Diagnosis, treatment and determinants of mortality in patients hospitalized with HIV-associated tuberculosis

Charlotte Schutz
MBChB; DipHIVMan(SA); MPH (Clinical Research)

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Supervisors:
Professor Graeme Meintjes
Professor Robert J. Wilkinson
Doctor Muki Shey

TABLE OF CONTENTS

DECLARATIONS:

I, Charlotte Schutz, declare that this thesis includes three published manuscripts (Chapter 3, 4 and 6). The content of each manuscript remains unchanged from that which has been published. The manuscripts are listed below, with a description of my contribution and the contribution of each co-author.

I confirm that I have been granted permission by the University of Cape Town's Doctoral Degrees Board to include the following publications in my PhD thesis and that my co-authors have reviewed and approved the co-author contributions as specified below and agreed that I may include these publications in my thesis:

1. Schutz C, Barr D, Andrade BB, et al. Clinical, microbiologic, and immunologic determinants of mortality in hospitalized patients with HIV-associated tuberculosis: A prospective cohort study. *PLoS Med* 2019;16(7):e1002840. (Chapter 3 of thesis)
2. Schutz C, Chirehwa M, Barr D, et al. Early antituberculosis drug exposure in hospitalized patients with human immunodeficiency virus associated tuberculosis. *Br J Clin Pharmacol*. 2020;86:966-978. https://doi.org/10.1111/bcp.14207. (Chapter 4 of thesis)
3. Schutz C, Ward A, Burton R, et al. False rifampicin resistant results using Xpert MTB/RIF on urine samples in hospitalised HIV-infected patients. *South Afr J HIV Med.* 2019;20(1):978 (Chapter 6 of thesis)

Clinical, microbiologic, and immunologic determinants of mortality in hospitalized patients with HIV-associated tuberculosis: a prospective cohort study: PLoS Med 2019;16(7):e1002840. (Chapter 3 of thesis)

Authors: Charlotte Schutz, David Barr, Bruno B. Andrade, Muki Shey, Amy Ward, Saskia Janssen, Rosie Burton, Katalin A. Wilkinson, Bianca Sossen, Kiyoshi F. Fukutani, Mark Nicol, Gary Maartens, Robert J. Wilkinson, Graeme Meintjes

Charlotte Schutz was the lead investigator on this study. She wrote the first draft of the clinical protocol, developed all study related documentation such as case record forms and SOPs, obtained University of Cape Town Faculty of Health Sciences Human Research Ethics Committee approval, initiated and provided oversight of day to day activities such as patient enrolment, sample collection, data collection and patient follow up. She performed laboratory assays (initially under supervision), was responsible for the database (with assistance) and performed most analyses, specifically the non-parametric comparisons, principal components and survival analyses. She drafted the first draft of manuscript, generated the data contained in all the figures and incorporated input from all co-authors, reviewers and the PLOS Medicine editorial team.

Graeme Meintjes conceived and obtained funding for this cohort study through a Wellcome Trust intermediate fellowship. He provided oversight for the duration of the study, funding for the laboratory work and he provided detailed input on the initial drafts of the manuscript and reviewers comments. Gary Maartens, Robert J Wilkinson, and Mark Nicol provided input on the study design, the early analyses of clinical and microbiological findings and provided input on the draft manuscript and reviewers comments.

David Barr initially oversaw the cleaning and curation of the database and taught Charlotte Schutz these skills during the study. Charlotte Schutz through self-directed learning upskilled herself in data cleaning and curation and did this independently using R in the final phases of data analysis. David Barr provided support with data base issues when needed and provided specific input on the principal components and survival analyses which Charlotte Schutz performed.

Bruno Andrade and Kyoshi Fukutani in collaboration with Charlotte Schutz and Graeme Meintjes used data provided by Charlotte Schutz to perform more sophisticated analyses of biomarkers and specifically created all heatmap figures and did network analyses.

Amy Ward, Saskia Janssen, David Barr, Rosie Burton and Bianca Sossen were involved in patient recruitment, data collection, completion of study documentation and provided input on the manuscript.

Muki Shey taught Charlotte Schutz how to perform laboratory experiments, supervised the initial experiments and provided hands-on oversight for all aspects of the laboratory experiments and analyses. Katalin Wilkinson provided input on planning of sample collection and processing, sample storage and the planning of experiments. She helped to troubleshoot any sample collection problems and experimental issues in real time. Laboratory assays were performed in the laboratory of Robert J Wilkinson and he provided laboratory space and facilities to conduct the experiments. Robert J Wilkinson and Katalin Wilkinson provided support and additional oversight of all the laboratory experiments. Muki Shey, Katalin Wilkinson and Robert J Wilkinson provided input on the manuscript.

Early antituberculosis drug exposure in hospitalized patients with human immunodeficiency virus associated tuberculosis; Br J Clin Pharmacol. 2020;86:966-978. https://doi.org/10.1111/bcp.14207. (Chapter 4 of thesis)

Authors: Charlotte Schutz, Maxwell Chirehwa, David Barr, Amy Ward, Saskia Janssen, Rosie Burton, Robert J. Wilkinson, Muki Shey, Lubbe Wiesner, Paolo Denti, Helen McIlleron, Gary Maartens, Graeme Meintjes

Charlotte Schutz was the lead investigator of the Khayelisha Hospital tuberculosis study in which this pharmacokinetics (PK) sub-study was nested. She (together with Amy Ward) set up the logistical aspects of the PK sub-study, recruited patients, performed the intensive PK sampling, collected the relevant data, processed and stored all samples within relevant time lines. Charlotte Schutz liaised with the clinical pharmacology laboratory about drug concentration measurements. She received, cleaned and analyzed all the PK results using non-compartmental analysis techniques that she learnt during the PhD. She received analysis advice from David Barr and Maxwell Chirehwa when needed. She wrote the first draft of the manuscript and incorporated all the co-author comments.

Graeme Meintjes obtained funding for this sub-study as part of a Wellcome Trust Intermediate fellowship. He provided oversight of the main study and the sub-study. His fellowship funded the laboratory assays.

Muki Shey provided support of all the laboratory aspects related to sample collection, processing and storage prior to measurement of drug concentrations and provided input on the manuscript.

Gary Maartens and Helen McIlleron provided input on the study design and together with Paolo Denti provided input on the logistics of sample collection, the analysis plan and the manuscript. Drug concentration measurements were performed at the Clinical Pharmacology Laboratory at Groote Schuur under the supervision of Lubbe Wiesner who provided expert input on these assays and provided input on the manuscript.

Saskia Janssen assisted with patient recruitment and Rosie Burton provided oversight of patient recruitment, both provided input on the manuscript. Robert J. Wilkinson provided laboratory facilities for processing and storage of samples, contributed to planning of the study and provided input on the manuscript.

False rifampicin resistant results using Xpert MTB/RIF on urine samples in hospitalised HIV-infected patients. South Afr J HIV Med. 2019;20(1):978 (Chapter 6 of thesis)

Authors: Charlotte Schutz, Amy Ward, Rosie Burton, Mark P. Nicol, Liz Blumenthal, Graeme Meintjes, Andrew D. Kerkhoff

Data from two comparable cohort studies were pooled for analysis in this paper. Charlotte Schutz was the lead investigator of the Khayelitsha Hospital tuberculosis study (KHTB) and lead patient recruitment, sample collection and data collection of the KHTB cohort. She also provided clinical support during recruitment of the Jooste Hospital tuberculosis (JTBS) study. Charlotte Schutz, together with Andrew Kerkhoff, Mark Nicol and Graeme Meintjes developed the research questions. Charlotte Schutz and Andrew Kerkhoff were responsible for the KHTB and JTBS databases

respectively and performed the analyses, wrote the first draft, subsequent drafts, and incorporated comments from all co-authors and reviewers.

Amy Ward assisted with recruitment, sample collection and data collection of the KHTB cohort. Rosie Burton and Graeme Meintjes provided clinical oversight of both studies. Liz Blumenthal provided clinical support and assisted with data collection for the JTBS cohort. Graeme Meintjes and Mark Nicol provided input on early drafts of the manuscript. All authors reviewed and approved the final draft of the manuscript.

I confirm that no part of this thesis has been submitted in the past, or is being, or is to be submitted for a degree at this or any other university. I hereby grant the University of Cape Town free license to reproduce this thesis in whole or part for the purposes of research or teaching.

Student: Charlotte Schutz **Student Number:** SCHCHA019

Signature: Signed by candidate **Date:** 24 December 2020

ABSTRACT:

Background: HIV-associated tuberculosis (HIV-TB) comprises 9% of global tuberculosis cases but contributes a disproportionate 17% of tuberculosis deaths. Tuberculosis is the leading cause of death, hospitalization and in-hospital death in HIV-positive patients world-wide with case fatality rates in hospitalized patients ranging between 13% and 32%. Underlying causes of mortality remain poorly characterized and better characterization of causes could inform development of novel management strategies to improve survival. This study aimed to assess determinants of mortality in hospitalized HIV-TB patients. I assessed the association of clinical, microbiologic and treatment factors, host soluble inflammatory mediators, markers of tuberculosis dissemination, antituberculosis drug concentrations and markers of microbial translocation with 12-week mortality in hospitalized HIV-TB patients.

Methods: We conducted a prospective observational cohort study and enrolled adult HIV-positive patients hospitalized with a new diagnosis of HIV-TB in Khayelitsha Hospital in Cape Town between 2014-2016. Detailed tuberculosis diagnostic testing was performed (including urine Xpert testing) and we collected clinical samples for analysis at baseline. We performed intensive pharmacokinetic studies in a subset of patients on the third day of antituberculosis therapy. Patients were followed up for 12 weeks to ascertain vital status.

Results: We enrolled 682 participants and included 576 patients with tuberculosis in the cohort analyses. Twelve-week mortality was 124/576 (21.5%) with 46/124 (37.1%) deaths occurring within 7 days of enrolment. Determinants of mortality included tuberculosis dissemination, rifampicin resistance and having features of sepsis syndrome. Using principal components analysis, we characterised an innate immune profile which was associated with mortality and with biomarkers of disseminated tuberculosis. A large proportion of patients had sub-optimal concentrations of rifampicin and isoniazid. Patients who presented with elevated lactate concentrations had higher rifampicin concentration and exposure. Opportunistic infections other than tuberculosis and microbial translocation did not have a significant association with mortality.

Conclusions: There was high early mortality in hospitalized HIV-TB patients. An innate immune profile was associated with tuberculosis dissemination and mortality. Rifampicin and isoniazid concentrations and exposure were sub-optimal. These findings provide novel pathophysiologic insight and provide rationale to test high dose rifampicin and immune modulatory therapy for safety and efficacy to improve survival in this patient population.

ACKNOWLEDGEMENTS:

The cohort study reported in this thesis was funded through a Wellcome Trust Intermediate Fellowship (098316) that was awarded to my supervisor Graeme Meintjes in 2012 for a period of five years. I received a full-time PhD scholarship from the South African Medical Research Council under the National Health Scholars Programme from 2015 for four years.

I thank my supervisor Professor Graeme Meintjes for the initial nudge into the world of clinical research, his support throughout every phase of the project and confidence in my ability to complete this. My co-supervisor, Professor Robert J Wilkinson for the opportunity to work in his laboratory, his critical input and thorough proof reading. My second co-supervisor Doctor Muki Shey for his hands-on teaching, long hours and patient help in the laboratory.

I thank the patients who participated in this study and the staff at the Khayelitsha Hospital and Ubuntu Clinic in Site B Khayelitsha where the study was conducted.
I thank my colleagues without whom the successful completion of this project would not have been possible. Specifically, Dr Amy Ward and Mr Mkhanyiseli Kenneth Mpalali (MK) who championed the recruitment and follow up stages of the study. Dr David Barr for the introduction to data management and regularly providing well-considered input. Dr Bruno Andrade for his exceptional ability to bring data to life visually. Kathryn Wood, René Goliath and Vanessa January for all their background work to keep the study logistics running smoothly. A special thank you to my colleague, fellow PhD student and fellow coffee enthusiast Dr Cari Stek for her support.

I thank my husband Pierre for his solid presence, patient support and enthusiasm which made it possible to complete this project. I thank my children Casey and Peter for their tolerance of my absence from their lives over the past years. I thank my parents for their support of my love of learning and encouragement in every new venture in my life.

"If I have seen further it is by standing on the shoulders of Giants." – Isaac Newton 1675

CHAPTER 1:

Introduction

Context and rationale

In 2018 ten million people developed active tuberculosis world-wide and an estimated 1.45 million people died due to tuberculosis. HIV-associated tuberculosis (HIV-TB) comprised an estimated 8.6% of global tuberculosis cases and contributed a disproportionate 17% of global tuberculosis mortality. The majority (72%) of HIV-TB patients live in Africa and an estimated 211000 HIV-TB deaths occurred in Africa in 2018, with 42000 of these deaths occurring in South Africa. In 2018 an estimated 484000 new cases of rifampicin resistant tuberculosis were reported world-wide, with 77000 cases in Africa and 11000 in South Africa (1). Patients hospitalized with HIV-associate tuberculosis have case fatality rates ranging from 11% to 32% and deaths occur early despite treatment (2-8). Patients with HIV-TB often present acutely ill with features of sepsis syndrome (9, 10), yet clinical services follow the same treatment guidelines which were developed for ambulant tuberculosis outpatients without HIV infection and no specific therapeutic interventions have been studied in severely ill hospitalized patients with disseminated HIV-TB. Rapid diagnostic tests using easily obtainable clinical samples (such as urine) and providing accurate sensitivity results (specifically the GeneXpert test) have not been sufficiently tested in non-sputum samples to guide routine clinical use. While public health services need to address upstream diagnostic challenges and treatment delay in patients with HIV-TB, patients are still presenting with advanced HIV infection and opportunistic infections (5, 11, 12) and patients hospitalized with HIV-TB will likely remain a common clinical problem in high burden settings into the foreseeable future. The pathophysiology and factors contributing to death in patients hospitalized with HIV-

TB are poorly understood and thus there is little evidence to provide rationale for improved therapeutic strategies in this vulnerable patient group.

We investigated factors associated with mortality and the pathophysiology underlying mortality in patients hospitalized with HIV-TB by addressing the following research questions, each of which could provide rationale for and potentially be amenable to improved therapeutic or diagnostic approaches.

Major research questions addressed in the PhD studies

1. What are the clinical contributors to mortality in patients hospitalized with severe HIV-TB?

   Rationale: *Mycobacterium tuberculosis* blood stream infection (MTB BSI) is the most common diagnosis in HIV-positive presenting to hospital with a clinical diagnosis of sepsis in high-burden settings and has high mortality (9, 10, 13). Post-mortem studies of HIV-positive patients who die in hospital show that 88% of patients have disseminated tuberculosis (14) and 50% are already on antituberculosis therapy at the time of death (8). Current rapid diagnostics (GeneXpert) rely on sputum samples which are difficult to obtain in very ill patients (15) or do not have sufficient sensitivity (lipoarabinomannan) (16) and because symptoms of disseminated tuberculosis may be vague and overlap with other opportunistic infections, delayed diagnosis of TB and/or rifampicin resistance and the resultant delay in treatment initiation may contribute to mortality.

   Hypothesis: Disseminated tuberculosis results in a higher mycobacterial load and this results in a clinical phenotype which resembles bacterial sepsis and contributes to early mortality together with other clinical factors such as

delayed tuberculosis diagnosis, delayed diagnosis of rifampicin resistance, delayed antituberculosis treatment initiation and co-infections.

2. Is fatal HIV-TB associated with an immune signature of worsening immunosuppression similar to what is seen in bacterial sepsis?
   Rationale: Severe HIV-TB may present with clinical features of bacterial sepsis (9, 17, 18). In bacterial sepsis an initial pro-inflammatory cascade occurs and this is followed by a compensatory anti-inflammatory response syndrome (CARS) which renders patients susceptible to secondary infections and mortality (19).
   Hypothesis: *Mycobacterium tuberculosis* blood stream infection causes an initial inflammatory cascade which is associated with a clinical sepsis syndrome. Similar to bacterial sepsis, this is followed by a compensatory anti-inflammatory response and we hypothesise that we will find an anti-inflammatory immune phenotype in hospitalized HIV-TB patients with a fatal outcome.

3. Is severe HIV-TB associated with subtherapeutic concentrations of antitubercular drugs during the critical early period of treatment?
   Rationale: Patients hospitalized with HIV-associated tuberculosis have high early mortality and up to 50% of deaths occur in patients already on antituberculosis therapy (8). Target therapeutic or reference ranges for first-line antituberculosis medication were determined in healthy volunteers, have not been validated in acutely ill HIV-positive patients with disseminated

tuberculosis and concentrations may not be adequate in the critical early treatment period (20).

Hypothesis: We hypothesized that compared to outpatients, there would be lower antituberculosis drug exposure in hospitalized HIV-TB patients, and amongst hospitalized patients exposure would be lower in patients who die or have high lactate (a sepsis marker).

4. Is fatal HIV-TB associated with translocation of bacteria and their products from the intestine into blood?

   Rationale: Translocation of microbial products from the gastrointestinal lumen into the systemic circulation contributes to chronic immune activation (21) and poor clinical outcomes in HIV infection and is associated with adverse clinical outcomes in chronic liver disease and chronic inflammatory bowel disease (22, 23).

   Hypothesis: We hypothesized that patients hospitalized with HIV-associated tuberculosis would have more gastrointestinal mucosal damage and higher levels of microbial translocation compared to outpatients with HIV infection and no active tuberculosis and that these markers would be associated with mortality.

5. What proportion of urine Xpert tests (a diagnostic assay with potential for expediting diagnosis in hospitalised HIV-TB patients) provide false positive rifampicin resistance results?

   Rationale: Samples which are easier to obtain than sputum samples, such as urine samples are advantageous in the setting of acutely ill hospitalized

patients. A small proportion of false rifampicin resistant results have previously been reported using GeneXpert MTB/RIF version G4 on sputum samples (24); however, this has not been investigated for urine samples in HIV-associated tuberculosis (TB).

Hypothesis: We hypothesized that we will find similar or higher proportions of false positive rifampicin resistant results using urine Xpert testing compared to sputum samples.

Overarching aim

We aimed to determine contributors to mortality in patients hospitalized with HIV-TB in order to provide an evidence base and rationale for the development of improved treatment strategies which could be tested in this patient population.

Overview of study design and setting

The PhD studies were nested within a prospective observational cohort study conducted at Khayelitsha Hospital, Cape Town from January 2013 until October 2016. Patients with a clinical suspicion of HIV-TB were enrolled as cases. Control patients were enrolled at Ubuntu clinic, Site B Khayelitsha. The antenatal HIV seroprevalence in Khayelitsha was 34% in 2015 (25) and TB notification rate in 2015 was 917/100 000 with 60% of cases being HIV-co-infected (Judy Caldwell, City of Cape Town Department of Health, 16 May 2016). ART and antituberculosis therapy (including treatment for drug resistant tuberculosis) are accessible at government clinics free of charge. Patients are referred to Khayelitsha Hospital from surrounding primary care community health clinics when they require hospitalization. Community health clinics have the capacity to initiate intravenous antibiotics and antituberculosis

therapy when indicated. A separate cohort study was previously conducted between June 2012 and October 2013 at GF Jooste Hospital in Manenberg, Cape Town and consecutive HIV-positive adults admitted to the medical wards were extensively investigated for tuberculosis (15). GF Jooste Hospital closed in 2013 and was replaced by two district hospitals, including Khayelitsha Hospital. Results from this cohort study was combined with the Khayelitsha Hospital cohort study in Chapter 6.

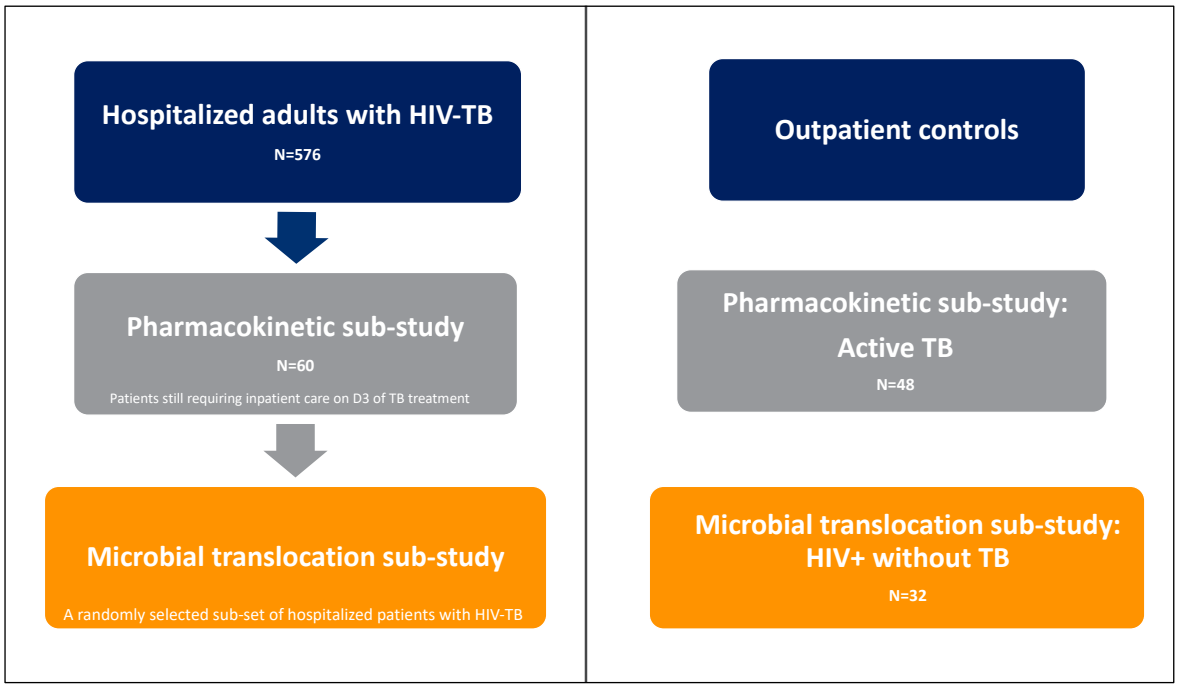
Participants

All patients in the Khayelitsha Hospital emergency room and medical wards were screened on weekdays. HIV-positive adults with a CD4 count <350 cells/μL and a high clinical suspicion of new tuberculosis (i.e. tuberculosis was considered the number one differential diagnosis on referral or after review by hospital or study staff) were eligible for enrolment. Pregnant patients, patients who received antituberculosis therapy within the past month or recently initiated and received 3 or more doses of antituberculosis therapy were not enrolled. A list of all potentially eligible patients was compiled daily and a random selection procedure was followed to enrol 2-4 patients daily and minimise selection bias. Clinical details, chest X-ray, sputum (spontaneous or induced if unable to produce sputum spontaneously), urine and blood samples were obtained at enrolment. Participants remained in routine clinical care and study results were made available to clinical teams. Participants were assessed daily in the ward and after discharge had telephonic follow up at week 4 and returned for a clinical assessment at week 12. Vital status at week 12 was also obtained through provincial health electronic records if the patient was lost to follow-up from the study.

We performed intensive pharmacokinetic (PK) studies in a sub-set of hospitalized patients on the third day of antituberculosis therapy. We enrolled outpatients initiating antituberculosis therapy at Ubuntu clinic, Site B Khayelitsha as controls and performed PK studies at the same time point (Chapter 4 of thesis). In a separate set of controls, HIV-positive outpatient controls were screened to exclude tuberculosis during a routine visit to the primary care antiretroviral clinic and served as control patients for the microbial translocation analysis (Chapter 5 of thesis).

In the GF Jooste Hospital cohort study unselected HIV-positive adults who were admitted to the medical wards in were recruited within 24 hours of admission regardless of TB treatment. Patients had a detailed tuberculosis diagnostic work-up, including a urine GeneXpert test and were followed up for vital status. We pooled the urine GeneXpert results of this cohort with the results of the Khayelitsha Hospital cohort study to assess numbers of false positive rifampicin resistance results on urine GeneXpert test (Chapter 6 of thesis).

Schematic of studies described in this thesis:



Study schematic: The main cohort was enrolled at Khayelithsa Hospital where HIV-positive adults with a clinical suspicion of new tuberculosis were enrolled and followed up for 12 weeks. A subset of 60 of these patients had intensive pharmacokinetic studies performed during the index admission on the third day of antituberculosis therapy. A random sample was selected from the cohort and stored samples were used to measure markers of microbial translocation. Outpatient controls were enrolled from Ubuntu clinic in Site B Khayelitsha. Outpatients with active tuberculosis initiating TB treatment were enrolled and had intensive pharmacokinetic studies performed on the third day of antituberculosis therapy. HIV-positive outpatients attending the clinic for routine care were screened for active tuberculosis and enrolled as controls if the tuberculosis screen was negative. Markers of microbial translocation were measured on stored samples.

Overview of laboratory assays

Tuberculosis tests were performed at the National Health Laboratory Services (NHLS). Sputum, blood and urine samples were tested for tuberculosis using Xpert MTB/RIF assay G4 cartridge (Cepheid) (sputum and urine samples) and liquid culture (sputum and blood samples) using the BACTEC MGIT 960 system (BD Diagnostic Systems, Sparks, MD, USA) and Myco/F Lytic bottles (Becton Dickinson Biosciences) respectively. Positive cultures (sputum and/or blood) had line probe assay performed using Genotype MTBDRplus assay (Hain Lifesciences) to identify

Mycobacterium tuberculosis and to determine drug susceptibility to rifampicin and isoniazid. Rifampicin resistant isolates had second line drugs susceptibility testing performed. Urine lipoarabinomannan (LAM) testing was performed retrospectively on frozen urine samples using the Determine™ LAM assay (Alere). CD4 count, HIV viral load, full blood count, differential count, renal function, liver function, C-reactive protein, procalcitonin, venous lactate and cytomegalovirus viral (CMV) load tests were performed on all participants by the NHLS. Serum cryptococcal antigen lateral flow assay was performed by study staff. Bacterial blood cultures were taken by the study team or hospital staff if intravenous antibiotics have not already been administered. Bacterial cultures from other anatomical sites were collected as clinically indicated and results were captured for all bacterial cultures during the index admission. Detail of tuberculosis and other laboratory tests are described in detail in relevant chapters of this thesis. Plasma was stored at -80°C for immunology, pharmacokinetic and microbial translocation assays. Once enrolment and follow up of all participants was completed, soluble inflammatory mediators were measured in stored plasma in a randomly selected subset of participants using the Biorad Bioplex 200 Luminex platform and Bio-Plex Pro Human Cytokine Standard 27-plex kit (Group 1). The analytes are described in Chapter 3. Rifampicin, isoniazid and pyrazinamide concentrations were measured on stored plasma in the sub-set of patients and a group of outpatient controls who had intensive pharmacokinetic studies performed. The assays are described in Chapter 4. Markers of microbial translocation were measured on stored samples of a randomly selected subset of patients and outpatient controls without tuberculosis. We measured two direct markers of microbial translocation and five indirect markers of microbial translocation which are described in Chapter 5. We combined the urine Xpert results of this cohort

with another well characterised hospitalized cohort from GF Jooste Hospital in Cape Town and determined the proportion of false positive urine Xpert results in Chapter 6.

Overview of data collection, definitions and data curation

Clinical data was obtained from the patient, hospital folder and clinical review at enrolment and captured on standard case record forms. Results of all study investigations, all tuberculosis tests and all bacterial cultures were captured. The primary outcome was vital status at week 12. Data was entered onto an Access database throughout the study period by data capturers. Once clinical follow up was complete, all folders were checked for completeness, data entry was completed, and the data from the Access database was exported to Excel spreadsheets and cleaned using R Statistical software. Based on results of all tuberculosis tests all participants were classified into mutually exclusive diagnostic groups: microbiologically confirmed tuberculosis, probable tuberculosis (clinical tuberculosis), possible tuberculosis and no tuberculosis. We quantified the degree of mycobacterial dissemination with a 3-point dissemination score previously described (26). Participants who had valid results for all three tests were allocated one point for each of the following: urine Xpert MTB/RIF assay positive for *M. tuberculosis*, urine Alere LAM test positive, mycobacterial blood culture positive and identified as *M. tuberculosis*, yielding a score ranging from 0-3. Early deaths were deaths which occurred within 7 days of enrolment and late deaths are all deaths which occurred after 7 days and within 12 weeks of enrolment. These definitions are described in more detail in Chapter 3 of this thesis.

Published pharmacokinetic reference ranges for maximum concentrations of rifampicin (8 μg/mL – 24 μg/mL), isoniazid (3 μg/mL – 6 μg/mL) and pyrazinamide (20 μg/mL – 60 μg/mL) were used to assess optimal maximum concentrations in a sub-group of patients who had intensive pharmacokinetic studies performed (Chapter 4 of thesis).

In Chapter 6 urine Xpert rifampicin resistance results were classified into one of the three mutually exclusive groups: (1) true rifampicin resistant urine Xpert, (2) false rifampicin resistant urine Xpert, or (3) unknown. True urine Xpert rifampicin resistance cases were classified as having heteroresistant if additional independent sample/s from the same clinical episode showed discordant rifampicin sensitivity results.

Brief overview of statistical analyses

Sample size calculations were performed for the major experiments in the original protocol. The planned sample size was 660. Each chapter of this thesis contains a detailed statistical analysis section. Data were analysed using R Statistical software, GraphPad Prism 7.0, JMP 14 (SAS), Gephi 0.9.1. and STATA 13.1. These analyses were performed to address the following research questions:

1. What are the clinical contributors to mortality in patients hospitalized with severe HIV-TB?

   We compared baseline clinical characteristics between patients hospitalized with HIV-TB who died within 12 weeks and patients who survived 12 weeks and explored the association of clinical factors such as time to initiation of antituberculosis therapy, rifampicin resistance, tuberculosis dissemination

markers, cytomegalovirus viraemia and other co-infections with mortality (Chapter 3 of thesis).

2. Is fatal HIV-TB associated with an immune signature of worsening immunosuppression similar to what is seen in bacterial sepsis?
   We measured 28 soluble markers of inflammation and performed cluster analyses, principal components analysis and Cox proportional hazards analysis to define the immune phenotype associated with mortality and we explored the association of this immune phenotype with markers of tuberculosis dissemination (Chapter 3 of thesis).
3. Is severe HIV-TB associated with subtherapeutic concentrations of antitubercular drugs during the critical early period of treatment?
   We performed intensive pharmacokinetic studies on the third day of antituberculosis therapy and measured rifampicin, isoniazid and pyrazinamide concentrations in a group of hospitalized patients with HIV-TB and in a group of outpatient controls being treated for TB and compared concentrations between hospitalized patients who died, patients who survived and outpatient controls using non-compartmental analysis (Chapter 4 of thesis).
4. Is fatal HIV-TB associated with translocation of bacteria and their products from the intestine into blood?
   We measured and compared concentrations of two direct and five indirect markers of microbial translocation on stored samples of hospitalized HIV-TB patients and outpatient controls with HIV infection and no tuberculosis. We compared hospitalized patients who died, hospitalized patients who survived and outpatient controls without tuberculosis (Chapter 5 of thesis).

5. What proportion of urine Xpert tests provide false positive rifampicin resistance results?

   We pooled urine Xpert results from this cohort with another cohort of hospitalized HIV-positive patients recruited in an overlapping geographical area with detailed tuberculosis diagnostic work-up. Urine Xpert tests are not routinely used yet, but are potentially a useful test for diagnosing TB in hospitalised HIV-positive patients. We determined the proportion of urine Xpert tests that provided false positive rifampicin resistant results and described the potential reasons for this (Chapter 6 of thesis).

Missing data was examined to assess if data was missing at completely at random or not. The major analyses (specifically the Cox regression analyses and the PCA analyses in Chapter 3) were performed using complete cases and repeated using multiple imputations and findings were compared. Multiple imputations did not change the size and direction of any associations, thus complete case analyses were used throughout.

Ethical approval

The study was approved by the University of Cape Town Human Research Ethics Committee (UCT HREC), reference number: 057/2013. Participants provided written informed consent. Eligible patients with a decreased level of consciousness were enrolled and followed up daily until they regained capacity to participate in the informed consent process and if not agreeable to participate, were withdrawn from the study. Permission was sought from the UCT HREC to use information of participants who died prior to providing informed consent. These procedures were all approved by UCT HREC.

Summary description of how results are presented

Chapter 2 provides detailed background to global and local burden of the HIV-associated tuberculosis and the associated high mortality.

Chapter 3 is the main cohort analysis which includes clinical and immunological findings. Results are presented as the published manuscript including all figures and supplementary material. The Appendix to Chapter 3 includes additional data and analyses which were not included in the publication or supplementary material.

Chapter 4 presents the findings of the non-compartmental analysis of the pharmacokinetic sub-study. The findings are presented in the format of the published manuscript including all figures and supplementary material. The Appendix to Chapter 4 includes additional data and analyses which were not included in the publication or supplementary material.

Chapter 5 presents findings of the microbial translocation markers and includes all analyses performed on the biomarkers of microbial translocation to date. These findings are presented as a chapter and will be condensed and prepared for a publication.

Chapter 6 presents findings of an evaluation of the frequency of false rifampicin resistance results when using the Xpert MTB/RIF assay on urine samples. We pooled urine Xpert MTB/RIF results from our cohort with results from a second cohort enrolled in the same geographical area where similarly extensive tuberculosis investigations were performed. These results are presented as the published manuscript.

Chapter 7 presents a summary of all the preceding chapters as conclusions to the thesis.

CHAPTER 2:

Background and literature review

A brief history of tuberculosis, HIV infection and HIV-TB co-infection

Tuberculosis is an ancient disease and despite significant progress in the fight against tuberculosis it remains the most successful and deadly pathogen in the world. Evidence compatible with tuberculosis have been found in Egyptian mummies dating back to 3000 BC. Archeological findings of bone tuberculosis in the skeletons of mainly young men and women in Britain date back to the Roman era and similar findings in Denmark and Italy date back a thousand years earlier. Clinical syndromes compatible with tuberculosis have been described as early as the second century AD, yet tuberculosis was only identified as an infectious disease as recently as 1882. Once some understanding of transmission dynamics developed, public health interventions were introduced, and various treatment options were offered at sanatoria which sprung up across the globe. Some of these interventions achieved moderate success and the tuberculosis mortality rate started declining prior to treatment becoming available in the form of streptomycin in the 1940s (27, 28).

The slowly progressive nature of tuberculosis gradually transformed the lives of its usually young victims and left loved ones and doctors as helpless bystanders. The presence of tuberculosis imprinted itself in art, literature and music over the ages, for example the artist Edvard Munch's work was heavily influenced by the death of his mother and sister due to tuberculosis. The world lost many notable authors such as Robert Louis Stevenson, David Herbert Lawrence, George Orwell, Henry David Thoreau, Franz Kafka, Kahlil Gibran, Anton Chekov and composers Frédéric Chopin, Igor Stravinsky and Niccolò Paganini to tuberculosis. After the discovery of

antibiotics the nearly inevitable death sentence of tuberculosis appeared to be something of the past, especially when multi-drug antibiotic regimens became available and rifampicin was introduced in the 1970s (27, 29). However, the emergence of the human immunodeficiency virus (HIV) infection and the HIV-TB epidemic which unfolded rapidly during the 1980s changed the epidemiology of tuberculosis.

In 1981 the first case series describing opportunistic infections in a small group of previously healthy, young gay men was published in the USA and many reports of similar cases followed (30). A clinical case definition was formulated by the Centers for Disease Control and Prevention (CDC) in the USA in 1982 which indicated a probable defect in cellular immunity . The vast majority of initial cases were reported in gay men and intravenous drug users. Despite the detection of similar cases in haemophiliacs, infants and heterosexual patients which followed in rapid succession, there was significant initial stigmatization around HIV infection and this affected much of the initial response to this rapidly spreading epidemic (31, 32). The retrovirus responsible for this immune deficit (human immunodeficiency virus or HIV) was first identified in France and then the USA in 1983 and 1984 respectively (33-36). HIV preferentially infects CD4+ T-cells and causes a profound decline in CD4+ T-cell numbers and deficits in function (37). This renders patients vulnerable to a range of viral, bacterial, fungal and protozoal opportunistic infections as well as opportunistic cancers which cause death. HIV infection is spread through contact with body fluids of a HIV-infected person, is preventable, but there is no cure currently.

The first documented cases of HIV infection in African patients were reported in 1983 in a group of Congolese immigrants in Belgium, although molecular evidence suggests HIV may have existed in Africa since the 1940s (38, 39). Subsequent epidemiological research in Africa in the 1980s confirmed a high prevalence of HIV infection in many African countries. Complicated sociopolitical issues in Africa and worldwide stigmatization of HIV-positive people lead to large-scale denial and a lack of political will to intervene early during the HIV epidemic in Africa. This together with the lack of effective treatment resulted in high mortality in adults and children with HIV infection across Africa during the early 1990s and a major burden on health care resources. This disease burden had devastating effects on communities. Persistent efforts from activist groups and civil society eventually positively impacted global African countries' responses to the HIV epidemic over the next decade (31).

Antiretroviral drugs suppress HIV replication, which allows recovery of CD4+ T-cell function and numbers, but resistance to treatment may develop. The first antiretroviral medication, zidovudine, was approved in 1987 in the USA (40). Single drug therapy was followed by combination therapy and after the approval of the first protease inhibitor in 1995, three drug combinations known as highly active antiretroviral therapy (HAART) (41) became the standard of care in the USA and is currently the global standard of care (32). The sociopolitical climate in Africa and initial prohibitive costs of antiretroviral therapy (ART) resulted in delayed access to treatment in Africa, but following mobilization by global agencies to address this in the early 2000s and decreases in the cost of ART, access was expanded and ART is currently widely accessible and affordable throughout the continent (31).

HIV infection increases susceptibility to active tuberculosis and the two infections modulate the host response to each other to the advantage of both pathogens. HIV infection is the most significant risk factor for development of active tuberculosis (42) and HIV-TB with the associated mortality is a major global public health challenge (1). In high income settings HIV infection caused a 'less rapid decline' in tuberculosis incidence and mortality (43) but there was major impact in Africa.

Global and local burden of HIV-associated tuberculosis and mortality

Between 1990 and 2005 the estimated global tuberculosis incidence more than doubled from 149 per 100000 population in 1990 to 343 per 100000 in 2005. Tuberculosis associated mortality nearly doubled from ~ 40 deaths per 100000 to 74 during the same time period (44, 45). In 2018 ten million people developed active tuberculosis world-wide, with ~25% of incident cases occurring in Africa, and ~300000 in South Africa. Whilst the most recent estimates show a steady decrease in tuberculosis incidence (231 cases per 100000 population) and mortality (20 deaths per 100000 in HIV-positive people in 2018 (46)), tuberculosis currently ranks amongst the top ten global causes of death and is the infectious disease responsible for most deaths annually (27, 29, 46). In 2018 HIV-TB comprised 8.6% of global tuberculosis cases, yet contributed a disproportionate 17% of global tuberculosis mortality. The majority (74%) of HIV-TB patients live in Africa (47) and in 2018 ~35% (211000) of all tuberculosis related deaths in Africa were HIV-TB related and 42000 HIV-TB deaths occurred in South Africa. Thus, despite massive scale up of HIV treatment and prevention programmes and widespread availability antituberculosis therapy, tuberculosis-related mortality remains a major public health problem and HIV-TB contributes disproportionately to tuberculosis mortality.

Case fatality rate is defined as the proportion of people with the disease who die which is approximated for tuberculosis by dividing the total number of deaths by the total number of incident cases. The global case fatality ratio of tuberculosis was 15% in 2018 which is lower than 22% in 2000 and 16% in 2015, but this varies widely from region to region. The case fatality ratio for tuberculosis in Africa was 608000/2450000 (~ 25%) and 63000/301000 (~ 21%) in South Africa in 2018. The case fatality ratio in patients with HIV-TB in Africa was 211000/615000 (~ 34%) and 42000/177000 (~ 23%) in South Africa in 2018 (calculated from Table 3.3 of WHO Global Tuberculosis Report 2019 (1)). Patients hospitalized with HIV-associate tuberculosis have high case fatality rates ranging from 11% to 32% and deaths occurs early despite treatment (Table 1) (2-8).

Causes of death in HIV infected patients

In the era of highly active antiretroviral therapy mortality in HIV-positive patients has decreased dramatically, however mortality remains higher than the general population (48). In high income countries AIDS defining illnesses and cancers, whilst still important causes of mortality, have been surpassed by non-AIDS related cancers and liver disease (49, 50). Cardiovascular disease and stroke are increasingly important contributors to mortality (48, 51). In resource limited settings with a high burden of HIV infection and communicable diseases, patients still present with advanced HIV infection (11). Although mortality in HIV-positive patients is decreasing in general, AIDS defining diseases still occur commonly and tuberculosis is the leading cause of death, hospitalization and in-hospital death (5, 52-56).

Table 1: Selected studies in high burden settings which reports mortality in adults hospitalized with HIV-associated tuberculosis during the ART-era:

Cohort	Country	Mortality	Days to death Median (IQR)	Follow up time
Hospitalised HIV-TB patients Subbarao et al (2) Patients enrolled in 2011	South Africa	32%	8 (3-39)	8 weeks
Hospitalised HIV-TB patients Bigna et al (3) Patient records from 2006-2013 were reviewed	Cameroon	32%	8.1 (3-25)	Duration of TB treatment
HIV-positive patients hospitalized with cough > 2 weeks Kyeyune et al (4) Patients enrolled from 2007-2008	Uganda	*31%	12 (4-37)	2 months
Sequential HIV-positive patients hospitalised to medical wards Meintjes et al (5) Patients enrolled from 2012-2013	South Africa	*21%	-	90 days
HIV-positive hospitalised with cough and any of the WHO danger signs Griesel et al (6) Patients enrolled from 2011 -2014	South Africa	*13%	-	56 days
HIV-positive patients admitted to 3 district hospitals **Alvarez-Uria et al Patients were enrolled from 2009-2011**	India	[a]25%	-	24 months

Table 1: HIV-TB: HIV associated tuberculosis.
*Mortality was calculated in patients with HIV-associated tuberculosis
a Three month mortality reported: 1-month mortality was 16%, 12-month mortality was 39% and 24-month mortality was 46%.

Diagnosis of HIV-associated tuberculosis in hospitalized patients

There are several diagnostic challenges in patients hospitalized with HIV-TB. Patients often present with non-specific symptoms and clinical findings in disseminated tuberculosis overlap with those of other opportunistic infections such as bacterial infections, disseminated fungal infections and HIV-associated malignancies (4, 57, 58). The chest X-ray is often normal in patients with HIV-TB, especially in patients with a low CD4 count (59). Another radiological diagnostic tool, focused assessment with sonography for HIV-TB (FASH) may be normal in

disseminated tuberculosis and there is overlap of tuberculosis-related abnormalities and other clinical conditions (60). Microbiological evidence for tuberculosis is required to make a definitive diagnosis and to determine sensitivity to antituberculosis drugs.

Samples are not easily obtained in acutely ill patients as patients are often unable to provide sputum specimens which is the specimen most commonly used to diagnose tuberculosis (15). Sputum induction may increase the yield of sputum samples, but this requires the correct equipment, trained staff, a safe space to conduct sputum induction and patients may not be able to tolerate sputum induction.

Culture-based diagnostics (from sputum, blood, urine or other clinical samples) are the gold standard for the microbiological diagnosis of tuberculosis and may also provide first line antituberculosis drug sensitivities, however culture-based tests require advanced laboratory infra-structure and may take 6-42 days to get a positive result, another day or two for speciation and 42 days to obtain a negative result. This limits the utility of culture-based results in informing treatment decisions in acutely ill patients. Sputum based acid fast bacilli smear and microscopy testing has been largely replaced by the molecular based sputum GeneXpert test in South Africa and more recently GeneXpert Ultra which is an improved version of GeneXpert. The Xpert platform has the advantage of higher sensitivity and specificity compared to microscopy, is easier to perform, provides results in two hours and provides rifampicin sensitivity results. However, GeneXpert tests cannot distinguish viable from non-viable mycobacteria, do not predict rifampicin resistance with 100% accuracy (61) and are dependent on obtaining the appropriate clinical sample, which is usually sputum. Samples which are easier to obtain for the majority of patients

such as urine and blood samples are advantageous in the setting of acutely ill hospitalized patients. GeneXpert test has been applied to other clinical samples such as cerebrospinal fluid, pleural fluid, blood and urine (62-65), but there is no current guidance on the use of GeneXpert on urine samples due to insufficient evidence of the assay's performance with these specimens (66).

Tuberculosis blood culture is a key diagnostic test in determining a diagnosis of disseminated tuberculosis or tuberculosis bloodstream infection. However, tuberculosis blood cultures are expensive to perform, require specialized laboratory infrastructure and may take 6-42 days for a positive result, plus a day or two for speciation and 42 days for a negative result. Urine based rapid diagnostics are very useful in this patient population. The urine lipoarabinomannan (LAM) is a rapid urine-based diagnostic test which tests for the presence of LAM which is a component of the mycobacterial cell wall. The assay uses a small volume of urine, is similar to a pregnancy test and takes 25 minutes to perform at the bedside. It gives a readout between 0 (negative) and 4 (strongly positive) which is semi-quantitative and performs better in patients with lower CD4 counts in whom making a diagnosis of tuberculosis is often the most difficult (67). Urine LAM positivity is independently associated with mortality in HIV-positive patients (68). However, urine LAM does not provide drug sensitivity results and even if urine LAM is positive additional tuberculosis diagnostics are needed to assess sensitivity to first line antituberculosis drugs. Urine LAM also does not distinguish between mycobacterial species and tests positive in non-tuberculous mycobacterial infections (69). The GeneXpert platform can also be used on urine samples, but there is inadequate evidence on the performance for it to be recommended as a routine investigation currently. In one study in South African patients the GeneXpert test used on urine samples correctly

diagnosed 4 rifampicin resistant cases, but also misdiagnosed 3 drug sensitive tuberculosis infections as rifampicin resistant (61).

In our study we performed sputum GeneXpert and culture (sputum obtained spontaneously or with induction), mycobacterial blood culture, urine GeneXpert and urine Alere LAM. We also recorded results of any additional tuberculosis tests performed in clinical care during the index admission in order to derive a robust diagnosis of microbiologically proven tuberculosis.

Pharmacokinetics in HIV-associated tuberculosis

Rifampicin is a key drug of first line antituberculosis therapy with potent bactericidal and sterilizing activity against *Mycobacterium tuberculosis*. Rifampicin binds the bacterial DNA-directed RNA polymerase enzyme and efficacy is concentration dependent (70, 71). Rifampicin and its active metabolite desacetylrifampicin are lipid soluble, have enterohepatic circulation, compete with bilirubin for biliary excretion and are excreted mainly in bile, but also in urine (72). Rifampicin is a potent inducer of drug metabolizing liver enzymes (72). Rifampicin was introduced in the 1970s at a dose of 10mg/kg mainly due to fear of hepatic toxicity but also due to cost considerations (73). Recent studies suggest that this dose is sub-optimal and doses of up to 35 mg/kg have been tested in clinical trials. Higher doses of rifampicin are well tolerated (74, 75), a small dose finding trial suggests improved survival in patients with tuberculosis meningitis when using high dose rifampicin (30 mg/kg) (76) and a small unpublished post hoc analysis of a clinical trial suggested improved survival in patients with HIV-TB with low CD4 counts (77). Due to liver enzyme induction and autoinduction, rifampicin settles into a steady state after administration of multiple doses when liver enzymes have reached maximal induction (78) and the

timing of pharmacokinetic studies are therefore important when interpreting results. The majority of rifampicin pharmacokinetic studies have been performed after administration of multiple doses when liver enzyme induction is advanced (79), yet mortality in hospitalized HIV-TB patients occurs early.

Isoniazid is another cornerstone of first line antituberculosis therapy. Isoniazid inhibits the synthesis of long chain mycolic acids in the mycobacterial cell wall (80, 81) and has excellent early bactericidal activity. Isoniazid is water soluble and is excreted in the urine. Isoniazid metabolism is genetically determined by polymorphisms in n-acetyltransferase-2 (NAT2) enzymes found in the liver and gastro-intestinal system, which results in slow, intermediate and fast metabolizers (82). Fast metabolizers are at risk of sub-optimal exposure, which has been associated with acquired resistance to rifamycins (83, 84) and slow metabolizers are at higher risk of side effects such as peripheral neuropathy and hepatotoxicity. The current recommended dose of 5mg/kg per dose achieves optimal early bactericidal activity (defined as 90% of the maximal early bactericidal activity) in the majority of fast metabolizers (85).

Pyrazinamide is a synthetic vitamin B3 (nicotinamide) analogue which is bactericidal against *M. tuberculosis.* Pyrazinamide is converted to its active metabolite pyrazinoic acid by bacterial pyrazinamidase (86) and this happens more successfully in an acidic environment (87). Pyrazinamide is most effective against slowly metabolizing bacteria inside macrophages (87) and is used during the first two months of first line antituberculosis therapy for its sterilizing activity. Pyrazinamide and its metabolite pyrazinoic acid are water soluble and are excreted in the urine (88).

Many studies have been conducted to assess the effect of HIV infection on antituberculosis drug concentrations, but there is significant heterogeneity between studies and results are conflicting. Several studies showed decreased exposure in HIV-infected patients compared to HIV-negative patients, but equally many studies showed no difference in exposure. A recent systematic review by Daskapan *et al* concluded that no dosing recommendations could be made but the review 'exposed the knowledge gaps of the effect of HIV infection on the pharmacokinetics of first line antituberculosis therapy' (79, 89, 90). Additionally the reference ranges used to determine whether first line antituberculosis medication concentrations were therapeutic were determined in healthy volunteers and it is unknown whether these ranges are generalizable to HIV-positive patients who are acutely ill with disseminated tuberculosis. In our cohort study we measured maximum concentrations, performed non-compartmental analysis and calculated the area under the concentration curve for rifampicin, isoniazid and pyrazinamide. We reviewed results of other studies which reported the same pharmacokinetic measures in cohorts which included HIV-infected patients in Africa. Studies were not included in the table if Cmax and AUC values were not reported and if results for HIV-positive participants could not be extracted separately.

Table 2: Summary of selected pharmacokinetic studies in HIV-positive adults in Africa and reported maximum concentration and/or area under the concentration curve values for first line antituberculosis drugs.

Study	Cohort	Comparisons	PK parameter	Rifampicin	Isoniazid	Pyrazinamide
Chideya Botswana 2009 (91) Patients enrolled from 1997-2000	Proven PTB; cough ≥ 2 weeks; abnormal CXR; >7 days TB treatment; HIV positive and HIV negative participants	CD4 <200 n=77	Cmax, µg/mL median (IQR)	4.4 (0.7–12.7)	4.3 (0.35–9.0)	46.9 (25.8–119)
		CD4 ≥ 200 n=67		5.7 (1.1–15.0)	4.2 (0.9–10.8)	49.9 (29.4–108)
		HIV negative n=66		4.6 (1.2–13.4)	4.1 (1.3–10.3)	52.3 (29.9–84.4)
Choudri Tanzania 1997 (92) Patients enrolled from 1994-1995	Smear positive PTB; not on antifungal treatment; no hepatic or renal failure; no previous GIT surgery; admitted to hospital for DOT; PK study on D14 HIV positive and HIV negative	HIV positive n=14	Cmax, mg/L mean	4.1 SD = 2.0	1.4 SD = 0.8	32.1 SD = 8.8
			$AUC_{0-12,}$ mg/(L*h) mean, SD	23.1, 12.9	7.9, 7.3	350, 111
		HIV negative n=15	Cmax, mg/L Mean, SD	4.3, 2.4	1.1, 0.4	33.1, 8.2
			$AUC_{0-12,}$ mg/(L*h) mean, SD	19.6, 10.6	6.3, 3.5	382, 143
Jeremiah Tanzania 2014 (93) Patients enrolled from 2010-2011	Sputum AFB smear positive; HIV positive not on ART and HIV negative; randomized to receiving nutritional support or no nutritional support	HIV positive Nutritional support n=26	Cmax, µg/mL Median (90% range)	6.4 (3.5; 11.2)	-	-
			$AUC_{0-24,}$ µg*h/ml Median (90% range)	31.6 (16.4; 60.3)		
		HIV positive No nutritional support n=24	Cmax, µg/mL Median (90% range)	5.6 (3.0; 9.9)	-	-
			$AUC_{0-24,}$ µg*h/ml median (90% range)	28.6 (15.0; 54.8)		

		HIV negative Nutritional support n=25	Cmax, µg/mL median (90% range)	7.4 (4.1; 12.9)	-	-
			AUC_{0-24}, µg*h/ml median (90% range)	36.5 (19.1; 69.8)		
		HIV negative No nutritional support n=25	Cmax, µg/mL median (90% range)	7.1 (3.9; 12.6)	-	-
			AUC_{0-24}, µg*h/ml median (90% range)	36.6 (19.1; 70.3)		
Taylor South Africa 1998 (94) Time period when patients enrolled not stated	Hospitalized adults with clinical tuberculosis admitted to tuberculosis hospital for inpatient treatment; no renal or hepatic dysfunction; HIV positive and negative; DOT for 3-5 days	HIV positive n=13	Cmax, µg/mL median	12.3	6.5	55.9
			AUC_{0-12}, µg/mL*min median	3604	1349	22392
		HIV negative n=14	Cmax, µg/mL median	7.4	6.1	56.9
			AUC_{0-12}, µg/mL*min median	1665	1062	23117
Tappero Botswana 2005 (95) Patients enrolled from 1997-1999	Adult outpatients with cough ≥ 2 weeks, abnormal chest X-ray and consented to HIV testing and taking TB trt for > 7 days	HIV positive n = 59	Cmax, µg/mL median (range)	5.60 (0; 13.71)	3.99 (0; 9.23)	48.7 (35.6; 118.8)
			AUC_{0-6}, µg/mL*hour median (range)	24.0 (7.0; 52.4)	15.1 (4.1; 35.0)	219 (156; 519)
		HIV negative n = 28	Cmax, µg/mL median (range)	5.96 (2.16; 14.63)	4.35 (0.72; 11.84)	55.5 (36.7–78.9)

			$AUC_{0-6,}$ µg/mL*hour median	21.7 (7.1; 52.9)	15.2 (2.6; 42.8)	241 (156; 372)
Vinnard Botswana 2017 (96) Time period when patients enrolled not stated	HIV-positive, ART naïve adults on TB trt between 5-28 days and repeated after 4 weeks on ART	HIV positive n = 40 pre-ART	Cmax, µg/mL median (range)	7.4 (2.56; 11.61)	-	-
			$AUC_{0-24,}$ mg*h/L median (range)	34.4 (8.2; 80.2)		
		HIV positive n = 24 on ART	Cmax, µg/mL median (range)	7.2 (3.63; 13.19)		
			$AUC_{0-12,}$ mg*h/L median (range)	28.0 (18.0; 61.2)		

Table 2: PK: Pharmacokinetic parameter; PTB: Pulmonary tuberculosis; CXR: chest X-ray; TB trt: antituberculosis therapy; Cmax: Maximum concentration; AUC: area under the concentration curve; IQR: interquartile range; SD: standard deviation; DOT: Directly observed therapy

There are few pharmacokinetic studies in HIV-TB which assess associations between antituberculosis drug exposure and clinical outcomes (91, 97). Suboptimal pyrazinamide exposure was linked to poor outcomes in two African cohorts and these findings are summarized in Table 3 below.

Table 3: Pharmacokinetic studies in Africa assessing association of first line tuberculosis drug concentrations with clinical outcomes:

Study	Outcome	Rifampicin	Isoniazid	Pyrazinamide
Chideya Botswana 2009 (91)	Treatment failure or death during treatment	No association	No association	Patients with low PZA Cmax had increased risk ratio of poor outcome: RR = 4.06, 95% CI: 2.72, 6.06)
Pasipanodya South Africa 2013 (97) CART analysis	Non-conversion of 2-month sputum	Cmax below 6.6 mg/L Ranked second	Cmax below 8.8 mg/L Ranked third	Cmax below 58.3 mg/L Ranked first (93% of patients who did not sputum convert had low PZA Cmax)
	Composite poor long-term outcome (microbiological failure, death or relapse)	AUC below 13 mg*h/L Ranked second	AUC below 52 mg*h/L Ranked third	AUC below 363 mg*h/L Ranked first

Table 3: Cmax: Maximum concentrations; AUC: area under the concentration curve; RR: risk ratio; CART: classification and regression tree analysis

Additional factors which may play a role in the pharmacokinetics of first line antituberculosis therapy in acutely ill patients hospitalized with HIV-TB are that many patients present with clinical features of bacterial sepsis (18, 98, 99). In high-burden settings *Mycobacterium tuberculosis* bloodstream infection (MTB BSI) is the most common diagnosis in HIV-infected patients presenting to hospital with a clinical syndrome of sepsis (9, 13, 17, 100). Analogous to sepsis there are many factors in severe HIV-TB which could potentially reduce drug exposure, such as impaired absorption of orally administered drugs due to delayed gastric emptying and decreased perfusion of the gastrointestinal tract, increased volume of distribution due to fluid shifts, and augmented renal clearance (101, 102). There are other factors in advanced HIV infection which could influence pharmacokinetic properties, such as intestinal tuberculosis, HIV-related enteropathy, and gastro-intestinal opportunistic infections and macro- or micronutrient deficiencies (93, 103, 104), that could contribute to reduced drug absorption and exposure. There are limited data suggesting that antitubercular drug exposure in critically ill patients is inadequate (105).

We measured rifampicin, isoniazid and pyrazinamide concentrations in a subset of patients hospitalized with HIV-TB and a group of outpatient controls on the third day of antituberculosis therapy (Chapter 4).

Microbial translocation in HIV-associated tuberculosis

Gastro-intestinal microbial translocation is defined as the non-physiological translocation of gastro-intestinal microbes and/or microbial products through the

gastro-intestinal epithelial barrier and the lamina propria into the portal venous system after damage to the mucosal barrier and local immune system (106). These bacterial products elicit an immune response. HIV infection causes early and profound damage to the structural and immunological barriers of the gastro-intestinal tract during acute infection which result in increased intestinal mucosal permeability and translocation of microbes and/or microbial products into the systemic circulation (107-110). Translocation of microbial products has been described in other clinical conditions such as inflammatory bowel disease, visceral leishmaniasis, graft versus host disease, patients with extensive burn wounds, haemorrhagic shock, acute alcohol intoxication and in chronic HIV infection (21, 111-116) and is associated with adverse clinical outcomes in patients with Crohn's disease and patients with liver cirrhosis (22, 23). In HIV infection damage to the gastro-intestinal barrier does not recover fully despite suppressive long-term antiretroviral therapy and can be averted by starting antiretroviral therapy during acute HIV infection (107). Microbial translocation contributes to chronic systemic inflammation and immune activation, which is associated with disease progression and mortality (21). Data from autopsy studies performed in hospitalized HIV-infected patients who died in medical wards show frequent concomitant bacterial infections which could support a hypothesis of translocation of viable bacteria across the gastro-intestinal barrier (117), although the route of bacterial entry may be from sources other than the gastro-intestinal system such as the lungs.

In patients hospitalized with HIV-TB co-infection there are additional factors which could contribute to decreased gastro-intestinal mucosal integrity. Patients commonly present with clinical features compatible with bacterial sepsis of which a feature is

intestinal hypoperfusion (18, 98, 99). Intestinal tuberculosis is present in 36- 43% of post-mortem HIV-TB cases (118, 119) and can affect the mucosa, sub-mucosal layers, lymph nodes and peritoneum. Malnutrition negatively affects gastro-intestinal mucosal integrity and could play a role in patients with severe HIV-TB.

Microbial product translocation can be measured directly by quantifying microbial products such as LPS or bacterial 16s ribosomal DNA (16s rDNA) in the peripheral blood. Indirect measures of microbial translocation are concentrations of the binding proteins and antibodies which are involved in the immune response to LPS, (soluble CD14, lipopolysaccharide binding protein (LBP), endotoxin core antibody (EndoCAB)) and markers of gastro-intestinal mucosal damage such as intestinal fatty binding protein (IFABP) and trefoil factor 3 (TFF3).

We hypothesized that in patients with severe HIV-TB who often present with a clinical syndrome of sepsis, there is significantly more bacterial translocation (due gastro-intestinal mucosal damage) than in patients with HIV alone and microbial translocation is associated with mortality. We hypothesized that a possible underlying mechanism of mortality is the translocation of viable bacteria from the gastro-intestinal system into the blood stream resulting in bacterial sepsis and death. We measured two direct markers and five indirect markers of microbial translocation in a subset of hospitalized patients with HIV-TB and a group of HIV-positive outpatient controls without tuberculosis (Chapter 5).

Other factors associated with mortality in HIV-associated tuberculosis

Globally a larger proportion of adult men with HIV-TB die every year compared to women. In 2018 of all patients with HIV-TB who died, 49% were men, 38% were women and 13% children (1). In Africa the difference is less pronounced with 45% of deaths occurring in men vs 41% of death occurring in women (1). A retrospective review of a large number of tuberculosis cases in South Africa identified female sex and older age as independent risk factors for mortality amongst patients with HIV-TB. The finding of female sex as a risk factor for mortality was in contrast to the majority of reports that have found male sex to be associated with increased mortality in tuberculosis. This finding may be due to unmeasured confounding factors such as different background gender based mortality rates in the community where the study was conducted (120). Some studies have shown associations of clinical factors with mortality in patients with HIV-TB. These clinical factors can be grouped together into factors associated with more advanced HIV infection such as lower CD4 count and higher WHO stage or factors associated with more severe acute illness at presentation such as poor ambulatory status, lower body mass index, lower haemoglobin concentration and higher white cell count (120-122). These factors are probably inter-related and patients with more advanced HIV infection are more likely to present with more severe illness and vice versa. Clinical measures of severity of illness such as abnormal vital signs, ambulatory status, level of consciousness and abnormal blood results could be influenced by a myriad of factors such as the patient's baseline clinical condition, severity of tuberculosis infection (degree of dissemination or mycobacterial load) and other concomitant infections.

Disseminated or extra-pulmonary tuberculosis occurs more commonly in patients with lower CD4 counts and is associated with higher mortality (123, 124). Post-mortem studies in patients with HIV-TB show evidence of disseminated tuberculosis in 88% of cases (14) and the most common diagnosis of HIV infected patients presenting to hospital with sepsis syndrome is tuberculosis bloodstream infection in Africa (9). Quantification of tuberculosis dissemination relies on indirect measures such as days to positivity of tuberculosis cultures, cycle thresholds of genotypic tests and urine LAM score and there is no standardized way to determine the degree of tuberculosis dissemination or mycobacterial load. Mycobacterial blood culture is the gold standard to diagnose mycobacterial blood stream infection. Urine LAM positivity is regarded as a marker of disseminated tuberculosis although the mechanism by which LAM is excreted in the urine is not fully understood. Urine LAM positivity is associated with renal tuberculosis on autopsy, the presence of mycobacteria in the urine (using GeneXpert test on urine samples) and with mycobacterial blood culture positivity (125-127). Thus urine LAM positivity probably indicates higher disseminated mycobacterial load and may explain why urine LAM positivity is an independent risk factor for mortality in patients with HIV-TB (68). The GeneXpert detects *Mycobacterium tuberculosis* DNA, thus indicating disseminated disease when it is positive in a urine sample. The combination of urine LAM and urine GeneXpert test results can rapidly detect patients with mycobacterial bloodstream infection (26).

Another important contributor to tuberculosis-related mortality is multi-drug resistant (MDR) and extremely drug resistant (XDR) tuberculosis strains. MDR tuberculosis indicates resistance to both rifampicin and isoniazid where XDR tuberculosis strains

are also resistant to a fluoroquinolone and an injectable tuberculosis drug. In 2018 an estimated 484000 new cases of rifampicin resistant tuberculosis were reported world-wide, with 77000 cases in Africa and 11000 in South Africa. The cure rate for drug resistant tuberculosis is only 55% and an estimated 214000 deaths due to drug resistant tuberculosis occurred in 2018 (1).

Apart from commonly finding tuberculosis in autopsy studies of HIV-infected patients who die in medical wards, there are also frequent bacterial infections, fungal infections and neoplasms reported. Of note, many patients who have tuberculosis also have evidence of other infections or neoplasms (117, 119). In one post mortem study by Wong et al, 62% of all patients with tuberculosis also had at least one other infectious or neoplastic cause of death (117).

Early initiation of antiretroviral therapy in patients with HIV-TB and CD4 count <50 cells/μL improves survival, but results in more cases of paradoxical tuberculosis immune reconstitution inflammatory syndrome (TB-IRIS) (128). Mortality due to paradoxical TB-IRIS is 2% among those patients diagnosed with paradoxical TB-IRIS (129), unless the central nervous system is involved when mortality is higher (130). Morbidity associated with paradoxical TB-IRIS is high with 25% of patients needing admission to hospital for clinical work up (129).

Immunological studies assessing associations with mortality in HIV-TB patients show the following associations with mortality: having higher concentrations of soluble markers of immune activation at baseline; failure to resolve baseline immune activation or increasing markers of immune activation after initiation of antiretroviral

therapy and failure to recover the cellular immune response to *M. tuberculosis* (131-133). One study which selected cases (HIV-TB patients with a CD4 count of < 50 μg/mL) from a larger cohort using a retrospective case-cohort design showed increased concentrations of baseline C-reactive protein, interferon gamma (IFN-γ), monocyte chemotactic protein 3 (MCP-3, or chemokine [C-C motif] ligand 7 (CCL7)), interleukin 15 (IL-15) and IL-17 were independently associated with 48-week mortality using Cox proportional hazards models adjusted for CD4 count and HIV viral load. This was a small cohort (n=51 with n=13 deaths) with deaths occurring between 2-40 weeks. In univariate analyses there were significantly higher baseline concentrations of IL1-receptor antagonist (IL1-Ra), IL-6, IL-8, macrophage inflammatory protein alpha (MIP-1α) and tumour necrois factor (TNF)-α in patients who died (132). A study in Malawi enrolled smear negative HIV-positive patients with at chronic weight loss, fever or diarrhoea and showed higher baseline C-reactive protein concentrations (a marker of inflammation) were independently associated with having tuberculosis, blood stream infection (other than tuberculosis) and 30-day mortality (134). An outpatient cohort study in Botswana measured immune markers longitudinally before and after ART initiation in HIV-TB patients. Participants who died had a slower increase in CD4 counts in response to ART, lower PPD-specific immune recovery (measured with enzyme-linked immunosorbent spot assays (ELISpot) assays on freshly isolated peripheral blood mononuclear cells (PBMCs)) and higher levels of immune activation as measured by IL-6, CRP and soluble CD14 (sCD14) (133). A more detailed immunological profiling of this cohort showed that HIV-TB patients with higher pre-ART levels of MCP-1/CCL2, eotaxin, IL-10, TNF-α, and IL-6 had higher 6-month mortality and that a significant increase in IL-1Ra, IFN-γ, and granulocyte-colony stimulating factor or colony stimulating factor 3 (G-

CSF/CSF 3) concentrations at 4 weeks after ART initiation was associated with mortality (131). Genotypic profiling of this cohort showed the presence of a single nucleotide polymorphism (SNP) involved in the inflammasome pathway, NOD-like receptor pyrin containing-3 (NLRP3) rs10754558-G, was independently associated with 6-month mortality and variations in the genotype at NLRP3 rs10754558 influenced participants' systemic inflammatory state pre-ART and at 4 weeks after ART initiation. The presence of this SNP appears to modulate inflammasome activation and contribute to increased inflammation, which may indicate a genetic predisposition to exaggerated inflammatory responses in some participants that is associated with poor outcome (135). A cohort study in Malawi enrolled outpatients with pulmonary tuberculosis initiating antituberculosis therapy and monitored patients for 56 days to determine vital status and episodes of acute deterioration. The majority of this cohort were (60%) HIV-positive and they performed a whole blood stimulation assay using LPS and heat-killed tuberculosis and measured TNF-α concentrations in stored supernatant. They found significantly lower TNF-α production in response to LPS and heat-killed tuberculosis in patients with poor outcome (including mortality or a life threatening deterioration which required hospitalization) (136).

We collected detailed baseline clinical characteristics (including demographic details, antiretroviral therapy and co-infections), performed detailed tuberculosis diagnostic tests and measured soluble markers of inflammation in peripheral blood collected at baseline in order to investigate clinical characteristics and the immunological phenotype associated with mortality in hospitalized patients with HIV-TB (Chapter 3).

Conclusion

Tuberculosis is the leading global cause of death by an infectious disease and ranks amongst the top ten causes of death world-wide. HIV-associated tuberculosis contributes disproportionately to tuberculosis mortality and will likely remain a common clinical problem in high burden settings in the foreseeable future. Patients hospitalized with disseminated HIV-TB are often acutely ill and have high early mortality, yet treatment guidelines are the same as for HIV-negative pulmonary tuberculosis patients and tuberculosis treatment trials have not included acutely ill hospitalized HIV-TB patients to date. The factors contributing to death and the underlying pathophysiology of death in patients hospitalized with HIV-TB are poorly understood and thus there is little evidence to provide a rationale for improved therapeutic strategies in this vulnerable patient group. We investigated determinants of mortality in patients hospitalized with HIV-TB with the aim to inform improved treatment strategies to test in prospective clinical trials in this patient group.

CHAPTER 3

Clinical, microbiologic, and immunologic determinants of mortality in hospitalized patients with HIV-associated tuberculosis: a prospective cohort study

PLOS MEDICINE

RESEARCH ARTICLE

Clinical, microbiologic, and immunologic determinants of mortality in hospitalized patients with HIV-associated tuberculosis: A prospective cohort study

Charlotte Schutz[1,2]*, David Barr[3], Bruno B. Andrade[1,4,5,6,7], Muki Shey[1,2], Amy Ward[1,2], Saskia Janssen[8], Rosie Burton[9], Katalin A. Wilkinson[1,2,10], Bianca Sossen[1,2], Kiyoshi F. Fukutani[4,5,11], Mark Nicol[12], Gary Maartens[13], Robert J. Wilkinson[1,2,10,14], Graeme Meintjes[1,2]

1 Wellcome Centre for Infectious Diseases Research in Africa (CIDRI-Africa), Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa, **2** Department of Medicine, University of Cape Town, Cape Town, South Africa, **3** Wellcome Trust Liverpool Glasgow Centre for Global Health Research, University of Liverpool, Liverpool, United Kingdom, **4** Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Brazil, **5** Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) Initiative, Salvador, Brazil, **6** Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, United States of America, **7** Universidade Salvador (UNIFACS), Laureate Universities, Salvador, Brazil, **8** Amsterdam University Medical Center, University of Amsterdam, Amsterdam, the Netherlands, **9** Khayelitsha Hospital, Department of Medicine, Cape Town, South Africa, **10** The Francis Crick Institute, London, United Kingdom, **11** Faculdade de Tecnologia e Ciências (FTC), Salvador, Brazil, **12** Division of Medical Microbiology, University of Cape Town and National Health Laboratory Services, Cape Town, South Africa, **13** Division of Clinical Pharmacology, Department of Medicine, University of Cape Town, Cape Town, South Africa, **14** Department of Medicine, Imperial College, London, United Kingdom

* Charlotte.Schutz@uct.ac.za

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Abstract

Background

In high-burden settings, case fatality rates are reported to be between 11% and 32% in hospitalized patients with HIV-associated tuberculosis, yet the underlying causes of mortality remain poorly characterized. Understanding causes of mortality could inform the development of novel management strategies to improve survival. We aimed to assess clinical and microbiologic determinants of mortality and to characterize the pathophysiological processes underlying death by evaluating host soluble inflammatory mediators and determined the relationship between these mediators and death as well as biomarkers of disseminated tuberculosis.

Methods and findings

Adult patients with HIV hospitalized with a new diagnosis of HIV-associated tuberculosis were enrolled in Cape Town between 2014 and 2016. Detailed tuberculosis diagnostic testing was performed. Biomarkers of tuberculosis dissemination and host soluble inflammatory mediators at baseline were assessed. Of 682 enrolled participants, 576 with tuberculosis (487/576, 84.5% microbiologically confirmed) were included in analyses. The median age

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Abbreviations: aHR, adjusted hazard ratio; ART, antiretroviral therapy; CCL, C-C motif chemokine ligand; CD4, cluster of differentiation 4; CMV, cytomegalovirus; CRAG, cryptococcal antigen; CSF2, colony stimulating factor 2; CSF3, colony stimulating factor 3; CXCL, C-X-C motif chemokine ligand; ELISA, enzyme-linked immunosorbent assay; FGF, basic fibroblast growth factor; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-γ, interferon gamma; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein; LAM, lipoarabinomannan; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MTB BSI, *Mycobacterium tuberculosis* bloodstream infection; NLRP3, NOD-like receptor pyrin containing-3; NHLS, National Health Laboratory Services; PC, principal component; PDGF, platelet-derived growth factor; PJP, *Pneumocystis jirovecii* pneumonia; PK, pharmacokinetic; RANTES, regulated on activation, normal T-cell expressed and secreted; ROC, receiver operating characteristic; SNP, single nucleotide polymorphism; SOFA, sequential organ failure assessment score; TGF-β1, transforming growth factor beta 1; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

was 37 years (IQR = 31–43), 51.2% were female, and the patients had advanced HIV with a median cluster of differentiation 4 (CD4) count of 58 cells/L (IQR = 21–120) and a median HIV viral load of 5.1 $\log_{10}$ copies/mL (IQR = 3.3–5.7). Antituberculosis therapy was initiated in 566/576 (98.3%) and 487/576 (84.5%) started therapy within 48 hours of enrolment. Twelve-week mortality was 124/576 (21.5%), with 46/124 (37.1%) deaths occurring within 7 days of enrolment. Clinical and microbiologic determinants of mortality included disseminated tuberculosis (positive urine lipoarabinomannan [LAM], urine Xpert MTB/RIF, or tuberculosis blood culture in 79.6% of deaths versus 60.7% of survivors, $p = 0.001$), sepsis syndrome (high lactate in 50.8% of deaths versus 28.9% of survivors, $p < 0.001$), and rifampicin-resistant tuberculosis (16.9% of deaths versus 7.2% of survivors, $p = 0.002$). Using non-supervised two-way hierarchical cluster and principal components analyses, we describe an immune profile dominated by mediators of the innate immune system and chemotactic signaling (interleukin-1 receptor antagonist [IL-1Ra], IL-6, IL-8, macrophage inflammatory protein-1 beta [MIP-1β]/C-C motif chemokine ligand 4 [CCL4], interferon gamma-induced protein-10 [IP-10]/C-X-C motif chemokine ligand 10 [CXCL10], MIP-1 alpha [MIP-1α]/CCL3), which segregated participants who died from those who survived. This immune profile was associated with mortality in a Cox proportional hazards model (adjusted hazard ratio [aHR] = 2.2, 95%CI = 1.9–2.7, $p < 0.001$) and with detection of biomarkers of disseminated tuberculosis. Clinicians attributing causes of death identified tuberculosis as a cause or one of the major causes of death in 89.5% of cases. We did not perform longitudinal sampling and did not have autopsy-confirmed causes of death.

Conclusions

In this study, we did not identify a major contribution from coinfections to these deaths. Disseminated tuberculosis, sepsis syndrome, and rifampicin resistance were associated with mortality. An immune profile dominated by mediators of the innate immune system and chemotactic signaling was associated with both tuberculosis dissemination and mortality. These findings provide pathophysiologic insights into underlying causes of mortality and could be used to inform the development of novel treatment strategies and to develop methods to risk stratify patients to appropriately target novel interventions. Causal relationships cannot be established from this study.

Author summary

Why was this study done?

- Patients with HIV who are hospitalized with new tuberculosis are at high risk of death even after starting on standard antituberculosis treatment, and the reasons for this are not well understood.
- To facilitate the development of novel treatment strategies that improve survival, a better understanding of the cause of these deaths is required.

What did the researchers do and find?

- We enrolled 576 patients with HIV hospitalized with a new diagnosis of tuberculosis and followed them up for 12 weeks.
- We systematically performed diagnostic tests for tuberculosis in blood and urine as a measure of tuberculosis dissemination (the spread of tuberculosis through the body via the bloodstream). We characterized the immune response in blood of these patients and compared these measures in patients who died with those who survived.
- In our study, 22% of patients died within 12 weeks, and those who had a greater number of positive measures for tuberculosis dissemination were more likely to die.
- An immune profile characterized by increased markers of the innate immune response and proteins that attract innate cells to tissue was associated with mortality, and this profile was also associated with more disseminated tuberculosis.

What do these findings mean?

- Our findings provide novel insights into the immune response associated with death in patients with HIV who are severely ill with tuberculosis.
- This knowledge can be used to design, evaluate, and monitor new treatment strategies, including immune-based therapies.
- Our study evaluated immune responses at a single time point prior to treatment, and future studies should evaluate these responses longitudinally on antituberculosis treatment.

Introduction

HIV-associated tuberculosis comprises 10% of global tuberculosis cases but contributes a disproportionate 22% of tuberculosis deaths [1]. Despite advances in diagnostics and widespread availability of treatment, tuberculosis remains the leading cause of death (40%), hospitalization (18%) and in-hospital death (25%) in patients with HIV worldwide [1–3]. In high-burden settings, hospitalized patients with HIV-associated tuberculosis have case fatality rates between 11% and 32% [4–10]. The underlying causes of mortality remain poorly characterized. In outpatient cohorts with HIV-associated tuberculosis, early mortality has been associated with high baseline immune activation [11,12], persistent or increased immune activation on antiretroviral therapy (ART) [12], and failure to recover cellular immune responses to *Mycobacterium tuberculosis* [13]. In Africa, disseminated tuberculosis is found in 88% of autopsies of patients dying with HIV-associated tuberculosis [14]. *M. tuberculosis* bloodstream infection (MTB BSI or mycobacteremia) is the most common microbiologic diagnosis in patients with HIV admitted with a clinical sepsis syndrome in Africa [15,16] and is associated with higher mortality [16–19]. Patients with MTB BSI may present with sepsis syndrome [19,20] and with septic shock, which has high mortality, especially if antituberculosis treatment is delayed [21].

Patients admitted with HIV-associated tuberculosis are frequently acutely ill, with inpatient deaths occurring at a median of 4–5 days after admission in autopsy series [22–24], despite

many receiving appropriate antituberculosis therapy [22]. Disseminated tuberculosis is challenging to diagnose, and while improved diagnostic tests like urine lipoarabinomannan (LAM) and urine Xpert MTB/RIF assays facilitate more rapid detection of disseminated tuberculosis [18], mortality in inpatients with suspected HIV-associated tuberculosis remains high despite implementation of these rapid tests [25,26].

Autopsy series also report frequent additional opportunistic infections, which may contribute to death [23,24,27]. However, autopsy studies do not characterize the dynamic pathophysiological processes underlying mortality.

There is an urgent need for improved, evidence-based, acute management strategies to increase survival in hospitalized patients with HIV-associated tuberculosis. An improved understanding of pathophysiological processes and contributors to mortality could facilitate the discovery of novel therapeutic targets and strategies, enable appropriate risk stratification to target new interventions appropriately, and aid monitoring of treatment responses.

In this prospective observational study, we followed a large cohort of participants with HIV who were diagnosed with active tuberculosis while admitted to hospital. We measured markers of tuberculosis dissemination and host soluble inflammatory mediators. Our first objective was to determine the 12-week case fatality rate and the contribution of antituberculosis drug resistance, dissemination of tuberculosis, and coinfections to these deaths. Our second objective was to characterize the pathophysiological processes underlying these deaths by specifically defining the relationship between biomarkers of tuberculosis dissemination and death, the relationship between host inflammatory mediators and death, and the relationship between biomarkers of tuberculosis dissemination and host inflammatory mediators.

Methods

Study design and setting

Patients were enrolled into a prospective observational cohort study at Khayelitsha Hospital, Cape Town, from January 2013 until October 2016. This 240-bed hospital has 60 medical beds and an emergency room. The antenatal HIV seroprevalence in Khayelitsha was 34% in 2015 [28] and the tuberculosis notification rate was 917/100,000 in 2015, with 60% of cases being HIV-coinfected (Judy Caldwell, City of Cape Town Department of Health, 16 May 2016). ART and antituberculosis therapy (including treatment for drug-resistant tuberculosis) are accessible at government clinics free of charge. Patients are referred from surrounding primary health clinics when they require inpatient care. Primary health clinics have the capacity to initiate intravenous fluids and intravenous antibiotic treatment while awaiting patient transfer to hospital. This study was conducted using a prospective protocol that included an analysis plan (S1 Document) and is reported according to the STROBE statement (S1 STROBE checklist).

Participants

All patients in the emergency room and medical wards were screened on weekdays. Adults with HIV with a cluster of differentiation 4 (CD4) count <350 cells/μL and a high clinical suspicion of new tuberculosis (i.e., tuberculosis was considered the number one differential diagnosis on referral or after review by hospital or study staff) were eligible for enrolment. Pregnant patients, patients who received antituberculosis therapy within the past month, or patients who were recently initiated and received three or more doses of antituberculosis therapy were not eligible for enrolment. A list of all potentially eligible patients was compiled daily, and a random selection procedure was followed to enroll 2–4 patients daily. Clinical details, chest X-ray, sputum (spontaneous or induced if unable to produce sputum spontaneously), urine, and blood samples were obtained at enrolment. Participants remained in routine

clinical care, and study results were made available to clinical teams. Treatment decisions were made by clinical teams, not study staff. Participants were assessed daily in the ward and after discharge were managed in primary care according to local guidelines. They had a telephonic follow-up at week 4 and returned for a clinical assessment at week 12.

Laboratory assays

Tuberculosis tests were performed at the National Health Laboratory Services (NHLS). Sputum (if obtained) was sent for tuberculosis culture and Xpert MTB/RIF assay (Cepheid). Urine Xpert MTB/RIF assay was performed on sediment from centrifuged urine as previously described [29]. Mycobacterial blood culture was performed by culturing 5 mL of whole blood in Myco/F Lytic (Becton Dickinson Biosciences) bottles for 42 days. The GenoType MTBDR*plus* assay (Hain Lifesciences) was used to identify *M*. *tuberculosis* complex from positive sputum and blood cultures and provided rifampicin and isoniazid resistance testing. Rifampicin-resistant isolates had susceptibility testing to second-line drugs performed at a local referral laboratory. Urine LAM testing was performed retrospectively on frozen urine samples using the Alere Determine TB LAM antigen test. Strips were read by two independent readers both blind to clinical details. CD4 count, HIV viral load, full blood count, differential count, renal function, liver function, C-reactive protein, procalcitonin, venous lactate, and cytomegalovirus (CMV) viral load tests were performed on all participants by the NHLS. CMV viral load >49 IU/mL on the Argene CMV R-gene platform was regarded as detectable. Serum cryptococcal antigen (CRAG) test was performed by study staff.

Bacterial blood cultures were performed by the study team if the patient had not received intravenous antibiotics at the time of enrolment. Results from all bacterial cultures (blood and other specimens) that were performed by the hospital staff were captured. Hospital staff sent additional samples for tuberculosis testing when required, and this included extrapulmonary samples such as pleural fluid, cerebrospinal fluid, and lymph node aspirates.

Plasma was stored at −80˚C for immunology assays. Soluble inflammatory mediators were tested in a randomly selected subset of participants (n = 507) on stored plasma at a 1:2 dilution using the Bio-Plex Pro Human Cytokine Standard 27-Plex kit (Group I) on the Biorad Bioplex 200 Luminex platform. The following analytes were measured: interleukin (IL)-1β, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, eotaxin, basic fibroblast growth factor (FGF), granulocyte colony stimulating factor (G-CSF)/colony stimulating factor 3 (CSF3), granulocyte-macrophage colony stimulating factor (GM-CSF/CSF2), interferon gamma (IFN-γ), interferon gamma-induced protein (IP-10)/C-X-C motif chemokine ligand 10 (CXCL10), monocyte chemoattractant protein-1 (MCP-1)/C-C motif chemokine ligand 2 (CCL2), macrophage inflammatory protein-1 alpha (MIP-1α/CCL3), MIP-1 beta (MIP-1β/CCL4), platelet-derived growth factor-BB (PDGF), regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF). ELISA testing (R&D Systems Quantikine) of undiluted stored plasma was used to determine transforming growth factor beta 1 (TGF-β1) concentrations.

Data collection and definitions

Clinical data were obtained from the patient, hospital folder, and clinical review at enrolment and captured on standard case record forms. Results of all study investigations and additional tuberculosis tests (if performed in-service) were captured. The primary outcome was vital status at week 12. If participants could not be contacted at week 12, extensive searches of regional electronic clinical, pharmacy, and laboratory records were conducted to ascertain vital status.

Participants with an entry indicating a clinic visit, collection of medication, or a laboratory test performed beyond week 12 were assumed to be alive at week 12. Based on results of all tuberculosis tests, all participants were classified into mutually exclusive diagnostic groups. Urine LAM more than or equal to grade 1 by two independent readers was regarded as positive. "Microbiologically confirmed tuberculosis" was defined as participants with *M*. *tuberculosis* on at least 1 culture or Xpert MTB/RIF test from any clinical sample. "Probable tuberculosis" was defined as participants without microbiologically confirmed tuberculosis who had positive urine LAM or had a compatible clinical and radiological picture and were treated for tuberculosis and without alternative primary diagnosis made during enrolment admission (S1 Table). "Possible tuberculosis" was defined as participants treated for tuberculosis and another opportunistic infection simultaneously, with indistinguishable clinical and radiological picture with neither infection proven (S2 Table). "No tuberculosis" was defined as participants who had proven alternative diagnosis during admission and not treated for tuberculosis. Possible tuberculosis and no tuberculosis were excluded from analyses.

MTB BSI was defined as having ≥one positive mycobacterial blood culture identified as *M*. *tuberculosis* during enrolment admission. Rifampicin-resistant tuberculosis was defined as rifampicin resistance on any clinical sample using either of the genotypic tests performed at the NHLS (Xpert MTB/RIF or GenoType MTBDRplus).

We quantified the degree of mycobacterial dissemination with a three-point dissemination score, previously described [18]. Participants were allocated one point for each of the following: urine Xpert MTB/RIF assay positive for *M*. *tuberculosis*, urine LAM test positive, mycobacterial blood culture positive and identified as *M*. *tuberculosis*, yielding a score ranging from 0 to 3. Participants who had all three tests performed, valid results for the tests, and known outcome at week 12 were allocated a score ($n = 457$).

Presentation to hospital was defined as the time the patient was triaged in the emergency room. Early deaths were deaths that occurred within 7 days of enrolment, and late deaths are all deaths that occurred after 7 days and within 12 weeks of enrolment.

Statistical analysis

Sample size calculations were performed for the major analyses in the original protocol (S1 Document). The planned sample size was 660. Our overarching immunology hypothesis was that patients who died would have evidence of a compensatory anti-inflammatory immune response. We changed the original analysis plan in two ways. Firstly, we expanded the repertoire of inflammatory mediators tested (from 12 to 27). Secondly, we added analyses to characterize immune profiles associated with markers of tuberculosis dissemination and mortality with hierarchical cluster analysis, principal components analysis, Cox proportional hazards analysis, and correlation analysis. The median values with IQRs were used as measures of central tendency. Categorical variables are presented using counts with percentage and compared using the Fisher exact or Pearson chi-squared test. Continuous variables were compared between the study groups using the Mann-Whitney *U* test (two-group comparisons) or the Kruskal-Wallis test with the Dunn multiple comparisons ad hoc test or nonparametric linear trend analysis (between three or more groups). Cox proportional hazards models were censored at 28 days to meet the proportional hazards assumption because there was a higher risk of death during the first 28 days after enrolment. Variables were first evaluated separately in unadjusted models and then adjusted for a priori selected variables. Patients lost to follow-up were censored on their last day of contact with health services.

Fluorescence intensity values of soluble inflammatory mediators were used in all the multidimensional analyses, including principal components analysis and hierarchical clustering

analyses. Using fluorescence values allows for the analysis of analytes of low abundance and does not require censoring or correction for background, and calculated concentrations are dependent on the distribution of the fluorescence values [30,31].

Inflammatory mediator values were log transformed, z-score normalized, and corrected for multiple comparisons (Holm-Bonferroni method), when appropriate. Correlations were examined using the Spearman rank test. Non-supervised hierarchical cluster analyses were performed using the Ward method and 100× bootstrap. Dendograms represent Euclidean distance. Principal components analysis (with maximum rotation) was performed using log transformed, scaled values of inflammatory mediators.

At the request of a reviewer, we performed a sensitivity analysis, excluding deaths occurring within 7 days of enrolment to exclude deaths that could potentially be attributable to missed bacterial infections at presentation. For this, we repeated the principal components analysis and the Cox proportional hazards analysis, which included the principal components values as variables.

Data were analyzed using R Statistical software, GraphPad Prism 7.0, JMP 14 (SAS), and Gephi 0.9.1.

Ethical approval

The study was approved by the University of Cape Town Human Research Ethics Committee (UCT HREC), reference number 057/2013. Participants provided written informed consent when possible. Eligible patients with a decreased level of consciousness were enrolled and followed up daily until they regained capacity to participate in the informed consent process, and if not agreeable to participate, were withdrawn from the study. The UCT HREC approved the use of information from participants who died prior to providing informed consent or could not provide consent by the end of study follow-up. This was consistent with the approved protocol. By the end of study follow-up, 47 patients did not provide written informed consent. Of these, 24/47 (51%) died prior to providing written informed consent. Survivors who did not provide written informed consent were participants who did not regain capacity to consent by the time of discharge and either did not return for a study follow-up in person or did not regain capacity to consent by the end of study follow-up.

Results

Participant characteristics and mortality

A total of 682 hospitalized patients with HIV and with suspected tuberculosis were enrolled a median of 2 days (IQR = 1–3 days, range = 0–10 days) after presentation to hospital. In this analysis, we include tuberculosis cases (n = 576), 487/576 (84.5%) of whom were microbiologically confirmed and 89/576 (15.5%) with probable tuberculosis (Fig 1), and we excluded possible and no tuberculosis cases. Twelve-week mortality was 124/576 (21.5%). Death occurred after a median of 12.5 days (IQR = 4–35 days) after enrolment, and 46/124 (37.1%) of deaths occurred within 7 days of enrolment. Nine participants (1.6%) were lost to follow-up.

Causes of death

Causes of death were clinician attributed, and in many participants it was not possible to attribute the cause of death to a single disease process. Tuberculosis was implicated as the sole underlying cause of death in 55/124 (44.4%) of cases but was implicated as a major cause of death (in combination with other factors, mostly suspected sepsis) in an additional 56/124 (45.2%) of deaths (Table 1). There were 12/124 (9.7%) deaths that were not attributed to tuberculosis and

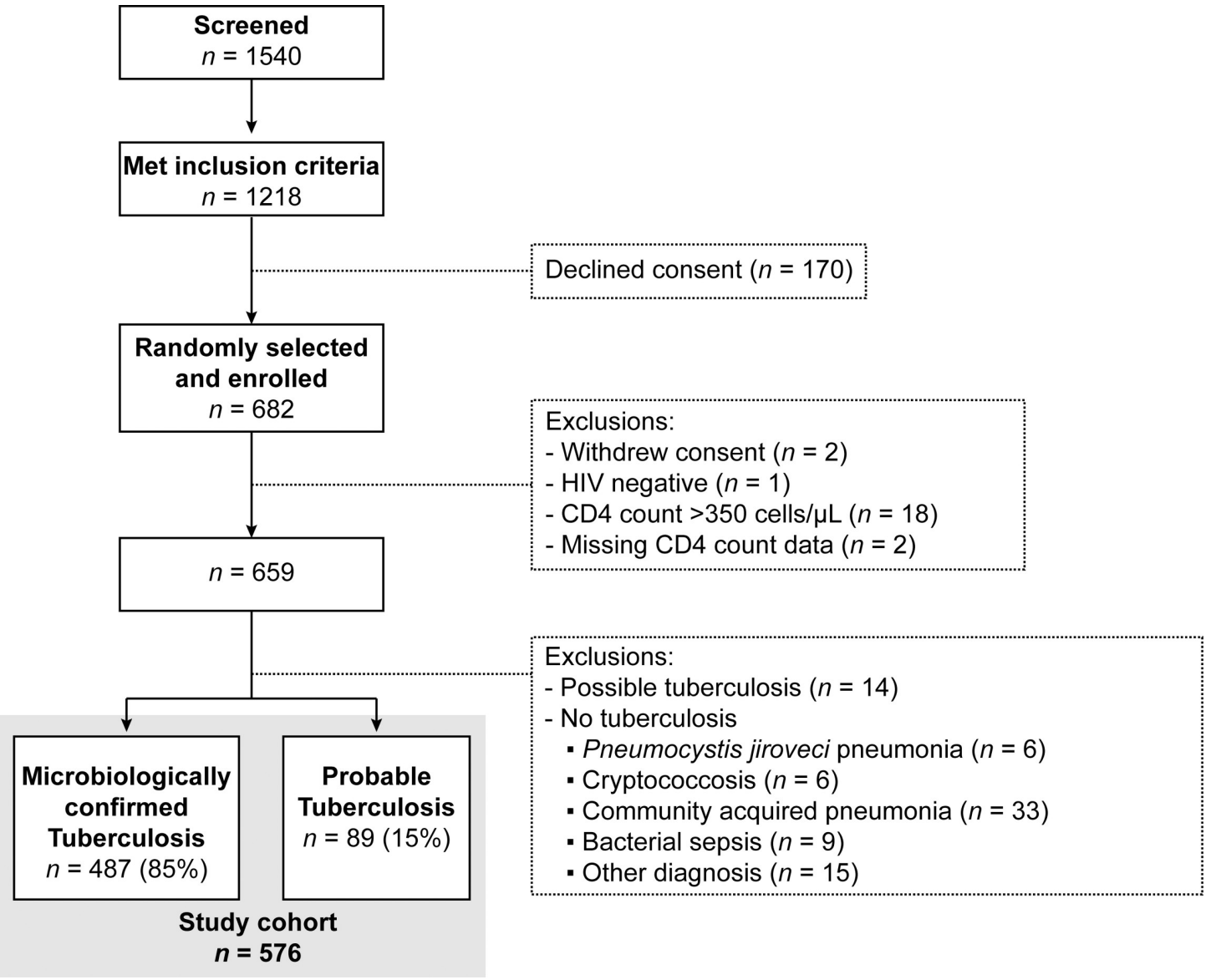

Fig 1. Study flowchart. Showing disposition of screened patients. Patients in the emergency room and medical wards were screened daily on weekdays, and potentially eligible patients were identified. Potentially eligible patients were randomly selected and approached for enrolment in the study. A total of 682 patients were enrolled, and 659 had HIV infection with a CD4 count <350 cells/μL. Tuberculosis was diagnosed in 576 participants (microbiologically confirmed and probable tuberculosis), and these participants are included in the analysis. CD4, cluster of differentiation 4.

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one with unknown cause of death. Rifampicin-resistant tuberculosis was present in 21/124 (16.9%) of deaths (Table 2) and tuberculosis was the sole or major cause of death in 19/21 (90.5%) of rifampicin-resistant participant deaths, or 19/124 (15.3%) of all deaths.

Co-morbidities, treatment access, and drug-resistant tuberculosis

In univariable comparisons, participants who died were older and had lower CD4, monocyte, and lymphocyte counts, but there was no difference in HIV viral load. Only 210/576 (36.5%) were taking ART and 70/576 (12.2%) were virologically suppressed, with no difference between outcome groups (15/124, 12.1% in those who died versus 53/443, 12.0%, $p = 1.000$). At the week 12 follow-up, 84% of the 443 survivors were on ART.

Table 1. Clinician-attributed causes of death in participants with HIV-associated tuberculosis.

Clinician-attributed cause of death	*n*
Direct consequence of tuberculosis, including • Central nervous system tuberculosis (*n* = 11) • TB-IRIS (*n* = 2)	55
Tuberculosis and/or bacterial sepsis	32
Tuberculosis and/or bacterial sepsis plus another condition, including • Venous thromboembolism (*n* = 2) • Drug toxicity (*n* = 4) • Renal failure (*n* = 4) • TB-IRIS (*n* = 2)	12
Tuberculosis plus condition other than bacterial sepsis, including • Drug toxicity (*n* = 2) • Cardiomyopathy (*n* = 1) • Venous thromboembolism (*n* = 1) • Cryptococcosis (*n* = 1) • *Pneumocystis jirovecii* pneumonia (*n* = 2) • Renal failure (*n* = 3)	12
Bacterial sepsis	3
Other causes, including • Venous thromboembolism (*n* = 4) • Cardiomyopathy (*n* = 1) • Chronic obstructive pulmonary disease (*n* = 1) • Drug toxicity (*n* = 1) • Carcinoma (*n* = 1) • HIV (*n* = 1)	9
Unknown	1

Abbreviation: TB-IRIS, tuberculosis immune reconstitution inflammatory syndrome.

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Antituberculosis therapy was started in 566/576 (98.3%) of participants, and 487/576 (84.5%) of participants started therapy within 48 hours of enrolment. Antituberculosis therapy was initiated after discharge from hospital in 19/576 (3.3%) participants. Ten participants 10/576 (1.7%) died before antituberculosis therapy was initiated, and these participants died early at a median of 2.5 days (IQR, 1–5 days) after enrolment.

There were 55 participants with rifampicin resistance: a significantly higher proportion who died had rifampicin resistance, 21/124 (16.9%), compared with 32/443 (7.2%) survivors, $p = 0.002$. For these participants, the initial antituberculosis therapy regimen was drug-resistant antituberculosis therapy in 19/55 (34.5%) (Table 2). We did not capture the switch to drug-resistant therapy after the initial treatment regimen but documented all medication received after enrolment. By the end of study follow-up, loss to follow-up, or death, therapy for drug-resistant tuberculosis was initiated in 74.6%–83.6% of participants as indicated by receipt of fluoroquinolone (46/55, 83.6%) or terizidone (41/55, 74.6%). Mortality in the rifampicin-resistant participants was 21/55 (38.2%).

To assess the contribution of bacterial infections to mortality, we reviewed results of all bacterial blood cultures taken during the enrolment admission. Bacterial blood cultures were performed in 296/576 (51.4%) participants, and 7/296 (2.4%) cultured a pathogen other than *M. tuberculosis* during the enrolment admission (1 *Cryptococcus neoformans*, 1 *Candida albicans*, 1 *Staphylococcus aureus*, 4 gram-negative bacteria); all seven had microbiologically confirmed tuberculosis. Four died: 1 with *S. aureus* and 3 with gram-negative bacteremia. Urine, pleural, and cerebrospinal fluid bacterial cultures performed in routine service yielded few positive results, with no difference between outcome groups (S5 Table). Sputum bacterial cultures

Table 2. Baseline characteristics of hospitalized participants with HIV-associated tuberculosis.

Characteristics		Died	Survived	*p*
		n = 124	*n* = 443	
Sex	Female	69 (55.6)	223 (50.3)	0.311
Age, years		39 [33–47]	35 [31–42]	**<0.001**
ART	Previously on ART	35 (28.9)	100 (22.6)	0.308
	Naive	42 (34.7)	179 (40.5)	
	Current ART	44 (36.4)	163 (36.9)	
Tuberculosis history	First episode	64 (53.8)	240 (56.3)	0.676
	Previous TB	55 (46.2)	186 (43.7)	
Ceftriaxone received during admission		108 (87.1)	391 (88.3)	0.755
[1]Antituberculosis therapy category at treatment initiation	Standard first-line regimen	97 (85.1)	421 (95.5)	**<0.001**
	Drug-resistant regimen	8 (7.0)	11 (2.5)	
Weight, kilograms		54 [44–61]	54 [48–63]	0.290
Glasgow Coma Scale <15		27 (21.8)	49 (11.1)	**<0.001**
CD4 count, cells/μL		40 [15–99]	63 [23–134]	**0.002**
HIV viral load, $\log_{10}$ copies/mL		5.0 [3.3–5.7]	5.1 [3.3–5.7]	0.655
Hemoglobin, g/dL		8.0 [6.7–10.0]	8.8 [7.4–10.5]	**0.005**
White cell count, $\times 10^9$/L		7.14 [4.18–10.87]	6.99 [4.51–10.29]	0.826
Absolute lymphocyte count, $\times 10^9$/L		0.41 [0.26–0.64]	0.69 [0.36–1.06]	**<0.001**
Absolute monocyte count, $\times 10^9$/L		0.23 [0.12–0.41]	0.36 [0.17–0.61]	**<0.001**
Absolute neutrophil count, $\times 10^9$/L		5.75 [3.29–8.96]	5.53 [3.20–8.59]	0.683
Platelet count, $\times 10^9$/L		232 [132–307]	273 [188–362]	**<0.001**
Random glucose, mmol/L		5.5 [4.9–6.6]	5.2 [4.7–6.0]	**0.009**
Venous lactate, mmol/L		2.3 [1.6–3.3]	1.7 [1.3–2.4]	**<0.001**
C-reactive protein, mg/L		179 [115–266]	148 [88–224]	**0.001**
Procalcitonin, μg/L		6.3 [1.3–31.2]	1.7 [0.3–6.6]	**<0.001**
[2]D-dimer, mg/L		2.4 [1.1–4.3]	1.2 [0.9–2.9]	**<0.001**
Alanine aminotransferase, U/L		24.5 [14.3–40.0]	27.0 [16.0–49.0]	0.229
Alkaline phosphatase, U/L		140.0 [96.0–218.5]	108.5 [77.0–170.3]	**<0.001**
Total bilirubin, μmol/L		9.0 [6.0–15.8]	7.0 [5.0–11.0]	**0.016**
Conjugated bilirubin, μmol/L		5.0 [3.0–9.0]	4.0 [3.0–7.0]	**0.018**
Total protein, g/L		73.0 [64.8–80.0]	76.0 [68.0–85.0]	**<0.001**
Albumin, g/L		22.0 [18.0–26.0]	25.0 [22.0–29.0]	**<0.001**
Creatinine, μmol/L		101.0 [63.8–183.5]	77.0 [59.0–106.5]	**<0.001**
CMV detectable		58 (49.6)	159 (36.4)	**0.011**
Serum CRAG lateral flow test	Positive	3 (2.4)	16 (3.6)	0.602
[3]*Mycobacterium tuberculosis* cultured from blood		64 (53.3)	153 (36.0)	**0.002**
[4]Urine lateral flow LAM test	Positive (≥1 by two readers)	46 (37.1)	150 (33.9)	0.287
[5]Urine Xpert MTB/RIF assay positive		58 (59.2)	164 (42.1)	**0.003**
Microbiologically confirmed tuberculosis		108 (87.1)	371 (83.7)	0.292
Rifampicin resistance on any clinical sample		21 (16.9)	32 (7.2)	**0.003**

Nine participants were lost to follow-up and not included in this table.

Categorical variables presented as count and percentage, *n* (%).

Continuous variables presented as median with IQR: median [IQR].

p-value comparing deaths to survivors using the Fisher exact test for categorical data or Wilcoxon rank sum test for continuous variables.

[1]Participants also started on other antituberculosis therapy regimens: *n* = 11 started on renally adjusted treatment regimen (ethambutol given on alternative days), *n* = 3 started a regimen adjusted due to liver dysfunction, and *n* = 4 started on rifabutin-containing regimens.

[2]D-dimer values missing for 100 participants.

[3]Percentages calculated for *n* = 554. No TB blood culture performed in 22 participants.

[4]No urine sample taken, or urine lateral flow LAM test not performed in 72 participants.

[5] Percentages calculated for *n* = 497. No urine sample taken, or urine Xpert MTB/RIF assay not performed in 79 participants.

Abbreviations: ART, antiretroviral treatment; CD4, cluster of differentiation 4; CMV, cytomegalovirus; CRAG, cryptococcal antigen; LAM, lipoarabinomannan; TB, tuberculosis.

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yielded 70/312 (22%) positive results, with 45/70 (64%) showing oral mixed flora, and the remaining positive results were not regarded as clinically significant in most cases (S5 Table).

Ceftriaxone was administered to 505/576 (87.7%) participants and 83/576 (14.4%) received the initial dose at a primary health clinic prior to transfer to hospital and thus before blood culture.

Serum lateral flow CRAG test was positive in 19 participants, and there was no difference in mortality in those who were CRAG test positive or negative (Table 2). CMV viral load was detectable in 223/576 (38.7%) participants, and a higher proportion was detectable in those who died (58/124, 49.6%) compared with survivors (159/443, 36.4%), $p = 0.011$. However, having a detectable CMV viral load was not independently associated with death in a Cox proportional hazards analysis after adjusting for potential confounders, including CD4 count (S3 Table).

Eight participants had clinical Kaposi sarcoma at the time of enrolment; of these, five had microbiologically confirmed tuberculosis, three had probable tuberculosis, and all eight survived.

Participants with a clinical diagnosis of *Pneumocystis jirovecii* pneumonia (PJP) were classified as no tuberculosis and excluded. Participants who were treated for PJP and tuberculosis simultaneously were classified as having possible tuberculosis and excluded from analyses (S2 Table).

Dissemination of tuberculosis

MTB BSI was diagnosed in 38.2% (220/576) of participants and in a significantly higher proportion of participants who died (51.6%, 64/124) versus survived (34.5%, 153/443) ($p = 0.002$) (Table 2 and Fig 2A).

A three-point dissemination score was used to explore tuberculosis dissemination further. A score was allocated to $n = 457$ ($n = 93$ deaths and $n = 364$ survivors) participants who had valid results for all three tests and known outcomes at 12 weeks. A high proportion of participants 295/457 (64.6%) had a tuberculosis dissemination score of ≥ 1. The distribution of the scores was as follows: a tuberculosis dissemination score of 0 in 162/457 (35.4%), a score of 1 in 106/457 (23.2%), a score of 2 in 102/457 (22.3%), and a score of 3 in 87/457 (19.0%). Of participants who died, 74/93 (79.6%) had a tuberculosis dissemination score of ≥ 1 versus 221/364 (60.7%) of

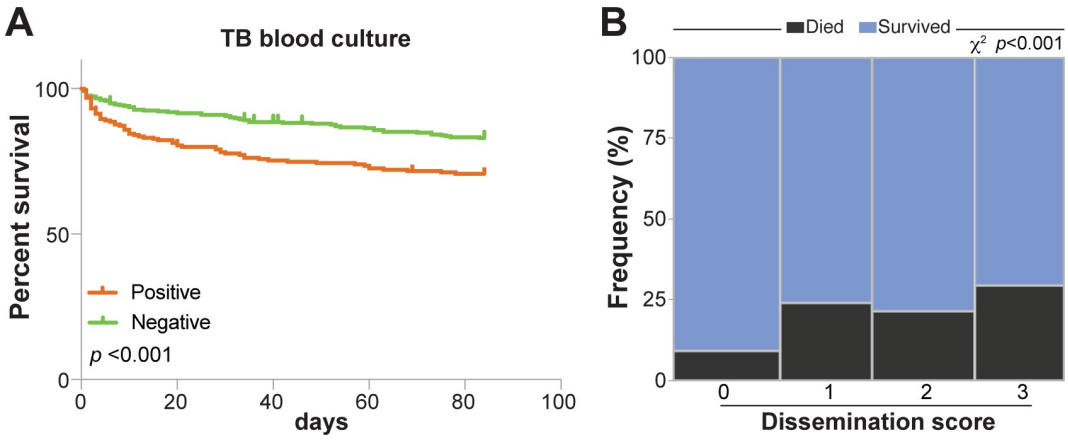

Fig 2. Association between tuberculosis dissemination and 12-week mortality. (A) Kaplan Meier curve showing percentage survival over 84 days for participants who tested positive for *Mycobacterium tuberculosis* blood culture versus those who tested negative. Curves were compared using log-rank (Mantel-Cox) test. **(B)** Frequencies of individuals who died, stratified by tuberculosis dissemination score values, were compared using the Pearson chi-squared test with linear trend. TB, tuberculosis.

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survivors, $p = 0.001$. We explored risk of mortality according to dissemination score; a higher dissemination score was associated with an increased risk of mortality (Figs 2B and S1, panel 3).

Features of sepsis syndrome

Many participants were acutely ill with features compatible with sepsis [32]. One third (195/576, 33.9%) had elevated lactate, which indicates strained cellular metabolism in sepsis [6,33]. A higher proportion of participants who died (63/124, 50.8%) compared with survivors (128/443, 28.9%, $p < 0.001$) had elevated lactate (Table 2). Similarly, in survival analysis, those with high lactate had significantly higher mortality (S1 Fig). Also compatible with sepsis syndrome was renal impairment (creatinine > 104 μmol/L), seen in 34.7% (177/576) of participants. Additional features that are used in the sequential organ failure assessment score (SOFA) to identify sepsis [32], such as elevated total bilirubin (>20 μmol/L), seen in 9.2% (53/576); decreased platelets ($<150 \times 10^9$/L), seen in 18.1% (104/576); and decreased level of consciousness (Glasgow Coma Scale <15), which was observed in 13.2% (76/576), are nonspecific in this context and may also be attributable to tuberculosis organ involvement. All these abnormalities were more common in participants who died (Table 2). Markers of inflammation used in clinical practice to monitor sepsis syndrome, such as C-reactive protein, procalcitonin, and D-dimer, were elevated in 557/576 (96.7%), 413/576 (71.7%), and 469/476 (98.5%) of participants, respectively, and were significantly higher in those who died (Table 2).

Host soluble mediators of inflammation and mortality

Many soluble mediators of inflammation were significantly elevated or reduced in univariable comparison (corrected for multiple comparisons) of participants who died to those who survived (Table 3 and S2 and S3 Figs). We compared fold differences in analyte values between participants who died and those who survived. In non-supervised two-way hierarchical cluster analysis, the mediators segregated most participants who died from survivors (Fig 3A). Biological pathways of mediators, which were significantly increased in participants who died (Figs 3B and S2), were related to the innate immune system and chemotactic signaling (IL-8, MIP-1β/CCL4, IP-10/CXCL10, and MIP-1α/CCL3), anti-inflammatory (IL-1Ra), and proinflammatory (IL-6). IL-1Ra was 8-fold higher in early deaths compared with survivors (median fluorescence intensity was 1,417 in early deaths versus 169.5 in survivors) (S4 Table). Functions of mediators that were significantly lower in participants who died (Figs 3B and S3) can be broadly classified as T-cell associated (IL-4, IL-17, RANTES/CCL5, IL-7, IL-12p70, IL-5, IFN-γ, IL-13) and growth factors (FGF, PDGF, TGF-β1).

Variance of host soluble inflammatory mediators

To explore covariance in host inflammatory mediators, we used principal components analysis with maximal rotation. Two major rotated principal components, principal component 1 and principal component 2 (PC1 and PC2) were identified, which accounted for 25% of total variability each (Fig 4A). Principal component 3 (PC3) accounted for 19% of variability. Functions of inflammatory mediators responsible for positive weighting in PC1 can be classified as mediators of the innate immune system and chemotaxis (IL-8, MIP-1β/CCL4, and MCP-1/CCL2), proinflammatory (IL-6), and anti-inflammatory (IL-1Ra). Inflammatory mediators responsible for positive weighting of PC2 were mainly T-cell associated (IL-13, IL-7, IL-12p70, TNF-α, IL-2, and IFN-γ). Mediators that contributed most to PC3 weighting were growth factors (PDGF, TGF-β1, FGF, VEGF) and T-cell associated (IL-17, IL-4) (Fig 4B).

To further explore the association of these immune profiles with mortality, the principal components (PC1, PC2, and PC3) were incorporated as variables in a Cox proportional

Table 3. Host soluble mediators of inflammation fluorescence intensity values in hospitalized participants with HIV-associated tuberculosis: Comparison between participants who died within 12 weeks and survivors.

Host soluble mediators of inflammation	Deaths	Survivors	*p*	Holms-Bonferroni *p*
	n = 108	*n* = 391		
Higher in participants who died				
IL-8	211.5 (110.4–410.8)	110.0 (78.5–165.5)	<0.001	<0.001
MIP-1β/CCL4	1,076.0 (570.5–2,501.0)	624.5 (397.5–1,087.5)	<0.001	<0.001
IL-1Ra	449.8 (145.1–1,425.3)	169.5 (93.0–397.5)	<0.001	<0.001
IL-6	361.3 (194.4–656.8)	208.0 (119.3–359.8)	<0.001	<0.001
IP-10/CXCL10	10,818.0 (6,326.9–16,913.8)	6,495.0 (3,301.5–11,846.3)	<0.001	<0.001
MIP-1α/CCL3	129.0 (73.0–295.0)	93.0 (65.8–156.3)	0.001	**0.027**
Lower in participants who died				
IL-5	22.00 (15.0–30.2)	31.0 (22.0–43.5)	<0.001	<0.001
RANTES/CCL5	12,688.0 (7,340.8–15,191.9)	15,369.5 (12,732.5–16,552.3)	<0.001	<0.001
IL-13	27.0 (18.0–39.8)	39.0 (29.0–59.5)	<0.001	<0.001
PDGF	93.5 (56.4–199.1)	201.0 (84.0–418.5)	<0.001	<0.001
FGF	45.3 (37.0–54.0)	54.0 (43.8–69.0)	<0.001	<0.001
IL-7	28.5 (22.0–37.0)	35.0 (28.0–45.3)	<0.001	<0.001
IL-12p70	44.5 (35.4–58.1)	56.0 (42.0–76.8)	<0.001	<0.001
IL-4	38.8 (26.8–55.1)	48.0 (36.8–63.3)	<0.001	<0.001
*TGF-β1	16.5 (12.0–36.2)	26.4 (15.7–55.4)	<0.001	**0.006**
IL-17	56.0 (41.8–78.3)	64.5 (48.8–90.3)	<0.001	**0.019**
IFNγ	45.0 (29.8–66.0)	54.0 (39.0–74.5)	0.001	**0.031**
No statistically significant difference between participants who died and those who survived				
TNFα	38.5 (30.0–52.5)	43.5 (36.0–54.3)	0.007	0.210
IL-2	62.3 (49.8–77.4)	68.0 (55.3–81.0)	0.019	0.522
MCP-1/CCL2	108.0 (76.5–159.5)	95.5 (75.0–138.0)	0.036	0.999
GM-CSF/CSF2	84.00 (64.5–109.3)	89.5 (72.0–113.0)	0.062	1.000
Eotaxin	61.3 (43.8–86.1)	66.0 (53.0–88.3)	0.065	1.000
IL-9	175.3 (113.9–243.0)	153.0 (121.0–205.0)	0.113	1.000
VEGF	107.0 (72.0–143.0)	107.0 (78.8–158.8)	0.237	1.000
G-CSF/CSF3	75.5 (47.8–117.1)	67.0 (54.0–90.5)	0.314	1.000
IL-15	90.0 (73.0–115.0)	89.5 (74.0–114.3)	0.923	1.000
IL-1β	64.0 (47.5–85.8)	64.0 (50.0–84.5)	0.950	1.000
IL-10	68.5 (51.5–91.5)	69.0 (55.0–85.0)	0.961	1.000

Host soluble mediators of inflammation were measured on stored plasma in a random selection of participants with HIV-associated tuberculosis (*n* = 507: *n* = 108 deaths, *n* = 391 survivors, *n* = 8 lost to follow-up) using the Biorad Bioplex 200 Luminex platform and fluorescence intensity values are presented.

*TGF-β1 concentrations were measured with ELISA and are presented in picograms per milliliter. Inflammatory mediators are arranged into three groups: mediators that were higher in participants who died, mediators that were lower in participants who died, and mediators that showed no difference between survival groups. Each group is ranked from lowest to highest *p*-values. Comparisons of participants who died with those who survived were made using the Wilcoxon rank sum test. *p*-values were corrected for multiple comparisons with Holms-Bonferroni correction. Bold *p*-values indicate mediators that remained significantly different after correction for multiple comparisons.

Abbreviations: CCL, C-C motif chemokine ligand; CSF2, colony stimulating factor 2; CSF3, colony stimulating factor 3; CXCL, C-X-C motif chemokine ligand; FGF, basic fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFNγ, interferon gamma; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T-cell expressed and secreted; TGF-β1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

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hazards model (Fig 5). PC1 was independently associated with mortality, aHR = 2.2 (95%

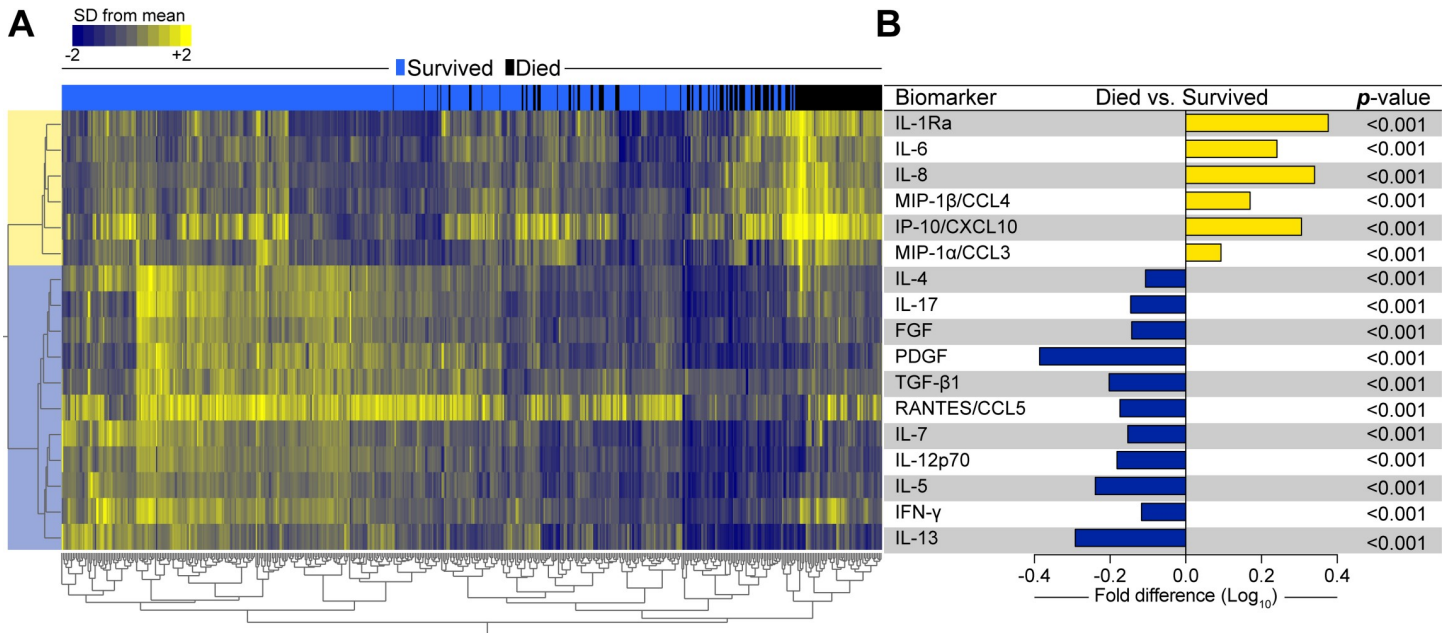

Fig 3. Host soluble inflammatory mediators associated with mortality in participants with hospitalized HIV-associated tuberculosis. (A) Values of inflammatory mediators were log transformed and z-score normalized. A non-supervised two-way hierarchical cluster analysis (Ward method with 100× bootstrap) was employed to test if simultaneous assessment of indicated mediators could group separately individuals that died from those who survived. Only mediators that were statistically different between the study groups after adjustment for multiple measurements (Holm-Bonferroni method) are shown. Data on other mediators are shown in S4 Table and S2 and S3 Figs. **(B)** Bars represent fold-difference values between participants that died versus those who survived, with Holm-Bonferroni *p*-values. Yellow bars indicate mediators that were significantly higher, whereas blue bars highlight mediators that were lower in participants who died compared with those who survived. FGF, basic fibroblast growth factor; IFNγ, interferon gamma; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein; CXCL10, C-X-C motif chemokine ligand 10; MIP-1α, macrophage inflammatory protein-1 alpha; CCL3, C-C motif chemokine ligand 3; MIP-1β, macrophage inflammatory protein-1 beta; CCL4, C-C motif chemokine ligand 4; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T-cell expressed and secreted; CCL5, C-C motif chemokine ligand 5; TGF-β1, transforming growth factor beta 1.

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CI = 1.9–2.7, $p < 0.001$), and PC2 was protective (aHR = 0.7 (95% CI = 0.5–1.0, $p = 0.018$). PC3 was not associated with mortality.

A sensitivity analysis excluding patients who died within 7 days yielded similar results. Amongst $n = 472$ patients, three principal components explained the majority of the variation. PC1 explained 26% of variance and was dominated by IL-13, IL-7, IL-12p70, TNF-α, IL-2, and IFN-γ. PC1 was protective, with HR for death = 0.63 (95%CI = 0.41–0.98, $p = 0.038$) in univariate analysis and aHR = 0.69 (95%CI = 0.45–1.05, $p = 0.080$). PC2 explained 22% of variance and was dominated by IL-8, IL-6, IL-1Ra, MIP-1β/CCL4, MCP-1/CCL2, and IP10/CXCL10. PC2 was associated with mortality with HR = 1.6 (95%CI = 1.2–2.2), $p = 0.002$) in univariate analysis and aHR = 1.57 (95%CI = 1.17–2.11, $p = 0.003$). PC3 explained 20% of variance and was dominated by PDGF, IL-17, TGFB-1, RANTES/CCL5, FGF, and VEGF. PC3 was not associated with mortality, with HR = 0.93 (95%CI = 0.65–1.3, $p = 0.680$) in univariate analysis and aHR = 1.03 (95%CI = 0.69–1.53, $p = 0.880$).

Association between host inflammatory mediators and leucocyte counts

Many of the inflammatory mediators are secreted by leucocytes. Participants who died had significantly lower lymphocyte and monocyte counts, but neutrophil counts were similar to those who survived (Figs 6A and S4). We explored the association of cell counts with each other and with soluble inflammatory mediators. Cell counts had significant positive correlations with each other (Fig 6B). We plotted the Spearman correlation coefficient of each inflammatory

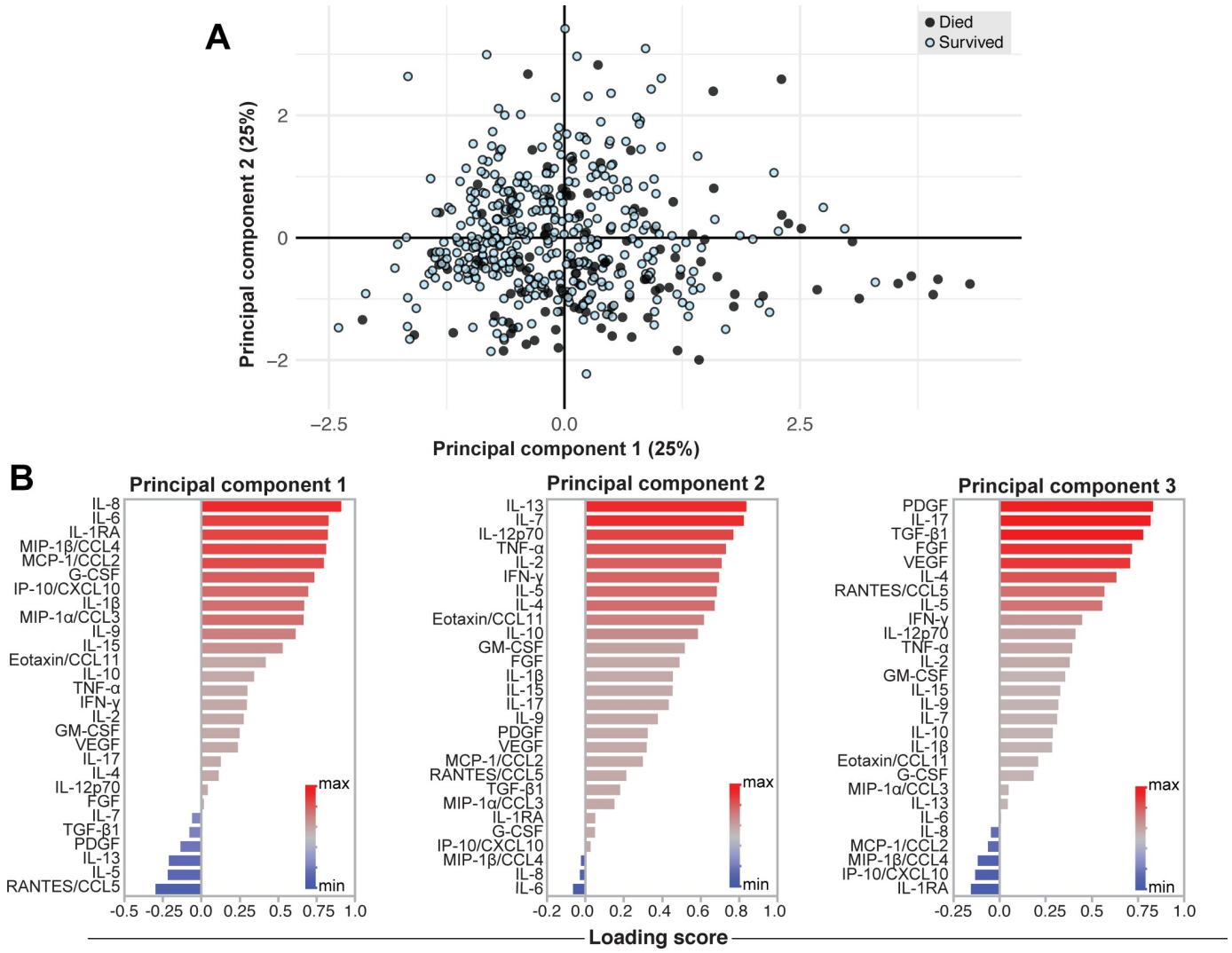

Fig 4. Principal components analysis of host soluble inflammatory mediators. (A) Principal components analysis with rotation was used to analyze the inflammatory mediators and explain the variance of the data distribution in the cohort. Participants are represented by dots and colored by outcome. The two axes represent principal components 1 (PC1 on the x-axis) and 2 (PC2 on the y-axis), and their contribution to the total data variance is shown as a percentage. PC3 contributed 19% of total variance and is not shown. **(B)** Variables contributing to PC1, PC2, and PC3 are shown with red bars indicating positive weighting and blue bars indicating negative weighting. CCL, C-C motif chemokine ligand; CSF2, colony stimulating factor 2; CSF3, colony stimulating factor 3; CXCL, C-X-C motif chemokine ligand; FGF, basic fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFNγ, interferon gamma; IL, interleukin; IL-1RA, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PC, principal component; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T-cell expressed and secreted; TGF-β1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

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mediator value with the neutrophil, monocyte, and lymphocyte counts (Fig 6C). Monocyte and lymphocyte counts showed significant negative correlations with IL-1Ra, IL-6, and mediators of chemotactic signaling IL-8, MIP-1β/CCL4, IP-10/CXCL10, and MIP-1α/CCL3. We explored the association of PC1 score with cell counts (Fig 6D). Lymphocytes and monocytes had significant negative correlation with PC1 score. PC2 was dominated by T-cell associated mediators, and we explored the association of PC2 with lymphocyte count and CD4 cell count. PC2 and lymphocytes had a weak positive correlation ($r = 0.1$, $p = 0.021$) and PC2 and CD4 cell count were not correlated ($r = 0.06$, $p = 0.196$) (S5 Fig).

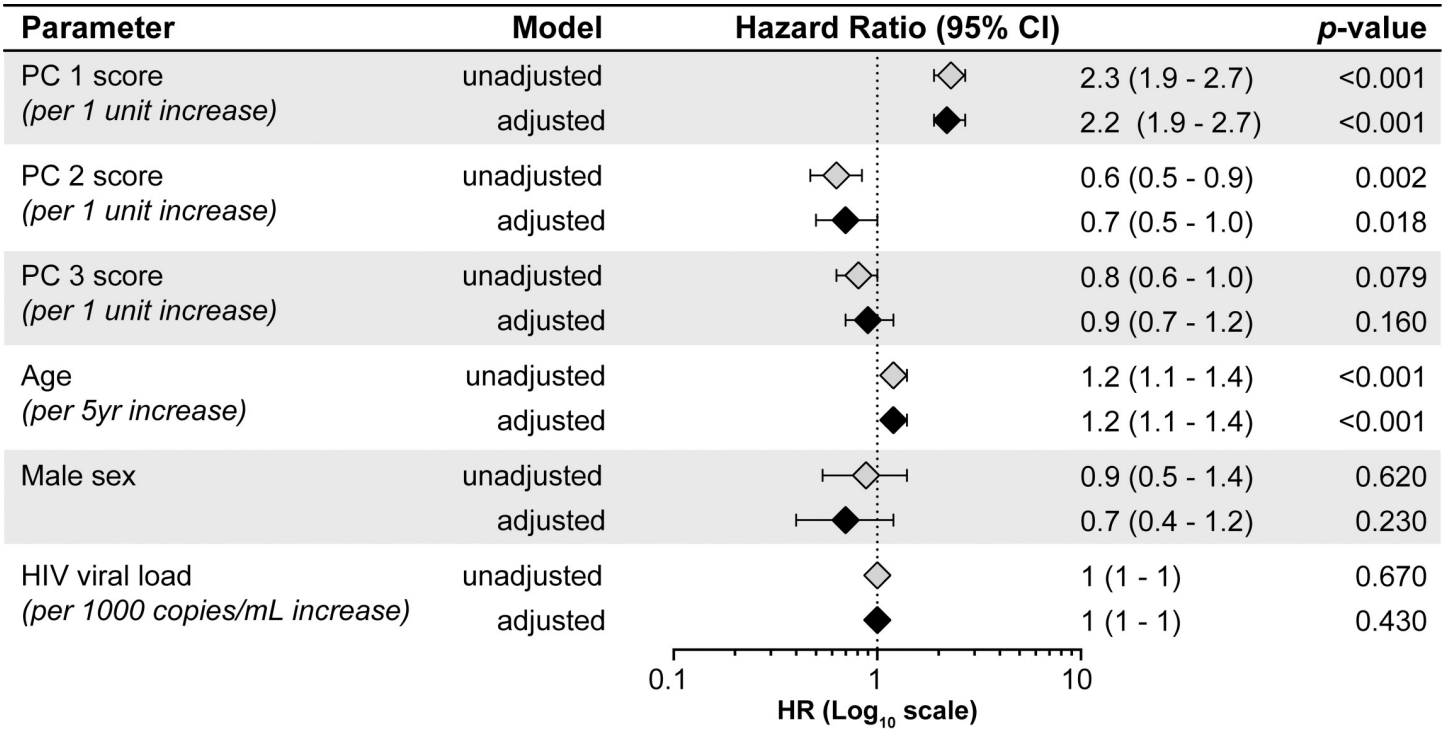

Fig 5. Cox-regression analysis to evaluate association between principal components score and 28-day mortality. Cox regression analysis was conducted with each variable individually (unadjusted) and then all variables were included in a multivariable model (adjusted). Age, sex, and HIV viral load were incorporated a priori to adjust for patient specific variance and HIV-related factors. The model was censored at 28 days to meet the proportional hazards assumption, and the global proportional hazards test for the multivariable model result was $p = 0.43$. PC, principal component.

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Dissemination of tuberculosis and soluble mediators of inflammation

Soluble inflammatory mediator values were calculated per tuberculosis dissemination score ($n = 457$ participants who had a tuberculosis dissemination score calculated) and illustrated in a heatmap using hierarchical clustering to determine which mediators grouped together (Fig 7A). Higher values of proinflammatory mediators associated with the innate immune response (IL-1β and IL-6), mediators of chemotactic signaling (IL-8, MIP-1β/CCL4, IP-10/CXCL10, MIP-1α/CCL3), and anti-inflammatory mediators (IL-1Ra, IL-10) grouped together and were higher in participants with a dissemination score of three. Higher PC1 score and neutrophil count, and lower lymphocyte and monocyte counts were associated with a high dissemination score.

Higher values of T-cell associated mediators (IL-4, -5, -7, -13, -17, -12p70; RANTES/CCL5, TNF-α) and growth factors (PDGF, FGF, and TGF-β1) grouped together in participants with a tuberculosis dissemination score of zero.

Next, we evaluated PC1 score values stratified by those who tested positive and negative for each of the three biomarkers that were used to calculate the tuberculosis dissemination score, and PC1 score was significantly higher in those who tested positive for each test (Fig 7B). Irrespective of outcome, PC1 score increased significantly with increased dissemination score (Fig 7C).

Association of inflammatory mediators with time to death

The distribution of time to death is shown in Fig 8A and 8B. Host soluble inflammatory mediators were explored in relation to time to death. Participants were ordered based on time to

death, and inflammatory mediators were ranked and colored from minimum to maximum values and illustrated in a heatmap. Higher values of certain inflammatory mediators grouped together (Fig 8C) in early deaths, and these mediators had significant negative correlations with time to death. These mediators were the anti-inflammatory IL-1Ra, mediators associated with the innate immune system, and chemotactic signaling (IL-8, MIP-1α/CCL3, MIP-1β/CCL4, IP-10/CXCL10) and one T-cell associated mediator (IL-9). We also explored the relationship between IL-1Ra and PC1 score with time to death, and both had significant nonlinear negative correlations with time to death (Fig 8D).

Inflammatory profile of early deaths and late deaths

We further explored the inflammatory profile of participants who died early. There was substantial overlap between inflammatory mediators that were significantly different in early and late deaths versus survivors. However, the magnitude of differences between early deaths and survivors was much larger (Fig 9A). A Venn diagram including the inflammatory mediators that had statistically significant higher or lower values between each death group compared with survivors revealed two modules of uniquely different mediators, one in early deaths and one in late deaths (Fig 9B). The ability of these modules to predict early or late death was explored with a receiver operating characteristic (ROC) curve. Of note, modules significantly associated with early deaths had an area under the curve of 0.86 ($p < 0.001$) to distinguish early deaths from survivors, whereas the discrimination accuracy for modules significantly associated with late deaths was weak to distinguish late deaths from survivors (Fig 9C). We also conducted exploratory networks analysis of the soluble inflammatory mediators and detected three main nodes that had high numbers of strong correlations in early and late deaths. Participants who died early showed higher numbers of strong correlations between mediators. TNF-α and IL-4 had the highest numbers of strong correlations in each outcome group (S6 Fig).

Discussion

We enrolled 576 hospitalized patients with HIV and newly diagnosed tuberculosis at presentation to hospital, collected samples at baseline, and followed patients for 12 weeks to ascertain vital status. We performed comprehensive tuberculosis investigations, measured host soluble inflammatory mediators, and compared patients who died with those who survived. We found high mortality (21.5%) despite timely initiation of antituberculous therapy. Clinician-attributed causes of death identified tuberculosis as the major contributor or one of the major contributors to death in 89.5% of cases. We observed disseminated tuberculosis in 64.6%, which was associated with mortality. One third of participants (33.9%) presented with features of sepsis syndrome, as indicated by elevated lactate, and amongst the patients in whom the clinician-attributed cause of death included tuberculosis as a major cause, 15.3% had rifampicin-resistant tuberculosis. We describe an immune profile identified by non-supervised hierarchical cluster analysis and principal components analysis that was associated with mortality in Cox proportional hazards analysis and was also associated with a higher tuberculosis dissemination score. The immune profile was dominated by soluble inflammatory mediators associated with the innate immune system and chemotactic signaling.

Tuberculosis is the leading cause of hospital admissions and in-hospital deaths in individuals with HIV in sub-Saharan Africa [2]. In our study, 576 hospitalized adults with HIV and a new diagnosis of tuberculosis were enrolled, and, like other studies [4–7], we observed a high case fatality rate of 21.5%. While 37.1% of deaths occurred within 7 days of enrolment, deaths continued to occur throughout the 12-week follow-up period.

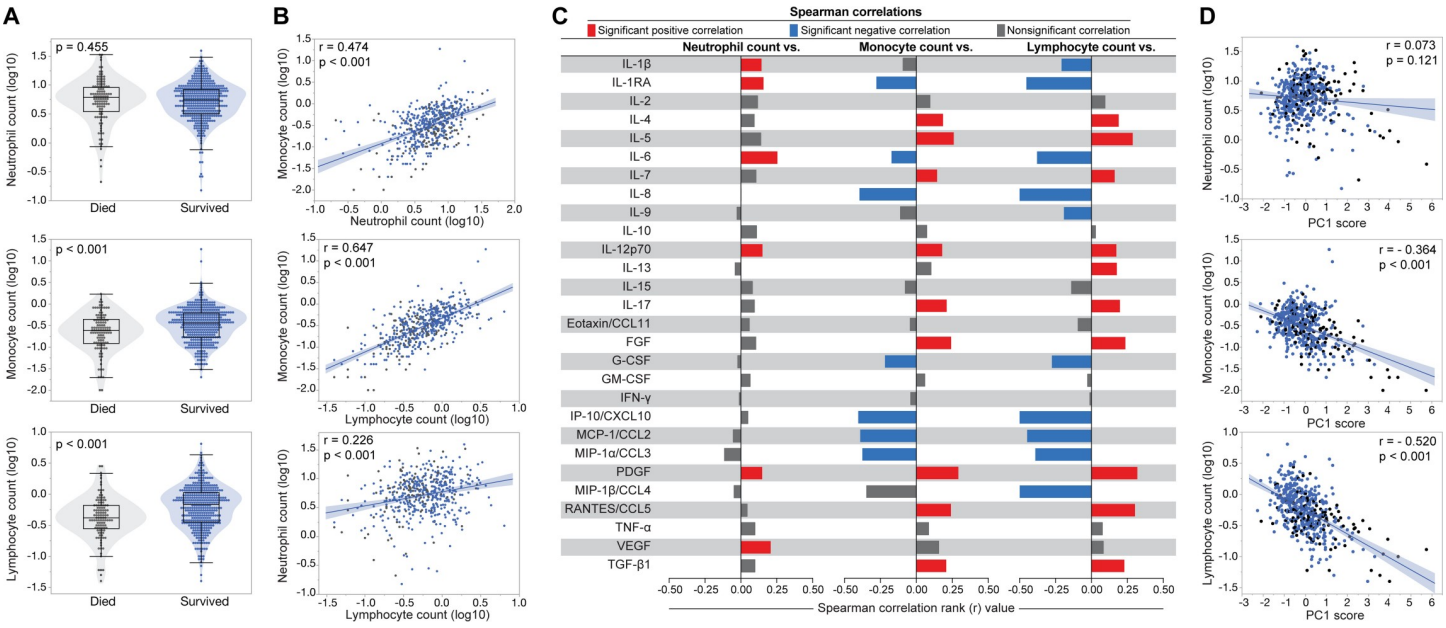

Fig 6. Associations between leukocyte counts in peripheral blood and systemic inflammation. (A) Absolute counts of indicated leukocytes were compared between the participants who died versus those who survived. Data are represented with violin plots, with scatter dots and box and whiskers indicating median values, IQRs, and maximum and minimum values excluding outliers, respectively. Groups were compared using the Mann-Whitney *U* test. **(B)** Spearman correlations between indicated cell counts are shown. Linear curve fit (with 95% CI) was used to illustrate trends of data distribution. **(C)** Spearman correlation analysis between cell counts and inflammatory mediator values in plasma. **(D)** Spearman correlations between indicated cell counts and PC1 score values are shown. Linear curve fit (with 95% CI) was used to illustrate trends of data distribution. CCL, C-C motif chemokine ligand; CSF, colony stimulating factor; CXCL, C-X-C motif chemokine ligand; FGF, basic fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFNγ, interferon gamma; IL, interleukin; IL-1RA, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PC1, principal component 1; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T-cell expressed and secreted; TGF-β1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

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We systematically assessed potential determinants of mortality, including the presence of rifampicin-resistant tuberculosis, time to initiation of antituberculosis therapy, concomitant infections, and disseminated tuberculosis. Tuberculosis was considered to be the major (or one of the major) contributors to death in the majority of cases, and 15.3% with tuberculosis-related deaths had rifampicin-resistant tuberculosis. A higher proportion of participants who died had CMV viremia, but this was not significant after adjustment for potential confounders. Ceftriaxone is the antibiotic recommended locally for patients with HIV with community-acquired pneumonia or suspected gram-negative infections. The majority of the cohort received ceftriaxone during the index admission. This was usually initiated in the emergency room, and 14% received ceftriaxone prior to arrival at hospital. There was, therefore, minimal delay in antibiotic initiation in patients with suspected sepsis. Infections with other opportunistic pathogens such as cryptococcosis and bacterial coinfections were not identified frequently.

Considering the low prevalence of bacterial coinfections identified and high numbers of very early deaths, it is possible that undiagnosed bacterial coinfections could have contributed to early deaths as well as the immune profiles that were associated with mortality. To address this, we performed a sensitivity analysis excluding patients who died within 7 days; in this sensitivity analysis, the association between the immune profiles and death were very similar to the main findings, suggesting that undiagnosed bacterial infections were not likely to be a major contributor to the immune profiles. This will need to be confirmed in future studies.

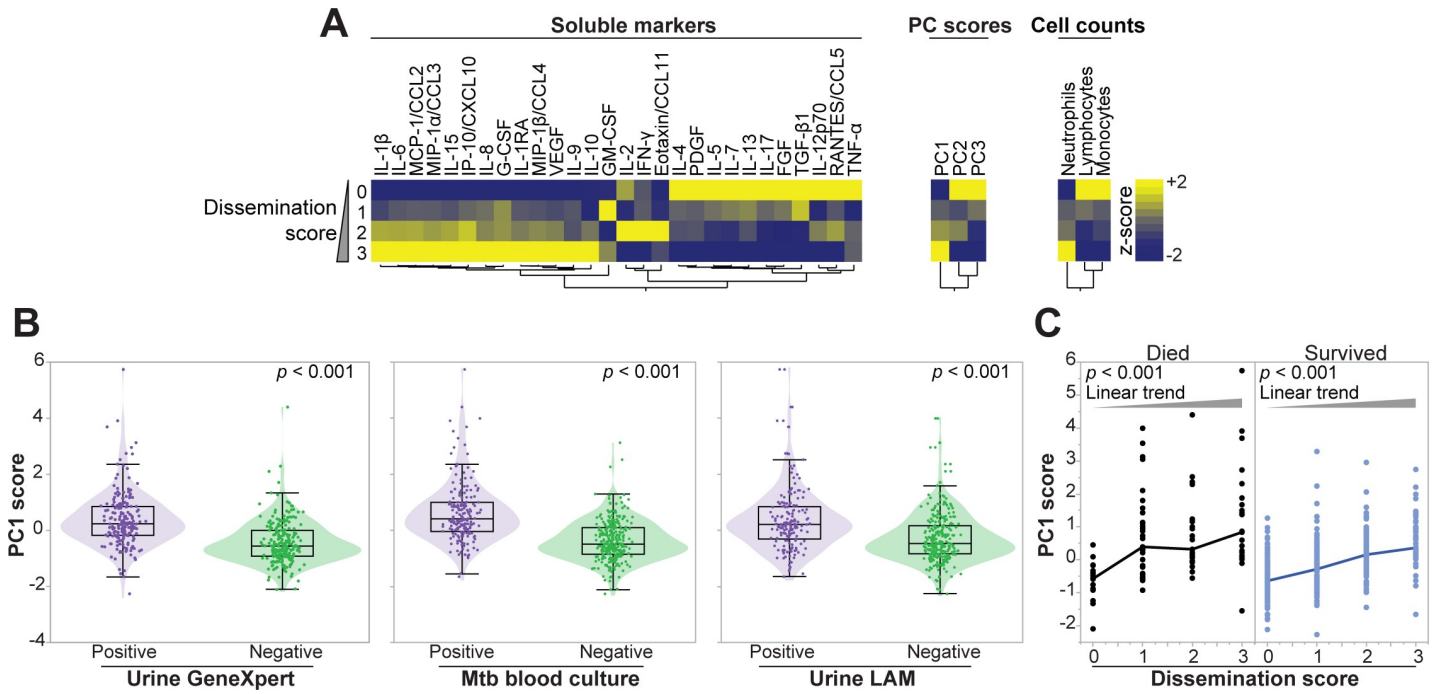

Fig 7. Associations between tuberculosis dissemination score, components thereof, and host soluble mediators of inflammation. (A) Mean values of log-transformed value of each plasma mediator per TB dissemination score values were calculated for all participants who had all three tests performed (urine LAM test, urine Xpert MTB/RIF test, mycobacterial blood culture), $n = 457$. Inflammatory mediator values were z-score normalized and illustrated in a heatmap in which inflammatory mediators were grouped using hierarchical clustering (Ward method with 100× bootstrap). Dendrograms represent Euclidean distance. **(B)** PC1 score values were compared between those who tested positive and negative for each of the three tests used to calculate the tuberculosis dissemination score using the Mann-Whitney *U* test. **(C)** PC1 score values were compared between participants presenting with increasing tuberculosis dissemination scores from 0 to 3 in both outcome groups, and values were compared using the Kruskal-Wallis test with the nonparametric linear trend ad hoc test. Lines connect median values. CCL, C-C motif chemokine ligand; CSF, colony stimulating factor; CXCL, C-X-C motif chemokine ligand; FGF, basic fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFNγ, interferon gamma; IL, interleukin; IP-10, interferon gamma-induced protein; LAM, lipoarabinomannan; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MTB, *Mycobacterium tuberculosis*; PC, principal component; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T-cell expressed and secreted; TB, tuberculosis; TGF-β1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

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Similar to autopsy studies [14] and previous cohort studies [19, 20], we observed a high frequency (62.6%) of participants with disseminated tuberculosis, which was associated with mortality. Many participants presented with sepsis syndrome: 33.9% had elevated lactate and 34.7% had renal impairment, and these abnormalities were associated with death. In Africa, MTB BSI is found frequently amongst patients with HIV admitted with febrile illness (7.6%) [17], HIV-associated tuberculosis (31%) [18], and hypotensive sepsis syndrome (23%) [16,19]. Closely associated with MTB BSI is the detection of a mycobacterial cell wall component LAM in urine. Urine LAM positivity is associated with the presence of renal tuberculosis on autopsy [34], the presence of mycobacteria in the urine [35], and with positive tuberculosis blood culture [18,36,37]. A positive urine LAM test can therefore be regarded as a marker of disseminated tuberculosis even though the mechanism has not been clearly elucidated [38]. Xpert MTB/RIF detects *M. tuberculosis* DNA and thus indicates disseminated disease when positive in urine. A combination of urine Xpert and LAM tests can rapidly detect patients with MTB BSI [18], but high mortality persists despite implementation of these diagnostics [25,26]. In our study, the degree of dissemination of tuberculosis was quantified with a simple dissemination score (previously described [18]) that relies only on non-sputum samples, and a higher dissemination score was associated with mortality.

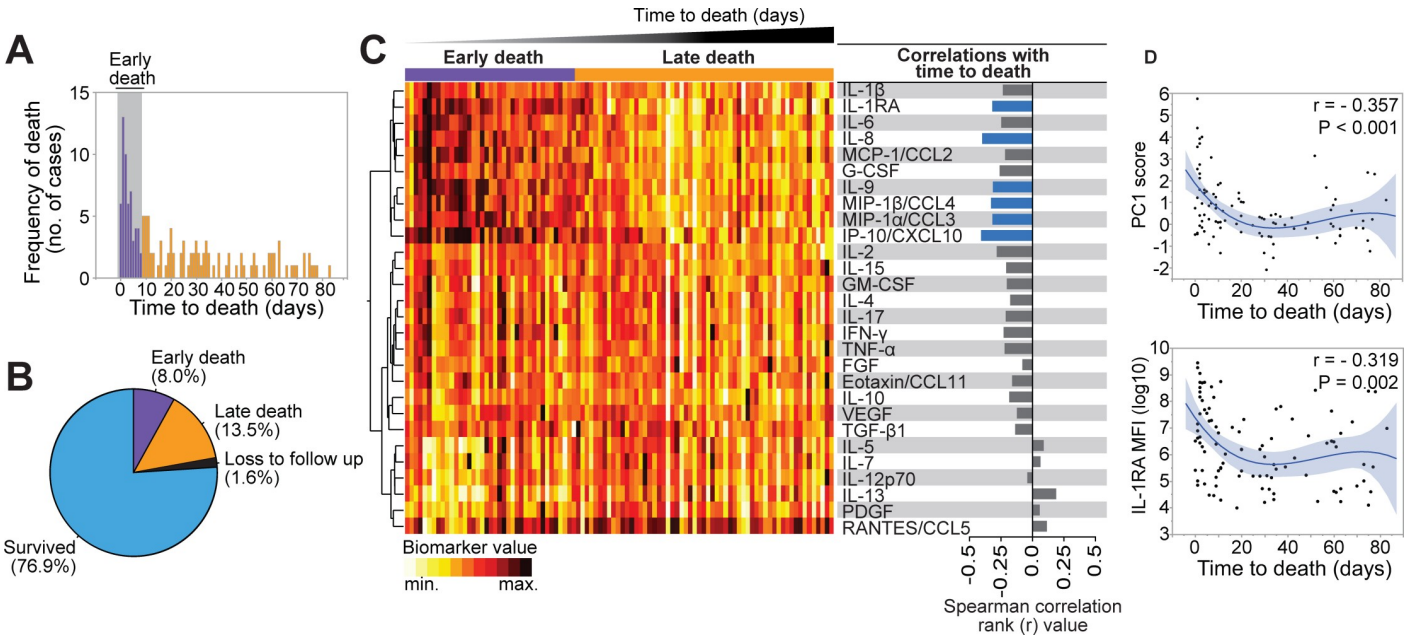

Fig 8. Inverse correlations between host soluble mediators of inflammation and time to death. (A) Histogram shows the frequency of participants who died over time. **(B)** Pie chart shows the frequency of participants who survived, those who died within 7 days of admission (early death), and those who died after 7 days (late death). **(C)** Left panel: data were log transformed and ranked and colored in a heatmap from minimum to maximum values detected for each inflammatory mediator. Participants were ordered based on time to death (in days), and plasma inflammatory mediators were clustered (Ward method with 100× bootstrap) according to the distribution profile in the study population. Dendrograms represent Euclidean distance. Right panel: Spearman correlations for each mediator and time to death. Blue bars indicate statistically significant correlations (which were all negative) after corrections for multiple measurements (Holm-Bonferroni method). **(D)** Spearman correlations between PC1 score values and IL1-Ra mean fluorescence intensity values and time to death are shown. Nonlinear curve fit (quadratic, with 95% CI) was used to illustrate trends of data distribution. CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; FGF, basic fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFNγ, interferon gamma; IL, interleukin; IL1-Ra, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein; max., maximum; MCP, monocyte chemoattractant protein; MFI, mean fluorescence intensity; min., minimum; MIP, macrophage inflammatory protein; no., number; PC1, principal component 1; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T-cell expressed and secreted; TGF-β1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

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Principal components analysis described two principal components that were each weighted by functionally distinct groups of soluble inflammatory mediators. Principal component 1 was dominated by mediators associated with the innate immune system and chemotactic signaling, and this was associated with 2-fold higher mortality in a Cox proportional hazards model. A higher dissemination score was also associated with elevation of the innate immune mediators comprising principal component 1. In contrast, a second principal component, which contained mostly T-cell associated mediators, was associated with lower mortality in a Cox proportional hazards model, and higher values of T-cell associated mediators and growth factors were associated with a tuberculosis dissemination score of zero in hierarchical cluster analysis.

Possible mechanistic interpretations of our findings are that the higher levels of innate and chemotactic mediators reflect increased activation and recruitment of innate cells into the tissue in response to multi-organ infection. This may be an appropriate, but ultimately ineffective, response, with significant immunologic and metabolic costs to the host. Recruitment of innate cells to tissue may result in tissue damage and organ dysfunction. Monocyte and lymphocyte depletion could render patients more susceptible to secondary bacterial infections [39,40]. Hyperlactatemia may represent metabolic switching to aerobic glycolysis in immune cells to meet increased energy demands of the inflammatory response, at the cost of increased acidosis [41]. Hepcidin production and iron sequestration may limit extracellular bacillary

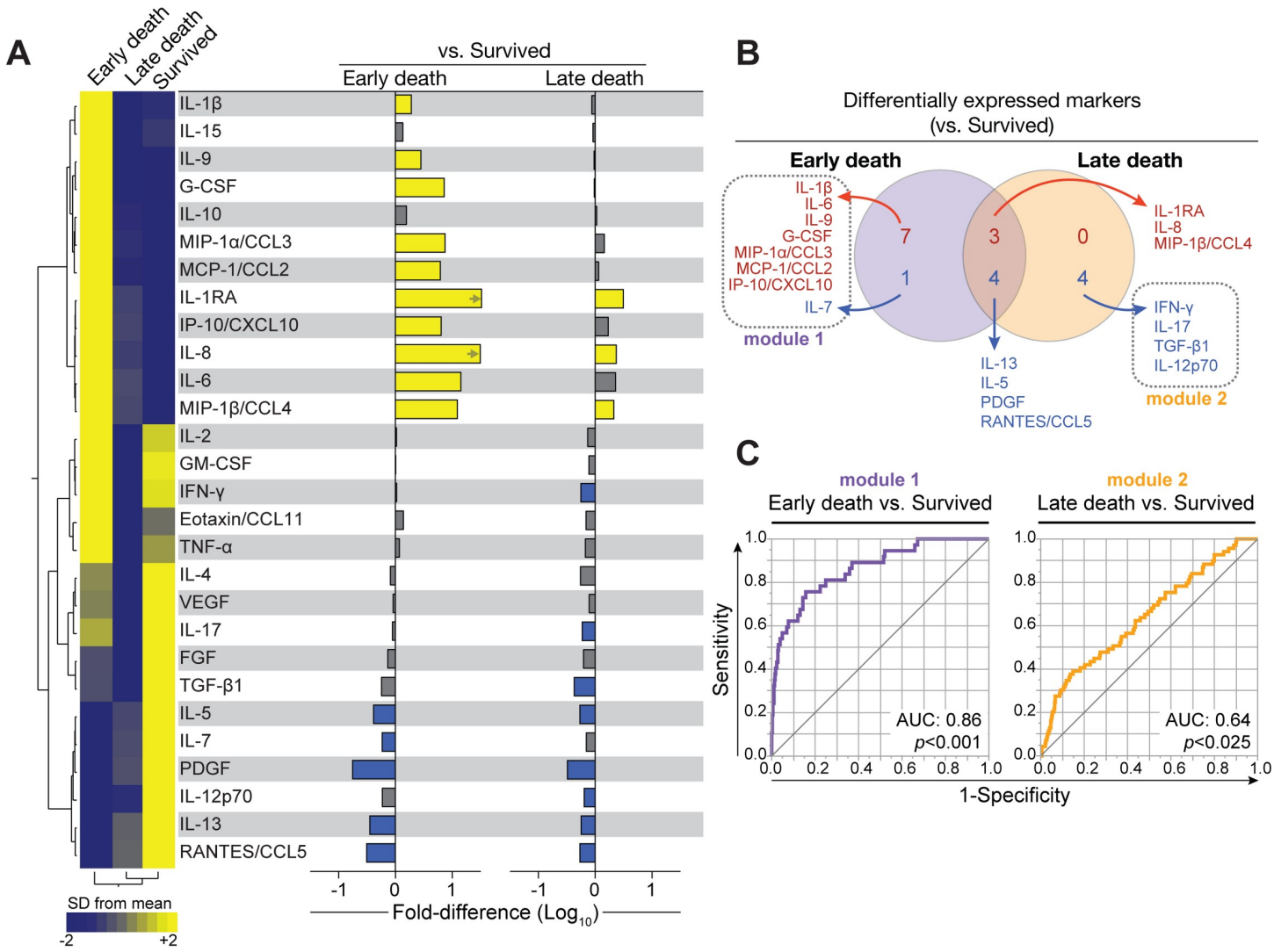

Fig 9. Participants who died early after admission exhibited a distinct inflammatory profile in plasma. (A) Left panel: mean values of log-transformed values of each soluble inflammatory mediator were calculated for early deaths (within 7 days of enrolment) and late deaths (after 7 days, within 12 weeks). Values were z-score normalized and illustrated in a heatmap in which inflammatory mediators were grouped using hierarchical clustering (Ward method with 100× bootstrap). Dendrograms represent Euclidean distance. Right panel: bars represent fold-difference values between participants who died early or late versus those who survived. Yellow bars indicate mediators that were significantly higher, whereas blue bars highlight mediators that were significantly lower in the groups of participants who died compared with those who survived, after adjustments for multiple measurements (Holm-Bonferroni method). Arrows indicate values higher than the upper limit of the axis. **(B)** Venn diagrams illustrate the inflammatory mediators that were significantly different between participants who died and survived. Mediators indicated in red were higher, whereas those in blue were lower in the groups of participants who died versus participants who survived. **(C)** ROC curve analyses of the combination of uniquely expressed mediators (module 1 or module 2) were used to test the power to predict early or late mortality versus survival. AUC, area under the curve; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; FGF, basic fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFNγ, interferon gamma; IL, interleukin; IL-1RA, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T-cell expressed and secreted; ROC, receiver operating characteristic; TGF-β1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

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growth at the cost of severe anemia [42]. Thus, the innate response may be initially protective, but it fails and potentially becomes harmful to the host due to overwhelming infection. From our data, it appears the major driver of mortality in these patients is disseminated tuberculosis itself, which triggers a pathophysiological process that results in death despite rapid initiation of standard antituberculosis therapy.

Our group and others have described suppressed or dysfunctional innate immune responses to bacterial stimuli, which were associated with mortality or clinical deterioration in critically ill participants with HIV-associated tuberculosis [43,44]. Both studies evaluated whole blood samples from HIV-associated tuberculosis participants, which were stimulated with bacterial antigens and heat-killed *M*. *tuberculosis*, and observed lower TNF-α production upon stimulation in participants with a poor outcome. This may indicate that innate immune cells in peripheral blood are already maximally stimulated in vivo.

We observed lower monocyte and lymphocyte counts in participants who died and significant negative correlations between these cell counts and IL-1Ra, IL-6, and IL-8.

Monocytes and lymphocytes are important sources of these mediators. These mediators are also secreted by several other cell types, which may account for the higher levels of mediators despite lower numbers of cells. Alternatively, despite lower cell numbers, the remaining monocytes and lymphocytes in participants who died may be producing increased amounts of these inflammatory mediators due to an enhanced inflammatory state. The higher levels of chemokines (IL-8, MIP-1β/CCL4, IP-10/CXCL10, and MIP-1α/CCL3) we observed in participants who died suggest that immune cells may be recruited to tissue and could be producing cytokines there, resulting in higher levels of innate mediators in blood.

We observed 3-fold higher IL-1Ra values in those who died compared with survivors but a striking 8-fold higher value in early deaths compared with survivors, with no difference in IL-1β values. IL-1Ra is secreted primarily by monocytes and macrophages and antagonizes the proinflammatory cytokines IL-1α and IL-Iβ. IL-1Ra binds to the IL-1R1 receptor and inhibits IL-1 signaling, thereby regulating inflammatory responses [45]. IL-1β is one of the key cytokines involved in the initial inflammatory response to *M*. *tuberculosis* infection, and IL-1Ra down-regulates and limits this immune response. IL-1Ra levels are elevated during *M*. *tuberculosis* infection, and gene polymorphisms of IL-1Ra have been associated with disease expression in tuberculosis [46] and mortality in meningococcal sepsis [47]. IL-1Ra is induced by HIV infection [48]. This finding may reflect an important anti-inflammatory signaling pathway, which debilitates the immune response to tuberculosis and predisposes patients to disseminated tuberculosis and death. This is a hypothesis that should be explored in future mechanistic studies.

Immune activation at baseline [11,49] and failure to resolve or increased immune activation after ART initiation have been described as risk factors for mortality in HIV-associated tuberculosis cohorts [12, 13, 50]. One of these studies was an outpatient cohort study that followed participants with HIV-associated tuberculosis and showed that higher pre-ART levels of MCP-1/CCL2, eotaxin, IL-10, TNF-α, and IL-6 were associated with 6-month mortality. It was also shown that a significant increase in IL-1Ra, IFNγ, and G-CSF concentrations at 4 weeks after ART initiation was associated with mortality [12]. The same group showed the presence of a single nucleotide polymorphism (SNP) involved in the inflammasome pathway, NOD-like receptor pyrin containing-3 (NLRP3) rs10754558-G, was independently associated with 6-month mortality, and variations in the genotype at NLRP3 rs10754558 influenced participants' systemic inflammatory state pre-ART and at 4 weeks after ART initiation. The presence of this SNP appears to modulate inflammasome activation and contribute to increased inflammation [51], which may indicate a genetic predisposition to exaggerated inflammatory responses in some participants and portend a worse outcome.

Our study has several limitations. First, samples were obtained only at a single time point, at time of enrolment, and there was no longitudinal sampling. Second, we may have underestimated the role of bacterial infections on mortality. Bacterial blood cultures had a low yield, as many participants were given intravenous antibiotics before enrolment. The contribution of bacterial infections to deaths that occurred after discharge could not be ascertained. Third,

there were no objective measures of adherence to antituberculosis therapy after discharge from hospital. We did not approach the analysis with training and validation subsets but used the entire data set to characterize biomarker profiles that can be tested and validated in future studies. Finally, we do not have autopsy information on causes of death.

Strengths of the study are that it is a large cohort, with extensive tuberculosis diagnostic testing and prospective follow-up and vital status known at 12 weeks for over 98% of participants. There was systematic ascertainment of other infections and contributors to mortality. Detailed clinical, laboratory, and immunologic assays and analyses provide insight into functional responses, unlike autopsy studies. We enrolled acutely ill patients with a decreased level of consciousness, which ensures that our results are generalizable to this vulnerable group.

Future research

Our findings provide a rationale to consider novel strategies such as host-directed therapies and higher-dose rifampicin in this patient population. Rifampicin efficacy is exposure dependent [52], and current treatment doses (10 mg/kg/day) are at the lower end of the dose-response curve. This dose may be insufficient, particularly in acutely ill patients with disseminated tuberculosis and high mortality risk. Rifampicin doses up to 35 mg/kg/day [53] have been evaluated in patients with pulmonary tuberculosis, but high-dose rifampicin should also be evaluated in patients with disseminated HIV-associated tuberculosis, in whom safety considerations may be different. Additional strategies to rapidly lower the mycobacterial load, such as the use of fluoroquinolones with excellent early bactericidal activity, should be investigated in this patient group.

We postulate that the innate immune profile is driven by a high disseminated mycobacterial load and contributes to mortality. This provides a rationale to test host-directed therapy that could modulate this innate immune response in addition to more intensive antimicrobial therapies strategies. Adjunctive corticosteroids reduce mortality in adults with severe pneumonia and sepsis [54,55], and adjunctive recombinant IL-7 is well tolerated in sepsis and results in more rapid and sustained recovery of sepsis-induced lymphopenia [56]. Our findings of elevated inflammatory and innate immune mediators together with lower lymphocytes and lymphocyte-associated mediators in participants who died provide a rationale to evaluate one or both these strategies. Additionally, our finding that an immune profile of lower T-cell associated markers is associated with mortality provides a rationale to consider immediate ART, started on the same day as antituberculosis therapy, together with corticosteroids. The corticosteroids could reduce the risk of paradoxical tuberculosis immune reconstitution inflammatory syndrome [57] and modulate the innate immune response. However, neither ART status nor HIV viral load were associated with mortality in this cohort, and this suggests that immediate treatment of HIV may not alter short-term outcomes. Pharmacokinetic (PK) studies of antituberculosis drugs should include hospitalized acutely ill patients with HIV-associated tuberculosis at early therapeutic time points. We performed intensive PK sampling to measure concentrations of rifampicin, isoniazid, and pyrazinamide in a subset of this cohort, and these findings will be reported in a subsequent manuscript.

The nesting of pathogenesis studies within such intervention trials would afford the opportunity to better define causal pathophysiological relationships.

Conclusions

In conclusion, high mortality in hospitalized patients with HIV associated tuberculosis is a critical public health problem requiring improved acute management strategies. In our study, disseminated tuberculosis (quantified by a 3-point dissemination score), features of sepsis syndrome, and rifampicin-resistant tuberculosis were associated with mortality. An immune profile dominated by elevated mediators of the innate immune system and chemotactic signaling was associated with mortality and a higher dissemination score. Even though causal relationships cannot be established from this study, the findings that an innate immune profile associates with both mortality and tuberculosis dissemination provide important insights into pathophysiological processes. These findings provide a rationale to evaluate immunomodulatory therapies and more rapidly bactericidal antituberculosis treatment strategies in future studies.

Supporting information

S1 Table. Criteria used to classify participants with probable tuberculosis. Participants who did not have microbiologically confirmed tuberculosis were assessed for features compatible with tuberculosis and classified as probable tuberculosis, possible tuberculosis, or no tuberculosis (see also S2 Table). Participants with probable tuberculosis were included in the analysis along with participants with microbiologically confirmed tuberculosis [58].
(DOCX)

S2 Table. Exclusions: Details of participants with no tuberculosis and possible tuberculosis. Participants without microbiologically confirmed tuberculosis were assessed for features compatible with tuberculosis and classified as probable tuberculosis, possible tuberculosis, or no tuberculosis (see also S1 Table). Participants with possible and no tuberculosis were excluded from analysis.
(DOCX)

S3 Table. Cox proportional hazards analysis evaluating the association of CMV viremia with mortality. The relationship between CMV infection and outcome may be confounded by HIV-related factors and immunosuppression. A Cox proportional hazards analysis was performed, and each variable was evaluated individually (unadjusted) and then in a multivariate model including age, sex, HIV viral load, and CD4 count to adjust for patient-specific variance and HIV-related factors. The model was censored at 28 days to meet the proportional hazards assumption, and the global proportional hazards test for the multivariable model result was $p = 0.75$. CD4, cluster of differentiation 4; CMV, cytomegalovirus.
(DOCX)

S4 Table. Host soluble mediators of inflammation values in hospitalized HIV-TB coinfected participants: Comparison between early deaths (within 7 days after enrolment) and survivors. Host soluble inflammatory mediators were measured in a random selection of participants with HIV-associated tuberculosis ($n = 46$ early deaths and $n = 391$ survivors) using the Biorad Bioplex 200 Luminex platform. Fluorescence index values are presented.*TGF-β1 concentrations were measured with ELISA and is presented in picograms per milliliter. This table shows differences between early deaths and survivors. Inflammatory mediators are arranged into three groups: mediators that were higher in early deaths, mediators that were lower in early deaths, and mediators that showed no difference between survival groups. Each group is ranked from lowest to highest *p*-values. Comparisons between early deaths and survivors were made using the Wilcoxon rank sum test. The *p*-values were corrected for multiple

comparisons with Holms-Bonferroni correction. Bold *p*-values indicate mediators that remained significantly different after correction for multiple comparisons. CCL, C-C motif chemokine ligand; CSF2, colony stimulating factor 2; CSF3, colony stimulating factor 3; CXCL, C-X-C motif chemokine ligand; FGF, basic fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFNγ, interferon gamma; IL, interleukin; IP-10, interferon gamma-induced protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; Ra, receptor antagonist; RANTES, regulated on activation, normal T-cell expressed and secreted; TGF-β1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.
(DOCX)

S5 Table. Hospitalized patients with HIV-associated tuberculosis: Bacterial culture results from urine, sputum, stool, and other anatomical sites. Results for all bacterial cultures that were performed in hospital were captured. The study team performed sputum bacterial cultures on patients when sufficient sputum was obtained to perform tuberculosis tests and bacterial culture. All other tests were performed in routine service by the medical teams, as clinically indicated. Results presented as *n* (%). The Fisher exact test was used to compare proportions. *n* = 105 patients had urine bacterial culture performed. *n* = 312 patients had sputum bacterial culture performed. *n* = 27 patients had pleural fluid bacterial culture performed; no sample had a positive bacterial culture. *n* = 154 patients had CSF bacterial culture performed; three patients had a positive culture. One patient with clinical tuberculosis cultured *Neisseria meningitidis* in CSF and survived. One patient with microbiologically proven TB cultured *Bacillus* species in CSF and survived. One patient with microbiologically proven TB cultured *Pseudomonas putida* in CSF and died. CSF, cerebrospinal fluid; TB, tuberculosis.
(DOCX)

S1 Fig. Kaplan Meier survival curves stratified by lactate level and tuberculosis dissemination score and parameters used to calculate dissemination score. Kaplan Meier survival curves representing percentage survival in participants with lactate above the upper limit of normal (in red) to those with normal lactate (in blue). Kaplan Meier survival curves representing percentage survival in participants with a tuberculosis dissemination score of 0–3. A score was allocated to participants who had valid results for all three tests used to calculate the tuberculosis dissemination score (urine LAM assay, urine Xpert MTB/RIF assay, mycobacterial blood culture) and known outcome at week 12 (*n* = 457, *n* = 93 deaths and *n* = 364 survivors). Kaplan Meier survival curves representing percentage survival in patients who tested positive for only TB blood culture, those who tested positive for both urine Xpert and urine LAM, those who tested positive for only one of the two urine tests, and those who did not test positive for any of the markers used to calculate the dissemination score. Curves were compared using log-rank (Mantel-Cox) test. LAM, lipoarabinomannan; TB, tuberculosis.
(TIF)

S2 Fig. Kaplan Meier survival curves for host soluble inflammatory mediators that were significantly increased in participants who died. Kaplan Meier survival curves showing percentage survival over time. All soluble inflammatory mediators that were significantly increased in participants who died are shown in this figure. Participants were stratified by those with values above or below the median fluorescence index values for each mediator. Curves were compared using log-rank (Mantel-Cox) test.
(TIF)

S3 Fig. Kaplan Meier survival curves for host soluble inflammatory mediators that were significantly lower in participants who died. Kaplan Meier survival curves showing percentage survival over time. All soluble inflammatory mediators that were significantly lower in participants who died are shown in this figure. Participants were stratified by those with values above or below the median fluorescence index values for each mediator. Curves were compared using log-rank (Mantel-Cox) test.
(TIF)

S4 Fig. Kaplan Meier survival curves: Neutrophil count, monocyte count, and lymphocyte count. Kaplan Meier survival curves showing percentage survival over time. Participants were stratified by those with cell counts above or below the median values for each type of cell. Curves were compared using log-rank (Mantel-Cox) test.
(TIF)

S5 Fig. Association of PC2 score with absolute lymphocyte count and CD4 T-cell count. We explored the association of PC2 with lymphocyte count and CD4 cell count. PC2 and lymphocytes had a very weak positive correlation and PC2 and CD4 cell count were not correlated. CD4, cluster of differentiation 4; PC2, principal component 2.
(TIF)

S6 Fig. Network analysis of host soluble inflammatory mediators. (A) Profiles of correlations between inflammatory mediators in different clinical groups were examined using network analysis of the Spearman correlation matrices. Networks represent strong Spearman correlations ($p < 0.001$; Spearman rank value >0.7 or <-0.7). Mediators were clustered based on a similarity index of the correlation profiles using a modularity algorithm and depicted with Fruchterman Reingold (force-directed graph drawing). Using this approach, three main nodes were detected. Both cytokines and cells counts were included. Only mediators that had strong correlations were plotted, to reduce visual pollution. **(B)** Node analysis was used to illustrate the number of strong correlations per mediator. Mediators were grouped according to the number of connections using hierarchical clustering (Ward method).
(TIF)

S1 Document. The original prospective protocol, including the analysis plan for this study. Ethical approval was obtained in 2013. Recruitment took place from 2014 to 2016, and laboratory work was conducted in 2017. Analysis was conducted in 2018.
(PDF)

S1 STROBE checklist. STROBE checklist completed, with section and paragraph details of where relevant information on the STROBE checklist can be found in the manuscript.
(DOCX)

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Author Contributions

Conceptualization: Mark Nicol, Gary Maartens, Robert J. Wilkinson, Graeme Meintjes.

Data curation: Charlotte Schutz, David Barr.

Formal analysis: Charlotte Schutz, Bruno B. Andrade, Kiyoshi F. Fukutani.

Funding acquisition: Graeme Meintjes.

Investigation: Charlotte Schutz, David Barr, Muki Shey, Amy Ward, Saskia Janssen, Rosie Burton, Katalin A. Wilkinson, Bianca Sossen, Graeme Meintjes.

Project administration: Charlotte Schutz, Amy Ward.

Resources: Robert J. Wilkinson, Graeme Meintjes.

Supervision: Muki Shey, Katalin A. Wilkinson, Graeme Meintjes.

Visualization: Bruno B. Andrade.

Writing – original draft: Charlotte Schutz.

Writing – review & editing: David Barr, Bruno B. Andrade, Muki Shey, Amy Ward, Saskia Janssen, Rosie Burton, Katalin A. Wilkinson, Bianca Sossen, Kiyoshi F. Fukutani, Mark Nicol, Gary Maartens, Robert J. Wilkinson, Graeme Meintjes.

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CHAPTER 3: Supplementary material:

S1 Table: Criteria used to classify participants with probable tuberculosis

Probable tuberculosis	Number
Tuberculosis not microbiologically confirmed and urine LAM positive	18
Tuberculosis not microbiologically confirmed; urine LAM negative; pleural effusion which was treated for tuberculosis or exudative pleural effusion with adenosine deaminase >30g/dL and no alternative diagnosis made	21
Tuberculosis not microbiologically confirmed; urine LAM negative; pericardial effusion treated for tuberculosis and no alternative diagnosis made	3
Tuberculosis not microbiologically confirmed; urine LAM negative; miliary tuberculosis on chest X-ray treated for tuberculosis and no alternative diagnosis made	6
Tuberculosis not microbiologically confirmed; urine LAM negative; features of tuberculosis on abdominal ultrasound (multiregion nodes ≥ 1 cm diameter or splenic microabcesses) treated for tuberculosis and no alternative diagnosis made	3
Tuberculosis not microbiologically confirmed; urine LAM negative; cerebrospinal fluid (CSF) picture compatible with probable tuberculous meningitis (TBM) with CSF score ≥2 out of 4 [1]), treated for TBM and no alternative diagnosis made.	9
Tuberculosis not microbiologically confirmed; urine LAM negative; computed tomography (CT) scan features of central nervous system tuberculosis, treated for tuberculosis with no alternative diagnosis made	1
Tuberculosis not microbiologically confirmed; urine LAM negative; compatible chest X-ray, treated for tuberculosis and remained on treatment	28
Total	89

S1 Table: Patients who did not have microbiologically confirmed tuberculosis were assessed for features compatible with tuberculosis and classified as probable tuberculosis, possible tuberculosis or no tuberculosis (see also Supplementary Table 2). Participants with probable tuberculosis were included in the analysis along with participants with microbiologically confirmed tuberculosis.

1. Marais S, Thwaites G, Schoeman JF, et al. Tuberculous meningitis: a uniform case definition for use in clinical research. Lancet Infect Dis **2010**; 10(11): 803-12.

S2 Table: Exclusions: Details of participants with no tuberculosis and possible tuberculosis:

No tuberculosis	Number
Tuberculosis not microbiologically confirmed; urine LAM negative and disseminated *cryptococcosis*	6
Tuberculosis not microbiologically confirmed; urine LAM negative and culture proven bacterial bloodstream infection	9
Tuberculosis not microbiologically confirmed; urine LAM negative and clinical diagnosis of community acquired pneumonia with no criteria for clinical tuberculosis and improved without antituberculosis treatment	33
Tuberculosis not microbiologically confirmed; urine LAM negative and treated for *Pneumocystis jiroveci* pneumonia, not treated for tuberculosis and improved or *P. jiroveci* diagnosed on post mortem	6
Tuberculosis not microbiologically confirmed; urine LAM negative and no criteria for clinical tuberculosis with alternative diagnosis confirmed (examples, malignancy or venous thromboembolism)	15
Possible tuberculosis	
Tuberculosis not microbiologically confirmed; urine LAM negative and clinical suspicion of tuberculosis together with or indistinguishable from a second infection; neither infection proven; treated for both.	14

S2 Table: Patients without microbiologically confirmed tuberculosis were assessed for features compatible with tuberculosis and classified as probable tuberculosis, possible tuberculosis or no tuberculosis (see also S1 Table). Participants with possible and no tuberculosis were excluded from analysis.

S3 Table: Cox proportional hazards analysis evaluating association of cytomegalovirus viraemia with mortality:

Variable	Hazard Ratio (95% CI)	p	Adjusted Hazard Ratio (95% CI)	p
CMV viral load detectable	1.50 (0.95-2.30)	0.081	1.37 (0.87-2.17)	0.171
Age (per 5-year increase)	1.30 (1.10-1.40)	<0.001	1.30 (1.17-1.44)	<0.001
Male sex	1.00 (0.65-1.60)	0.980	0.94 (0.60-1.48)	0.790
HIV Viral Load (per 1000 copies/ml increase)	1.00 (1.00-1.00)	0.370	1.00 (1.00-1.00)	0.310
CD4 count (per 10 cells increase)	0.96 (0.93-0.99)	0.014	0.96 (0.93-0.99)	0.014

S3 Table: The relationship between cytomegalovirus infection and outcome may be confounded by HIV related factors and immunosuppression. A Cox proportional hazards analysis was performed and each variable was evaluated individually (unadjusted) and then in a multivariate model including for age, sex, HIV viral load and CD4 count to adjust for patient specific variance and HIV-related factors. The model was censored at 28 days to meet the proportional hazards assumption and the global proportional hazards test for the multivariable model result was p=0.75.

S4 Table: Host soluble mediators of inflammation values in hospitalized HIV-TB co-infected participants: Comparison between early deaths (within 7 days after enrolment) and survivors

	Early Deaths n=35	Survivors n=391	p	Holms-Bonferroni p
Higher in participants who died early				
IL-8	428.5 [195.5, 1197.8]	110.0 [78.5, 165.5]	<0.001	**<0.001**
IL-1Ra	1417.0 [449.8, 3866.0]	169.5 [93.0, 397.5]	<0.001	**<0.001**
MIP-1β/CCL4	1809.0 [917.0, 4051.3]	624.5 [397.5, 1087.5]	<0.001	**<0.001**
IP-10/CXCL10	16911.0 [8412.5, 22836.3]	6495.0 [3301.5, 11846.3]	<0.001	**<0.001**
IL-6	590.0 [244.0, 1092.3]	208.0 [119.3, 359.8]	<0.001	**<0.001**
RANTES/CCL5	10728.5 [4928.8, 13834.0]	15369.5 [12732.5, 16552.3]	<0.001	**<0.001**
MIP-1α/CCL3	248.0 [92.3, 462.0]	93.0 [65.8, 156.3]	<0.001	**<0.001**
MCP-1/CCL2	153.0 [87.0, 453.0]	95.5 [75.0, 138.0]	<0.001	**0.012**
IL-9	189.0 [146.0, 435.5]	153.0 [121.0, 205.0]	0.001	**0.029**
Lower in participants who died early				
IL-13	24.0 [17.5, 32.5]	39.0 [29.0, 59.5]	<0.001	**<0.001**
PDGF	72.0 [55.5, 133.3]	201.0 [84.0, 418.5]	<0.001	**<0.001**
IL-5	18.0 [13.5, 25.0]	31.0 [22.0, 43.5]	<0.001	**<0.001**
IL-7	26.0 [22.0, 31.8]	35.0 [28.0, 45.3]	<0.001	**0.004**
IL-12p70	43.5 [34.5, 56.8]	56.0 [42.0, 76.8]	0.001	**0.037**
No difference between early deaths and survivors				
FGF	44.0 [38.0, 57.0]	54.0 [43.8, 69.0]	0.004	0.118
G-CSF/CSF3	91.0 [51.5, 218.3]	67.0 [54.0, 90.5]	0.015	0.407
*TGF-β1	18.1 [14.2, 32.4]	26.4 [15.7, 53.9]	0.020	0.571
IL-4	41.0 [28.5, 57.8]	48.0 [36.8, 63.3]	0.059	1.000
IL-15	104.0 [75.0, 133.5]	89.5 [74.0, 114.3]	0.078	1.000

IL-1β	69.5 [57.3, 122.0]	64.0 [50.0, 84.5]	0.104	1.000
IL-17	56.0 [42.5, 85.8]	64.5 [48.8, 90.3]	0.181	1.000
IL-10	80.5 [53.5, 115.5]	69.0 [55.0, 85.0]	0.201	1.000
VEGF	114.5 [64.5, 150.5]	107.0 [78.8, 158.8]	0.446	1.000
Eotaxin	69.5 [45.0, 136.3]	66.0 [53.0, 88.3]	0.592	1.000
IFNγ	52.0 [33.5, 79.5]	54.0 [39.0, 74.5]	0.663	1.000
GM-CSF/CSF2	88.0 [68.3, 116.3]	89.5 [72.0, 113.0]	0.895	1.000
TNFα	43.0 [31.5, 60.0]	43.5 [36.0, 54.3]	0.914	1.000
IL-2	66.0 [50.8, 87.0]	68.0 [55.3, 81.0]	0.988	1.000

S4 Table: Host soluble inflammatory mediators were measured in a random selection of participants with HIV-associated tuberculosis (n= 35 early deaths and n=391 survivors) using the Biorad Bioplex 200 Luminex platform, except for transforming growth factor beta 1 (TGF-β1) concentrations which were measured with enzyme-linked immunosorbent assay (ELISA). This table shows differences between early deaths and survivors. Inflammatory mediators are arranged into three groups: Mediators which were higher in early deaths, mediators which were lower in early deaths and mediators which showed no difference between survival groups. Each group is ranked from lowest to highest p-values. Fluorescence index values are presented for all mediators except *TGF-β1 which is presented in picogram per millilitre. Comparisons between early deaths and survivors were made using the Wilcoxon rank sum test. P-values were corrected for multiple comparisons with Holms-Bonferroni correction. Bold p-values indicate mediators which remained significantly different after correction for multiple comparisons.

IL: interleukin, MIP: monocyte inflammatory protein, CCL: chemokine (C-C motif) ligand, Ra: receptor antagonist, IP-10: interferon gamma induced protein, CXCL: C-X-C motif chemokine, MIP: macrophage inflammatory protein, MCP: monocyte chemoattractant protein, RANTES: regulated on activation, normal T cell expressed and secreted, PDGF: platelet-derived growth factor , FGF: *fibroblast growth factor*, IFNγ: interferon gamma, TNFα: tumour necrosis factor alpha, GM-CSF: granulocyte-macrophage colony-stimulating factor, CSF2: colony stimulating factor 2, VEGF: vascular endothelial growth factor, G-CSF: granulocyte-colony stimulating factor, CSF3: colony stimulating factor 3

S5 Table: Hospitalized patients with HIV-associated tuberculosis: Bacterial culture results from urine, sputum, stool and other anatomical sites

		Died n=124	Survived n=443	p
Urine bacterial culture	*Enterococcus* species	2 (1.6)	3 (0.7)	0.924
	Gram negative organism	2 (1.6)	2 (0.5)	
	Yeast	5 (4)	9 (2)	
Sputum bacterial culture	*Candida albicans*	2 (1.6)	6 (1.4)	0.202
	Gram negative organism	3 (2.4)	3 (0.7)	
	Moraxella catarrhalis	0	1 (0.2)	
	Rhizopus species	0	1 (0.2)	
	Staphylococcus aureus	0	9 (2.0)	
	Mixed oral flora	7 (5.6)	38 (8.6)	
Clostridium difficile test in stool	Positive	2 (1.6)	6 (1.4)	1.000

S5 Table: Results for all bacterial cultures which were performed in hospital were captured. The study team performed sputum bacterial cultures on patients when sufficient sputum was obtained to perform tuberculosis tests and bacterial culture. All other tests were performed in routine service by the medical teams as clinically indicated.

Results presented as n (%).

Fisher's exact test used to compare proportions.

n= 105 patients had urine bacterial culture performed

n= 312 patients had sputum bacterial culture performed

n= 27 patients had pleural fluid bacterial culture performed: no sample had a positive bacterial culture

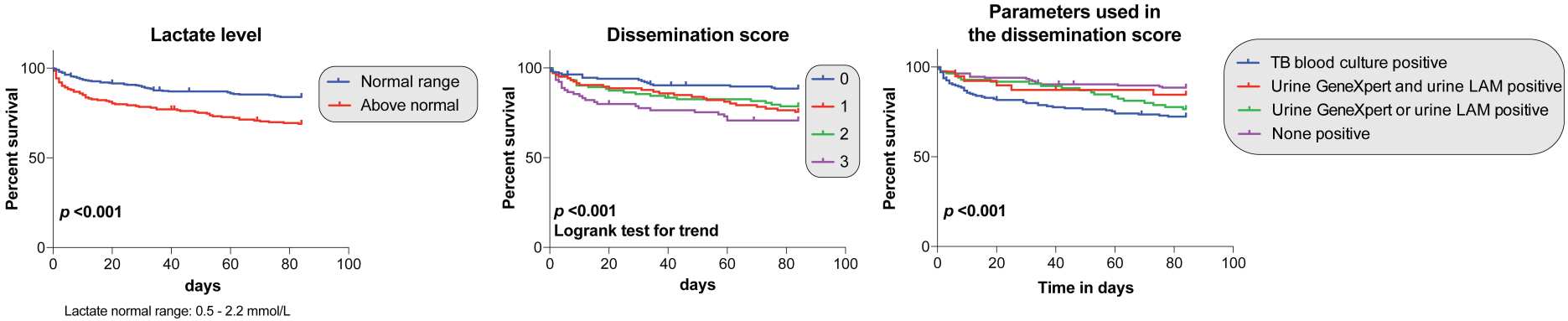
n= 154 patients had cerebrospinal fluid (CSF) bacterial culture performed: 3 patients had a positive culture:

One patient with clinical tuberculosis cultured *Neisseria meningitidis* in CSF and survived.

One patient with microbiologically proven TB cultured *Bacillus* species in CSF and survived.

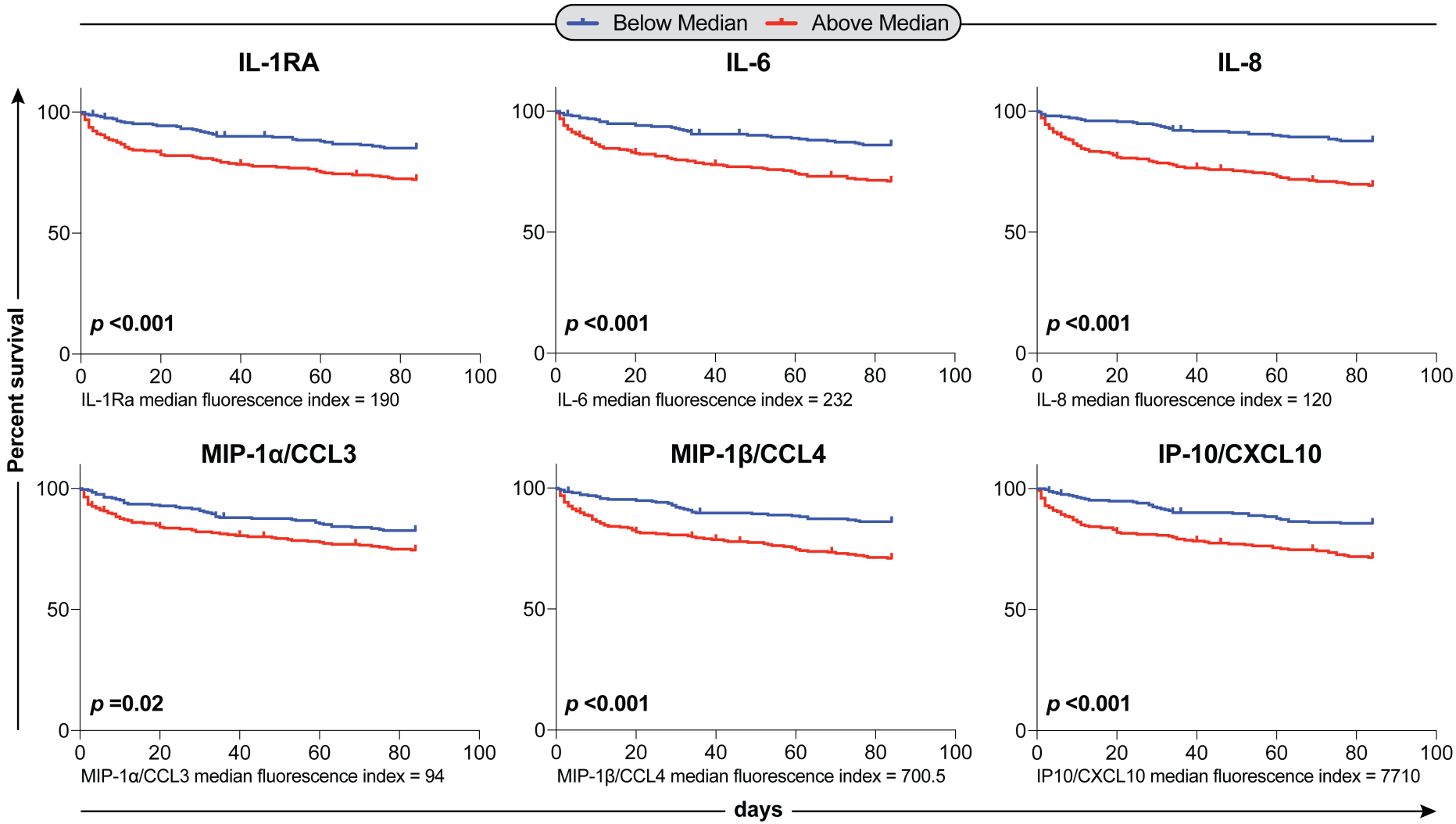
One patient with microbiologically proven TB cultured *Pseudomonas putida* in CSF and died.

S1 Fig: Kaplan Meier survival curves stratified by lactate level and tuberculosis dissemination score and parameters used to calculate dissemination score.



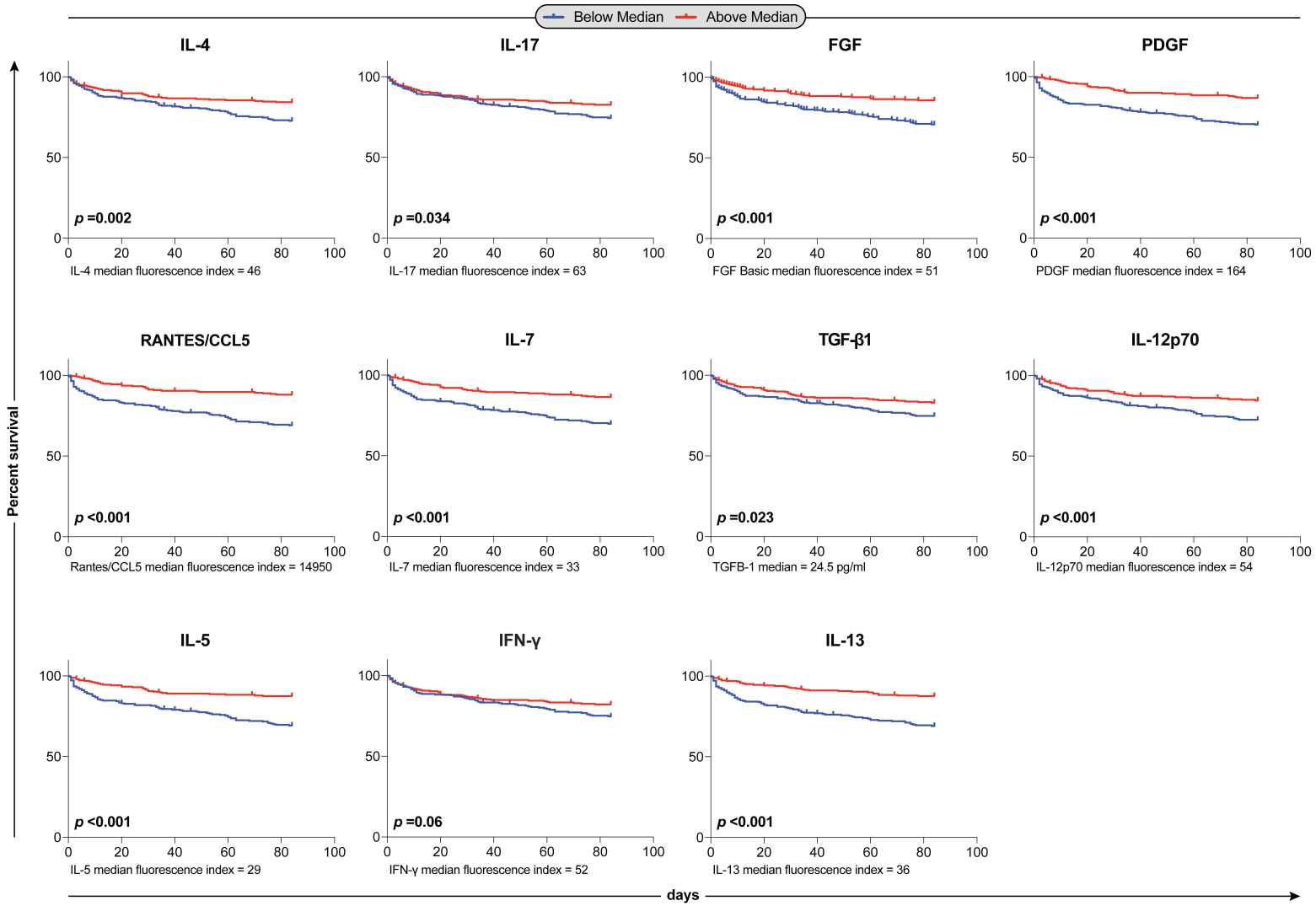
S1 Fig: Kaplan Meier survival curves representing percentage survival in participants with lactate above the upper limit of normal (in red) to those with normal lactate (in blue). Kaplan Meier survival curves representing percentage survival in participants with a tuberculosis dissemination score of 0–3. A score was allocated to participants who had valid results for all three tests used to calculate the tuberculosis dissemination score (urine LAM assay, urine Xpert MTB/RIF assay, mycobacterial blood culture) and known outcome at week 12 (*n* = 457, *n* = 93 deaths and *n* = 364 survivors). Kaplan Meier survival curves representing percentage survival in patients who tested positive for only TB blood culture, those who tested positive for both urine Xpert and urine LAM, those who tested positive for only one of the two urine tests, and those who did not test positive for any of the markers used to calculate the dissemination score. Curves were compared using log-rank (Mantel-Cox) test. LAM, lipoarabinomannan; TB, tuberculosis.

S2 Fig: Kaplan Meier survival curves for host soluble inflammatory mediators that were significantly increased in participants who died.



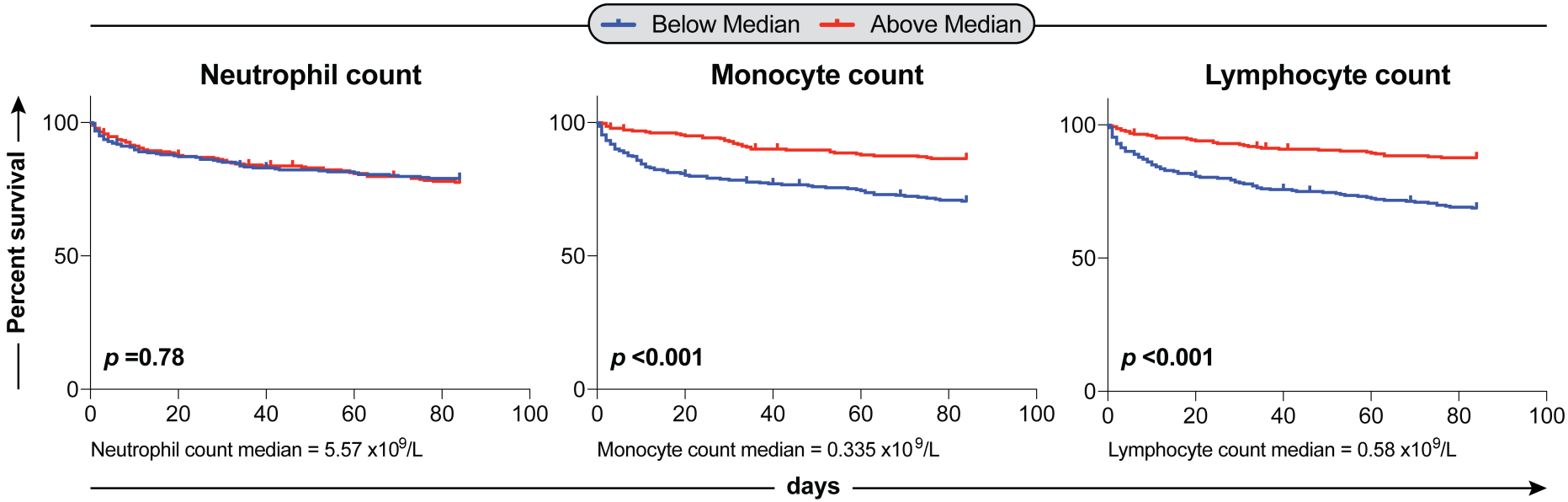
S2 Fig: Kaplan Meier survival curves showing percentage survival over time. All soluble inflammatory mediators that were significantly increased in participants who died are shown in this figure. Participants were stratified by those with values above or below the median fluorescence index values for each mediator. Curves were compared using log-rank (Mantel-Cox) test.

S3 Fig: Kaplan Meier survival curves for host soluble inflammatory mediators that were significantly lower in participants who died.



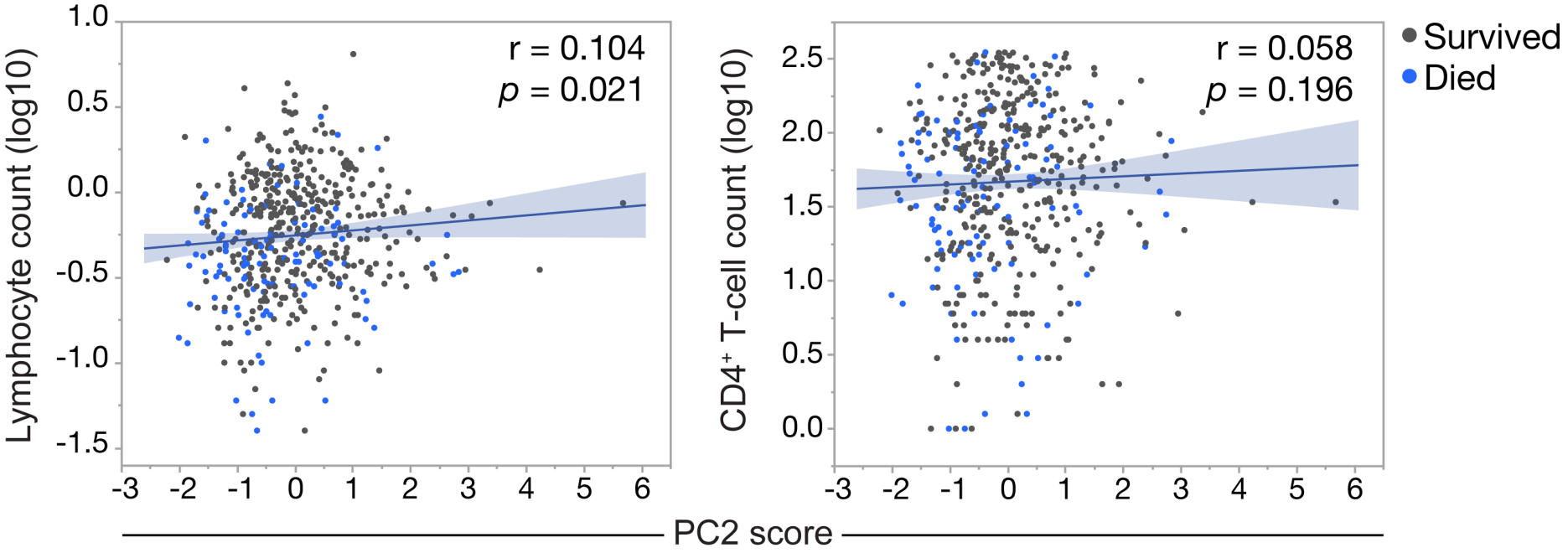
S3 Fig: Kaplan Meier survival curves showing percentage survival over time. All soluble inflammatory mediators that were significantly lower in participants who died are shown in this figure. Participants were stratified by those with values above or below the median fluorescence index values for each mediator. Curves were compared using log-rank (Mantel-Cox) test.

S4 Fig: Kaplan Meier survival curves: Neutrophil count, monocyte count, and lymphocyte count.



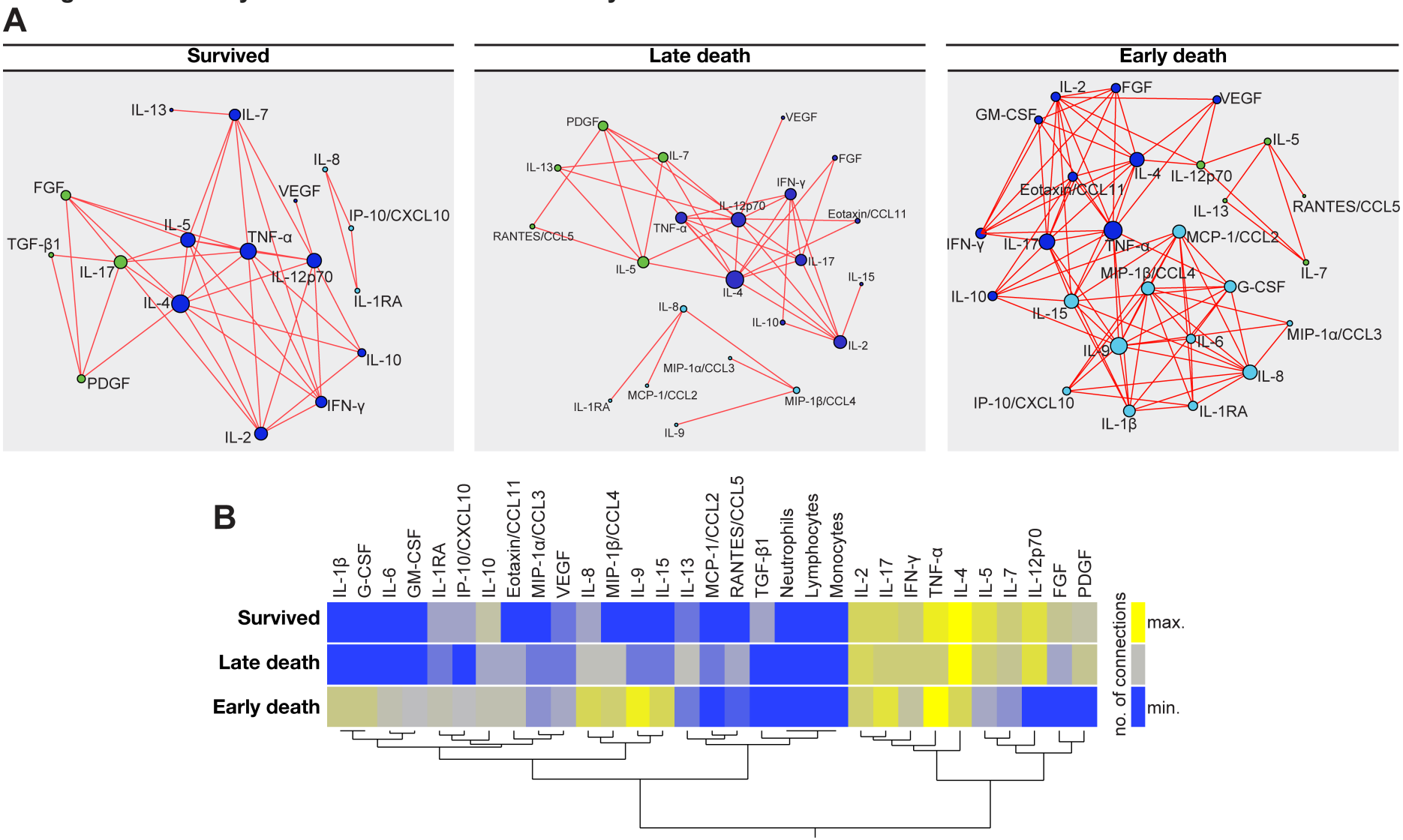
S4 Fig: Kaplan Meier survival curves showing percentage survival over time. Participants were stratified by those with cell counts above or below the median values for each type of cell. Curves were compared using log-rank (Mantel-Cox) test.

S5 Fig: Association of PC2 score with absolute lymphocyte count and CD4 T-cell count.



S5 Fig: We explored the association of PC2 with lymphocyte count and CD4 cell count. PC2 and lymphocytes had a very weak positive correlation and PC2 and CD4 cell count were not correlated. CD4, cluster of differentiation 4; PC2, principal component 2.

S6 Fig: Network analysis of host soluble inflammatory mediators.



S6 Fig: (A) Profiles of correlations between inflammatory mediators in different clinical groups were examined using network analysis of the Spearman correlation matrices. Networks represent strong Spearman correlations ($p < 0.001$; Spearman rank value >0.7 or <−0.7). Mediators were clustered based on a similarity index of the correlation profiles using a modularity algorithm and depicted with Fruchterman Reingold (force-directed graph drawing). Using this approach, three main nodes were detected. Both cytokines and cells counts were included. Only mediators that had strong correlations were plotted, to reduce visual pollution. **(B)** Node analysis was used to illustrate the number of strong correlations per mediator. Mediators were grouped according to the number of connections using hierarchical clustering (Ward method).

Supplementary material not included in the thesis but available online:

S1 Document: The original prospective protocol, including the analysis plan for this study.
Ethical approval was obtained in 2013. Recruitment took place from 2014 to 2016, and laboratory work was conducted in 2017. Analysis was conducted in 2018.
https://doi.org/10.1371/journal.pmed.1002840.s012 (PDF)

S1 STROBE checklist: **STROBE checklist completed, with section and paragraph details of where relevant information on the STROBE checklist can be found in the manuscript.**
https://doi.org/10.1371/journal.pmed.1002840.s013 (DOCX)

Appendix to Chapter 3

Missing data analyses

Missing data was examined at each step of the main analyses in Chapter 3, specifically the Cox regression and principal components analyses. We considered whether data was missing completely at random or not by evaluating the number of missing datapoints and by comparing the clinical characteristics of patients with missing samples. The variables used in these analyses had few missing values and the missing values were missing completely at random. We used complete case analyses throughout our analyses. In addition to the complete cases analyses we imputed missing values by creating 5 datasets with imputed values and then pooling these results to obtain an average imputed value. We repeated the principal components and Cox regression analyses including the imputed values. The effect sizes were only marginally different and the directions of associations were unchanged, thus we presented only the complete cases analyses.

The association of TB dissemination score, mortality and PC1 score

TB dissemination and PC1 score are both related to outcome and to each other. The underlying mechanism, size and direction of the associations are not known. The associations between these variables will not be appropriately assessed using traditional multivariable analyses and even more sophisticated causal pathway analyses may not accurately determine these associations using this cross sectional dataset. In order to assess this association the data were stratified into dissemination score, a PC1 score above and below the median and a PC1 score in the upper quartile and the lower three quartiles. The proportions of deaths were compared in each group.

Table 1: Comparison of the proportion of deaths in patients with different tuberculosis dissemination scores and a principal component 1 value above and below the median and a principal component 1 value in the upper quartile compared to the lower three quartiles.

Tuberculosis dissemination score	n	PC1 above median n=229		PC1 below median n=228		p
		Deaths	Survivors	Deaths	Survivors	
Diss score = 0	144	2	28	14	100	0.570
Diss score = 1	126	24	34	11	57	**0.003**
Diss score = 2	103	20	51	3	29	**0.020**
Diss score = 3	76	21	45	2	8	0.615
Diss score >=1	310	65	129	16	94	**<0.001**
		PC1 in upper quartile n=114		PC1 in lower 3 quartiles n=343		
		Deaths	Survivors	Deaths	Survivors	
Diss score = 0	147	0	8	16	120	1.00
Diss score = 1	127	16	14	19	77	**0.003**
Diss score = 2	104	11	24	12	56	0.207
Diss score = 3	79	14	24	9	29	0.318
Diss score >=1	310	41	62	40	162	**<0.001**

Table 1: Diss score: Tuberculosis dissemination score, a score was allocated to all patients who had a valid result for a urine Xpert MTB/RIF assay, urine Alere LAM test and mycobacterial blood culture. Each positive test was allocated one point and the score ranges from 0 to 3.
PC1: Principal component 1 value. PC1 was dominated with markers of the innate immune system and chemotactic signalling.
The p-value represents the result of the Chi squared or Fisher's Exact test comparing the proportion of deaths between the groups.

Patients with a tuberculosis dissemination score of 1 and 2 and a PC1 value above the median had significantly higher mortality than patients with the same dissemination score and PC1 value below the median. Grouping together patients who tested positive for any of the tuberculosis dissemination score markers, mortality was significantly higher in the patients who had a PC1 value above the median. Dividing PC1 value into quartiles and comparing patients with PC1 value in the upper quartile to those in the lower three quartiles, there were significantly more deaths in the group who had any tuberculosis dissemination score test positive and a PC1 value in the upper quartile, compared to patients who had PC1 values in the lower 3 quartiles. This suggests that a combination of greater tuberculosis dissemination

together with a high PC1 value is associated with mortality. By comparison patients with disseminated tuberculosis together with a lower PC1 score have lower mortality. Thus, the innate immune phenotype underlying the PC1 score appears to play a role in mortality when tuberculosis is more disseminated, but not when all three tests for disseminated tuberculosis are negative.

The Cox model in Chapter 3 was repeated and dissemination score was added as a variable.

Table 2: Cox-regression analysis to evaluate association between principal component values and 28-day mortality.

	Univariable analysis		Multivariable analysis	
	HR (95% CI)	**p**	**Adjusted HR (95% CI)**	**p**
PC1	2.30 (1.90-2.70)	**<0.001**	2.29 (1.87-2.82)	**<0.001**
PC2	0.62 (0.46-0.84)	**0.002**	0.73 (0.54-0.99)	**0.045**
PC3	0.81 (0.64-1.00)	0.094	0.91 (0.68–1.23)	0.555
Age	1.30 (1.10-1.40)	**<0.001**	1.24 (1.08-1.42)	**0.002**
Sex	1.00 (0.65-1.60)	0.98	0.83 (0.48-1.43)	0.495
HIV Viral load	1.0 (1.00 – 1.00)	0.37	1.00 (1.00 –1.00)	0.455
Diss score	1.4 (1.10 – 1.70)	**0.002**	1.13 (0.85-1.48)	0.405

Table 2: Cox regression analysis was conducted with each variable individually (unadjusted) and then all variables were included in a multivariable model (adjusted). Age, sex, and HIV viral load were incorporated a priori to adjust for patient specific variance and HIV-related factors. The model was censored at 28 days to meet the proportional hazards assumption, and the global proportional hazards test for the multivariable model result was p = 0.48. PC, principal component; HR: Hazard ratio; Age: per 5 year increase; Sex: Male to female; Diss score: Tuberculosis dissemination score: per 1 unit increase.

In this Cox regression analysis dissemination score is significantly associated with mortality in univariable analysis, but not in multivariable analysis. PC1 and PC2 values remain significantly associated with mortality and survival respectively in multivariable analysis after adjusting for dissemination score. Formal testing of

interaction between PC1 and dissemination score, PC2 and dissemination score and PC3 and dissemination score using a Cox model censored at 28 days and a logistic regression model showed no significant interaction. However, these variables are related to each other and to the outcome and these findings are likely due to effect modification or confounding and should be further investigated with a causal pathway analysis, ideally with longitudinal data to better inform the hypotheses regarding the direction of the associations between the variables.

Cotrimoxazole administration:

Patients were frequently too ill to provide a detailed and reliable medical history at enrolment, thus the quality of our previous medical history data is poor. Cotrimoxazole prophylaxis prior to admission was documented in 8/576 (1.4%) of patients. Cotrimoxazole prophylaxis during admission was administered in 176/576 (30.6%) of patients during index admission. Cotrimoxazole treatment was administered to 55/576 (9.6%) of patients during index admission.

Contribution of drug toxicities to mortality:

Drug toxicities are difficult to diagnose in patients with advanced HIV who are hospitalized and acutely ill. Patients with advanced HIV infection who are admitted with tuberculosis frequently present with a clinical syndrome resembling bacterial sepsis and often receive multiple medications. We followed patients up closely for 12 weeks and documented episodes of deterioration, readmissions to hospital and drug toxicity. Causes of death were ascertained by the study clinicians as described in Chapter 3. One death was attributed to drug toxicity only. This was a patient who presented with acute renal failure two weeks after initiating tenofovir based ART. Drug toxicity contributed to 6 deaths (See Table 1 in Chapter 3: Clinician-attributed

causes of death in participants with HIV-associated tuberculosis). Amongst these 6 deaths, 3 drug toxicities were related to antituberculosis therapy, 2 with hepatotoxicity and 1 with severe thrombocytopenia and 3 were related to nephrotoxicity secondary to tenofovir based ART. These patients had complex clinical presentations and drug toxicity was one of multiple causes considered and managed by the clinical teams.

CHAPTER 4

Early antituberculosis drug exposure in hospitalized patients with human immunodeficiency virus associated tuberculosis

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ORIGINAL ARTICLE

Early antituberculosis drug exposure in hospitalized patients with human immunodeficiency virus-associated tuberculosis

Charlotte Schutz[1,2] | Maxwell Chirehwa[3] | David Barr[4] | Amy Ward[1,2] | Saskia Janssen[5] | Rosie Burton[2,6] | Robert J. Wilkinson[1,2,7,8] | Muki Shey[1] | Lubbe Wiesner[3] | Paolo Denti[3] | Helen McIlleron[1,3] | Gary Maartens[1,3] | Graeme Meintjes[1,2]

[1]Wellcome Centre for Infectious Diseases Research in Africa, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Observatory, South Africa

[2]Department of Medicine, University of Cape Town, Observatory, South Africa

[3]Division of Clinical Pharmacology, Department of Medicine, University of Cape Town, Cape Town, South Africa

[4]Wellcome Trust Liverpool Glasgow Centre for Global Health Research, University of Liverpool, Liverpool, UK

[5]Amsterdam University Medical Centre, University of Amsterdam, Amsterdam, Netherlands

[6]Khayelitsha Hospital, Department of Medicine, Cape Town, South Africa

[7]Department of Infectious Diseases, Imperial College, London, UK

[8]The Francis Crick Institute, London, UK

Correspondence
Charlotte Schutz, IDM, Faculty of Health Sciences, Anzio Road, Observatory, Cape Town, 7925, South Africa.
Email: charlotte.schutz@uct.ac.za

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Aims: Patients hospitalized at the time of human immunodeficiency virus-associated tuberculosis (HIV-TB) diagnosis have high early mortality. We hypothesized that compared to outpatients, there would be lower anti-TB drug exposure in hospitalized HIV-TB patients, and amongst hospitalized patients exposure would be lower in patients who die or have high lactate (a sepsis marker).

Methods: We performed pharmacokinetic sampling in hospitalized HIV-TB patients and outpatients. Plasma rifampicin, isoniazid and pyrazinamide concentrations were measured in samples collected predose and at 1, 2.5, 4, 6 and 8 hours on the third day of standard anti-TB therapy. Twelve-week mortality was ascertained for inpatients. Noncompartmental pharmacokinetic analysis was performed.

Results: Pharmacokinetic data were collected in 59 hospitalized HIV-TB patients and 48 outpatients. Inpatient 12-week mortality was 11/59 (19%). Rifampicin, isoniazid and pyrazinamide exposure was similar between hospitalized and outpatients (maximum concentration [C_{max}]: 7.4 *vs* 8.3 μg mL^{-1}, P = .223; 3.6 *vs* 3.5 μg mL^{-1}, P = .569; 50.1 *vs* 46.8 μg mL^{-1}, P = .081; area under the concentration–time curve from 0 to 8 hours: 41.0 *vs* 43.8 mg h L^{-1}, P = 0.290; 13.5 *vs* 12.4 mg h L^{-1}, P = .630; 316.5 *vs* 292.2 mg h L^{-1}, P = .164, respectively) and not lower in inpatients who died. Rifampicin and isoniazid C_{max} were below recommended ranges in 61% and 39% of inpatients and 44% and 35% of outpatients. Rifampicin exposure was higher in patients with lactate >2.2 mmol L^{-1}.

Conclusion: Mortality in hospitalized HIV-TB patients was high. Early anti-TB drug exposure was similar to outpatients and not lower in inpatients who died. Rifampicin and isoniazid C_{max} were suboptimal in 61% and 39% of inpatients and rifampicin exposure was higher in patients with high lactate. Treatment strategies need to be optimized to improve survival.

The authors confirm that the PI for this paper is Graeme Meintjes and that he had direct clinical responsibility for patients.

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KEYWORDS

human immunodeficiency virus, tuberculosis, treatment, pharmacokinetics

1 | INTRODUCTION

Tuberculosis (TB) is the leading cause of hospitalization and in-hospital death in human immunodeficiency virus (HIV)-infected people worldwide.[1,2] In high-burden settings hospitalized patients with HIV-associated TB (HIV-TB) have case fatality rates between 11 and 32%.[3-8] The majority of these deaths occur within 2 weeks[3-5,8] and in postmortem series inpatient HIV-TB deaths are reported at a median of 4–5 days after admission[9,10], with 50% of deaths occurring in patients already on anti-TB therapy.[11]

Severe HIV-TB may present with clinical features of bacterial sepsis.[12-14] In high-burden settings *Mycobacterium tuberculosis* bloodstream infection is the most common diagnosis in HIV-infected patients presenting to hospital with a clinical syndrome of sepsis.[15-18] Analogous to sepsis, there are many factors in severe HIV-TB that could reduce drug exposure, such as impaired absorption of orally administered drugs due to delayed gastric emptying and decreased perfusion of the gastrointestinal tract, increased volume of distribution due to fluid shifts, and augmented renal clearance.[19,20] Other factors in advanced HIV infection such as intestinal TB, HIV-related enteropathy, and gastrointestinal opportunistic infections and macro- or micronutrient deficiencies[21-23] could contribute to reduced drug exposure. Limited existing data suggest that anti-TB drug exposure in critically ill patients is inadequate.[24] Elevated blood lactate is used as a marker of sepsis severity[25] and is associated with mortality in hospitalized patients with HIV-TB.[5]

HIV infection has a variable effect on anti-TB drug concentrations across studies, with some studies showing lower concentrations than in HIV-negative patients.[26-28] There are few pharmacokinetic studies in HIV-TB that assess relationships between drug exposure and clinical outcomes.[29-31]

Rifampicin is a potent inducer of drug metabolizing liver enzymes[32] and also undergoes auto-induction.[33] The majority of rifampicin pharmacokinetic studies have been performed after administration of multiple doses when autoinduction is advanced,[27] yet mortality in hospitalized HIV-TB patients occurs early. In the parent cohort of this pharmacokinetic (PK) study 37% of deaths occurred within 7 days of enrolment.[34] Preliminary evidence suggest that higher-dose than the currently recommended 10 mg kg^{-1} daily may improve survival in HIV-TB patients with low CD4 counts.[35]

We performed intensive PK studies on the third day of anti-TB therapy, administered at standard doses, in hospitalized patients with HIV-TB and outpatient controls and determined 12-week mortality in hospitalized patients. We compared exposure of rifampicin, isoniazid and pyrazinamide between inpatients and outpatients, between inpatients who survived and those who died within 12 weeks, and between inpatients presenting with an elevated lactate (a marker of sepsis severity) and those presenting with normal lactate. We hypothesized that exposure to rifampicin, isoniazid and pyrazinamide would be lower in inpatients than outpatients; lower in inpatients who died within 12 weeks compared to survivors, and lower in inpatients presenting with elevated venous lactate compared to those presenting with normal lactate.

What is already known about this subject

- Patients hospitalized with human immunodeficiency virus-associated tuberculosis (HIV-TB) have high mortality despite treatment and often present with a clinical picture compatible with sepsis.
- Deaths occur early and there is paucity of data regarding antitubercular drug exposure in hospitalized critically ill HIV-TB patients.

What this study adds

- Rifampicin, isoniazid and pyrazinamide exposure in hospitalized HIV-TB patients and outpatients on day 3 of standard treatment are described.
- Hospitalized HIV-TB patients do not have lower exposure than outpatients; however, many have suboptimal concentrations, which could play a role in mortality.
- This could inform treatment strategies in hospitalized HIV-TB patients.

2 | METHODS

2.1 | Study design and study population

We enrolled hospitalized HIV-infected adults with a CD4 count of ≤350 cells μL^{-1} starting treatment for active TB at Khayelitsha Hospital and ambulant outpatients (HIV-infected and uninfected) at Ubuntu clinic, Site B Khayelitsha, Cape Town, South Africa between November 2014 and November 2016. Inpatients were recruited as part of an observational cohort study investigating causes of mortality in hospitalized patients with HIV-TB. HIV-infected adults aged

18 years or older, with a suspected new diagnosis of TB were enrolled at presentation to hospital and PK studies were performed in a subgroup within the routine hospital service on the third day of anti-TB therapy. Patients who survived to the third day of TB treatment were enrolled sequentially for PK studies, provided they still required inpatient care, did not require transfer to a tertiary care facility for intensive care or investigations and there were adequate staff to fulfil the parent study's operational requirements and perform PK study. Outpatients were enrolled at treatment initiation and returned for PK studies on the third day of therapy. Patients were enrolled regardless of antiretroviral therapy status or type. Outpatients were HIV-infected or HIV-uninfected. Clinical data and baseline blood tests were obtained at enrolment. Twelve-week vital status was ascertained for inpatients.

2.1.1 | Anti-TB therapy and PK study methods

Standard combination anti-TB therapy for drug sensitive TB was administered according to weight as per the South African Department of Health National Tuberculosis Management guidelines[36] and consisted of 4-drug fixed-dose combination (FDC) tablets containing rifampicin (150 mg), isoniazid (75 mg), pyrazinamide (400 mg) and ethambutol (275 mg). In the first 8 weeks of treatment, patients weighing 30–37 kg received 2 FDC tablets per dose, while those weighing 38–54, 55–70 or >70 kg received 3, 4 or 5 tablets respectively. One inpatient had crushed tablets (mixed with water) inserted via a nasogastric tube. Two inpatients with renal impairment received separate rifampicin, isoniazid, pyrazinamide and ethambutol tablets to allow alternate day dosing of ethambutol. Patients received the FDC formulation in use at the hospital and clinic at the time the study was conducted. All outpatient controls and 31/59 (52.5%) of hospitalized patients received Rifafour e-275 (Sanofi) and the remaining hospitalized patients received RITIB (Pharmacare Limited).

Participants were fasted overnight and were offered a standardized breakfast after the 1-hour sample and a standardized lunch between the 4- and 6-hour samples. The study team administered the third dose of anti-TB therapy and collected samples immediately before (0 h) and at 1, 2.5, 4, 6 and 8 hours after the dose. Timing of samples were calculated from the time the dose was administered and all samples were collected within a 10-minute window (±5 min). A cold chain was maintained by placing blood samples in crushed ice immediately after collection, spinning in a cold centrifuge (8°C) and flash freezing plasma aliquots in dry ice within 30 minutes of collection. Plasma aliquots were transported and stored in a −80°C freezer at the end of each day.

Rifampicin, isoniazid and pyrazinamide concentrations were measured on stored plasma using high-performance liquid chromatography coupled to tandem mass spectrometry at the Division of Clinical Pharmacology Laboratory, University of Cape Town. The combined accuracy and precision statistics of the low-, medium-, and high-quality control samples during analysis (n = 22) of the rifampicin assay were between 99.7% and 100.8%, and 4.7% and 7.7%, respectively. The combined accuracy and precision statistics of the low-, medium- and high-quality control samples during analysis (n = 22) of the isoniazid assay were between 98.3% and 100.4%, and 3.0% and 5.1%, respectively. The combined accuracy and precision statistics of the low-, medium- and high-quality control samples during analysis (n = 22) of the pyrazinamide assay were between 88.1% and 92.3%, and 2.9% and 3.6%, respectively. Baseline blood tests including venous lactate measurements were performed at the National Health Laboratory Services.

2.2 | Ethical approval

The study was approved by the University of Cape Town Human Research Ethics Committee (UCT HREC reference: 057/2013) and written informed consent was obtained for the PK substudy. Eligible inpatients with a decreased level of consciousness were enrolled and followed up daily until they regained capacity to participate in the informed consent process. Permission was sought from the UCT HREC to use information of participants who died prior to providing informed consent.

2.3 | Statistical analysis

Noncompartmental analysis was performed using Stata/SE 13.1 for Mac (StataCorp, College Station, TX, USA) and all other comparative statistics were performed and plots created using R version 3.4.4 and the R Studio interface version 1.0.143.[37,38] Maximum concentration (C_{max}) is defined as the maximum plasma concentration reached after administration of the third dose of anti-TB therapy and within 8 hours. Comparisons of C_{max} and area under the concentration–time curve from 0 to 8 hours (AUC_{0-8}) were made between inpatients and outpatients, between inpatients who survived and those who died, and between inpatients presenting with elevated lactate concentrations and those presenting with normal lactate. We compared groups using the Wilcoxon rank sum, Kruskal–Wallis, Pearson's χ^2 or Fisher's exact test, where appropriate, and report median values with interquartile range (IQR) or number and percentage. We compared HIV-positive outpatient controls to HIV-negative outpatient controls. There were no differences in PK parameters of HIV-positive *vs* HIV-negative outpatients (Table S5) and this group was not disaggregated for any of the other analyses. Lactate was also compared to PK variables as a continuous variable. Correlations were performed on log or square root transformed variables using Pearson's correlation test or Spearman's rank correlation where appropriate. In hospitalized patients we calculated the odds ratio for survival per doubling of lactate concentration using a logistic regression model and log2 transformed lactate concentration. We did not adjust for other clinical variables. We performed correlation tests (Pearson or Spearman's correlation) to assess relationships between PK variables, creatinine clearance and conjugated bilirubin, and pyrazinamide exposure with 2 inflammatory markers (C-reactive protein and procalcitonin). Concentrations below the lower limit of quantification (LLQ) were imputed at half the value of the LLQ. Missing concentrations were imputed using the slope of

the relevant drug's log concentration curve for the patient when possible (Tables S1 and S2). The LLQ for rifampicin, isoniazid and pyrazinamide was 0.117, 0.105 and 0.203 μg mL^{-1}, respectively.

Drug concentrations were log-transformed and the geometric mean was calculated by exponentiating the mean of the log-transformed values. We used published reference ranges of drug concentrations that can be expected after administration of standard doses of anti-TB therapy for comparison for comparison of our C_{max} results (8–24 μg mL^{-1} for rifampicin, 3–6 μg mL^{-1} for isoniazid and 20–60 μg mL^{-1} for pyrazinamide).[31-33]

3 | RESULTS

3.1 | Outcomes of the parent study and baseline characteristics

The parent study enrolled 576 hospitalized patients with HIV-TB and the 12-week mortality was 124/576 (22%) at a median of 12.5 days from enrolment.[34]

Intensive PK studies were performed in a subgroup of 60 inpatients and in 48 outpatients with TB. One inpatient was excluded due to a high CD4 count and an alternative diagnosis of mycetoma. We analysed data from 59 inpatients and 48 outpatients (Figure 1). Outpatients included 19/48 (40%) HIV-uninfected patients. The median CD4 counts for inpatients and HIV-infected outpatients were 58 and 146 cells μL^{-1}, respectively (Table 1). Twelve-week mortality for inpatients was 11/59 (19%) with median days from PK study to death = 40 days (interquartile range = 8–60 days). One inpatient was lost to follow up after 2 months.

On baseline blood tests there were significant differences between inpatients and outpatient controls, including significantly lower CD4 count, haemoglobin, creatinine clearance and albumin, and significantly higher liver enzymes and C-reactive protein in inpatients (Table 1). Inpatients and outpatients received similar doses (mg kg^{-1}) of rifampicin, isoniazid and pyrazinamide and there was a similar distribution of patients in different weight categories (Table 1). There were fewer differences between hospitalized patients who died and those who survived 12 weeks of follow up (Table S3).

3.2 | C_{max}

Comparing hospitalized patients to outpatients, neither the median rifampicin C_{max} (7.4 *vs* 8.3 μg mL^{-1}, P = .223), nor the median isoniazid C_{max} (3.6 *vs* 3.5 μg mL^{-1}, P = .569) were significantly different. The median pyrazinamide C_{max} in hospitalized patients was higher than outpatients (50.1 *vs* 46.8 μg mL^{-1}, P = .081) but this did not reach statistical significance (Table 2 and Figure 2). Rifampicin C_{max} was below the minimum threshold of the reference range of 8 μg mL^{-1} in 36/59

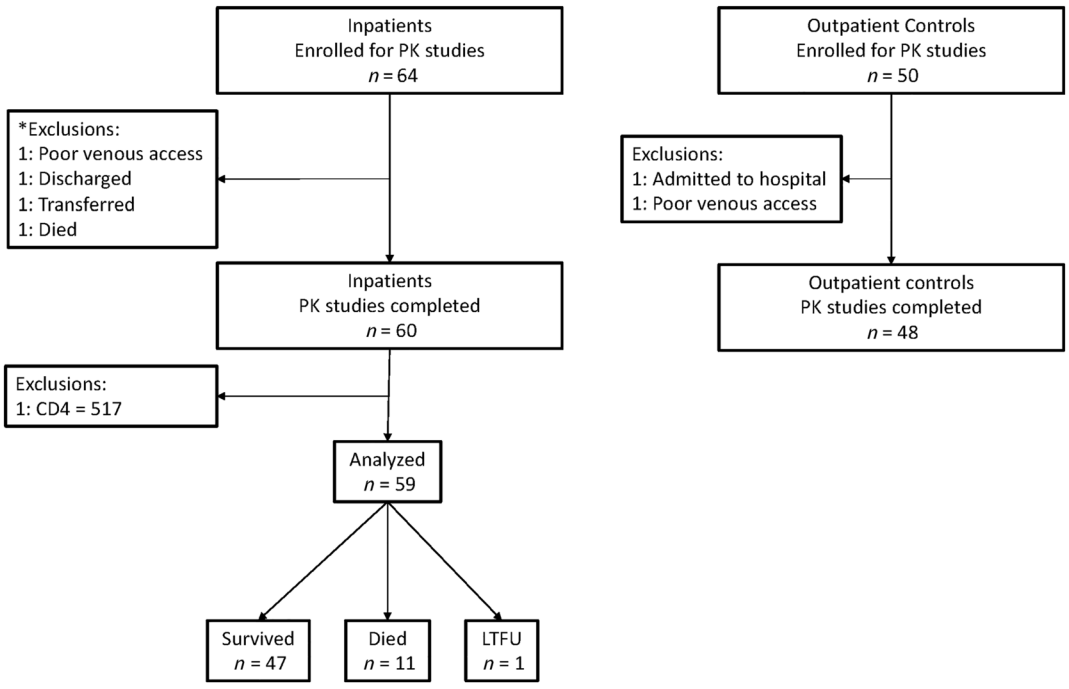

FIGURE 1 Study flow chart: hospitalized human immunodeficiency virus (HIV)-infected adults with a CD4 count of ≤350 cells μL^{-1} starting tuberculosis treatment in hospital and ambulant outpatients (HIV-infected and uninfected) were enrolled for intensive pharmacokinetic (PK) studies. Inpatients were enrolled at presentation and PK studies were performed within the routine hospital service on the third day of antituberculosis therapy, provided they still required inpatient care and did not need transfer for intensive care. Outpatients were enrolled at treatment initiation and returned for PK studies on the third day of therapy. Twelve-week mortality was ascertained for inpatients. *Exclusions are only listed if participants had consented to take part in the study and PK study could not be performed. We did not document all patients who qualified to take part in the PK study and could not be included due to logistical reasons such as early deaths, transfers to tertiary facilities and staff availability

TABLE 1 Baseline characteristics of outpatient controls with TB and hospitalized patients with HIV-TB who had intensive pharmacokinetic studies performed on the third day of anti-TB therapy

	Outpatient controls	Hospitalized	
	n = 48	*n* = 59	*P*
First episode of TB	32 (71.1)	35 (59.3)	.286
Sex, male	36 (75.0)	28 (47.5)	**.005**
Age, years	36 [32, 42]	37 [32, 41]	.980
HIV infected	29 (60.4)	59 (100)	-
[a]HIV viral load, log copies mL^{-1}	5.4 [4.3, 5.5]	5.0 [3.3, 5.7]	.684
[b]CD4 count, cells μL^{-1}	146 [37, 233]	53 [16, 129]	**.007**
[c]Current antiretroviral therapy	5 (17.2)	20 (34.5)	.169
[d]MTB on TB blood culture	-	16 (28.1)	-
Glasgow coma score <15 at presentation	0 (0.0)	12 (20.3)	**.003**
[e]Height, m	1.69 [1.64, 1.75]	1.64 [1.59, 1.70]	**.002**
Weight, kg	57.2 [52.0, 62.3]	54.5 [48.0, 60.8]	**.039**
[f]Body mass index, kg m^{-2}	19.6 [18.3, 21.8]	19.3 [17.6, 22.4]	.561
Body mass index <18.5 kg m^{-2}	15 (31.2)	21 (41.2)	.681
Body mass index 18.5 – 24.9 kg m^{-2}	29 (60.4)	26 (51.0)	
Body mass index >25 kg m^{-2}	4 (8.3)	4 (6.8)	
Random glucose, mmol L^{-1}	4.5 [4.1, 5.2]	5.2 [4.7, 5.9]	**.002**
Lactate, mmol L^{-1}	-	1.6 [1.1, 2.3]	-
C-reactive protein, mg L^{-1}	78.5 [46.8, 134.5]	192.0 [105.1, 264.5]	**<.001**
Procalcitonin, $\mu g\ mL^{-1}$	-	3.7 [0.6, 17.2]	-
Aspartate amino transferase, U L^{-1}	29.0 [21.0, 49.8]	50.0 [34.0, 82.3]	**<.001**
Alanine amino transferase, U L^{-1}	19.5 [13.0, 29.0]	27.0 [18.0, 47.0]	**.012**
Gamma-glutamyl transferase, U L^{-1}	49.5 [35.8, 93.3]	77.0 [46.5, 144.5]	**.005**
Alkaline phosphatase, U L^{-1}	88.0 [74.0, 117.5]	106.0 [75.5, 154.3]	**.043**
Total bilirubin, $\mu mol\ L^{-1}$	10.0 [6.8, 14.0]	10.0 [6.0, 14.5]	.967
Conjugated bilirubin, $\mu mol\ L^{-1}$	6.0 [4.0, 8.0]	6.0 [3.0, 9.0]	.607
Total protein, g L^{-1}	81.0 [75.0, 87.5]	80.0 [69.5, 85.0]	.068
Albumin, g L^{-1}	33.5 [32.0, 37.0]	25.0 [21.0, 29.5]	**<.001**
Creatinine, $\mu mol\ L^{-1}$	62.0 [51.50, 72.5]	95.0 [63.0, 142.0]	**<.001**
Creatinine clearance, mL $minute^{-1}$	120.5 [96.9, 145.7]	68.2 [45.2, 96.1]	**<.001**
Haemoglobin, g dL^{-1}	11.1 [9.6, 12.0]	8.7 [7.1, 9.9]	**<.001**
White cell count, $\times 10^9\ L^{-1}$	7.1 [5.7, 9.6]	7.2 [5.1, 9.8]	.770
Platelets, $\times 10^9\ L^{-1}$	424.0 [316.0, 505.0]	291.0 [199.0, 355.0]	**<.001**
Absolute neutrophil count, $\times 10^9\ L^{-1}$	4.80 [3.53, 6.91]	5.43 [3.58, 8.41]	.241
Absolute lymphocyte count, $\times 10^9\ L^{-1}$	1.36 [1.04, 1.82]	0.66 [0.36, 0.95]	**<.001**
Absolute monocyte count, $\times 10^9 L^{-1}$	0.69 [0.50, 0.83]	0.34 [0.18, 0.62]	**<.001**
Rifampicin dose, mg kg^{-1}	10.3 [9.2-10.9]	10.0 [9.2, 11.1]	.607
Isoniazid dose, mg kg^{-1}	5.2 [4.6-5.5]	5.0 [4.6, 5.6]	.754
Pyrazinamide dose, mg kg^{-1}	27.6 [24.4-29.1]	26.7 [24.4, 29.6]	.525

Continuous variables are presented as median with [interquartile range] and categorical variables as *n* (%).
P-value represents result of the nonparametric test comparison (Wilcoxon rank sum test for continuous variables and Fisher's exact or Pearson's χ^2 test for categorical variables).
TB: tuberculosis; HIV: human immunodeficiency virus; CD4: cluster of differentiation 4; MTB: *Mycobacterium tuberculosis*
[a]HIV viral load for hospitalized patients (*n* = 59) and HIV-infected outpatients (*n* = 29)
[b]CD4 count for all hospitalized patients (*n* = 59) and HIV-infected outpatients (*n* = 29)
[c]Current antiretroviral therapy indicated as a proportion of HIV-infected outpatients (*n* = 29).
[d]Mycobacterial blood culture was not performed in outpatients.
[e]Height was missing for 8 hospitalized patients: 4 survivors, 3 patients who died, 1 lost to follow up.
[f]Body mass index was not calculated for patients with missing height.

(62%) and 21/48 (44%), of hospitalized patients and outpatients (95% confidence interval of the difference in proportions [95% CI]: −3.4, 37.9; P = .079), respectively. Isoniazid C_{max} was below the minimum recommended 3 μg mL^{-1} in 23/59 (39%) and 17/48 (35%) of hospitalized and outpatients, respectively (95% CI: −16.7, 23.8; P = .841). No pyrazinamide C_{max} below the minimum reference range of 20 μg mL^{-1} was observed.

Comparing hospitalized patients who survived to those who died within 12 weeks, there were no significant differences in the median C_{max} for rifampicin (7.2 *vs* 7.5 μg mL^{-1}, P = .655), isoniazid (3.9 *vs* 3.2 μg mL^{-1}, P = .394) or pyrazinamide (48.0 *vs* 55.1 μg mL^{-1}, P = .302; Table 3 and Figure 2). Comparing inpatients who survived to those who died, there was no difference in the proportion with low rifampicin C_{max} 29/47 (62%) *vs* 7/11 (64%; 95%CI: −35.5, 31.6; P > .999) and low isoniazid C_{max} 18/47 (38%) *vs* 5/11 (46%; 95%CI: −45.3, 31.0; P = .738; Figure 2 and Table 3).

3.3 | AUC_{0-8}

Comparing hospitalized patients to outpatients, the AUC_{0-8} for rifampicin (41.0 *vs* 43.8 mg h L^{-1}, P = .290), isoniazid (13.5 *vs* 12.4 mg h L^{-1}, P = .630) and pyrazinamide (316.5 *vs* 292.2 mg h L^{-1}, P = .164) were not significantly different (Table 2 and Figure 3).

Hospitalized patients who survived and those who died within 12 weeks had similar AUC_{0-8} for rifampicin (40.0 *vs* 43.2 mg h L^{-1}, P = .684), isoniazid (13.4 *vs* 13.7 mg h L^{-1}, P = .976) and pyrazinamide (310.9 *vs* 356.1 mg h L^{-1}, P = .128; Table 3 and Figure 3).

3.4 | Patients presenting with an elevated lactate concentration

In hospitalized patients, venous lactate was performed at enrolment in 58/59 (98%) patients. One patient who survived did not have lactate performed.

Lactate was elevated (>2.2 mmol L^{-1}) at presentation in 16/59 (27%). The median lactate for all inpatients was 1.6 mmol L^{-1}, and the median was 1.45 mmol L^{-1} in patients who survived *vs* 2.4 mmol L^{-1} in patients who died, P = .078 (Table S3). The odds of survival decreased by 60% with doubling of the lactate concentration (odds ratio for survival per doubling of lactate: 0.41; 95% CI: 0.14, 1.08; P = .078). The proportion of patients presenting with an elevated lactate was 10/47 (21%) in survivors and 6/11 (55%; 95% CI: −4.0, 70.5; P = .079) in patients who died.

Comparing clinical characteristics of patients presenting with an elevated lactate to patients with normal lactate, patients with elevated lactate had significantly higher random glucose (Table S4). Lactate concentration was positively correlated with random glucose and conjugated bilirubin concentrations (Table S4). Lactate concentration was positively correlated with rifampicin C_{max} and AUC_{0-8} with Spearman's rho of 0.329, P = .012 and 0.376, P = .004 respectively (Figure 4 and Table 4). Patients with an elevated lactate at presentation (>2.2 mmol L^{-1}) had significantly higher rifampicin C_{max} (median = 9.0 *vs* 6.5 μg mL^{-1}; P = .002) and AUC_{0-8} (median = 47.3 *vs* 36.7 mg h L^{-1}; P = .006; Table 4) with a nonsignificant trend towards higher isoniazid and pyrazinamide

TABLE 2 Rifampicin, isoniazid and pyrazinamide area under the time-concentration curve from 0 to 8 hours and maximum concentration: comparison of outpatient controls and hospitalized patients with human immunodeficiency virus-associated tuberculosis

		Outpatient controls	Hospitalized	
Drug	**PK parameter**	***n* = 48**	***n* = 59**	***P***
Rifampicin	[a]AUC	43.8 [35.3, 53.8]	41.0 [28.3, 49.7]	.290
	[b]AUC	41.4 (1.5)	37.2 (1.8)	.291
	C_{max}	8.3 [6.8, 9.5]	7.4 [6.1, 9.3]	.223
	Low C_{max}	21 (43.8)	36 (62.1)	.079
Isoniazid	[a]AUC	12.4 [8.6, 18.9]	13.5 [8.9, 18.7]	.630
	[b]AUC	12.5 (1.6)	13.1 (1.7)	.632
	C_{max}	3.5 [2.4, 4.5]	3.6 [2.6, 5.0]	.569
	Low C_{max}	17 (35.4)	23 (39.0)	.841
Pyrazinamide	[a]AUC	292.2 [272.2, 319.3]	316.5 [255.4, 359.1]	.164
	[b]AUC	291.6 (1.8)	311.8 (1.3)	.165
	C_{max}	46.8 (41.9, 51.1)	50.1 [44.1, 58.4]	.081
	Low C_{max}	0 (0.0)	0 (0.0)	-

PK: pharmacokinetic

[a]AUC: area under the time-concentration curve from 0 to 8 hours in mg h L^{-1}: median and interquartile range.

[b]AUC: area under the time-concentration curve from 0 to 8 hours in mg h L^{-1}: geometric mean and geometric standard deviation (approximate coefficient of variation).

C_{max}: maximum concentration in μg mL^{-1}: median and interquartile range.

Low C_{max}: number and percentage of patients with maximum concentrations below minimum threshold of reference ranges: 8 μg mL^{-1} for rifampicin, 3 μg mL^{-1} for isoniazid and 20 μg mL^{-1} for pyrazinamide.[31]

P value represents the result of the nonparametric comparison (Wilcoxon rank sum test) for the numerical values or the Pearson's χ^2 test for categorical variables, comparing outpatient controls to hospitalized patients with human immunodeficiency virus-associated tuberculosis.

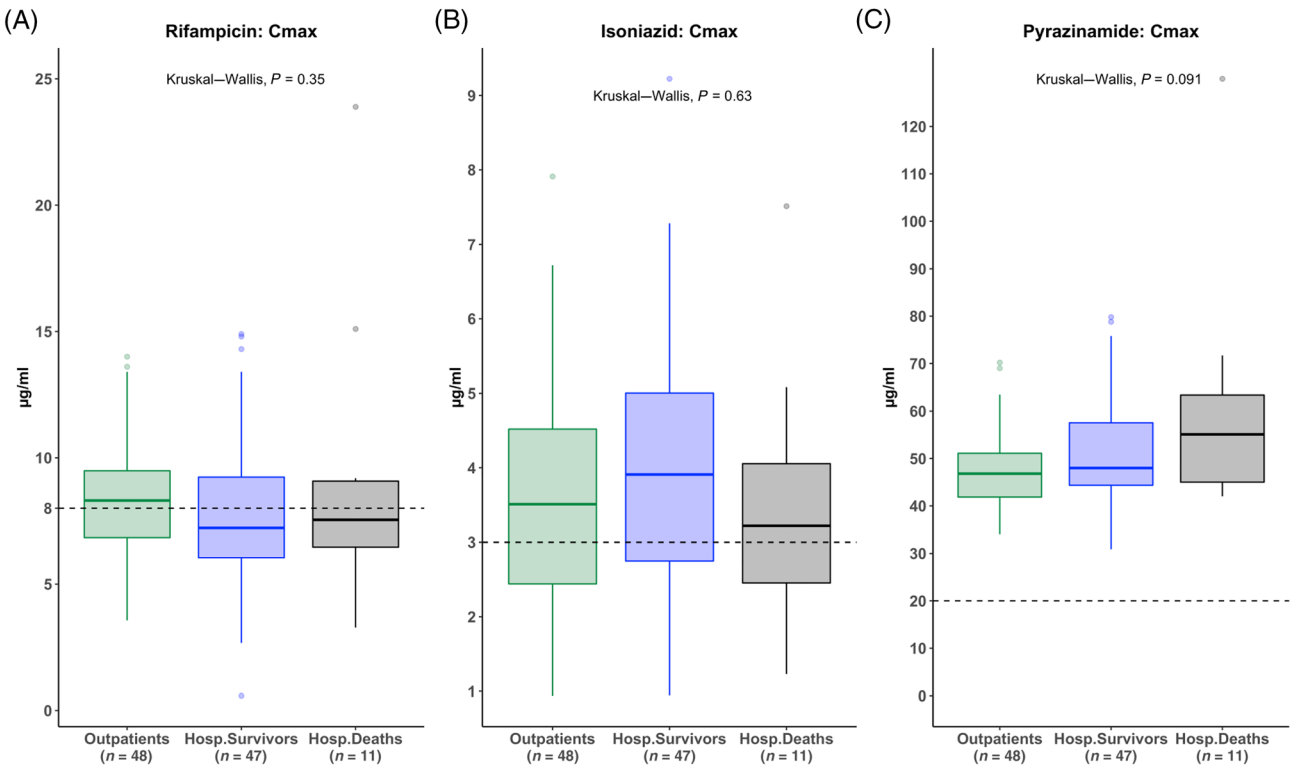

FIGURE 2 Rifampicin, isoniazid and pyrazinamide peak concentrations (C_{max}) on the third day of antituberculosis therapy: boxplots of rifampicin, isoniazid and pyrazinamide maximum concentrations are presented in µg mL^{-1} for outpatients (green), hospitalized patients who survived 12-week follow up (blue) and hospitalized patients who died within 12 weeks (black). *P* value: Kruskal–Wallis test comparing C_{max} values across 3 groups. Dashed horizontal lines represent the minimum threshold of the reference range: 8 µg mL^{-1} for rifampicin, 3 µg mL^{-1} for isoniazid and 20 µg mL^{-1} for pyrazinamide. C_{max}: maximum (peak) concentration; Outpatients: ambulant tuberculosis patients attending outpatient clinic for treatment; Hosp.Survivors: hospitalized survivors; Hosp.Deaths: hospitalized patients who died within 12 weeks of enrolment

C_{max} and AUC_{0-8} (Table 4). These findings are contrary to our hypothesis that patients presenting with elevated lactate would have lower exposure to TB drugs.

3.4.1 | Associations of PK findings with selected clinical variables

Based on findings from previous studies and the physicochemical properties of the drugs we measured, we next performed an exploratory analysis to assess the relationship of selected clinical variables with our findings. In hospitalized patients we explored the correlations of PK variables with creatinine clearance and conjugated bilirubin concentrations. The only significant findings were a positive correlation of rifampicin AUC_{0-8} and C_{max} with creatinine clearance (Pearson's correlation coefficient [r] = 0.27, *P* = .040 and *r* = 0.29, *P* = .025 respectively) and a positive correlation of rifampicin AUC_{0-8} with conjugated bilirubin concentration (*r* = 0.38, *P* = .004). Rifampicin C_{max} and conjugated bilirubin showed a positive trend, *r* = 0.26, *P* = .055 (Figure S1).

Pyrazinamide exposure was not correlated with C-reactive protein (AUC_{0-8}: *r* = 0.02, *P* = .839, C_{max}: *r* = 0.01, *P* = .885) or procalcitonin concentrations (AUC_{0-8}: *r* = −0.01, *P* = .947, C_{max}: *r* = 0.07, *P* = .585).

4 | DISCUSSION

We measured concentrations of rifampicin, isoniazid and pyrazinamide on the third day of anti-TB therapy in hospitalized adults with a new diagnosis of HIV-TB and in outpatient controls. We found high 12-week mortality of 19% for inpatients and no significant difference in C_{max} or AUC_{0-8} of rifampicin, isoniazid or pyrazinamide between hospitalized patients and outpatients, or between hospitalized patients who survived and those who died. Rifampicin and isoniazid peak concentrations were below reference ranges in 61% and 39% of inpatients and 44% and 35% of outpatients. All patients attained pyrazinamide concentrations within the reference range. We found significantly higher rifampicin C_{max} and AUC_{0-8} amongst patients presenting with elevated venous lactate, taken as a marker of sepsis severity.

We observed high 12-week mortality despite treatment and patients died at a median of 40 days after the PK study. This time to death is longer than the median days to death in the main study,

TABLE 3 Rifampicin, isoniazid and pyrazinamide area under the time-concentration curve from 0 to 8 hours and maximum concentration: comparison of hospitalized patients with human immunodeficiency virus-associated tuberculosis who survived or died within 12 weeks

		Hospitalized survivors	Hospitalized deaths	
Drug	**PK parameter**	***n* = 47**	***n* = 11**	***P***
Rifampicin	[a]AUC	40.0 [27.8, 49.0]	43.2 (30.7, 49.4]	.684
	[b]AUC	35.5 (1.8)	41.5 (1.7)	.696
	C_{max}	7.2 [6.1, 9.2]	7.5 [6.5, 9.1]	.655
	Low C_{max}	29 (61.7)	7 (63.6)	>.999
Isoniazid	[a]AUC	13.4 [9.0, 18.4]	13.7 [7.1, 22.5]	.976
	[b]AUC	13.1 (1.7)	13.0 (2.0)	.984
	C_{max}	3.9 [2.8, 5.0]	3.2 [2.5, 4.1]	.394
	Low C_{max}	18 (38.1)	5 (45.5)	.738
Pyrazinamide	[a]AUC	310.9 [251.1, 354.2]	356.1 [293.0, 437.1]	.124
	[b]AUC	303.8 (1.3)	359.3 (1.4)	.128
	C_{max}	48.0 [44.4, 57.6]	55.1 [45.0, 63.4]	.302
	Low C_{max}	0 (0.0)	0 (0.0)	-

Patients were followed up for 12 weeks to ascertain vital status. One patient was lost to follow up at 2 months and is not included in this table.
PK: pharmacokinetic
[a]AUC: area under the time-concentration curve from 0 to 8 hours in mg h L^{-1}: median and interquartile range.
[b]AUC: area under the time-concentration curve from 0to 8 hours in mg h L^{-1}: geometric mean and geometric standard deviation (approximate coefficient of variation).
C_{max}: maximum concentration in μg mL^{-1}: median and interquartile range.
Low C_{max}: number and percentage of patients with maximum concentrations below minimum threshold of reference ranges: 8 μg mL^{-1} for rifampicin, 3 μg mL^{-1} for isoniazid and 20 μg mL^{-1} for pyrazinamide.[31]
P value represents the result of the nonparametric comparison (Wilcoxon rank sum test) for the numerical values or the Pearson's χ^2 test for categorical variables, comparing hospitalized patients with human immunodeficiency virus-associated tuberculosis who survived the 12-weeks and those who died within 12 weeks.

which was 12.5 days from enrolment.[34] This PK study was performed within the routine clinical service. Critically ill patients requiring intensive care were transferred to a tertiary facility or died and stable patients were often discharged before the third day of anti-TB therapy and could thus not be included in the PK study.

A large proportion of all patients had suboptimal rifampicin and isoniazid peak concentrations. Low concentrations of anti-TB medications have been reported in other studies[29,42] and low exposure to pyrazinamide in particular have been associated with poor clinical outcomes. One study conducted intensive PK studies at 2 months on treatment and monitored 2-year outcomes in South African pulmonary TB patients.[31] They used classification and regression tree analysis, which identified pyrazinamide AUC_{0-24} < 363 mg h L^{-1} as the highest-ranking factor associated with poor 2-year outcomes (relapse, death or therapy failure). A predominantly HIV-infected pulmonary TB cohort from Botswana had PK studies performed after at least 7 days on treatment and were followed for the duration of treatment. Lower peak concentrations of pyrazinamide (<35 μg mL^{-1}) was the only PK variable associated with poor outcome and was associated with 3-fold increased risk of poor outcome.[29] In our cohort, pyrazinamide Cmax was <35 μg mL^{-1} in 6 patients (5 inpatients who survived and 1 outpatient) and there was a trend towards higher exposure in hospitalized patients. One potential mechanism for a trend towards higher pyrazinamide AUC_{0-8} in inpatients who died is impaired renal clearance due to acute kidney injury. Pyrazinamide and its main metabolite pyrazinoic acid are excreted in the urine[43] and, although hospitalized patients and specifically inpatients who died had higher creatinine, we observed no significant correlation between pyrazinamide exposure and creatinine clearance. Pyrazinamide clearance was shown to be inversely correlated to chronic cellular immune activation in HIV-TB patients in Botswana.[44] We did not measure human leucocyte antigen-DR expression on CD8 T cells in our study and, even though hospitalized patients and specifically patients who died had higher C-reactive protein and procalcitonin, there was no significant correlation between pyrazinamide exposure and either of these markers.

In a previous study, optimal early bactericidal activity was associated with an isoniazid C_{max} and $AUC_{0-\infty}$ of >2.19 and 10.52 mg h L^{-1}, respectively. In our study 10/59 (17%) and 23/59 (40%) of hospitalized patients had isoniazid C_{max} and AUC_{0-8} below these values respectively. Optimal early bactericidal effect may be important for survival in this patient group.

In hospitalized patients, we found a median rifampicin AUC_{0-8} of 41.0 mg h L^{-1}, which is higher than the predicted AUC_{0-24} (30.7 mg h L^{-1}) previously reported in South African pulmonary TB patients and HIV-infected pulmonary TB patients in Botswana at steady state (36.3 and 34.4 mg h L^{-1}).[42,45,46] Higher AUC_{0-8} values in our study are expected because auto-induction with the resulting drop in AUC value

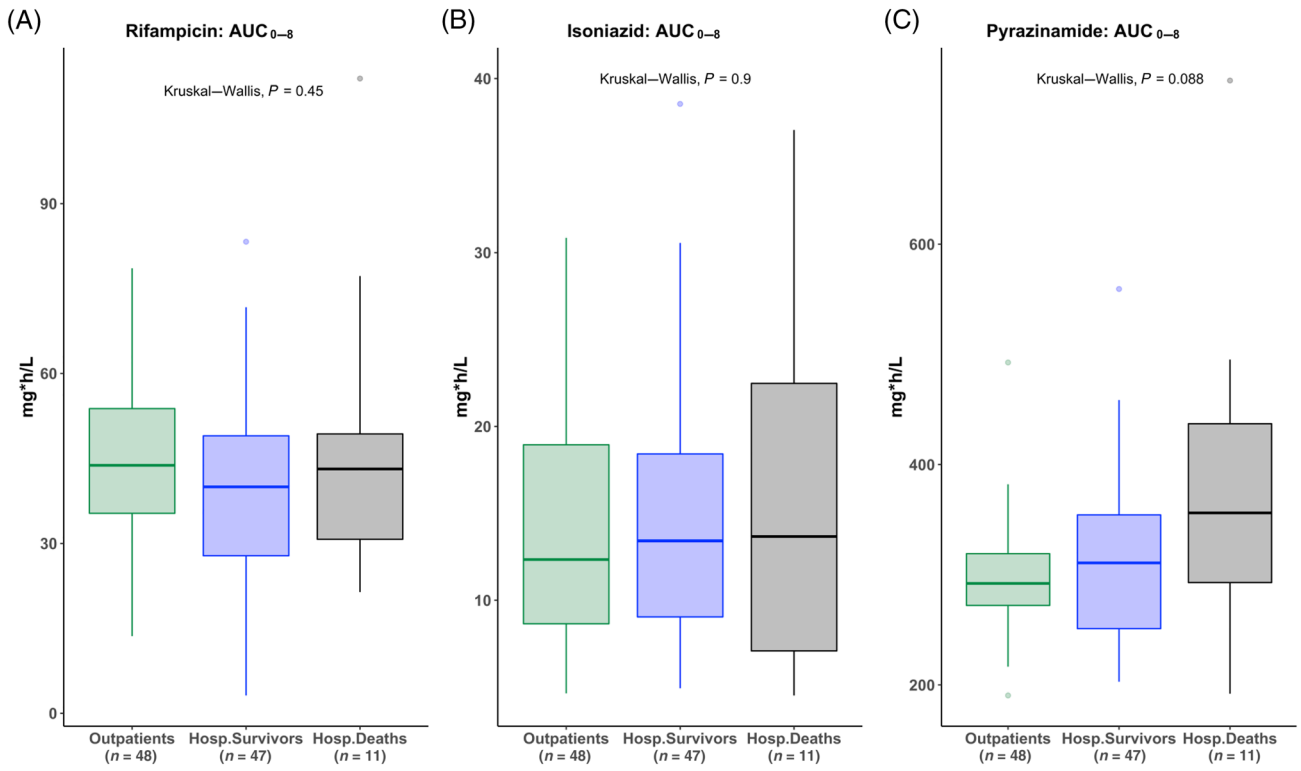

FIGURE 3 Rifampicin, isoniazid and pyrazinamide $AUC_{0\text{-}8}$ on the third day of antituberculosis therapy: boxplots of rifampicin, isoniazid and pyrazinamide $AUC_{0\text{-}8}$ presented in mg h L^{-1} for outpatients (green), hospitalized patients who survived 12 week follow up (blue) and hospitalized patients who died within 12 weeks (black). *P* value: Kruskal–Wallis test comparing $AUC_{0\text{-}8}$ values across 3 groups. AUC (0–8 h): area under the time-concentration curve from 0 to 8 hours; Outpatients: ambulant tuberculosis patients attending outpatient clinic for treatment; Hosp. Survivors: hospitalized survivors; Hosp.Deaths: hospitalized patients who died within 12 weeks of enrolment

takes 4 weeks[47,48] and would not have been complete at this early therapeutic time point (3 days).

We found significantly higher C_{max} and $AUC_{0\text{-}8}$ for rifampicin in inpatients presenting with elevated lactate, which is contrary to our hypothesis, and a positive correlation of rifampicin C_{max} and $AUC_{0\text{-}8}$ with conjugated bilirubin. Lactate is used as a marker of sepsis severity and probably reflects increased aerobic glycolysis on cellular level due to adrenergic stimulation[49] and metabolic switching of activated innate immune cells to aerobic glycolysis in critically ill patients.[50,51] The largest proportion of tissue resident macrophages (Kupffer cells) are present in the liver and these cells play a critical role in the innate immune response to pathogens, which involves activation and metabolic switch to a proinflammatory (M1-macrophage) phenotype and have important antimicrobial activity.[52] Rifampicin and its active metabolite desacetylrifampicin are lipid soluble, have enterohepatic circulation, competes with bilirubin for biliary excretion and are excreted mainly in bile and but also in urine.[32] High pretreatment bilirubin levels in patients with advanced liver cirrhosis are associated with higher rifampicin exposure.[53] It is possible that the cellular metabolic changes which underly higher lactate concentrations could play a role in the higher rifampicin exposure we observed in these patients. Neither of the hydrophylic drugs (isoniazid or pyrazinamide) were correlated with creatinine clearance, but rifampicin exposure was positively correlated with creatinine clearance. The mechanism for this is unclear.

One third of hospitalized patients in this cohort had *M. tuberculosis* blood stream infection; however, inpatients achieved concentrations and exposures similar to ambulant outpatients. The high mortality amongst hospitalized patients and the high proportion with maximum concentrations below minimum thresholds of reference ranges suggest that these concentrations and exposures may not be adequate in critically ill patients.

The parent study demonstrated an association between mortality and a higher number of mycobacterial dissemination markers being positive as well as an immune profile dominated by innate mediators.[34] These findings together with our findings of suboptimal rifampicin and isoniazid concentrations in hospitalized HIV-TB patients provide directions to consider for improving treatment strategies. One objective of treatment optimization studies should be to evaluate if more rapid reduction of disseminated mycobacterial infection load can be achieved and whether this improves survival. Strategies to accomplish this could include higher dose rifampicin, higher dose isoniazid or the addition of another rapidly bactericidal drug such as a fluoroquinolone. The safety and efficacy of these strategies would need to be tested in clinical trials in this patient population. Other strategies to evaluate could include those that modulate the immune response associated with mortality. Treatment optimization research in hospitalized HIV-TB patients should consider and could draw on the experience of treatment

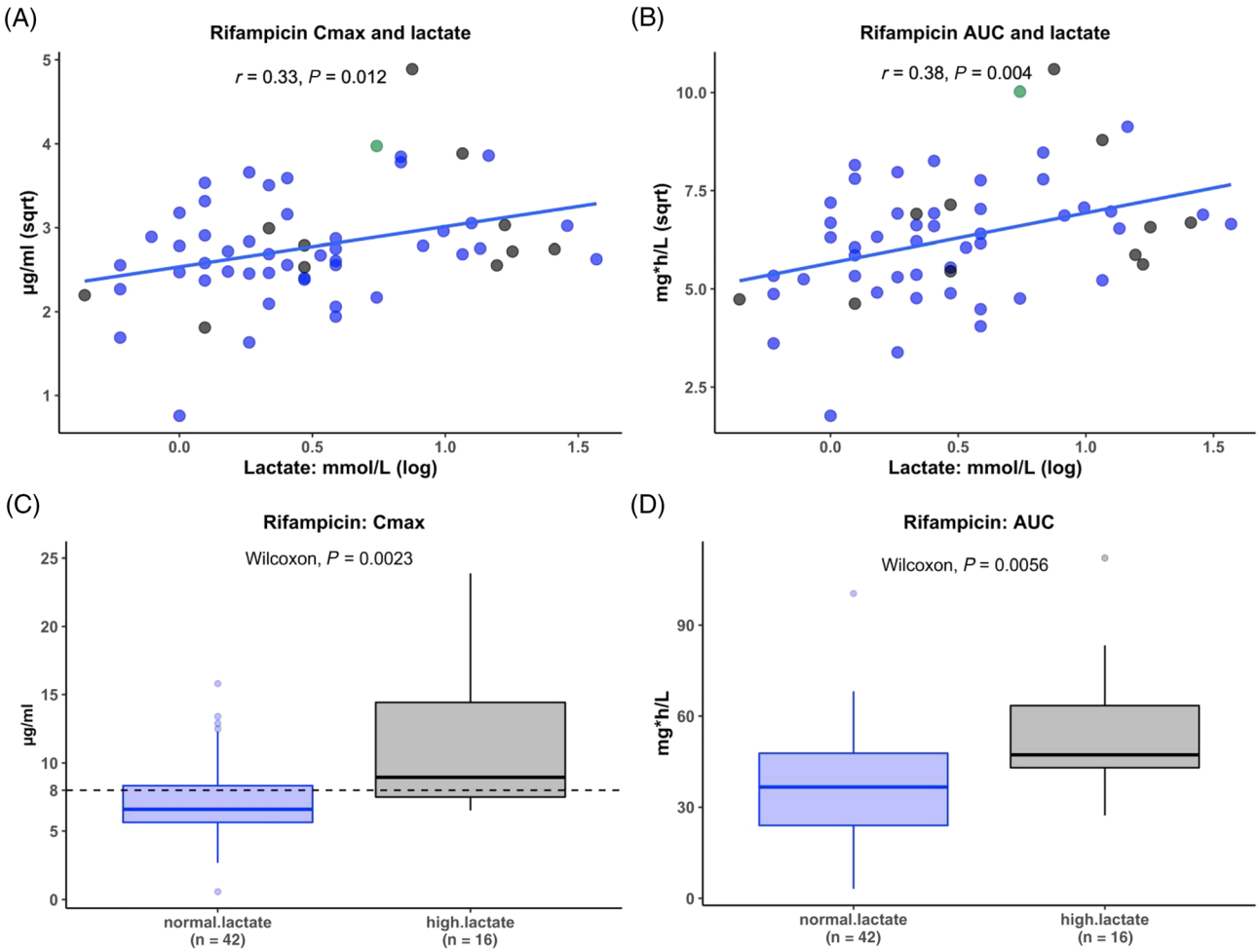

FIGURE 4 Rifampicin peak concentrations (C_{max}) and area under the time-concentration curve from 0 to 8 hours (AUC_{0-8}) on the third day of antituberculosis therapy compared with lactate concentration at presentation. Rifampicin exposure was compared to lactate concentration, a marker of sepsis severity, at presentation. Rifampicin maximum concentration and area under the concentration time curve (AUC_{0-8}) was first correlated with lactate concentration at presentation (panels A and B). The blue line indicates the linear regression line of best fit. The blue dots indicate patients who survived to 12 weeks, the black dots indicate patients who died within 12 weeks and the green dot indicates 1 patient who was lost to follow up at 8 weeks. Panels C and D are boxplots comparing rifampicin concentration in µg mL^{-1} between hospitalized patients who presented with a normal lactate concentration to those presenting with high lactate (>2.2 mmol L^{-1}) concentrations. *r*: result of Spearman's rank correlation test in panel A and Pearson's correlation coefficient in panel B. C_{max}: maximum (peak) concentration; Sqrt: square root transformed; log: log transformed; normal.lactate: patients presenting with normal lactate concentrations; high.lactate: patients presenting with high lactate (>2.2 mmol L^{-1})

optimization research in the field of tuberculous meningitis, in particular, findings regarding the efficacy and safety of higher dose rifampicin and isoniazid.[54]

Strengths of this study are that we performed intensive PK sampling in acutely ill hospitalized patients in a routine care setting at an early therapeutic time point and in a control group with pulmonary TB from the same geographical area at the same therapeutic time point. These results could help to inform future treatment optimization strategies in hospitalized HIV-TB patients. The study has several limitations. Firstly, performing the PK studies on the third day of anti-TB therapy probably introduced survival bias because some critically ill patients were transferred or died before the third day of anti-TB therapy. Half of the hospitalized patients received an FDC from a different manufacturer and this may have introduced variation in the drug concentrations. We did not calculate AUC_{0-24} and reported only AUC_{0-8}. We did not perform genotyping to assess patients' isoniazid acetylator status. Potential unmeasured differences in distribution of acetylator status across the comparator groups may have biased our analysis of the PK of isoniazid. The associations of PK variables with selected clinical variables could be due to other underlying mechanisms than the potential mechanisms we explored.

In conclusion, rifampicin and isoniazid peak concentrations were below reference ranges in 62% and 39% of hospitalized patients with HIV-TB, respectively. Isoniazid peak concentration and exposure were below the levels associated with optimal early bactericidal activity in 17% and 40% of inpatients, respectively. Inadequate exposure to key

TABLE 4 Rifampicin, isoniazid and pyrazinamide area under the time-concentration curve from 0 to 8 hours and maximum concentration: comparison of patients presenting with normal lactate and with high lactate concentrations

		Normal lactate	High lactate			
Drug	**PK parameter**	***n* = 42**	***n* = 16**	**^1P**	**Correlation coefficient**	**^2P**
Rifampicin	[a]AUC	36.7 [24.0, 47.8]	47.3 [43.0, 63.4]	**.006**	0.376	**.004**
	[b]AUC	32.9 (1.8)	50.6 (1.5)	**.003**	-	-
	C_{max}	6.6 [5.6, 8.3]	9.0 [7.5, 14.4]	**.002**	0.329	**.012**
	Low C_{max}	29 (70.7)	7 (43.8)	.073	-	-
Isoniazid	[a]AUC	13.2 [8.4, 17.8]	16.6 [9.7, 25.1]	.244	0.144	.281
	[b]AUC	12.6 (1.7)	15.2 (1.8)	.250	-	-
	C_{max}	3.6 [2.6, 5.0]	4.1 [2.7, 5.2]	.424	0.096	.474
	Low C_{max}	16 (38.1)	6 (37.5)	>.999	-	-
Pyrazinamide	[a]AUC	302.9 [234.7, 359.7]	333.1 [285.9, 369.2]	.117	0.162	.224
	[b]AUC	299.5 (1.3)	344.8 (1.3)	.120	-	-
	C_{max}	47.4 [41.8, 57.6]	54.1 [47.2, 63.1]	.073	0.179	.180
	Low C_{max}	0 (0.0)	0 (0.0)	-	-	-

Lactate is used as a marker of sepsis severity and we divided patients into those presenting with high lactate (>2.2 mmol L^{-1}, n = 16) and those presenting with normal lactate (n = 41) concentration. One patient who survived had no lactate performed and is not included in this table.
Pharmacokinetic parameters were compared between groups using a nonparametric comparison (Wilcoxon rank sum test) for the numerical values or the Pearson's χ^2 test for categorical variables.
In addition, lactate was treated as a continuous variable and correlation tests (Spearman's rank correlation (distribution not normal) or Pearson's correlation test (normal distribution)) were used to correlate lactate concentrations with PK variables.
PK: pharmacokinetic
[a]AUC: area under the time-concentration curve from 0 to 8 hours in mg h L^{-1}: median and interquartile range.
[b]AUC: area under the time-concentration curve from 0to 8 hours in mg h L^{-1}: geometric mean and geometric standard deviation (approximate coefficient of variation).
C_{max}: maximum concentration in μg mL^{-1}: median and interquartile range.
Low C_{max}: number and percentage of patients with maximum concentrations below minimum threshold of reference ranges: 8 μg mL^{-1} for rifampicin, 3 μg mL^{-1} for isoniazid and 20 μg mL^{-1} for pyrazinamide.[31]
Correlation coefficient: Spearman's ρ or Pearson's correlation coefficient.

anti-TB drugs during initial therapy may contribute to the high mortality observed in acutely ill patients hospitalized with HIV-TB. While upstream public health interventions are needed to prevent diagnostic and treatment delays, TB treatment strategies in patients hospitalized with HIV-TB need to be optimized to improve survival. Novel therapeutic strategies should be evaluated for safety and efficacy in this patient population.

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COMPETING INTERESTS

There are no competing interests to declare.

CONTRIBUTORS

Gr.M. conceptualized the study with input from Ga.M., R.J.W., H.M. and P.D. Gr.M. funded the study. C.S. and A.W. recruited patients and performed PK sampling with assistance from S.J. and D.B. Gr.M. and R.B. provided clinical oversight of recruitment. R.J.W. provided laboratory facilities for storages of samples and MS provided laboratory oversight. L.W. provided oversight of

measurement of drug concentrations. C.S. curated drug concentration results and analysed data with assistance of D.B. and M.C. C.S. wrote the manuscript and all coauthors reviewed and contributed to the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in ZivaHub Open Data UCT by FigShare] at https://doi.org/10.25375/uct.9541991.v1, title: Khayelitsha Hospital TB study PK variables: Non compartmental analysis.

ORCID

Charlotte Schutz https://orcid.org/0000-0001-8329-6158

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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CHAPTER 4: Supplementary Material:

Supplementary Table 1: Missing pharmacokinetic study time points: Samples not collected:

PK time point	Medication	Samples not taken n/N N=107	Value imputed n/missing	Outpatient controls n=48	Inpatient Survivors n=47	Inpatient Deaths n=11
0 hour	Rifampicin	0 (0%)	-	-	-	-
	Isoniazid	0 (0%)	-	-	-	-
	Pyrazinamide	0 (0%)	-	-	-	-
1 hour	Rifampicin	0 (0%)	-	-	-	-
	Isoniazid	0 (0%)	-	-	-	-
	Pyrazinamide	0 (0%)	-	-	-	-
2.5 hour	Rifampicin	2 (2%)	0 (0%)	1 (2%)	1 (2%)	0 (0%)
	Isoniazid	2 (2%)	0 (0%)	1 (2%)	1 (2%)	0 (0%)
	Pyrazinamide	2 (2%)	0 (0%)	1 (2%)	1 (2%)	0 (0%)
4 hour	Rifampicin	2 (2%)	0 (0%)	2 (4%)	0 (0%)	0 (0%)
	Isoniazid	2 (2%)	0 (0%)	2 (4%)	0 (0%)	0 (0%)
	Pyrazinamide	2 (2%)	0 (0%)	2 (4%)	0 (0%)	0 (0%)
6 hour	Rifampicin	0 (0%)	-	-	-	-
	Isoniazid	1 (1%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)
	Pyrazinamide	0 (0%)	-	-	-	-
8 hour	Rifampicin	11 (10%)	10 (91%)	6 (13%)	2 (4%)	2 (18%)
	Isoniazid	11 (10%)	11 (100%)	7 (15%)	2 (4%)	2 (18%)
	Pyrazinamide	11 (10%)	11 (100%	7 (15%)	2 (4%)	2 (18%)

Legend for Supplementary Table 1: PK time point: Time at which the samples were collected in relation to administration of the study medication.
Medication: Indicates antituberculosis drug at each time point.
Samples not taken: Indicate the total number of samples at each time which were not collected and indicates the percentage value of the missing samples to expected samples at each time point.
N= Number of expected samples at each time point which is 107.
Value imputed: Indicates number of values which were imputed using the log concentration curve for each patient and gives the percentage of imputed values to missing values at each time point.
Outpatient controls, Inpatient Survivors and Inpatient Deaths indicates the spread of missing samples by group and percentage values of missing samples to the total expected samples in each group.

Supplementary Table 2: Missing pharmacokinetic study time points: Value below the lower limit of quantification:

PK time point	Medication	Below LLQ n/N N= 107	Outpatient Controls n=48	Inpatient Survivors n=47	Inpatient Deaths n=11
0 hour	Rifampicin	30 (28%)	13 (27%)	16 (34%)	1 (9%)
	Isoniazid	76 (71%)	30 (62%)	36 (77%)	9 (82%)
	Pyrazinamide	1 (1%)	0 (0%)	1 (2%)	0 (0%)
1 hour	Rifampicin	1 (1%)	0 (0%)	1 (2%)	0 (0%)
	Isoniazid	2 (2%)	1 (2%)	1 (2%)	0 (0%)
	Pyrazinamide	0 (0%)	-	-	-
2.5 hour	Rifampicin	0 (0%)	-	-	-
	Isoniazid	0 (0%)	-	-	-
	Pyrazinamide	0 (0%)	-	-	-
4 hour	Rifampicin	0 (0%)	-	-	-
	Isoniazid	0 (0%)	-	-	-
	Pyrazinamide	0 (0%)	-	-	-
6 hour	Rifampicin	0 (0%)	-	-	-
	Isoniazid	1 (1%)	0 (0%)	1 (2%)	0 (0%)
	Pyrazinamide	0 (0%)	-	-	-
8 hour	Rifampicin	0 (0%)	-	-	-
	Isoniazid	8 (8%)	2 (4%)	6 (13%)	0 (0%)
	Pyrazinamide	0 (0%)	-	-	-

Supplementary Table 2: PK time point: Time at which the samples were collected in relation to administration of the study medication.
Medication: Indicates antituberculosis drug at each time point.
Below LLQ: Indicates the total number of samples at each time point which were below the lower limit of quantification. The percentage value is the number of samples below the LLQ to total expected samples at each time point.
N= Number of expected samples at each time point which is 107.
Outpatient controls, Inpatient Survivors and Inpatient Deaths indicates the spread of samples below the LLQ by group. The percentage indicates number of samples below LLQ to the total expected samples in each group.
LLQ: Lower limit of quantification,
LLQ for rifampicin = 0.117 μg/ml
LLQ for isoniazid = 0.105 μg/ml
LLQ for pyrazinamide = 0.203 μg/ml
Half of the value of the LLQ was imputed for all concentrations below LLQ for the analysis.

Supplementary Table 3: Baseline characteristics of hospitalized patients with HIV-associated tuberculosis who had intensive pharmacokinetic studies performed on the third day of antituberculosis therapy: Comparison between patients who died within twelve weeks and those who survived:

	Hospitalized Survivors n=47	Hospitalized Deaths n=11	p
First episode of TB	26 (56.5)	8 (72.7)	0.592
Sex, Male	23 (48.9)	5 (45.5)	1.000
Age, years	38 [32, 40]	35 [31, 50]	0.960
HIV viral load, log copies ml^{-1}	4.9 [2.9, 5.6]	5.6 [5.3, 6.0]	0.051
CD4 count, cells μL^{-1}	77 [18, 132]	32 [13, 94]	0.275
Current antiretroviral therapy	19 (41.3)	1 (9.1)	**0.049**
MTB on TB blood culture	13 (28.9)	3 (27.3)	1.000
Glasgow coma score <15 at presentation	7 (14.9)	5 (45.5)	**0.039**
[a]Height, meters	1.64 [1.59, 1.70]	1.64 [1.59, 1.71]	0.736
Weight, kilograms	54.0 [48.0, 60.5]	55.0 [47.5, 60.0]	0.843
[b]Body mass index	19.3 [17.4, 22.3]	21.7 [18.6, 23.1]	0.300
Body mass index <18.5 kg per m^2	19 (44.2)	2 (25.0)	0.340
Body mass index 18.5 – 24.9 kg per m^2	21 (48.8)	5 (62.5)	
Body mass index >25 kg per m^2	3 (6.4)	1 (.1)	
Random glucose, mmol L^{-1}	5.1 [4.6, 5.5]	6.4 [5.3, 7.3]	**0.006**
Lactate, mmol L^{-1}	1.45 [1.10, 1.80]	2.40 [1.50, 3.35]	0.078
C-reactive protein, mg L^{-1}	175.0 [101.1, 246.0]	231.3 [182.5, 316.8]	0.076
Procalcitonin, µg mL^{-1}	2.3 [0.5, 18.2]	10.1 [4.0, 15.5]	0.129
Aspartate amino transferase, U L^{-1}	47.0 [33.5, 83.5]	64.5 [36.3, 81.0]	0.632
Alanine amino transferase, UL^{-1}	30.0 [20.0, 49.5]	22.0 [15.0, 30.5]	0.100
Gamma-glutamyl transferase, U L^{-1}	77.0 [50.5, 126.5]	90.0 [40.0, 393.0]	0.388
Alkaline phosphatase, U L^{-1}	99.0 [73.0, 148.5]	129.0 [113.5, 263.5]	0.015
Total bilirubin, µmol L^{-1}	9.0 [5.0, 14.0]	12.0 [9.5, 15.0]	0.136
Conjugated bilirubin, µmol L^{-1}	5.0 [2.0, 8.0]	8.0 [6.0, 10.0]	**0.033**
Total protein, g L^{-1}	80.0 [72.0, 86.0]	80.0 [67.0, 81.0]	0.262
Albumin, g L^{-1}	26.0 [22.0, 30.5]	22.0 [16.5, 25.0]	**0.031**
Creatinine, µmol L^{-1}	90.0 [63.0, 131.5]	136.00 [78.0, 315.5]	0.102
Creatinine clearance, mL $minute^{-1}$	74.8 [47.9, 101.6]	50.1 [27.4, 75.4]	0.079
Haemoglobin, g dL^{-1}	9.0 [7.5, 10.1]	7.7 [6.0, 8.8]	0.022
White cell count, $x10^9$ L^{-1}	7.1 [5.5, 9.5]	7.7 [4.1, 12.4]	0.913
Platelets, $x10^9$ L^{-1}	299.0 [213.5, 360.0]	204.0 [175.5, 318.5]	0.262
Absolute neutrophil count, $x10^9$ L^{-1}	5.3 [3.6, 8.0]	7.0 [3.6, 10.3]	0.781
Absolute lymphocyte count, $x10^9$ L^{-1}	0.71 [0.4, 1.2]	0.4 [0.3, 0.7]	0.100
Absolute monocyte count, $x10^9$ L^{-1}	0.4 [0.2, 0.7]	0.2 [0.1, 0.4]	0.065
Rifampicin dose, mg kg^{-1}	10.0 [9.3 - 11.1]	10.0 [8.9 - 10.7]	0.766
Isoniazid dose, mg kg^{-1}	5.0 [4.7 - 5.6]	5.0 [4.5 - 5.4]	0.662

Pyrazinamide dose, mg kg^{-1}	26.7 [24.9 - 29.6]	26.2 [23.7 - 28.6]	0.713

Supplementary Table 3: Baseline characteristics of hospitalized patients comparing patients who survived 12 weeks of follow up and patients who died within 12 weeks. One patient was lost to follow up at 2 months and is not included in this table. Continuous variables are presented as median with interquartile range and categorical variables as number with percentage.
p-value represents result of the non-parametric test comparison (Wilcoxon rank sum test for continuous variables and Fisher's exact or Pearson's Chi squared test for categorical variables)
TB: Tuberculosis; HIV: Human immunodeficiency virus; CD4: Cluster of differentiation 4; MTB: *Mycobacterium tuberculosis*
[a]Height was missing in 8 patients: 4 survivors, 3 patients who died, 1 lost to follow
[b]BMI was not calculated for patients with missing height

Supplementary Table 4: Baseline characteristics of hospitalized patients with HIV-associated tuberculosis who had intensive pharmacokinetic studies performed on the third day of antituberculosis therapy: Comparison between patients with high lactate (> 2.2 mmol L^{-1}) and those with normal lactate at presentation.

Clinical characteristic	High lactate n=16	Normal lactate n=42	[1]p	Correlation coefficient	[2]p
First episode of TB	13 (81.2)	21 (50.0)	0.084	-	0.176
Sex, Male	7 (43.8)	21 (50.0)	0.772	-	0.870
Age, years	38 [31, 45]	37 [32, 40]	0.808	0.027	0.842
HIV viral load, log copies ml^{-1}	5.3 [4.6, 5.8]	4.9 [3.3, 5.6]	0.350	0.141	0.301
CD4 count, cells μL^{-1}	64 [19, 115]	45 [14, 142]	0.801	-0.009	0.944
Current antiretroviral therapy	4 (25.0)	15 (36.6)	0.171	-	0.166
MTB on TB blood culture	4 (26.7)	11 (26.8)	1.000	-	0.677
Glasgow coma score <15 at presentation	6 (37.5)	6 (14.3)	0.072	-	0.346
[a]Height, meters	1.64 [1.59, 1.69]	1.64 [1.59, 1.70]	0.931	-0.074	0.612
Weight, kilograms	57.1 [44.0, 65.5]	54.0 [48.0, 59.0]	0.450	0.074	0.584
[b]Body mass index	20.5 [16.1, 23.6]	19.3 [17.8, 21.8]	0.666	0.056	0.700
Body mass index <18.5 kg per m^2	6 (42.9)	15 (41.7)	0.083	-	0.325
Body mass index 18.5 – 24.9 kg per m^2	5 (35.7)	20 (55.6)			
Body mass index >25 kg per m^2	3 (18.8)	1 (2.4)			
Random glucose, mmol L^{-1}	6.1 [4.7, 7.3]	5.1 [4.7, 5.4]	**0.038**	**0.367**	**0.005**
C-reactive protein, mg L^{-1}	183.5 [104.8, 252.5]	197.0 [109.3, 296.8]	0.596	-0.052	0.696
Procalcitonin, µg mL^{-1}	3.4 [0.9, 10.9]	4.0 [0.6, 22.5]	0.657	0.016	0.903
Aspartate amino transferase, U L^{-1}	78.0 [35.0, 89.0]	43.5 [34.0, 70.0]	0.268	0.187	0.189
Alanine amino transferase, U L^{-1}	27.5 [17.3, 36.3]	26.5 [18.5, 49.0]	0.632	-0.066	0.624
Gamma-glutamyl transferase, U L^{-1}	83.5 [37.8, 170.5]	75.0 [52.5, 132.8]	0.876	-0.054	0.690

Alkaline phosphatase, U L^{-1}	125.5 [76.5, 191.8]	99.0 [74.0, 147.0]	0.174	0.133	0.324
Total bilirubin, µmol L^{-1}	10.5 [6.8, 14.0]	10.0 [6.0, 15.0]	0.944	0.142	0.288
Conjugated bilirubin, µmol L^{-1}	7.0 [4.0, 9.0]	5.0 [3.0, 9.0]	0.304	**0.267**	**0.051**
Total protein, g L^{-1}	80.0 [70.0, 83.3]	79.0 [71.0, 86.0]	0.882	-0.039	0.769
Albumin, g L^{-1}	23.5 [19.8, 29.8]	25.5 [22.0, 29.8]	0.519	-0.102	0.445
Creatinine, µmol L^{-1}	80.5 [59.0, 189.5]	69.1 [44.7, 96.8]	0.958	-0.148	0.269
Creatinine clearance, mL $minute^{-1}$	66.4 [48.2, 87.4]	69.1 [44.7, 96.8]	0.972	0.073	0.588
Haemoglobin, g dL^{-1}	7.90 [7.07, 8.93]	9.1 [7.5, 10.3]	0.117	-0.136	0.307
White cell count, $x10^9$ L^{-1}	7.45 [3.58, 12.39]	7.2 [5.5, 9.4]	0.767	-0.019	0.889
Platelets, $x10^9$ L^{-1}	253 [159, 306]	303 [219, 361]	0.167	-0.129	0.335
Absolute neutrophil count, $x10^9$ L^{-1}	7.60 [3.18, 10.73]	5.4 [4.1, 7.6]	0.808	0.123	0.359
Absolute lymphocyte count, $x10^9$ L^{-1}	0.50 [0.25, 0.77]	0.70 [0.37, 1.07]	0.220	-0.170	0.201
Absolute monocyte count, $x10^9$ L^{-1}	0.23 [0.15, 0.51]	0.38 [0.20, 0.64]	0.189	-0.177	0.183
Died within 12 weeks	6 (37.5)	5 (11.9)	0.079	-	-

Supplementary Table 4: One patient who survived had no lactate performed and is not included in this table. Median and interquartile range are presented for continuous variables and number with percentage for categorical variables. TB: Tuberculosis; HIV: Human immunodeficiency virus; CD4: Cluster of differentiation 4; MTB: *Mycobacterium tuberculosis*. Patients were divided into those presenting with raised (n=16) or normal lactate (n=41) and baseline characteristics were compared using non-parametric (Wilcoxon rank sum test for continuous variables and Fisher's exact or Pearson's Chi squared test for categorical variables) tests.

[1]p represents the result of the comparison of the baseline characteristic values between patients presenting with high or normal lactate.In addition, lactate was treated as a continuous variable and correlation tests (Spearman's rank correlation or Pearson's correlation test) were used to correlate lactate concentrations with continuous baseline clinical variables. Non-parametric tests (Wilcoxon rank sum or Kruskal Wallis test) were used to compare lactate concentrations across different levels of categorical variables. Correlation coefficient: Spearman's rho (if normal distribution not achieved by log or square root transformation) or Pearson's correlation coefficient (if both variables were normally distributed).

[2]p represents the result of the correlation test or the non-parametric test comparing lactate concentration to the baseline characteristic.

[a]Height was missing in 2 patients with high lactate and 6 patients with normal lactate.

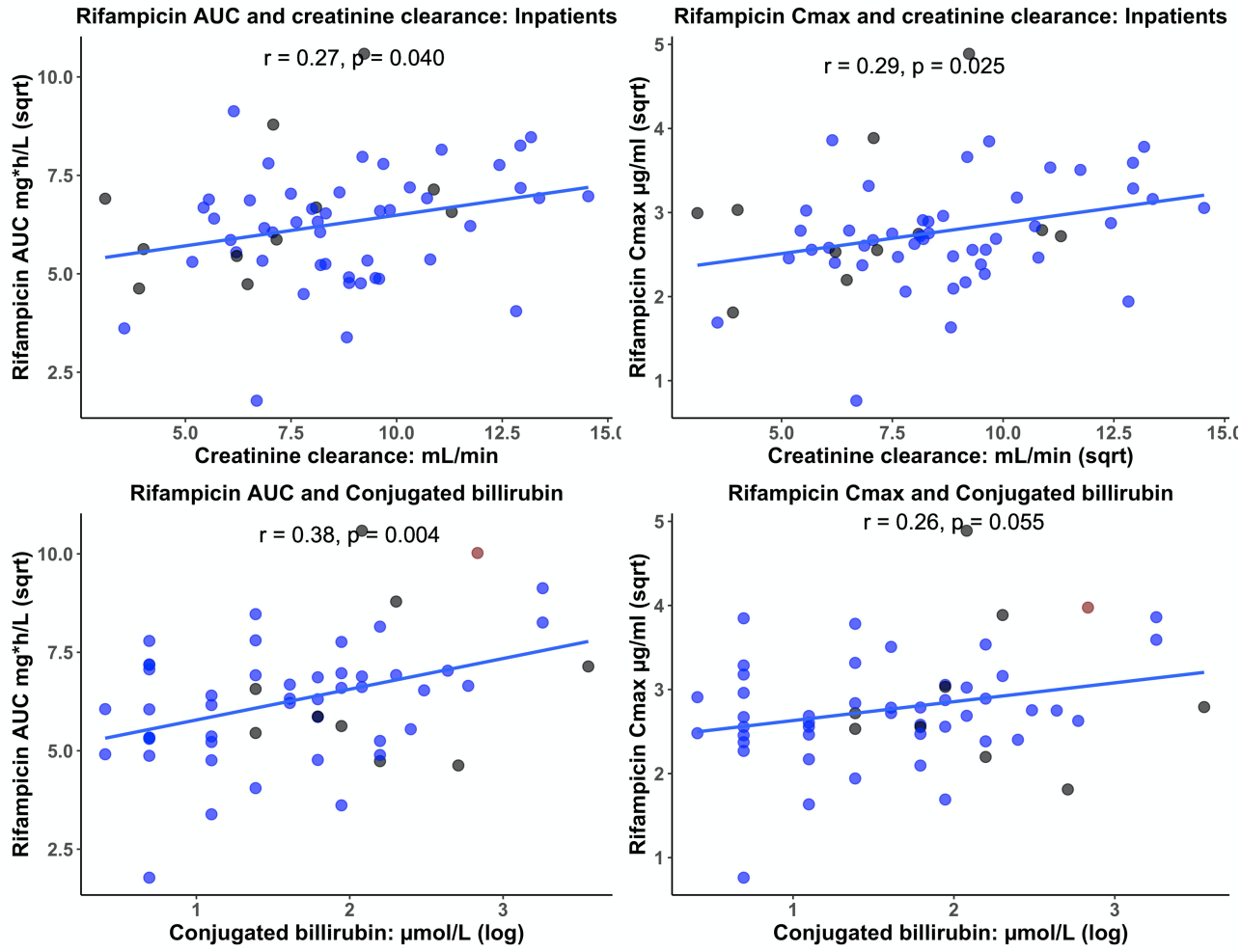
[b]BMI was not calculated for patients with missing height.

Supplementary Table 5: Rifampicin, isoniazid and pyrazinamide area under the concentration curve (0-8 hours) and maximum concentration: Comparison of outpatient controls tuberculosis stratified by HIV-status:

Drug	PK parameter	Outpatient Control HIV negative, n=19	Outpatient Control HIV positive, n=29	p
Rifampicin	AUC	40.6 [33.9, 50.1]	47.1 [35.8, 56.7]	0.202
	C_{max}	8.2 [6.5, 8.9]	8.8 [6.9, 10.5]	0.255
	Low C_{max}	9 (47.4)	12 (41.4)	0.770
Isoniazid	AUC	12.3 [9.0, 20.0]	12.8 [8.1, 17.0]	0.728
	C_{max}	3.7 [2.4, 4.6]	3.4 [2.5, 4.5]	0.628
	Low C_{max}	6 (31.6)	11 (37.9)	0.762
Pyrazinamide	AUC	290.8 [272.5, 305.8]	292.4 [272.3, 327.7]	0.487
	C_{max}	45.7 [42.6, 52.1]	47.1 [41.9, 50.8]	0.874
	Low C_{max}	0 (0.0)	0 (0.0)	-

Supplementary Table 5: PK: Pharmacokinetic; n= number; AUC: Area under the concentration curve: 0 – 8 hours in mg·h L^{-1}: Median and interquartile range are presented. C_{max}: Maximum concentration in µg ml^{-1}: Median and interquartile range are presented. Low C_{max}: Number and percentage of patients with maximum concentrations below minimum threshold of reference ranges: 8 µg mL^{-1} for rifampicin, 3 µg mL^{-1} for isoniazid and 20 µg mL^{-1} for pyrazinamide (reference: Alsultan A, Peloquin CA. Therapeutic drug monitoring in the treatment of tuberculosis: an update. Drugs. 2014;74(8):839-54). P value represents the result of the non-parametric comparison (Wilcoxon rank sum test) for the numerical values or the Pearson's Chi squared test for categorical variables, comparing outpatient controls with tuberculosis who were HIV negative to those who were HIV positive.

Supplementary Figure 1: Rifampicin maximum concentration and area under the concentration curve: Correlation with creatinine clearance and conjugated bilirubin.



Supplementary Figure 1: Rifampicin area under the concentration time curve (AUC_{0-8}) and maximum concentration (C_{max}) were correlated with creatinine clearance and conjugated bilirubin concentration.
The blue line indicates the linear regression line of best fit. The blue dots indicate patients who survived to 12 weeks, the black dots indicate patients who died within twelve weeks and the orange dot indicates one patient who was lost to follow up at 8 weeks.
r: Result of Spearman's rank correlation or Pearson's correlation coefficient.
Cmax: Maximum (peak) concentration; Sqrt: square root transformed; log: log transformed

Appendix to Chapter 4:

Baseline characteristics of patients with rifampicin maximum concentration below and above the minimum threshold of the recommended therapeutic range (including all hospitalized and outpatients).

	Rifampicin concentration <8mg/kg n=57	Rifampicin concentration ≥8mg/kg n=50	p
Sex, Male	39 (68.4)	25 (50.0))	0.075
Age, years	36 [32, 40]	38 [32, 44]	0.272
HIV viral load, log copies ml^{-1}	4.9 [3.3, 5.6]	5.3 [4.3, 5.6]	0.459
CD4 count, cells µL^{-1}	64 [23, 143]	88 [21, 188]	0.409
[*]MTB on TB blood culture	11 (32.4)	5 (21.7)	0.549
Glasgow coma score <15 at presentation	6 (10.5)	6 (12.0)	1.000
[a]Height, meters	1.66 [1.61, 1.72]	1.65 [1.60, 1.71]	0.353
Weight, kilograms	55.0 [49.0, 61.0]	56.0 [52.0, 61.9]	0.326
[b]Body mass index	19.4 [18.1, 21.4]	20.2 [18.1, 22.9]	0.161
Random glucose, mmol L^{-1}	5.0 [4.5, 5.5]	4.9 [4.3, 5.8]	0.802
[*]Lactate, mmol L^{-1}	1.6 [1.1, 1.8]	1.5 [1.3, 2.7]	0.380
C-reactive protein, mg L^{-1}	162.0 [69.0, 230.8]	110.0 [69.0, 172.0]	0.091
Alanine amino transferase, UL^{-1}	25.0 [17.0, 37.0]	20.5 [13.3, 43.8]	0.344
Total bilirubin, µmol L^{-1}	10.0 [6.0, 15.0]	10.0 [6.0, 12.0]	0.614
Conjugated bilirubin, µmol L^{-1}	6.0 [3.0, 8.5]	6.0 [4.0, 8.8]	0.779
Albumin, g L^{-1}	28.0 [23.0, 33.0]	31.5 [25.5, 35.0]	0.102
Creatinine, µmol L^{-1}	80.0 [61.0, 117.0]	62.0 [48.5, 79.8]	**0.007**
Haemoglobin, g dL^{-1}	9.7 [7.7, 11.4]	9.4 [7.9, 11.2]	0.987
Hospitalized patients	36 (63.2)	23 (46.0)	0.083
Rifampicin dose, mg kg^{-1}	10.0 [9.0 - 11.1]	10.2 [9.3 - 10.9]	0.680
Isoniazid dose, mg kg^{-1}	5.0 [4.5 - 5.6]	5.1 [4.7 - 5.4]	0.844
Pyrazinamide dose, mg kg^{-1}	26.7 [24.0 - 29.6]	27.3 [24.8 – 29.0]	0.591

Supplementary Table 3: Baseline characteristics comparing patients with rifampicin maximum concentration below and within the therapeutic range. Continuous variables are presented as median with interquartile range and categorical variables as number with percentage.
p-value represents result of the non-parametric test comparison (Wilcoxon rank sum test for continuous variables and Fisher's exact or Pearson's Chi squared test for categorical variables)
HIV: Human immunodeficiency virus; CD4: Cluster of differentiation 4; MTB: *Mycobacterium tuberculosis*
[a]Height was missing in 8 patients: 5 patients with low rifampicin maximum concentration and 3 patients with normal concentration.
[b]BMI was not calculated for patients with missing height
[*]Mycobacterial blood culture and lactate were only performed in hospitalized patients

Baseline characteristics of patients with isoniazid maximum concentration below and above the minimum threshold of the recommended therapeutic window (including all hospitalized and outpatients).

	Isoniazid concentration <3mg/kg n=39	Isoniazid concentration ≥3mg/kg N=68	p
Sex, Male	24 (61.5)	40 (58.8)	0.840
Age, years	35 [32, 40]	38 [32, 44]	0.185
HIV viral load, log copies ml^{-1}	5.3 [4.7, 5.6]	5.0 [3.3, 5.6]	0.634
CD4 count, cells µL^{-1}	44 [23, 160]	92 [20, 153]	0.914
[*]MTB on TB blood culture	8 (38.1)	8 (22.2)	0.232
Glasgow coma score <15 at presentation	5 (12.8)	7 (10.3)	0.755
[a]Height, meters	1.65 [1.61, 1.73]	1.66 [1.59, 1.72]	0.666
Weight, kilograms	55.1 [50.6, 62.2]	55.9 [48.6, 61.5]	0.491
[b]Body mass index	19.4 [18.2, 22.5]	19.7 [18.0, 21.9]	0.741
Random glucose, mmol L^{-1}	4.9 [4.4, 5.5]	4.9 [4.4, 5.7]	0.795
[*]Lactate, mmol L^{-1}	1.5 [1.1, 2.3]	1.6 [1.2, 2.3]	0.993
C-reactive protein, mg L^{-1}	127.0 [64.5, 220.2]	130.5 [69.3, 202.3]	0.913
Alanine amino transferase, UL^{-1}	26.0 [20.0, 35.5]	20.0 [14.0, 43.0]	0.185
Total bilirubin, µmol L^{-1}	10.0 [6.0, 15.0]	10.0 [6.0, 14.0]	0.753
Conjugated bilirubin, µmol L^{-1}	6.0 [4.0, 8.0]	5.0 [3.0, 9.0]	0.453
Albumin, g L^{-1}	32.0 [25.0, 33.0]	30.0 [23.0, 34.0]	0.785
Creatinine, µmol L^{-1}	77.0 [58.0, 127.0]	62.5 [53.0, 96.3]	0.234
Haemoglobin, g dL^{-1}	9.3 [8.3, 11.6]	9.7 [7.7, 11.2]	0.426
Hospitalized patients	22 (56.4)	37 (54.4.0)	1.00
Rifampicin dose, mg kg^{-1}	9.8 [9.0 - 10.9]	10.0 [9.4 – 11.2]	0.131
Isoniazid dose, mg kg^{-1}	4.9 [4.5 - 5.5]	5.1 [4.7 - 5.6]	0.096
Pyrazinamide dose, mg kg^{-1}	25.4 [24.0 - 29.1]	26.7 [25.0 – 30.0]	0.126

Supplementary Table 3: Baseline characteristics comparing patients with isoniazid maximum concentration below and within the therapeutic range.
Continuous variables are presented as median with interquartile range and categorical variables as number with percentage. P-value represents result of the non-parametric test comparison (Wilcoxon rank sum test for continuous variables and Fisher's exact or Pearson's Chi squared test for categorical variables). HIV: Human immunodeficiency virus; CD4: Cluster of differentiation 4; MTB: *Mycobacterium tuberculosis*
[a]Height was missing in 8 patients: 1 patients with low isoniazid maximum concentration and 7 patients with normal concentration.
[b]BMI was not calculated for patients with missing height
[*]Mycobacterial blood culture and lactate were only performed in hospitalized patients

Additional discussion points:

The correlation between rifampicin and creatinine clearance may be a chance finding as it does not align well with what is known about rifampicin elimination. Patients with higher lactate concentrations had lower conjugated bilirubin levels (Chapter 4 Supplementary Table 4) which raises the possibility of a problem with hepatic conjugation capacity as an explanation for the association between rifampicin pharmacokinetics and high lactate concentrations.

The likely impact of each study limitation

Survival bias: Findings in patients who died earlier or were so ill they needed transfer to ICU facilities may be very different to our findings. We cannot be sure if survival bias impacted our findings or the direction of such impact. A small ICU based study which did not report HIV status, patients received at least 7 prior doses of TB medication and received medication via nasogastric tube showed rifampicin proportion under the reference range in 6/10 (60%) and low isoniazid in only 2/10 (20%) (105). We thus hypothesize that at a very early time point, in acutely ill hospitalized HIV-TB patients who died or were transferred, both rifampicin and isoniazid concentrations would be lower than what we found in our study. PK studies in such patients are needed to inform appropriate dosing strategies.

Fixed dose combination (FDC) from different manufacturer: Studies have shown up to 20% difference in drug concentrations when using different FDC formulas (137) and the fact that the FDC formulation changed during the study period could have affected findings.

No estimated 24 hour AUC: This could have influenced our AUC findings, but we chose to report what was measured instead of estimating the 24 hour AUC and to examine this data in more detail in a separate population pharmacokinetic model.

No genotyping to assess acetylator status: Unmeasured differences in acetylator status could have assisted the interpretation of the INH concentration results.

CHAPTER 5

The association of microbial translocation with mortality in hospitalized patients with HIV-associated tuberculosis

Abstract:

Background and objectives: HIV-associated tuberculosis remains the leading cause of mortality in HIV positive people world-wide and pathophysiology underlying mortality is poorly understood. Translocation of microbial products from the gastrointestinal lumen into the systemic circulation contributes to chronic immune activation and poor clinical outcomes in HIV infection and is associated with adverse clinical outcomes in chronic liver disease and chronic inflammatory bowel disease. We hypothesized that patients hospitalized with HIV-associated tuberculosis would have more gastrointestinal mucosal damage and higher levels of microbial translocation compared to outpatients with HIV infection and no active tuberculosis and that these markers would be associated with mortality.

Patients and Methods: We measured lipopolysaccharide [LPS], three LPS binding proteins/antibodies: soluble CD14 [sCD14]; lipopolysaccharide binding protein [LBP]; endotoxin core antibody IgM [endoCAB]; and two markers of gastrointestinal mucosal damage: trefoil factor 3 [TFF3] and intestinal fatty acid binding protein 2 [IFABP]) in 373 patients hospitalized with a new diagnosis of HIV-associated tuberculosis and 32 outpatients with HIV infection and no active tuberculosis. We ascertained vital status at 12 weeks for hospitalized patients and compared hospitalized patients to outpatient controls and hospitalized patients who died within 12 weeks to survivors. We measured 16s rDNA concentrations in a subset of 235

patients. We investigated associations with 12-week mortality, markers of tuberculosis dissemination and bacterial 16s rDNA concentrations.

Results: sCD14 and TFF3 concentrations were significantly higher and EndoCAB concentrations significantly lower in hospitalized patients with HIV-associated tuberculosis compared to outpatient HIV-infected controls and in hospitalized patients who died within 12 weeks compared to survivors. TFF3 was independently associated with mortality (adjusted odds ratio = 1.035, $p < 0.001$ per 1 ng/mL increase). IFABP was (unexpectedly) significantly higher in outpatient controls. We found low LPS concentrations overall with no difference between groups. Bacterial 16s rDNA concentration was higher in hospitalized patients with HIV-associated tuberculosis compared to HIV-infected outpatients. There was no difference between hospitalized patients who died compared to patients who survived and no association with mortality in survival analysis. LBP concentration had a significant positive association with 16s rDNA concentration in multivariable linear regression analysis. Soluble CD14, TFF3 concentrations were significantly higher and EndoCAB concentrations significantly lower in patients who tested positive for markers of tuberculosis dissemination.

Conclusion: Our findings support the hypothesis that there is more gastrointestinal mucosal damage and higher levels of microbial translocation in hospitalized patients with HIV-associated tuberculosis compared to outpatient HIV infected controls and in hospitalized patients with HIV-associated tuberculosis who died. TFF3 was independently associated with mortality and higher 16s rDNA concentration in hospitalized patients was not associated with mortality. Our findings also suggest a relationship between gastrointestinal mucosal damage, microbial translocation and the mycobacterial load or burden. It is not clear from our findings whether

gastrointestinal mucosal damage and microbial translocation plays an active role in the pathophysiology underlying mortality and should be considered as a therapeutic target in this context. This should be explored in future studies.

Background

Despite widespread availability of treatment, tuberculosis is the leading cause of hospitalization and death in HIV-positive people world-wide (55, 56), causing an estimated 251 000 deaths in 2018, with 211 000 of these deaths occurring in Africa (1). In high burden settings case fatality rates in patients hospitalized with HIV-associated tuberculosis range between 13% and 32% (2-7). The majority of deaths occur early (within two weeks of hospitalization) and many deaths occur after initiation of antituberculosis therapy. The causes of death and underlying pathophysiology associated with death in HIV-associated tuberculosis are poorly understood.

Gastrointestinal microbial translocation is defined as the non-physiological translocation of gastrointestinal microbes and/or microbial products through the gastrointestinal epithelial barrier and the lamina propria into the portal venous system after damage to the mucosal barrier and local immune system (106). These bacterial products (such as lipopolysaccharide and flagellin) are not generally associated with bacteraemia but elicit a systemic immune response and microbial product translocation has been described in various clinical conditions such as inflammatory bowel disease, visceral leishmaniasis, graft versus host disease, patients with extensive burn wounds, haemorrhagic shock, acute alcohol intoxication and in chronic HIV infection (21, 111-116). HIV infection causes early and profound

damage to the structural and immunological barriers of the gastrointestinal tract during acute infection. Mucosal structural integrity is compromised by enterocyte apoptosis and disruption of tight junctions. The immunological barrier is compromised by profound early depletion of CD4+ T cells within the gastrointestinal mucosa, preferential depletion of Th-17 cells, local mucosal inflammation and exhaustion of intestinal macrophages' phagocytic function. Together, these changes result in increased intestinal mucosal permeability and translocation of microbes and/or microbial products into the systemic circulation (107-110). In patients with Crohn's disease the presence of bacterial DNA in blood is independently associated with relapse within 6 months and with other adverse clinical endpoints such as risk of hospitalization, switch of treatment and initiation of steroid treatment (23). In liver cirrhosis the presence of microbial DNA in the blood is associated with mortality (22).

In HIV, damage to the gastrointestinal barrier, which occurs early during acute HIV infection, recovers slowly on antiretroviral therapy, but does not recover fully despite suppressive long-term antiretroviral therapy and can be averted by starting antiretroviral therapy during acute HIV infection (107). In HIV infection, microbial translocation contributes to chronic systemic inflammation and immune activation, which is associated with disease progression and mortality (21). It is unknown if this occurs in the setting of advanced HIV-associated tuberculosis, but data from autopsy studies show frequent concomitant bacterial infections which could support this hypothesis (117), albeit the route of bacterial entry may be from sources other than the gastrointestinal system such as the lungs.

In HIV-TB co-infection there are additional factors which could contribute to

decreased gastrointestinal mucosal integrity. Patients with severe HIV-associated tuberculosis often present with clinical features compatible with bacterial sepsis of which a feature is intestinal hypoperfusion (18, 98, 99). Intestinal tuberculosis is present in 36- 43% of post-mortem HIV-TB cases (118, 119) and can affect the mucosa, sub-mucosal layers, lymph nodes and peritoneum. Malnutrition negatively affects gastrointestinal mucosal integrity and could play a role in patients with severe HIV-associated tuberculosis.

Microbial product translocation can be measured directly by quantifying microbial products such as LPS or bacterial 16s ribosomal DNA (16s rDNA) in the peripheral blood. Indirect measures of microbial translocation infer the degree of microbial translocation by measuring concentrations of the binding proteins and antibodies which are involved in the immune response to LPS, such as soluble CD14, lipopolysaccharide binding protein (LBP) and endotoxin core antibody (EndoCAB). Markers of damage to the gastrointestinal mucosal lining have also been used to infer the degree of microbial translocation. Microbial translocation is limited during acute HIV infection when there are low concentrations of LPS and raised sCD14, LBP and EndoCAB concentrations, whereas EndoCAB concentrations decrease and LPS concentrations increase during chronic HIV infection (109).

We measured LPS concentrations and also bacterial 16s ribosomal DNA concentrations in a subset of patients as direct measures of microbial translocation. Lipopolysaccharide (LPS or endotoxin) is an integral part of the gram-negative bacterial cell wall and is a potent stimulator of the immune system. LPS stimulates monocyte/macrophages by binding to cell-surface CD14 receptors. CD14 binds LPS

to the toll-like receptor 4 (TLR4)/MD2 complex which triggers a cascade that results in the production of inflammatory cytokines and type 1 interferon (IFN). An exaggerated immune response to LPS may be potentially harmful and effective control of the cellular immune response is vital, thus there are also efficient mechanisms to clear LPS from the circulation without initiating an immune response (138). Bacterial 16s ribosomal DNA (16s rDNA) is part of the chromosomal DNA in bacteria and is a highly conserved universal DNA sequence in bacterial species which encodes the 16s ribosomal RNA which is essential for bacteria to function. It is widely used to identify and classify bacteria (139).

We measured three indirect markers of lipopolysaccharide activity. Soluble CD14 (sCD14) is produced by activated monocytes and macrophages and either facilitates a pro-inflammatory immune response via membrane bound CD14 and the TLR4/MD2 complex or clears LPS from the system without stimulating an immune response (140). Higher concentration of sCD14 is a non-specific marker of monocyte and macrophage activation and is regarded as an indirect marker of the presence of LPS immune stimulation. Lipopolysaccharide binding protein (LBP) is an acute phase protein which, similar to sCD14, can facilitate an immune response to LPS or clear it from circulation without stimulating an immune response. Higher concentrations of LBP are associated with higher concentrations of LPS in the circulation. Endotoxin core antibody (EndoCAB) is an antibody which is involved in clearing LPS from systemic circulation. EndoCAB which is bound to LPS is not measurable by the EndoCAB assay, thus lower concentrations of EndoCAB are regarded as indicating higher concentrations of LPS in the circulation. We also measured two markers of gastrointestinal mucosal damage, trefoil factor 3 (TFF3)

and intestinal fatty acid binding protein (IFABP). TFF3 is a secretory protein which is produced by mucous secreting cells of the intestinal mucosa (141) and concentrations increase when gastrointestinal mucosal damage occurs. Intestinal fatty acid binding proteins are cytoplasmic proteins which are present in intestinal epithelial cells and are rapidly released into systemic circulation when enterocytes necrosis occurs (142).

In the setting of severe HIV-associated tuberculosis we postulated an association between microbial translocation and mortality. In patients severely ill with HIV-associated tuberculosis, the increased systemic inflammatory response to products of microbial translocation may contribute to mortality. It is also plausible that higher concentrations of microbial products may be the harbinger of the translocation of whole viable bacteria from the gastrointestinal tract into the bloodstream which may result in sepsis and mortality.

We measured direct and indirect markers of microbial translocation and markers of gastrointestinal epithelial damage in hospitalized patients with HIV-associated tuberculosis and HIV-infected outpatient controls without tuberculosis and determined 12-week mortality in hospitalized patients. The objective of our study was to determine if there are higher levels of microbial translocation in hospitalized HIV-TB patients compared to HIV-infected outpatients and to determine the association of markers of microbial translocation with mortality.

We hypothesized that in hospitalized patients with severe HIV-associated tuberculosis, intestinal immunity and mucosal integrity are impaired and result in:

a) higher levels gastrointestinal mucosal damage and more microbial translocation, compared to HIV infected outpatients without tuberculosis,

b) higher levels of gastrointestinal mucosal damage and more microbial translocation would be associated with 12-week mortality in patients hospitalized with HIV-associated tuberculosis.

Patients and methods

Study Design and Study population

We performed a prospective observational cohort study and enrolled non-pregnant, HIV-positive adults with CD4 count of ≤350 cells/μl admitted to Khayelitsha Hospital with a new diagnosis of tuberculosis (described in detail in Chapters 1 and 3) and ambulant HIV-positive outpatients within a similar CD4 count range without tuberculosis at Ubuntu clinic, Site B Khayelitsha, between January 2014 and November 2016. Outpatient controls were enrolled during a routine visit to the primary care antiretroviral clinic and screened to exclude tuberculosis. They completed a symptom screen and had sputum (if able to produce sputum) and urine Xpert MTB/RIF testing performed at enrolment. Markers of microbial translocation were measured in a randomly selected sub-group of hospitalized patients with HIV-associated tuberculosis and in controls. Hospitalized patients were followed up for 12 weeks to ascertain survival status.

Laboratory methods:

Peripheral blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) at the time of enrolment (baseline), and transported to the laboratory in cooled transport boxes. Samples were centrifuged at

3000 rpm for 5 minutes and plasma was collected and stored at -80°C. Translocation markers were measured in a randomly selected subset of participants from the parent study and in outpatient control patients with HIV infection and no tuberculosis. To limit freeze-thaw cycles, the samples were firstly identified and the sample positions on each plate were planned for each marker we planned to test. The samples were then thawed and the required volume of sample was transferred onto a plate for each planned assay. These plates were clearly labelled and stored at -80°C. The plates were thawed on the day the assay was performed.

Lipopolysaccharide (LPS):

We measure LPS concentrations using the Limulus Amebocyte Lysate (LAL) QCL-1000 assay (Lonza) assay. This test uses the activation of the Limulus Polyphemus or Tachypleus species of the horseshoe crab amebocyte clotting cascade to measure LPS concentration (143). The clotting cascade is activated by the lipid A moiety of the LPS structure and this LPS detection system was introduced and has been in use since the 1970s (144) . We followed an optimized protocol using pyrogen free consumables. Samples (20 µL) were diluted to a 1:10 dilution with endotoxin-free reagent water, heat-inactivated at 70 °C for 15 minutes and centrifuged. The E.Coli O111:B4 stock vial was reconstituted and diluted to a strength of 0.5 EU/ml using the Certificate of Analysis accompanying the kit. Standard dilutions were prepared in glass pyrogen-free dilution tubes and ranged from 0.5 EU/ml to 0 EU/ml. A volume of 50 µL standards and samples were loaded onto the plates in triplicate, equilibrated at 37°C for 5 minutes. Reconstituted LAL reagent (50 µL) was added, plates incubated for 12 minutes and 100 µL of chromogenic substrate added. Plates were incubated for a final 7 minutes, 25%

acetic acid was added as a stop solution and plates were read at 415 nm. Standards and samples were loaded in triplicate, and LAL reagent and substrate were added to the first two wells and the third was used to measure background. We used a linear regression curve to create a standard curve. Endotoxin Units (EU) are a measure of the biological activity of the endotoxin and is not based on mass, which does not account for different potency of different endotoxin preparations. Using EU/mL allows comparison of results of different types of LAL tests (145).

Markers involved in the immune response to LPS: sCD14; LBP and EndoCAB:

Soluble CD14 is a glycoprotein produced by activated macrophages in response to LPS stimulation. We measured sCD14 in stored plasma using R&D Quantikine ELISA kits. We followed manufacturer's instructions and used a 1:200 sample dilution, but we found >30% of samples had sCD14 concentrations higher than the standard range of 250 to 16000 pg/mL. We performed a dilution experiment and a 1:1000 sample dilution was optimal for our samples and was used for all the sCD14 assays. We used a quadratic curve to create a standard curve.

Lipopolysaccharide binding protein (LBP) is an acute phase protein which binds to LPS and was measured using Hycult Biotech ELISA kits. We followed the manufacturer's instructions and used a 1:1000 sample dilution, however the majority of samples had concentrations which were higher than the standard range of 0 to 50 ng/mL. We then performed a dilution experiment and a 1:10000 sample dilution was found to be optimal and used for all the LBP assays. We used a 5-parameter curve to create a standard curve.

Endotoxin core antibody IgM was tested using Hycult Biotech ELISA kit with a 1:100 sample dilution as per manufacturer's instructions and this dilution was optimal to

detect EndoCAB in the standard range of 0 to 3.5 MMU/mL. We used a 5-parameter curve to create a standard curve.

Markers of gastrointestinal endothelial damage: TFF3 and IFABP:

Trefoil factor 3 is involved in gastrointestinal mucosal repair and we measured concentrations using R&D Quantikine ELISA kits. We performed a dilution experiment and found that manufacturer's instruction of a 1:50 sample dilution was optimal to detect TFF3 concentrations in the standard range of 39 to 25000 pg/mL. We created a standard curve with a 4-paramenter curve.

Intestinal fatty acid binding protein is released from necrotic enterocytes and we measured concentrations with the Hycult Biotech ELISA, using kit with the recommented 1:2 sample dilution, which was optimal to measure concentrations within the standard range of 0 to 3000 pg/mL. We used a 5-parameter curve to create the standard curve.

All ELISA assays were corrected for the dilution factor and concentrations which were out of the range of the standard curve were imputed. Concentrations which were higher than the range of the standard curve were imputed as the highest value of the standard curve and concentrations below the range of the standard curve were imputed as half of the lowest value of the standard curve.

Bacterial 16s ribosomal DNA concentrations:

Bacterial 16s ribosomal DNA (16s rDNA) is part of the chromosomal DNA in bacteria and is a universal DNA sequence in prokaryotes. In a subset of patients bacterial 16s ribosomal DNA (16s rDNA) quantitation was performed on stored whole blood by a commercial laboratory, Vaiomer (Labège, France). The subset of samples

consisted of all hospitalized patients who died and a randomly selected group of hospitalized survivors and outpatient controls with available stored whole blood samples. Vaiomer is a biotech company with expertise in tissue and blood microbiota. Vaiomer was founded in 2011 and investigates the role of the tissue and blood microbiome in various clinical conditions such as cardiometabolic diseases, neurodegenerative disorders and infectious diseases (146-149). They described the blood microbiome profile of healthy blood donors (150), in chronic kidney disease (151) and a case of polymicrobial bacteraemia (152). DNA was extracted from the samples using an optimized tissue-specific technique. The total bacterial 16s rDNA concentrations in the samples were measured by quantitative PCR in triplicate and normalized using a plasmid-based standard scale. The amount of bacterial DNA was assessed using the "Universal 16S Real Time qPCR" workflow established by Vaiomer (Vaiomer SAS, Labège, France).

Other clinical samples:

Baseline blood tests (CD4 count, HIV viral load, C-reactive protein) were performed by the National Health Laboratory Service (NHLS) as previously reported (described in detail in Chapter 3). Three tests were used to calculate a tuberculosis dissemination score (described in the data collection and definitions section below). Urine samples were tested at the NHLS laboratory using the Xpert MTB/RIF assay G4 version 5. Thirty to forty millilitre of urine was centrifuged at 3000 g for 15 minutes on the day of collection, the supernatant was removed and the pellet resuspended in the remaining urine (approximately 0.5 mL). No phosphate buffer was added and 0.75 ml of this concentrated urine was tested after the addition of Xpert sample reagent as per manufacturer's instructions and previously reported (15,

153, 154). Mycobacterial blood cultures were performed at the NHLS using Myco/F Lytic bottles (Becton Dickinson Biosciences). Five millilitre of whole blood was inoculated and bottles were incubated for 42 days. The Genotype MTBDRplus assay (Hain Lifesciences) was used to identify *Mycobacterium tuberculosis* complex from positive blood cultures. Urine lipoarabinomannan (LAM) testing was performed by an independent laboratory on frozen urine samples using Alere Determine™ TB LAM antigen test. The test strips were read by two independent readers who were blinded to clinical details and outcome.

Data collection and definitions:

Clinical data and baseline blood tests were obtained at enrolment and captured on standard case record forms. We used a three-point dissemination score previously described (26) to quantify the degree of mycobacterial dissemination. We allocated a dissemination score to all patients who had a valid result for the urine Xpert MTB/RIF assay, urine Alere LAM test and mycobacterial blood culture. Each positive test was allocated one point if positive for *M. tuberculosis*, yielding a score ranging from 0 to 3.

Statistical Analysis:

Comparisons were made between hospitalized patients admitted with a new diagnosis of HIV-associated tuberculosis and outpatient controls who were HIV-infected and had no active tuberculosis, and also between hospitalized patients who died within 12 weeks and those who survived. Analyses were performed using R statistical software, Graphpad Prism and JMP Statistical Software (developed by SAS Institute). We used non-parametric tests (Kruskal Wallis or Wilcoxon rank sum

test where appropriate) for comparisons. We report median values with interquartile range as measures of central tendency and number and percentage for categorical variables.

We plotted and compared LPS concentrations in the initial exploratory analyses (comparisons between hospitalized and outpatient groups and hospitalized patients who died versus those who survived) but we did not include LPS concentrations in subsequent analyses because we found low concentrations overall and no difference between groups. We included patients with a complete set of indirect markers of microbial translocation in the cluster analysis (n=341). We performed non-hierarchical 2-way cluster analysis using Ward's method and microbial translocation marker values were standardized to range from a minimum value of zero and a maximum value of one. We performed cluster analysis including all patients. We assessed the distribution of the different clusters across these groups. We repeated the cluster analysis including only hospitalized patients with known outcome. Comparisons of markers of microbial translocation between groups were made using all patients with valid results for a specific marker of microbial translocation.
We performed correlation analyses of the indirect markers of microbial translocation and selected clinical variables to assess for strong collinearity before performing univariable and multivariable logistic regression analyses. We performed logistic regression analysis including markers of microbial translocation and selected clinical variables (age, sex, HIV viral load) to assess the effect of markers of microbial translocation on 12-week mortality.

Increased numbers of positive markers of tuberculosis dissemination were associated with a linear increase in mortality in the parent study, so we plotted the microbial translocation marker concentrations against the dissemination score to assess the association of these markers with the number of positive markers of tuberculosis dissemination. We performed correlation analyses of the indirect markers of microbial translocation, selected clinical variables and bacterial 16s rDNA concentration to assess for strong collinearity before multivariable analyses. We also plotted the markers of microbial translocation against bacterial 16s rDNA concentrations to assess these associations visually. We performed univariable and multivariable linear regression analyses to assess the association of indirect markers of microbial translocation with 16s rDNA concentrations. We included selected clinical variables, age, sex, HIV viral load and C-reactive protein (a non-specific marker of inflammation). We plotted bacterial 16s rDNA concentrations against the three-point dissemination score and plotted Kaplan Meier curves to assess the relationship between 16s rDNA concentration and survival.

Ethical approval

The study was approved by the University of Cape Town Human Research Ethics Committee (UCT HREC reference: 057/2013). Additional detail provided in Chapter 1 and Chapter 3.

Results

Outcomes and baseline characteristics

The parent study enrolled 576 hospitalized patients with HIV-associated tuberculosis and 35 outpatients with HIV infection, without tuberculosis (Figure 1). Markers of

microbial translocation were tested in a randomly selected sub-group of hospitalized patients. There were n=341 hospitalized patients and n=32 outpatient controls who had a complete set of microbial translocation markers measured (Figure 1, Table 1). The number of patients who had each marker measured is presented in Table 1. Twelve-week mortality for hospitalized patients was 71/341 (21%) with median days from enrolment to death = 11 days (interquartile range: 6-34 days). 5 patients were lost to follow up. The median CD4 count was 59 cells/μl and 167 cells/μl ($p < 0.001$) for hospitalized and outpatients respectively, and 35 cells/μl vs 68 cells/μl ($p < 0.001$) in hospitalized patients who died and those who survived (Table 2 and 3).

Figure 1: Study flow chart:

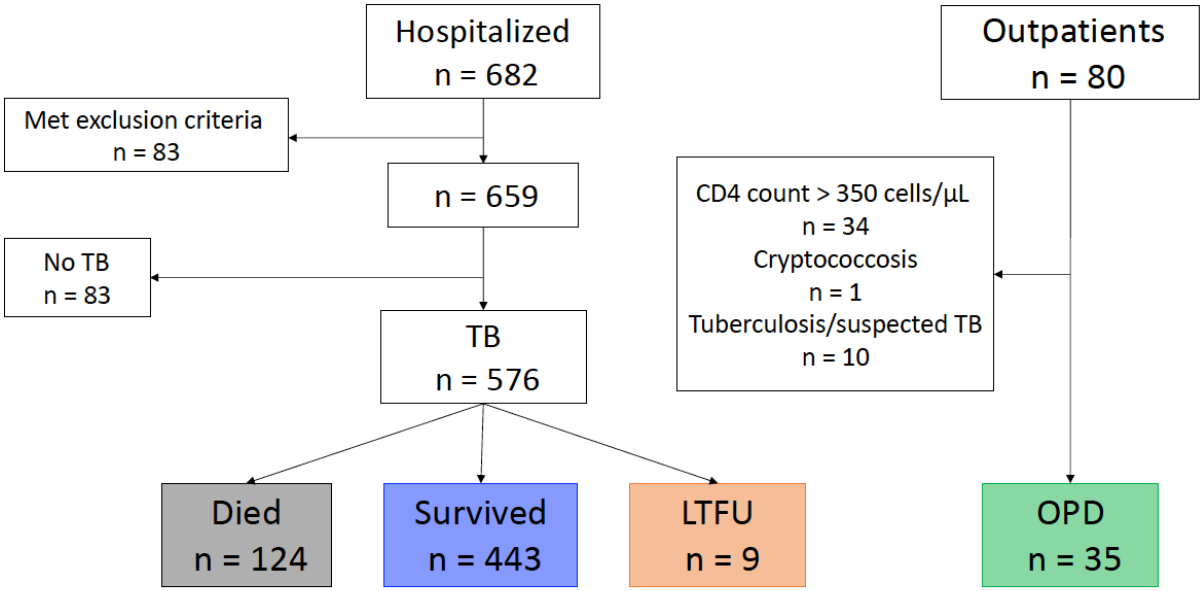


Figure 1: Study flow chart showing numbers of patients enrolled and 12 week outcome in hospitalized patients.

Table 1: Randomly selected patients who had translocation markers tested and 16s ribosomal DNA quantified:

Marker	n tested	Died	Survived	LTFU	OPD
LPS	392	79	274	7	32
sCD14	454	86	329	7	32
LBP	379	72	269	6	32
EndoCAB IgM	373	71	265	5	32
TFF3	379	72	269	6	32
I-FABP	379	72	269	6	32
All MTL markers	373	71	265	5	32
16s rDNA	235	111	104	-	20
All MTL markers & 16s rDNA	152	62	70	-	20

Table 1: LPS: lipopolysaccharide; sCD14: soluble CD14; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; TFF3: trefoil factor 3; IFABP: intestinal fatty acid binding protein; MTL: microbial translocation markers; 16s rDNA: bacterial 16s ribosomal DNA quantified from stored whole blood; n = number of patients who had the specific marker quantified; Died: hospitalized patients with HIV-associated tuberculosis who

died within 12 weeks of enrolment; Survived: hospitalized patients with HIV-associated tuberculosis who survived up to 12 weeks after enrolment; LTFU: Hospitalized patients with HIV-associated tuberculosis who were lost to follow up within 12 weeks of enrolment; OPD: Outpatient controls with HIV infection and no tuberculosis.

Table 2: Baseline characteristics of patients who had microbial translocation markers measured: Comparison of hospitalized patients and outpatient controls.

	Hospitalized n = 341	OPD n = 32	p
Sex, Female	167 (49)	23 (71.9)	**0.022**
Age, years	36 [31, 44]	35 [30, 42]	0.678
CD4 count, cells/μL	59 [22, 116]	168 [102, 229]	**< 0.001**
HIV viral load, log_{10} copies/mL	5.10 [3.30, 5.74]	4.27 [1.59, 4.82]	**0.004**
C-reactive protein, mg/L	148.05 [95.28, 220.73]	-	-

Table 2: Hospitalized: Hospitalized patients with HIV-associated tuberculosis; OPD: Outpatient controls with HIV infection and no tuberculosis. Outpatient controls did not have C-reactive protein concentrations performed.

Table 3: Baseline characteristics of hospitalized patients with HIV-associated tuberculosis who had microbial translocation markers measured: Comparison of patients who died within twelve weeks and those who survived.

	Hospitalized: Died n = 71	Hospitalized: Survived n = 265	p
Sex, Female	37 (52.1)	130 (49.1)	0.746
Age, years	41 [55, 47]	35 [31, 43]	**0.002**
CD4 count, cells/μL	35 [17, 77]	68 [26, 127]	**< 0.001**
HIV viral load, log_{10} copies/mL	4.98 [3.29, 5.74]	5.11 [3.43, 5.75]	0.567
C-reactive protein, mg/L	174.70 [109.35, 248.85]	145.75 [89.55, 211.25]	**0.008**

Table 3: Hospitalized: Died: Hospitalized patients with HIV-associated tuberculosis who died within 12 weeks after enrolment; Hospitalized: Survived: Hospitalized patients with HIV-associated tuberculosis who survived until 12 weeks after enrolment;
Five patients who were lost to follow up are not included in this table.

Comparing concentrations of microbial translocation markers in hospitalized patients with HIV-TB to outpatients with HIV infection, two of the markers involved in the immune response to LPS; sCD14 and LBP were both significantly higher in

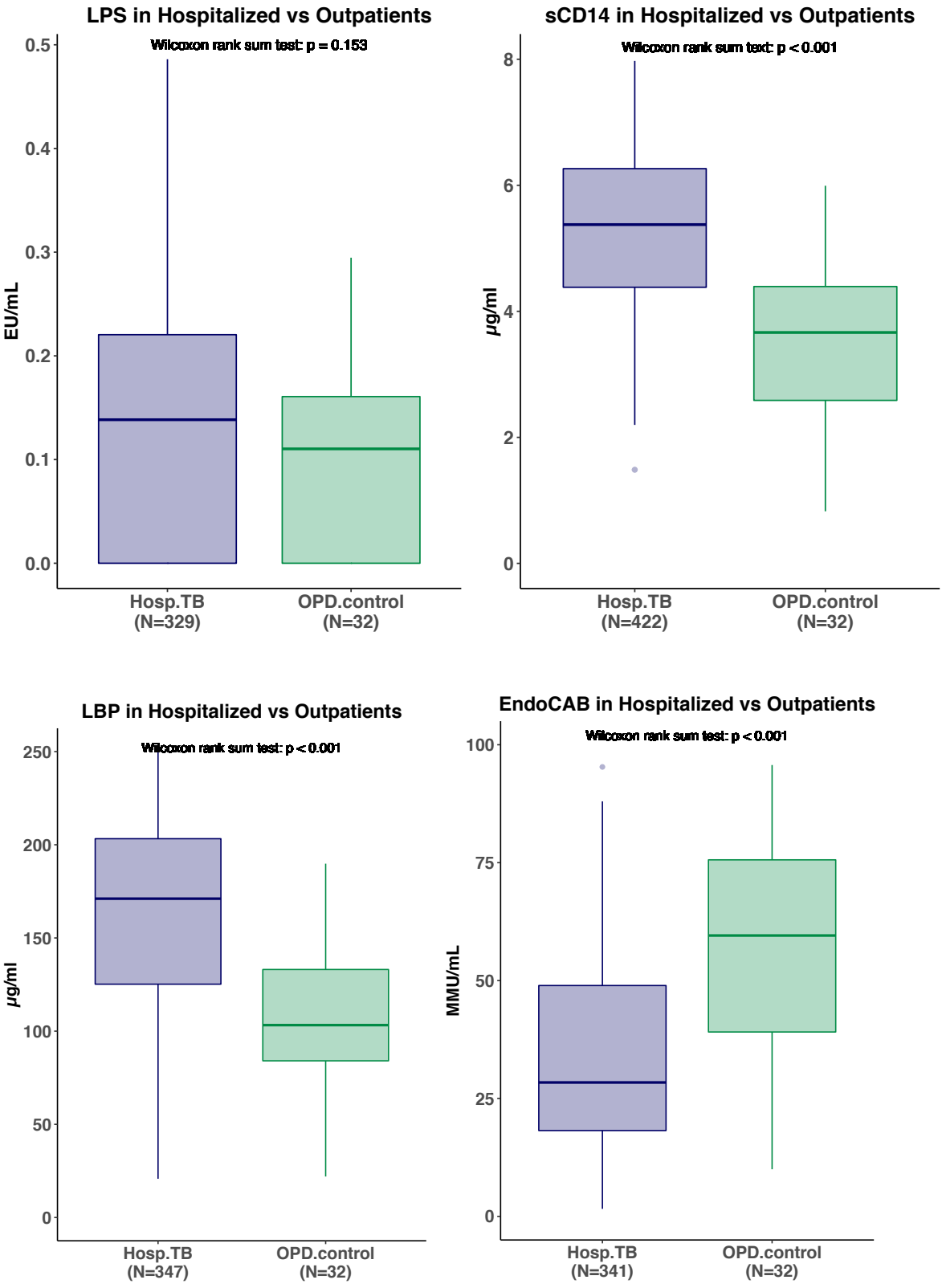
hospitalized patients and EndoCAB was significantly lower as hypothesised. There was no difference in LPS concentration and overall LPS concentrations were low. One marker of gastrointestinal mucosal damage, TFF3 concentrations was significantly higher in hospitalized patients, which was consistent with our hypothesis but IFABP concentrations were significantly higher in outpatient controls, which was the opposite of what we expected to find (Table 4 and Figure 2).

Table 4: Microbial translocation marker concentrations: Hospitalized patients with HIV-associated tuberculosis compared to HIV-infected outpatients without tuberculosis.

Marker	n	Hospitalized	Outpatient controls	p
LPS, EU/mL	373	0.14 [0.00, 0.22]	0.11 [0.00, 0.16]	0.153
sCD14, µg/mL	454	5.68 [4.60 - 7.03]	3.66 [2.59 - 4.39]	**<0.001**
LBP, µg/mL	379	188.11 [142.80 - 248.79]	103.23 [84.07 - 133.07]	**<0.001**
EndoCAB, MMU/L	373	33.50 [19.30 - 57.90]	100.35 [62.73 - 208.45]	**<0.001**
TFF3, ng/mL	379	15.35 [11.10 - 24.51]	10.97 [9.71 - 13.22]	**<0.001**
IFABP, pg/mL	379	200.21 [73.54 - 430.37]	610.76 [385.69 - 937.29]	**<0.001**

Table 4: Median and interquartile range presented; n: number of patients in the cohort who had the marker tested; EU/mL: Endotoxin standard units per millliliter; µg/mL: microgram per milliliter; MMU/mL: standard median units per millliliter; ng/mL: nanogram per millliliter; pg/mL: picogram per milliliter; LPS; lipopolysaccharide; sCD14: soluble CD14; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; TFF3: trefoil factor 3; IFABP: intestinal fatty acid binding protein; Hospitalized: patients hospitalized with HIV-associated tuberculosis; Outpatient controls: outpatients with HIV infection and no tuberculosis.

Figure 2: Microbial translocation marker concentrations in hospitalized patients with HIV-associated tuberculosis versus HIV-infected outpatients without tuberculosis:



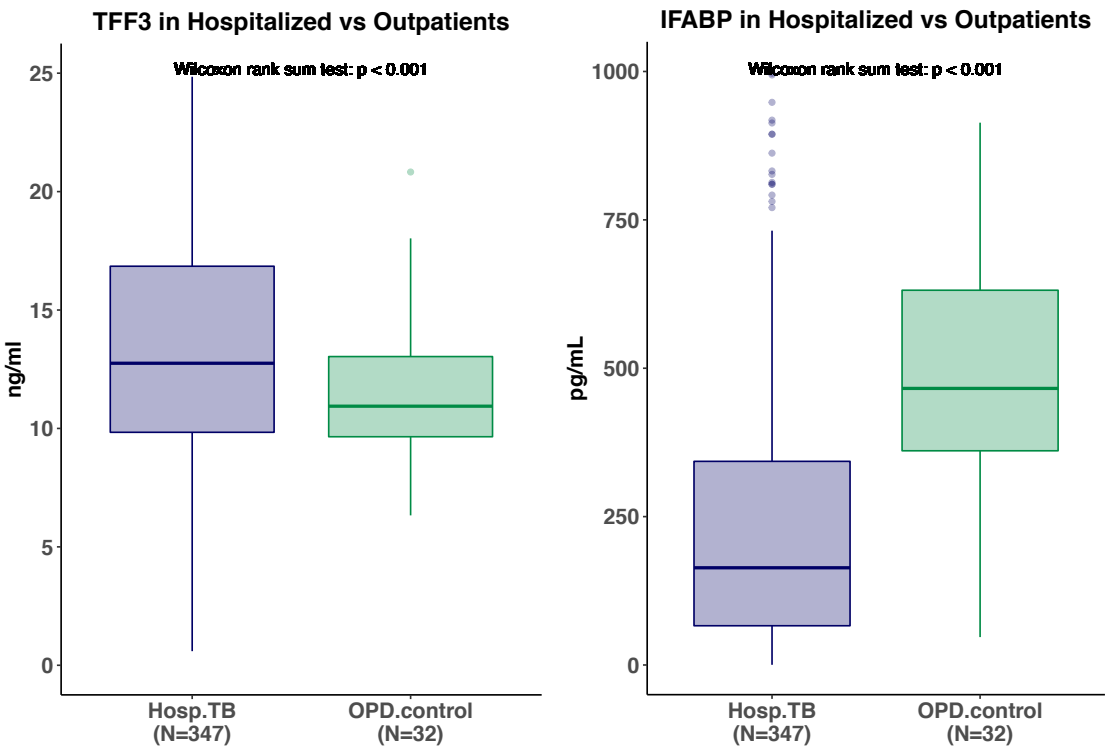


Figure 2: Hosp.TB: Patients hospitalized with HIV-associated tuberculosis; OPD.control: Outpatients with HIV infection and no tuberculosis; EU/mL: Endotoxin standard units per milliliter; µg/mL: microgram per milliliter; MMU/mL: standard median units per milliliter; ng/mL: nanogram per milliliter; pg/mL: picogram per milliliter; LPS; lipopolysaccharide; sCD14: soluble CD14; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; TFF3: trefoil factor 3; IFABP: intestinal fatty acid binding protein.

Comparing hospitalized patients with HIV-TB who died within 12 weeks and those who survived, sCD14 concentrations were significantly higher and EndoCAB concentrations were significantly lower in patients who died, which is consistent with our hypothesis. LPS and LBP concentrations showed no difference. TFF3, which indicates gastrointestinal mucosal damage, was significantly higher in patients who died and IFABP concentrations showed no difference between outcome groups (Table 5 and Figure 3).

We unexpectedly found very low LPS concentrations in all samples. LPS concentrations were not detected in 30% of all samples tested. We re-tested non-

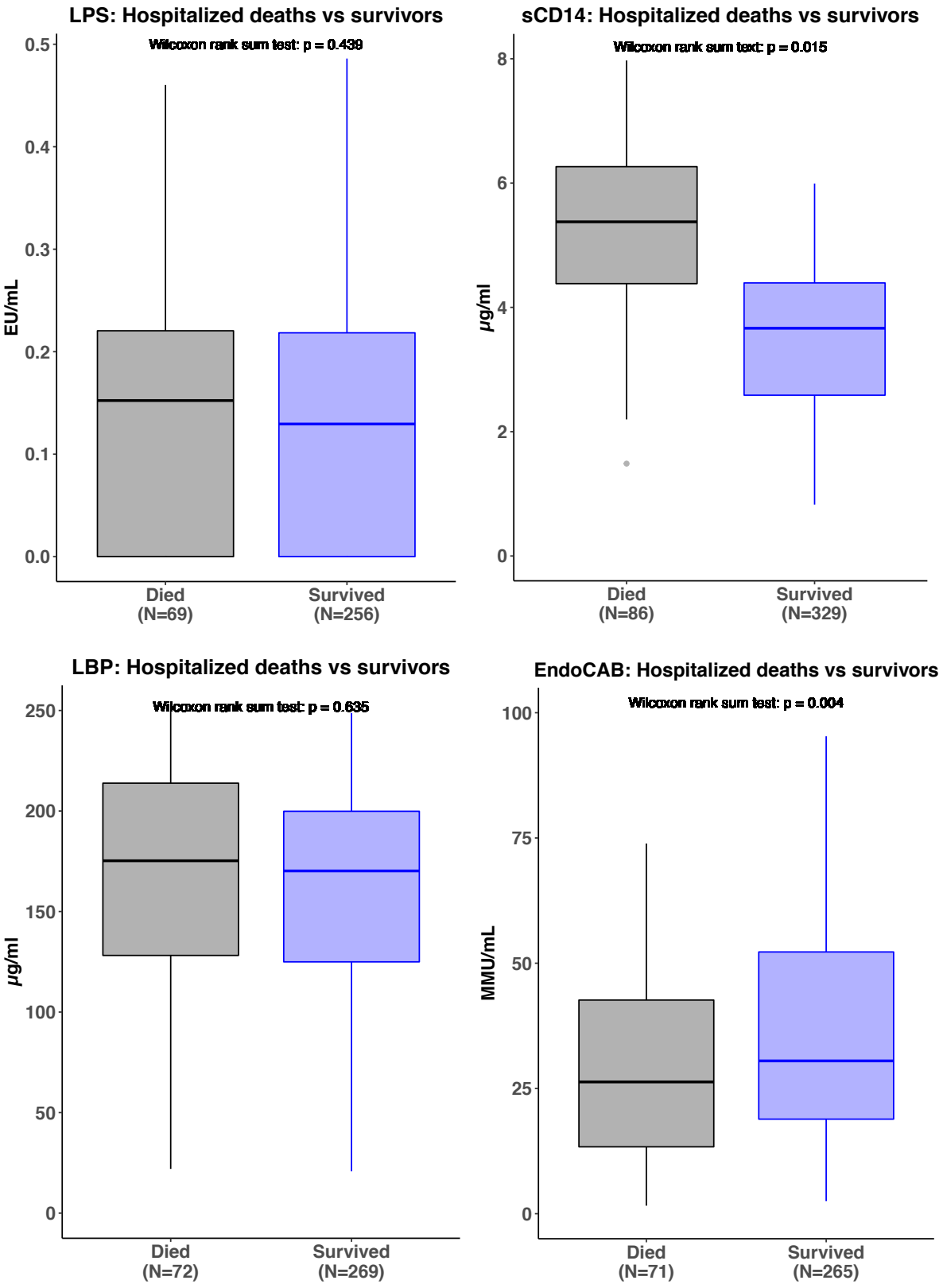
precious samples from the same cohort at a 1:2 dilution, extended the time of heat-inactivation and still found low LPS concentrations. We re-tested the first two batches of samples together with a senior laboratory scientist who has extensive experience with these assays to assess if there were any technical problems with performance of the assays. We found similarly low LPS concentrations and no technical problems with performance of the assays. However, the samples which were re-tested had more than 2 freeze-thaw cycles at the time of re-testing. We then repeated the experiment with samples which had no previous freeze-thaw cycles and found similarly low LPS concentrations. We concluded that there was likely a biological explanation for the low LPS concentrations. We explored other ways to measure LPS, but none of the options available at the time were feasible to perform in our laboratory.

Table 5: Microbial translocation marker concentrations: Hospitalized patients with HIV-associated tuberculosis who died within 12 weeks compared to patients who survived.

Marker	n*	Hospitalized: Died n = 71	Hospitalized: Survived n = 265	p
LPS, EU/mL	336	0.15 [0.00, 0.22]	0.13 [0.00, 0.22]	0.439
sCD14, µg/mL	415	6.21 [4.76 - 8.11]	5.54 [4.52 - 6.95]	**0.015**
LBP, µg/mL	341	185.02 [141.20 - 236.66]	188.11 [142.97 - 251.19]	0.635
EndoCAB MMU/L	336	26.60 [13.50 - 43.80]	35.70 [20.00 - 61.70]	**0.004**
TFF3, ng/mL	341	25.06 [16.40 - 71.73]	13.52 [10.09 - 20.54]	**<0.001**
IFABP, pg/mL	341	234.12 [58.21 - 564.89]	190.85 [78.92 - 392.10]	0.362

Table 5: Median and interquartile range presented. n*: Number of hospitalized patients who had this marker tested. Patients who were lost to follow up are not included. EU/mL: Endotoxin standard units per milliliter; µg/mL: microgram per milliliter; MMU/mL: standard median units per milliliter; ng/mL: nanogram per milliliter; pg/mL: picogram per milliliter; LPS; lipopolysaccharide; sCD14: soluble CD14; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; TFF3: trefoil factor 3; IFABP: intestinal fatty acid binding protein

Figure 3: Markers of microbial translocation concentrations in hospitalized patients with HIV-associated tuberculosis who died within 12 weeks and patients who survived:



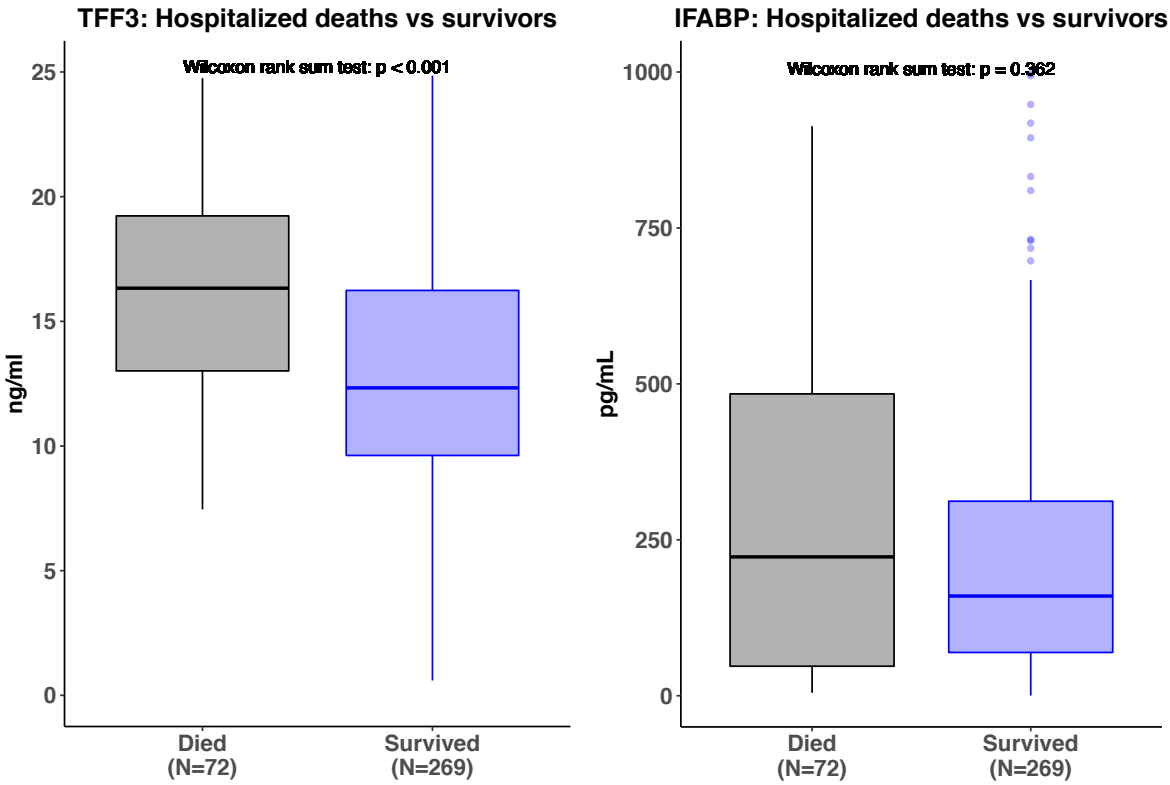


Figure 3: Died: Hospitalized patients with HIV-associated tuberculosis who died within 12 weeks after enrolment; Survived: Hospitalized patients with HIV-associated tuberculosis who survived 12 weeks; EU/mL: Endotoxin standard units per milliliter; µg/mL: microgram per milliliter; MMU/mL: standard median units per milliliter; ng/mL: nanogram per milliliter; pg/mL: picogram per milliliter; LPS; lipopolysaccharide; sCD14: soluble CD14; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; TFF3: trefoil factor 3; IFABP: intestinal fatty acid binding protein

Comparison with other African cohorts

We compared our findings to other African cohorts which included patients with HIV infection and or patients with HIV-associated tuberculosis (Table 6). Our sCD14 and LBP concentrations in hospitalized and outpatients patients were higher than other cohorts from South Africa and Uganda (155-158). EndoCAB concentrations in our cohort were lower than concentrations in an Ugandan cohort (157). Our IFABP concentrations in the hospitalized patients were five-fold lower than concentrations in Ugandan HIV-TB patients who were monitored for the development of TB-IRIS (158) and comparable with hospitalized HIV-TB patients in South Africa who had early mortality(2). We found higher IFABP concentrations in outpatient controls.

Table 6: Comparison of markers of microbial translocation concentrations to results from other African cohorts which included HIV infected patients and/or patients with HIV-associated tuberculosis:

	LPS EU/mL	sCD14 µg/mL	LBP µg/mL	EndoCAB MMU/mL	IFABP pg/mL
Our cohort results: Median (IQR) Hospitalized: HIV-TB	0.14 [0.00, 0.22]	5.68 (4.60 - 7.03)	188.11 (142.80 - 248.79)	33.50 (19.30 - 57.90)	200.21 (73.54 - 430.37)
Our cohort results Median (IQR) Outpatient HIV infected; no TB	0.11 [0.00, 0.16]	3.66 (2.59 - 4.39)	103.23 (84.07 - 133.07)	100.35 (62.73 - 208.45)	610.76 (385.69 - 937.29)
South Africa (Cassol et al) HIV infected Median, SD	2.14, 0.57	2.47, 0.78	-	-	-
South Africa (Cassol et al) HIV infected with OI Median, SD	2.42, 0.34	-	-	-	-
South Africa (Cassol et al) HIV infected without OI Median, SD	2.03, 0.43	-	-	-	-
South Africa (Cassol et al) HIV negative Median, SD	1.1, 0.26	1.61, 0.44	-	-	-
Uganda (Toossi et al) HIV-TB, CD4 <350 cells/µL *Median	~20 pg/mL	~ 3.0	~ 80	-	-
Uganda (Toossi et al) HIV-TB, CD4 >350 cells/µL *Median		~ 4.0	-	--	-
Uganda (Toossi et al) HIV infected; no TB	~ 100 pg/mL	~ 2.0	-	-	-

***Median**					
Uganda (Toossi et al) HIV negative with TB *Median	-	~ 2.0	-	-	-
Uganda (Toossi et al) HIV negative; no TB *Median	-	~ 1.0	~ 60	-	-
Uganda (Redd, et al) HIV infected *Median	~100 pg/mL	~ 2.0	-	~ 400	-
Uganda (Goovaerts et al) HIV-TB: Developed TB-IRIS Median (IQR)	16.2 (11.5-19.5)	3.4 (2.5 – 3.9)	29.7 (15.42-37.1)	-	1000 (500-1500)
Uganda (Goovaerts et al) HIV-TB: No TB-IRIS Median (IQR)	17.7 (15.3 – 20.4)	3.3 (2.5 – 4.2)	42.3 (25.1 – 53.5)	-	900 (500 – 1700)
South Africa (Subbarao et al) Hospitalized HIV-TB: Died Median (IQR)	93 pg/mL (65.8– 163.0)	-	-	-	131.5 (0 – 2033)
South Africa (Subbarao et al) Hospitalized HIV-TB: Survived Median (IQR)	57 pg/mL (0.0– 100.3)	-	-	-	0 (0.0– 6751.0)

Table 6: LPS: lipopolysaccharide; sCD14: soluble CD14; LBP: lipolopolysaccharide binding protein; EndoCAB: Endotoxin core antibody IgM; IFABP: intestinal fatty acid binding protein; EU/mL: Endotoxin standard units per milliliter; µg/mL: microgram per milliliter; MMU/mL: standard median units per milliliter; pg/mL: picogram per milliliter; HIV-TB: HIV-associated tuberculosis

*Results not reported in paper, but extracted from figures.

LPS column pg/mL values: 100 pg/mL is equivalent to 1 EU/mL (reference: Toossi et al)

Trefoil factor 3 not included in this table because no other African cohorts reported trefoil factor 3 results to compare our results to.

Fold differences between groups:

We calculated fold differences in translocation markers and compared hospitalized patients with HIV-TB to outpatient controls and hospitalized patients with HIV-TB who died to those who survived. We also compared hospitalized patients who died and hospitalized patients who survived to outpatient controls respectively. Comparing hospitalized patients with HIV-TB to outpatient controls (Figure 4: panels A, C and D), sCD14, TFF3 and LBP were higher in hospitalized patients and EndoCAB and IFABP were lower, with the most pronounced differences between hospitalized patients who died and outpatient controls. Comparing hospitalized HIV-TB patients who died to survivors (Figure 4: panel B), sCD14, TFF3 and IFABP were higher and EndoCAB and LBP were lower in patients who died compared to survivors.

Figure 4: Fold differences in markers of microbial translocation:

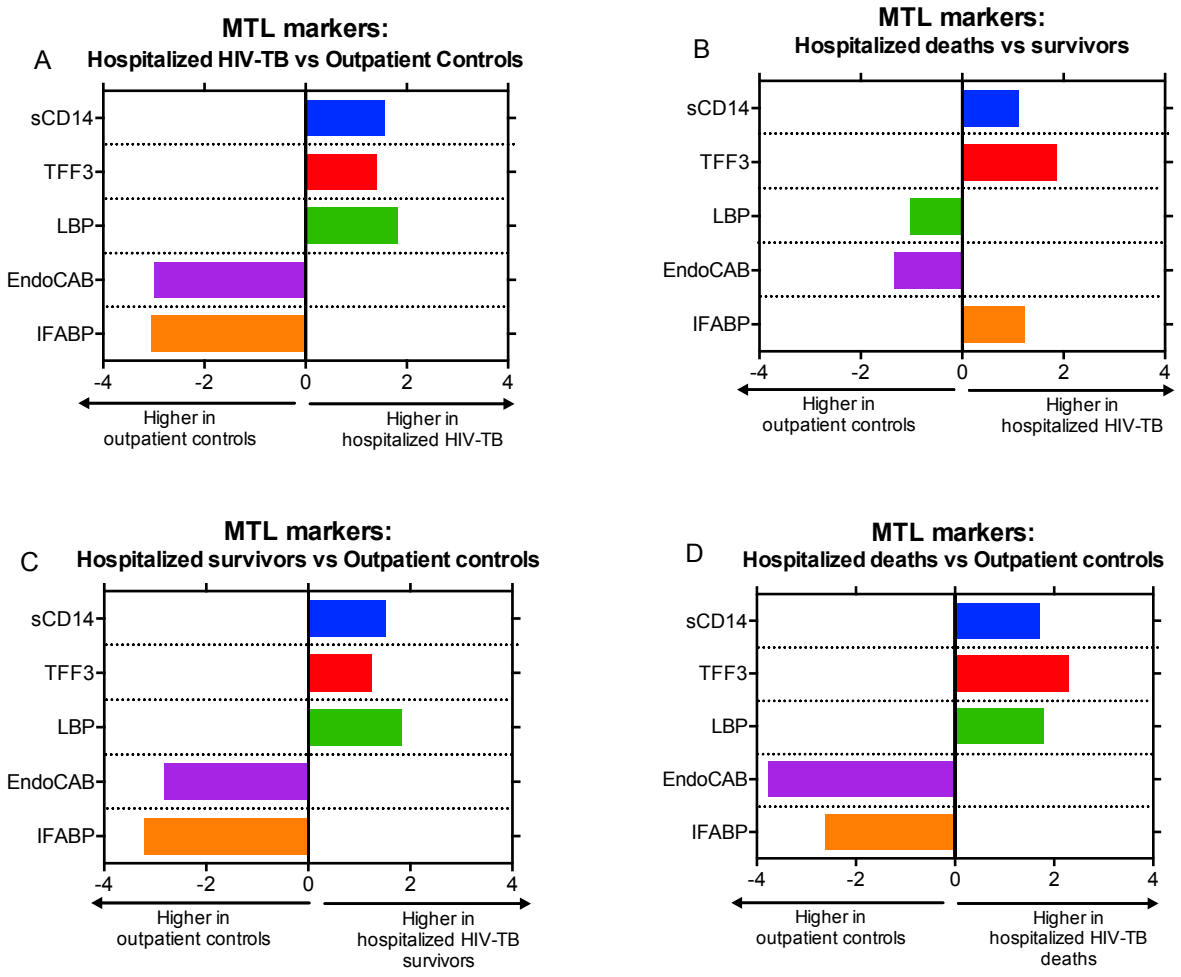


Figure 4: This figure shows fold differences in microbial translocation marker concentrations between different groups: A) hospitalized patients with HIV-associated tuberculosis versus outpatient controls with HIV infection and no tuberculosis, B) Hospitalized patients with HIV-associated tuberculosis who died within 12 weeks versus those who survived, C) hospitalized patients with HIV-associated tuberculosis who survived 12 weeks versus outpatient controls with HIV infection and no tuberculosis, D) hospitalized patients with HIV-associated tuberculosis who died within 12 weeks versus outpatient controls with HIV infection and no tuberculosis. sCD14: soluble CD14; TFF3: Trefoil factor 3; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody; IFABP: intestinal fatty acid binding protein.

Cluster analysis

We performed non-supervised two-way hierarchical cluster analysis including the five indirect markers of microbial translocation to see if these markers could distinguish hospitalized patients who died, those who survived and outpatient controls.

Cluster analysis identified 17 separate clusters within the data. The microbial translocation markers did not clearly separate the different patient groups from each other. The different clusters were fairly evenly distributed within the different groups (hospitalized deaths, hospitalized survivors, patients lost to follow up and outpatient controls, Figure 6). We also performed non-supervised two-way hierarchical cluster analysis including only the hospitalized patients and again the markers of microbial translocation did not clearly separate the patients who died within 12 weeks from those who survived (Figure 7).

Figure 5: Hierarchical cluster analysis of markers of microbial translocation:

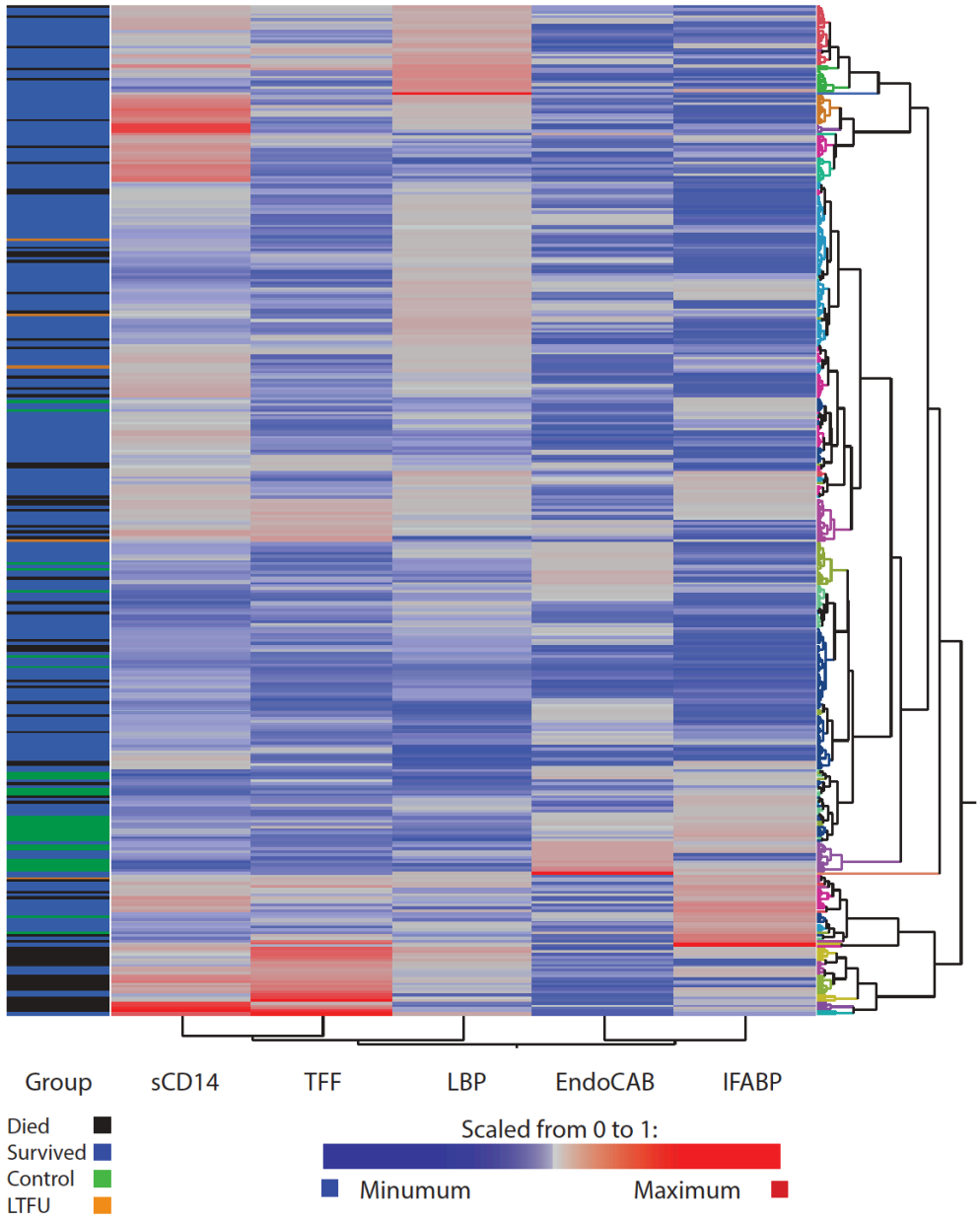


Figure 5: Non-supervised two-way hierarchical cluster analysis Hierarchical cluster analysis of microbial translocation markers and patient groups. Translocation markers were scaled from 0-1 with blue indicating the minimum value and red the maximum value of markers. Patient groups are indicated in the left hand column. Dendograms represent Euclidian distance. Group: Indicates patient group; Died: Hospitalized patients with HIV-associated tuberculosis who died within 12 weeks; Survived: Hospitalized patients with HIV-associated tuberculosis who survived 12 weeks; Control: HIV-infected outpatient without tuberculosis; LTFU: hospitalized patient with HIV-associated tuberculosis who was lost to follow up before 12 weeks; sCD14: soluble CD14; TFF3: Trefoil factor 3; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody; IFABP: intestinal fatty acid binding protein.

Figure 6: Distribution of the clusters identified during hierarchical cluster analysis by patient group:

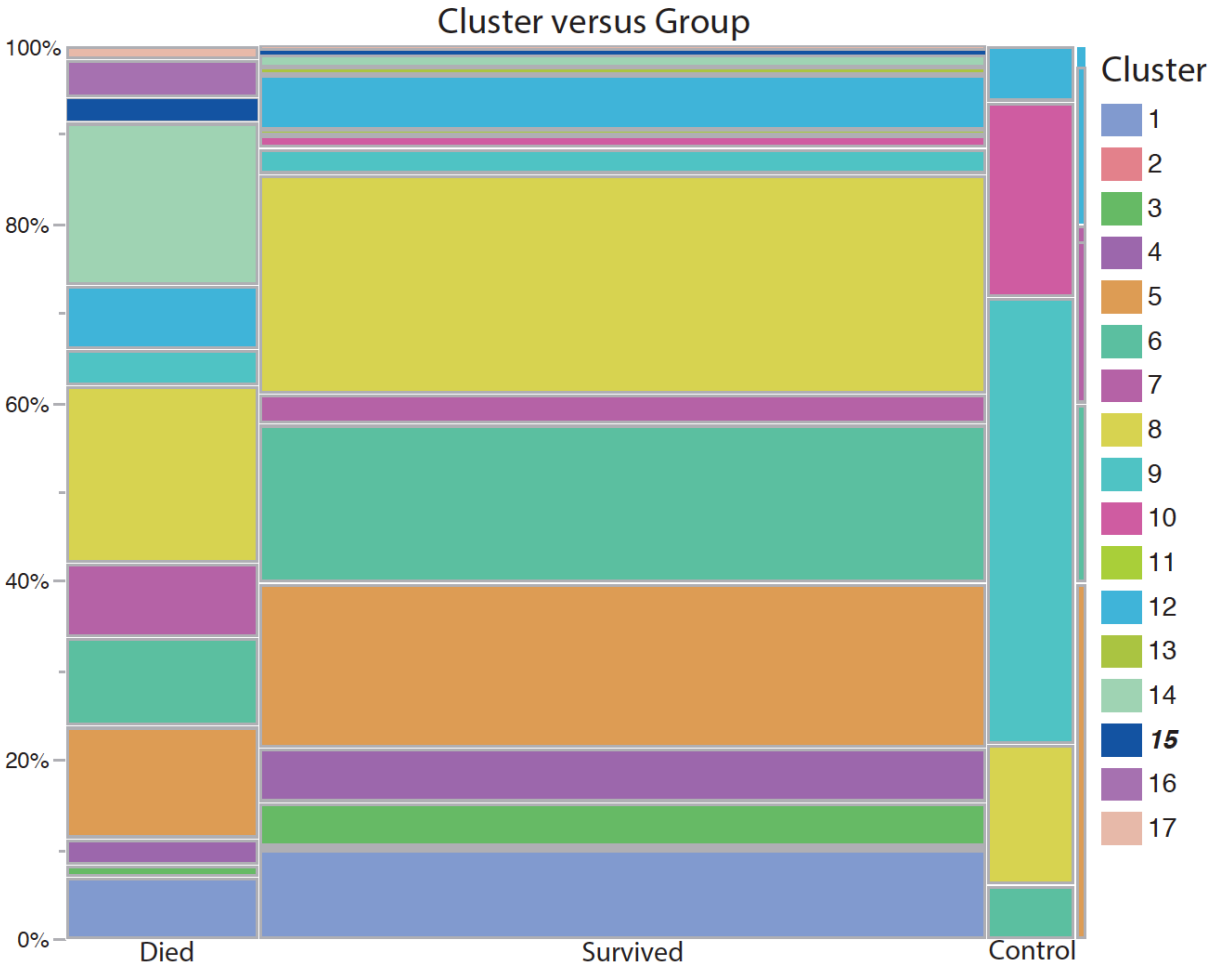


Figure 6: This figure shows the distribution of different clusters by outcome group. Died: Hospitalized patients with HIV-associated tuberculosis who died within 12 weeks; Survived: Hospitalized patients with HIV-associated tuberculosis who survived 12 weeks; Control: HIV-infected outpatient without tuberculosis. The width of the column is representative of the number of patients in each group. The vertical height of each coloured block indicates the proportion of the cluster represented in each column. The narrow column on the right hand side of the figure represents the hospitalized patients who were lost to follow up (n=9).

Figure 7: Hierarchical cluster analysis of markers of microbial translocation in hospitalized patients with HIV-associated tuberculosis:

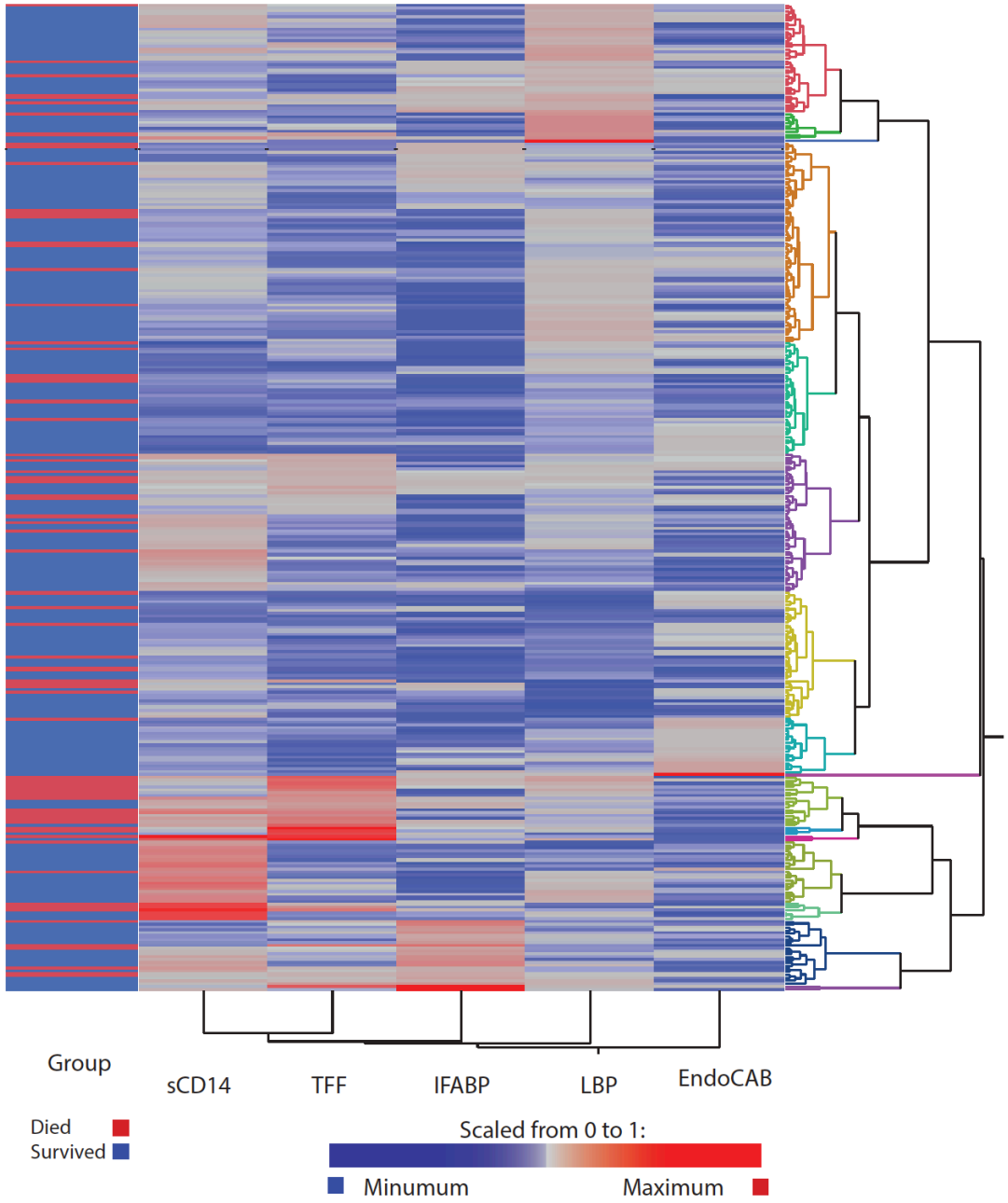


Figure 7: Non-supervised two-way hierarchical cluster analysis Hierarchical cluster analysis of microbial translocation markers and patient groups. Translocation markers were scaled from 0-1 with blue indicating the minimum value and red the maximum value of markers. Patient groups are indicated in the left hand column. Dendograms represent Euclidian distance. Group: Indicates patient group; Died: Hospitalized patients with HIV-associated tuberculosis who died within 12 weeks; Survived: Hospitalized patients with HIV-associated tuberculosis who survived 12 weeks; sCD14: soluble CD14; TFF3: Trefoil factor 3; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody; IFABP: intestinal fatty acid binding protein.

Logistic regression analyses:

We performed logistic regression analyses to assess the independent association of the indirect markers of microbial translocation with mortality in hospitalized patients. We adjusted for *a priori* selected demographic variables, age, sex and HIV viral load. To assess bivariate associations and possible collinearity we first performed correlation analyses of the microbial translocation variables (sCD14, TFF3, LBP, EndoCAB and IFABP) and the numerical demographic variables (age and HIV viral load). The only moderately strong correlation (correlation coefficient of > 0.3) was a positive correlation between sCD14 and TFF3 (Figure 8). In the absence of strong collinearity all variables were included in the multivariable analysis as planned.

Figure 8: Correlation plot of microbial translocation markers and demographic variables used in the logistic regression analysis with the outcome of death vs survival:

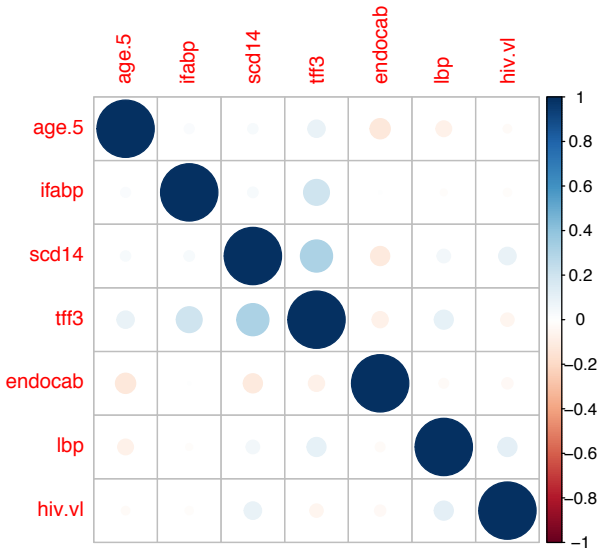


Figure 8: Correlation plot showing the correlation of markers of microbial translocation with each other and with age and HIV viral load. Correlation coefficients range from +1 (perfect positive correlation) to -1 (perfect negative correlation). A positive correlation is indicated by a blue colour and a negative correlation is indicated in red. The size of the circle represents the strength of the correlation. age.5: Age per five year increase; ifabp: intestinal fatty acid binding protein; scd14: soluble CD14; tff3: trefoil factor 3; endocab: endotoxin core antibody; lbp: lipopolysaccharide binding protein; hiv.vl: HIV viral load.

We performed univariable logistic regression analysis of each variable and then included all the variables in a multivariable model. Results are presented in Table 7. In univariable analysis, for each unit increase in sCD14 and TFF3 concentrations, and for every 5 year age increase, there was significantly higher odds of death. For each unit increase of EndoCAB concentrations, there was significantly lower odds of death. When all the translocation markers plus age, sex and HIV viral load were included in a multivariable model, only increased TFF3 concentrations remained significantly associated with mortality.

Table 7: Logistic regression analysis of microbial translocation markers in hospitalized HIV-TB patients: Outcome: 12-week mortality:

	Univariate model estimate	Odds ratio (95% CI)	p	Multivariate model estimate	Odds ratio (95% CI)	p
sCD14, µg/mL	0.096	1.1 (1.007,1.202)	**0.034**	-0.037	0.964 (0.857,1.085)	0.542
TFF3, ng/mL	0.031	1.031 (1.02,1.042)	**<0.001**	0.034	1.035 (1.021,1.049)	**<0.001**
LBP, µg/mL	-0.001	0.999 (0.996,1.002)	0.492	-0.002	0.998 (0.994,1.001)	0.152
EndoCAB, MMU/L	-0.011	0.989 (0.980,0.998)	**0.014**	-0.008	0.992 (0.983,1.001)	0.072
IFABP, pg/mL	0.000	1.000 (1.000,1.000)	0.849	-0.000	1.000 (1.000,1.000)	0.123
Age, per 5 year increase	0.189	1.208 (1.062,1.374)	**0.004**	0.122	1.13 (0.979,1.304)	0.096
Sex, Male versus Female	-0.122	0.885 (0.524,1.495)	0.647	-0.311	0.733 (0.403,1.334)	0.309
HIV viral load, per 1000 copies/mL increase	-0.000	1.000 1.000, 1.000)	0.471	-0.000	1.000 (1.000,1.000)	0.889

Table 7: sCD14: soluble CD14, TFF3: trefoil factor 3; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; µg/mL: microgram per milliliter; MMU/mL: standard median units per milliliter; ng/mL: nanogram per milliliter; pg/mL: picogram per milliliter. The univariable model odds ratio interpretation (sCD14 used as an example): For every 1 µg/mL increase in sCD14, the odds of death increases with 10%, which is statistically significant with p = 0.034. Multivariable model odds ratio interpretation(sCD14 used as an example): Keeping all the other variables constant, for every 1 µg/mL increase in sCD14 the odds of death decreases with 3.6% and this is not statistically significant with p = 0.542.

We also performed logistic regression analyses with each marker of microbial translocation individually and correcting for only the clinical variables and not the other markers of translocation (Tables 7a- 7e). Higher concentrations of sCD14 and TFF3 were associated with higher odds of 12-week mortality and higher concentrations of EndoCAB was associated with lower odds of 12-week mortality when correcting only for clinical variables. Increasing age was significantly associated with mortality in all of these models.

Table 8a: Multivariable logistic regression analysis of soluble CD14 in hospitalized HIV-TB patients: Outcome 12-week mortality:

	Multivariate model estimate	Odds ratio	p
sCD14, µg/mL	0.098	1.103	**0.035**
Age, per 5 year increase	0.185	1.204	**0.005**
Sex, Male versus Female	-0.103	0.902	0.710
HIV viral load, per 1000 copies/mL increase	-0.000	1.000	0.436

Table 8b: Multivariable logistic regression analysis of trefoil factor 3 in hospitalized HIV-TB patients: Outcome 12-week mortality:

	Multivariate model estimate	Odds ratio	p
TFF3, ng/mL	0.029	1.030	**<0.001**
Age, per 5 year increase	0.164	1.178	**0.019**
Sex, Male versus Female	-0.253	0.776	0.400
HIV viral load, per 1000 copies/mL increase	-0.000	1.000	0.837

Table 8c: Multivariable logistic regression analysis of lipopolysaccharide binding protein in hospitalized HIV-TB patients: Outcome 12-week mortality:

	Multivariate model estimate	Odds ratio	p
LBP, µg/mL	-0.000	1.000	0.753
Age, per 5 year increase	0.185	1.203	**0.005**
Sex, Male versus Female	-0.139	0.871	0.616
HIV viral load, per 1000 copies/mL increase	-0.000	1.000	0.558

Table 8d: Multivariable logistic regression analysis of endotoxin core antibody IgM in hospitalized HIV-TB patients: Outcome 12-week mortality:

	Multivariate model estimate	Odds ratio	p
EndoCAB, MMU/L	-0.010	0.990	**0.030**
Age, per 5 year increase	0.152	1.164	**0.024**

Sex, Male versus Female	-0.211	0.810	0.445
HIV viral load, per 1000 copies/mL increase	-0.000	1.000	0.474

Table 8e: Multivariable logistic regression analysis of intestinal fatty acid binding protein in hospitalized HIV-TB patients: Outcome 12-week mortality:

	Multivariate model estimate	Odds ratio	p
IFABP, pg/ml	0.000	1.000	0.979
Age, per 5 year increase	0.019	1.206	**0.004**
Sex, Male versus Female	-0.146	0.864	0.593
HIV viral load, per 1000 copies/mL increase	-0.000	1.000	0.537

Tables 8a-8e: Multivariable model odds ratio interpretation (sCD14 used as an example): Keeping the clinical variables constant, for every 1 µg/mL increase in sCD14 the odds of death increases with 10.3% and this is statistically significant with p = 0.035. sCD14: soluble CD14, LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; TFF3: trefoil factor 3; IFABP: intestinal fatty acid binding protein; µg/mL: microgram per milliliter; MMU/mL: standard median units per milliliter; ng/mL: nanogram per milliliter; pg/mL: picogram per milliliter.

Markers of microbial translocation and the dissemination of tuberculosis:

We next explored the microbial translocation markers in relation to a tuberculosis dissemination score in hospitalized patients. We used a 3 point dissemination score previously described (26, 153) where patients scored 1 point for each positive test amongst mycobacterial blood culture, urine lipoarabinomannan (LAM) and urine Xpert tests. Patients who had valid results for all three tests were allocated a score ranging from 0-3 and we plotted concentrations of each translocation marker by tuberculosis dissemination score in n=304 patients (Figure 9a-e).

Soluble CD14 was significantly higher in patients who tested positive for any marker of dissemination compared to patients who tested negative for all three markers. There were no significant differences between patients who tested positive for only one marker of dissemination compared to those who tested positive for two or three markers of dissemination.

Figure 9a: Soluble CD14 plotted against 3-point tuberculosis dissemination score:

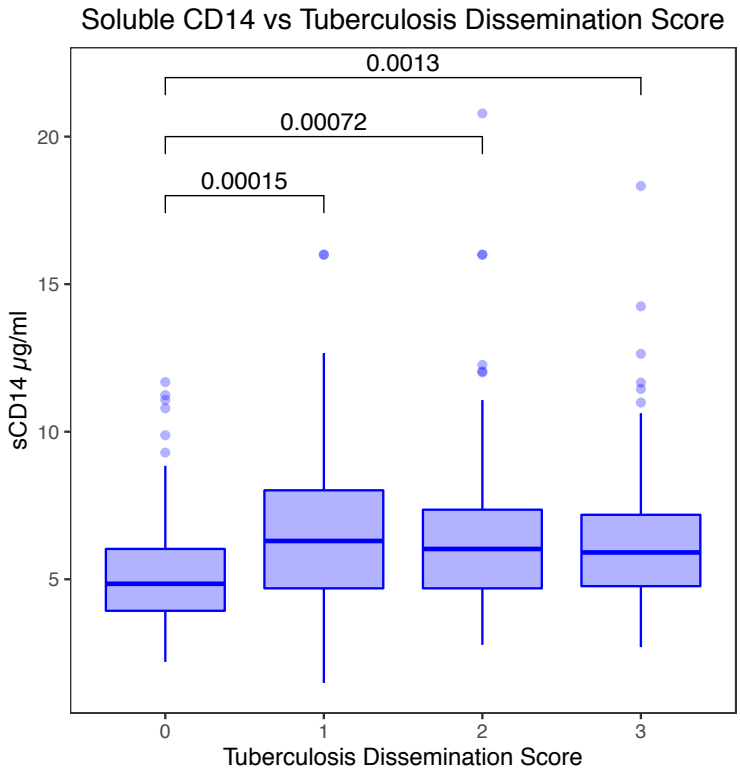


Figure 9a: Soluble CD14 concentrations were plotted against a 3 point tuberculosis dissemination score which was calculated by allocating one point for each positive test amongst mycobacterial blood culture, urine lipoarabinomannan (LAM) and urine Xpert tests. sCD14: Soluble CD14; µg/mL: microgram per milliliter. n = 304 patients who had valid results for all three tests were included.

TFF3 was also significantly higher in patients who tested positive for any marker of dissemination compared to patients who tested negative for all three markers. There were no significant differences between patients who tested positive for a single dissemination marker compared to patients those who tested positive for more than one.

Figure 9b: Trefoil factor 3 plotted against 3-point tuberculosis dissemination score:

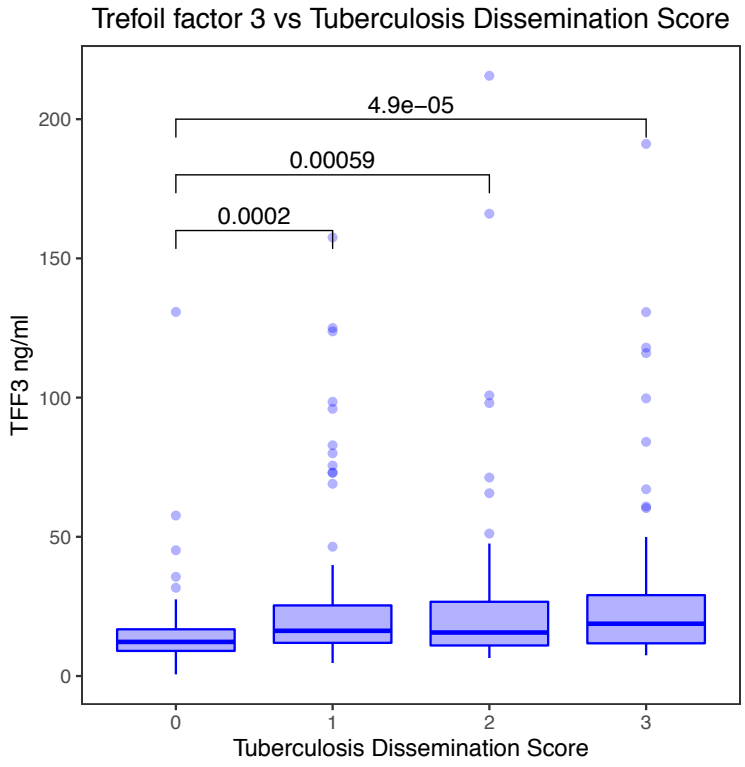


Figure 9b: Trefoil factor 3 concentrations were plotted against a 3 point tuberculosis dissemination score which was calculated by allocating one point for each positive test amongst mycobacterial blood culture, urine lipoarabinomannan (LAM) and urine Xpert tests. TFF3: trefoil factor 3; ng/mL: nanogram per milliliter. n = 304 patients who had valid results for all three tests were included.

LBP was significantly higher in patients who tested positive for 2 or 3 markers of disseminated tuberculosis compared to patients who tested negative for all three markers and patients who tested positive for a single marker.

Figure 9c: Lipopolysaccharide binding protein plotted against 3-point tuberculosis dissemination score:

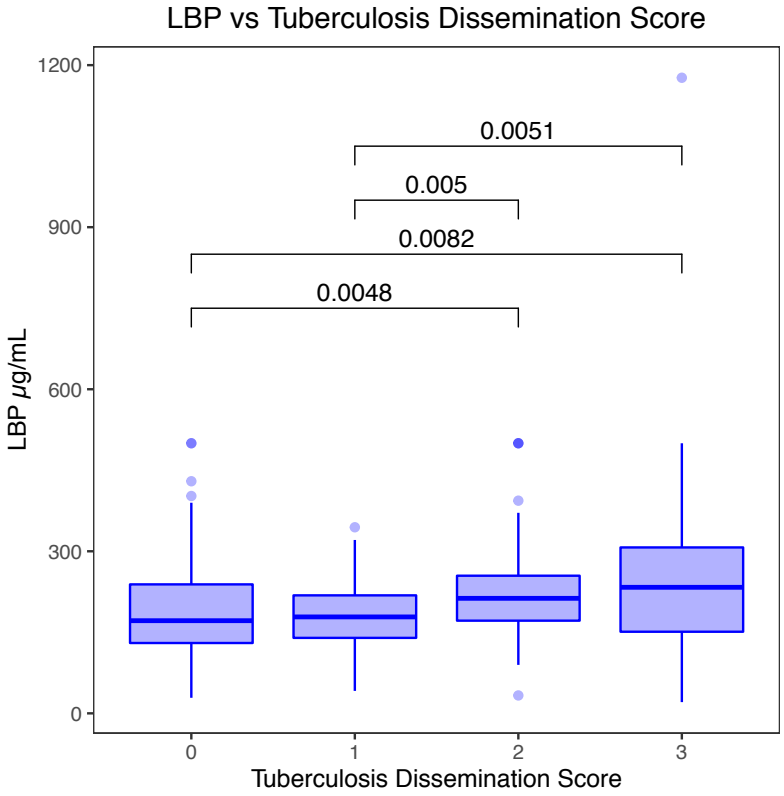


Figure 9c: Lipopolysaccharide binding protein concentrations were plotted against a 3 point tuberculosis dissemination score which was calculated by allocating one point for each positive test amongst mycobacterial blood culture, urine lipoarabinomannan (LAM) and urine Xpert tests. LBP: Lipopolysaccharide binding protein; µg/mL: microgram per milliliter. n = 304 patients who had valid results for all three tests were included.

Patients who tested positive for any markers of dissemination had significantly lower concentrations of EndoCAB compared to patients who tested negative for all three dissemination markers. There were no significant differences between patients who tested positive for a single marker compared to patients who tested positive for more than one.

Figure 9d: Endotoxin core antibody IgM plotted against 3-point tuberculosis dissemination score:

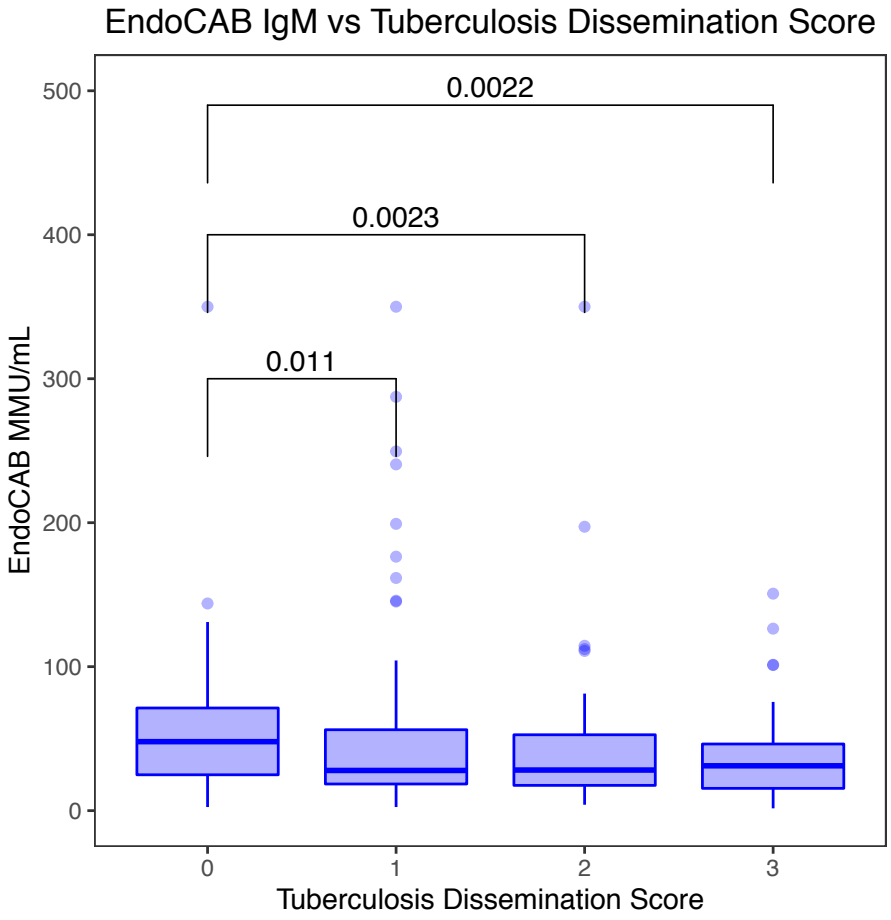


Figure 9d: Endotoxin core antibody IgM concentrations were plotted against a 3 point tuberculosis dissemination score which was calculated by allocating one point for each positive test amongst mycobacterial blood culture, urine lipoarabinomannan (LAM) and urine Xpert tests. EndoCAB: Endotoxin core antibody IgM; MMU/mL standard median units per milliliter. n = 304 patients who had valid results for all three tests were included.

Patients who tested positive for all three dissemination markers had significantly lower concentrations of IFABP compared to patients who tested positive for a single marker. There were no significant differences between patients who tested negative for all three markers and patients who tested positive for one, two or three dissemination markers.

Figure 9e: Intestinal fatty acid binding protein plotted against 3-point tuberculosis dissemination score:

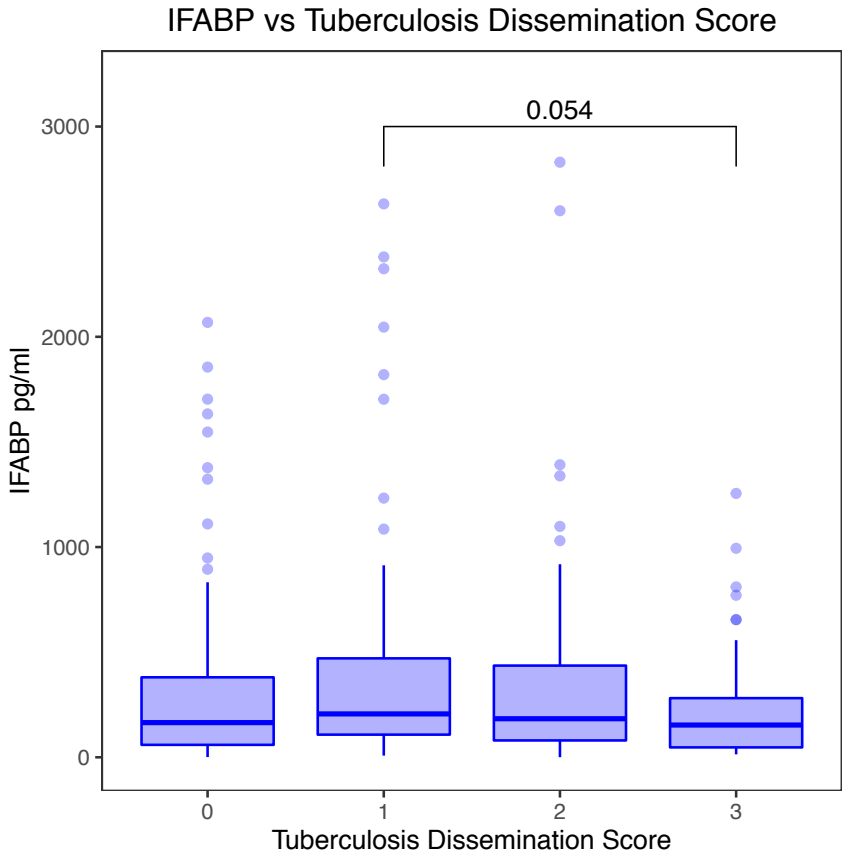


Figure 9e: Intestinal fatty acid binding protein concentrations were plotted against a 3 point tuberculosis dissemination score which was calculated by allocating one point for each positive test amongst mycobacterial blood culture, urine lipoarabinomannan (LAM) and urine Xpert tests. IFABP: Intestinal fatty acid binding protein; pg/mL picogram per milliliter. n = 304 patients who had valid results for all three tests were included.

Bacterial 16s rDNA quantitation in whole blood:

We had 16s rDNA quantitation performed on stored whole blood samples of 235 patients (n= 20 outpatient controls, n= 111 hospitalized deaths and n = 104 hospitalized survivors). The median concentration of 16s rDNA was 3736.7 copies/μL whole blood (interquartile range [IQR] = 2223.3 - 6421.7 copies/μL). The median concentrations for outpatients, hospitalized deaths and hospitalized survivors were 2196.667 copies/μL (IQR: 1458.333 - 3468.333 copies/μL), 4076.667 copies/μL (IQR: 2333.333 - 7831.667 copies/μL) and 3783.333 copies/μL (IQR: 2390.833 - 6146.250 copies/μL), respectively. Bacterial 16s rDNA concentrations in hospitalized patients who died were not significantly different from hospitalized patients who survived, but hospitalized patients had significantly higher concentrations compared to outpatient controls (Figure 10). In the parent cohort we found low rates of gram-negative bacterial co-infections. Amongst 296/576 (51.4%) of patients who had bacterial blood cultures performed only 7 patients cultured an organism other than *Mycobacterium tuberculosis* and 4 were gram-negative organisms. None of the patients with gram-negative infections had very high 16s rDNA concentrations (1416.67 copies/mL, 3245 copies/mL, 3723.33 copies/mL and 5593.33 copies/mL).

Figure 10: Bacterial 16s ribosomal DNA concentrations in patients hospitalized with HIV-associated tuberculosis and outpatient controls:

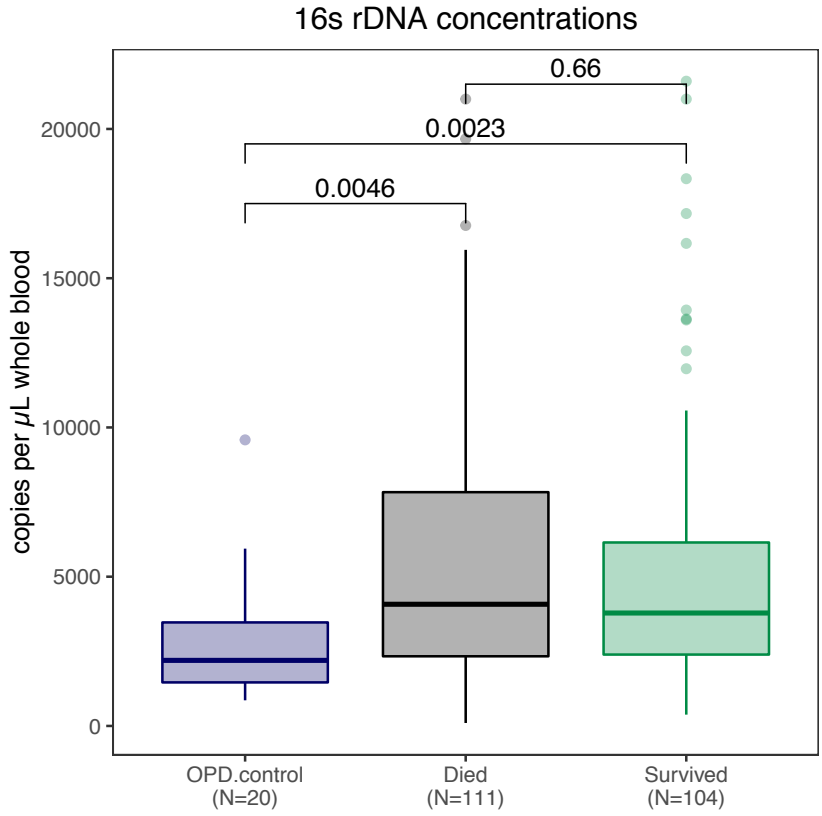


Figure 10: Bacterial 16s rDNA concentrations were measured in n = 235 patients. All hospitalized patients with HIV-associated tuberculosis who died within 12 weeks with available stored whole blood samples were included (n = 111), a group of randomly selected hospitalized patients who survived (n = 104) plus 20 outpatient controls with HIV and no tuberculosis. OPD.control: outpatients with HIV infection and no tuberculosis; Died: patients hospitalized with HIV-associated tuberculosis who died within 12 weeks of enrolment; Survived: patients hospitalized with HIV-associated tuberculosis who survived 12 weeks.

We performed linear regression analysis to assess whether translocation markers and pre-selected clinical predictors were associated with 16s rDNA concentrations. There were n = 152 patients who had 16s rDNA concentrations measured and a full set of indirect markers of microbial translocation (n= 20 OPD controls, n= 62 hospitalized deaths, n= 70 hospitalized survivors). We included the indirect markers of microbial translocation, age, sex, HIV viral load and C-reactive protein (CRP) in the linear regression model. We first assessed for collinearity by performing a

correlation analysis of these variables. The only markers with a moderately strong correlation were a positive correlation between sCD14 and TFF3 (correlation coefficient (rho) = 0.406) and CRP had moderately strong positive correlation with age (rho = 0.264), sCD14 (rho = 0.278), TFF3 (rho = 0.347), 16s rDNA (rho = 0.271), LBP (rho = 0.300), and a negative correlation with EndoCAB (rho: -0.285) (Figure 11). In the absence of strong collinearity all variables were included in the multivariable analysis as planned.

Figure 11: Correlation plot of microbial translocation markers and selected clinical variables used in the linear regression analysis 16s rDNA concentrations:

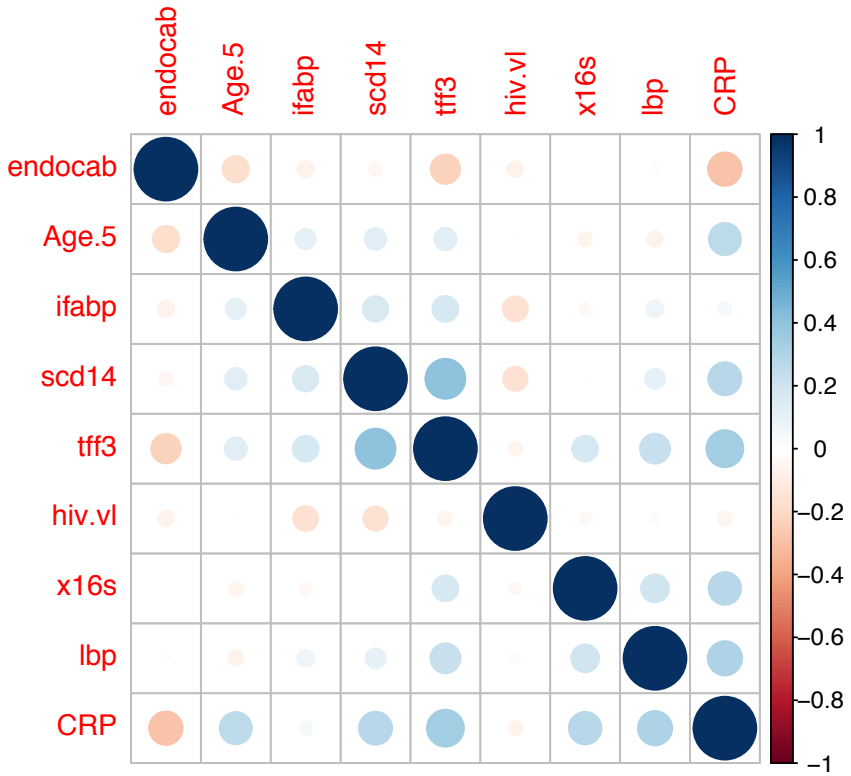
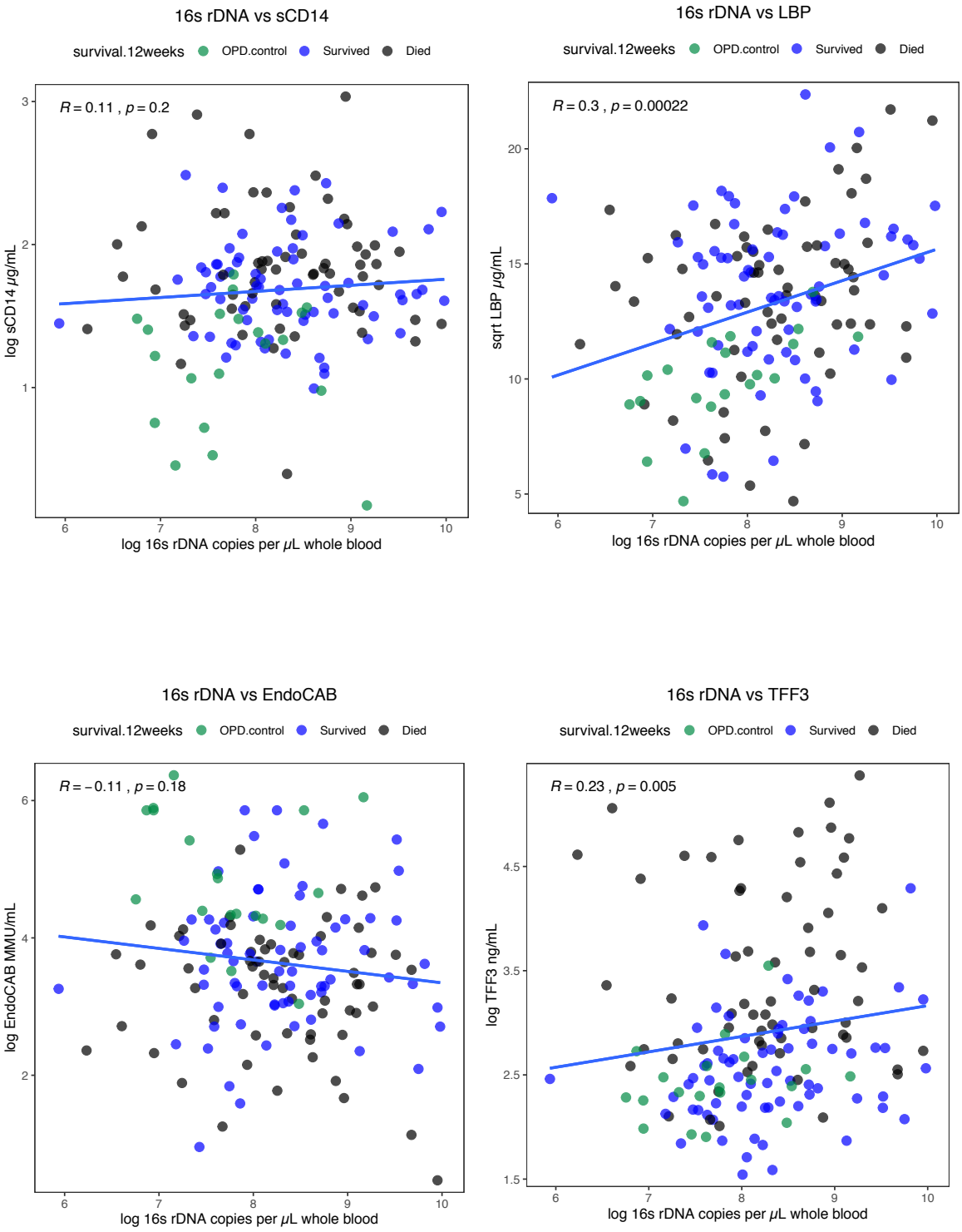


Figure 11: Correlation plot showing the correlation of markers of microbial translocation, 16s ribosomal DNA and selected clinical markers. Correlation coefficients range from +1 (perfect positive correlation) to -1 (perfect negative correlation). A positive correlation is indicated by a blue colour and a negative correlation is indicated in red. The size of the circle represents the strength of the correlation. endocab: endotoxin core antibody; hiv.vl: HIV viral load; x16s: bacterial 16s ribosomal DNA; lbp: lipopolysaccharide binding protein; CRP: C-reactive protein; ifabp: intestinal fatty acid binding protein; Age.5: Age per five year increase; scd14: soluble CD14; tff3: trefoil factor 3.

We also plotted the markers of microbial translocation and clinical markers against bacterial 16s rDNA concentrations individually to examine the bacterial 16s rDNA correlations in more detail. We expected to find a positive correlation between bacterial 16s rDNA concentrations and the markers of intestinal mucosal damage TFF3 and IFABP. We found a significant positive correlation between bacterial 16s rDNA concentrations and TFF3, but not IFABP. There was also a significant positive correlation between bacterial 16s rDNA and CRP (Figure 12).

Figure 12: Individual correlation plots of microbial translocation markers and clinical variables used in the linear regression analysis with 16s rDNA concentrations:



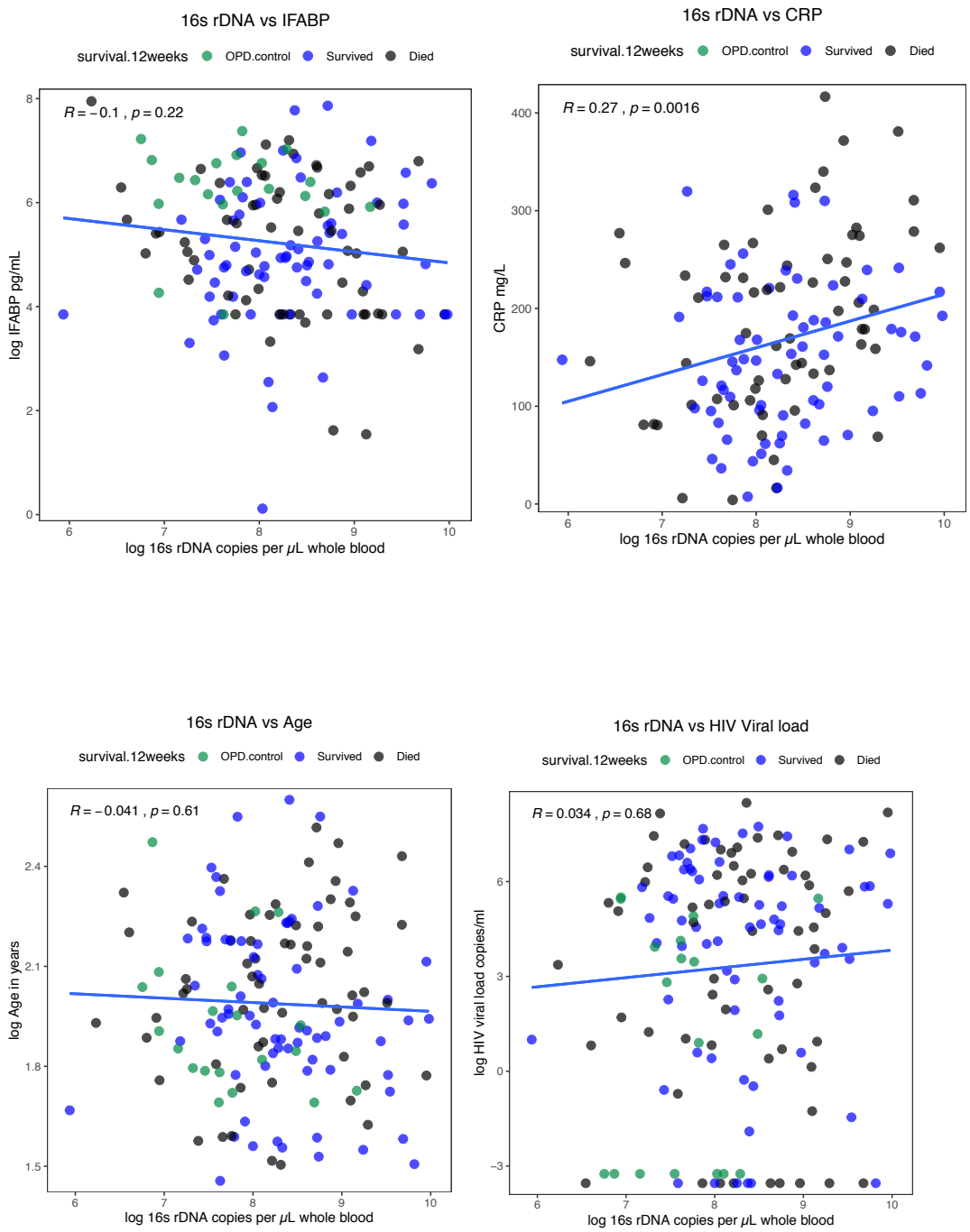


Figure 12: Correlation plots of individual markers of microbial translocation, clinical variables used in the linear regression analysis and bacterial 16s ribosomal DNA concentrations. Variables were log or square root transformed before correlation was performed. Outpatient controls did not have C-reactive protein concentrations measured. R: Spearman's rank correlation rho; 16s rDNA: bacterial 16s ribosomal DNA concentration; sCD14: soluble CD14; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; TFF: trefoil factor 3; IFABP: intestinal fatty acid binding protein; CRP: C-reactive protein; Age: Age in years per five year increase

The results of the linear regression model are presented in Table 9. We first performed univariable analysis with each variable included in a model individually and then we included all variables in a multivariable model. Increased concentrations of LBP and CRP were significantly associated with increased 16s rDNA concentrations in univariable analysis. In multivariable analysis increased LBP and CRP concentrations remained significantly associated with increased 16s rDNA concentrations. We also included mycobacterial blood culture in univariable and multivariable models (results not presented) and having a positive mycobacterial blood culture had no significant relationship to 16s rDNA concentrations in univariable or multivariable analysis and had no influence on the association of 16s rDNA with LBP and CRP.

Table 9: Linear regression analysis of microbial translocation markers and clinical variables with 16s rDNA concentrations (copies per microlitre whole blood):

	Intercept	Univariate model estimate	p	Multivariate model estimate	p
sCD14, µg/mL	4968.120	18.350	0.881	-0.022	0.130
TFF3, ng/mL	4708.160	13.320	0.200	7.541	0.530
LBP, µg/mL	2198.236	15.386	**<0.001**	9.522	**0.034**
EndoCAB, MMU/L	5423.936	-5.115	0.189	-0.056	0.993
IFABP, pg/mL	5437.218	-0.958	0.210	-0.287	0.732
C-reactive protein	3125.610	13.621	**0.003**	1.312	**0.012**
Age, per 5 year increase	6167.2	-144.6	0.431	-0.025	0.221
Sex, Male vs Female	5506.700	-848.3	0.231	-0.002	**0.030**
HIV viral load	4820.180	0.644	0.195	0.325	0.517

Table 9: The intercept is the value of 16s rDNA in copies per milliliter of whole blood if the specific variable is equal to zero. Example of the interpretation of the intercept: The value of 16s DNA will be 4968.12 copies/µL if sCD14 equals zero µg/mL. Example of the interpretation of univariate model estimate: For each unit (µg/mL) increase in sCD14 there will be an 18.350 unit (copies/µL blood) increase in 16s rDNA concentration, which is not statistically significant with p = 0.881. Example of the interpretation of multivariable model estimate: Keeping all other variables constant, for each unit (µg/mL) increase in sCD14 there will be an 0.022 (copies/µL blood) decrease in 16s rDNA concentration, which is not statistically significant with p = 0.130.

We also performed linear regression analysis where we included each indirect marker of microbial translocation individually and corrected for the clinical variables in each model. Increasing LBP concentrations and increasing CRP concentrations remained significantly associated with higher 16s rDNA concentrations (Tables 10a – 10e).

Table 10a : Linear regression analysis of soluble CD14 and clinical variables with 16s rDNA concentrations (copies per microlitre whole blood):

	Intercept	Multivariate model estimate	p
	6238.861		
sCD14, µg/mL	-	-6.360	0.960
Age, per 5 year increase	-	-125.585	0.503
Sex, Male versus Female	-	-921.593	0.209
HIV viral load, per 1000 copies/mL increase	-	0.733	0.148

Table 10b: Linear regression analysis of lipopolysaccharide binding protein and clinical variables with 16s rDNA concentrations (copies per microlitre whole blood):

	Intercept	Multivariate model estimate	p
	3270.104		
LBP, µg/mL	-	16.350	**<0.001**
Age, per 5 year increase	-	-86.909	0.620
Sex, Male versus Female	-	-1488.173	**0.033**
HIV viral load, per 1000 copies/mL increase	-	0.399	0.401

Table 10c: Linear regression analysis of endotoxin core antibody IgM and clinical variables with 16s rDNA concentrations (copies per microlitre whole blood):

	Intercept	Multivariate model estimate	p
	7083.649		
EndoCAB, MMU/mL	-	-6.3291	0.120
Age, per 5 year increase	-	-165.4563	0.376
Sex, Male versus Female	-	-1141.5743	0.122
HIV viral load, per 1000 copies/mL increase	-	0.631	0.208

Table 10d: Linear regression analysis of trefoil factor 3 and clinical variables with 16s rDNA concentrations (copies per microlitre whole blood):

	Intercept	Multivariate model estimate	p
	6110.857		
TFF3, ng/mL	-	15.191	0.152
Age, per 5 year increase	-	-162.302	0.385
Sex, Male versus Female	-	-1002.002	0.168
HIV viral load, per 1000 copies/mL increase	-	0.695	0.164

Table 10e: Linear regression analysis of intestinal fatty acid binding protein and clinical variables with 16s rDNA concentrations (copies per microlitre whole blood):

	Intercept	Multivariate model estimate	p
	6446.222		
IFABP, pg/mL	-	-0.838	0.277
Age, per 5 year increase	-	-117.495	0.528
Sex, Male versus Female	-	-873.980	0.229
HIV viral load, per 1000 copies/mL increase	-	0.698	0.163

Tables 10a – 10e: The intercept is the value of 16s rDNA in copies per milliliter of whole blood if the specific variable is equal to zero. Example of the interpretation of the intercept: The value of 16s DNA will be 6446.222 copies/µL if IFABP concentrations equals zero pg/mL. Interpretation of the multivariable model estimate: Keeping the other variables constant, for each unit (pg/mL) increase in IFABP there will be an 0.838 (copies/µL blood) decrease in bacterial 16s rDNA concentration, which is not statistically significant, p = 0.277. sCD14: soluble CD14, LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; TFF3: trefoil factor 3; IFABP: intestinal fatty acid binding protein; µg/mL: microgram per milliliter; MMU/mL: standard median units per milliliter; ng/mL: nanogram per milliliter; pg/mL: picogram per milliliter.

Bacterial 16s rDNA concentrations and tuberculosis dissemination score:

We plotted bacterial 16s rDNA concentrations against the tuberculosis dissemination score to investigate the relationship of 16s rDNA concentration with increasing number of positive tests for disseminated tuberculosis. There were no significant differences in 16s rDNA concentrations between patients who tested negative for all three tests and those who tested positive for one, two or three markers of tuberculosis dissemination (Figure 13). Comparing patients who tested negative to

all three tests (dissemination score = 0) to all patients who tested positive for one or more (dissemination score ≥ 1), there was no significant difference (p = 0.767).

Figure 13: Bacterial 16s rDNA concentrations plotted against 3-point tuberculosis dissemination score:

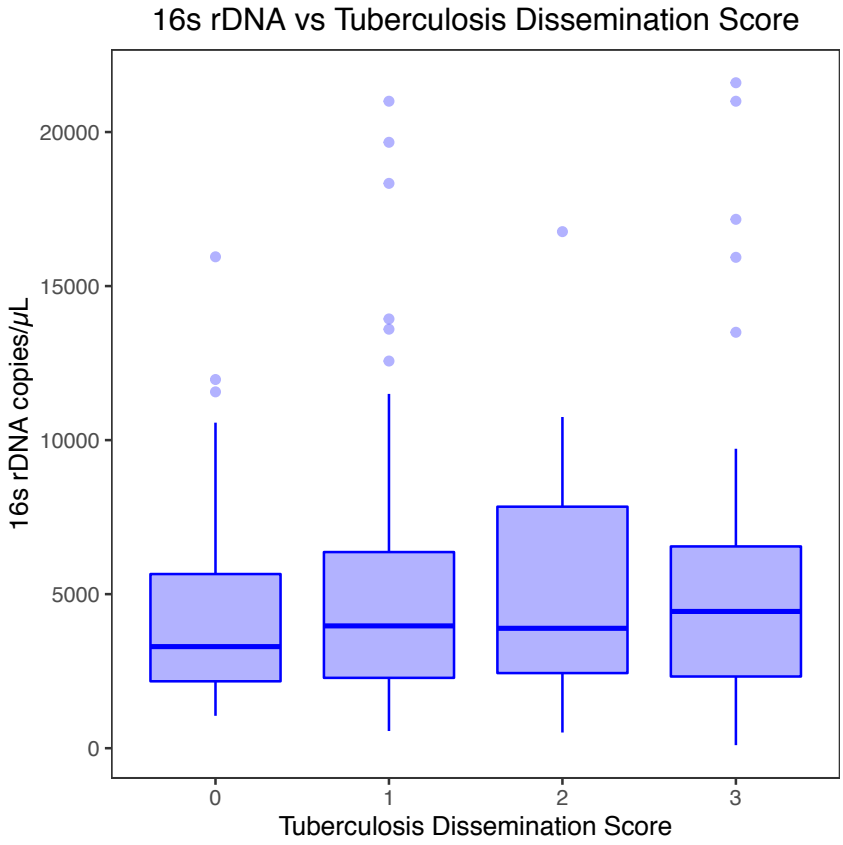


Figure 13: Concentrations of 16s ribosomal DNA were plotted against a 3 point tuberculosis dissemination score which was calculated by allocating one point for each positive test amongst mycobacterial blood culture, urine lipoarabinomannan (LAM) and urine Xpert. 195 patients who had valid results for all three tests and 16s rDNA concentration measured were included.

Survival analysis: 16s rDNA concentrations in whole blood:

We further examined the association of bacterial 16s rDNA concentrations and mortality by plotting Kaplan Meier curves. All patients with 16s rDNA were included: outpatient controls, hospitalized patients who died and those who survived. Patients were then divided into those with 16s rDNA levels above or below the median

concentration (Figure 14). There was no significant difference between the survival curves. We also plotted Kaplan-Meier curves excluding outpatient controls and there was no difference in the survival curves (Figure 15). To further examine this association, we also divided 16s rDNA concentrations into quartiles and firstly plotted Kaplan Meier curves for all 4 quartiles and secondly we compared patients with 16s rDNA concentrations in the uppermost quartile (Q4) against the other three quartiles. We found no significant differences (Figure 16 and 17).

Figure 14: Kaplan-Meier curve of patients stratified by those with 16s rDNA concentrations above or below the median values:

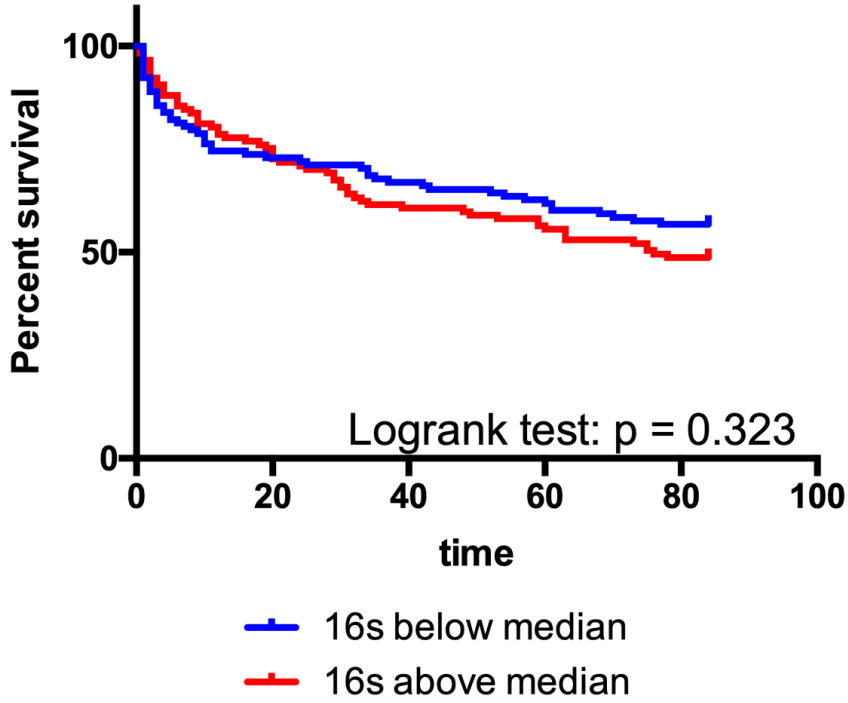


Figure 14: Percentage survival on y-axis. Time in days on x-axis. 16s: bacterial 16s ribosomal DNA. These curves include all hospitalized patients (n= 111 patients who died and n= 104 survivors) and outpatient controls (n= 20). Patients were divided into those with bacterial 16s rDNA concentrations above or below the median concentration. The logrank test compares the curves.

Figure 15: Kaplan-Meier curve of hospitalized patients stratified by those with 16s rDNA concentrations above or below the median values (excluding outpatient controls):

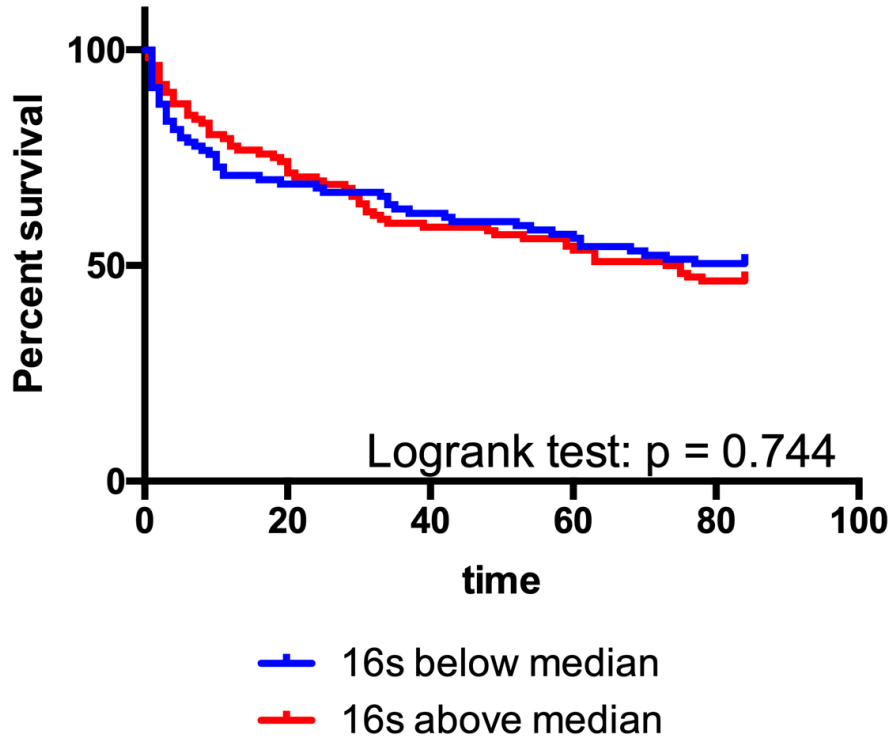


Figure 15: Percentage survival on y-axis. Time in days on x-axis. 16s: bacterial 16s ribosomal DNA. These curves include all hospitalized patients, n= 111 patients who died and n= 104 survivors and no outpatient controls. Patients were divided into those with bacterial 16s rDNA concentrations above or below the median concentration. The logrank test compares the curves.

Figure 16: Kaplan-Meier curve of hospitalized patients stratified into quartiles of 16s rDNA concentration:

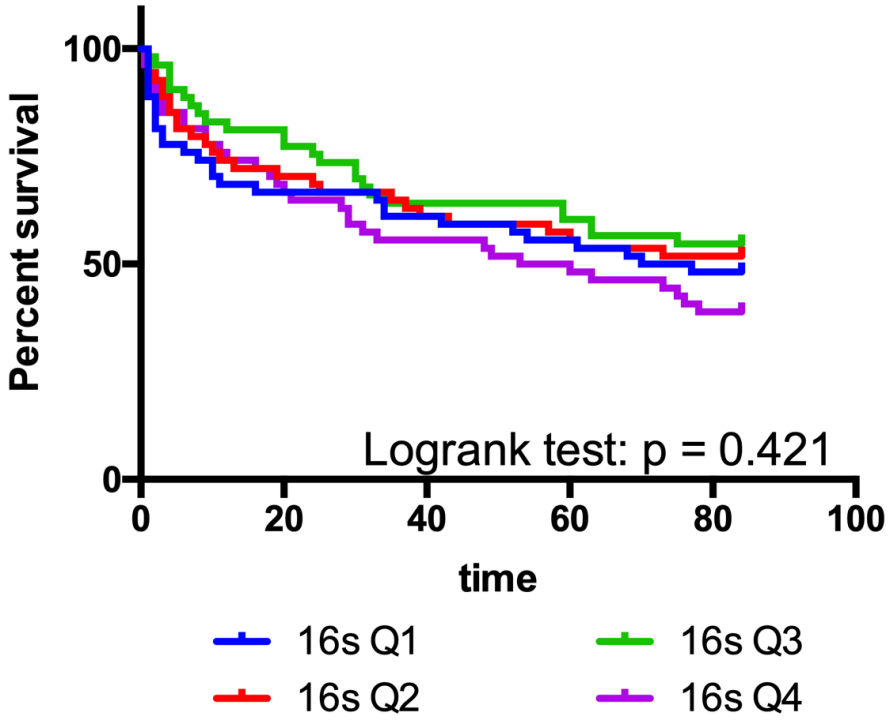


Figure 16: Percentage survival on y-axis. Time in days on x-axis. 16s: bacterial 16s ribosomal DNA. These curves include all hospitalized patients (n= 111 patients who died and n= 104 survivors) and outpatient controls (n= 20). Q1: patients with 16s rDNA values in the first quartile (lowest); Q2: patients with 16s rDNA values in the second quartile; Q3: patients with 16s rDNA values in the third quartile; Q4: patients with 16s rDNA values in the fourth quartile (highest). The logrank test compares the curves.

Figure 17: Kaplan-Meier curve of hospitalized with patients stratified into quartiles of 16s rDNA concentration: Quartile 4 compared to quartile 1-3:

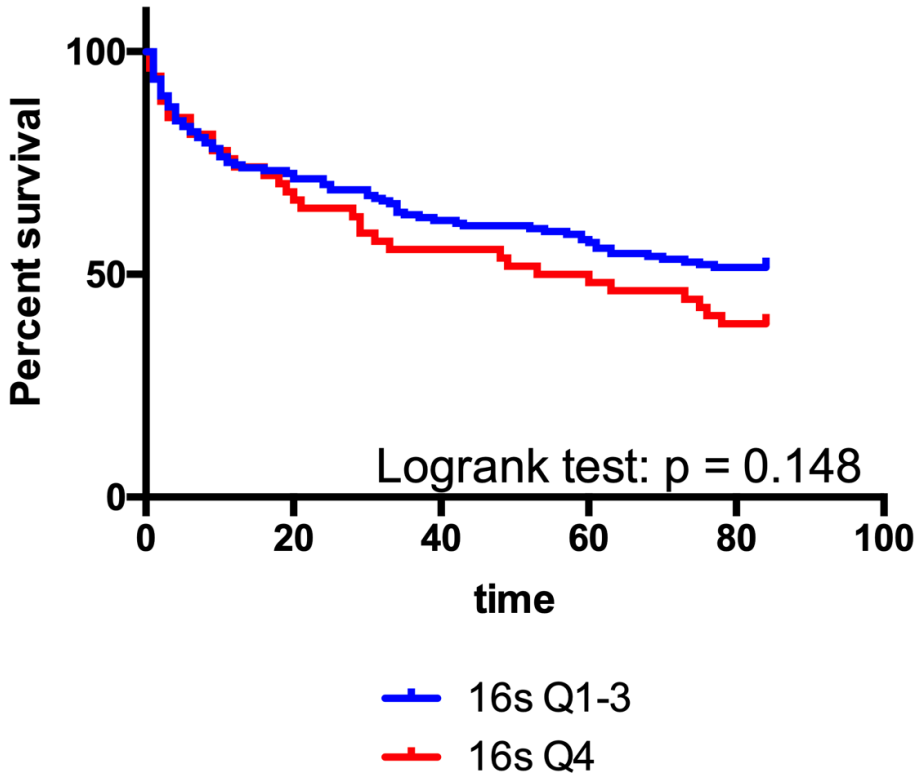


Figure 17: Percentage survival on y-axis. Time in days on x-axis. 16s: bacterial 16s ribosomal DNA. These curves include all hospitalized patients (n= 111 patients who died and n= 104 survivors) and outpatient controls (n= 20). Q1-3: patients with 16s rDNA values in the first, second and third quartile (lower 3 quartiles); Q4: patients with 16s rDNA values in the fourth quartile (highest). The logrank test compares the curves.

Discussion:

Pathophysiology underlying the high mortality in patients hospitalized with HIV-associated tuberculosis is poorly understood. HIV infection causes early, severe damage to the gastrointestinal structural and immunological barriers. The translocation of microbial products across this damaged gastrointestinal lumen into systemic circulation is one of the mechanisms underlying chronic systemic immune activation in HIV infection which in turn is associated with disease progression and mortality (21, 159). In the setting of chronic inflammatory bowel disease, the presence of bacterial DNA in the blood is associated with relapse episodes and an increased risk of hospitalization, need for treatment change or steroid initiation (23). In chronic liver cirrhosis the presence of bacterial DNA in the blood is associated with mortality (22). In the setting of severe HIV-associated tuberculosis we postulated an association between microbial translocation and mortality. Higher levels of microbial translocation and its effect on the systemic immune response may contribute to mortality and it is also plausible that higher concentrations of microbial products may be the harbinger of the translocation of whole viable bacteria from the gastrointestinal tract into the bloodstream which may result in sepsis and mortality. We determined if there were higher levels of gastrointestinal mucosal damage and microbial translocation in hospitalized HIV-TB patients compared to HIV-infected outpatients and if this was associated with 12-week mortality in hospitalized patients.

Comparing outpatients with HIV infection (and no tuberculosis) to hospitalized patients with HIV-associated tuberculosis, we found significantly higher concentrations of sCD14, LBP, and lower EndoCAB concentrations, which is compatible with higher concentrations of lipopolysaccharide in circulation. We also

found significantly higher concentrations of TFF3, which is a marker of gastrointestinal endothelial damage, which suggests more gastrointestinal mucosal damage. We unexpectedly found significantly higher concentrations of IFABP (a marker of gastrointestinal enterocyte necrosis) in outpatients.

Amongst hospitalized patients with HIV-associated tuberculosis, we found significantly higher concentrations of sCD14 and significantly lower concentrations of EndoCAB in patients who died within 12 weeks compared with survivors, which is compatible with higher concentrations of lipopolysaccharide in circulation. Patients who died also had significantly higher TFF3 concentrations compared to patients who survived, which is compatible with more gastrointestinal mucosal damage. In multivariable logistic regression higher concentrations of TFF3 was independently associated with 12-week mortality. We found significantly higher concentrations of sCD14 and LBP and significantly lower concentrations of EndoCAB in patients who tested positive for one or more markers of disseminated tuberculosis, compared to those who tested negative for these markers of dissemination, which suggests higher levels of microbial translocation in patients with more disseminated tuberculosis. We also found higher concentrations of TFF3 in patients with positive markers of disseminated tuberculosis, which indicates more gastrointestinal endothelial damage in patients with disseminated tuberculosis.

We quantified bacterial 16s rDNA in a subset of patients and found significantly higher concentrations in hospitalized patients compared to HIV-infected outpatients without tuberculosis. In a multivariable linear regression model, higher LBP concentrations and higher C-reactive protein concentrations were significantly

associated with higher bacterial 16s rDNA concentrations. We found no difference in bacterial 16s rDNA concentrations amongst patients who tested positive for markers of tuberculosis dissemination and those who tested negative for these markers. Higher concentration of bacterial 16s rDNA was not associated with mortality in survival analysis. We found low LPS concentrations in the whole cohort and no differences between groups.

Our findings relating to the indirect markers of microbial translocation suggest more gastrointestinal mucosal damage and microbial translocation occurs in hospitalized HIV-associated tuberculosis patients compared to HIV-infected outpatients without tuberculosis. Amongst the hospitalized patients who died versus patients who survived, the findings were similar but the differences were less pronounced. The finding of higher bacterial 16s rDNA concentrations in hospitalized patients support the findings of the indirect markers, however 16s rDNA concentrations were not associated with mortality and LPS concentrations were low in the entire cohort.

Lipopolysaccharide (LPS) is a potent stimulator of the immune system and is rapidly recognized by antibodies and binding proteins. Depending on the concentration, LPS triggers pro-inflammatory responses or is cleared from circulation. To initiate an immune response LPS is recognized by sCD14 or LBP and transferred to membrane bound CD14 which acts as an LPS receptor on myeloid cells. Membrane bound CD14 binds LPS to the TLR4/MD2 complex, which activates nuclear factor-κB (NF-κB) through a myeloid differentiation primary response gene 88 (MyD88) dependent or MyD88-independent pathway to mediate secretion of pro-inflammatory mediators such as interleukin-1β (IL-1β), IL-6, TNF-α and type 1 interferon (160). To clear LPS

from circulation, sCD14 or LBP transfer LPS to high density (HDL) or other lipoproteins in plasma and these bind LPS in such a way that it cannot interact with the TLR4/MD2 complex (143). LPS in the plasma is difficult to measure and the interpretation of LPS findings is challenging. All LPS assays, including the LAL assay we used to measure LPS, are technically challenging, sensitive to interference and have caveats in the interpretation. The LPS molecules produced by bacteria, the LPS used to prepare assay standards and the LPS measured in plasma are heterogenous molecules. LPS consists of a lipid A, core and O-antigen, but LPS molecules (even LPS extracted from pure cultures) have variation in the polysaccharide chain length, acetylation of lipid A and the extent to which molecules such as phosphate, ethanolamine and arabinose are attached to the lipid A or core oligosaccharide (143). Some LPS molecules may stimulate (or activate blood cells), some LPS molecules do not stimulate blood cells and some may even be inhibitory. None of the LPS assays can distinguish these types of LPS molecules and this greatly limits the interpretation of measured LPS concentrations (143, 161). LPS concentrations are elevated in the context of HIV-infection and previous cohort studies which measured LPS concentrations in HIV-TB patients showed high LPS concentrations , thus we expected to find high concentrations of LPS in this cohort of acutely ill patients hospitalized with HIV-associated tuberculosis (2, 21, 156). However, there are several points to consider. Firstly, the LAL assay is technically difficult to perform and it cannot be ruled out that there was a technical issue with performance of the assay. Secondly, it is possible that a constituent of plasma present in high abundance in our patient population which was not neutralized by heat inactivation interfered with the activation of LAL and detection of LPS in our samples. Thirdly, the findings may accurately reflect low concentrations of LPS due

to successful clearance from the peripheral blood by sCD14 and LBP, both of which we found in high concentrations and both of which have been shown to remove LPS from cell surfaces and to reduce the bioactivity of LPS (140, 162-165). High concentrations of LBP have also been shown to prevent LPS from activating LAL (166).

sCD14 is produced by activated monocytes and macrophages and is a non-specific marker of monocyte/macrophage activation (167). Higher sCD14 concentrations are associated with disease progression and poor outcome in patients with chronic HIV infection (159, 168) but this is unlikely a causal relationship. Higher concentrations of sCD14 are taken to indicate a state of chronic immune activation and microbial translocation is one of the underlying causes of immune activation in HIV. LBP is an acute phase protein which is produced by the liver as part of the immune response to gram negative bacteria. Both sCD14 and LBP have the ability to stimulate or inhibit the immune response to LPS and the nature of the response depends on the concentration of both markers and their environment (163). High concentrations of LBP and sCD14 during acute infection inhibits the cellular immune response to LPS, whereas low concentrations at the site of infection may enhance the LPS-associated immune activation of monocytes and macrophages (163, 169). sCD14 can remove LPS from cellular CD14 and transfer LPS to plasma lipoproteins (140). Both sCD14 and LBP concentrations are used as surrogate markers of microbial translocation in the peripheral blood, however both markers are non-specific and could be increased due to the acute phase response to other infections such as bacterial infections or TB-related inflammation. We found higher concentrations of both markers in hospitalized HIV-TB patients compared to outpatient controls and higher sCD14 concentrations in hospitalized patients who died. Our findings of low LPS

concentration and high concentrations of LBP and sCD14 are similar to a study in Uganda which also found unexpectedly low LPS concentrations in patients with HIV-associated tuberculosis compared to patients with only HIV infection and a CD4 count < 350 cells/μL. They found higher concentrations of LBP in HIV-associated tuberculosis, but no negative correlation between plasma LBP concentration and LPS concentration, so postulated that LBP and other LPS binding proteins were implicated in their finding of lower than expected LPS concentrations (156).

Endotoxin Core antibody (EndoCAB) is involved in clearing LPS from systemic circulation and EndoCAB IgA, IgM and IgG can be measured in healthy individuals (170). The measurement unit (standard median units) was developed based on the values found in 1000 healthy adults in a single geographical location. Several studies show a drop in EndoCAB levels after major surgery and this is thought to be due to EndoCAB consumption by binding to LPS that is released into the circulation during the postoperative period (171). EndoCAB which is bound to LPS is not measurable by the EndoCAB assay. Studies have also shown that people with low pre-operative EndoCAB values and sepsis patients with low EndoCAB concentrations have poor clinical outcomes compared to people with normal values (172-174). This has been interpreted as an indication that patients with low EndoCAB concentrations do not have the same capacity or reserves to deal with the post-operative or sepsis related systemic release of LPS as patients with normal concentrations. EndoCAB increases during acute HIV infection and decreases during chronic HIV infection (21, 159). We found significantly lower EndoCAB concentrations in hospitalized patients compared to controls and hospitalized patients who died had significantly lower concentrations compared to survivors.

Trefoil factor 3 (TFF3) is a stable secretory protein which is produced by the gastrointestinal mucosal cells. It is part of the trefoil family of proteins which are secreted by mucin-producing gastrointestinal cells and play a key role in the maintenance and repair of the gastrointestinal mucosa (141). TFF3 expression increases when gastrointestinal mucosal damage occurs and is used as a marker of gastrointestinal mucosal damage. We found higher concentrations of TFF3 in hospitalized patients and higher concentrations in hospitalized patients who died compared to those who survived. TFF3 was independently associated with 12 week mortality in hospitalized patients. Intestinal fatty acid binding protein (IFABP) has been the most commonly studied marker of gastrointestinal mucosal damage in the context of HIV infection and microbial translocation to date. Fatty acid binding proteins are low molecular weight intracellular proteins which play a role in the metabolism and transport of long chain fatty acids and are tissue specific. Intestinal fatty acid binding protein is present in the epithelial cells (enterocytes) of the gastrointestinal system and is rapidly released into systemic circulation when enterocyte necrosis occurs (142). We found significantly higher concentrations of IFABP in outpatient controls, which was an unexpected finding. We found IFABP ~200 pg/mL in hospitalized patients and ~ 600 pg/mL in outpatient controls. Another South African cohort of hospitalized HIV-TB patients found median I-FABP concentrations of 137 pg/mL in patients who died and 0 pg/mL in survivors (2). Another cohort of HIV-TB outpatients found a median of 345 pg/mL in ART naïve patients and 612 pg/mL in a sub-group of patients at 4 weeks on ART (96). This suggests that there may be other mechanisms responsible for variation in IFABP concentrations and further research is needed to assess the meaning and utility of IFABP concentrations in the context of HIV, HIV-associated tuberculosis and other

co-infections. TFF3 could be considered as an alternative marker of gastrointestinal mucosal damage.

Bacterial 16s rDNA can be measured in whole blood, is present in healthy people (150) and has been linked to pathological conditions in which microbial translocation is thought to play a role in the pathophysiology of disease, such as liver fibrosis in patients with severe obesity and non-alcoholic fatty liver disease (149). A case study reported high 16s rDNA concentrations in a patient with an episode of probable bacterial blood stream infection (152). There are not many studies which report 16s rDNA concentrations in HIV-infected patients. Our finding of a median 3736.7 copies/µL of whole blood [IQR: 2223.3 - 6421.7 copies/µL] is higher than 16s rDNA concentrations found in the plasma of HIV-infected patients in the USA with median 16s rDNA concentrations of 132 copies/µL of plasma (175) and higher than HIV-hepatitis C co-infected patients in Spain with median 198.87 copies/µL of plasma [IQR: 89.14 - 355.98 copies/µL]) (176). However, a direct comparison of results from whole blood and plasma is likely inappropriate due to technical differences of the PCR assays, standard curves and normalization of the values. We did not find association between bacterial 16s rDNA concentrations and mortality in this cohort, which is similar to findings from the SMART-trial which showed no association of 16s rDNA with poor clinical outcome (159). The lack of association with mycobacterial blood culture is counter-intuitive, but this is likely because 16s rDNA measures DNA from all types of bacteria. *Mycobacterium tuberculosis*, even though present in blood of many patients on blood culture, did not appear to represent a large proportion of the bacterial DNA in blood. Prospective studies will need to measure 16s rDNA concentrations longitudinally in hospitalized patients with HIV-TB to establish

whether 16s rDNA could play a role in identifying pathogenic bacterial infections or whether longitudinal measurement identifies patients at higher risk of mortality. In the parent cohort 88% of patients received ceftriaxone during the index admission and this may have played a role in our bacterial 16s rDNA concentration findings.

In summary, our findings of higher sCD14 and TFF3 concentrations together with lower EndoCAB concentrations in hospitalized patients with HIV-associated tuberculosis compared to outpatients with HIV infection only, and similar findings in hospitalized HIV-associated tuberculosis patients who died within 12 weeks compared to patients who survived, support the hypothesis that there is more gastrointestinal mucosal damage and more microbial translocation in hospitalized HIV-TB patients and specifically in patients who die within 12 weeks. TFF3 was independently associated with mortality. Our findings of higher sCD14 and TFF3 concentrations and lower EndoCAB concentrations in patients with positive markers of tuberculosis dissemination suggests a relationship between gastrointestinal mucosal damage, microbial translocation and the mycobacterial bacillary load or degree of tuberculosis dissemination. Soluble CD14 concentrations may reflect tuberculosis related inflammation or be driven by LPS translocation. LBP concentrations were significantly higher in hospitalized patients and in patients with two or more positive markers of tuberculosis dissemination. This suggests that LBP increases as an acute phase protein and that this may be driven by several inter-related processes: microbial translocation and systemic immune activation, higher mycobacterial load and tuberculosis-related inflammation. IFABP concentrations were unexpectedly higher in outpatient HIV-infected control patients this suggests an incomplete understanding of what factors contribute to increased concentrations of

IFABP. Bacterial 16s rDNA concentrations were raised in hospitalized HIV-TB patients, but was not associated with mortality and was not associated with biomarkers of tuberculosis dissemination. This supports higher levels of microbial translocation in severely ill hospitalized patients, but a limited role in the processes driving mortality. A limitation, though, is that we did not undertake longitudinal measurement.

An improved understanding of the causes of mortality and underlying pathophysiology of mortality in hospitalized patients with HIV-associated tuberculosis is a research priority and is needed to develop improved treatment strategies in this patient population. Our findings support the hypothesis of increased microbial translocation and suggests an association between microbial translocation and tuberculosis dissemination. It is important to understand whether microbial translocation is in the causal pathway contributing to mortality (and should therefore be considered as a therapeutic target) or whether it is a ‘bystander’ finding which will improve with effective anti-tuberculosis treatment. This study advances our knowledge, but raises many additional questions regarding the role of gastro-intestinal integrity and microbial translocation in the pathogenesis of HIV-TB.

Appendix to Chapter 5

Association of markers of microbial translocation and immune phenotypes:

Markers of microbial translocation concentrations and the principal components values derived from principal components analysis of 28 soluble mediators of inflammation were plotted in a correlation matrix and compared using Spearman's correlation test.

Figure 1: Correlation plot of markers of microbial translocation against principal components values:

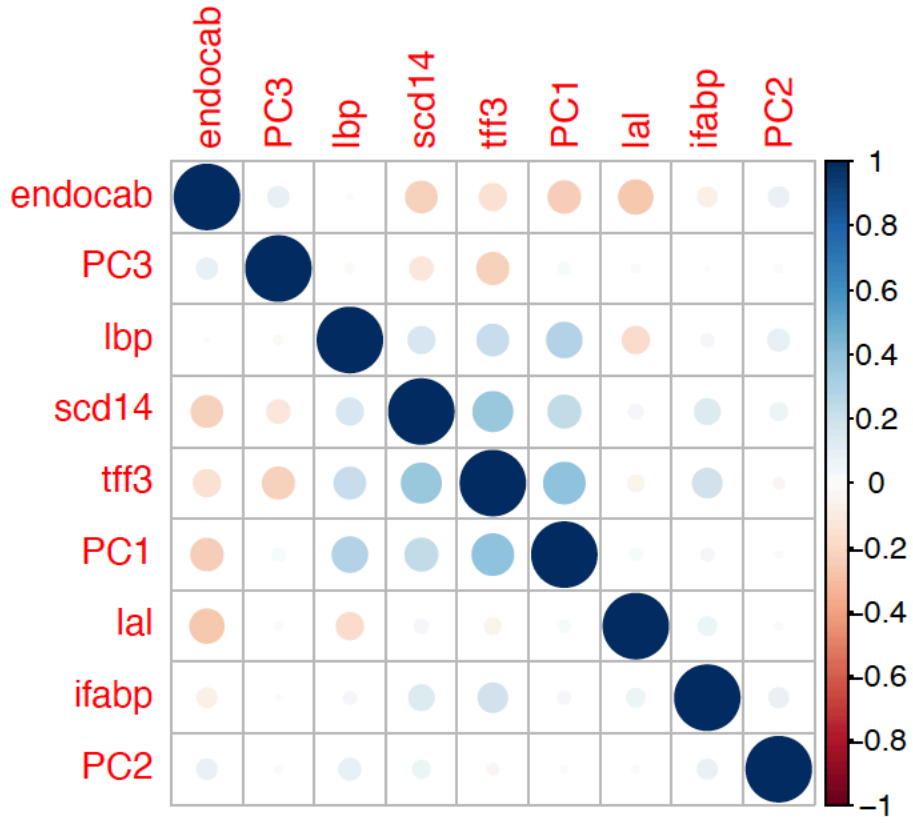


Figure 1: endocab: Endotoxin core antibody IgM; PC3: principal component score 3; lbp: lipopolysaccharide binding protein; scd14: soluble CD14; tff3: trefoil factor 3; PC1: principal component score1; lal: lipopolysaccharide; ifabp: intestinal fatty acid binding protein; PC2: principal component score 2. The right hand y-axis represents Spearman's rho and the size of the colour intensity is related to the strength of the association. Blue indicates a positive association and red a negative association.

PC1 had positive correlation with trefoil factor 3 (rho = 0.39), lipopolysaccharide binding protein (rho = 0.28), soluble CD14 (rho = 0.24) and a negative correlation

with endotoxin core antibody IgM (rho = -0.24). PC2 did not have strong correlations with markers of microbial translocation and PC3 had a negative association with trefoil factor 3 (rho = -0.23).

Microbial translocation in patients with abdominal tuberculosis

This analysis was restricted to hospitalised patients with HIV-TB and who had a markers of microbial translocation concentrations measured. Abdominal ultrasound (USS) findings were recorded for all patients who had abdominal USS performed in routine care during the index admission This variable reflects any findings compatible with abdominal tuberculosis, including multiple abdominal lymphnodes larger 1 cm in diameter, splenic microabcesses and ascites with stranding. Amongst hospitalized TB patients 277/576 (48%) had abdominal ultrasounds performed during their index admission and 242/277 (87%) had findings compatible with abdominal tuberculosis.

Figure 2: Makers of microbial translocation plotted against abdominal ultrasound findings:

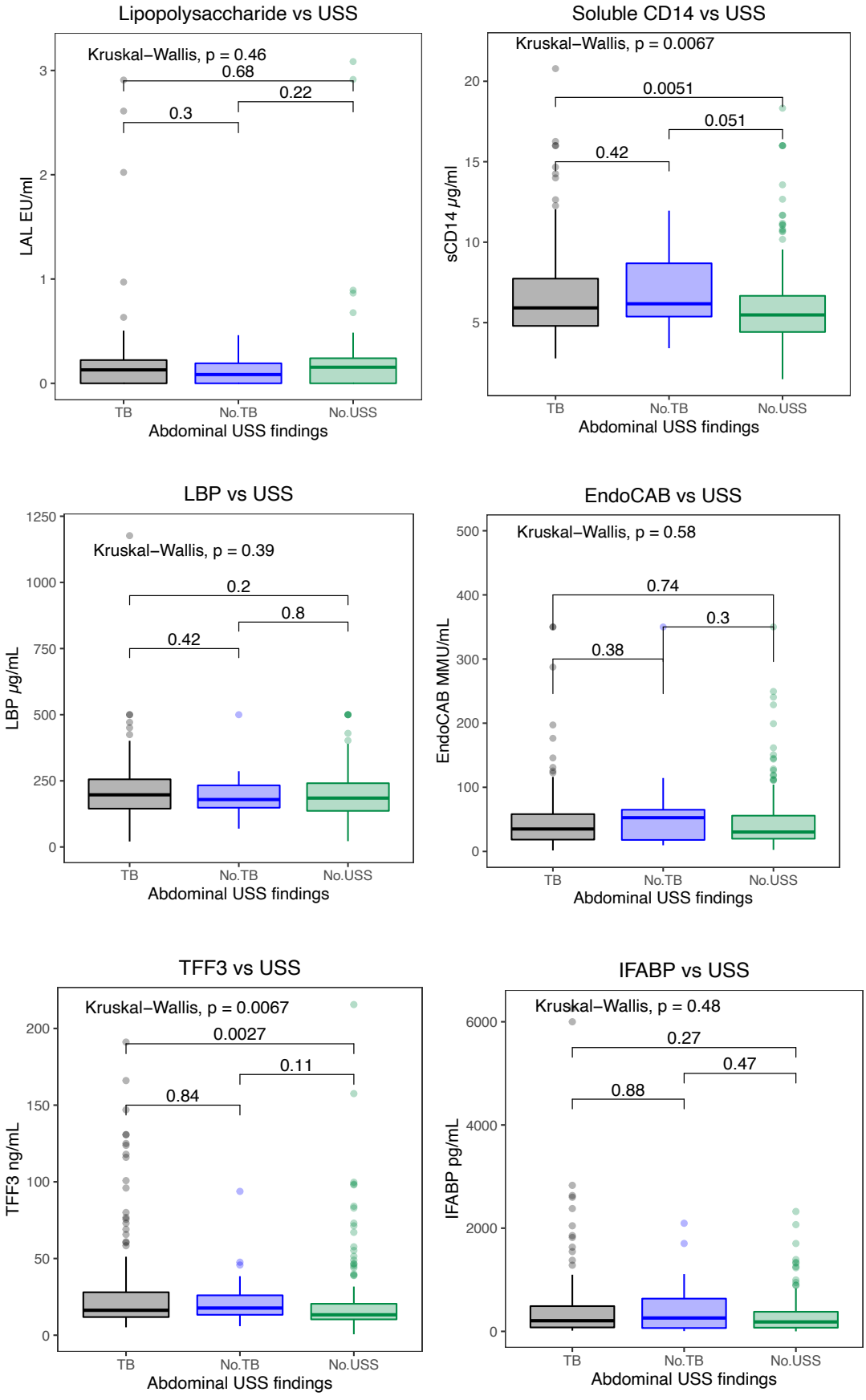


Figure 2: TB: Hospitalized patients with HIV-TB who had an abdominal ultrasound performed during index admission. TB (coloured black): Patients with abdominal ultrasound findings compatible with tuberculosis; No.TB: (coloured blue) Hospitalized patients with no ultrasound features of tuberculosis; No.USS (coloured green): No abdominal ultrasound performed; EU/mL: Endotoxin standard units per milliliter; µg/mL: microgram per milliliter; MMU/mL: standard median units per milliliter; ng/mL: nanogram per milliliter; pg/mL: picogram per milliliter; LPS; lipopolysaccharide; sCD14: soluble CD14; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; TFF3: trefoil factor 3; IFABP: intestinal fatty acid binding protein. The p-values represent Wilcoxon rank sum comparisons between groups and Kruskal-Wallis test across groups where indicated.

Hospitalised patients with HIV-TB who had abdominal ultrasound features compatible with tuberculosis had significantly higher soluble CD14 and trefoil factor 3 concentrations than patients who had no ultrasound performed. However there were no differences between patients who had ultrasound performed and had features compatible with tuberculosis *versus* those with no features compatible with tuberculosis. The clinical significance of this is unclear.

Microbial translocation in patients with enteric organisms on bacterial blood culture during index admission

Four patients hospitalised with HIV-TB also cultured gram negative pathogens on blood culture during index admission. Two of these patients had markers of microbial concentration measured. Their values are tabulated below, but it is not possible to make any statistical comparisons in view of the small sample size.

Table 1: Microbial translocation marker concentrations in patients hospitalized with HIV-associated tuberculosis and a gram negative pathogen in blood:

Patient	Marker	Value
1st Patient	LPS, EU/mL	0
	sCD14, µg/mL	11.96
	LBP, µg/mL	247.89
	EndoCAB MMU/L	9.6
	TFF3, ng/mL	93.77
	IFABP, pg/mL	327.61

2nd Patient	LPS, EU/mL	0.19
	sCD14, µg/mL	4.19
	LBP, µg/mL	142.63
	EndoCAB MMU/L	61.8
	TFF3, ng/mL	14.20
	IFABP, pg/mL	91.63

Table 5: **Median concentration presented**
EU/mL: Endotoxin standard units per milliliter; µg/mL: microgram per milliliter; MMU/mL: standard median units per milliliter; ng/mL: nanogram per milliliter; pg/mL: picogram per milliliter; LPS; lipopolysaccharide; sCD14: soluble CD14; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; TFF3: trefoil factor 3; IFABP: intestinal fatty acid binding protein

Microbial translocation in patients with *Mycobacterium tuberculosis* blood stream infection (MTB BSI)

Markers of microbial translocation concentrations were plotted in patients who cultured MTB on blood culture and those who were blood culture negative.

The majority of patients had mycobacterial blood culture performed 556/576 (96.2%). Two patients cultured non-tuberculous mycobacteria and 22 patients had no blood cultures performed and these patients were not included in this analysis. Patients who cultured *Mycobacterium tuberculosis* (MTB) in blood had significantly higher sCD14, LBP and TFF3 concentrations together with significantly lower EndoCAB concentrations, which supports the hypothesis that there may be more microbial translocation occurring in patients with MTB bloodstream infection compared to patients without MTB BSI.

Figure 3: Makers of microbial translocation plotted against mycobacterial blood culture results:

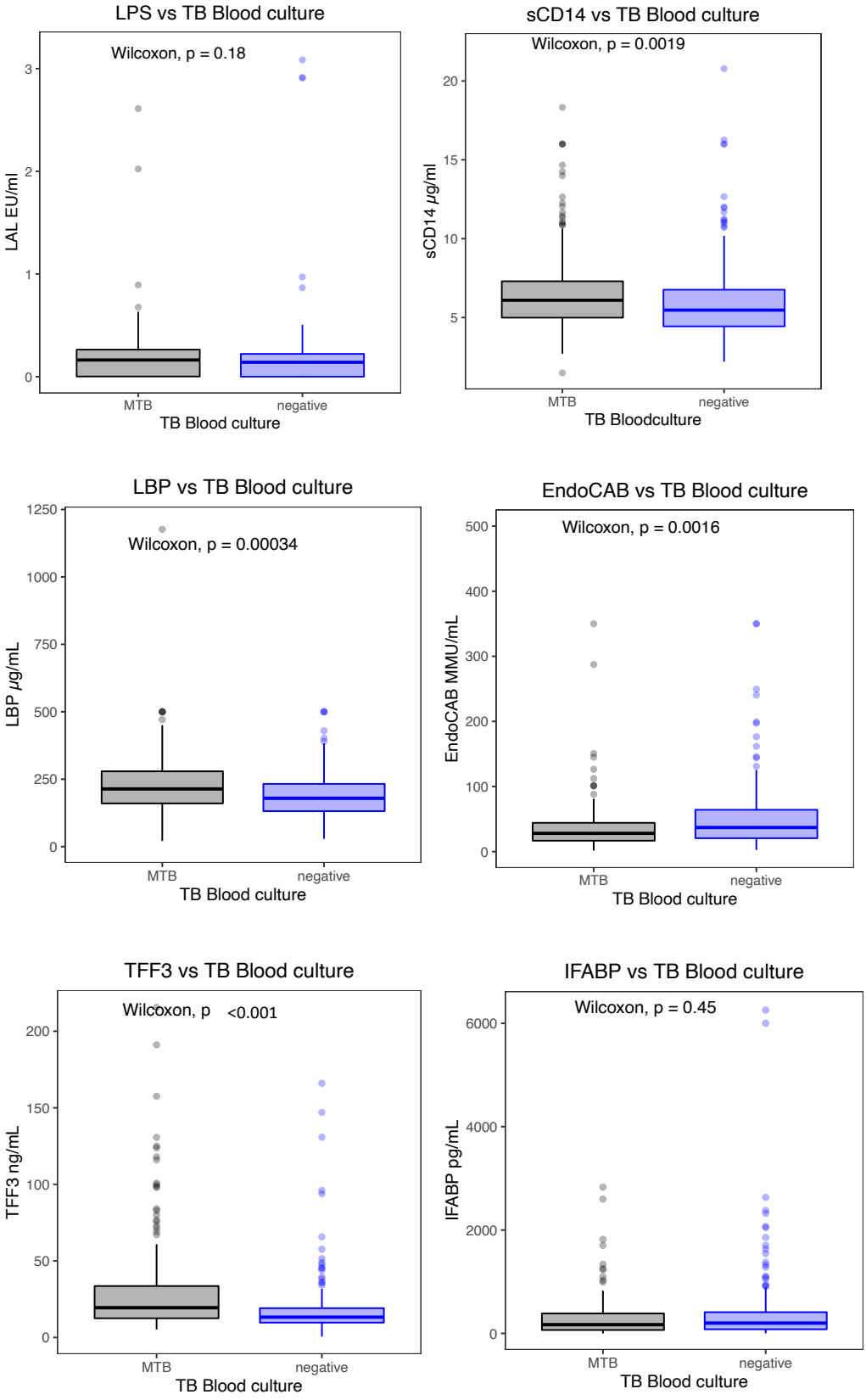


Figure 3: TB: Hospitalized patients with HIV-TB who mycobacterial blood culture performed. N = 552, 2 patients who cultured non tuberculous mycobacteria were not included. 22

patients had no mycobacterial blood culture performed. MTB (coloured black): *Mycobacterium tuberculosis* identified on blood culture; negative (coloured blue): Negative TB blood culture result; EU/mL: Endotoxin standard units per milliliter; µg/mL: microgram per milliliter; MMU/mL: standard median units per milliliter; ng/mL: nanogram per milliliter; pg/mL: picogram per milliliter; LPS; lipopolysaccharide; sCD14: soluble CD14; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; TFF3: trefoil factor 3; IFABP: intestinal fatty acid binding protein

Microbial translocation in patients with extensive changes on chest X-ray

All patients had chest radiograph (CXR) performed during their index admission. To assess microbial translocation markers in patients who had extensive CXR changes compared to other patients we analysed patients who had a miliary infiltration on CXR and compared these patients to all other patients. In total 95/576 (16.5%) had a CXR image compatible with miliary tuberculosis. CXRs were assessed by the enrolling clinician and have not been reported by a radiologist. The were no differences in microbial marker concentrations between patients who had a CXR with a miliary picture compared to those with other findings.

Figure 4: Makers of microbial translocation plotted against chest radiograph findings:

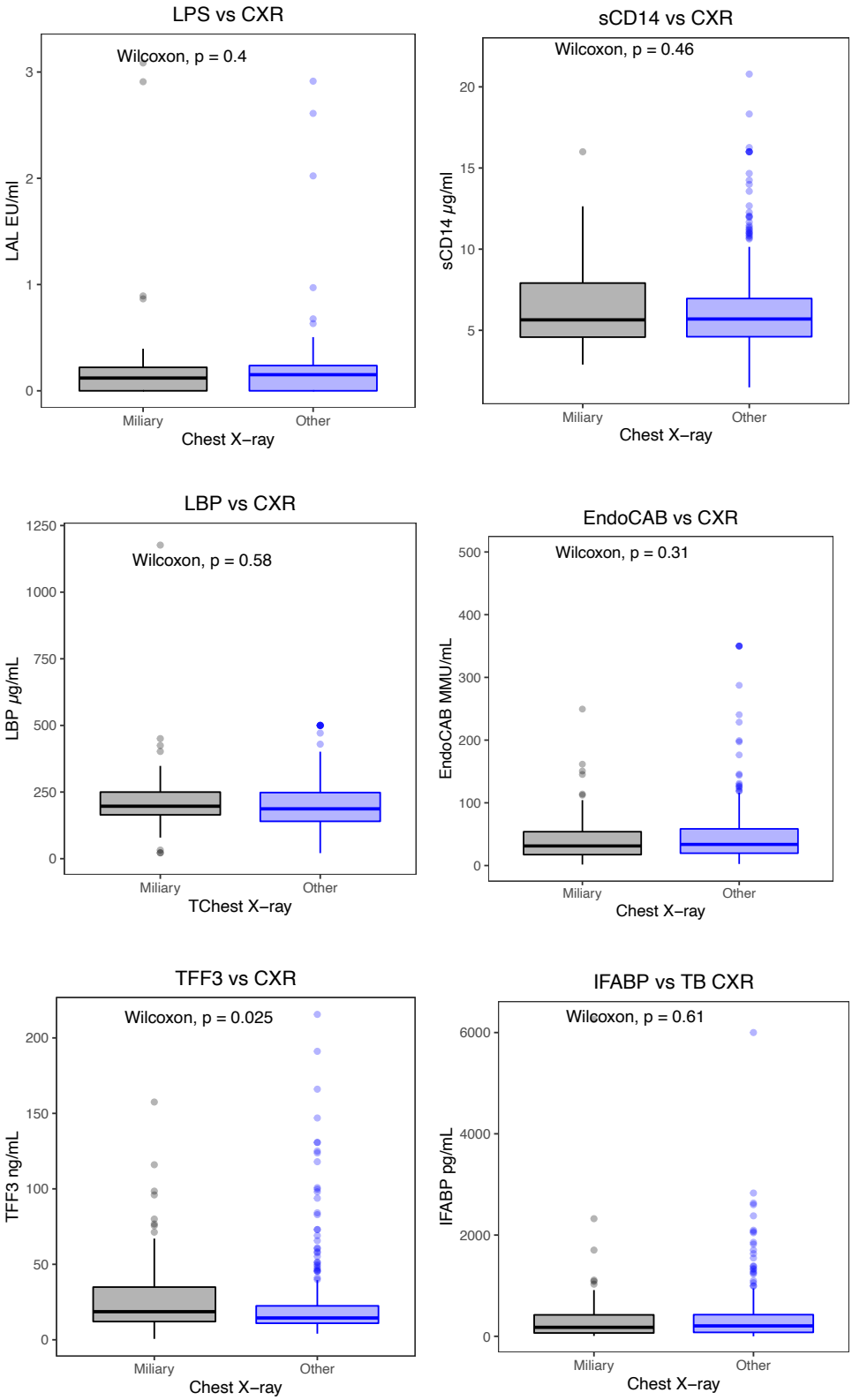


Figure 4: CXR: Chest radiograph. Miliary (coloured in black): Chest radiograph image compatible with miliary tuberculosis. Other (coloured in blue): All other radiograph findings. EU/mL: Endotoxin standard units per milliliter; µg/mL: microgram per milliliter; MMU/mL: standard median units per milliliter; ng/mL: nanogram per milliliter; pg/mL: picogram per

milliliter; LPS; lipopolysaccharide; sCD14: soluble CD14; LBP: lipopolysaccharide binding protein; EndoCAB: endotoxin core antibody IgM; TFF3: trefoil factor 3; IFABP: intestinal fatty acid binding protein

CHAPTER 6:

False rifampicin resistant results using Xpert MTB/RIF on urine samples in hospitalized HIV-infected patients

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False rifampicin resistant results using Xpert MTB/RIF on urine samples in hospitalised HIV-infected patients

Authors:
Charlotte Schutz[1]
Amy Ward[1]
Rosie Burton[2]
Mark P. Nicol[3]
Liz Blumenthal[1]
Graeme Meintjes[1]
Andrew D. Kerkhoff[4]

Affiliations:
[1]Wellcome Centre for Infectious Diseases Research in Africa (CIDRI-Africa), Institute of Infectious Disease and Molecular Medicine (IDM) and Department of Medicine, University of Cape Town, Cape Town, South Africa

[2]GF Jooste Hospital; Department of Medicine, University of Cape Town, Cape Town, South Africa

[3]Division of Medical Microbiology, University of Cape Town and National Health Laboratory Services, Cape Town, South Africa

[4]Division of HIV, Infectious Diseases and Global Medicine at Zuckerberg San Francisco General Hospital and Trauma Center, Department of Medicine, University of California, San Francisco, United States

Corresponding author:
Andrew Kerkhoff,
andrew.kerkhoff@ucsf.edu

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Background: A small proportion of false rifampicin resistant results have previously been reported using GeneXpert MTB/RIF version G4 on sputum samples; however, this has not been investigated for urine samples in HIV-associated tuberculosis (TB).

Objectives: We sought to determine the proportion of false rifampicin resistant results using Xpert MTB/RIF version G4 on urine samples among HIV-infected inpatients investigated for TB.

Methods: Hospitalised HIV-infected patients undergoing systematic TB testing from two cohorts in Cape Town, South Africa, were enrolled. All patients with ≥1 urine Xpert result available were included. Rifampicin resistant urine Xpert results were classified into three mutually exclusive groups: (1) true rifampicin resistance, (2) false rifampicin resistance or (3) unknown after review of available microbiologic and clinical data.

Results: Overall, 1171 patients were included, from whom a total of 1704 urine Xpert results were available on unconcentrated and/or concentrated urine samples. There were 416 samples positive for TB (24.4% [95% CI 22.4–26.5]), of which 43/413 (10.4% [95% CI 7.6–13.8]) were rifampicin resistant (after excluding three results that were falsely positive due to contamination). Of 43 rifampicin resistant Xpert results (among 40 patients), 30 were classified as true resistance, 11 as false resistance and 2 could not be classified. Excluding unclassifiable results, 30/41 results were confirmed as true-positive urine Xpert rifampicin resistance (positive predictive value: 73.2% [95% CI 57.1–85.8]).

Conclusion: Urine Xpert testing showed a high proportion of false rifampicin resistance results. Urine Xpert rifampicin resistant results should be interpreted cautiously and confirmed when possible.

Keywords: HIV; AIDS; Tuberculosis; Xpert; Rifampicin resistance; False resistance.

Introduction

Tuberculosis (TB) remains the leading cause of death in people living with HIV, contributing to one-in-three AIDS-related deaths.[1] Timely diagnosis of TB in such patients remains challenging because of non-specific presentations and disseminated disease.[2,3,4] Gene Xpert MTB/RIF, an automated nucleic acid amplification test, is capable of providing results in a few hours and represents an important breakthrough for diagnosing HIV-associated TB. Importantly, Xpert also rapidly detects rifampicin resistance, without need for an additional sample or cartridges. It has been endorsed by the World Health Organization (WHO) since 2010. Sputum Xpert (or Xpert Ultra where available) is currently recommended by the WHO as the initial diagnostic test in patients with suspected HIV-associated TB or multi-drug resistant (MDR) TB.[5] In those with microbiologically confirmed TB, Xpert MTB/RIF is also recommended by the WHO as a first-line assay for the rapid detection of rifampicin-resistance. It is therefore an important tool in tackling the growing global health challenge of drug resistant (DR)-TB. However, the WHO does not currently have a recommendation regarding the use of Xpert MTB/RIF in urine owing to an insufficient amount of data on the performance and utility of this assay in urine specimens.[6]

In concordance with WHO guidelines, sputum Xpert was implemented as the initial diagnostic evaluation in those with suspected TB and DR-TB in South Africa as well as other countries[7] and in South Africa, it has now been replaced with the updated Xpert Ultra cartridge. Although

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Xpert has not been associated with a mortality reduction in most trials to date,[8,9,10] its implementation has been associated with overall shorter times to starting anti-TB therapy, including DR TB.[8,11,12,13] It has also increased the diagnostic yield by 1.4% – 15% (compared to sputum microscopy) in clinical trials in Sub-Saharan Africa, Brazil and Indonesia.[8,9,10,11,13,14,15,16]

Against the backdrop of improved case detection, previous studies have reported on false rifampicin resistance results associated with the Xpert MTB/RIF assay, and meta-analyses found the overall specificity of the Xpert for rifampicin-resistance in sputum samples to be 98% (i.e. 2% showed false rifampicin resistant results) and 99% in extra-pulmonary samples.[17,18] This however appeared to be associated in part with earlier Xpert cartridge generations.[19] An implementation study from South Africa found the Xpert G4 cartridge to have excellent positive predictive value for rifampicin resistance of 99.5% (95% CI 98.5–100) in sputum samples.[20]

We have previously found that among HIV-patients requiring acute medical hospitalisation, testing of a single concentrated urine sample detected 2.2 times more TB cases than sputum Xpert testing, largely because of the inability of sick inpatients to produce a sputum sample.[21] Additionally, a recent randomised, multi-country trial found that the addition of rapid urine-based assays (including urine Xpert) to sputum Xpert testing was associated with reduced mortality among hospitalised HIV-infected patients in sub-group analyses.[22] This suggests that urine-based testing using Xpert may have an important role in the TB diagnostic algorithm among hospitalised patients with advanced HIV, especially those too ill to produce a sputum sample. However, the proportion of false-positive rifampicin resistance results using Xpert on urine samples has not been reported. We sought to determine the proportion of urine Xpert false rifampicin resistance results among hospitalised HIV-infected patients being investigated for HIV-associated TB in Cape Town, South Africa.

Methods

Patients and setting

Patients from two parent cohort studies were included. In the first, patients were recruited at GF Jooste Hospital, South Africa from June 2012 to October 2013. Unselected HIV-infected patients admitted to the medical wards were recruited within 24 h of admission, regardless of TB treatment at the time of admission.[21,23] GF Jooste Hospital was closed at the end of 2013 and two new hospitals (including Khayelitsha Hospital) were opened serving the same communities at the time the second study was conducted. The second study was undertaken at Khayelitsha Hospital from January 2014 until October 2016 and recruited HIV-infected patients with a low CD4 T-cell count (< 350 cells/μL) admitted to hospital with a suspected new diagnosis of TB. Patients already on TB treatment were excluded from this study. Sputum, blood and urine samples were systematically obtained (when possible) as part of both study protocols and submitted for mycobacteriology (TB culture and/or Xpert). Information about any additional specimens, that were clinically indicated and collected by the medical teams were also recorded – for example, lymph node aspirates, cerebrospinal fluid TB cultures, pleural TB cultures and urine TB cultures.

In the first study, two cases of false urine rifampicin resistance occurred 3 months after study initiation (Appendix Table 1-A1 – patients JTBS097 & JTBS099). Both patients' urine Xpert samples were collected after a sample was taken from an MDR patient earlier on the same day. It was determined that both samples were likely contaminated due to inadequate cleaning of the reusable bedpan, although laboratory cross-contamination could not be ruled out. We subsequently introduced single-use disposable bed pans (Litha Healthcare Group, Johannesburg, South Africa) and these were used for the remainder of the Jooste Hospital TB study and the duration of the Khayelitsha Hospital TB study. There were no repeat episodes of suspected cross-contamination. Urine was transferred to a polypropylene tube using a sterile syringe. Patients from both cohort studies had urine Xpert testing performed. Demographic details and clinical symptoms were recorded for all patients at study entry. Patients were managed by the hospital and clinic staff, and all TB diagnostic test results were made available by study staff and could be utilised to inform patient care.

Laboratory methods

Urine Xpert testing for both studies was performed at the Groote Schuur Hospital National Health Laboratory Service laboratory using Xpert MTB/RIF Assay G4 version 5. All specimens were processed using standardised protocols and quality assurance procedures as previously described.[24] In brief, for the GF Jooste Hospital study, Xpert testing of urine samples was conducted in two ways on each sample. The first method (unconcentrated) utilised 2.0 mL of fresh urine that was centrifuged, resuspended in 0.75 mL phosphate buffer and then tested using Xpert.[25] The second method (concentrated) used a 30 mL – 40 mL urine sample that was centrifuged at 3000 g for 15 min. The resultant supernatant was removed and the pellet was resuspended in the residual urine volume (without the addition a phosphate buffer); 0.75 mL was then tested using Xpert.[21] For both methods, Xpert sample reagent (1.5 mL) was added to the samples as per manufacturer's instructions. The Khayelitsha Hospital study only used Xpert testing on concentrated urine samples and was undertaken using the same methods as described above. The reference standard for drug resistance, including rifampicin resistance for both studies, was a molecular line probe assay (MTBDR plus; Hain Lifescience Nehren, Germany) undertaken on culture isolates from any clinical specimen (not necessarily urine).

Analysis

Patient populations were from overlapping referral areas in the Cape Town townships and both cohorts included

HIV-infected patients requiring medical admission and had detailed TB investigations performed. Urine Xpert rifampicin resistance results were classified by two authors independently by first assessing all available microbiological results (including culture, Xpert and line probe assay) on all clinical samples. In cases where it was not possible to classify urine Xpert rifampicin resistance results by assessing microbiological results from other clinical samples, the type of TB treatment, response to treatment and vital status at 12 weeks were also considered. All patients with urine Xpert rifampicin resistant results were assigned to one of the three mutually exclusive groups: (1) true rifampicin resistant urine Xpert (patients who had rifampicin-resistant TB confirmed by culture or Xpert on other clinical samples) (2) false rifampicin resistant urine Xpert (patients who did not have rifampicin-resistant TB present on additional clinical samples and had a clinical course that was not compatible with drug-resistant TB), (3) unknown (insufficient microbiological and clinical evidence to classify a patient's urine Xpert rifampicin resistant result). Furthermore, patients with true urine Xpert rifampicin resistance were classified as having heteroresistance if additional independent sample/s from the same clinical episode demonstrated both a rifampicin-susceptible and a rifampicin-resistant *Mycobacterium tuberculosis* (MTB) isolate, i.e. discordant results from two different clinical specimens in the same patient. Two patients (contributing three urine Xpert rifampicin resistance results) were determined to have false urine Xpert rifampicin resistance; this occurred within 3 months of initiating the first study, and was prior to the introduction of single use disposable bedpans (see details above). This led to the introduction of single-use disposable bedpans and avoided further such cases.

Ethical consideration

Approval for both studies was obtained from the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee and patients provided written informed consent according to the approved study protocols.

Results

There were 585 patients from the GF Jooste Hospital cohort and 586 patients from the Khayelitsha Hospital cohort with urine Xpert results available for a total of 1171 hospitalised HIV-infected patients. Overall 1704 urine Xpert results were available from 1171 patients, of which 554 were performed on unprocessed urine samples and 1150 on concentrated urine samples (Figure 1). Baseline characteristics of the two cohorts were similar. (Table 1).

Among 1704 urine Xpert results, there were 416 (24.4% [95% CI 22.4–26.5]) samples that tested positive for MTB and 46 results indicating rifampicin resistance among 42 patients (Figure 1). After excluding three results (from two patients) that were determined to be caused by contamination, 43 results from 40 patients remained (n = 43/413; prevalence 10.4% [95% CI 7.6–13.8]) and were further classified. The majority of rifampicin resistance results (n = 30/43; 69.8% [95% CI 53.9–82.8]) were classified as true urine Xpert rifampicin resistance based on the results from other independent clinical samples. Eleven (11/43, 25.6% [95% CI 13.5–41.2]) results were classified as false rifampicin resistance and two further results (one from each study) could not be classified. Thus, by the most conservative estimate (excluding 2 unknown results), n = 30/41 results were confirmed as true urine Xpert rifampicin resistant results, for a positive predictive value of 73.2% (95% CI 57.1–85.8). Comprehensive details for each patient with urine Xpert rifampicin resistance were reported in Appendix Table 1-A1.

TABLE 1: Baseline characteristics of Jooste Hospital tuberculosis study and Khayelitsha Hospital tuberculosis study patients.

Variable	Jooste Hospital study (n = 585)		Khayelitsha Hospital study (n = 586)	
	n	% or IQR	n	% or IQR
Sex				
Female	338	57.8	307	52.4
Male	247	42.2	279	47.6
Age, years	35.3	28.9, 41.4	35.9	30.8, 43.9
ART status				
Defaulted ART	113	19.3	140	24.1
ART naive	209	35.7	222	38.3
Currently on ART	263	45.0	218	37.6
TB history				
Previous TB	263	45.1	268	45.7
Unknown TB history	2	0.3	23	3.9
CD4, cells/mL	134	53, 275	66	24, 138
HIV viral load, log copies/mL	4.2	1.6, 5.5	5.2	3.8, 5.7
Established on TB treatment at enrolment	158	27	-	-

ART, Antiretroviral therapy; TB, Tuberculosis.

Continuous variables presented as median and interquartile range and categorical variables as number and percentage.

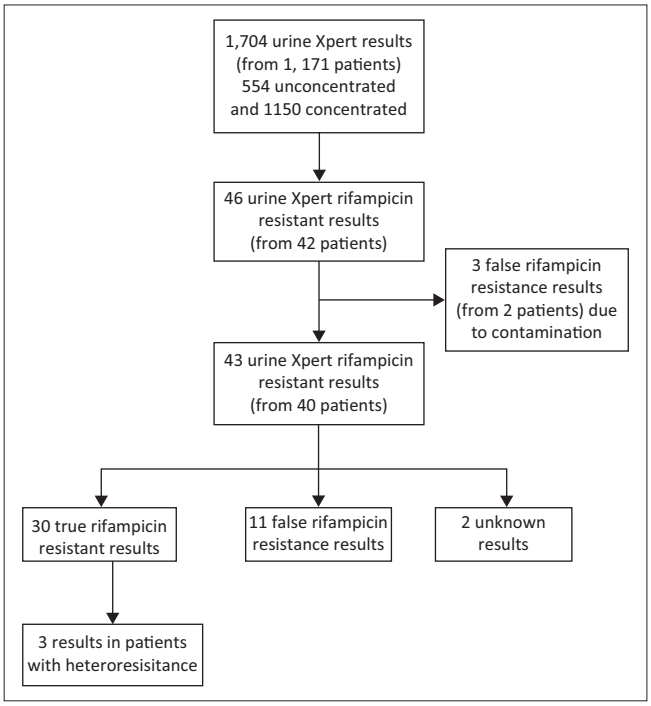

FIGURE 1: Overview of urine Xpert rifampicin resistance results from two cohorts of hospitalised HIV-patients in Cape Town, South Africa.

False urine rifampicin resistance results were more commonly observed in the Jooste Hospital study: 9/18 (50%) results compared with 2/25 (8%) in the Khayelitsha Hospital study (Figure 2). The Jooste Hospital study enrolled not only patients not yet on TB treatment but also those already established on TB treatment, whereas the Khayelitsha Hospital study excluded patients who were already on TB treatment at the time of admission. In the Jooste Hospital study, there were n = 14 results (one unknown rifampicin resistant result) from patients on TB treatment at enrolment and n = 4 results from patients not on TB treatment at enrolment and among these, n = 7/13 (53.8%) and n = 2/4 (50%) had false rifampicin resistant urine Xpert results, respectively. Therefore, in both cohorts and excluding two results that could not be classified, among patients not on TB therapy, n = 24/28 (85.7% [95% CI 67.3–96.0] had true positive urine Xpert resistance results compared to n = 7/13 (53.8% [95% CI 25.1–80.8] among those receiving TB therapy at study enrolment. This suggests that the positive predictive value of Xpert MTB/RIF for rifampicin resistance is higher among those not on TB treatment compared with those who were already established on TB treatment.

Twelve-week mortality for patients with urine Xpert rifampicin resistant results was 30% (n = 12/40) and 7.5% (n = 3/40) were lost to follow-up. No deaths were observed among the 10 patients (accounting for 11 results) with false urine Xpert rifampicin resistance. Limited details regarding

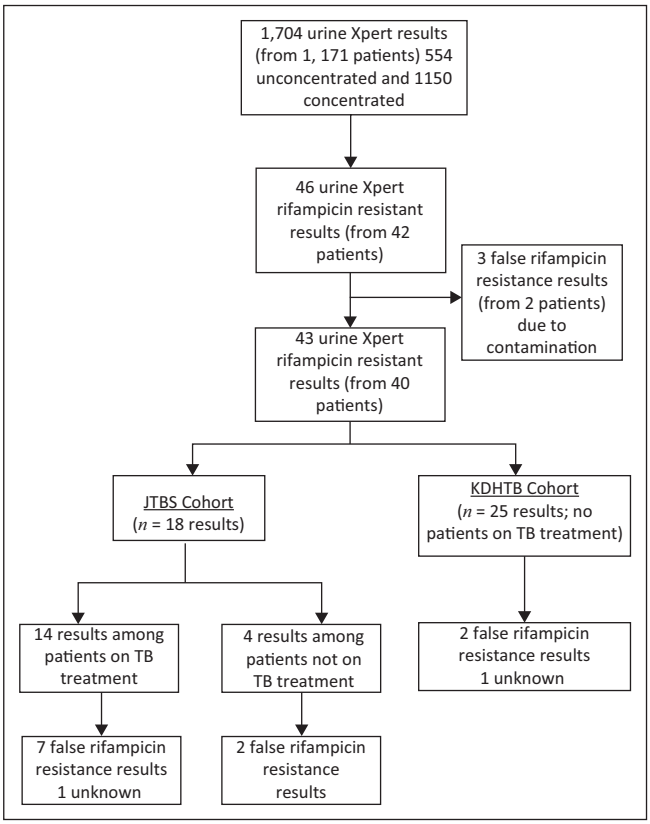

FIGURE 2: Urine Xpert rifampicin resistance results separated by cohort and tuberculosis treatment status.

Xpert probe features for the two patients with false rifampicin resistance in the Khayelitsha Hospital study were available. The clinical microbiologists' comment for patient KDHTB479 indicated that there was a very low load with a double mutation detected by a delay in probes D and E and that the result was likely false positive. In patient KDHTB439 there was a failure of probe D to bind in the isolate and a repeat sample was requested that demonstrated RIF susceptibility. We were unable to obtain information about the probe features for samples of the JTBS study.

Three patients (n = 3/40, 7.5%) with a confirmed rifampicin resistant urine Xpert result had evidence of likely heteroresistant infection. The first patient (Appendix Table 1-A1 – KDHTB203) cultured a drug susceptible isolate from blood (MycoF/lytic bottle), sputum and urine samples but also a rifampicin resistant isolate from sputum during the same admission. The second patient (KDHTB531) cultured a drug-sensitive isolate from blood as well as a drug-resistant isolate from sputum during the same admission. The third patient (JTBS463) was originally started on drug-sensitive TB treatment after a prior sputum Xpert and abscess aspirate culture both showed rifampicin susceptible isolates. One month after starting TB treatment, the patient was admitted for TB immune reconstitution inflammatory syndrome (IRIS). Shortly after discharge, the patient was readmitted for gastroenteritis and was clinically deteriorating despite drug-sensitive TB treatment. At this time, two urine Xpert results showed rifampicin resistance; however, the patient died shortly after receipt of urine Xpert results.

Discussion

In this study, which included hospitalised HIV-infected patients systematically investigated for TB, the overall proportion of urine Xpert rifampicin resistance results was 10.4% (n = 43/413); however, the positive predictive value of urine Xpert MTB/RIF for rifampicin resistance was only 73.2% (n = 30/41).

The correct identification of drug-resistant TB has important implications for both the individuals' health as well as for public health. For the patient, a false rifampicin resistance result may result in not only over-treatment with more toxic drugs that are less efficacious for drug-sensitive TB, but also significantly and unnecessarily prolong treatment times. In high burden, under-resourced settings, a false rifampicin resistance may have important resource implications by resulting in additional drug susceptibility testing, significantly more expensive treatment costs and unnecessary community contact tracing.[26] Thus, any test that detects DR TB should ideally have very high specificity. Under the best-case scenario when results were restricted to those not receiving TB treatment, we found that Xpert testing of rifampicin resistance on urine samples did not achieve sufficiently high positive predictive value (86%) to be the sole/

definitive test for drug-resistant TB identification. This, however, needs to be evaluated in additional settings.

Xpert Ultra is an updated, next-generation sample cartridge for the Xpert platform that is now recommended by the WHO as a replacement for the current Xpert MTB/RIF cartridge[27,28] and has been implemented in South Africa. It provides increased sensitivity for the detection of MTB in sputum (especially smear-negative and pauci-baciliary disease). Xpert Ultra utilises a new melt curve analysis to detect RIF-resistance; however, its diagnostic accuracy (including specificity) for the detection of rifampicin resistance is similar to that of Xpert.[29] The results of this study suggest that urine Xpert Ultra rifampicin resistance results should be interpreted cautiously and confirmed by alternative drug susceptibility testing (either phenotypic or alternative genotypic assays) until the specificity of Xpert Ultra for rifampicin resistance detection has been confirmed to be adequately high to warrant stand-alone testing on urine samples.

Of interest, in this cohort we describe three patients with a confirmed urine Xpert rifampicin resistance result who also had drug-sensitive strains from independent samples during the same admission suggesting likely heteroresistance (either polyclonal infection or acquired heteroresistance). The prevalence of heteroresistance in MTB infections has previously been described.[30,31,32] Although not well-studied, these are likely associated with increased rates of treatment failure for the individual[31] and could complicate TB control efforts at a population level. Xpert may miss heteroresistance if used as a stand-alone test for the detection of rifampicin resistance, however, early studies show that Xpert Ultra may detect heteroresistance when the resistant DNA comprises 5% or more of the sample.[28]

Strengths of this study include a large number of urine Xpert rifampicin results from two geographically and clinically comparable cohorts where patients were prospectively recruited and underwent systematic testing for TB. Additionally, all TB assays including urine Xpert testing were performed at the same laboratory according to standard protocols. After an error yielded two likely false Xpert rifampicin resistant urine cases due to contamination soon after recruitment initiation, disposable bedpans (single-use) were implemented for the duration of both studies. We therefore recommend that clinicians use single-use specimen collection bedpans and containers when utilising Xpert or Xpert Ultra testing on urine to prevent DNA-cross-contamination between samples.

The reason(s) for the high proportion of false positive urine rifampicin resistance is not entirely clear, but the proportion was higher among those already receiving TB therapy. A limitation of this study is that we did not have data available to systematically evaluate the Xpert probe features associated with our classification of false rifampicin resistance. Different methods of drug susceptibility testing could explain discrepant results in some cases.[33,34] The majority of drug susceptibility testing on cultured isolates in both studies was PCR-based; however, we also captured results of all TB tests performed in-service and cannot reliably differentiate between drug susceptibility testing performed with other methods such as liquid or solid media for all samples for the duration of the study. Because of the early implementation of disposable bedpans, we do not suspect undetected contamination beyond that described above. Furthermore, because most patients with positive urine rifampicin results did not have paired urine culture isolates available for further genotypic or phenotypic drug-susceptibility testing, patients classified as having false positive rifampicin results may have had heteroresistance with compartmentalised true rifampicin-resistant urinary TB and rifampicin-susceptible TB at other anatomic sites. However, the favourable clinical course of most of these patients on first-line drug-sensitive TB treatment counts against this possibility. Notably, a large proportion of false positive rifampicin results were among those already receiving anti-TB therapy, where 50% of urine Xpert rifampicin resistance results were classified as false resistance; this suggests that further caution should be applied when interpreting urine Xpert rifampicin resistance results in treatment-experienced patients.

An additional limitation of the study is that sequencing of isolates was not performed as part of either study. Sequencing of the rpoB gene would have been particularly useful in the cases that we could not classify as true or false resistance and the heteroresistant cases. Furthermore, urine TB cultures were not routinely performed in either study and it may have been useful to compare drug susceptibility results on isolates cultured from urine samples collected at the same time as the urine Xpert samples.

In conclusion, urine testing using Xpert provides important diagnostic yield for hospitalised HIV-infected patients being investigated for HIV-associated TB, especially in those unable to produce sputum samples. Although the overall proportion of patients with urine Xpert rifampicin resistance in this cohort was relatively low, the proportion of those classified as false rifampicin resistance was substantially higher than has previously been reported on sputum. Urine Xpert rifampicin resistant results should therefore be interpreted with caution, repeated on a second sample in patients at low-risk for drug resistant TB (as currently recommended by the WHO for sputum samples) and confirmed using additional culture-based or molecular assays when possible. Whether these findings apply to Xpert Ultra is an issue that requires further study.

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Competing interests

The authors have no conflict of interests.

Authors' contributions

G.M. R.B., C.S., L.B. A.W. and G.M. were responsible for patient recruitment and overseeing the individual study sites. A.D.K. and C.S. were responsible for the database. C.S. and A.D.K. designed and performed the analyses with input from G.M. and M.P.N. was responsible for the mycobacteriology. C.S. and A.D.K. wrote the first draft of the article and G.M. gave input to further drafts. All other authors commented on a draft and approved the final version of the article.

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Data availability statement

The data sets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Disclaimer

The views expressed in the article are those of the authors and not an official position of the institution or funder.

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Appendix 1

TABLE 1-A1: Patients with positive urine Xpert rifampicin resistant results – details of additional tuberculosis tests and clinical course.

Study ID	*n*	Sample type	Test	Organism	Susceptibility to rifampicin	Susceptibility to isoniazid	12-week outcome	Classification of urine Xpert rifampicin resistance†	Heteroresistance	Reason for classification
KDHTB067	1	Blood	Culture	MTB	-	-	Died	Unknown	No	No samples could confirm resistance. Patient initially improved on drug-sensitive TB treatment and then experienced rapid neurological deterioration and died on day 3 of therapy.
	1	Blood	Culture	Neg	-	-				
KDHTB086	2	Blood	Culture	Neg	-	-	Died	True Resistance	No	Urine culture sample confirmed rifampicin resistance and sputum Xpert indicated rifampicin resistance.
	1	CSF	Culture	Neg	-	-				
	1	Sputm	Xpert	MTB	Resistant	-				
	1	Urine	Culture	MTB	Resistant	Resistant				
KDHTB110	1	Blood	Culture	Neg	-	-	Alive	True Resistance	No	One blood and three urine culture samples confirmed rifampicin resistance.
	1	Blood	Culture	MTB	Resistant	Resistant				
	1	CSF	Culture	Neg	-	-				
	1	Sputum	Culture	Neg	-	-				
	3	Urine	Culture	MTB	Resistant	Resistant				
KDHTB203	1	Blood	Culture	MTB	-	-	Alive	True Resistance	Yes	Sputum culture result showed rifampicin resistance and three other cultures (sputum, blood and urine) showed drug-sensitive MTB.
	1	Blood	Culture	MTB	Sensitive	Sensitive				
	1	Sputum	Culture	MTB	Sensitive	-				
	1	Sputum	Culture	MTB	Resistant	Sensitive				
	1	Urine	Culture	MTB	Sensitive	Sensitive				
KDHTB238	1	Blood	Culture	MTB	Resistant	Sensitive	LTFU	True Resistance	No	Five cultures and one additional Xpert confirmed rifampicin resistance.
	4	Sputum	Culture	MTB	Resistant	Sensitive				
	1	Sputum	Xpert	MTB	Resistant	-				
KDHTB242	1	Blood	Culture	Neg	-	-	Alive	True Resistance	No	Four cultures confirmed rifampicin resistance.
	1	Pleural	Culture	MTB	Resistant	Sensitive				
	2	Sputum	Culture	MTB	Resistant	Sensitive				
	1	Urine	Culture	MTB	Resistant	Sensitive				
KDHTB264	2	Blood	Culture	Neg	-	-	Alive	True Resistance	No	One culture result confirmed rifampicin resistance.
	1	CSF	Culture	Neg	-	-				
	1	Pleural	Culture	MTB	Resistant	Resistant				
	2	Urine	Culture	Neg	-	-				
KDHTB398	2	Blood	Culture	MTB	Resistant	Resistant	Died	True Resistance	No	Two culture results confirmed rifampicin resistance.
	1	CSF	Culture	Neg	-	-				
KDHTB416	1	Blood	Culture	Neg	-	-	LTFU	True Resistance	No	Two culture results confirmed rifampicin resistance.
	1	Sputum	Culture	Neg	-	-				
	2	Sputum	Culture	MTB	Resistant	Resistant				
KDHTB439	1	Blood	Culture	Neg	-	-	Alive	False Resistance	No	One culture and three Xpert results showed rifampicin sensitivity.
	1	Pleural	Xpert	MTB	Sensitive	-				
	1	Sputum	Xpert	MTB	Sensitive	-				
	1	Sputum	Culture	MTB	Sensitive	Sensitive				
	1	Urine	Culture	Contam	-	-				
	1	Urine	Xpert	MTB	Sensitive	-				
KDHTB444	2	Blood	Culture	MTB	-	-	Died	True Resistance	No	One culture result confirmed rifampicin resistance.
	1	Blood	Culture	MTB	Neg	-				
	1	Sputum	Culture	MTB	Resistant	Resistant				
KDHTB445	1	Blood	Culture	Neg	-	-	Alive	True Resistance	No	Three culture results confirmed rifampicin resistance.
	3	Sputum	Culture	MTB	Resistant	Resistant				
KDHTB477	1	Blood	Culture	MTB	Resistant	Sensitive	Alive	True Resistance	No	Three culture results confirmed rifampicin resistance.
	2	Sputum	Culture	MTB	Resistant	Sensitive				
KDHTB479	1	Blood	Culture	Neg	-	-	Alive	False Resistance	No	Two culture results showed rifampicin sensitivity.
	2	Sputum	Culture	MTB	Sensitive	Sensitive				
KDHTB524	1	Blood	Culture	Neg	-	-	Alive	True Resistance	No	One culture and two Xpert results confirmed rifampicin resistance.
	1	Sputum	Culture	Neg	-	-				
	2	Sputum	Xpert	MTB	Resistant	-				
	1	Sputum	Culture	MTB	Resistant	Sensitive				

Table 1-A1 continues on the next page →

TABLE 1-A1 (Continues...): Patients with positive urine Xpert rifampicin resistant results – details of additional tuberculosis tests and clinical course.

Study ID	*n*	Sample type	Test	Organism	Susceptibility to rifampicin	Susceptibility to isoniazid	12-week outcome	Classification of urine Xpert rifampicin resistance†	Heteroresistance	Reason for classification
KDHTB531	1	Blood	Culture	MTB	Sensitive	Sensitive	Died	True Resistance	Yes	One culture result confirmed rifampicin resistance and one showed rifampicin sensitivity.
	1	Sputum	Culture	MTB	Resistant	Sensitive				
KDHTB555	1	Blood	Culture	MTB	Resistant	Resistant	Alive	True Resistance	No	Three culture results confirmed rifampicin resistance.
	2	Blood	Culture	Neg	-	-				
	2	Sputum	Culture	MTB	Resistant	Resistant				
KDHTB556	1	Abscess	Culture	MTB	Resistant	Resistant	Died	True Resistance	No	Two culture results confirmed rifampicin resistance.
	1	Blood	Culture	Neg	-	-				
	1	Sputum	Culture	MTB	Resistant	Resistant				
KDHTB560	2	Blood	Culture	Neg	-	-	Alive	True Resistance	No	One culture result confirmed rifampicin resistance.
	1	Sputum	Culture	MTB	Resistant	Resistant				
KDHTB598	1	Blood	Culture	MTB	Resistant	Sensitive	Died	True Resistance	No	Three culture results confirmed rifampicin resistance.
	1	CSF	Culture	MTB	Resistant	Sensitive				
	1	Sputum	Culture	MTB	Resistant	Sensitive				
KDHTB616	1	Abscess	Culture	MTB	Resistant	Sensitive	Alive	True Resistance	No	Four culture results confirmed rifampicin resistance.
	2	Blood	Culture	MTB	Resistant	Sensitive				
	5	Blood	Culture	Neg	-	-				
	1	Blood	Culture	MTB	Resistant	Sensitive				
KDHTB627	1	Blood	Culture	MTB	Resistant	Sensitive	Died	True Resistance	No	One culture result confirmed rifampicin resistance.
KDHTB631	1	Blood	Culture	Neg	-	-	Alive	True Resistance	No	One Xpert result and two culture results confirmed rifampicin resistance.
	2	Sputum	Culture	MTB	Resistant	Resistant				
	1	Sputum	Xpert	MTB	Resistant	-				
	1	Urine	Culture	Neg	-	-				
KDHTB660	6	Blood	Culture	Neg	-	-	Died	True Resistance	No	Two culture results confirmed rifampicin resistance.
	2	Blood	Culture	MTB	Resistant	Sensitive				
KDHTB668	1	Blood	Culture	Neg	-	-	Alive	True Resistance	No	Treated for MDR-TB, improved and survived.
JTBS021	1	Blood	Culture	Neg	-	-	Alive	Unknown	No	No samples confirmed rifampicin resistance. Patient started drug-sensitive TB treatment. Inadequate information on improvement or deterioration.
	1	Pleural eff	Culture	Neg	-	-				
JTBS074	1	Urine	Xpert	Neg	-	-	Alive	False Resistance	No	No sample confirmed resistance and patient improved on drug-sensitive TB treatment.
	1	Blood	Culture	Neg	-	-				
	1	CSF	Culture	Neg	-	-				
JTBS090	2	Urine	Culture	MTB	Resistant	Resistant	LTFU	True Resistance	No	Three culture results confirmed rifampicin resistance.
	1	Sputum	Culture	MTB	Resistant	Resistant				
JTBS097†	2	Blood	Culture	MTB	Sensitive	Sensitive	Died	Contamination	No	Two culture results showed rifampicin sensitivity. Patient started drug-sensitive TB treatment, then switched to MDR-TB treatment on basis of urine Xpert result; however, died before other drug susceptibility results were available.
	1	Sputum	Culture	Neg	-	-				
	1	Sputum	Culture	MTB	Sensitive	Sensitive				
	1	Urine	Culture	Neg	-	-				
	1	Sputum	Culture	Contam	-	-				
	1	CSF	Culture	Contam	-	-				
JTBS098	1	Urine	Xpert	Neg	-	-	Died	True Resistance	No	No samples from enrolment admission confirmed rifampicin resistance; however, a 7-month-old result showed rifampicin resistance on pleural fluid, which was untreated. It is unclear why the patient was not treated for MDR-TB and he died shortly after admission to hospital.
	1	Blood	Culture	Neg	-	-				
	2	CSF	Culture	Neg	-	-				
	1	Sputum	Culture	Contam	-	-				
JTBS099†	4	Tracheal aspirate	Culture	MTB	Sensitive	Resistant	Alive	Contamination	No	Four culture results showed rifampicin sensitivity and patient responded to drug-sensitive TB treatment.
JTBS159	1	Urine	Xpert	Neg	-	-	Alive	False Resistance	No	No cultures confirmed rifampicin resistance and one sputum Xpert showed rifampicin sensitivity. Patient treated with drug-sensitive TB treatment and improved.
	1	Blood	Culture	Neg	-	-				
	2	Sputum	Culture	Neg	-	-				
	1	Sputum	Xpert	MTB	Sensitive	-				
	1	Sputum	Culture	Contam	-	-				
	1	Urine	Culture	Neg	-	-				

Table 1-A1 continues on the next page →

TABLE 1-A1 (Continues...): Patients with positive urine Xpert rifampicin resistant results – details of additional tuberculosis tests and clinical course.

Study ID	*n*	Sample type	Test	Organism	Susceptibility to rifampicin	Susceptibility to isoniazid	12-week outcome	Classification of urine Xpert rifampicin resistance†	Heteroresistance	Reason for classification
JTBS160	1	Blood	Culture	Neg	-	-	Alive	False Resistance (on two results)	No	Two cultures showed rifampicin sensitivity and patient improved on drug-sensitive TB treatment.
	2	Sputum	Culture	MTB	Sensitive	Sensitive				
	1	Urine	Culture	Neg	-	-				
JTBS181	1	Urine	Xpert	MTB	Resistant	-	Alive	True Resistance (on two results)	No	Two culture results confirmed rifampicin resistance.
	2	Blood	Culture	Neg	-	-				
	1	Sputum	Culture	MTB	Resistant	-				
	2	Ascites	Culture	Neg	-	-				
	1	Urine	Culture	MTB	Resistant	Resistant				
JTBS192	1	Sputum	Culture	MTB	Resistant	-	Died	True Resistance	No	Three culture results confirmed rifampicin resistance.
	1	Urine	Xpert	Neg	-	-				
	1	Urine	Culture	MTB	Resistant	Resistant				
	1	Sputum	Culture	MTB	Resistant	Resistant				
	1	Sputum	Culture	Contam	-	-				
	1	Sputum	Culture	Neg	-	-				
JTBS202	1	Urine	Xpert	Neg	-	-	Alive	False Resistance	No	One culture result showed rifampicin sensitivity and patient improved on drug-sensitive TB treatment
	1	Blood	Culture	Neg	-	-				
	1	Sputum	Culture	Neg	-	-				
	1	Sputum	Culture	MTB	Sensitive	Sensitive				
JTBS211	9	Sputum	Culture	Neg	-	-	Alive	False Resistance	No	No confirmation of rifampicin resistance patient improved on drug-sensitive TB treatment.
	1	Sputum	Culture	Contam	-	-				
JTBS249	1	Urine	Xpert	Neg	-	-	Alive	False Resistance	No	No confirmation of rifampicin resistance and patient improved on drug-sensitive TB treatment.
	1	Blood	Xpert	Neg	-	-				
	5	CSF	Culture	Neg						
JTBS358	1	Urine	Xpert	MTB	Sensitive	-	Alive	False Resistance	No	No cultures confirmed rifampicin resistance and patient improved on drug-sensitive TB treatment.
	1	Urine	Xpert	Neg	-	-				
	1	Sputum	Culture	MTB	-	-				
	1	Blood	Culture	Neg	-	-				
JTBS463	1	Urine	Xpert	MTB	Resistant	-	Died	True Resistance (from two results)	Yes	Two urine Xpert results showed rifampicin resistance, but one abscess culture and one sputum Xpert (2 months prior) showed rifampicin sensitivity. Patient started on drug-sensitive TB treatment. About 1 month after starting TB treatment the patient was admitted for TB IRIS and anaemia. Patient was then readmitted shortly thereafter with gastroenteritis and was deteriorating despite drug-sensitive TB treatment; it was at this time two urine Xpert results showed rifampicin resistance; however the patient died shortly after receipt of urine Xpert results.
	1	Abscess	Culture	MTB	Sensitive	Sensitive				
JTBS534	1	Urine	Xpert	Neg	-	-	Died	True Resistance	No	No samples confirmed rifampicin resistance. Patient presented with ascites and pleural fluid of which all TB tests were negative. Ascitic fluid had high ADA and patient was started on drug-sensitive TB treatment. About 4 weeks later, the patient was readmitted while clinically deteriorating on drug-sensitive TB treatment. A urine Xpert result showed rifampicin resistance; however, the patient died shortly after receipt of urine Xpert result.
	1	Blood	Culture	Neg	-	-				
	1	Ascites	Culture	Neg	-	-				
	1	Pleural	Culture	Neg	-	-				
	1	Sputum	Culture	Neg	-	-				
JTBS574	1	Urine	Xpert	Neg	-	-	Alive	False Resistance	No	One culture and two Xpert results showed rifampicin sensitivity and patient improved on drug-sensitive TB treatment.
	1	Blood	Culture	Neg	-	-				
	1	Sputum	Culture	MTB	Sensitive	Sensitive				
	2	Sputum	Xpert	MTB	Sensitive	-				

n = number of samples; – Susceptibility unknown or not available. Neg, negative; Contam, Contaminated; MTB, Mycobacterial tuberculosis; LTFU, lost to follow-up; TB, tuberculosis; Pleural, Pleural effusion; Abscess, Abscess aspirate; Drug-sensitive TB treatment = Intensive phase with isoniazid, rifampicin, ethambutol and pyrazinamide, followed by consolidation phase with isoniazid and rifampicin.

†, Two patients had false urine Xpert rifampicin resistance due to contamination (accounting for three results). These patients were excluded from analysis.

For JTBS patients, nearly all had their urine specimens tested by Xpert using two different methods (unconcentrated and concentrated). This accounts for why patients may have two urine Xpert results. However, in the table above, rifampicin resistance was only detected on one urine Xpert result, unless stated otherwise. Some patients may have had an additional urine Xpert performed for confirmation after receiving a rifampicin resistant result.

Of note, the table includes all other TB cultures and Xperts conducted on all clinical specimens, including repeat urine Xpert tests conducted.

CHAPTER 7:

Conclusions

Despite widespread availability of antiretroviral and antituberculosis therapy patients in high burden settings still present with severe forms of tuberculosis in the context of advanced HIV disease (11) and HIV-associated tuberculosis contributes disproportionately to global tuberculosis related mortality (1). Patients who are hospitalized at the time of tuberculosis diagnosis have high case fatality rates despite treatment (2). The underlying pathophysiology and causes of mortality in patients hospitalized with HIV-TB are poorly understood. Acutely ill hospitalized HIV-TB patients with disseminated disease and clinical features of sepsis have not been included in treatment trials to date and are currently treated following the same treatment guidelines as ambulant HIV negative patients with pulmonary tuberculosis. While diagnostic and treatment delays in HIV-TB contribute to morbidity and mortality and need to be addressed, there is an urgent need for novel evidence based, treatment strategies to improve survival in hospitalized patients newly diagnosed with HIV-TB. We conducted a prospective observational cohort study and enrolled HIV positive patients admitted to hospital at the time of tuberculosis diagnosis. We systematically investigated contributors to 12-week mortality.

Clinical and immunologic contributors to mortality are reported in **Chapter 3**. Patients who presented with a high lactate concentration (which is used as a marker of sepsis) had higher mortality. We used a three-point dissemination score consisting of 3 biomarkers of tuberculosis dissemination (Alere urine LAM, urine GeneXpert and mycobacterial blood culture) and patients who had a higher score had higher

mortality. Rifampicin resistant tuberculosis contributed to mortality, but we did not ascertain a major contribution from bacterial or fungal co-infections. We examined 28 host soluble inflammatory mediators in the peripheral blood and described an immunological profile dominated by markers of the innate immune system and chemotaxis (IL-1Ra, IL-6, IL-8, MIP-1β/CCL4, IP-10 and MIP-1α/CCL3) which was associated with 12-week mortality and also with tuberculosis dissemination.

Next, we hypothesized that acutely ill patients hospitalized with HIV-TB who die within 12 weeks would have lower concentrations of first line antituberculosis therapy and we performed intensive pharmacokinetic studies on the third day of treatment in a sub-group of patients within the main cohort study. Our results (**Chapter 4**) show that hospitalized patients had rifampicin, isoniazid and pyrazinamide concentrations similar to ambulant outpatients on the third day of antituberculosis therapy. However, rifampicin and isoniazid concentrations were below recommended reference ranges in 53% and 37% of all patients; in acutely ill hospitalized patients this may contribute to poor outcome. Patients who presented with high lactate concentrations (used as a marker of sepsis) had significantly higher exposure to rifampicin. All patients achieved pyrazinamide concentrations within recommended reference ranges.

We then investigated the association of microbial translocation with mortality in this cohort (**Chapter 5**) and measured two direct (LPS and bacterial 16s rDNA) and five indirect markers of microbial translocation (3 markers which are involved in the immune response to LPS [sCD14, LBP, EndoCAB] and 2 markers of gastro-intestinal epithelial damage [TFF3, IFABP]) in a subset of patients. Higher concentrations of TFF3, bacterial 16s rDNA, sCD14 and lower concentrations of

EndoCAB suggested more gastro-intestinal epithelial damage and higher levels of microbial translocation in hospitalized HIV-TB patients compared to outpatient HIV positive controls. TFF3 (higher concentrations) was independently associated with mortality. We found low concentrations of LPS overall. Bacterial 16s rDNA concentrations were not associated with mortality. We found significantly higher sCD14 and TFF3 concentrations and lower EndoCAB concentrations in patients with positive markers of tuberculosis dissemination, which suggests a relationship between gastrointestinal mucosal damage, microbial translocation and the mycobacterial bacillary load. The clinical significance of gastro-intestinal mucosal damage and microbial translocation in this setting needs to be explored further.

Diagnosing disseminated tuberculosis in severely ill hospitalized HIV-positive patients is challenging and accurate rapid diagnostic tests which can be used in readily obtained samples and provide reliable susceptibility results for first line antituberculosis drugs are urgently needed. Such tests may contribute to reducing mortality by allowing timely initiation of appropriate anti-tuberculosis treatment. Urine GeneXpert is one such test but misdiagnosis of rifampicin resistance in drug sensitive tuberculosis strains has been reported (61). In **Chapter 6** we pooled data from two large cohorts recruited from the same geographical area and reported the frequency of false rifampicin resistance results using urine GeneXpert tests. Although there was a low frequency of false rifampicin resistance results overall, there was higher frequency in patients already on antituberculosis therapy and we recommend that rifampicin resistant results from GeneXpert tests on urine samples should always be confirmed. Whether this same issue will arise with the GeneXpert Ultra assay needs to be investigated in future studies.

Translational implications of findings and future research

Our findings confirm high mortality in patients hospitalized with HIV-TB despite appropriate investigation and timely initiation of antituberculosis therapy. We postulate that the innate immune profile which we described in Chapter 3 is driven by a high disseminated mycobacterial load and may contribute to mortality. We also postulate that sub-optimal rifampicin and isoniazid concentrations described in Chapter 4 contribute to mortality in patients who are hospitalized at the time of tuberculosis diagnosis. These key findings provide rationale to inform novel treatment strategies such as host-directed therapies that could modulate the innate immune response as well as more intensive initial antimicrobial therapy in this vulnerable patient population. Additionally, improving rapid diagnostic tests such as urine LAM testing and Xpert Ultra could be utilized in combination with immune biomarkers to develop a clinical tool that could rapidly identify patients at high risk for early mortality who may benefit from intensified treatment strategies.

Host directed therapy strategies to consider are immediate antiretroviral therapy, adjunctive corticosteroids and adjunctive recombinant IL-7. Early ART initiation (within 1-4 weeks of initiation of antituberculosis therapy) is known to improve survival in patients HIV-TB who have CD4 count < 50 cells/μL (128). Immediate ART initiation (on the same day as antituberculosis therapy initiation) in patients hospitalized with HIV-TB is a possible strategy to consider. This will likely result in an increased risk for paradoxical immune reconstitution inflammatory syndrome (TB-IRIS) and adjunctive corticosteroids would be needed to mitigate this risk (177). However, neither ART status nor HIV viral load were associated with mortality in this

cohort, which indirectly suggests that immediate ART may not alter acute outcomes. Adjunctive corticosteroids reduce mortality in adults with severe pneumonia and sepsis (178, 179) and have been safely used in high risk patients with advanced HIV and active TB to reduce the incidence of paradoxical TB-IRIS (177). Corticosteroids are readily available and their side effect profile is well known, which makes it a feasible adjunctive treatment option in high burden settings. Recombinant IL-7 is still in early phases of testing in clinical trials for sepsis treatment and has thus far been well tolerated and resulted in more rapid and sustained recovery of sepsis-induced lymphopenia (180). This would be a novel adjunctive treatment strategy in hospitalized HIV-TB patients but is not readily available and the safety profile in this patient group would need to be established.

More intensive antimicrobial treatment strategies to consider are high dose rifampicin and high dose isoniazid. Rifampicin doses up to 35 mg/kg/day have been evaluated in patients with pulmonary tuberculosis (74) and such strategies should be evaluated in patients hospitalized with disseminated HIV-TB. High dose rifampicin has been well tolerated in phase 2 randomized controlled trials to date, but these trials have not systemically included a wide spectrum of severely ill hospitalized HIV-TB patients with disseminated tuberculosis and safety will need to be established in this patient group. Safety considerations such as potential hepatoxicity, gastro-intestinal disturbances and cutaneous drug reactions should be considered in the context of the high mortality risk in severely ill hospitalized HIV-TB patients. High dose isoniazid (10-15 mg/kg) may need to be guided by individual patients' genotypic acetylator status to avoid toxicity, although higher doses have been successfully used in the setting of drug resistant tuberculosis (181) and the mechanism of isoniazid

hepatotoxicity may be idiosynchratic and not dose related (182). However early bactericidal activity studies suggest that maximum activity of isoniazid is likely reached at current doses (183), whereas that is not the case for rifampicin. The PanACEA MAMS-TB trial showed that a regimen including rifampicin 35mg/kg in patients with pulmonary tuberculosis resulted in significantly faster sputum conversion in liquid culture by 12 weeks. Patients in the rifampicin 35 mg/kg arm achieved culture conversion at a median of 48 days compared to 62 days in the control arm with an adjusted HR 1.78, 95% CI 1.22-2.58; p=0.003 (74). The addition of fluoroquinolones which have excellent early bactericidal activity could also be considered (184).

Taken together, our findings support the critical need to conduct treatment trials in severely ill patients hospitalized with HIV-associated tuberculosis to optimize treatment strategies aimed at improving survival. Novel treatment strategies such as high dose rifampicin together with corticosteroids and the addition of other rapidly bactericidal drugs such as fluoroquinolones should be tested for safety and efficacy in this patient population. Nested pathogenesis and pharmacokinetic studies are critical to further advance understandings of pathophysiology underlying mortality in this population. The findings described in this thesis have provided the rationale for obtaining funding for such a clinical trial which will commence in 2021.

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