course of the COVID-19 pandemic, raised questions as to whether SARS-CoV2 should be investigated as a causative organism, particularly in patients presenting with a meningitis or encephalitis in a setting where SARS-CoV2 infection rates are high, both in those with and without a confirmed diagnosis of COVID-19. In our cohort who were enrolled sequentially in a tertiary setting for COVID-19 care, despite detailed examination of the CSF for presence of SARS-CoV2 using PCR primers for genomic and subgenomic RNA, no patients were found to have evidence of direct neurotropic invasion to the CNS. This finding is important in shaping the direction of clinical care in the management of patients presenting with neurological symptoms in the COVID-19 era, particularly in resource limited settings where the prioritisation of investigations is an important consideration in clinical care.

In our study, one patient had a clinical presentation consistent with myelitis alongside a diagnosis of COVID-19, however in this case an explanation other than SARS-CoV2 infection was thought more likely to account for their clinical presentation and no evidence of direct neurotropic invasion of SARS-CoV2 was found. Acute myelitis (Kang Zhao) is one of many cases reported in the literature where the mechanism was thought due to a post- or para-infectious inflammatory response to SARS-CoV2. Other cases

which may suggest inflammatory sequelae occurring during or following SARS-CoV2 infection include: acute necrotising hemorrhagic encephalopathy (Rossi, 2008, Poyiadji et al., 2020), Guillain Barre Syndrome (Sedaghat and Karimi, 2020, El Otmani et al., 2020, Alberti et al., 2020, Toscano et al., 2020b) and Miller Fisher Syndrome (Gutierrez-Ortiz et al., 2020), and acute disseminated encephalomyelitis (Parsons et al., 2020, Novi et al., 2020), all of which occurred without evidence of SARS-CoV2 in the CSF. Given that within our cohort direct neurotropic invasion of the CNS was not found, these results might suggest that greater emphasis should now turn towards understanding the role of inflammation at the time of, or following, SARS-CoV2 infection in a subset of patients.

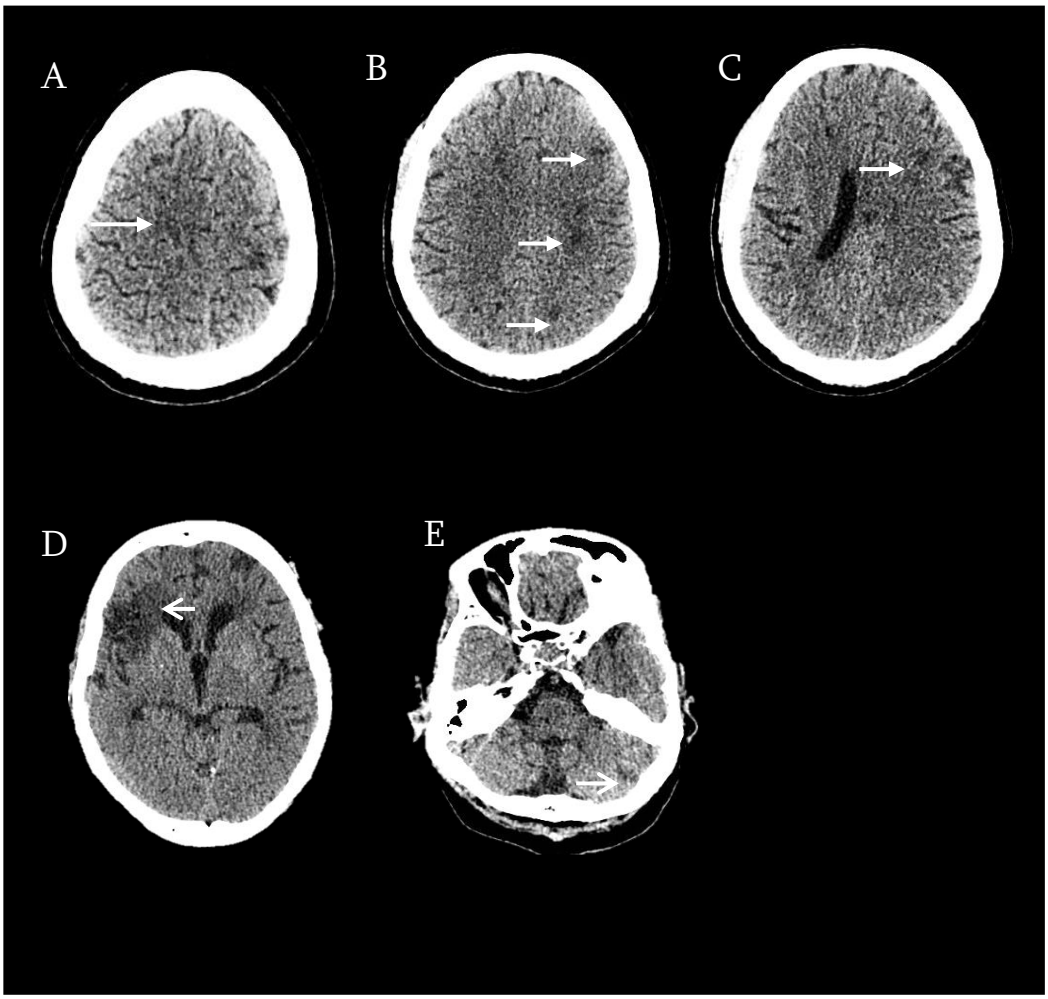
Moreover, the proportional contribution of the now well-described coagulopathy leading to endothelial dysfunction and eventual end organ damage is unknown (Hess et al., 2020). This is particularly important to understand in the context of stroke in patients with COVID-19; now frequently reported to occur where otherwise no clear vascular risk factors exist (Goldberg et al., 2020, Avula et al., 2020). In our cohort, two patients presented with presumed stroke alongside COVID-19 pneumonia. In both cases, vascular risk factors co-existed and may in part or completely explain the vascular complications. Further research is required to understand the interplay of the presumed coagulopathy both on pre-existing vascular risk factors such as hypertension and diabetes, and other stroke risk factors such as HIV, particularly within the South African context.

There were several limitations to this study. Given the pragmatic nature of its design, only data on investigations indicated as part of routine clinical care were available for analysis, resulting in an incomplete laboratory and

radiological data set. This includes cerebral imaging, which was not performed in all participants, and in instances where it was, contrast was not given in the majority (23/26) of cases. Factors related to coagulation, such as D-Dimer levels, would have provided interesting comparison of patients presenting with complications such as stroke with and without confirmed COVID-19. In contrast, laboratory procedures related to the discovery of SARS-CoV2 in CSF were thorough and robust, which is reassuring that despite multiple runs, the negative findings are reliable.

Although small, this pragmatic observational cohort study contributes knowledge to our increasing understanding of COVID-19 management. Through systematic analysis of CSF in patients presenting with neurological symptoms in a context where incidence of SARS-CoV2 infection is high, we have demonstrated that although cases within the literature exist, direct neurotropic invasion of the CNS is uncommon. This includes suspected cases of meningitis and encephalitis, syndromes most aligned to direct neurotropic mechanism. This considered, neurological presentations in cases of COVID-19 continue to be reported, and lead to morbidity and mortality in patients affected. The results from our study suggest that further emphasis must now turn towards understanding the role of inflammation and coagulopathy in the development of neurological syndromes. This includes studies to assess the efficacy of proven anti-inflammatory drugs such as corticosteroids and tocilizumab, and therapeutics to manage acute stroke in the treatment of patients who develop neurological symptoms due to SARS-CoV2 infection.

Figure A.1: Neuroimaging in patients with confirmed diagnosis of COVID-19



Axial unenhanced CT head imaging in two patients where a diagnosis of COVID-19 was confirmed (PCR for SARS-CoV2 positive on NP swab). Top row demonstrates poorly-defined multifocal cortical and subcortical hypodensities in keeping with subacute left middle cerebral artery territory (A,C) and anterior deep borderzone territory (B) infarcts. Bottom row demonstrates multi-territory mature infarcts in the right middle cerebral artery territory (D) and left posterior inferior cerebellar artery territory (E).

Table A.1 Baseline demographics and clinical characteristics in HIATUS-3

	All	SARS-CoV2 -ve on NP swab or unknown SARS-CoV2 status	SARS-CoV2 +ve on NP swab
n	39	32	7
Gender			
Male	15	11	4
Female	24	21	3
Age (years)			
Median (range)	44 (27-84)	42 (27-79)	47 (42-84)
HIV seropositivity			
HIV co-infected	22	19	3
On ART ART naïve/defaulted	11 11	8 11	3 0
HIV uninfected	13	11	2
Not known	4	2	2
HIV-1 VL (copies/ml) (n=13, median ; IQR)	75831; 28,0141	253,344; 258,249	205; 206
CD4 Cell count (cells/mm3) (nr 332-1642) (n=16, median; IQR)	35.5; 186	32; 99	260; 69
Comorbidities			
Cardiovascular Disease	2	2	0
Hypertension	13	9	4
Diabetes Mellitus	4	4	2
Obesity	15	11	4
Underlying Respiratory Disease	5	5	0
Chronic Kidney Disease	1	0	1
Baseline Symptoms			
Cough	17	12	5
Fever	7	3	4
Dyspnoea	11	7	4
Lethargy	10	9	1
Nausea	3	3	0
Diarrhoea	11	10	1
Headache	12	11	1
Neck Stiffness	14	13	1
Seizures	7	6	1
Agitation/psychosis	10	9	1

Confusion	27	23	4
Anosmia/Dysgeusia	1	0	1
Days symptomatic median (IQR)	7 (13)	8 (13)	7 (5)
Baseline Neurological Examination			
Hemiplegia	5	4	1
Paraplegia	1	0	1
Cerebellar ataxia	0	0	0
Tremor	0	0	0
Seizures	1	1	0
Cranial Nerve Abnormality	4	4	0
Glasgow Coma Score (Median)	14	14	14

Table A.2 Blood and CSF analysis

	Value expressed as	Performed in (n)	Normal range	All participants	SARS-CoV2 -ve on NP swab or unknown SARS-CoV2 status	SARS-CoV2 +ve on NP swab	p values
Blood							
Hemoglobin (g/dL)	**Median**; IQR	39	12-15	**11.5**; 4	**11.1**; 2	**12.3**; 2	
Platelets (cells x109/L)		37	186-454	**281**; 140	**281**; 116	**271**; 168	
White cells (cells x109/L Neutrophils (cells x10^9/L) Lymphocytes (cells x10^9/L) Eosinophils (cells x10^9/L)		39 15 15 15	3.9 – 12.6 1.6 – 8.3 1.4 – 4.5 0.0 – 0.4	**8.4**; 9 **6.0**; 0 **1.12**; 12 **0.02**; 0	**8.3**; 7 **7.8**; 7 **(In 13/15), 1.12**; 1 **0.02**; 0	**8.4**; 9 **4.4**; 1 **(in 2/15), 8.56**; 8 **0.01**; 0	
C-Reactive Protein (mg/L)			<10	**68**; n/a	**47**; n/a	**110**; n/a	*p=0.36*
Erythrocyte Sedimentation Rate (mm/hr)		5	0-10	**96**; n/a	**96**; 67	**n/a**; n/a	
Ferritin (µg/L)		3	11-307	**83**; n/a	**83**; 492	**n/a**; n/a	
D Dimer (ng/mL)		14	0 – 0.25	**1.25**; 2	**1.32**; 1	**0.66**; 2	
ALT (IU/l)		32	19-25	**29.5**; 15	**32**; 27	**19**; 16	
Total Bili (µmol/L)		32	5-21	**12**; 8	**11**; 12	**15**; 6	
Na+ (mmol/L)		39	136-145	**136**; 9	**135**; 10	**137**; 6	
K+ (mmol/L)		39	3.5-5.1	**3.9**; 0	**4.0**; 1	**3.8**; 0	
Creatinine (µmol/L)		39	49-90	**79.0**; 24	**82.5**; 64	**72.0**; 18	
Total Protein (g/L)		24	6-78	**75.0**; 14	**75.0**; 13	**76.5**; 10	
HbA1C (%)		7	4-5.6	**7.9**; 6	**7.25**; 2	**13.0**; 0	***p=0.02***
Cerebrospinal Fluid							
Polymorphonuclear cells (cells/µL)	**Median**; IQR; range	38	<3	**0**; 6; 0-118	**0**; 1; 0-118	**0**; 0; 0-0	*p=0.39*
Lymphocytes (cells/µL)		38	<3	**0**; 6; 0-2073	**0**; 9; 0-2073	**2**; 4; 0-12	*p=0.62*
Erythrocytes (cells/µL)		38	<3	**5**; 48; 3-349	**3**; 17; 0-349	**62**; 148; 0-311	*P=0.28*
Protein (g/L)		39	0.15-0.45	**0.39**; 1; 0.13-4.82	**0**; 1; 0.13 – 4.82	**0**; 0; 0.17 – 2.19	*p=0.56*
Glucose (mmol/L)		39		**3**; 3; 0.1-10.2	**3**; 2; 0.1-7.0	**5**; 5; 2.8 – 10.2	***p=0.01***
Bacterial Culture	n, (% positive)	39		5/39 (12.8)	4/32 (12.5)	1/7 (14.2)	*p=0.90*
CLAT	n, (% positive)	32		5/32 (15.6)	5/29 (17.2)	0/3 (0)	
GXPU	n, (% positive)	22		1/22 (45.5)	1/18 (5.5)	0/4 (0)	
PCR for SARS-CoV2 in CSF							

Positive CT value for E		39		0/39	0/32	0/7	
Positive CT value for sgRNA		39		0/39	0/32	0/7	

Na+: sodium; K+: potassium; CLAT: cryptococcal latex agglutination titre; GXPU: GeneXpert Ultra; CT (cycle threshold); sgRNA: subgenomic RN.

Table A.3 Radiological analysis

Radiological Finding	All participants	SARS-CoV2 -ve on NP swab or unknown SARS-CoV2 status	SARS-CoV2 +ve on NP swab
Leptomeningeal Enhancement	1/26	1/24	0/2
Hydrocephalus	3/26	3/26	0/2
of which communicating	3/26	3/26	n/a
Radiological evidence of infarcts	14/26	13/26	1/2
of which new	3/14	3/13	0/1
single	10/14	9/13	1/1
multiple	4/14	4/13	0/1
Territory			
Middle cerebral artery territory	9/14	8/13	1/1
Anterior cerebral artery territory	0/14	0/13	0/1
Posterior cerebral artery territory	0/14	0/13	0/1
Ring enhancing lesions	0/26	0/24	0/2
Effacement	1/26	1/24	0/2
Of which local sulcal	1/26	1/24	0/2
Of which hemispheric sulcal	1/26	1/24	0/2
Of which global sulcal	0/26	0/24	0/2
Of which basal cistern	0/26	0/24	0/2

Table A.4 Detailed clinical presentations, laboratory findings in patients presenting with COVID-19 and neurological symptoms

Age range, Gender	HIV status, VL, CD4	Co-morbidities	Baseline symptoms and examination findings	Significant blood and CSF findings (Units as described in table 2)*	Radiological findings	Final diagnosis
50-50 M	Neg	Nil	2 day history of cough, fever, seizures (unclear if new onset) and confusion. GCS 14 on assessment	Blood: Hb 10.7, WCC 23 (eosinophils 291), CRP 110, Na 132. CSF: Lymphocytes 2, erythrocytes 13, protein 0.17, glucose 4.7, normal gram stain, negative bacterial culture	Not done	COVID pneumonia with confusion +/- new onset/worsening seizure frequency
40-50 F	UNK	Hypertension, DM, Obesity	2 day history of shortness of breath, anosmia, worsening confusion and agitation. GCS 7 on assessment with no focal neurological abnormalities	Blood: WCC 15, CRP 77, Na 147 CSF: Lymphocytes 1, Erythrocytes 62, protein 0.19, glucose 7.8, occasional lymphocytes on gram stain, negative bacterial culture	Not done	COVID pneumonia, with DKA/HHS
40-50 M	Pos, VL undetectable, CD4 329, unknown treatment status	Obesity	7 day history of cough, fever, sore throat, headache and neck stiffness. GCS 15 and unremarkable neurological examination.	Blood: WCC 5, CRP 74 CSF: Lymphocytes 2, erythrocytes 76, protein 0.42, glucose 5.5, negative gram stain and bacterial culture, GXPU trace on first LP, negative on second. TB culture negative.	Not done	COVID pneumonia, no cause for neurological symptoms identified
40-50 M	Neg	Hypertension	7 day history of cough, shortness of breath, sore throat and fever, new onset right sided weakness. Examination revealed reduced power (MRC 3/5 throughout) in right upper and lower limbs. GCS 15.	Blood: WCC 14, CRP 196. CSF: WCC 0, CSF protein 0.3, CSF glucose 3.0 (no blood glucose comparator), negative gram stain, no growth on bacterial culture, negative GXPU and CLAT.	Subacute left MCA territory infarct	COVID-19 pneumonia and stroke
60-70 F	Pos, unknown VL, CD4 191, on ART	Hypertension, DM, Obesity	7 day history of cough, shortness of breath and increasing confusion. GCS 14.	Blood: WCC 7, CRP 453, Na 130, D Dimer 4.94 CSF lymphocytes 4, erythrocytes 311, CSF protein 0.3, CSF glucose 10.2 (no blood glucose comparator), occasional lymphocytes seen on gram stain, negative bacterial culture, negative GXPU and CLAT.	Multi-territory mature infarcts in both cerebral hemispheres and in the cerebellum.	COVID-19 pneumonia and radiological diagnosis of multifocal infarcts

40-50 F	Pos, VL 411, CD4 unknown, on ART	Obesity	20 day history of GI symptoms with increasing weakness in both lower limbs. Pyramidal distribution weakness, hyperreflexia, T8 sensory level	CSF: Lymphocytes 12, erythrocytes 161, protein 2.19, glucose 2.8, Viral PCR positive for Epstein-Barr virus, HIV-1 viral load 838368 copies/mL	No brain imaging done MRI spine reported as showing expanded poorly enhancing STIR/T2W hyperintense T7-T12 spinal cord associated with subtle enhancement of the adjacent leptomeninges. Arachnoiditis. Impression of cauda equina syndrome.	Viral myelitis with HIV CSF escape
80-90 F	Unknown,	Nil	7 day history of cough, fever, shortness of breath, lethargy, increasing confusion	Blood: Hb 10.0 CSF: WCC 0. protein 0.3, glucose 4.5, GS occasional lymphocytes, bacterial culture negative	No brain imaging done	COVID-19 pneumonia with new onset confusion

Table A.5 Results of CSF PCR analysis for detection of SARS-CoV2

Sample CODE	Primers	CT (run 22.1.21)	Freshly isolated RNA from 300ul CSF (run 1)	Freshly isolated RNA from 1ml CSF (run 2)
NTC	E/sgRNA/RP	U/U/U		
negative RNA	E/sgRNA/RP	U/U/19.64	Not run	Not run
positive RNA	E/sgRNA/RP	29.588/35.725/23.228		
A	E/sgRNA/RP	U/U/27.838	U/U/28.543	Not run
B	E/sgRNA/RP	U/U/32.167	U/U/31.723	Not run
C	E/sgRNA/RP	U/U/30.423	U/U/30.291	Not run
D	E/sgRNA/RP	U/U/33.769	U/U/34.833	Not run
E	E/sgRNA/RP	U/U/29.596	U/U/28.904	Not run
F	E/sgRNA/RP	U/U/29.508	U/U/29.433	Not run
G	E/sgRNA/RP	U/U/30.766	U/U/31.742	Not run
H	E/sgRNA/RP	U/U/28.1	U/U/27.882	Not run
J	E/sgRNA/RP	U/U/28.301	U/U/26.715	Not run
K	E/sgRNA/RP	U/U/30.967	U/U/31.610	Not run
L	E/sgRNA/RP	U/U/32.565	U/U/29.195	Not run
M	E/sgRNA/RP	U/U/29.434	U/U/31.586	Not run
N	E/sgRNA/RP	U/U/30.329	U/U/30.767	Not run
P	E/sgRNA/RP	U/U/32.947	U/U/32.885	Not run
Q	E/sgRNA/RP	U/U/32.693	U/U/33.181	Not run
R	E/sgRNA/RP	U/U/31.684	U/U/31.898	Not run

S	E/sgRNA/RP	U/U/31.920	U/U/32.882	Not run
T	E/sgRNA/RP	U/U/30.518	U/U/30.682	Not run
U	E/sgRNA/RP	U/U/21.694	U/U/21.572	Not run
V	E/sgRNA/RP	U/U/31.449	U/U/32.053	Not run
W	E/sgRNA/RP	U/U.27.471	U/U/26.683	Not run
X	E/sgRNA/RP	U/U/26.666	U/U/27.990	Not run
Y	E/sgRNA/RP	U/U/28.835	U/U/28.412	Not run
Z	E/sgRNA/RP	U/U/27.502	Not run	Not run
A2	E/sgRNA/RP	U/U/28.626	Not run	Not run
B2	E/sgRNA/RP	U/U/29.912	Not run	Not run
C2	E/sgRNA/RP	U/U/31.769	Not run	Not run
D2	E/sgRNA/RP	U/U/23.736	Not run	
E2	E/sgRNA/RP	37.867/39.461/30.956	Not run	U/U/29.642
F2	E/sgRNA/RP	U/U/24.674	Not run	U/U/30.549
G2	E/sgRNA/RP	U/38.837/31.717	Not run	U/U/30.549
H2	E/sgRNA/RP	40.430/U/27.301	Not run	U/U/24.015
J2	E/sgRNA/RP	U/U/26.379	Not run	Not run
K2	E/sgRNA/RP	U/U/29.545	Not run	Not run
L2	E/sgRNA/RP	U/38.438/23.510	Not run	U/U/21.109
M2	E/sgRNA/RP	U/U/29.039	Not run	Not run
N2	E/sgRNA/RP	U/U/19.058	Not run	Not run
P2	E/sgRNA/RP	U/38.087/27.014	Not run	U/U/29.229

Abbreviations: CT (cycle threshold), CSF (cerebrospinal fluid), NTC (no template control), RP (RNAseP), U (Undetermined)

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